Isolation of stem/progenitor cells from normal lung tissue of adult humans

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Abstract

Objectives: This study aimed to isolate and characterize stem/progenitor cells, starting from normal airway epithelia, obtained from human adults.

Materials and methods: Cultures of multicellular spheroids were obtained from human lung tissue specimens after mechanical and enzymatic digestion. Tissue-specific markers were detected on their cells by immunohistochemical and immunofluorescent techniques. Ultrastructural morphology of the spheroids (termed as bronchospheres) was evaluated by electron microscopy, gene expression analysis was performed by reverse transcription–polymerase chain reaction, and gene down-regulation was analysed by an RNA interference technique.

Results: Bronchospheres were found to be composed of cells with high expression of stem cell regulatory genes, which was not or was only weakly detectable in original tissues. Morphological analysis showed that bronchospheres were composed of mixed phenotype cells with type II alveolar and Clara cell features, highlighting their airway resident cell origin. In addition to displaying specific pulmonary and epithelial commitment, bronchospheres showed mesenchymal features. Silencing of the *Slug* gene, known to play a pivotal role in epithelial–mesenchymal transition processes and which was highly expressed in bronchospheres but not in original tissue, led bronchospheres to gain a differentiated bronchial/alveolar phenotype and to lose the stemness gene expression pattern.

Conclusions: Ours is the first study to describe *ex vivo* expansion of stem/progenitor cells resident

in human lung epithelia, and our results suggest that the epithelial–mesenchymal transition process, still active in a subset of airway cells, may regulate transit of stem/progenitor cells towards epithelial differentiation.

Introduction

The lung originates from endodermal cells of the floor of the foregut between the pharyngeal pouches, and invades the splanchnic mesoderm after about 1 month of human embryonic development. Alveoli begin to develop during the 6–7th month of gestation in humans and alveoli reach up to 300 million in number by the ages of 2–4 years (1,2), becoming the main structure of the lung. Their cell surface is directly exposed to potentially injurious inhaled gases, particles and pathogens, and they must therefore be able to respond quickly and effectively to cell damage (3,4). However, little is still known about regulation of cell turnover and renewal of alveoli in adults after acute lung injury.

Studies in animal models have shown that after chemical ablation of epithelial cells in different lung epithelial compartments, regenerative foci are maintained in airways. This has led to the hypothesis of the existence of stem or progenitor cells capable of repairing injured tissue, such as cytokeratin 5-positive basal cells, responsible for mucosal gland development and renewal of the branched epithelium of the trachea (5,6), Clara cells, responsible for bronchiole renewal (7), and type 2 pneumocytes, responsible for alveolar renewal (8). The same experimental models have also highlighted the involvement of various stem-cell regulatory transcription factors, including Sox family members (in particular, SOX-2) (9,10), Oct 3/4 (11), and β -catenin/TCF (Lef) (12), as well as of the surface molecule BCRP-1 (13), which belongs to the ABC-transporter family and is associated with a highly enriched stem cell subpopulation. Recently, a primitive putative adult stem cell group with mesenchymal and epithelial potential was identified in lung tissue from a

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murine experimental model (14). Furthermore, it has been hypothesized that circulating mesenchymal progenitor CD45⁺ CXCR4⁺ CK5⁺ cells derived from bone marrow, may be recruited for lung repair and regeneration (15). However, as yet no animal or human models have provided definitive evidence of reconstitution of lung alveolar epithelial cells by bone marrow-derived or purified haematopoietic stem cells (16).

The present work reports, for the first time, on isolation and *in vitro* expansion of stem or progenitor cells from human adult lung tissue, using the gold standard methodology of multicellular spheroids (17). In particular, this study has focused on characterizing the stemness transcriptional profile of isolated cells and on tracing their origin back to resident lung epithelial cells by assessing their morphological features.

Materials and methods

Tissue disaggregation

Tumour tissue was obtained from 28 patients (18 men, 10 women), median age 65.5 years (range, 38–81 years), with primary lung cancer who underwent lobectomy. Written informed consent was obtained from all patients. Normal tissue was sampled by a pathologist at least 1 cm away from neoplastic lesions (under sterile conditions) and the healthy nature of the tissue was microscopically confirmed. Tissues were processed within 30 min of surgery, as previously described (18). Briefly, specimens were minced and incubated for 6–12 h in the presence of collagenase/hyaluronidase enzyme mix in Epicult medium (StemCell Technologies, Vancouver, BC, Canada). After digestion, cell clumps were sieved through a 40- μ m cell strainer (Falcon) to obtain single cell suspensions. When the number of single cells was < 99%, the cell suspension was re-sieved.

