Regulation of human lung alveolar multipotent cells by a novel p38α MAPK/miR-17-92 axis

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Feride Oeztuerk-Winder, Anna Guinot, Anna Ochalek and Juan-Jose Ventura*

CSCR (Wellcome Trust Centre for Stem Cell Research), Cambridge, UK

The cellular and molecular mechanisms that control lung homeostasis and regeneration are still poorly understood. It has been proposed that a population of cells exists in the mouse lung with the potential to differentiate into all major lung bronchioalveolar epithelium cell types in homeostasis or in response to virus infection. A new population of E-Cad/Lgr6+ putative stem cells has been isolated, and indefinitely expanded from human lungs, harbouring both, self-renewal capacity and the potency to differentiate in vitro and in vivo. Recently, a putative population of human lung stem cells has been proposed as being c-Kit+. Unlike Integrin-α6+ or c-Kit+ cells, E-Cad/Lgr6+ single-cell injections in the kidney capsule produce differentiated bronchioalveolar tissue, while retaining self-renewal, as they can undergo serial transplantations under the kidney capsule or in the lung. In addition, a signalling network involving the p38α pathway, the activation of p53 and the regulation of the miR-17-92 cluster has been identified. Disruption of the proper cross-regulation of this signalling axis might be involved in the promotion of human lung diseases.

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Introduction

The existence of a population of lung cells that could be the source of all main cell types of the lung bronchioalveolar epithelium is controversial and has been the focus of many recent investigations. Unlike in other tissues, the understanding of the cellular and molecular mechanisms that maintain adult lung homeostasis and may be involved in regeneration is in its infancy (Morrisey and Hogan, 2010; Rock and Hogan, 2011; Weiss et al, 2011). Recently, a number of groups have reported the existence of certain populations of putative stem cells in mouse lungs (Giangreco et al, 2002; Kim et al, 2005; Ventura et al, 2007; Teisanau et al, 2009; Chapman et al, 2011). These cells have the potential to differentiate into the major bronchiolar (Clara) and Alveolar type-1 (AT1) or type-2 (AT2) cells. However, if the cellular hierarchy of the mouse alveolar epithelium is still poorly known, the human lung biology has been even more neglected. A putative population of human lung stem cells (HLSCs) has been proposed as being c-Kit+ (Kajstura et al, 2011). This population would have the potential to differentiate not only into lung epithelial cells but also into mesenchymal and endothelial tissues. Nevertheless, this proposed cell type has been the subject of a much greater controversy and its origin and defined profile remain unclear (Anversa et al, 2011). Other stem cell markers have been extensively used to detect and isolate stem cells from various epithelial tissues (Kumar et al, 2011). Among the most specific markers found to label epithelial stem cells are the members of the family of leucine-rich repeat-containing G-protein-coupled receptors (Lgr), and in particular Lgr5 and Lgr6 (Barker et al, 2007; Barker and Clevers, 2010; Snippert et al, 2010).

Several extracellular signals have been linked to the regulation of embryonic lung stem cells during development (Que et al, 2007; Lange et al, 2009; Morrisey and Hogan, 2010; Rock et al, 2011). However, the intracellular mechanisms involved in adult lung homeostasis and regeneration are still mostly unknown. One intracellular signal involved in mouse lung homeostasis is the MAPK p38α pathway (Ventura et al, 2007). The activity of this kinase is necessary to maintain the differentiation mechanisms and control the self-renewal of mouse lung stem cells (Hui et al, 2007; Ventura et al, 2007; Liu et al, 2008). p38α regulates transcription factors (e.g., C/EBPα) involved in lung differentiation (Efimova et al, 2002; Martis et al, 2006) and downregulates several factors involved in stem/progenitor cell proliferation (Sugahara et al, 2001; Ventura et al, 2007). Nevertheless, the mediators and cross-talking pathways that may be involved in maintaining HLSCs homeostasis are not yet known.

Besides kinase pathways, the role of some microRNAs in the regulation of lung development and cancer has been reported (Qian et al, 2008). The miR-17-92 cluster has been found to play roles in both processes (Mendell, 2008), with a relevant function in the lung. The miR-17-92 cluster is associated with a negative regulation of lung development (Lu et al, 2007; Ventura et al, 2008) and a promotion of cancer cell proliferation (Hayashita et al, 2005). One way to regulate the activity of this cluster is by controlling its levels, and miR-17-92 promoter activity can be suppressed by the transcription factor p53, downregulating pri-miR-17-92 expression (Yan et al, 2009).

