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(54) Title: PROGENITOR CELLS FOR REGENERATION OF LUNG TISSUE

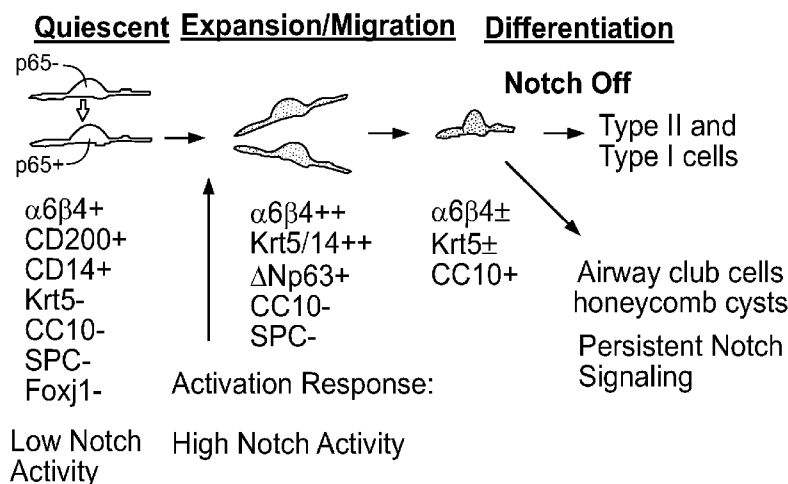


FIG. 7B

(57) Abstract: The present disclosure is generally related to methods for isolating epithelial progenitor cells from mammalian lung tissue. In addition, the present disclosure provides compositions comprising the isolated epithelial progenitor cells, and methods of use for repairing injured lung.

PROGENITOR CELLS FOR REGENERATION OF LUNG TISSUE**STATEMENT REGARDING FEDERALLY SPONSORED
RESEARCH OR DEVELOPMENT**

[0001] This invention was made with government support under UO1 HL111054 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] The application claims the benefit of U.S. Provisional Application No. 62/094,883, filed December 19, 2014, which is incorporated by reference in its entirety.

FIELD

[0003] The present disclosure is generally related to methods for isolating epithelial progenitor cells from mammalian lung tissue. In addition, the present disclosure provides compositions comprising the isolated epithelial progenitor cells, and methods of use for repairing injured lung.

BACKGROUND

[0004] Acute and chronic lung diseases are among the leading causes of morbidity, mortality and health care costs. Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease characterized by the development of scar tissue in the lungs, which limits oxygen exchange. IPF prognosis is poor, with a median survival after diagnosis of only about 3 years (Blackwell et al., Am J Respir Crit Care Med, 189:214-222, 2014). Although a number of pharmacological interventions have been explored, many of these are no longer considered to be effective treatment options. Lung transplantation in patients capable of undergoing major surgery reduces risk of death in the majority of recipients. However, the availability of donor lungs is limited. Thus, there remains a need in the art for therapies to restore lung function.

SUMMARY

[0005] The present disclosure is generally related to methods for isolating epithelial progenitor cells from mammalian lung tissue. In addition, the present disclosure provides compositions comprising the isolated epithelial progenitor cells, and methods of use for repairing injured lung.

[0006] Specifically, the present disclosure provides methods of isolating epithelial progenitor cells, comprising: (a) isolating primary epithelial cells from mammalian lung tissue

by positive selection for expression of E-cadherin; and (b) isolating epithelial progenitor cells from the primary epithelial cells by positive selection for expression of integrin beta-4 (ITGB4), CD14, and CD200. In some embodiments, the methods further comprise a step before (a) of obtaining the lung tissue from a mammalian subject. In some embodiments, the lung tissue is adult lung tissue. In some embodiments, the mammalian lung is human lung. In some methods, step (a) further comprises removing hematopoietic cells from the lung tissue by negative selection for expression of CD45, and one or both of CD16 and CD32. In some preferred embodiments, the isolated progenitor cells are among a cell population that is substantially devoid of one or more of Krt5, CC10, SPC and FoxJ1. Some methods further comprise (c) culturing the epithelial progenitor cells in vitro under suitable conditions for obtaining an expanded population of epithelial progenitor cells. In some embodiments, the expanded population comprises is at least 2 fold (at least 2X, 3X, 4X, 5X, etc. more cells) greater or from about 2 to 20 fold greater than the starting population of isolated progenitor cells. The present disclosure also provides methods comprising isolating a population of epithelial progenitor cells from a population of mammalian primary epithelial cells by positive selection for expression of integrin beta-4 (ITGB4), CD14, and CD200, wherein the epithelial progenitor cells have the ability to differentiate into mature epithelial cells. In some embodiments, the selection is by flow cytometry or immunomagnetic separation. In some preferred embodiments, the selection involves the use of a monoclonal antibody specific for ITGB4, a monoclonal antibody specific CD14, and a monoclonal antibody specific for CD200.

[0007] Also provided by the present disclosure are pharmaceutical compositions comprising a pharmaceutically acceptable excipient and the progenitor cells obtained by any method of the preceding paragraph. In some preferred embodiments, the pharmaceutical composition is a suspension of the progenitor cells in a sterile, isotonic solution. Additionally, the present disclosure provides methods for treating an injured lung, comprising administering the pharmaceutical composition to a mammalian subject in need thereof so as to treat the injured lung. In some embodiments, the mammalian subject is a human patient. In some embodiments, the administration is intrapulmonary administration. In some embodiments, the intrapulmonary administration is by intranasal administration. In some embodiments, the mammalian subject has a chronic fibrosing lung injury, a smoking related lung injury or an acute/subacute lung injury. In some embodiments, the chronic fibrosing lung injury is idiopathic pulmonary fibrosis. In some embodiments, the acute/subacute injury is as a result of a viral infection or a bacterial infection. In some embodiments, the epithelial progenitor cells have the ability to give rise to one or both of SPC+ cells and KRT5+ cells. Some methods further comprising administering a

Notch signaling pathway inhibitor to the mammalian subject after the progenitor cells have engrafted in the injured lung. In some embodiments, the Notch signaling pathway inhibitor is a γ -secretase inhibitor. In some embodiments, the Notch signaling pathway inhibitor is an antibody that neutralizes one of the group consisting of Notch1, Notch 2, Notch 3, Notch4, and ligands thereof. In some embodiments, the Notch signaling pathway inhibitor is a compound selected from the group consisting of genistein, sulforaphane, quercetin, curcumin, and resveratrol.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] FIG. 1A is a schematic depicting lineage analysis methodology. FIG. 1B-1D shows a quantification of lineage tracing, expressed as percentage of cells counted bearing the respective lineage tag (GFP / tdTomato).

[0009] FIG. 2A shows FACS segregation of epithelial (EpCam+) cells by β 4 expression and a CC10-CreERT2 lineage tag (GFP), demonstrating a β 4+ population distinct from club cells. FIG. 2B shows the hierarchical clustering / heat map of RNA-seq transcriptomes from single CC10- β 4+ cells ($\circ$) and distal Krt5-CreERT2 traced cells (Δ) (columns). Listed genes (rows) were selected from >1200 differentially expressed genes identified by ANOVA. FIG. 2C is a schematic depicting orthotopic cell transplantation methodology. FIG. 2D-2E shows FACS isolation of β 4+ CD200+ CD14+ LNEPs. FIG. 2F shows FACS isolation of Krt5-CreERT2-labeled LNEPs.

[0010] FIG. 3A shows quantification of Krt5+ colonies in influenza-injured mice after exposure to BALF in conjunction with DAPT. FIG. 3B shows Krt5+ cell activation / expansion after DAPT treatment as measured by fraction of lung section area. FIG. 3C shows a quantification of SPC expression in LNEPs *in vitro*. FIG. 3D shows a quantification of SPC expression in Krt5-Cre traced cells following intranasal administration of DBZ.

[0011] FIG. 4 shows a quantification of Hess1+ cells at late time points post-influenza in SPC+ type II cells in hyperplastic foci and Krt5-CreERT2-traced (tdTomato+) cells in cystic structures.

[0012] FIG. 5 shows levels of Krt5 protein from whole lung mouse lysate after either influenza injury or bleomycin treatment for 11 or 17 days.

[0013] FIG. 6A shows cytopins of sorted CC10- β 4+ cells with multiciliated cells (green, acetylated tubulin+) and Δ Np63+ cells (red). FIG. 6B shows a PCA plot of cells sequenced in

FIG. 2B. The CC10- β 4+ population is within the dotted outline. FIG. 6C shows the gating on CD14 expression within the Epcam+ β 4+ CD200+ population and exclusion of the CC10-expressing cells. FIG. 6D shows qRT-PCR analysis of mature lineage genes and genes of interest in all populations.

[0014] FIG. 7A shows that distinct epithelial cell types contribute to regeneration depending on the severity of parenchymal injury. Examples of each are referenced. FIG. 7B shows how Notch signaling regulates the activation, expansion, and differentiation of LNEPs.

DESCRIPTION

[0015] The present disclosure is generally related to methods for isolating epithelial progenitor cells from mammalian lung tissue. In addition, the present disclosure provides compositions comprising the isolated epithelial progenitor cells, and methods of use for repairing injured lung.

[0016] Broadly, tissue regeneration is achieved in two ways: by proliferation of common differentiated cells and/or by deployment of specialized stem/progenitor cells. Which of these pathways applies is both organ and injury-specific¹⁻⁴. Current paradigms in the lung posit that epithelial repair can be attributed to cells expressing mature lineage markers⁵⁻⁸. In contrast we here define the regenerative role of previously uncharacterized, rare lineage-negative epithelial stem/progenitor (LNEPs) cells present within normal distal lung. Quiescent LNEPs activate a Δ Np63 / cytokeratin 5 (Krt5+) remodeling program after influenza or bleomycin injury. Activated cells proliferate and migrate widely to occupy heavily injured areas depleted of mature lineages, whereupon they differentiate toward mature epithelium. Lineage tracing revealed scant contribution of pre-existing mature epithelial cells in such repair, whereas orthotopic transplantation of LNEPs, isolated by a definitive surface profile identified through single cell sequencing, directly demonstrated the proliferative capacity and multipotency of this population. LNEPs require Notch signaling to activate the Δ Np63/Krt5+ program whereas subsequent Notch blockade promotes an alveolar cell fate. Persistent Notch signaling post-injury led to parenchymal micro-honeycombing, indicative of failed regeneration. Lungs from fibrosis patients show analogous honeycomb cysts with evidence of hyperactive Notch signaling. Our findings indicate distinct stem/progenitor cell pools repopulate injured tissue depending on the extent of injury, and the outcomes of regeneration or fibrosis may ride in part on the dynamics of LNEP Notch signaling.

