Mimicking the niche of lung epithelial stem cells and characterization of several effectors of their *in vitro* behavior

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Abstract

The niche surrounding stem cells regulate their fate during homeostasis and after injury or infection. The 3D organoid assay has been widely used to study stem cells behavior based on its capacity to evaluate self-renewal, differentiation and the effect of various medium supplements, drugs and co-culture with supportive cells. We established an assay to study both lung and trachea stem cells *in vitro*. We characterized their proliferation and differentiation spectrum at baseline then evaluated the effect of co-culturing with fibroblasts and endothelial cells and/or treating with several biologically relevant substances as possible contributors to their niche. We found that lung epithelial (but not tracheal basal) stem cells require co-culture with stromal cells to undergo clonal proliferation and differentiation. Fibroblasts were more efficient than endothelial cells in offering this support and the pattern of support varied based on the tissue origin of the stromal cells. Treating distal lung epithelial or basal stem cells with FGF2, FGF9, FGF10, LIF as well as ALK5 and ROCK inhibitors increased their colony formation efficiency and resulted in variable effects on colonies number, size and differentiation spectrum. This model and findings pave the way for better understanding of lung stem cell niche components and factors that can manipulate lung stem cell behavior.
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Introduction

The ability of adult tissues and organs to maintain itself and repair after injury is dependent on the activity of tissue-resident stem cells. These tissue stem cells respond to exogenous cues from the surrounding environment, the niche, by quiescence, proliferation and/or differentiation. Understanding how this niche regulate the stem cell behavior is essential in order to be able to exploit stem cells for various therapeutic applications (Wagers, 2012). The lung is a complex organ with multiple functions and extensive branching. It is composed of three major distinct regions the tracheobronchial, bronchiolar airways and the alveolar regions. Each of these regions consists of distinct epithelial cell types with unique cellular physiologies and stem cell compartments. Several recent studies have identified and characterized the stem cells of these regions during homeostasis and after injury (Hegab et al., 2011, 2012; Rock et al., 2009; Barkauskas et al., 2013; Rawlins et al., 2009; Zheng et al., 2012; Tata et al., 2013; Giangreco et al., 2002). Cells lining the ducts of submucosal gland of the tracheobronchial airways have been shown to be responsible for maintaining and repairing submucosal glands after injury (Hegab et al., 2011, 2012). Basal cells are the stem cells of trachea as they self-renew and differentiate into club (previously called Clara) and ciliated cells (Rock et al., 2009) while Alveolar type II cells are the stem cells of the alveolar region (Barkauskas et al., 2013). Club cell is the main and well-characterized stem cell of the intrapulmonary airways and it can self-renew and differentiate into ciliated cells (Rawlins et al., 2009). Under severe injury conditions, club cells can differentiate into alveolar cells (Barkauskas et al., 2013; Zheng et al., 2012) or "de-differentiate" into basal cells (Tata et al., 2013). The presence of a subpopulation of club cells at branching points, close to neuroepithelial bodies and at terminal bronchioles close to bronchiolaveolar duct junction (BADJ) that are more resistant to injury and have more superior stem cell characteristics has been repeatedly described (Giangreco et al., 2002; Chen et al., 2012; Reynolds et al., 2000). Pulmonary neuroendocrine cells also self-renew and contribute to club and ciliated cells after injury (Reynolds et al., 2000; Song et al., 2012). However, to the contrary to several other organs like the heart (Christalla et al., 2012) and skin (Solanas and Benitah, 2013), in which extensive knowledge regarding their stem cell niche have accumulated over the past years, our knowledge regarding what niche components are influencing lung regenerative potential and what are the mechanisms regulating the various lung stem cells' differentiation and repair are lacking. A stem cell niche would usually comprise several of these components: Cellular components, which may include epithelial, endothelial, mesenchymal (especially fibroblasts) and/or hematopoietic cells. These cells interact with the stem cell through signaling components which may be paracrine (e.g. sonic hedgehog, Wnt), autocrine or juxtacrine (contact) signals. In addition, substances present in the extracellular matrix represent another component (e.g. collagen, lamenin and fibronectin) that plays an active role in the interaction with stem cells (Wagers, 2012).

The use of the extracellular matrix substitute; Matrigel, in a 3D in vitro culture system to characterize and manipulate stem cells have helped in advancing our knowledge regarding lung stem cells. Both proximal and distal lung stem cells are known to proliferate and form sphere-like colony structures when cultured in this system (Hegab et al., 2011, 2012; Rock et al., 2009; Barkauskas et al., 2013; Tata et al., 2013). It is also suitable for dissecting various potential niche components by cell co-culture and/or treatment with different biologically active substances then observing for their effect on colony's size, number, morphology, type selection and lineage differentiation.

Although the identity of lung stem cells have been clearly identified in vivo using lineage tracing and injury models (Hegab et al., 2011, 2012; Rock et al., 2009; Barkauskas et al., 2013; Rawlins et al., 2009; Zheng et al., 2012; Tata et al., 2013), apart from airway basal stem cells (ABSC), simple and reproducible methods to purely isolate individual lung stem cells are lacking. Differences and controversy regarding the methods for lung processing and markers used for their flow cytometry isolation, in addition to the variable terms and characteristics used to describe the colonies resulting from their culture have made accurate assessment of factors influencing their in vitro behaviors more complicated and challenging (Teisanu et al., 2009, 2011; McQualter et al., 2009, 2010; Raiser and Kim, 2009; Driscoll et al., 2012). On the other hand, isolation and in vitro characterization of basal ABSCs are now more established (Hegab et al., 2011, 2012, 2014; Rock et al., 2009).

In this study, we describe a simple and easily reproducible technique for lung stem cell 3D in vitro culture assay. Then, we identify the morphologicl characteristics of the different colony types growing in this assay and their differentiation spectrum at baseline culture conditions. Finally, we show the effect of manipulating several potential niche components and growth factors on lung stem cells' activation, proliferation and differentiation.

Materials and methods

Mice

8-12 weeks old wild-type C57BL/6 mice were used in all in vitro experiments (Charles River). SP-C/GFP mice (CBA/Ca x C57BL6J) were obtained from Brigid Hogan, Duke University. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Keio University.

Lung digestion and isolation of mouse lung epithelium by Fluorescence-Activated Cell Sorting (FACS)

Mice were euthanized by CO2 or ketamine—xylazine administration then abdominal aorta was exposed and exsanguinated. The thoracic cavity was opened, and the lungs were exposed. Blood was flushed out of the lung vasculature by perfusing it with 10 mL of sterile cold PBS through the right ventricle. The trachea was cannulated with 24G cannula then 1.2 mL of 10U dispase (BD) was injected into the lungs. The trachea and lungs were removed from the chest en-block and incubated for 20 minutes at room temperature (RT). Lung lobes were dissected from the trachea, heart and rest of mediastinal structures then finely minced, and incubated for more 10 minutes in extra 2 ml of dispase.

The suspension was passaged through an 18G needle 4-5 times to help open lung compartments. If sticky DNA was detected, 10-30 ul of the 4 mg/ml DNase I (Sigma) was added to the cell suspension and incubated at 37 °C for 5 minutes. Cells were filtered through a 100 micron cell strainer (BD) to obtain single-cell suspensions (SCS). Red blood cells were lysed using RBC lysing buffer. Cell were simultaneously stained with CD45 and CD31 microbeads (Miltenyi Biotec) then hematopoietic and endothelial cells were depleted on the AutoMACS system according to manufacturer's protocol. CD45/31 negative (whole lung cells; WLC) were stained with EpCAM-Alexa Fluor-647 (Biolegend) and epithelial cells were sorted on a MoFlo sorter (Beckman Coulter).

In preliminary studies, we stained the whole enzyme-digested lung cell suspension with anti-CD45, CD31 and EpCAM antibodies then we gated and sorted the EpCAM positive epithelial cells, excluding the hematopoietic and endothelial cells (Fig. S1). However, because of the large number of these cells within the lung, we found that their initial depletion step using MACS beads significantly shortens the sort time.

Isolation of fibroblasts and endothelial cells

Lung fibroblasts were obtained by culturing the CD45-/CD31-/EpCAM- cells sorted from WLCs on uncoated plastic plates for 1-2 hours then medium was changed to remove unattached cells. Fibroblasts were allowed to grow to confluence then were either passaged, frozen or used as feeder cells. Feeder cells were prepared by treating cultured fibroblasts with 10 μg/ml Mitomycin-C (Sigma) for 2 hours. For liver endothelial cells, liver is minced and digested by collagenase/dispase mixture for 20 minutes. Endothelial cells were isolated from lung or liver SCSs through positive selection with CD31 microbeads. Purity of the selected populations was confirmed by FACS analysis (Fig. S1F). To avoid selection of a subpopulation or change in their characteristics, fibroblasts were used for co-culture experiments at passage 1 and primary endothelial cells were isolated and used immediately. Phenotypic characterization of the fibroblasts used was performed by staining for Sca-1, CD166 and alpha smooth muscle Actin (aSMA) (Fig. S2).