Understanding the mechanisms involved in the proper function of HLSCs, and defining potential markers that could be used to detect and isolate a specific and homogeneous population of lung stem cells are absolutely essential, prior to considering any possible cellular or molecular therapy involving stem cells in the human lung.
Results

Isolation and characterization of a human alveolar E-Cad/Lgr6 multipotent population

The lack of studies investigating adult lung homeostasis prompted us to focus on the identification and isolation of a population of putative HLSCs. Using a similar protocol as Kim et al. (2005) but CD34 – (Ventura et al., 2007), cells have been isolated from mouse lungs that could be indefinitely expanded in vitro while retaining their self-renewal and differentiation potential (Ventura et al., 2007). A similar, modified approach was used in an attempt to detect and isolate HLSCs.

The stem cell marker Sca-1 was previously used to isolate mouse stem cells (Ventura et al., 2007). However, it is not present in humans, and other markers that might be specifically expressed in human lung cells were investigated (Holmes and Stanford, 2007). The expression, in human lungs, of published putative epithelial and stem cell markers, which may be suitable to be used as targets, such as E-Cadherin, c-Kit, Integrin-α6, Lgr5 and Lgr6, was tested. Only Lgr6 was found to be restricted to a discrete population of E-Cadherin-positive cells (Figure 1A and B; Supplementary Figure S1A and B) that did not express other lung differentiation markers. They localized mainly near small bronchioles (Figure 1A; Supplementary Figure S1E) and endothelium (Supplementary Figure S1A and D) and co-expressed Lgr5 (Supplementary Figure S1C). c-Kit (Figure 1C) and Integrin-α6 (Figure 1D) were expressed in a heterogeneous number of cell types, including haematopoietic and endothelial lineages, and Lgr5 was also expressed in Clara cells (Supplementary Figure S2D). Lgr6 and c-Kit did not co-express (Figure 1C; Supplementary Figure S2A and B) labelling distinct cells. Based on the previous results, several populations were sorted using a preliminary negative selection to avoid mesenchymal, endothelial or haematopoietic (Lin−) contaminants (Figure 1E). Single cells (from four human lung samples) from the different populations were used to test for clonal capacity in serial dilution assays (Supplementary Figure S2E). E-Cad−/Lgr6− and e-Kit+ single cells failed to grow clonally in vitro and only two clones (15%) of single Integrin-α6− cells grew after four passages (Figure 1F). However, 13 of 25 (52%) E-Cad/Lgr6+ single-cell clones were successfully expanded for >15 passages (Figure 1F). E-Cad/Lgr6+ cells expressed Integrin-α6 (Figure 1D; Supplementary Figure S2C) and could be considered as a sub-population within the lung Integrin-α6 heterogeneous population.

Clonally derived E-Cad/Lgr6+ cells (HLSCs) grew in vitro forming aggregates that could be expanded for >50 passages while expressing lung-specific (SP-C, CC-10, AQ5), epithelial (E-Cad) and stem cell markers (Sox9, Lgr5/6, Integrin-α6) (Supplementary Figure S3). Although in vitro E-Cad/Lgr6+ cells did not express the AT2 (SP-C) and Clara (CC-10) cell markers, the in vitro aggregates were positive for these lung markers (Figure 2A). In general, there was a reduction of lung-specific and epithelial markers, and an increase in mRNA expression of stem cell markers in the clonally expanded (HLSCs) and the freshly isolated E-Cad+/Lgr6+ cells, compared to E-Cad+/Lgr6− (Figure 2B; Supplementary Figure S3). In vitro, HLSCs responded to matrices morphologically, forming monolayers, and molecularly, differentially expressing AT2 (SP-C) or Clara (CC-10) cell markers (Figure 2C). HLSCs carrying an EGFP reporter under the control of the CC-10 promoter maintained promoter activity and CC-10 protein levels in fibronectin but lost SP-C expression (Figure 2D, upper). However, on laminin HLSCs shutdown the CC-10 promoter but maintained the alveolar SP-C (Figure 2D, lower) and AQ5 (Supplementary Figure S2F) expression.