[0017] Briefly, the present disclosure provides methods of isolating mammalian epithelial progenitor cells, comprising: isolating epithelial progenitor cells from primary epithelial cells by positive selection for expression of integrin beta-4 (ITGB4), CD14, and CD200. In some embodiments, the methods further comprise the step of isolating primary epithelial cells from mammalian lung tissue by positive selection for expression of E-cadherin.

[0018] As used herein, the terms “epithelial cadherin,” “E-cadherin,” “CDHE,” “ECAD,” “cadherin-1,” “CDH1,” “uvomorulin,” “UVO,” and “CD324” refer to human protein set forth as UniProtKB - P12830 (CADH1_HUMAN), and mammalian counterparts thereof. The term E-cadherin and the like also encompass variants that are at least 95% identical to the amino acid sequence of the extracellular domain of the exemplary human protein. In some preferred embodiments, positive selection for expression of E-cadherin is accomplished using a monoclonal antibody that binds to the extracellular domain of E-cadherin, which extends from positions 155-709 of the 882 residue exemplary human protein.

[0019] As used herein, the terms “integrin beta-4,” “ITGB4,” and “CD104” refer to a human protein as set forth as UniProtKB - P16144 (ITB4_HUMAN) and mammalian counterparts thereof. The term ITGB4 and the like also encompass variants that are at least 95% identical to the amino acid sequence of the extracellular domain of the exemplary human protein. In some preferred embodiments, positive selection for expression of ITGB4 is accomplished using a monoclonal antibody that binds to the extracellular domain of ITGB4, which extends from positions 28-710 of the 1822 residue exemplary human protein.

[0020] As used herein, the terms “monocyte differentiation antigen CD14,” “CD14,” and “myeloid cell-specific leucine-rich glycoprotein” refer to a human protein set forth as UniProtKB - P08571 (CD14_HUMAN) and mammalian counterparts thereof. The term CD14 and the like also encompass variants that are at least 95% identical to the amino acid sequence of the extracellular domain of the exemplary human protein. In some preferred embodiments, positive selection for expression of CD14 is accomplished using a monoclonal antibody that binds to the extracellular domain of CD14, which extends from positions 20-345 of the 375 residue exemplary human protein.

[0021] As used herein, the terms “OX-2 membrane glycoprotein,” “OX2,” and “CD200” refer to a human protein set forth as UniProtKB - P41217 (OX2G_HUMAN) and mammalian counterparts thereof. The term CD200 and the like also encompass variants that are at least 95% identical to the amino acid sequence of the extracellular domain of the exemplary human protein.

In some preferred embodiments, positive selection for expression of CD200 is accomplished using a monoclonal antibody that binds to the extracellular domain of CD200, which extends from positions 31-232 of the 278 residue exemplary human protein.

[0022] The terms “treating” or “treatment” of a disease refer to executing a protocol, which may include administering one or more pharmaceutical compositions to an individual (human or other mammal), in an effort to alleviate signs or symptoms of the disease. Thus, “treating” or “treatment” does not require complete alleviation of signs or symptoms, does not require a cure, and specifically includes protocols that have only a palliative effect on the individual. As used herein, and as well-understood in the art, “treatment” is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results include, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival of an individual not receiving treatment.

[0023] “Palliating” a disease or disorder means that the extent and/or undesirable clinical manifestations of the disease or disorder are lessened and/or time course of progression of the disease or disorder is slowed, as compared to the expected untreated outcome.

[0024] The term “Notch signalling pathway inhibitor” refers to compounds that reduce the activity or expression of Notch receptors. Notch signalling pathway inhibitors that reduce activity may bind to Notch receptors (e.g., Notch1, Notch2, Notch3 or Notch 4) or their ligands (e.g., Jagged1/2, DLL1, DLL3, etc.). These inhibitors may include neutralizing antibodies, fragments or derivatives thereof, as well as decoys, gamma-secretase inhibitors, blocking peptides and natural compounds (see, e.g., Espinoza and Miele, *Pharmacol Ther*, 139:95-110, 2013).

[0025] The terms “isolating” and “purifying” as used in reference to cells or compounds refer to cells or compounds that are removed from their natural environment, such that they are at least 75%, 90%, 95%, 98% or 99% free from other components with which they are naturally associated. For instance, a substantially pure population of epithelial progenitors cells expressing ITGB4, CD15 and CD200, may include up to 1%, 2%, 5%, 10% or 25% by number of cells of other phenotypes (e.g., cells devoid of one or more of ITGB4, CD15 and CD200).

[0026] Likewise, the term “removing” and “depleting” as used herein in reference to cells or compounds refer to cells or compounds that are removed from a composition containing the cells or compounds, such that the composition is at least 75%, 90%, 95%, 98% or 99% free of the cells or compounds. For instance, removing hematopoietic cells from a cell suspension derived from lung tissue, is intended to result in a cell suspension that includes only up to 1%, 2%, 5%, 10% or 25% by number of hematopoietic cells (e.g., cells expressing CD45 and one or both of CD16 and CD32).

[0027] The term “expanded” as used herein in reference to a population of cells refers to a population of cells that has been increased by at least two-fold in number in comparison to the starting population of cells (e.g., epithelial cell population prior to culture in vitro or growth in vivo). In some embodiments, the expansion is by from 2-fold to 100-fold, preferably 5-fold to 50-fold, more preferably from 10-fold to 25-fold.

[0028] As used herein and in the appended claims, the singular forms “a,” “an” and “the” include plural referents unless otherwise indicated or clear from context. For example, “a polynucleotide” includes one or more polynucleotides.

[0029] Reference to “about” a value or parameter describes variations of that value or parameter. For example, the term about when used in reference to 1×10^6 epithelial progenitor cells encompasses 0.9×10^6 to 1.1×10^6 epithelial progenitor cells.

[0030] It is understood that aspects and embodiments described herein as “comprising” include “consisting of” and “consisting essentially of” embodiments.

EXAMPLES

[0031] Abbreviations: BALF (bronchioalveolar lavage fluid); IPF (idiopathic pulmonary fibrosis); and LNEP (lineage-negative epithelial stem/progenitor).

EXAMPLE 1

[0032] This examples describes the identification of rare lineage-negative epithelial stem/progenitor (LNEP) cells in normal lung with a high proliferative capacity and multipotency..

Materials and Methods

[0033] *Animals.* SPC-CreERT2 (Sftpc^{tm1(cre/ERT2,rtTA)Hap}), Krt5-CreERT2 (Krt5^{tm1.1(cre/ERT2)Blh}), CC10-CreERT2 (Scgb1a1^{tm1(cre/ERT)Blh}), FoxJ1-CreERT2 (Tg^{(Foxj1-cre/ERT2)1Blh}), and Cp-eGFP (Tg(Cp-EGFP)25Gaia) mice are previously described.^{17,22-24} All of these strains were bred to either mTmG (Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo})²⁵ or Ai14-tdTomato (Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze})²⁶ mice in order to generate mice expressing a fluorophore in Cre-expressing cells. For transplant experiments, mTmG and/or Ub-GFP (Tg(UBC-GFP)30Scha)²⁷ were used for donor cells. For all experiments, 6-8 week old animals of both sexes were used in equal proportions. Investigators were not blinded to mouse identity. All studies were approved by UCSF IACUC, protocol AN088356-03.

[0034] For lineage analysis for the cell of origin of Krt5+ cells, mice were administered three doses (Krt5-CreERT2) or five doses (SPC-CreERT2 and CC10-CreERT2) of 0.25 mg / g body weight tamoxifen in 50 µl corn oil. A chase period >21 days was employed to insure the absence of residual tamoxifen prior to injury.

[0035] *Injury (Influenza, Bleomycin).* Mice were administered 280 FFU of Influenza A/H1N1/Puerto Rico/8/34 (PR8) intra-nasally. PR8 virus dissolved in 30 µl of PBS was pipetted onto the nostrils of heavily anesthetized mice (visual confirmation of agonal breathing), whereupon mice aspirated the fluid directly into their lungs. The mice were allowed to recover and weighed twice a week. For experiments analyzing the lineage fate of Krt5+ cells, a single dose of 0.125 mg / g body weight tamoxifen was administered at day 10 post-PR8 infection.

[0036] Infective viral particles were assayed by inoculation of either stock virus or homogenate (in 1 ml PBS) of left lung, spleen, and brain onto 96 well plates of confluent MDCK cells. After one hour, samples were decanted and replaced with serum-free media containing TPCK trypsin at 100 µg/ml. Fifteen hours later, the cells were fixed in 100% methanol, and then underwent indirect immunocytochemistry using Millipore mouse anti-influenza A (MAB 8257) at 1.25 µg/ml, followed by Vector® 102 biotinylated horse anti-mouse, and the biotin/avidin system (PK-4002) with diaminobenzidine as a chromogen. Samples were processed in triplicate over dilutions, and foci were counted in wells that yielded 30-100 discrete foci.

[0037] 1.7 U / kg body weight bleomycin was administered intra-tracheally. Mice were weighed twice a week. For lineage tracing Krt5+ cells post-bleomycin, a single dose of 0.125 mg / g body weight tamoxifen was administered at day 17 post-bleomycin.

[0038] *Treatment of animals with γ -secretase inhibitors.* For DAPT administration, mice received 50 mg / kg body weight DAPT in 20 μ l DMSO per intraperitoneal injection, for the indicated periods. For intranasal DBZ administration, 30 μ moles / kg body weight DBZ was suspended in 50 μ l sterile PBS and sonicated in a Bioruptor UCD-200 for 15 min total, 30 sec intervals on ice. In DBZ experiments, both DBZ and vehicle group also received 2.5 μ g / g body weight dexamethasone (Sigma) in the intranasal solution and 10 mg / kg 3-Isobutyl-1-methylxanthine (IBMX, Sigma) i.p. daily. Both DAPT and DBZ were obtained from Toronto Research Chemicals. >7500 Krt5-CreERT2-labeled cells were quantified for SPC expression in at least 2 individual lobes from each mouse.