In vitro 3D organoid culture, treatments and quantification of colonies

Lung epithelial cells alone or mixed with feeder cells in 1:1 ratio were resuspended in MTEC/Plus medium (You et al., 2002), and mixed 2:1 with growth factor-reduced matrigel (BD) and 150 ul were placed in 0.4 μm transwell inserts (Corning) in 24-well plates. 600 ul MTEC/Plus medium was supplied to the lower chamber and medium was changed every other day. Descriptions of medium constituents, incubator conditions and preliminary experiments to decide on seeding densities are provided in the Supplementary materials and Methods. 100 ng/ml recombinant fibroblasts growth factor (FGF)-9 (peprotech and R&D), FGF10 (R&D) or FGF2, 10 uM Rho-associated protein kinase inhibitor (ROCK-I) Y-27632, transforming growth factor-beta superfamily type I activin receptor-like kinase-5 inhibitor (Alk5-I) SB-431542 or 10 ng/ml Leukemia Inhibitory Factor (LIF) (Life Technologies) or FGF

Receptor (FGFR) inhibitor PD173074 (Merck KGaA) were supplied in the lower chamber medium. The numbers of colonies per insert were counted on day 7, 14 or 21. Both fluorescent and phase contrast images were taken on Leica DMi6000B microscope. Quantification of colony numbers was done by direct visual counting under the microscope and from digital images of all colonies in each well. Colonies diameters were measured based on the images scale bar. For irregular-shaped colonies, the longest distance between 2 outer points on each colony was measured.

Isolation of ABSCs and trachea fibroblasts

Isolation of ABSCs was performed as described previously (Hegab et al., 2011, 2014). In brief, tracheas were dissected and cleaned from all non-tracheal tissues under dissecting microscope. Tracheas were longitudinally cut open and incubated in dispase for 30 minutes. Mouse tracheal epithelial cells (MTEC) were peeled off with fine forceps and trypsinized into SCS. MTEC were either cultured as such or further stained with ITGA6 (Abcam and Biolegend), NGFR (Abcam) or CD44 (Biolegend) and Trop2 (R&D) or EpCAM then basal cells were FACS sorted (Fig. S3). The remaining tracheal stroma after epithelial stripping were minced then cultured on plastic plate for 10 days until fibroblasts formed colonies out of the tracheal pieces. The plate was trypsinized and contents were sieved then passaged once to expand.

Collection of the matrigel 3D colonies and processing for histological examination

200ul of HistoGel (Thermo Scientific), that was liquefied by warming at 50°C; were added into a cryo-mold then the membrane of the matrigel/colonies-bearing transwell was cut from below with a scalpel. The matrigel/colonies disc was pushed from above into the HistoGel mold. Additional 200ul Histogel were added to completely cover the matrigel/colonies disc then was allowed to cool down to RT. The now-hardened histogel block was removed from the mold, immersed in 70% ethanol then embedded in paraffin. Paraffin-embedded gel/colonies were cut into 6-um sections and stained for H&E and microscopically examined (Olympus BX53).

Histology, Immunofluorescence and quantification of cellular markers expression

Paraffin sections from all cultures were stained for panel of antibodies in different combinations to confirm identities of different cells. Deparaffinized slides were immersed in citrate buffer then placed in a water bath (95−99°C) for 20 min then allowed to cool down to RT. Slides were then incubated in PBS with Triton x 0.25% for 10 minutes then blocked with serum-free protein block (Dako). Primary antibodies were diluted in protein block and incubated overnight at 4 °C. The primary antibodies used were rabbit cytokeratin-5 (K5) (Covance), goat Sftpc and CC10, mouse PCNA (Santa Cruz), rabbit Sftpc (Millipore), rabbit aquaporin-5 (AQP5) (Abcam), mouse E-cadherin (BD), rabbit lysozyme (Novus Biologicals), mouse MUC5AC (Thermo Scientific),

rabbit calcitonin gene-related peptide (CGRP) and mouse acetylated α-tubulin (Sigma). The appropriate Alexa-Fluor-coupled secondary antibodies (Invitrogen) were used in double and triple staining sections. Sections were counterstained with DAPI (Vector labs) and analyzed (Zeiss AxioImager). Quantifications of cellular markers expression were performed by direct visual counting of positively stained cells per total number of DAPI-stained nuclei. At least 20 colonies were examined per slide from 3 replicates of at least 3 independently reproduced experiments. Range of percentages, rather than an average and standard deviation of marker-expressing cells within a colony type are shown to affirm the variability of percentages of cell types from colony to colony in the same well and across the replicates.

Statistics

All culture wells were in triplicates and every treatment was run on at least three independent occasions. Numbers of colonies counted visually from the bright-field microscope images from the triplicate wells were averaged. Then average and standard deviation of the three (or more) independent experiments were used to construct the shown bar graphs. Similarly, average of all measured diameters of each colony type per triplicate per experiment were used in the graphs. Significance was evaluated by Student's t-test with P<0.05 considered statistically significant.

Results

Physical contact with fibroblasts is essential for distal lung stem cells proliferation and differentiation

We first wanted to establish a simple and easily reproducible assay to study lung stem cells behavior and niche interactions *in vitro*. Pervious work revealed that distal lung epithelial stem cells require co-culture with fibroblasts to be able to proliferate and form clonal spheres *in vitro* (Barkauskas et al., 2013; Chen et al., 2012; McQualter et al., 2010; Teisanu et al., 2011). In these studies, autologous lung EpCAM-/Sca-1+ cells (McQualter et al., 2010), neonatal mouse lung fibroblast cell line; MLg (Chen et al., 2012; Teisanu et al., 2011) or lung lipofibroblasts enriched from *Pdgfra-H2B/GFP* transgenic mice (Barkauskas et al., 2013) were used. Seeding densities in these studies ranged from 3000-10,000 epithelial cells co-cultured with 100,000–200,000 fibroblasts per transwell of the 24-well plate (approximately 1:20 ratio). However, because fibroblasts from different sources as well as subsets of fibroblasts from a single tissue may show different phenotypes (Rinn et al., 2006), we elected to use adult mouse lung whole fibroblasts to become more physiologically relevant and to facilitate future reproducibility and follow up studies. To identify the optimum ratio for co-culture with lung epithelial cells, we co-cultured fixed number of EpCAM+ lung epithelial cells with an increasing numbers of fibroblasts. We found that small epithelial colonies could be observed at 10:1 ratio. A 1:1 ratio gave the highest colony formation efficiency (CFE) (Fig. 1A-C). 1:5 or 1:10 ratios did not cause further increase in colony formation efficiency but resulted in earlier

development of apoptotic changes due to overcrowding. We also found that prior treatment of the fibroblasts with mitomycin-C to render them mitotically inactive allowed keeping the culture for longer observation periods without impairing fibroblasts' ability to support colony formation. Finally, we characterized the used fibroblasts to identify its basic phenotype and confirm the absence of major shift in it during brief expansion in culture (Fig. S3). Lung fibroblasts at 1 week after plating expressed 95+/-2% Sca-1, 1-2% aSMA and 8+/-3% CD166. Passage 1 fibroblasts expressed 80+/-5% Sca-1, 4-6% aSMA and 25+/-5% CD166.

Under these culture conditions, lung epithelial cells yielded three morphologically distinct colony types (Fig. 1D) that were similar to those described before (Chen et al., 2012). We established these morphological characteristics for identification of the different colony types: Type A is big (>100 um diameter at 2-weeks), rounded shape with big lumen and thin wall (1-3 layers). Type B is big (>100 um), irregular shape (locular, pudding, partially rounded or a combination of these shapes) with big or small lumen(s) but thick wall (2-10 layers). Type C is small (<100 um), rounded or oval shape with no or small lumen.

To determine the differentiation potential of each colony type, we stained sections from culture wells harvested at 2-3 weeks for K5, CC10, MUC5AC, lysozyme, CGRP, acetylated α-Tubulin, AQP5 and Sftpc (markers for basal, club, goblet, serous, neuroendocrine, ciliated, AT-I and AT-II cells). The presence of a large lumen in type A colonies reflects the presence of secretory cells. Indeed, all type A colonies expressed one or more of the 3 markers of airway secretory cells; CC10, MUC5AC and lysozymes, in 20-70% of cells. 20-40% of cells were ciliated cells. 5-20% of cells expressed the basal cell marker K5. 5-15% of cells expressed Sftpc indicating alveolar cell differentiation. Type B colonies, on the other hand, although had differentiation spectrum similar to Type A, percentages of cellular subtypes were different. Type B colonies mainly expressed alveolar cell markers in 60-80% of cells while expression of secretory and ciliated cell markers was at 20-30%. K5 expression was less than 5%. Interestingly, 10-15% of cells expressed CGRP indicating the presence of neuroendocrine-like cells. Type C colonies expressed Sftpc and AQP5 in 100% of cells suggesting that they originate from alveolar type II progenitor/stem cells and they have limited differentiation potential under these culture conditions (Fig. 1-E).