Bronchosphere culture

After filtration, single cells were plated in ultralow attachment plates (Corning Inc., Corning, NY, USA) at a density of 20 000 viable cells/ml in serum-free DMEM-F12 (Cambrex, Walkersville, MA, USA) supplemented with B27 supplement (StemCell Technologies), 20 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich, Milan, Italy), 10 ng/ml epidermal growth factor (EGF), 10 μ g/ml insulin, 10⁻⁶ M hydrocortisone, and *ad hoc* aliquots of gentamicin and amphotericin (Cambrex). Under these conditions an adequate number of non-adherent spherical cell clusters (primary bronchospheres) was obtained from 26 of the 28 samples. To obtain secondary bronchospheres, primary spheres were gently centrifuged, enzymatically

dissociated with 1 $\times$ Trypsin–EDTA solution (Celbio, Milan, Italy) for 1–3 min and, after two washes in complete growth medium, were re-sieved through a 40- μ m nylon mesh.

Bronchosphere cell composition

Immunohistochemistry Immunostaining of tissue-specific markers was performed on serial sections obtained from collagen-included bronchospheres (StemCell Technologies), fixed in 10% buffered formalin and embedded in paraffin wax. Four-micrometre sections were mounted on positively-charged slides (Bio-Optica, Milan, Italy), deparaffinized with xylene, rehydrated, and endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide solution. Antigen unmasking was performed for SP-A, CC10, and TTF-1 by epitope retrieval solution (0.01 M citrate buffer pH 6.0) in a water bath at 98.5 °C for 40 min, followed by a 20-min cooling period at room temperature.

Sections were then treated for non-specific antigen binding with 3% bovine serum albumin in phosphate-buffered saline (PBS) for 20 min, after which they were incubated with the following primary antibodies: CD45 (ready-to-use solution, Dako Corporation, Carpinteria, CA, USA) positive-charged slides (Bio-Optica); factor VIII (related antigen polyclonal antibody, dilution 1 : 30, Dako Corporation) in PBS for 30 min at room temperature; vimentin (1 : 100, Novocastra, Newcastle upon Tyne, UK) for 30 min at room temperature; TTF-1 (1 : 150, Novocastra) for 1 h at 25 °C; SP-A (1 : 200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at room temperature; and CC10 (1 : 100, Santa Cruz Biotechnology) for 1 h at room temperature.

Primary antibody staining reaction was detected using the avidin-biotin technique (LSAB+ kit; Dako Corporation) with diaminobenzidine/hydrogen peroxidase chromogen solution (DAB+ liquid substrate–chromogen solution; Dako Corporation). Slides were counterstained blue with Mayer's Hemalum and mounted in Eukitt (Bio-Optica).

Immunofluorescence Spheroids obtained were embedded in 2% agar in mammary epithelial cell growth medium (Cambrex) and then frozen in Optimal Cutting Temperature Compound (OCT, Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) using isopentane, precooled in liquid nitrogen. Eight-micrometre sections were cut with a cryostat (Kryostat 1720; Ernst Leitz Wetzlar GmbH, Wetzlar, Germany), dried at room temperature for 30 min and then stained with haematoxylin and eosin.

For immunofluorescence analysis, samples were fixed in cold acetone for 5 min and then dried for 30 min at room temperature. Sections were washed in PBS (pH 7.4)

and were incubated with 1% bovine serum albumin in PBS for 30 min at room temperature to block non-specific binding. Sections were then incubated for 45 min at 37 °C with the following monoclonal antibodies: CD133 (1 : 80, Miltenyi Biotech, Gladbach, Germany), CD44 (1 : 50, BD Pharmingen, Milan, Italy), CD90 (1 : 50, BD Pharmingen), CD105 (1 : 20, BD Pharmingen), vimentin (1 : 50, BD Pharmingen), cytokeratin 5 (1 : 100, DakoCytomation, Dako Milan, Italy), cytokeratin 8 (1 : 100, DakoCytomation), cytokeratin 19 (1 : 100, DakoCytomation) SP-A (1 : 200, Santa Cruz Biotechnology), CC10 (1 : 100, Santa Cruz Biotechnology), and Oct 3/4 (1 : 100, Santa Cruz Biotechnology). Negative controls were obtained by omitting the primary antibodies. Isotypic control mouse IgG₁, κ (BD Pharmingen) at the above corresponding dilutions was used for CD105, CD44, CD90, vimentin, CD133, cytokeratin 5, cytokeratin 8 and cytokeratin 19. Rabbit polyclonal IgG (Beckman Coulter, Fullerton, CA, USA) was used as isotypic control at the above corresponding dilutions for CC10, SP-A and Oct 3/4 (Figs S1 and S2, Supporting Information). Sections were then washed three times in PBS and were incubated for 45 min at 37 °C with fluorescein isothiocyanate-conjugated polyclonal rabbit anti-mouse immunoglobulin (1 : 250, DakoCytomation, Hamburg, Germany). Mounting and nuclei counterstaining were performed using the 'Pro Long Antifade Reagent with DAPI' (Molecular Probes, Milan, Italy) and the samples were then examined under a fluorescence microscope (Axiovert 40, Carl Zeiss, Oberkochen, Germany).