[0039] *Orthotopic lung transplantation.* Left lung transplants were carried out using the method described by Okazaki and colleagues.²⁸ The donor animal was anesthetized and injected with heparin (50 units) immediately prior to perfusion of the lung vasculature with 5 ml of ice cold Perfadex solution (Xvivo Perfusion, Goteborg Sweden), clamping of the hilar structures, and removal from the donor animal. The left lung was transplanted into the recipient animal using the cuff anastomosis technique.

[0040] *Orthotopic tracheal transplantation.* The donor animal was anesthetized and with the aid of microscopic dissection, a segment of trachea composed of 5-7 tracheal rings was removed. The recipient animal was anesthetized and the donor trachea was interposed using proximal and distal anastomoses¹¹.

[0041] *Immunofluorescence analysis of tissue.* After euthanasia, lungs were either immediately inflated with OCT and flash frozen or inflated with 4% PFA and fixed for 1 hour at room temperature and subsequently embedded in OCT. 7 μ m sections were cut on a cryostat, with fresh frozen tissue immediately fixed for 5 min in 4% paraformaldehyde at room temperature. All sections were subsequently incubated for 3 x 10 minute intervals with 1 mg/ml sodium borohydride (Sigma) in PBS to reduce aldehyde-induced background fluorescence. Slides were subsequently blocked $\geq$ 1 hour in PBS + 1% bovine serum albumin (Affymetrix), 5% nonimmune horse serum (UCSF Cell Culture Facility), 0.1% Triton X-100 (Sigma), and 0.02% sodium azide (Sigma). Slides were incubated overnight in primary antibodies listed below, diluted in block solution. Slides were washed three times with PBS + 0.1% Tween 20, and incubated with secondary antibodies (typically Alexa Fluor conjugates, Life Sciences) at a 1:2000 dilution $\geq$ 1 hour. Finally, slides were again washed, incubated with 1 μ M DAPI for 5 min, and mounted using Prolong Gold (Life Sciences).

[0042] The following antibodies were used: rabbit anti-proSPC (1:3000; Millipore, AB3786), goat anti-proSPC (1:2000; Santa Cruz, M-20), goat anti-CC10 (1:10,000, a gift from Barry Stripp), rabbit anti-Krt5 (1:1000; Covance, PRB-160P), chicken anti-Krt5 (1:1000; Covance, SIG-3475), rabbit anti- Δ Np63 (1:100; Biolegend, POLY6190), rat anti-CD45 (1:200, BD 30-F11), sheep anti-eGFP (1:500; Pierce, 10396164), rabbit anti-phospho histone H3 (1:500; Millipore, 06-570), rabbit anti-Hes1 (1:1000; Cell Signaling, D6P2U), rabbit anti-activated Notch1 (1:1000; Abcam, ab8925), mouse anti-acetylated tubulin (1:500, Sigma, 6-11B-1).

[0043] *Quantification of lineage tracing.* Samples were prepared for immunofluorescence staining. Quantification at day 11 post-influenza is the result of counting >2900 cells (CC10 trace), >4000 cells from (SPC trace), or >1300 (Krt5 trace) from at least 3 mice per genotype. Cells were counted from >5 sections per mouse and included at least 3 individual lobes. Mutual exclusivity of CC10-traced and Krt5+ cells at day 7-8 was determined with a smaller sample size, n=2 mice, 12 Krt5+ airways / >500 cells examined. Only mice possessing the appropriate genotype were used in studies.

[0044] *Epithelial cell isolation and flow cytometry.* Lung epithelial cells were isolated as previously described¹⁷, with the following modifications. After installation with agarose and subsequent hardening by a brief incubation on ice, each lobe was cut away from the mainstem bronchi. The proximal-most ¼ of each lobe surrounding the bronchi was then cut away to minimize the inclusion of basal cells in the cell preparation, and the previous protocol was followed from this point on.

[0045] For FACS analysis, single cell preparations were incubated 30-45 minutes at 4°C with the following primary antibodies: PE, Alexa Fluor 488, or BV421-conjugated rat anti-mouse EpCAM (1:500; Biolegend, G8.8), Alexa Fluor 647 or PE-conjugated rat anti-mouse integrin β 4 (1:75; BD, 450-9D), Alexa 647-conjugated CD200 (1:100, Biolegend, OX-90), and PE/Cy7 conjugated CD14 (1:100, Biolegend, Sa14-2) . Antibody incubations were done in DMEM (without phenol red) + 2% FBS and cells were washed twice with PBS after antibody incubations. Sorting and analysis was performed on BD FACS Aria cytometers.

[0046] *Orthotopic cell transplantation.* Recipient C57BL/6 mice were infected with PR8. At 9 days post-infection, donor cells were sorted from mTmG or Ub-GFP mice and resuspended in 50ul sterile PBS. Recipient mice received cell solution intranasally as described above for influenza administration. The total number of β 4+ cells ranged from 150,000 to 350,000 per transplant (n=6), and equivalent numbers of β 4- cells were always transplanted into injured

littermates for comparison. For transplantation of Krt5-CreERT2-labeled cells, 1000 cells were transplanted per recipient ($n = 2$). For $\beta 4+$ CD14+ CD200+ cell transplants, 3000-10,000 cells were transplanted per mouse ($n=3$). FoxJ1-CreERT2 or CC10-CreERT2-labeled cell transplants were performed in $n=3$ or 4 mice each ($1-3 \times 10^5$ cells per mouse). Endpoint analysis was performed at day 21 post-infection unless otherwise noted. For analysis of proliferation, recipient mice were administered 50 mg / kg body weight Edu (Santa Cruz) in PBS daily. Edu was detected with Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen).

[0047] *Primary cell culture.* Isolated primary lung epithelial cells were plated and cultured on Matrigel as follows. 8-well chamber slides were coated with 150 μ l Matrigel per well, allowed to solidify at 37°, and then equilibrated SABM (Lonza) for at least 30 minutes prior to cell plating. 15 to 40,000 cells were plated in each well of and maintained in “baseline” media consisting of SAGM (Lonza) supplemented with 5% charcoal-stripped FBS and 10 ng / ml KGF (FGF-7, Peprotech). Other growth factors were included in the media only when indicated and are summarized in Supplementary Table 2.

[0048] Bronchioalveolar lavage fluid (BALF) was harvested from injured animals for cell culture as follows. Euthanized mice were intratracheally intubated prior to cardiac perfusion and 1 ml of baseline media was lavaged. The lungs were repeatedly lavaged with the media at least three times. BALF was then centrifuged three times for 5 min spins at 1500xG to remove the cells and other debris. Clarified BALF was finally filtered through a 0.25 μ m Spin-X filter (Sigma) to remove any additional debris and to ensure a cell-free preparation. BALF prepared in this way was either added to cells immediately or frozen in aliquots at -80° C and was added to cultured cells without dilution.

[0049] *Long term cell culture.* Cells isolated as above were maintained in SAGM as above, with the addition of 10 μ M Y-27632 (Sigma) and 50 ng/ml murine noggin (Peprotech). Cells were passaged every 7-10 days by initial incubation with 25 U/ml Dispase at 37° for 20 minute to liberate colonies. Single cell dissociation was performed by additional 10 minute incubation with 2 mM EDTA in PBS in combination with mechanical disaggregation by pipetting.

[0050] *γ -secretase treatment of LNEPs in vitro.* LNEPs maintained as above were dissociated and re-plated directly into SAGM baseline media with added DAPT or GSI-X (Calbiochem) at 40 or 20 μ M concentrations (unless otherwise indicated). For SPC induction experiments, IBMX was added when indicated. LNEPs were cultured for 7-10 days and then analyzed by immunofluorescent staining.

[0051] *Immunofluorescence analysis of cultured cells.* Cells grown on matrigel were fixed 5-10 minutes in IHC Zinc Fixative (BD) and subsequently stained as indicated above, except that all staining solutions were prepared with TBS as the zinc fixative reacts with phosphate.

[0052] *Live slice imaging.* Krt5-CreERT2 / tdTomato mice were administered 280 FFU PR8 (as above) and received a single 0.25 mg/kg dose of tamoxifen 24 hours prior to sacrifice at the indicated time points. Injured mice were euthanized and perfused and lavaged with PBS. Lungs were instilled with 2% low-melting point agarose and ~300 um slices were prepared on a vibratome. Lung slices were maintained in SAGM + 10 ng/ml KGF during imaging with the addition of 500 nM hydroxytamoxifen (Sigma) in order to induce recombination in all Krt5-expressing cells. Slices were imaged continuously for 12 hours in a 37° C chamber on an inverted stage with a Leica SP5 confocal microscope. Images obtained were deconvoluted with Bitplane Imaris for presentation.

[0053] *Quantitative reverse transcriptase PCR.* RNA was isolated from sorted cells using the Promega RNA Reliprep kit. cDNA was synthesized and amplified using the Ovation PicoSL WTA V2 kit (NuGen). RT-PCR reactions were performed using Faststart Universal SYBR green Master Mix (Roche) and run on an Eppendorf Realplex² thermocycler. Primer sequences are as listed in Table 1-1.