To confirm that type C colonies originate from alveolar cells, we used lung epithelial cells from the *SP-C/GFP* transgenic reporter line. In this mouse, GFP is brightly expressed in all alveolar type II cells and dimly expressed in few cells at bronchioalveolar duct junction or in terminal bronchioles just proximal to it (Fig. S4). When lung epithelial cells from this mouse were co-cultured with wt fibroblasts, only Type C colonies were GFP+ while all type A and B colonies were GFP- (Fig. 1-F and F′).

On the other hand, type A colonies express the basal cell marker K5 in 5-20% of cells and are morphologically similar to the previously-described ABSC spheres (tracheo-spheres) (Hegab et al., 2011; Rock et al., 2009). To confirm that Type A colonies did not originate from a putative intrapulmonary basal cell, we compared ABSC spheres grown from MTEC with Type A colonies and found these exclusive differences. ABSC spheres expresses K5 in all basally-positioned cells of all spheres. They never differentiate into Sftpc+ cells and
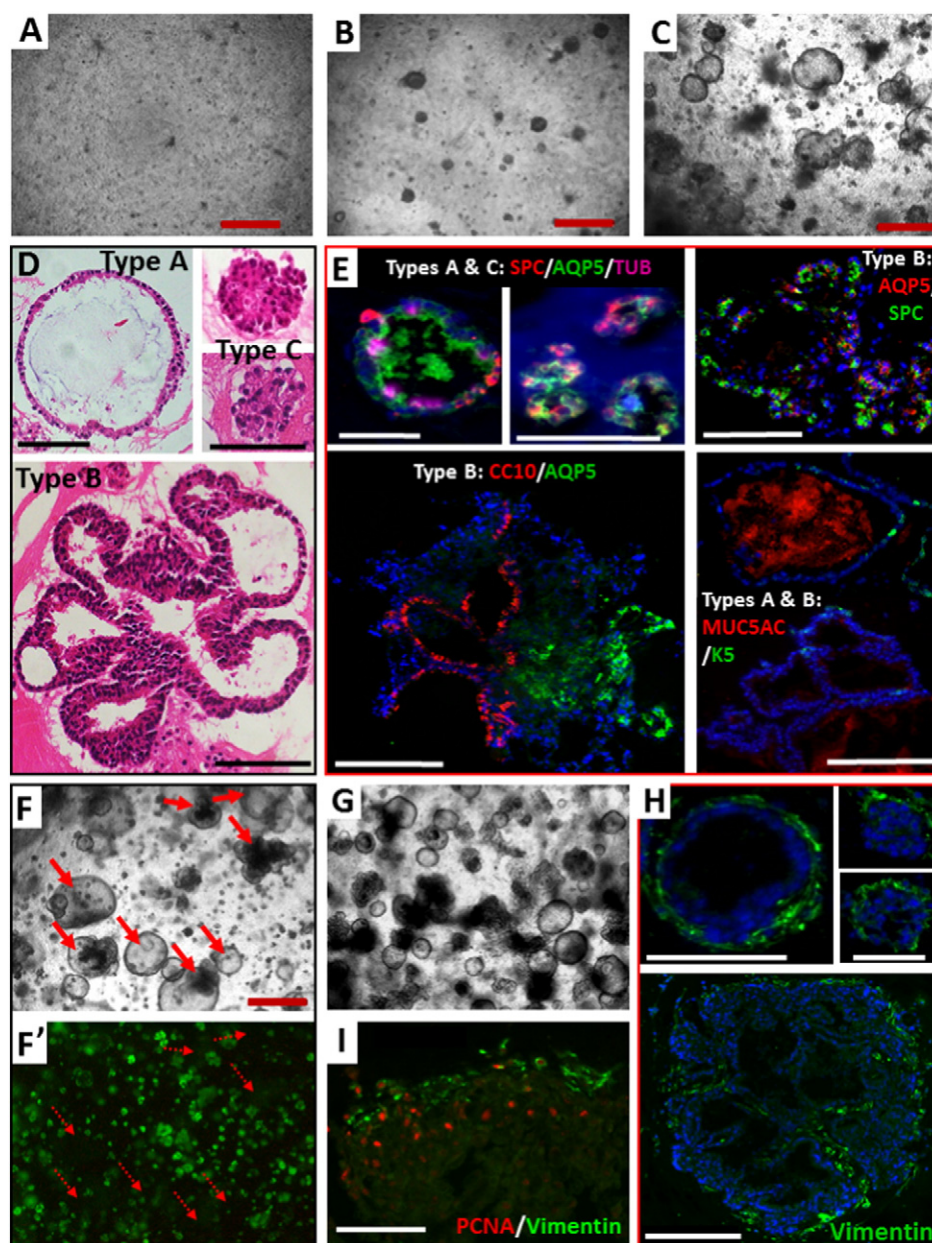
Figure 1 Establishing the baseline culture conditions for an assay to study lung stem cells behavior and niche interactions *in vitro*. 50,000 lung epithelial cells were cultured in the *in vitro* 3D-organoid/sphere culture system alone (A), or were co-cultured with 10:1 (B) or 1:1 (C) mouse lung fibroblasts and observed for 2 weeks. (D) Colonies growing in the 1:1 co-culture wells consistently revealed three different morphologies, named types A, B and C. (E) Immunofluorescent staining of the three colony types for the different lung epithelial cell markers revealed their differentiation spectrum. (F and F′) Lung epithelial cells from *SP-C/GFP* transgenic mice were co-cultured with wt lung fibroblasts, only Type C colonies were GFP+ while all type A and B colonies were GFP− (red arrows in (F) point to some Type A and B colonies. Dotted arrows in (F′) point to position of same colonies showing they are GFP−). (G) Lung epithelial cells were co-cultured with tracheal fibroblasts. This favored the growth of more type A colonies than types B and C; compared to lung fibroblasts co-culture. (H) Staining of the three colony types for the mesenchymal cell marker Vimentin showed that fibroblasts wrap themselves tightly around the 3 different colony types. (I) Staining for vimentin and PCNA showed that epithelial cells next to the fibroblasts wrapping are proliferating while more central cells are not. Scale bars: (A-C, F, G) 500 μm; all others are 50μm. See also supplemental Fig. S4.

are not dependent on fibroblasts co-culture for their growth (see below). Furthermore, we confirmed by immunostaining the absence of K5+ basal cells in mice intra-pulmonary bronchi and in cytospin preparations from the lung epithelium single cells (data not shown). Thus, the presence of K5+ basal-like cells in Type A&B colonies is most probably through (de-)differentiation of an airway (secretory) stem/progenitor cells, as recently described (Tata et al., 2013).

We speculate that Type A colonies are more likely to be originating from more proximal airways with more secretory

phenotype (single big lumen) and ability to (de-)differentiate into basal cells, while Type B colonies originate from more distal airways with a secretory and tendency-to-branching phenotype; and ability to differentiate into alveolar cells.

Indeed, co-culture with fibroblasts isolated from mouse trachea resulted in more type A colonies than types B and C compared to the lung fibroblasts co-culture (Fig. 1-G). Furthermore, analysis of the tracheal fibroblast co-culture showed a change in differentiation pattern within Type A and B colonies with cells positive for basal, secretory and ciliated cell markers increasing and cells with alveolar markers decreasing compared to co-culture with lung fibroblasts (data not shown).

To identify the spatial relationship between fibroblasts and the different colony types, culture sections were stained for the mesenchymal cell marker vimentin. We found that fibroblasts wrapped tightly around the 3 different colony types (Fig. 1-H). To identify if the role of fibroblasts is due to direct cell-cell contact or through secretion of paracrine signaling factors, we seeded equal numbers of cells either in direct co-cultures, indirect; where fibroblasts were seeded in the lower chamber below the transwell or with fibroblasts-conditioned medium (FCM). No epithelial colonies formed with indirect co-culture or FCM confirming the essentialness of -close proximity to fibroblasts- for lung epithelial stem/progenitor cells colony formation. Furthermore, co-staining for vimentin and proliferating cell nuclear antigen (PCNA) revealed that only cells in close proximity to the fibroblasts are proliferating while cells towards the center of the colony were not (Fig. 1-I).

As we have now established and characterized baseline culture conditions for an *in vitro* assay to study lung stem/progenitor cells behavior and niche interactions, we began to study the effect of manipulating several biologically relevant growth factors and cytokines on colony type, number, size and differentiation potential. As lung epithelium turnover is known to be very slow, it is inferred that most lung epithelial stem cells are in a state of quiescence and thus have very low CFEs *in vitro*. Accordingly, an increase in CFE in response to a treatment or co-culture is indicative of an activation of quiescent stem cells. Conversely, a decrease in CFE would mean a shift towards quiescence or an injury of the active stem cells. In addition, compared to the naïve baseline control, bigger size of colonies with colony wall thickening will mean an induction of the rate of proliferation of the active stem cell; while bigger sizes with thinning of the colony wall will mean an increase in the rate of secretion and/or number of differentiated secretory cells. On the other hand, smaller colonies is interpreted as slowing in the stem cell proliferation and differentiation.