Ultrastructure The spheroids were pelleted and fixed at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 4 h. Specimens were washed, fixed with 1% buffered osmium tetroxide, dehydrated through graded series (70–100%) of alcohol and were embedded in epoxy resin. One-micrometre sections were stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope (Philips 400T). (Check with authors – this seems very thick.)

Culture in differentiating conditions

Single sphere suspensions were seeded into flasks and were grown for approximately 2 weeks in Bronchial Epithelial Cell Basal Medium supplemented with bovine pituitary extract (2 ml), hydrocortisone (0.5 ml), hEGF (0.5 ml), epinephrine (0.5 ml), transferrin (0.5 ml), insulin (0.5 ml), retinoic acid (0.5 ml), triiodothyronine (0.5 ml), and GA-1000 (0.5 ml) (Cambrex).

Cells were then trypsinized and washed twice with PBS. Approximately $7\text{--}8 \times 10^5$ cells were plated onto microscope slides using the Cytospin cell preparation

system (Thermo-Shandon, Pittsburgh, PA, USA). Fixed samples were rehydrated and endogenous peroxidase activity was blocked using 3% hydrogen peroxide solution. Incubation of sections with primary antibodies specific for lung tissue (TTF-1, SP-A, CCSP), and detection of immunoreaction were performed as described above.

Gene expression analysis: RNA extraction, reverse transcription and cDNA amplification

Total RNA was extracted from cells using TRIzol[®] Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) reactions were performed in a 20- μ l volume with 800 ng of total RNA using iScript[™] cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). cDNA obtained from the RT reaction was amplified using a thermal cycler PTC 200 (M-Medical Genenco, Florence, Italy) in 25- μ l reaction mixtures containing 2 μ l of cDNA template, 1 unit/ μ l of TaqAra, 1 $\times$ Ex-Taq buffer, 250 μ M of dNTPs mixture (TaKaRa ex Taq[™], Takara Bio Inc., Shiga, Japan) and 1 μ M of each oligonucleotide primer. The reaction mixture was subjected to different annealing temperatures in relation to the different primers used (Table S1, Supporting Information). Polymerase chain reaction (PCR) conditions were as follows: 94 °C for 1 min, the appropriate annealing temperature for 1 min, 72 °C for 1 min for 30 cycles followed by a final extension step at 72 °C for 5 min. To verify that equal amounts of cDNA were added in each PCR, the housekeeping gene *GAPDH* was amplified from each cDNA sample. PCR amplification products were analysed by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining viewed under ultraviolet light.

Real-time RT-PCR analysis

One microgram of RNA was reverse-transcribed into cDNA using iScript (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's recommendations and analysed by real-time RT-PCR (MyiQ System, Bio-Rad) to evaluate level of expression of BCRP-1, CD133, Oct 3/4, Bmi-1, CD44, Slug and Lef-1. The standard reaction volume was 25 μ l and contained 1 $\times$ SYBR GREEN SuperMix (Bio-Rad), 5 μ M of each primer and 2 μ l of cDNA template. The mixture was subjected to the following cycling conditions: 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 30 s. Primers for Oct 3/4, CD133, CD44 and Slug were the same as those used for RT-PCR analysis. Primer sequences used for BCRP-1, Bmi-1 and Lef-1 are listed in Table S5 (Supporting Information). The amount of mRNA of each marker was normalized to

endogenous reference β_2 -microglobulin using Gene Expression Macro Software, version 1.1 (Bio-Rad).

Slug gene silencing by siRNA technique

Slug and control (scramble, SCR) short interference siRNA oligonucleotides were purchased from Invitrogen. Slug siRNA sequences were: AUCAGAAUGGGUCUG-CAGAUGAGCC and GGCUCAUCUGCAGACCCAUCUCUGAU.

Stealth negative control RNAi (SCR siRNA) with medium GC (Invitrogen) was used as negative control. Slug/SCR siRNA transfection into spheres was performed using *in vitro* JET-PEI (Polyplus Transfection, Illkirch, France) in a 5 : 1 reagent siRNA proportion following the manufacturer's instructions. Efficiency of gene silencing in spheres ranged from 70% to 90%.

Statistical analysis

Statistical analysis for categorical variables was performed by McNemar's test for non-independent samples and by paired sample *t*-test for continuous variables using the SPSS 10.1 Package (SPSS, Milan, Italy).