Table 1-1. Primer Sequences

Marker	Primer 1	Primer 2
SPC	ATGGACATGAGTAGCAAAGAGGT (SEQ ID NO:1)	CACGATGAGAAGGCGTTTGAG (SEQ ID NO:2)
CC10	ATGAAGATCGCCATCACAATCAC (SEQ ID NO:3)	GGATGCCACATAACCAGACTCT (SEQ ID NO:4)
Keratin 5	TCCAGTGTGTCCTTCCGAAGT (SEQ ID NO:5)	TGCCTCCGCCAGAACTGTA (SEQ ID NO:6)
Δ Np63	ATGTTGTACCTGGAAAACAATGCC (SEQ ID NO:7)	CAGGCATGGCACGGATAAC (SEQ ID NO:8)
Jagged1	CCTCGGGTCAGTTTGAGCTG (SEQ ID NO:9)	CCTTGAGGCACACTTTGAAGTA (SEQ ID NO:10)
Jagged2	CAATGACACCACTCCAGATGAG (SEQ ID NO:11)	GGCAAAGAAGTCGTTGCG (SEQ ID NO:12)
Hey1	GCGCGGACGAGAATGGAAAA (SEQ ID NO:13)	TCAGGTGATCCACAGTCATCTG (SEQ ID NO:14)
Aqp5	AGAAGGAGGTGTGTTTCAGTTGC (SEQ ID NO:15)	GCCAGAGTAATGGCCGGAT (SEQ ID NO:16)
ABCA3	GCTTGAAGATCCAGTCGGAGA (SEQ ID NO:17)	CATAGCGAATGTAGTCCTCGAAG (SEQ ID NO:18)
CGRP	GCGGGCTCTAGCTTGGACAG (SEQ ID NO:19)	AAGGTGTGAACTTGTTGAGGT (SEQ ID NO:20)
Sox2	GCGGAGTGGAACTTTTGTTTC (SEQ ID NO:21)	GGGAAGCGTGTACTTATCCTTCT (SEQ ID NO:22)
Foxj1	CATCTACAAGTGGATCACGGAC (SEQ ID NO:23)	GAGCAGGCGCTCTGCGTACTG (SEQ ID NO:24)

[0054] *Single Cell RNA-seq.* Distal lung epithelial cells were isolated and FACS sorted as described above from CC10-CreERT2 / mTmG mice. In addition, tdTomato+ cells were sorted from tamoxifen-treated Krt5-CreERT2 / tdTomato mice. Sorted single cells were captured on a medium-sized (10–17 μ m cell diameter) microfluidic RNA-seq chip (Fluidigm) using the Fluidigm C1 system. All downstream steps (lysis, cDNA synthesis / amplification, library preparation, sequencing, and raw data processing) were performed exactly as previously described.¹² FPKM files for each cell were analyzed using Fluidigm Singular® software running in R.

[0055] *Human tissues.* All human tissue samples were obtained from UCSF Interstitial Lung Disease Blood and Tissue Repository and are classified as Non-identifiable Otherwise Discarded Human Tissues.

[0056] *Statistics.* For calculations involving single cell RNA-seq, Fluidigm Singular® software running in R was used. All other statistical calculations were performed using Graphpad Prism. P values were calculated from two-tailed t-tests (paired or unpaired depending on experimental design) or ANOVA for multivariate comparisons. Variance was analyzed at the time of t-test analysis.

Results

[0057] Influenza infection challenges pulmonary regenerative capacity due to the widespread ablation of epithelial cells in substantial areas of lung⁸. A robust expansion of regenerative Krt5+ cells in the lung parenchyma following influenza infection has been observed in mice⁸, which we confirmed. In addition we directly observed migration and identified coexpression of integrin $\alpha 6\beta 4$. These cells also appear variably after bleomycin injury, where ~1/3 of the Krt5+ cells resolved into type II pneumocytes by 50 days post-injury. A cellular origin and mechanistic framework for expansion after influenza, and potential parallels in human lung injury, remain unknown.

[0058] To define the cell-of-origin, we lineage traced mature cell types implicated in epithelial repair. Krt5+ cells appearing by day 11 post influenza infection were essentially completely untraced using CC10- or SPC-CreERT2 drivers (FIG. 1B-D). Analysis at 7-8 days post-injury confirmed mutual exclusivity of CC10-Cre labeled cells and the Krt5+ cells (FIG. 1B). Conflicting results in other reports are likely caused by tamoxifen persistence.

[0059] A small fraction (13%) of expanded Krt5+ cells bear the Krt5-CreERT2 lineage label (FIG. 1D), raising the possibility that tracheal basal cells might migrate distally during injury. Short chase time after tamoxifen administration to CC10-CreERT2 mice results in significant trace (GFP+) in Krt5+ cells. We transplanted sections of fluorescent trachea into syngeneic animals and a non-fluorescent left lung into a fluorescent mouse¹¹. Abundant Krt5+ cells arose after infection but none were fluorescent. Upper-airway basal cells therefore do not contribute to this phenomenon and instead implicate a lineage-negative epithelial progenitor(s) (LNEPs) as the major source of Δ Np63+/Krt5+ cells.

[0060] To characterize quiescent LNEPs we used $\beta 4$ expression in CC10-CreERT2 mice to segregate LNEPs from club cells in uninjured lungs (FIG. 2A) and confirmed minimal expression of mature lineage markers (FIG. 6D). The CC10- $\beta 4$ + (LNEP containing) population uniquely expressed Δ Np63 (FIG. 6D). Δ Np63+ cells were identified *in situ* scattered

sporadically throughout distal airways by immunofluorescent staining. These cells did not express detectable Krt5 protein. In a total of 65 small airways examined in two mice, we identified 24 Δ Np63+ cells. Only 7 of the 24 cells were labeled in Krt5-CreERT2 mice, likely explaining the small fraction of post-injury Krt5+ cells bearing the Krt5-CreERT2 lineage label (FIG. 1D).

[0061] Given the infrequency of Δ Np63+ cells we suspected progenitor activity of the CC10- β 4+ population might be restricted to a smaller subset. Immunostaining revealed multicilia in 78% of this population, whereas Δ Np63+ cells were less than 1%. To address this heterogeneity, we performed single cell RNA-seq on CC10- β 4+ cells and rare Krt5-CreERT2-labeled cells, a subset of this population (FIG. 2F). Δ Np63 transcript was detected in several cells in the CC10- β 4+ population (red $\circ$, far left) as well as the Krt5-traced cells (green Δ) (FIG. 2B). ANOVA comparison between putative LNEPs (Krt5-traced cells combined with all p63-expressing cells) and the remaining cells revealed enrichment of ~900 genes (>2 fold change, >1 FPKM, $p \leq 0.05$) in the LNEP group. We note enrichment for pluripotency-associated transcription factors (Myc, Klf4) in the LNEP group (FIG. 2B) while many genes enriched in the remaining cells (FIG. 2B top rows) are known markers of ciliated cells¹². Surprisingly, Δ Np63+ CC10- β 4+ cells most closely related to the Krt5-traced cells also expressed cilia-associated genes (FIG. 2B, denoted by *). Cytospins of CC10- β 4+ cells revealed primary cilia on Δ Np63+ cells and additional cells without discernible Δ Np63, suggesting the LNEP profile extends to a larger fraction of Δ Np63 low/neg cells. The presence of primary cilia has been linked to a stem / progenitor phenotype^{13,14}.

[0062] To assess the potential of LNEPs *in vivo*, we devised a transplantation assay by which $\sim 10^5$ fluorescent CC10- β 4+ cells were delivered orthotopically into influenza-injured mice (FIG. 2C). Seeded LNEPs developed into multicellular structures in two patterns seemingly dependent on location: areas of type II cells virtually indistinguishable from surrounding endogenous type II cells and engraftments expressing both Krt5 and CC10 near endogenous Krt5+/CC10+ structures. β 4- type II cells engrafted infrequently in small clusters (<8 cells), and expressed only alveolar markers such as SPC. CC10+ cells could engraft but exhibited scant differentiation, even losing CC10 expression. Transplantation of multi-ciliated cells resulted in only occasional persistence of single cells without structures, consistent with their lack of progenitor properties^{15,16}.

[0063] Transplantation of mixed eGFP and tdTomato-expressing LNEPs demonstrated engraftments to be largely non-overlapping. Abundant incorporation of Edu shows engraftments are also highly proliferative, arguing for near-clonal expansion. Although mature type II cells do not express integrin $\beta 4^{17}$, clones derived from donor LNEPs exhibited $\beta 4$ /SPC co-expression 5 days after transplant, confirming their LNEP origin. These data demonstrate multipotency of LNEPs as well as the viability of orthotopic cell transplantation as a functional tool.

[0064] We interrogated the RNA-Seq analysis and identified enrichment for CD14 in $\Delta Np63+$ CC10- $\beta 4+$ cells (*, FIG. 2B). In combination with CD200, which further selects against multi-ciliated cells (FIG. 6C), CD14+ cells were isolated and transplanted. $\beta 4+$ CD200+ CD14+ cells (~3000) (FIG. 2D) phenocopied the larger (150,000) CC10- $\beta 4+$ population by differentiating into both SPC+ and Krt5+ cells, validating this small population as the active LNEPs. Utilizing a complementary approach, distal Krt5-CreERT2-labeled $\Delta Np63+$ cells within the LNEP fraction were transplanted (1000 cells per mouse) (FIG. 2F). Multipotency was again observed, though we noted fewer SPC expressing cells. We posit that isolation using Krt5-driven Cre enriches for LNEPs that have undergone partial commitment to the Krt5 program, whereas surface marker-based selection represents a less biased approach. This is consistent with lineage analysis (FIG. 1D) indicating Krt5-CreERT2-traced cells can only account for a small fraction of the Krt5+ expansion.

[0065] Accordingly, LNEPs cultured *ex vivo* did not express Krt5 even when treated with various trophic / morphogenic factors. However, bronchoalveolar lavage fluid (BALF) from injured mice induced dramatic proliferation and Krt5 expression. $77 \pm 13\%$ of colonies treated with the BALF stained positive for Krt5 (FIG. 3A) whereas type II cells treated with the same BALF did not respond.

[0066] Although the active principle(s) in injury BALF is uncertain, a screen of pathway inhibitors implicated a critical role of Notch. The γ -secretase inhibitor DAPT in conjunction with active BALF attenuated intensity and Krt5+ colony fraction ($22.7 \pm 13\%$) (FIG. 3A). This prompted us to analyze Notch activity *in vivo*. Notch1 ICD and the canonical Notch target gene Hes1 were evident in the nucleus of parenchymal Krt5+ cells post-influenza. Notch activity was further validated using a Notch reporter mouse (Cp-eGFP). Krt5+ cells arising in distal airways expressed GFP in Notch reporter mice 7 days after influenza infection. When DAPT was administered to mice post-influenza, the fraction of lung area bearing Krt5+ cells by day 11 was markedly reduced (FIG. 3B).