Manipulation of FGFs and FGFRs can modulate lung stem/progenitor cells activation and differentiation

During embryonic development, several FGFs and their receptors; FGFRs are expressed in the developing lung. They play an important role in guiding lung branching morphogenesis and maturation. They are expressed in the epithelium, mesenchyme or both (Ornitz and Yin, 2012). To determine if FGFs are involved in the interaction between adult lung epithelial stem/progenitor cells and fibroblasts, and whether FGFs can replace the requirement for fibroblasts support of epithelial colony formation, lung epithelial cells were treated with recombinant FGF2, FGF9 or FGF10

with and without fibroblasts co-cultures. Treating lung epithelial cells with FGFs failed to support colony formation in the absence of fibroblast co-culture (Fig. 2A). On the other hand, treating the co-culture wells with FGFs significantly increased the number and size of all colony types (Fig. 2B-E, J-K). To further confirm the involvement of FGFs and/or FGFRs, FGFs-treated co-cultures were treated with the FGFR blocker PD173074. Blocking FGFRs abolished the inducing effect of FGF10 and FGF2; but not FGF9 on colonies' size and number (Fig. 2F-H, J-K). Treating control wells with FGFR blocker resulted in marked inhibition of colony formation (Fig. 2I-K). Notably, neither FGFs nor FGFR blocker treatments influenced the tight wrapping of fibroblasts around the epithelial colonies (Fig. S5). Analysis of the differentiation spectrum of FGFs-treated colonies revealed that FGF treatment caused near-complete disappearance of the basal cell marker K5 and an increase in the alveolar cell marker; Sftpc expressions in type A and B colonies. Type C colonies grew bigger and developed lumens. Conversely, the few small colonies that grew in FGFR blocker-treated culture well expressed less Sftpc but more K5 and MUC5AC in colony types A and B. Type C colonies were few and small (Fig. S5). These data indicate that FGFs can activate quiescent lung stem cells, enhance their proliferation and promote their differentiation towards alveolar lineage.

Effect of LIF, ALK5 and ROCK inhibitors on lung stem/progenitor cells activation and differentiation

Then, we studied several biologically relevant factors as potential contributors to lung stem cell niche. The dependence of lung stem/progenitor cells on fibroblasts is similar to the previously described dependence of mouse embryonic stem cells (ESCs) on fibroblast feeder cells. Later on, it was identified that a single cytokine, LIF, could maintain mESCs' self-renewal and pluripotency in the absence of fibroblast feeder cells (Williams et al., 1988). Also, signaling via *Alk5* is known to control club cell differentiation (Xing et al., 2010) and ALK5 inhibitor has been used previously to enhance colony-forming efficiency (Chen et al., 2012; Teisanu et al., 2011). Additionally, the ROCK family of G proteins are known to regulate cell migration, proliferation and actin cytoskeleton (Hall, 1988). ROCK inhibitor have been shown to inhibit apoptosis and thus have been widely used to enhance ESC survival *in vitro* (Watanabe et al., 2007). Furthermore, combining ROCK inhibitor with fibroblast feeder cells in epithelial cell culture resulted in unlimited propagation of epithelial cells from various organs including lung epithelium, both human and mice (Liu et al., 2012; Horani et al., 2013) although one study described ROCK-I to have no effect on distal lung colony formation (Teisanu et al., 2011).

Treating lung epithelial cells with LIF, ALK5-I or ROCK-I without fibroblasts co-culture failed to support colony formation (Fig. 3A). However, applying them to the co-culture wells significantly increased the number and size of colonies compared to the co-culture alone. Interestingly, each of these factors caused variable effects on colony types and differentiation spectrum. LIF increased all the 3 colony types with most of the A and B colonies shifting from Sftpc-expressing towards K5-expressing cells. ALK5-I favored the growth of types A and B colonies and caused them to express 60-90% Sftpc with
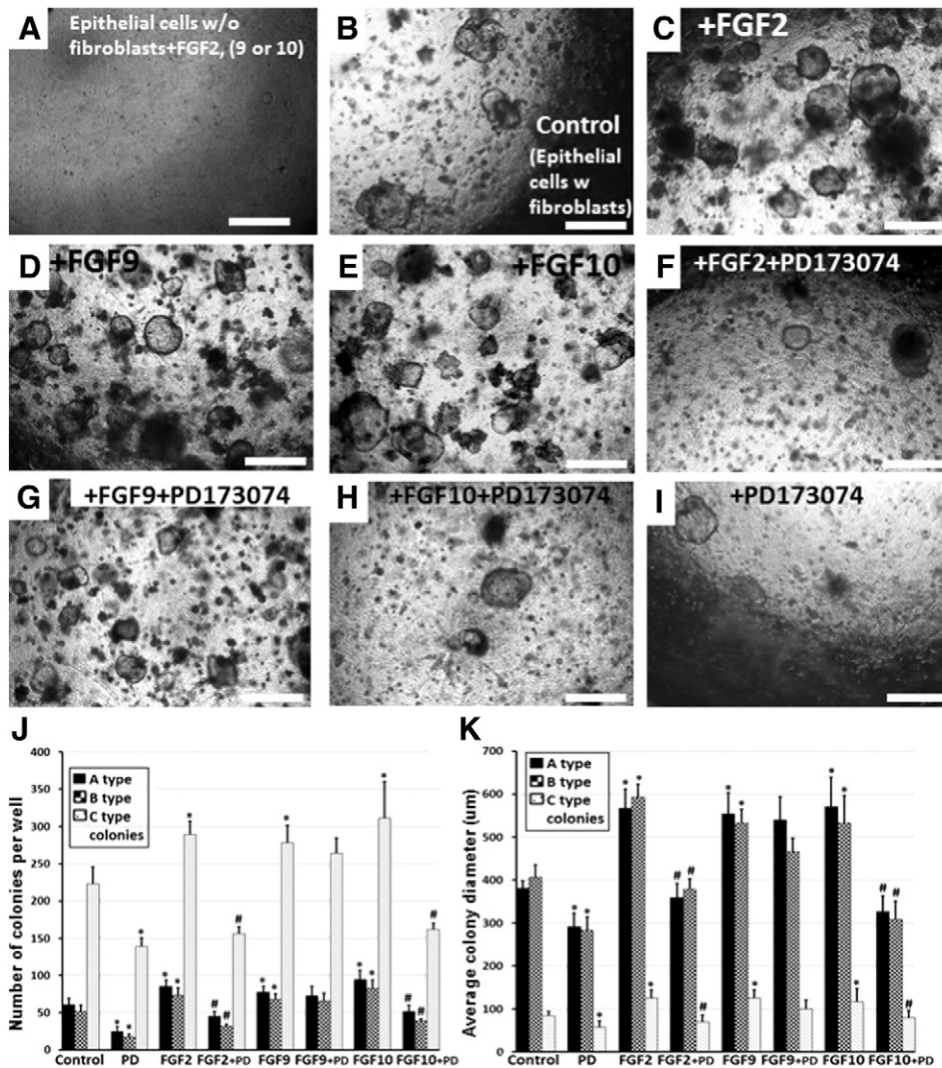
Figure 2 Effect of FGFs and FGFR blocker on lung stem/progenitor cells. 50,000 lung epithelial cells without fibroblast co-culture were treated with FGF2, FGF9 or FGF10. No colonies grew. (A) Shows a representative image of culture wells at 2 weeks. Lung epithelial cells co-cultured with fibroblasts were treated with FGF2 (C), FGF9 (D) or FGF10 (E). All treatments caused significant increase in number and size of all colony types compared to vehicle treated control (B). Similar co-culture wells were co-treated with FGFs and FGFRs blocker; PD173074. Blocking FGFRs resulted in smaller and fewer colonies in FGF2 (F) and FGF10 (H) co-treatment; but not with FGF9 (G). (I) Treating lung epithelial/fibroblast co-culture with FGFR blocker markedly reduced colony formation. (J, K) Quantification for number and average diameter of all colonies from 3 independent experiments. (*$p<0.05$ compared to control, #$p<0.05$ compared to FGF-treated parent). Scale bars: 500 μm. See also supplemental Fig. S5.

near-complete disappearance of K5-expressing cells. While ROCK-I obviously markedly induced the growth of type A colonies and caused marked increase in K5-expressing cells within them leaving very few colonies expressing Sftpc (Fig. 3B-F and S6). None of the treatments abolished the tight wrapping of fibroblasts around the epithelial colonies (not shown).