Results

Multicellular bronchospheres are generated from normal adult human lung tissue

Single cells from normal lung tissue were plated at a density of 20 000 viable cells and cultured in serum-free medium containing EGF and bFGF. After approximately 7–10 days, floating spheres, termed as bronchospheres, were obtained from 26 of the 28 cases (92%). Primary bronchospheres, once enzymatically disaggregated into single cells and replated at a density of 1000 cells/ml in ultralow attachment plates, always gave rise to secondary spheres within 5–7 days of culture (Fig. 1).

BPE express high levels of stem cell genes compared to original lung tissue

A panel of stem cell regulatory genes including Bmi-1, CD133, Oct 3/4, Lef-1 and the surface molecule BCRP-1 was evaluated by RT-PCR in bronchospheres and in matched original tissue (Table S2, Supporting Information). Expression of Oct 3/4, CD133, and Bmi-1 was detected in a notable number of original tissue samples but was 2- to 3-fold higher in bronchospheres. Moreover, Lef-1, which was only occasionally expressed in original lung tissue samples, was detected in more than half of bronchospheres, and BCRP-1 expression was also observed in twice as

Figure 1. Multicellular bronchospheres can be reliably generated from normal adult lung tissues. Inverted phase-contrast microscopy (Olympus IX51) of primary bronchospheres (I) obtained from lung tissue, and secondary bronchospheres (II) derived from primary bronchospheres cultured in serum-free medium. Scale bar, 100 μ m.

many bronchospheres as in matched original tissue samples (Fig. 2a). Furthermore, RT-PCR and real-time RT-PCR analyses highlighted that mRNA expression level of the above genes was always significantly higher in bronchospheres than in the original tissue samples (Fig. 2b). Conversely, other stem cell-associated genes, such as SOX-2 and CXCR4, were similarly expressed in original tissues and in bronchospheres (87.5% vs. 91.7%, and 100% vs. 87.5%, respectively).

Immunofluorescence analysis confirmed the higher percentage of cells expressing Oct 3/4 and a similar percentage of cells expressing CD133 in bronchospheres (Fig. 2c).

Bronchospheres derive from undifferentiated airway cells

Transmission electron microscopical analysis revealed that bronchospheres were composed of cells showing type II pneumocyte features (presence of lipid droplets, intracellular lamellar bodies and extracellular concentric lamellae, resembling surfactant deposition areas) and Clara cells (presence of dilated cisternae and electron-dense granules, resembling Clara bodies) (Fig. 3a). Bronchosphere cells also showed mesenchymal features, revealing cytoplasmic collections of vimentin-like intermediate filaments and well-developed tight junctions (Fig. 3b). Scanty microvilli were observed in peripheral cells of bronchospheres (Fig. 3a,b).

Figure 2. Bronchospheres (BS) express higher levels of stem cell regulatory genes than original tissue. (A) Number of primary BS and original tissue samples expressing stem cell markers (*P*-value according to McNemar's test) and mean expression levels of stem cell markers (*P*-value according to paired sample *t*-test). (B) mRNA levels of stem cell markers analysed by RT-PCR in original tissue and in BS (representative samples). *GAPDH* was used as a loading control. Images represent similar results. (C) Immunofluorescence analysis of Oct 3/4 and CD133 in BS. Scale bar, 20 μ m.

Immunofluorescence and immunohistochemical analyses confirmed that almost all of the cells expressed vimentin and that the majority also expressed lung-specific transcription factor TTF-1 (Fig. 3c).

Furthermore, immunofluorescence analysis also showed that all of the cells composing bronchospheres expressed the type II pneumocyte gene CD44, and a large proportion expressed cytokeratin 5, an antigen present in basal bronchial cells, alveolar type II pneumocyte gene surfactant protein A (SP-A), and the Clara cell-specific antigen CC10 (Fig. 3d). Simultaneously, RT-PCR and real-time RT-PCR analyses revealed negligible levels of CD44 mRNA and an absence of cytokeratin 5 mRNA expression in original tissue samples, in contrast to high expression of both mRNAs (65.4% and 92%, respectively)

in bronchospheres (Fig. 3e and Table S3). RT-PCR also detected the expression of alveolar SP-A gene in bronchospheres but not in original tissues (Fig. 3e). These data indicate that bronchospheres originate from undifferentiated airway cells, showing type II pneumocyte/Clara cell phenotype with the presence of mesenchymal features.

Bronchospheres are composed of mesenchymal stem cells showing epithelial differentiation markers

Immunofluorescence analysis showed that bronchospheres were composed of cells expressing mesenchymal stem cell antigens, CD90 and CD105. The presence of mature or precursor endothelial and myeloid cells was excluded by performing immunofluorescence and immunohistochemical

analyses of factor VIII, CD34 and CD45 expression (Fig. 4a). At the same time, immunofluorescence and RT-PCR assays revealed that bronchospheres were composed of cells expressing epithelial genes, specifically cytokeratin 8, cytokeratin 18 and cytokeratin 19 (Fig. 4b). These data indicate that cells in bronchospheres have a mixed mesenchymal/epithelial phenotype.