[0067] During development Notch signaling is known to suppress alveolar differentiation in both the lung and mammary gland^{18,19}. When LNEPs were cultured in the presence of γ -secretase inhibitors we observed strong induction of SPC expression, further promoted by IBMX²⁰ (FIG. 3C). Therefore, persistent Notch signaling prevents alveolar differentiation whereas removal of this signal promotes maturation toward type II cells. This result proved relevant to the long-term outcome of regeneration in the influenza injury model.

[0068] Although regions of relatively normal histology bearing the Krt5-CreERT2 trace develop after resolution of bleomycin injury, we were surprised to find few traced SPC+ type II cells after influenza. Instead, large regions of Krt5-Cre traced epithelial cysts were present in all mice examined between day 52 and day 200 post-injury (n=7 mice). These cysts consisted of CC10+ cells, scattered Krt5+ cells, otherwise nondescript epithelial cells, but very few SPC+ cells, raising the possibility of ongoing Notch activity in cystic epithelium. Strong Hes1 expression persisted in Krt5-CreER traced cysts cells indefinitely, whereas it is normally undetectable in alveolar epithelium. The same correlation was observed in transplant experiments: LNEP-derived Krt5+ & CC10+ areas exhibited strong Hes1 whereas it was low or absent in areas of type II cell differentiation. Notch antagonism *in vivo* via intranasal delivery of DBZ (in conjunction with dexamethasone and IBMX) resulted in a significant increase in the number of cyst-derived SPC+ cells (12.3% vs 1.6%) (FIG. 3D).

[0069] Persistent cysts bear a strong resemblance to “micro-honeycombing” in lungs of Idiopathic Pulmonary Fibrosis (IPF) patients. These lungs (n=10) showed almost all cystic epithelia were comprised of Krt5+ cells surrounded by either additional metaplastic Krt5+ cells or pseudostratified epithelium with ectopic but otherwise typical basal cells²¹. Distinct foci of hyperplastic SPC+ cells were also present. Notch activity correlated with Krt5+ cysts but was absent in most hyperplastic SPC+ cells (FIG. 4) and in normal alveolar regions.

[0070] In lungs from scleroderma patients (n = 7) fibrotic areas displayed the IPF pattern of persistent Krt5+ / Hes1+ cystic structures. However, in 3 less fibrotic specimens, we observed extensive Krt5 / SPC double positive cells lining alveoli. Although the origin of this Krt5+ expansion is uncertain in humans, we note Δ Np63+/Krt5- cells in normal terminal airways, analogous to LNEPs in mice.

[0071] These experiments identify a rare, undifferentiated epithelial population that is the major responder in distal lung following severe damage (FIG. 7A). Notch signaling modulates the quiescence, activation, and differentiation state of murine LNEPs (FIG. 7B), providing a

signaling paradigm to frame the dynamic aspects of LNEP function. The persistently abnormal parenchymal structures that derive from LNEPs following influenza infection represent a failed regenerative process, promoted at least in part by ongoing Notch activity. The striking parallels to the currently inexplicable micro-honeycombing that characterizes progressive fibrotic lung disease, including hyperactive Notch, suggest inappropriate Notch signaling may also be a major contributor to failed regeneration in chronic lung disease.

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See also Rawlins, Nature, 517:556-557, 2015; Vaughan et al., Nature, 517:621-625, 2015; and Zuo et al., Nature, 517:616-220, 2015.

CLAIMS

We claim:

1. A method of isolating epithelial progenitor cells, comprising:
 - (a) isolating primary epithelial cells from mammalian lung tissue by positive selection for expression of E-cadherin; and
 - (b) isolating epithelial progenitor cells from the primary epithelial cells by positive selection for expression of integrin beta-4 (ITGB4), CD14, and CD200.
2. The method of claim 1, further comprising a step before (a) of obtaining the lung tissue from a mammalian subject.
3. The method of claim 1, wherein the lung tissue is adult lung tissue.
4. The method of claim 1, wherein the mammalian lung is human lung.
5. The method of claim 1, wherein step (a) further comprises removing hematopoietic cells from the lung tissue by negative selection for expression of CD45, and one or both of CD16 and CD32.
6. The method of claim 1, wherein the isolated progenitor cells are among a cell population that is substantially devoid of one or more of Krt5, CC10, SPC and FoxJ1.
7. The method of claim 1, further comprising step (c) culturing the epithelial progenitor cells in vitro under suitable conditions for obtaining an expanded population of epithelial progenitor cells.
8. A method comprising isolating a population of epithelial progenitor cells from a population of mammalian primary epithelial cells by positive selection for expression of integrin beta-4 (ITGB4), CD14, and CD200, wherein the epithelial progenitor cells have the ability to differentiate into mature epithelial cells.
9. The method of any one of claims 1-8, wherein the selection is by flow cytometry or immunomagnetic separation.
10. A pharmaceutical composition comprising a pharmaceutically acceptable excipient and the progenitor cells obtained by the method of claim 9.

11. A method for treating an injured lung, comprising administering the pharmaceutical composition of claim 10 to a mammalian subject in need thereof so as to treat the injured lung.
12. The method of claim 11, wherein the administration is intrapulmonary administration.
13. The method of any one of claims 12, wherein the mammalian subject has a chronic fibrosing lung injury, a smoking related lung injury or an acute/subacute lung injury.
14. The method of claim 13, wherein the chronic fibrosing lung injury is idiopathic pulmonary fibrosis.
15. The method of claim 13, wherein the acute/subacute injury is as a result of a viral infection or a bacterial infection.
16. The method of claim 12, wherein the epithelial progenitor cells have the ability to give rise to one or both of SPC+ cells and KRT5+ cells.
17. The method of claim 12, further comprising administering a Notch signaling pathway inhibitor to the mammalian subject after the progenitor cells have engrafted in the injured lung.
18. The method of claim 17, wherein the Notch signaling pathway inhibitor is a γ -secretase inhibitor.
19. The method of claim 17, wherein the Notch signaling pathway inhibitor is an antibody, fragment or derivative that neutralizes one of the group consisting of Notch1, Notch 2, Notch 3, Notch4 and ligands thereof.
20. The method of claim 17, wherein the Notch signaling pathway inhibitor is a compound selected from the group consisting of genistein, sulforaphane, quercetin, curcumin, and resveratrol.

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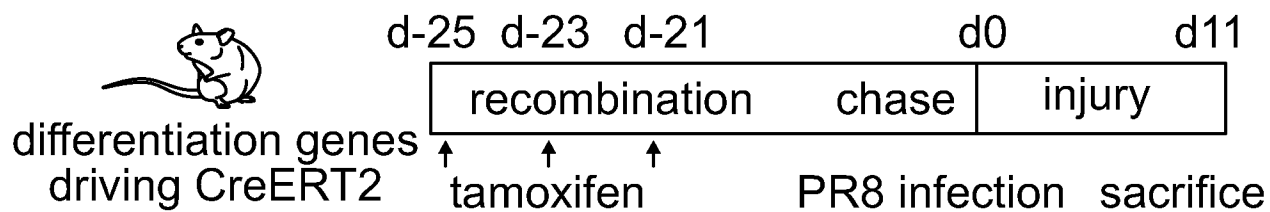