Regenerative potential of E-Cad/Lgr6+ cells

Ex-vivo and in-vivo approaches were used to functionally test the stem cell potential of E-Cad/Lgr6+ (double positive) cells using a bleomycin-induced lung injury model (Aso et al., 1976). E-Cad/Lgr6+ or HLSCs carrying a PGK-EGFP reporter were injected into human lung explants that had been treated with bleomycin in vitro (Supplementary Figures S4A and S5B). The injected cells migrated from the site of injection to the epithelium (Supplementary Figure S4B). HLSCs replenished the dead cells at the damaged alveoli (Figure 3A). The stem cells differentiated and acquired the morphology of AT1 or AT2 cells in the alveoli (Figure 3B). HLSCs were not only migrated but they also got integrated into the endogenous human tissue, mixed with the remaining surviving cells, and differentiated into polygonal AT2 (SP-C positive), elongated AT1 (AQ5), or cuboidal Clara (CC-10) cells, regenerating the bronchioalveolar tissue (Figure 3C; Supplementary Figures S4C and S5). Human lungs have a reduced proportion of bronchial tissue compared to mouse, so the contribution to Clara cell (CC-10 positive) differentiation was marginal.

An in-vivo model of bleomycin-induced lung injury was further used to study the potential of E-Cad/Lgr6+ cells to regenerate bronchioalveolar tissue. Bleomycin was injected into the tail vein of nude mice prior to the injection of HLSCs. Mouse tail vein injection (TVI) has been extensively used to deliver cells to the lungs (Kennedy et al., 2003), allowing HLSCs to migrate into the damaged alveolar epithelium (Supplementary Figure S4D). After 10 days, the animals were sacrificed and the lungs were histologically examined by immunofluorescence or used to isolate the resident HLSCs. EGFP+ HLSCs contributed to regenerate the damaged tissue forming small bronchioles (Clara cells) or alveoli (AT1 and AT2 cells), and the engrafted cells expressed specific bronchial (CC-10) or alveolar (SP-C, AQ5) markers (Supplementary Figures S4E and S6). The human origin of the engrafted cells was confirmed with a specific anti-human mitochondrial antibody (Supplementary Figure S4F).

Single E-Cad/Lgr6+ cell ability to produce epithelium and recruit a niche

The in-vivo stem cell potential of E-Cad/Lgr6+ cells was further examined using kidney capsule engraftments, which have been used to test other tissue stem cells (Eirew et al., 2008). Different dilutions of sorted E-Cad+/Lgr6+, E-Cad−/Lgr6− or clonal HLSCs were injected under the kidney capsule, and the grafts were examined at different times, showing dose- and time-dependent growth (Figure 4A; Supplementary Figure S7A). The engrafted human E-Cad+/Lgr6+ cells were distinguishable from the mouse kidney using a commercial specific human nuclear antibody (Figure 4B). E-Cad+/Lgr6+ and clonal HLSCs cells engrafted in the kidney, even with single-cell injections, but E-Cad−/Lgr6− cells failed (Figure 4C). Only the single-cell injections were carried out in matrigel to avoid the spillage of the content. Injections of higher number of cells were performed with the cells in PBS solutions. As shown in previous clonal
assays, comparative analysis showed a superior stem cell potential of E-Cad+/Lgr6+/ c-Kit+ or Integrin-α6+ cells in single-cell kidney grafts (Figure 4D). Clonal EGFP expressing cells were used for further characterization of the kidney grafts, allowing better tracking of HLSCs differentiation (Supplementary Figure S7B). After 8 weeks, grafts from single EGFP+ HLSCs injections still harboured small pools of Lgr6+ undifferentiated cells (Figure 4E). Retention of self-renewal potential was demonstrated with serial transplantation of sorted EGFP+ cells from kidney engraftments or cross-transplantation with cells from lung or kidney grafts (Figure 4F).