Bronchosphere-derived cells differentiate into mature bronchial/alveolar cells in adherent culture conditions

Bronchospheres were seeded into adherent flasks in the presence of bronchial epithelia-differentiating media.

Under these culture conditions, bronchospheres adhered to the surface of the flask and gave rise to the formation of sheaths of epithelial cells that expressed, as shown by immunohistochemical and RT-PCR analyses, TTF-1 (pulmonary epithelium marker), SP-A (alveolar type II-like cells) and CC10 (Clara-like cells), confirming the multilineage differentiation capacity of bronchospheres (Fig. 5a). RT-PCR analysis confirmed that bronchosphere-derived epithelial cells expressed higher mRNA levels of epithelial-specific markers (E-cadherin, cytokeratin 18, cytokeratin 19), TTF-1 and SP-A compared to bronchospheres (Fig. 5b). These data suggest that bronchospheres give rise to cells that can differentiate into pneumocytes and Clara cells.

Figure 4. Bronchospheres (BS) show a mixed mesenchymal/epithelial phenotype. (A) Immunofluorescence analysis of CD105, CD90, CD34, and CD45, and immunohistochemical analysis of factor VIII and CD45 protein. (B) Immunofluorescence and RT-PCR analyses of cytokeratins 8 and 19.

Figure 5. Bronchospheres (BS) differentiate into bronchial/alveolar cells. (A) Comparison between BS and BS-derived epithelia (BSDE): phase contrast analysis (a, e), IHC analysis of TTF-1, SP-A, and CC10 in BS (b, c, d) and in BSDE (f, g, h). (B) mRNA levels of E-cadherin, cytokeratin 8, cytokeratin 19, TTF-1, and SP-A analysed by RT-PCR in BS and BSDE. β2-microglobulin was used as loading control.

Slug gene governs epithelial–mesenchymal transition in bronchospheres

The *Slug* gene plays a pivotal role in the process that governs transition from mesenchyme to epithelium during

embryogenesis. RT-PCR and real-time RT-PCR analyses showed that *Slug* expression was infrequent and weak in original tissue samples, very frequent (92%) and high in bronchospheres, and down-regulated in bronchosphere-derived epithelial cells (Fig. 6a,b; Table S4, Supporting

Figure 6. Slug governs transition from epithelial to mesenchymal phenotype in bronchospheres (BS). (A) RT-PCR and real-time RT-PCR analyses of Slug mRNA expression in BS and original tissues. (B) RT-PCR analysis of Slug mRNA expression in BS and BSDE. (C) RT-PCR analysis of cytokeratin 8, cytokeratin 18, TTF-1, SP-A, Oct 3/4, CD133 and Lef-1 mRNA levels in BS exposed to Slug-specific or control (SCR) siRNA for 72 h. (D) Inverted phase-contrast microscopy of 10-day secondary BS obtained from primary BS exposed to Slug-specific or control (SCR) siRNA.

Information). To verify the functional involvement of *Slug* in the acquisition of differentiation markers, bronchospheres were transfected with Slug or SCR-specific siRNA. It was seen that Slug siRNA caused mRNA up-regulation of all the epithelial markers (cytokeratin 8, cytokeratin 18, TTF-1, SP-A) and down-regulation of stemness markers (Oct 3/4, CD133, Lef-1) (Fig. 6c). Furthermore, after *Slug* silencing, bronchospheres lost their capacity to originate second-generation spheres (Fig. 6d). These data would seem to indicate that *Slug* down-regulation accompanies the differentiation process of lung stem/progenitor cells.

Discussion

Multicellular spheroid methodology has successfully been used to isolate and expand *in vitro* stem progenitor

cells from some solid adult tissues, such as nervous or mammary tissue, but not previously from pulmonary tissue (18–20). Starting from normal adult human-derived airway epithelia and using this methodology and specific culture conditions, we obtained spheroids, termed bronchospheres, composed of cells expressing high levels of stem cell regulatory genes that are not, or are only negligibly, expressed in original tissue. Among these, we observed high expression of *Oct 3/4* gene, which was also recently found to be expressed in murine foetal lung stem/progenitor cells (1) and *Lef-1* gene, downstream effector of β -catenin, a pathway which is fundamental for ontogenesis and lung epithelial stem cell survival (21–23). We also detected expression of *Bmi-1* and *CD133* genes, which have been shown to be expressed in stem/progenitor cells of several tissues (24–31), but not previously of lung tissue.

Absence of immunostaining for factor VIII, CD45 and CD34 markers led us to exclude the possibility of a circulating haematopoietic cell origin of bronchospheres, in contrast to the hypothesis formulated by some authors on lung stem cell origin in studies performed on mice (15,34) and on patients who underwent transplantation of bone marrow (33–35).