Endothelial cells are less efficient than fibroblasts in supporting distal lung stem cells proliferation and differentiation

Endothelial cells have been shown recently to be capable of supporting a putative lung stem cell population defined by

expression of EpCAM and Sca1 (Lee et al., 2014). Sca1 is known to be expressed on lung mesenchymal cells; mainly fibroblasts as well as on distal airway epithelial cells (Teisanu et al., 2009; McQualter et al., 2009). We examined the effect of endothelial cell (isolated from mouse lung or liver) co-cultures with lung epithelium in our *in vitro* assay in comparison to fibroblasts. Morphologically, type C colonies were rarely seen with endothelial cells co-culture indicating that endothelial cells are less efficient than fibroblasts in supporting lung alveolar progenitors. On the other hand, both types of endothelial cells could support the growth of type A and B colonies and their size and complexity were more enhanced with liver than lung endothelial cells (Fig. 3G-H). On analysis of their differentiation spectrum, type A and B colonies from both liver and lung endothelial
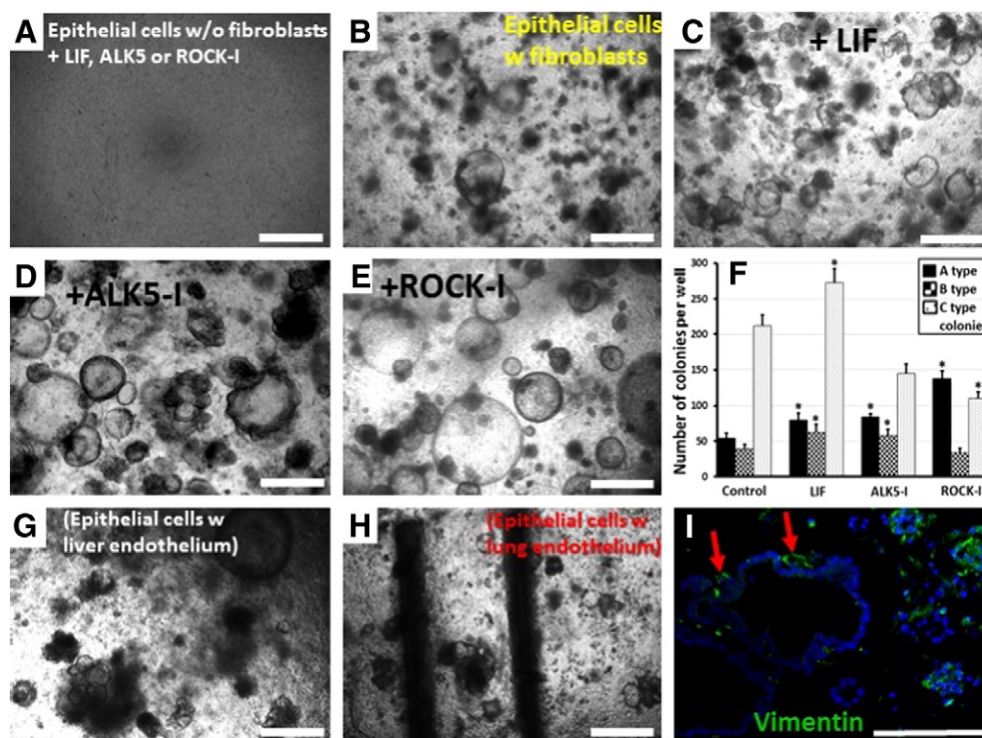
Figure 3 Effect of LIF, ALK5-I, ROCK-I or endothelial cells on lung stem/progenitor cells. 50,000 lung epithelial cells without fibroblast co-culture were treated with LIF, ALK5-I or ROCK-I. No colonies grew. (A) Shows a representative image of culture wells at 2 weeks. Lung epithelial cells co-cultured with fibroblasts were treated with LIF (C), ALK5-I (D) or ROCK-I (E). All treatments caused significant increase in colonies' number and size compared to untreated control (B). (F) Quantification for number of colony types from 3 independent experiments. *p<0.05 compared to control. Lung epithelial cells were co-cultured with liver (G) or lung (H) endothelial cells. Endothelial cells could support type A and B colonies but not type C compared to fibroblasts (B). Epithelial colonies in liver endothelium co-culture showed enhanced size and complexity compared to lung endothelium. (I) Vimentin staining showed that few endothelial cells came in close proximity with the epithelial colonies (arrows) but did not wrap around them. Many endothelial cells formed clusters nearby. Scale bars: A–E, G, H: 500 μm, (I) 50 μm. See also supplemental Fig. S6.

cell co-cultures showed limited differentiation spectrum compared to fibroblast co-culture. They extensively expressed the secretory cell markers MUC5AC and CC10. They showed reduced expression of tubulin and K5 while Sftpc was completely absent (data not shown). Some endothelial cells were seen in close proximity to epithelial colonies while others formed clusters in between, but the tight wrapping seen with fibroblasts was not detected (Fig. 3I). Taken together, these data suggest that endothelial cells have the ability (though a limited one) to support airway epithelial stem/progenitor cells self-renewal and differentiation into airway lineages but lacks the fibroblasts ability to induce alveolar differentiation.

Fibroblasts and endothelial cells are not required but can influence ABSCs' proliferation and differentiation *in vitro*

The 3D *in vitro* colony/sphere formation assay for mouse ABSCs has been established (Hegab et al., 2011; Rock et al., 2009) and already used to study factors influencing basal cells' niche and interactions with common environmental hazards like aldehydes and reactive oxygen species (Hegab et al., 2014; Paul et al., 2014). Contrary to the distal lung stem/progenitor cells, ABSC can self-renew and form spheres in 3D

culture without fibroblasts co-culture (Hegab et al., 2011; Rock et al., 2009). However, the culture medium used in these studies (MTEC/plus) is supplemented with Epidermal Growth Factor (EGF) and bovine pituitary extract (BPE) which may have replaced the role played by fibroblast co-culture. BPE itself is known to contain anti oxidants, growth hormones and growth factors that include FGF2 and other tyrosine kinase inhibitor-sensitive factors (Kent and Bomser, 2003). Therefore, we first studied the essentialness of EGF and PBE for ABSC sphere formation. We found that removal of EGF, BPE or both from the medium did not cause any noticeable effect on sphere number or size (Fig. 4A-D). Then we examined the effect of co-culture with endothelial cells and fibroblasts. Even though ABSC does not require co-culture with stromal cells to form clonal spheres, co-culturing them with endothelial cells or fibroblasts resulted in marked induction of their CFE, more with fibroblasts. (Fig. 4E-H). To the contrary of lung stem cells, co-culturing ABSC with lung endothelial cells resulted in higher CFE than liver endothelial cells co-culture. Furthermore, immunostaining for various cellular markers showed that co-culture with fibroblasts resulted in more efficient differentiation while endothelial cells caused spheres with thinner walls and less differentiated cells (Fig. S7). Notably, neither fibroblasts nor endothelial cells wrapped around the spheres (Not shown).