Figure 1 Isolation, clonal expansion and in vitro characterization of human lung stem cells. (A) Confocal section, of a 3D image, showing E-Cad/Lgr6+ cells nearby small bronchioles (SB) in the human lung. (B) E-Cad/Lgr6+ cells (yellow arrows) in the epithelium and E-Cad+ epithelial cells (green arrows). (C) Immunofluorescent staining of human lung tissue with c-Kit+ (green) and Lgr6+ (red) labelling different cells. (D) A small number of Integrin-α6+ cells (green) express Lgr6 (yellow) in the human lung bronchioalveolar epithelium. (E) Lung cells isolated from human lung tissue (from three different patients), and then negative sorted for CD45, CD31, CD73 and CD34 (Lin−) and positive for E-Cad and Lgr6, c-Kit or Integrin-α6 and were used for functional assays or clonal expansion. (F) Clonogenicity assay of E-Cad/Lgr6+, c-Kit+ or Integrin-α6+ single sorted cells. Cells from three different patients were used. Positive colonies (red) and total single cells seeded (black). Images of E-Cad/Lgr6+ single cell first division at day 1 and clonal aggregate at day 7 (see also Supplementary Figure S1).
EGFP+ HLSCs remained epithelial in the kidney grafts, but were able to recruit connective and endothelial tissues to the graft to generate a microenvironment (Supplementary Figure S7C). The engrafted tissue resembled a bronchioalveolar epithelium (Supplementary Figure S7D), with alveolar- and bronchiolar-like (Clara cells) structures formed by cells with AT1 and AT2 or Clara cell morphologies (Supplementary Figure S7E). Alveolar and bronchiolar (AT2, AT1 or Clara) cells expressed the specific markers SP-C, AQ5 and CC-10, respectively (Figure 4G; Supplementary Figures S7F and S8A). The human origin of the engrafted epithelium was confirmed with a specific human nuclear DNA antibody (Supplementary Figure S8B).

**p38α activity in HLSCs regulates miR-17-92 levels to maintain homeostasis**

p38αMAPK has been previously shown to regulate the homeostasis of mouse lung stem cells (Ventura et al., 2007). However, the intermediate molecules and cross-talking pathways involved in this physiological control remain elusive. As it was not possible to use the same genetic deletion strategy to study human cells, RNAi was employed to dissect the mechanisms involved in p38α regulation of HLSCs (WT HLSCs carried a scrambled siRNA in all RNAi experiments). Four siRNAs against p38α were tested in HLSCs (Supplementary Figure S9A). Two siRNAs to generate shp38α lentiviral constructs capable of stable p38α knockdown in clonal HLSCs (SH2/SH3) (Figure 5A). These cells had increased proliferation similar to p38α−/− mouse lung cells (data not shown). Protein analysis showed that lack of p38α in HLSCs disrupted the expression of both lung markers and integrins (Figure 5B; Supplementary Figure S9C–E). However, the stem cell marker Sox9 was expressed at higher levels.

Possible mediators and pathways potentially involved in p38α function were then investigated. One focus of interest was the miR-17-92 cluster of microRNAs, as it has been reported to have an opposing role to p38α, acting as a suppressor of lung differentiation while promoting lung cell proliferation (Hayashita et al., 2005; Qian et al., 2008; Ventura et al., 2008). Analysis of RNA showed upregulation of this cluster in HLSCs lacking p38α (Figure 5C). A potential cross-talk between these two signals was then investigated. Interestingly, it has been reported that p53 may repress miR-17-92 expression (Van et al., 2009). Furthermore, it is also known that the downstream p38α target MK2 (MAPKAPK-2) (Stokoe et al., 1992) can phosphorylate p53 (Ser20), switching on p53 transcriptional activity (She et al., 2002; Hsu et al., 2011). A similar RNAi strategy was used to study MK2, and its expression was downregulated by specific siRNAs (Supplementary Figure S9B). Knockdown of MK2 in HLSCs resulted in the loss of p53 Ser20 phosphorylation (Figure 5D). Either lack of MK2 (Figure 5E) or inhibition of p53 transcriptional activity (Figure 5F) correlated with increased pri-miR-17-92 levels. Direct repression by p53 was confirmed using a luciferase reporter vector controlled by the human miR-17-92 promoter containing a p53-binding site that has been linked to miR-17-92 repression (Van et al., 2009). Chemical inhibition of p53 transcriptional activity in HLSCs induced miR-17-92 promoter activity, which was already constitutively active in SH3 (Figure 5G). All mature miR-17-92 components were upregulated in SH3 cells (Figure 5H). This demonstrated a cross-talk between the p38α pathway and a microRNA cluster in HLSCs.