We also obtained exhaustive data based on a combination of ultrastructural, immunofluorescence, immunohistochemical and RT-PCR analyses, showing that cells present in bronchospheres have features of two distinct pulmonary cell types: alveolar type II pneumocytes (TTF⁺ CD44⁺ SP-A⁺ cells) (36) located at bronchial–alveolar junctions, and Clara cells (CC10⁺ SP-A⁺ cells) (37–39) resident in the bronchial-tree, both of which have previously been ascribed as stem/progenitor cells (7,8). Interestingly, a recent study provided evidence that a subset of airway cells, termed *bronchioalveolar stem cells* by the authors, expressed markers specific for both types of pulmonary cell in murine lung models (40). Thus, our results further strengthen the hypothesis that lung progenitor/stem cells have these mixed features and are also present in human adult lung tissue. It is also possible that local microenvironment may drive them toward a definitive mature phenotype.

Molecular mechanisms of epithelial–mesenchymal transition, primarily studied in epithelial cell lines, include loss of epithelial cell polarity and acquisition of a variety of mesenchymal phenotypic traits. This type of conversion occurs over a number of critical steps of metazoan embryogenesis, but is also involved in tissue repair processes in the adult (41). Our data have highlighted that epithelial and mesenchymal features coexist in bronchospheres. In fact, cells with mixed bronchial and alveolar features also expressed high levels of vimentin, a mesenchymal cell marker, and CD90 and CD105, two markers currently used to identify mesenchymal stem cells (42). Intriguingly, these cells also expressed epithelial genes such as cytokeratin 8, cytokeratin 18 and cytokeratin 19, strongly confirming their epithelial commitment. Cells in bronchospheres would therefore seem to be blocked in an immature state, which recalls the transition phase between mesenchyme and epithelium. Several authors have suggested a pivotal role for *Slug* and other members of the Snail family in epithelial–mesenchymal transition and in the maintenance of undifferentiated mesenchymal states during development (43,44). In the present study, we observed high *Slug* gene expression in bronchospheres, but not in original tissue. The hypothesis of a dynamic involvement of *Slug* in maintenance of a stem/progenitor phenotype was confirmed by the fact that *Slug* gene silencing induced both increased expression of epithelial (cytokeratins) and differentiation (TTF1, SPA) markers in

bronchospheres and a decrease in Oct 3/4, CD133 and Lef-1 expression. This suggests that *Slug* gene may drive transition from an undifferentiated/stem cell-like cell to a differentiated epithelial cell, and indicates for the first time, a potential relationship between epithelial–mesenchymal transition and adult stem cell phenotype.

Furthermore, our findings show that all three phenotypes attributed to lung stem cells (Clara cells, type II alveolar cells and mesenchymal cells) are contained in bronchospheres, suggesting that circulating mesenchymal-like and epithelial stem/progenitor cells may actually represent two steps of the same differentiating process. Indeed, our data indicate that epithelial–mesenchymal transition may play a role in adult stem/progenitor function, which is aimed at tissue repair. Interestingly, we did not observe any decrease in capacity of generating bronchospheres when elderly patients were compared to younger ones, suggesting that stem/progenitor cells' reservoir is maintained with ageing and, at least *in vitro*, its efficiency is comparable to that of adults. These findings are in agreement with the notion that the repair response to injury in adult lung recapitulates several aspects of embryonic lung development (45,46). They also indicate potential involvement of epithelial–mesenchymal transition processes in maintaining life-long stem cell phenotype and could help us to understand the aetio-pathogenesis of pulmonary diseases where substitution of normal epithelia with fibrotic tissue occurs. It has been reported that idiopathic pulmonary fibrosis may depend upon aberrant activation of Wnt/ β -catenin signalling and on consequent anomalous epithelial–mesenchymal transition (47), a process we have shown to be of pivotal importance for stem/progenitor cell function.

From a clinical point of view, isolation and characterization of stem/progenitor lung cells from adult lung also addresses the issue of regenerative medicine in patients with acute and chronic lung disorders, including acute respiratory distress syndrome, pulmonary hypertension, chronic obstructive pulmonary disease (such as emphysema), and interstitial lung disease (such as pulmonary fibrosis). Although the natural course of these pathologies is unpredictable, their progression dramatically worsens clinical outcome, and the only therapeutic option that currently exists to improve patient survival is lung transplantation. All these patients would therefore benefit from strategies aimed at promoting correct differentiation of stem cells.