FGFs induce ABSCs proliferation more than differentiation, more with FGF9

We also examined the effect of FGFs on ABSCs. Treating ABSCs with FGFs not only increased number of spheres but also their size. This indicates that FGFs activated more dormant ABSCs to form colonies (thus increasing number of spheres) and also increased their rate of proliferation and differentiation towards secretory lineages (thus increasing size). We noticed that 20-40% of spheres are having thicker
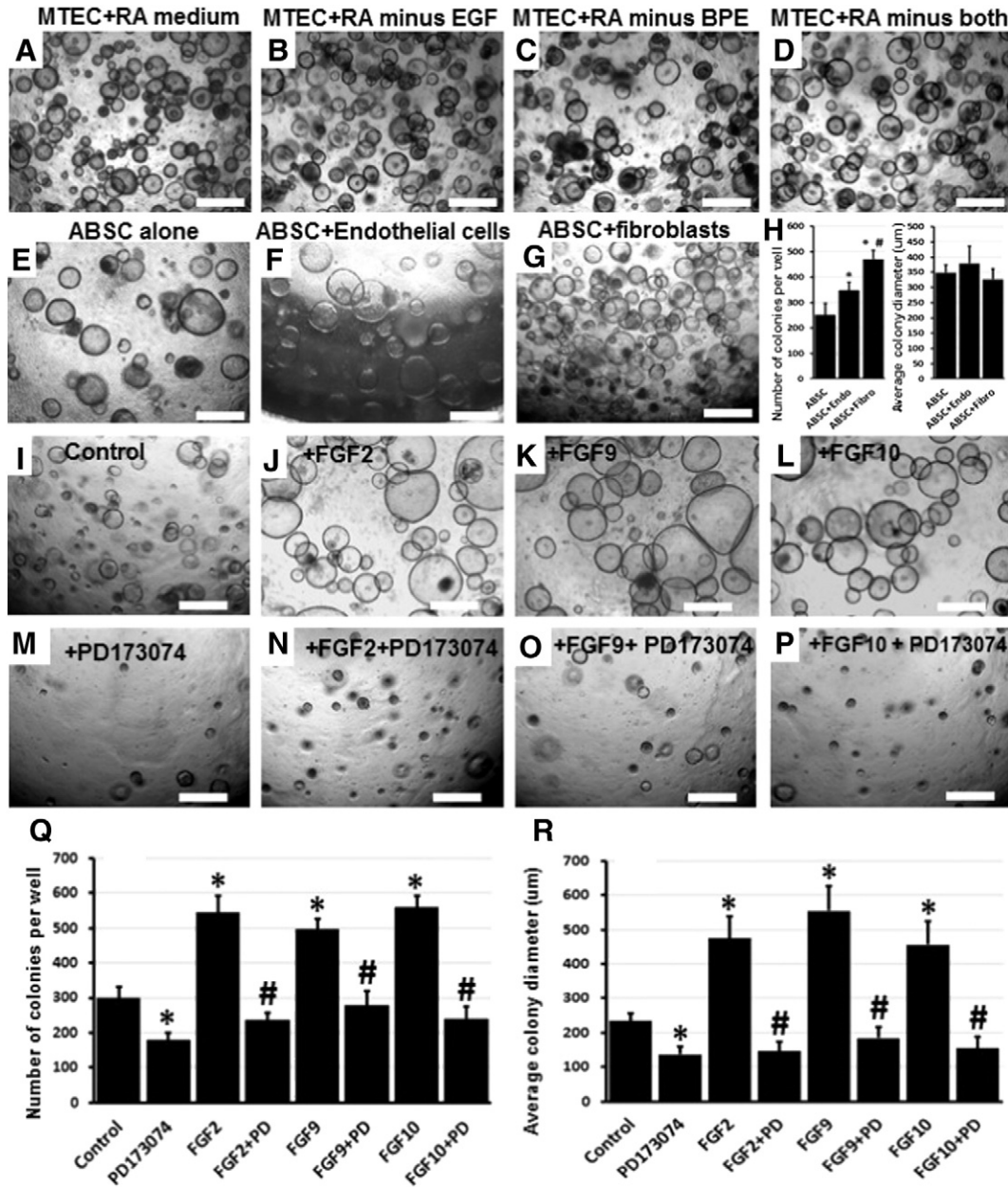

Figure 4   Characterizing the effect of medium supplements, endothelial cells and fibroblasts co-culture, FGFs and FGFR blocker on ABSCs *in vitro*. -80,000/well ABSCs were cultured in the *in vitro* 3D-organoid/sphere culture system using the standard MTEC/plus medium (A), or using MTEC/plus medium without EGF (B), PBE (C) or both supplementations (D). No difference in sphere number or size was observed. 50,000/well ABSCs were cultured in MTEC/plus medium alone (E), or with 1:1 endothelial cells (F) or fibroblast co-culture (G). Both co-cultures induced CFE with fibroblasts inducing higher efficiency. (H) Quantification for number and average diameter of all colonies from 3 independent experiments. (*p<0.05 compared to control). 50,000/well ABSCs were treated with FGF2, FGF9 or FGF10 alone (J-L) or with the FGFRs blocker; PD173074 alone (M) or in co-treatment with FGFs (N-P). All FGF treatments caused induction of both spheres number and size compared to vehicle-treated control (I). Treating ABSCs with FGFR blocker alone markedly reduced colony formation and co-treatment abolished the FGF-induced increase in spheres' number and size. (Q, R) Quantification for number and average diameter of all colonies from 3 independent experiments. (*p<0.05 compared to control, #p<0.05 compared to FGF-treated parent). Scale bars: 500 μm. See also supplemental Figs. S7 and S8.

walls (3-7 layers of nuclei) that mainly consisted of K5+ basal cells, fewer differentiated/non-basal cells and smaller lumens; specially with FGF9 indicating that FGFs might be "uncoupling" proliferation of stem cells from differentiation thus causing "basal cell hyperplasia". FGFR blocker reduced both number and size of untreated spheres, and abolished the FGFs-inducing effect on sphere numbers indicating the involvement of signaling through the FGF receptors in the baseline and FGF-induced sphere formation (Fig. 4I-R and S8).

Effect of LIF, ALK5 and ROCK inhibitors on ABSC activation and differentiation

Then we examined the effect of LIF, ALK5-I and ROCK-I on ABSCs. All treatments significantly increased the number and size of colonies compared to ABSCs alone (Fig. 5A-D). ALK5-I-treated colonies were >LIF-treated colonies; and these were >ROCK-I-treated colonies (Fig. 5A-D, F-G). In contrast to the effect of FGFs, most spheres had thin walls and did not show basal cell hyperplasia, indicating induction of rate of secretion and/or increased differentiation towards the secretory lineage (ALK5-I>LIF>ROCK-I) rather than proliferation (Fig. S9). Indeed, number of ciliated cells showed a reversed trend as their number in ROCK-I-treated colonies was >LIF-treated colonies; and these were >ALK5-I-treated colonies (Fig. S9). Combining Rock-I with fibroblasts caused marked additional increase in number of spheres but resulted in a further shift of differentiation towards ciliated lineage resulting in spheres with thicker walls and smaller lumens (Fig. 5E-G and Fig. S6).

Discussion

The future use of stem cells for therapeutic applications requires proper understanding of their proliferation and differentiation potentials and identification of factors that can control and direct these processes in the desired direction. Our study establishes a simple and reproducible in vitro model to study both distal and proximal lung epithelial stem cells and their niches. We describes the morphology and differentiation profile of each colony type at the baseline culture conditions then demonstrate the effects caused by supplementing several potential niche components or factors that can influence lung stem cell behavior.

The requirement of distal lung epithelial stem cells for fibroblast co-culture to proliferate and form clonal spheres in vitro has been previously identified (Barkauskas et al., 2013; Reynolds et al., 2000; McQualter et al., 2010; Teisanu et al., 2011). However, the mechanism by which fibroblasts adhere to the proliferating/differentiating stem cell and the exact nature of their interaction remains to be elucidated. McQualter et al reported that supplementing the medium with FGF10 or HGF could replace the requirement for fibroblast support of colonal growth; without specifying the effect of these factors on colony type or number (McQualter et al., 2010). In our hands, without fibroblasts co-culture, using 50-100 ng/mL FGF10 (or any other supplement) failed to induce the growth of any epithelial colony while using higher concentrations of FGFs (300 ng/mL) resulted in appearance of few and small colonies, mostly type A (not-shown data). Indeed, the colonies shown by McQualter et al growing in response to growth factors replacement are also rounded and luminal type A-like colonies. We speculate that the interactions between fibroblasts and each stem cell/colony type are complex and varies in its nature based on stem cell/colony type. It is unlikely that single factor supplementation will be able to replace fibroblasts support.

The presence of major differences among fibroblasts isolated from various organs and tissues have been described several decades ago. Castor et al have described striking differences between fibroblasts from skin, periosteum, pericarpdium and synovial tissue in terms of proliferative potential, cell cycle and rate of mucopolysaccharide formation (Castor et al., 1962). In addition, the presence
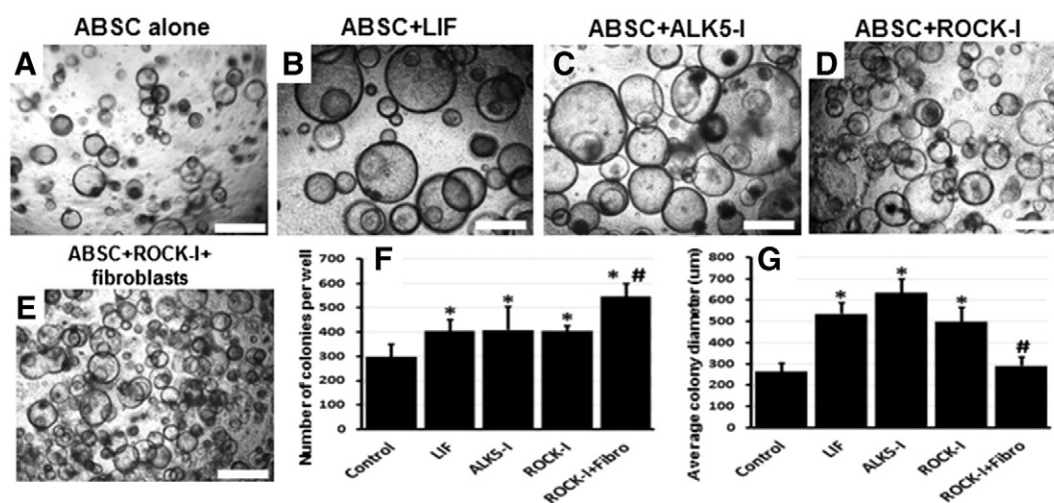