**miR-17-92 regulates lung transcription factors and HLSCs differentiation**

Having demonstrated a negative regulation of miR-17-92 expression by p38α, the cellular and physiological consequences of that cross-talk were investigated. RNAi was used to study the downstream mediators of miR-17-92. Downregulation of this microRNA cluster and p38α with shRNAs allowed further examination of the role of miR-17-92 in HLSCs (Figure 5E and H). As has been previously shown in mouse lung, p38α deficiency is accompanied by reduced expression of lung differentiation factors, although the mechanism supporting this correlation was still unknown (Ventura et al., 2007). Interestingly, among the predicted targets for miR-17-92 found in public databases (TargetScan Human5.1, PicTar) are several lung differentiation transcription factors, including C/EBPα and GATA6. C/EBPα is downregulated in the lungs of p38α−/− mice (Ventura et al., 2007). C/EBPα and GATA6 were found to be downregulated in SH3 cells in a miR-17-92-dependent manner, as normal protein and mRNA levels could be rescued following
the concurrent knockdown of miR-17-92 (Figure 6A and B). miR-17-92 did not regulate other non-target lung differentiation transcription factors, such as TTF-1 (Figure 6A and B). Direct repression of C/EBPα and GATA6 by miR-17-92 was confirmed using luciferase reporters carrying the 3′-UTR of each gene containing the putative target site for miR-92, a member of the miR-17-92 cluster. Luciferase activity was downregulated in SH3 cells, but not in a 3′-UTR carrying a non-binding mutated target sequence, and that activity was restored by concomitant knocking down of miR-17-92 (Figure 6C). Conversely, the TTF-1 3′-UTR was not sensitive to miR-17-92 levels (Figure 6C). Downregulation and rescue of these transcription factors levels correlated with changes in the expression of lung-specific markers and stem cell markers (Figure 6D), together with misexpression of integrins (Figure 6E; Supplementary Figure S9F).

To assess the functional role of the p38α/miR-17-92 network in HLSCs, their in-vivo stem cell potential was...
examined using kidney capsule engraftments. SH3 cell injections failed to produce kidney engraftments (Figure 7C). The inability of SH3 to form kidney grafts was further confirmed by co-injecting a mix of HLSCs, WT (EGFP) and SH3 (H2B-cherry) cells (Figure 7A). Only WT, but not SH3 cells differentiated and produced alveolar-like tissue (Figure 7A). The involvement of the miR-17-92 cluster mediating the functional role of p38α was examined using p38α/miR-17-92 concomitant knockdown cells (SH3/miR). Restoration of miR-17-92 levels to normal in SH3 cells rescued the potential to produce lung-like epithelium in kidney grafts (Figure 7B). The stem cell potential of HLSCs to differentiate in vivo was controlled by p38α and miR-17-92 in a coordinated way (Figure 7C). It could be concluded that p38α repressed miR-17-92, via p53 activation, in order to maintain the proper balance between differentiation and self-renewal potential in HLSCs (Figure 7D).
Figure 5 Mechanism of regulation of HLSC homeostasis by p38α involving the MK2-p53-dependent downregulation of pri-miR-17-92 expression. (A) HLSCs stably expressing a p38α short hairpin lentiviral vector (shp38α) (SH3) knocked down p38α protein levels. (B) Western blot analysis of lung-specific and stem markers and integrins in total lysates of WT or SH3 cell in vitro. SH3 cells had disrupted levels of lung and stem cell markers. (C) HLSCs lacking p38α (SH3) showed overexpression of pri-miR-17-92. Values are depicted as mean ± standard error of the mean (s.e.m.) from four different experiments. (D) Protein blots of WT, SH3, SH3 + si-miR or WT + si-MK2 cells. SH3 (lacking P-MK2 activation) and HLSCs deficient in the p38α downstream target MK2, and p53 Serine 20 phosphorylation. (E) HLSCs lacking MK2 overexpressed pri-miR-17-92. A representative experiment of five replicates is depicted as the mean of five different values ± s.e.m. (F) Chemical inhibition of p53 (p53I) increased pri-miR-17-92 levels but reduced the p53 target gene p21. A representative experiment of three replicates is depicted as the mean of five different values ± s.e.m. (G) p53 chemical inhibition or p38α knockdown (SH3) induced pri-miR-17-92 promoter activity. Data are mean of three independent experiments ± s.e.m. (H) miR-17-92 components were overexpressed in SH3 cells but the levels were restored to normal by compound knockdown with a pri-miR-17-92 siRNA.