FIG. 1A

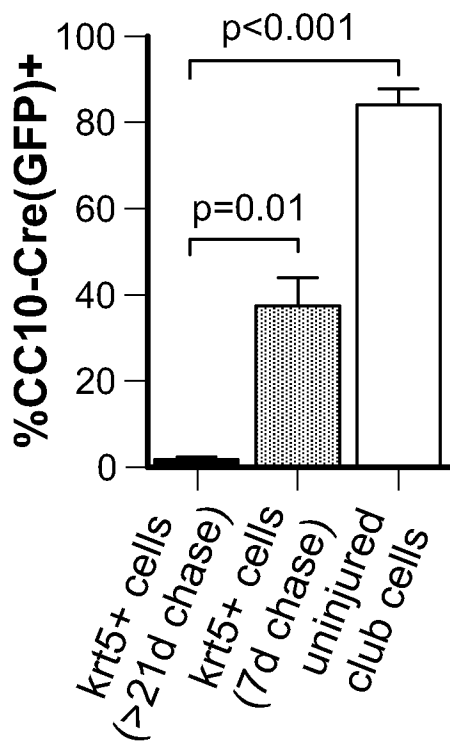


FIG. 1B

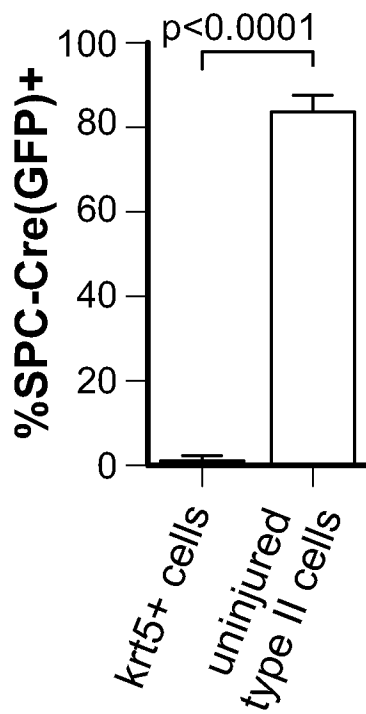


FIG. 1C

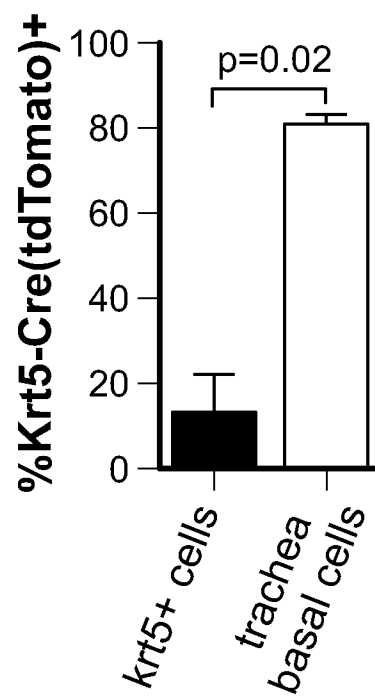


FIG. 1D

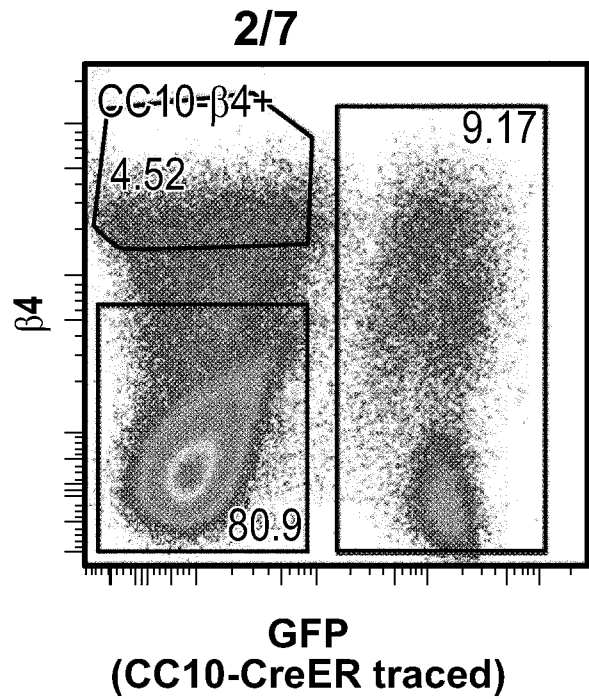


FIG. 2A

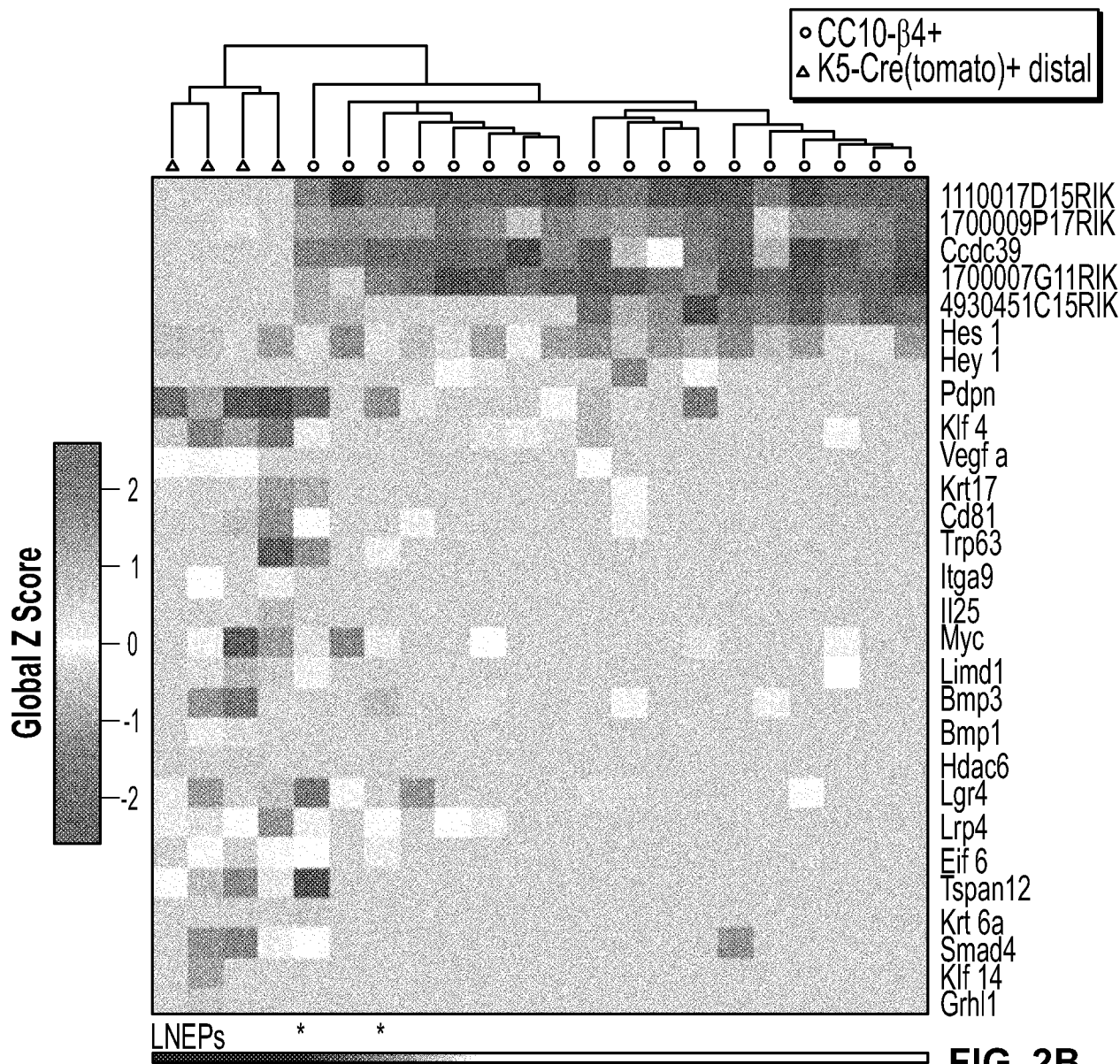


FIG. 2B

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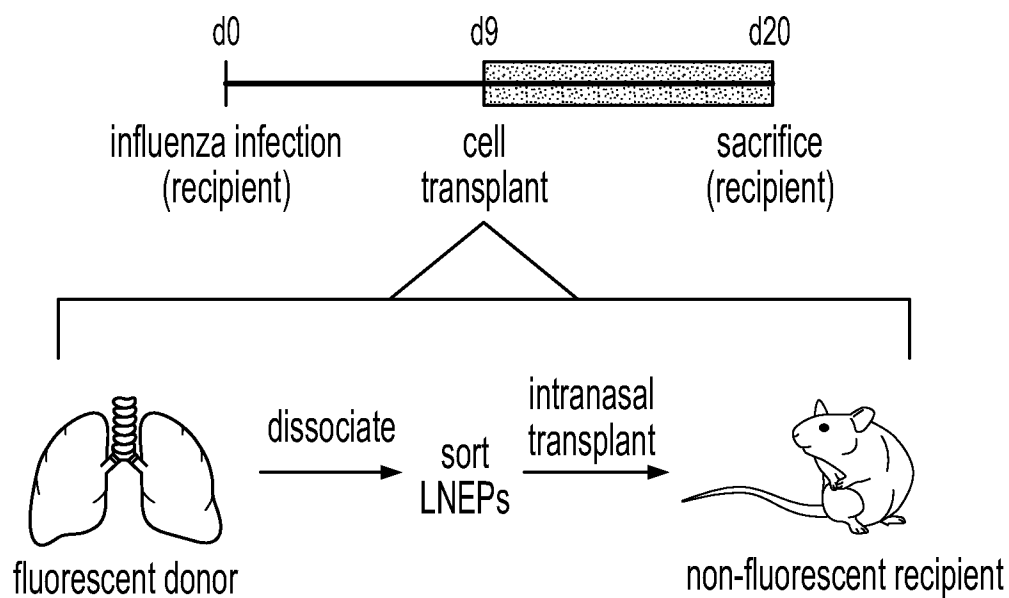