Figure 5 Effect of LIF, ALK5-I, ROCK-I or ROCK-I+fibroblasts on ABSCs *in vitro*. 50,000/well ABSCs were cultured in MTEC/plus medium alone (A), or treated with LIF (B), ALK5-I (C) or ROCK-I (D). All treatments enhanced colonies number and size compared to untreated control. Combining ROCK-I treatment with fibroblasts co-culture resulted in further increase in colony number but not size (E). (F, G) Quantification for number and average diameter of all colonies from 3 independent experiments. (*$p<0.05$ compared to control, #$p<0.05$ compared to ROCK-I alone). Scale bars: 500 μm. See also supplemental Fig. S9.

of subtypes of fibroblasts within the same organ, including the lung, has also long been documented. Lipid-containing or non-containing (Brody and Kaplan, 1983), and Thy1+ or Thy1- (Phipps et al., 1990) are subtypes of lung fibroblasts that have been identified to have different morphologies, ability to synthesize matrix components and immunological function. It is likely that different stem cells in the lung are associated with positionally and phenotypically different subtypes of lung fibroblasts. Indeed, lung lipofibroblasts – which are closely associated with alveolar cells- were more efficient in supporting the clonal proliferation and sphere formation of alveolar cells *in vitro* compared to MLg (Barkauskas et al., 2013). Additional aspect of the use of fibroblasts in niche-mimicking co-culture has been recently revealed by showing that the ex vivo expansion of fibroblasts alters their ability to support lung epithelial colonies in culture. Their prolonged expansion in culture seemed to promote their 'differentiation' and result in reduction of their colony-supportive capacity (McQualter et al., 2013). Also, in the current study, we show that tracheal fibroblasts preferentially supported type A colonies –which most probably originate from more proximal intra-pulmonary airways- and shifted their differentiation pattern into more of airway and less of alveolar phenotype.

Because of the lack of specific surface markers to purely and specifically isolate individual lung stem cells, in this study we had to culture the whole lung epithelial cells, which obviously comprise minority of multiple stem cell types mixed with majority of non-stem epithelial cells. We acknowledge that the use of heterogeneous epithelial cell population from distal lung casts doubt on our conclusions concerning lung stem cell fate specification and regulation because of the possible interactions among the non-stem epithelial cells and the studied component. However, the presence of non-stem epithelial cells in the culture might be -on its own- an additional niche contributor. Hegab et al showed that non-basal epithelial cells expressing high aldehyde dehydrogenases (ALDHs) had enhanced ability to induce ABSCs clonal proliferation and sphere formation compared to ALDH low non-basal cells or ABSCs alone (Hegab et al., 2012, 2014).

The ability of colony types A and B to differentiate into basal, secretory, ciliated and alveolar-like cells support the recent assumption of the presence of a multipotent stem cell in the adult lung. Integrin alpha-6 has been suggested as a marker to identify these cells *in vivo* and to sort them for *in vitro* characterization (Chapman et al., 2011; Chernaya et al., 2014). Their exact location, function during lung homeostasis or repair after various types of injury/disease and their relation to the K5+/p63+ basal-like cells detected in the repairing lung parenchyma after H1N1 influenza infection; requires further experimental testing (Chernaya et al., 2014; Kumar et al., 2011; Li et al., 2013; Zuo et al., 2015).

The finding that treating both ABSCs and distal lung stem cells with FGFs resulted in higher CFE and induced their rate of proliferation is intriguing. We also showed that ABSCs treated with FGF9 resulted in thick-walled and basal cell hyperplasia-like colonies and that FGFR blocker failed to reverse the FGF9-inducing effect on distal lung stem cell colonies. We have previously published data showing that in a mouse model with transgenic expression of FGF9 in the lung, epithelial cells rapidly proliferated and formed adenocarcinoma-like nodules that did not regress after FGF9 withdrawal (Yin et al., 2013). Further characterization of this FGF9 transgenic mouse model showed that these adenocarcinomas developed from both proximal and distal airway epithelium as well as from alveolar cells (Arai et al., 2015). Taken together, these data suggest that increased signaling through the FGF9/FGFR axis may be involved in "transforming" lung stem cells from their controlled quiescence/proliferation/differentiation behavior into more of activation/proliferation that eventually leads to malignant transformation. Further studies are warranted to determine how the FGFR downstream signaling cascade interfere with lung stem cells' cell-cycle regulation and differentiation.

A recent study showed that mesenchymal ALK5 regulates the balance between smooth muscle versus lipofibroblast differentiation and that ALK5 inhibition shifts the balance towards lipofibroblast (Li et al., 2014). Thus, it is possible that the enhancing effect of ALK5-I we observed in our culture system is –at least in part- due to differentiating the lung fibroblasts into the more support-efficient "lipofibrblasts" (Barkauskas et al., 2013).

The formation and maintenance of airway surface liquid layer is vital to mucociliary clearance. In cystic fibrosis, the formation of luminal fluid and its composition are disturbed (Wood et al., 1976). In this study, both proximal and distal lung stem cells made luminal colonies that contained watery fluids and several treatments and co-culture influenced the volume and probably the composition of this fluid, which makes this model especially useful to study diseases affecting airway secretion and the specific effect/mechanism of drugs that enhanced the secretion in a "stem cell" context.

In conclusion, the stem cell assay we described and the characterization of several components that can enhance or suppress lung stem cells clonal growth and differentiation provides insight into potential regulatory mechanisms, which might be relevant to normal tissue maintenance and repair *in vivo*. Further *in vitro* studies of these components and their downstream signals will provide valuable knowledge of both the intrinsic and niche-related mechanisms that control lung stem cell behavior in health and disease.

Author contributions

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.05.005.

References

Arai, D., Hegab, A.E., Soejima, K., Kuroda, A., Ishioka, K., Yasuda, H., Naoki, K., Kagawa, S., Hamamoto, J., Yin, Y., Ornitz, D.M., Betsuyaku, T., 2015. Characterization of the cell of origin and propagation potential of the fibroblast growth factor 9-induced mouse model of lung adenocarcinoma. J. Pathol. 235, 593–605.

Barkauskas, C.E., Cronce, M.J., Rackley, C.R., Bowie, E.J., Keene, D.R., Stripp, B.R., Randell, S.H., Noble, P.W., Hogan, B.L., 2013. Type 2 alveolar cells are stem cells in adult lung. J. Clin. Invest. 123, 3025–3036.

Brody, J.S., Kaplan, N.B., 1983. Proliferation of alveolar interstitial cells during postnatal lung growth. Evidence for two distinct populations of pulmonary fibroblasts. Am. Rev. Respir. Dis. 127, 763–770.

Castor, C.X., Prince, R.K., Dorstewitz, E.L., 1962. Characteristics of human "fibroblasts" cultivated in vitro from different anatomical sites. Lab. Invest. 11, 703–713.

Chapman, H.A., Li, X., Alexander, J.P., Brumwell, A., Lorizio, W., Tan, K., Sonnenberg, A., Wei, Y., Vu, T.H., 2011. Integrin alpha6beta4 identifies an adult distal lung epithelial population with regenerative potential in mice. J. Clin. Invest. 121, 2855–2862.

Chen, H., Matsumoto, K., Brockway, B.L., Rackley, C.R., Liang, J., Lee, J.H., Jiang, D., Noble, P.W., Randell, S.H., Kim, C.F., Stripp, B.R., 2012. Airway epithelial progenitors are region specific and show differential responses to bleomycin-induced lung injury. Stem Cells 30, 1948–1960.

Chernaya, O., Shinin, V., Liu, Y., Minshall, R.D., 2014. Behavioral heterogeneity of adult mouse lung epithelial progenitor cells. Stem Cells Dev. 23, 2744–2757.

Christalla, P., Hudson, J.E., Zimmermann, W.H., 2012. The cardiogenic niche as a fundamental building block of engineered myocardium. Cells Tissues Organs 195, 82–93.

Driscoll, B., Kikuchi, A., Lau, A.N., Lee, J., Reddy, R., Jesudason, E., Kim, C.F., Warburton, D., 2012. Isolation and characterization of distal lung progenitor cells. Methods Mol. Biol. 879, 109–122.

Giangreco, A., Reynolds, S.D., Stripp, B.R., 2002. Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. Am. J. Pathol. 161, 173–182.

Hall, A., 1988. Rho GTPases and the actin cytoskeleton. Science 279, 509–514.

Hegab, A.E., Ha, V.L., Gilbert, J.L., Zhang, K.X., Malkoski, S.P., Chon, A.T., Darmawan, D.O., Bisht, B., Ooi, A.T., Pellegrini, M., Nickerson, D.W., Gomperts, B.N., 2011. Novel stem/progenitor cell population from murine tracheal submucosal gland ducts with multipotent regenerative potential. Stem Cells 29, 1283–1293.