Discussion

There have been extensive efforts and reports in the search for common stem/progenitors of the adult lung bronchioalveolar epithelium. However, adult lung stem cell research is still in its infancy and most previous studies have focused on the proximal airways (Johnson and Hubbs, 1990; Engelhardt et al., 1995; Hong et al., 2004) or isolated cells of an uncertain origin and controversial stem cell potential (Fujino et al., 2011; Kajstura et al., 2011). In particular, the knowledge about cell hierarchy in human lungs is still very limited. Here, we show the existence of a distinct population of human alveolar stem cells, with a defined signature, that can be isolated and clonally expanded in culture while maintaining their potential to differentiate. This population could be considered as a sub-population of the number of Integrin-α6+ cells in the human lung. They harbour self-renewal, shown by in-vitro indefinite expansion and in-vivo serial transplantation experiments and differentiation capacity, as would be expected from a stem cell. The in-vitro and in-vivo stem cell potential of this population has been demonstrated with some of the most commonly used techniques to test tissue stem cells, including kidney capsule or lung injury engraftments. Unlike other reported putative HLSCs (Kajstura et al., 2011), our HLSC population has an epithelial origin and does not differentiate into mesenchymal or endothelial cells. E-Cad/Lgr6+ but not c-Kit+, single-cell injections produce lung epithelium in the kidney, and they did not require an extra-stromal compartment like the reported Integrin-α6/β4 mouse lung cells (Chapman et al., 2011). This comparative
study has shown the superior stem cell potential of human lung E-Cad/Lgr6 \(^+\) cells. Indefinite expansion of lung E-Cad/Lgr6 \(^+\) cells brings new possibilities, such as their use in pharmacological screenings, generation of disease models or genetic manipulation to repair defective mutations.

In addition, it could be demonstrated that regulation of HLSCs relies on an integrated network involving p38\(\alpha\) and the miR-17-92 cluster. Both signals have been previously related to lung differentiation and proliferation, but the molecular insights of this function remained unknown. A negative regulation of miR-17-92 expression has been discovered through the transcriptional activation of p53 by the p38\(\alpha\) pathway. Defects in the fine-tuning between these two signaling pathways may result in disease (e.g., cancer) or defective regeneration (e.g., lung fibrosis) (Hayashita et al., 2005; Mendell, 2008). p38\(\alpha\) deficiency produces an unbalanced increase of miR17-92 levels that downregulate lung-specific transcription factors and results in misexpression of integrins. Integrins are involved in the response of stem cells to extracellular matrix-directed terminal differentiation (Watt, 2002). Thus, the p38\(\alpha\)/miR-17-92 axis regulates the intracellular machinery and the response to extracellular signals involved in lung stem cell fate decision. Disruption of this network in p38\(\alpha\)-deficient cells causes loss of the potential of HLSCs to engraft and differentiate in the kidney capsule. HLSCs differentiate into epithelium, but they can also recruit endothelium and connective tissue to create a proper microenvironment. The contribution of differentiation factors and/or integrins in establishing a lung stem cell niche is still unclear. Delineation of the molecular mediators and functional effectors in this network may contribute to a better understanding of the regulation of HLSCs in homeostasis and disease.

The E-Cad/Lgr6 \(^+\) population adds pieces to the cellular puzzle regulating lung homeostasis and the regenerative response to injury. This population appears to have a larger stem cell potential, ranking higher in the lung bronchioalveolar hierarchy than other of the previously reported cell types, although the existence of cells of a higher stemness in the bronchioalveolar epithelium cannot be discounted (as it is in the haematopoietic system).

It highlights new molecular targets that may help to easy detection and isolation of human lung bronchioalveolar multipotent cells. Overall, our work provides a reliable model for in-vitro studies, a more complete understanding of the cellular mechanisms regulating lung cell fate decision, and a leap forward in the search for potential lung cellular and molecular targets for regenerative therapies.

Materials and methods

Isolation and culture of human lung progenitor cells

Human lung tissue was obtained from patients undergoing lung resection (Papworth Hospital, UK). This study was approved by the Ethics Committee at Cambridge University and the Papworth Hospital. All subjects gave informed consent. All samples collected were healthy lung tissue biopsies from cancer patients, used in the clinic as a control. No samples from patients with COPD or other inflammatory diseases were used to isolate stem cells.