FIG. 2C

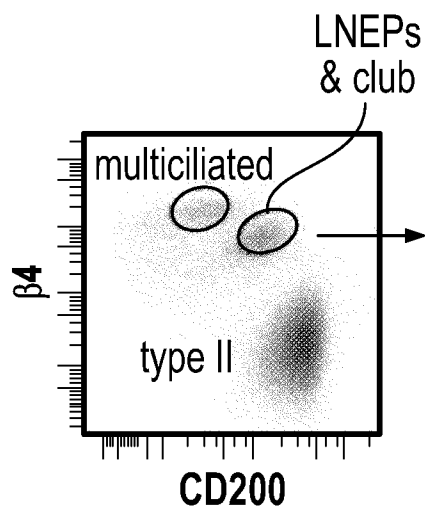


FIG. 2D

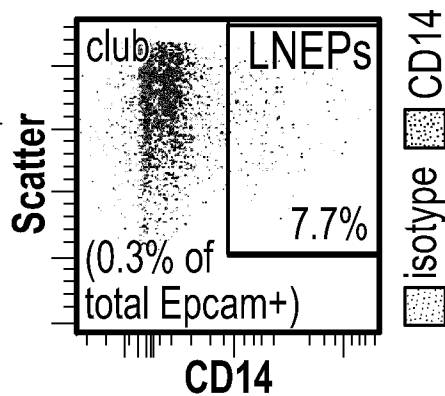


FIG. 2E

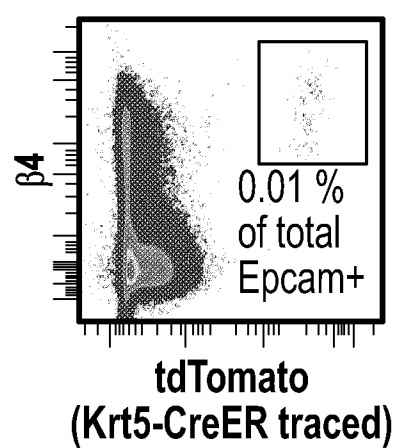


FIG. 2F

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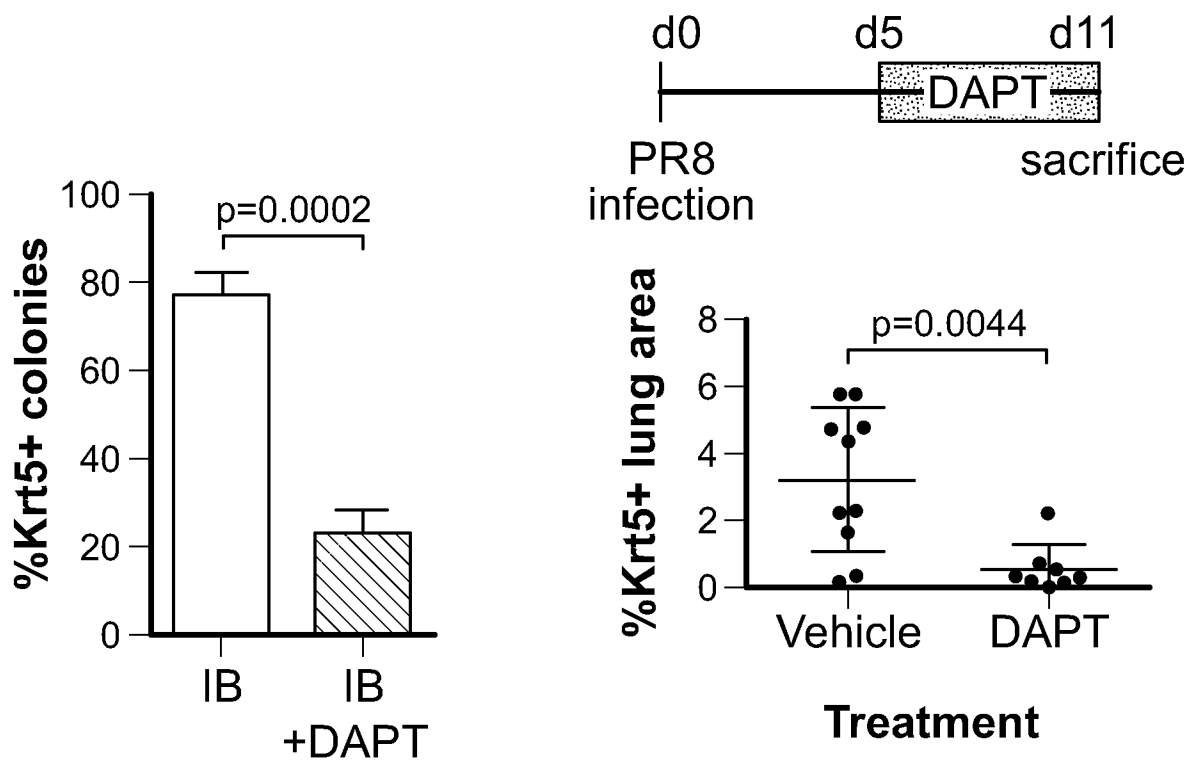


FIG. 3A

FIG. 3B

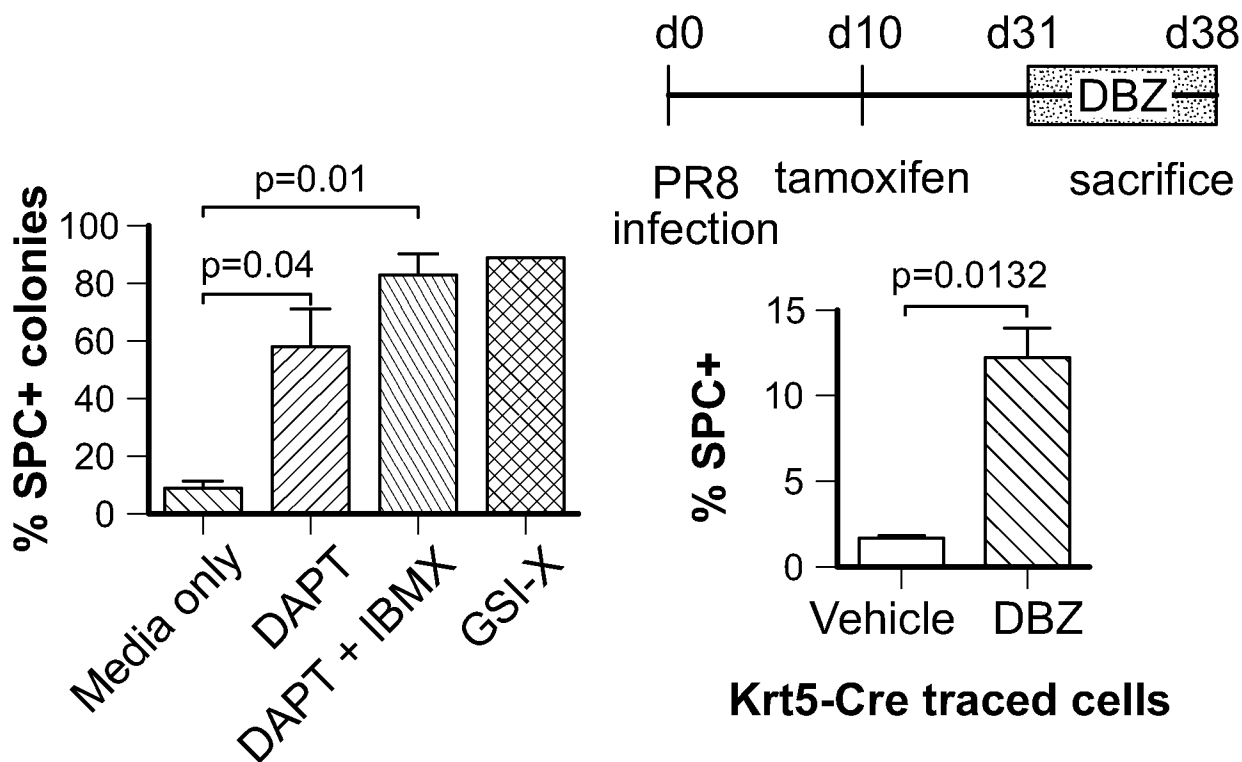


FIG. 3C

FIG. 3D

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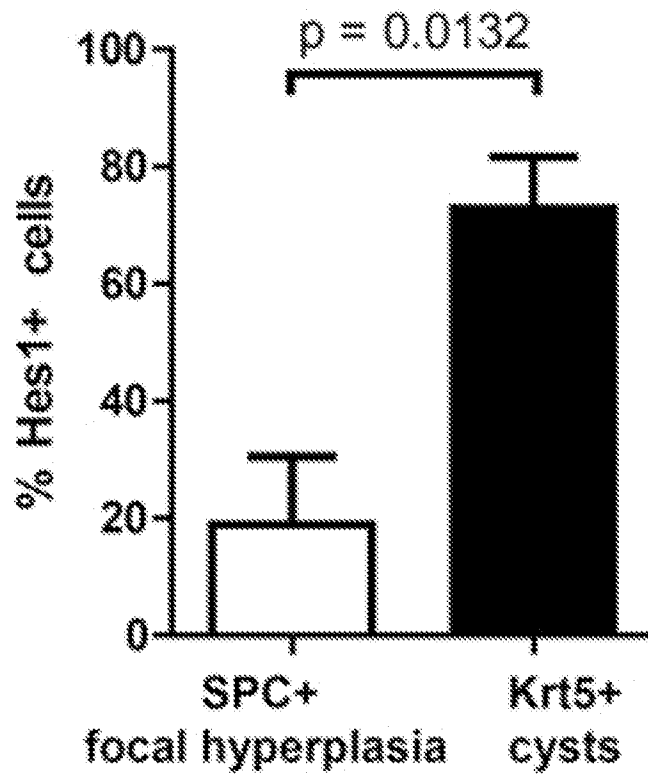


FIG. 4



FIG. 5

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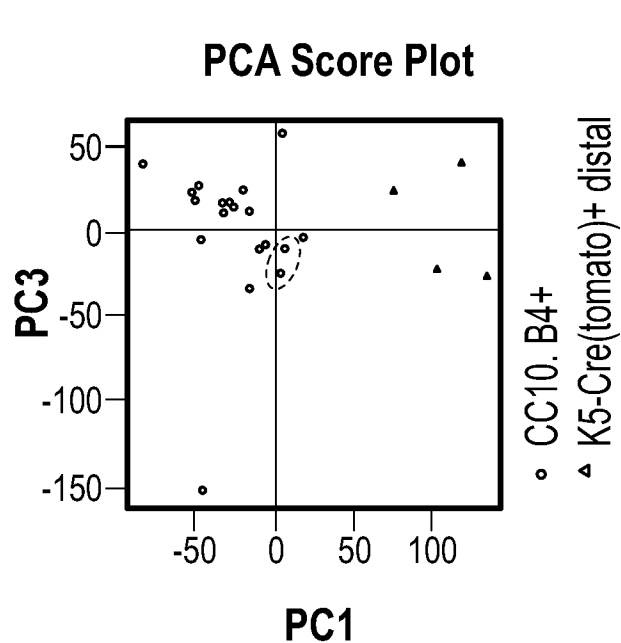