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References

- 1 Perl AK, Wert SE, Nagy A, Lobe CG, Whitsett JA (2002) Early restriction of peripheral and proximal cell lineages during formation of the lung. *Proc. Natl. Acad. Sci. USA* **99**, 10482–10487.
- 2 Jeffery PK, Hislop AA (2003) Embryology and growth. In: Gibson GJ, Geddes DM, Costabel U, eds. *Respir. Med.*, pp. 51–63. London: W.B. Saunders.
- 3 Rawlins EL, Ostrowski LE, Randell SH, Hogan BL (2007) Lung development and repair: contribution of the ciliated lineage. *Proc. Natl. Acad. Sci. USA* **104**, 410–417.
- 4 Rawlins EL, Hogan BL (2006) Epithelial stem cells of the lung: privileged few or opportunities for many? *Development* **133**, 2455–2465.
- 5 Borthwick DW, Shahbazian M, Krantz QT, Dorin JR, Randell SH (2001) Evidence for stem-cell niches in the tracheal epithelium. *Am. J. Respir. Cell Mol. Biol.* **24**, 662–670.
- 6 Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR (2004) Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am. J. Pathol.* **164**, 577–588.
- 7 Giangreco A, Reynolds SD, Stripp BR (2002) Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am. J. Pathol.* **161**, 173–182.
- 8 Reddy R, Buckley S, Doerken M, Barsky L, Weinberg K, Anderson KD *et al.* (2004) Isolation of a putative progenitor subpopulation of alveolar epithelial type 2 cells. *Am. J. Physiol. Lung. Cell Mol. Physiol.* **286**, L658–L667.
- 9 Park KS, Wells JM, Zorn AM, Wert SE, Laubach VE, Fernandez LG *et al.* (2006a) Transdifferentiation of ciliated cells during repair of the respiratory epithelium. *Am. J. Respir. Cell Mol. Biol.* **34**, 151–157.
- 10 Park KS, Wells JM, Zorn AM, Wert SE, Whitsett JA (2006b) Sox17 influences the differentiation of respiratory epithelial cells. *Dev. Biol.* **294**, 192–202.
- 11 Ling TY, Kuo MD, Li CL, Yu AL, Huang YH, Wu TJ *et al.* (2006) Identification of pulmonary Oct +4 stem/progenitor cells and demonstration of their susceptibility to SARS coronavirus (SARS-CoV) infection in vitro. *Proc. Natl. Acad. Sci. USA* **103**, 9530–9535.
- 12 Duan D, Sehgal A, Yao J, Engelhardt JF (1998) Lef1 transcription factor expression defines airway progenitor cell targets for *in utero* gene therapy of submucosal gland in cystic fibrosis. *Am. J. Respir. Cell Mol. Biol.* **18**, 750–758.
- 13 Summer R, Kotton DN, Sun X, Ma B, Fitzsimmons K, Fine A (2003) Side population cells and Bcrp1 expression in lung. *Am. J. Physiol. Lung. Cell Mol. Physiol.* **285**, L97–L104.
- 14 Giangreco A, Shen H, Reynolds SD, Stripp BR (2004) Molecular phenotype of airway side population cells. *Am. J. Physiol. Lung. Cell Mol. Physiol.* **286**, L624–L630.
- 15 Gomperts BN, Belperio JA, Rao PN, Randell SH, Fishbein MC, Burdick MD *et al.* (2006) Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury. *J. Immunol.* **176**, 1916–1927.
- 16 Kotton DN, Fabian AJ, Mulligan RC (2005) Failure of bone marrow to reconstitute lung epithelium. *Am. J. Respir. Cell Mol. Biol.* **33**, 328–334.
- 17 Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1–13.
- 18 Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ *et al.* (2003) *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.* **17**, 1253–1270.
- 19 Storci G, Sansone P, Trere D, Tavolari S, Taffurelli M, Ceccarelli C *et al.* (2008) The basal-like breast carcinoma phenotype is regulated by SLUG gene expression. *J. Pathol.* **214**, 25–37.
- 20 Rietze RL, Reynolds BA (2006) Neural stem cell isolation and characterization. *Methods Enzymol.* **419**, 3–23.
- 21 Veeman MT, Axelrod JD, Moon RT (2003) A second canon. Functions and mechanisms of β -catenin-independent Wnt signaling. *Dev. Cell* **5**, 367–377.
- 22 Blanpain C, Horsley V, Fuchs E (2007) Epithelial stem cells: turning over new leaves. *Cell* **128**, 445–458.
- 23 Reynolds SD, Zemke AC, Giangreco A, Brockway BL, Teisanu RM, Drake JA *et al.* (2008) Stabilization of β -catenin expands the pool of lung stem cells. *Stem Cells* **26**, 1337–1346.
- 24 Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV *et al.* (2000) Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci. USA* **97**, 14720–14725.
- 25 Bhatia M (2001) AC133 expression in human stem cells. *Leukemia* **15**, 1685–1688.
- 26 Park IK, Qian D, Kiel M, Becker MW, Pihlaja M, Weissman IL *et al.* (2003) Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**, 302–305.
- 27 Bussolati B, Bruno S, Grange C, Buttiglieri S, Deregibus MC, Cantino D *et al.* (2005) Isolation of renal progenitor cells from adult human kidney. *Am. J. Pathol.* **166**, 545–555.
- 28 Molofsky AV, He S, Bydon M, Morrison SJ, Pardoll R (2005) Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes Dev.* **19**, 1432–1437.
- 29 Rizzo S, Attard G, Hudson DL (2005) Prostate epithelial stem cells. *Cell Prolif.* **38**, 363–374.
- 30 Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW *et al.* (2006) Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res.* **66**, 6063–6071.
- 31 Laurson J, Selden C, Clements M, Mavri-Damelin D, Coward S, Lowdell M *et al.* (2007) Putative human liver progenitor cells in explanted liver. *Cells Tissues. Organs.* **186**, 180–191.
- 32 Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R *et al.* (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369–377.
- 33 Kleeberger W, Versmold A, Rothamel T, Glockner S, Bredt M, Haverich A *et al.* (2003) Increased chimerism of bronchial and alveolar epithelium in human lung allografts undergoing chronic injury. *Am. J. Pathol.* **162**, 1487–1494.
- 34 Suratt BT, Cool CD, Serls AE, Chen L, Varella-Garcia M, Shpall EJ *et al.* (2003) Human pulmonary chimerism after hematopoietic stem cell transplantation. *Am. J. Respir. Crit. Care Med.* **168**, 318–322.
- 35 Mattsson J, Jansson M, Wernerson A, Hassan M (2004) Lung epithelial cells and type II pneumocytes of donor origin after allogeneic hematopoietic stem cell transplantation. *Transplantation* **78**, 154–157.
- 36 Griffiths MJ, Bonnet D, Janes SM (2005) Stem cells of the alveolar epithelium. *Lancet* **366**, 249–260.
- 37 Auten RL, Watkins RH, Shapiro DL, Horowitz S (1990) Surfactant apoprotein A (SP-A) is synthesized in airway cells. *Am. J. Respir. Cell Mol. Biol.* **3**, 491–496.
- 38 Reynolds SD, Giangreco A, Power JH, Stripp BR (2000) Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. *Am. J. Pathol.* **156**, 269–278.