Normal lung was used for cell isolation and histological evaluation. After plura was separated bluntly, lung specimens were finely minced and resuspended in collagenase (0.5–3 mg/ml, Whorthington)/dispase (1 mg/ml, Invitrogen) containing DMEM (Invitrogen) and incubated for 30–45 min at 37°C in a shaking incubator. The suspension was spun for 5 min at 1200 r.p.m. and the supernatant removed. The pellet was resuspended in fresh DMEM containing 0.1 mg/ml DNase (optional) and incubated for further 5–10 min. The suspension was washed with PBS, filtered through cell strainers (100, 70 \(\mu\)m, BD) and treated with red blood
cell lysis buffer (Roche Applied Science). Following further filtration (40 μm mesh) and centrifugation (5 min at 1200 r.p.m.), the isolated cells were cultured in RH-B (Stem Cell Science) medium containing 2% FCS, with additional insulin (5 μg/ml, Pepro Tech), EGF (10 ng/ml, Pepro Tech) and FGF2 (20 ng/ml, Pepro Tech) for 2 days. This was then replaced with fresh, serum-free medium containing growth factors (37 °C in a 7% humidified CO₂ incubator).

**Lentiviral vector preparation**

The lentiviral vector pSINPGKEGFP was used to generate pSINhCC10EGFP, containing the human CC10 promoter. Lentiviral particles were produced by co-transfecting 293T cells with pSINPGKEGFP or pSINhCC10EGFP, pCMV Δ8.9 and pMD.G (encoding VSV-G), as previously described (Naldini et al., 1996; Capowski et al., 2007).

Four siRNAs (Thermo Scientific) were tested to knockdown p38α shRNA n.3 was cloned into the PLKO.1-TRC (Sigma) lentiviral vector to generate a p38α knockdown construct (SH3). Infectious virus was added to cells in the presence of 8 mg/ml polybrene (hexadimethrine bromide, Sigma) and incubated for 6 h.

A commercial (Applied Biosystems) siRNA was used to knock-down the pri-miR-17-92: sense-GGAGAGCUAAUCUGCACAtt. Luciferase assays

To test miR-17-92 promoter activity and transcription factors 3′-UTRs processing, pGL3 reporter vectors containing the firefly luciferase were used (with a pGL3 basic for the promoter and the pGL3-Vector control for the 3′-UTR as activity controls). As a control of the transfection efficiency a vector expressing the Renilla luciferase was used.

The 0.5 and 0.7 kb fragments of the miR-17-92 promoter were obtained by PCR using specific primers (see Supplementary data), and cloned in the promoterless pGL3 basic vector after NheI/XhoI restriction digestion. The 3′-UTRs of the C/EBPα, GATA6 and TTF1 genes were obtained by PCR using specific primers (see Supplementary data) and cloned in the pGL3-Vector control after XbaI digestion.

Mutant 3′-UTRs were created introducing a mutation in the miR-92a target sequence of the C/EBPα and GATA6 3′-UTRs using the appropriate oligos (see Supplementary data).

**In-vitro assessment of differentiation potential**

Cells were grown to low confluence on 10 μg/ml fibronectin (Millipore) or laminin- (in PBS, Sigma) coated tissue culture dishes (24- or 6-well plates). All differentiation cultures were maintained for 10–15 days, medium (containing 2–5% FCS) being renewed every 72 h.

**Immunofluorescent staining**

Cultured cytospin (800 g, 3 min) cells were fixed with 4% paraformaldehyde (PFA) for 15–20 min at RT. The remaining human tissues were also fixed with 4% PFA for 24 h and embedded in paraffin or OCT compound (Sakura, UK) after 30% sucrose treatment (at 4°C for 24 h). Samples were blocked and permeabilized with 0.1% Triton-X/4% goat serum/PBS for 60 min at RT. Cells were incubated with primary antibodies overnight at 4°C and then washed three times with PBS at room temperature (5 min per wash). Secondary antibody (Alexa Fluor 488 and/or Alexa Fluor 594 secondary
antibodies, Invitrogen) incubation took place for 1 h at RT. Cells were visualized using DAPI (4',6-Diamidino-2-phenylindole) counterstaining. Images were collected using a Leica SP5 confocal microscope.