FIG. 6A

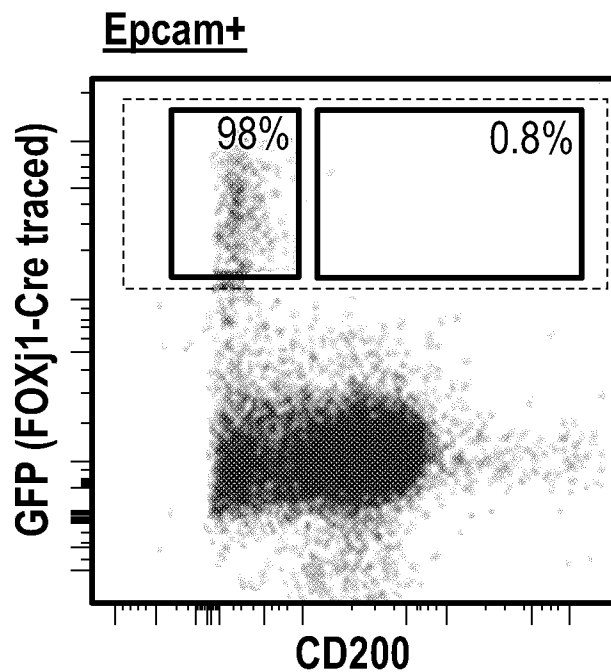


FIG. 6B

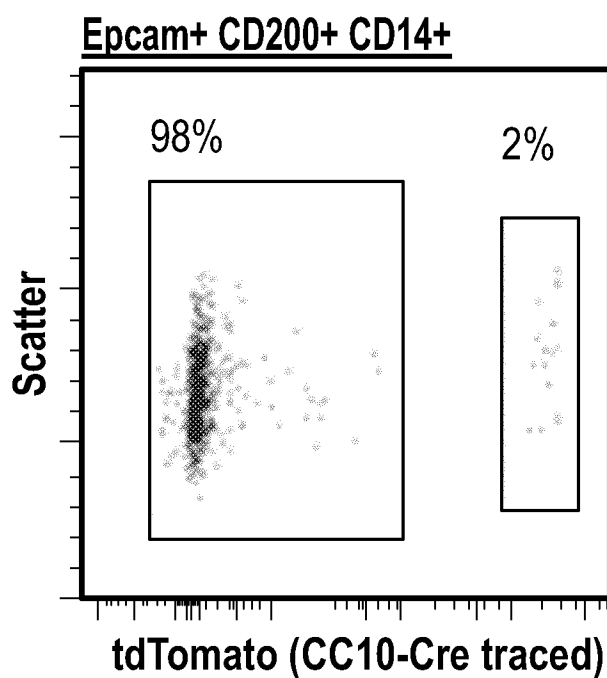


FIG. 6C

fold change vs. type II cells

	clara cells	CC10- β 4+
SPC	0.25 ± 0.1	0.023 ± 0.01
ABCA3	0.46 ± 0.06	0.05 ± 0.01
CC10	89.2 ± 25.5	7.2 ± 2.1
Acp5	0.49 ± 0.04	0.017 ± 0.01
CGRP	1.12 ± 0.12	0.19 ± 0.06
FoxJ1	85.8 ± 25.8	271 ± 73
ΔNp63	ND	detected
Hey1	66.2 ± 14.0	161 ± 82.0
Jag2	1.22 ± 0.41	4.46 ± 1.51
Sox2	481 ± 51	1444 ± 152

FIG. 6D

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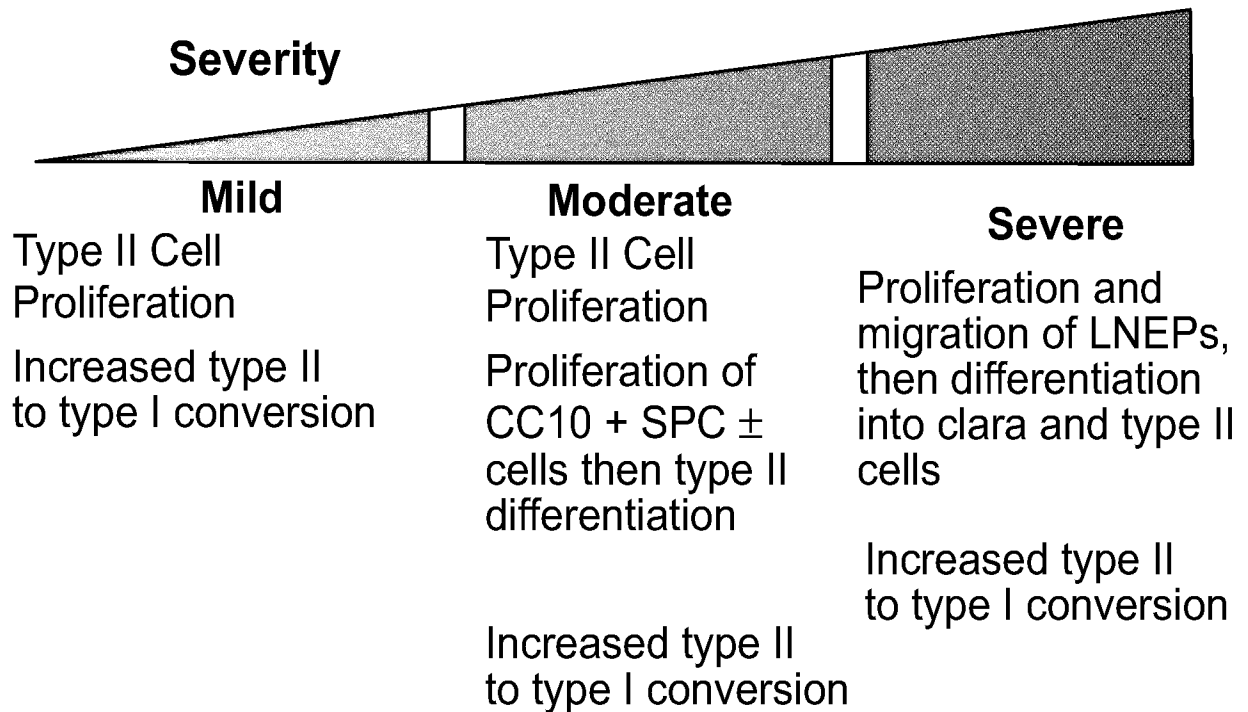


FIG. 7A

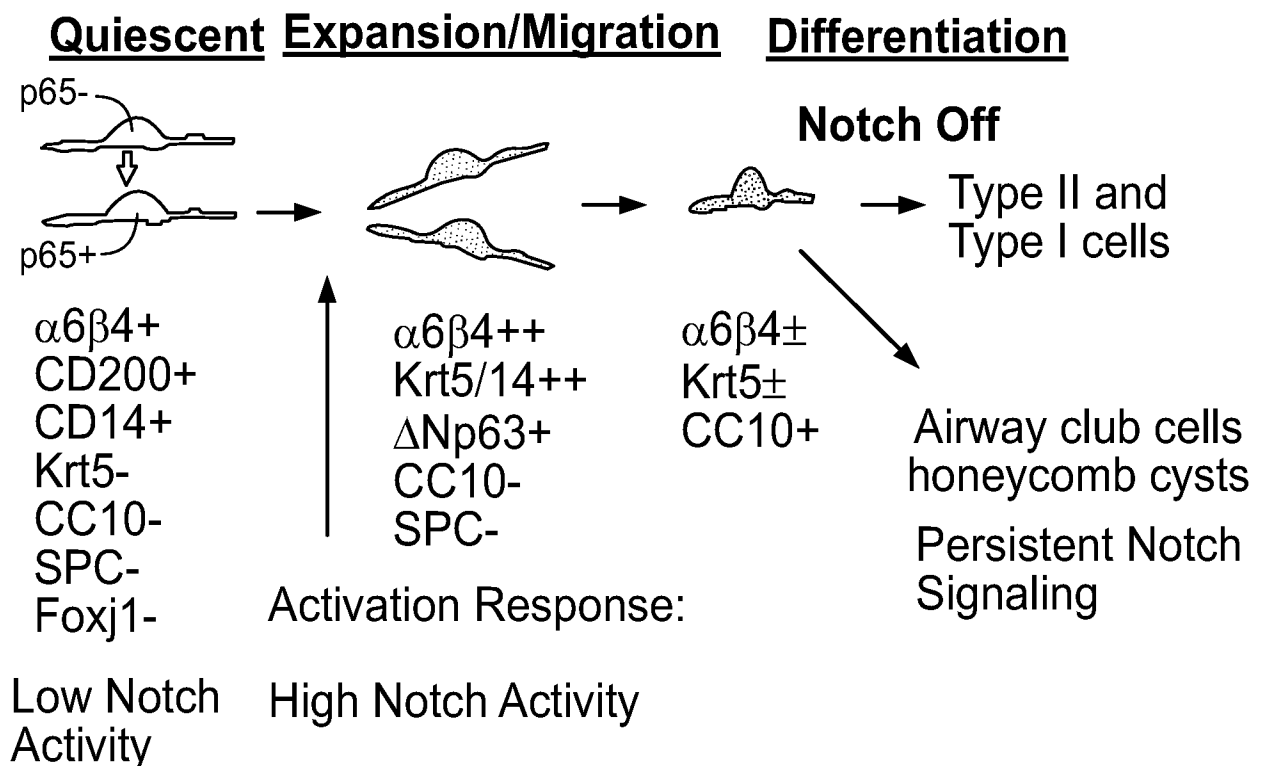


FIG. 7B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066950**A. CLASSIFICATION OF SUBJECT MATTER****C12N 5/071(2010.01)i, G01N 15/14(2006.01)i, G01N 33/569(2006.01)i, A61K 35/36(2006.01)i, A61K 39/395(2006.01)i, A61K 31/05(2006.01)i, A61K 31/12(2006.01)i, A61K 31/26(2006.01)i, A61K 31/35(2006.01)i, A61P 11/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 5/071; A61K 35/42; A61K 48/00; G01N 15/14; G01N 33/569; A61K 35/36; A61K 39/395; A61K 31/05; A61K 31/12; A61K 31/26; A61K 31/35; A61P 11/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: epithelial progenitor cells, isolation, E-cadherin, integrin beta-4, CD14, CD200, lung tissue

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007-149447 A2 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 27 December 2007 See abstract; and claims 1-25.	1-10
A	WO 2010-085842 A1 (AUSTRALIAN STEM CELL CENTRE LIMITED) 05 August 2010 See abstract; and claims 1-32.	1-10
A	SCHNIEDERMANN, JUDITH et al., 'Mouse lung contains endothelial progenitors with high capacity to form blood and lymphatic vessels', BioMed Central (BMC) Cell Biology, 2010, Vol.11, No.50 (internal pages 1-13) See the whole document.	1-10
A	DRISCOLL, BARBARA et al., 'Isolation and characterization of distal lung progenitor cells', Author Manuscript, Methods in Molecular Biology, 2012, Vol.879, pp.109-122 See the whole document.	1-10
A	WO 2013-106677 A1 (THE GENERAL HOSPITAL CORPORATION) 18 July 2013 See the whole document.	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

29 April 2016 (29.04.2016)

Date of mailing of the international search report

04 May 2016 (04.05.2016)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2015/066950

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	VAUGHAN, ANDREW E. et al., 'Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury' , Nature, 29 January 2015, Vol.517, 7536, pp.621-625 See the whole document.	8-10

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2015/066950**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 11-20
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 11-20 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2015/066950

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007-149447 A2	27/12/2007	US 2011-0061115 A1 WO 2007-149447 A3	10/03/2011 30/10/2008
WO 2010-085842 A1	05/08/2010	None	
WO 2013-106677 A1	18/07/2013	CA 2861068 A1 EP 2802647 A1 EP 2802647 A4 JP 2015-504676 A US 2014-0329318 A1	18/07/2013 19/11/2014 25/11/2015 16/02/2015 06/11/2014