- 39 Madsen J, Tornøe I, Nielsen O, Koch C, Steinhilber W, Holmskov U (2003) Expression and localization of lung surfactant protein A in human tissues. *Am. J. Respir. Cell Mol. Biol.* **29**, 591–597.
- 40 Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S *et al.* (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* **121**, 823–835.
- 41 Thiery JP (2003) Epithelial-mesenchymal transitions in development and pathologies. *Curr. Opin. Cell Biol.* **15**, 740–746.
- 42 Hennrick KT, Keeton AG, Nanua S, Kijek TG, Goldsmith AM, Sajjan US *et al.* (2007) Lung cells from neonates show a mesenchymal stem cell phenotype. *Am. J. Respir. Crit. Care Med.* **175**, 1158–1164.
- 43 Locascio A, Manzanares M, Blanco MJ, Nieto MA (2002) Modularity and reshuffling of Snail and Slug expression during vertebrate evolution. *Proc. Natl. Acad. Sci. USA* **99**, 16841–16846.
- 44 Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. *Nat. Rev. Mol. Cell Biol.* **3**, 155–166.
- 45 Warburton D, Tefft D, Mailleux A, Bellusci S, Thiery JP, Zhao J *et al.* (2001) Do lung remodeling, repair, and regeneration recapitulate respiratory ontogeny? *Am. J. Respir. Crit. Care Med.* **164**, S59–S62.
- 46 Demayo F, Minoo P, Plopper CG, Schuger L, Shannon J, Torday JS (2002) Mesenchymal–epithelial interactions in lung development and repair: are modeling and remodeling the same process? *Am. J. Physiol. Lung. Cell Mol. Physiol.* **283**, L510–L517.
- 47 Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, Pedron S, Bertaso M, Scarpa A, Murer B, Cancellieri A, Maestro R, Semenzato G, Doglioni C (2003) Aberrant Wnt/ β -catenin pathway activation in idiopathic pulmonary fibrosis. *Am. J. Pathol.* **162**, 1495–1502.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Isotypic control mouse IgG₁, κ . Isotypic control used at the highest concentration.

Figure S2. Isotypic control rabbit polyclonal IgG. Isotypic control used at the highest concentration.

Table S1. Primers

Table S2. Comparison of stem cell marker expression in bronchospheres and in origin tissues

Table S3. Comparison of basal bronchial cells (cytokeratin 5) and pneumocyte II (CD44) marker expression in bronchospheres and in origin tissues

Table S4. Comparison of Slug gene expression in bronchospheres and in origin tissues

Table S5. Primers for real-time RT-PCR

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