Hegab, A.E., Ha, V.L., Darmawan, D.O., Gilbert, J.L., Ooi, A.T., Attiga, Y.S., Bisht, B., Nickerson, D.W., Gomperts, B.N., 2012. Isolation and in vitro characterization of basal and submucosal gland duct stem/progenitor cells from human proximal airways. Stem Cells Transl. Med. 1, 719–724.

Hegab, A.E., Ha, V.L., Bisht, B., Darmawan, D.O., Ooi, A.T., Zhang, K.X., Paul, M.K., Kim, Y.S., Gilbert, J.L., Attiga, Y.S., Alva-Ornelas, J.A., Nickerson, D.W., Gomperts, B.N., 2014. Aldehyde

dehydrogenase activity enriches for proximal airway basal stem cells and promotes their proliferation. Stem Cells Dev. 23, 664–675.

Horani, A., Nath, A., Wasserman, M.G., Huang, T., Brody, S.L., 2013. Rho-associated protein kinase inhibition enhances airway epithelial Basal-cell proliferation and lentivirus transduction. Am. J. Respir. Cell Mol. Biol. 49, 341–347.

Kent, K.D., Bomser, J.A., 2003. Bovine pituitary extract provides remarkable protection against oxidative stress in human prostate epithelial cells. In Vitro Cell. Dev. Biol. Anim. 39, 388–394.

Kumar, P.A., Hu, Y., Yamamoto, Y., Hoe, N.B., Wei, T.S., Mu, D., Sun, Y., Joo, L.S., Dagher, R., Zielonka, E.M., Wang de, Y., Lim, B., Chow, V.T., Crum, C.P., Xian, W., McKeon, F., 2011. Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. Cell 147, 525–538.

Lee, J.H., Bhang, D.H., Beede, A., Huang, T.L., Stripp, B.R., Bloch, K.D., Wagers, A.J., Tseng, Y.H., Ryeom, S., Kim, C.F., 2014. Lung stem cell differentiation in mice directed by endothelial cells via a BMP4-NFATc1-thrombospondin-1 axis. Cell 156, 440–455.

Li, X., Rossen, N., Sinn, P.L., Hornick, A.L., Steines, B.R., Karp, P.H., Ernst, S.E., Adam, R.J., Moninger, T.O., Levasseur, D.N., Zabner, J., 2013. Integrin α6β4 identifies human distal lung epithelial progenitor cells with potential as a cell-based therapy for cystic fibrosis lung disease. PLoS One 8, e83624.

Li, A., Ma, S., Smith, S.M., Xing, Y., Li, S., Lee, M.K., Borok, Z., Li, C., Minoo, P., 2014. Lung mesodermal alk5 mediated tgfβ signaling controls the balance between smooth muscle versus lipofibroblast cell differentiation. Am. J. Respir. Crit. Care Med. 189, A1217.

Liu, X., Ory, V., Chapman, S., Yuan, H., Albanese, C., Kallakury, B., Timofeeva, O.A., Nealon, C., Dakic, A., Simic, V., Haddad, B.R., Rhim, J.S., Dritschilo, A., Riegel, A., McBride, A., Schlegel, R., 2012. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. Am. J. Pathol. 180, 599–607.

McQualter, J.L., Brouard, N., Williams, B., Baird, B.N., Sims-Lucas, S., Yuen, K., Nilsson, S.K., Simmons, P.J., Bertoncello, I., 2009. Endogenous fibroblastic progenitor cells in the adult mouse lung are highly enriched in the sca-1 positive cell fraction. Stem Cells 27, 623–633.

McQualter, J.L., Yuen, K., Williams, B., Bertoncello, I., 2010. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. Proc. Natl. Acad. Sci. U. S. A. 107, 1414–1419.

McQualter, J.L., McCarty, R.C., Van der Velden, J., O'Donoghue, R.J., Asselin-Labat, M.L., Bozinovski, S., Bertoncello, I., 2013. TGF-β signaling in stromal cells acts upstream of FGF-10 to regulate epithelial stem cell growth in the adult lung. Stem Cell Res. 11, 1222–1233.

Ornitz, D.M., Yin, Y., 2012. Signaling networks regulating development of the lower respiratory tract. Cold Spring Harb. Perspect. Biol. 4 (5).

Paul, M.K., Bisht, B., Darmawan, D.O., Chiou, R., Ha, V.L., Wallace, W.D., Chon, A.T., Hegab, A.E., Grogan, T., Elashoff, D.A., Alva-Ornelas, J.A., Gomperts, B.N., 2014. Dynamic changes in intracellular ROS levels regulate airway basal stem cell homeostasis through Nrf2-dependent notch signaling. Cell Stem Cell 15, 199–214.

Phipps, R.P., Penney, D.P., Keng, P., Silvera, M., Harkins, S., Derdak, S., 1990. Immune functions of subpopulations of lung fibroblasts. Immunol. Res. 9, 275–286.

Raiser, D.M., Kim, C.F., 2009. Commentary: Sca-1 and cells of the lung: a matter of different sorts. Stem Cells 27, 606–611.

Rawlins, E.L., Okubo, T., Xue, Y., Brass, D.M., Auten, R.L., Hasegawa, H., Wang, F., Hogan, B.L., 2009. The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. Cell Stem Cell 4, 525–534.

Reynolds, S.D., Giangreco, A., Power, J.H., Stripp, B.R., 2000. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am. J. Pathol. 156, 269–278.

Rinn, J.L., Bondre, C., Gladstone, H.B., Brown, P.O., Chang, H.Y., 2006. Anatomic demarcation by positional variation in fibroblast gene expression programs. PLoS Genet. 2, e119.

Rock, J.R., Onaitis, M.W., Rawlins, E.L., Lu, Y., Clark, C.P., Xue, Y., Randell, S.H., Hogan, B.L., 2009. Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc. Natl. Acad. Sci. U. S. A. 106, 12771–12775.

Solanas, G., Benitah, S.A., 2013. Regenerating the skin: a task for the heterogeneous stem cell pool and surrounding niche. Nat. Rev. Mol. Cell Biol. 14, 737–748.

Song, H., Yao, E., Lin, C., Gacayan, R., Chen, M.H., Chuang, P.T., 2012. Functional characterization of pulmonary neuroendocrine cells in lung development, injury, and tumorigenesis. Proc. Natl. Acad. Sci. U. S. A. 109, 17531–17536.

Tata, P.R., Mou, H., Pardo-Saganta, A., Zhao, R., Prabhu, M., Law, B.M., Vinarsky, V., Cho, J.L., Breton, S., Sahay, A., Medoff, B.D., Rajagopal, J., 2013. Dedifferentiation of committed epithelial cells into stem cells in vivo. Nature 503, 218–223.

Teisanu, R.M., Lagasse, E., Whitesides, J.F., Stripp, B.R., 2009. Prospective isolation of bronchiolar stem cells based upon immunophenotypic and autofluorescence characteristics. Stem Cells 27, 612–622.

Teisanu, R.M., Chen, H., Matsumoto, K., McQualter, J.L., Potts, E., Foster, W.M., Bertoncello, I., Stripp, B.R., 2011. Functional analysis of two distinct bronchiolar progenitors during lung injury and repair. Am. J. Respir. Cell Mol. Biol. 44, 794–803.

Wagers, A.J., 2012. The stem cell niche in regenerative medicine. Cell Stem Cell 6, 362–369.

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S., Muguruma, K., Sasai, Y., 2007. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat. Biotechnol. 25, 681–686.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A., Gough, N.M., 1988. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336, 684–687.

Wood, R.E., Boat, T.F., Doershuk, C.F., 1976. Cystic fibrosis. Am. Rev. Respir. Dis. 113, 833–878.

Xing, Y., Li, C., Li, A., Sridurongrit, S., Tiozzo, C., Bellusci, S., Borok, Z., Kaartinen, V., Minoo, P., 2010. Signaling via Alk5 controls the ontogeny of lung Clara cells. Development 137, 825–833.

Yin, Y., Betsuyaku, T., Garbow, J.R., Miao, J., Govindan, R., Ornitz, D.M., 2013. Rapid induction of lung adenocarcinoma by fibroblast growth factor 9 signaling through FGF receptor 3. Cancer Res. 73, 5730–5741.

You, Y., Richer, E.J., Huang, T., Brody, S.L., 2002. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. Am. J. Physiol. Lung Cell. Mol. Physiol. 283, L1315–L1321.

Zheng, D., Limmon, G.V., Yin, L., Leung, N.H., Yu, H., Chow, V.T., Chen, J., 2012. Regeneration of alveolar type I and II cells from Scgb1a1-expressing cells following severe pulmonary damage induced by bleomycin and influenza. PLoS One 7, e48451.

Zuo, W., Zhang, T., Wu, D.Z., Guan, S.P., Liew, A.A., Yamamoto, Y., Wang, X., Lim, S.J., Vincent, M., Lessard, M., Crum, C.P., Xian, W., McKeon, F., 2015. p63+Krt5+ distal airway stem cells are essential for lung regeneration. Nature 517, 616–620.