Mouse experiments
All mouse experiments were performed according to UK Home Office Regulations. CD-1<sup>+</sup> nude mice (Charles River) were maintained under standard pathogen-free conditions.

Kidney capsule engraftments
Six- to eight-week-old mice were anaesthetized with isoflurane (10:5:2%). Cells were dissociated with accutase to generate a single-cell suspension (1–1 × 10<sup>6</sup> cells in 20 μl PBS) and this suspension was injected under the kidney capsule. Mice were killed 2, 4, and 6 weeks later and the kidneys harvested to examine in-vivo differentiation of the injected cells. The engraftments were removed and prepared for immunofluorescent microscopy.

For transplantation experiments, the GFP engrafted cells were sorted prior to be injected into kidney capsule or use for TVI for lung co-transplantations.

Bleomycin treatment of mice and lung stem cell transplantation
Six- to eight-week-old mice were given one tail vein (t.v.) injection of 5 U/kg of bleomycin in 100 μl PBS. Control animals were given an equivalent volume of PBS. Control groups received PBS and experimental groups received bleomycin. Forty-eight hours later, the experimental group received the stem cells (t.v.). Each group had six mice (repeated three times), which were analysed 10 days post bleomycin/progenitor cell injection. Lungs were fixed overnight with buffered neutral formalin 10% (VWR) or 4% PFA at room temperature. Tissues were then processed for paraffin embedding or for cryosectioning. Slides were stained in Mason’s Trichrome stain (Fisher Scientific) and H&E (Dako), according to manufacturer’s instructions. Cryosections were analysed for the presence of human lung cells by microscopy.

For co-transplantation experiments, the GFP cells engrafted in mouse lungs were sorted prior to be used for further injections.

Bleomycin treatment of mice and human lung explants ex vivo
Human or adult mouse lungs were cultured as slices (200–800 μm thickness) and exposed to bleomycin (3 days) in vitro. After injury, EGFP-labelled HLSCs were microinjected into the lungs and cultured for 7–10 days. Lungs were fixed overnight with buffered neutral formalin 10% (VWR) or 4% PFA at room temperature. Tissues were then processed for paraffin embedding or for cryosectioning for the presence and differentiation of the HLSCs.

Colony assays
To isolate and expand clonogenic cells, cells were dissociated with Accutase (PAA). Single cells that were seeded into 96-well plates by limited dilutions. The cells were maintained in stem cell restricted medium RH-B (Stem Cell Science) containing 2% FCS, with added limited dilutions. The cells were maintained in stem cell restricted media with fresh, serum-free medium containing growth factors (37 °C, 7% humidified CO<sub>2</sub> incubator). After 14 days, the number of wells with colonies was counted. Every assay was repeated four times.

Total RNA isolation and quantitative RT–PCR
Total RNA was extracted using TRIzol (Invitrogen) and was DNAase I (Promega) treated. In all, 1 μg RNA was reverse transcribed per sample (Bio-Rad), according to manufacturer’s instructions. Quantitative Real-Time PCR (qPCR) was used to determine the expression levels of the different genes using human-specific primer pairs (Eppendorf, Realplex<sup>®</sup>). Reaction conditions for amplification were as follows: first step of 95 °C 20 s, then 40 cycles of three-step 95 °C 1 s, 60 °C 30 s and 68 °C 20 s with 2 μl of cDNA per reaction in 10 μl SYBR Green PCR Master Mix (Applied Biosystems). Specificity of PCR products was tested by dissociation curves. Threshold cycles of primer probes were normalized to a housekeeping gene (GAPDH or HPRT) and relative values calculated (Livak and Schmittgen, 2001).

Preliminary expression was quantified by TaqMan qPCR and normalized to GAPDH-expression (see Supplementary data). In all, 2 μl of cDNA was used per 10 μl of Taqman Fast Universal PCR Master Mix (2×). The cycles were first step of 95 °C 20 s, then 40 cycles two-step 95 °C for 1 s and 60 °C 20 s (default set-up in StepOne machine). VIC-labelled human GAPDH from Applied Biosystems was used as internal control.

Immunoblot analysis
Proteins were extracted and analysed as previously described (Ventura et al., 2007), to confirm that observed changes in mRNA expression were reflected in the amount of protein present.

Antibodies
For western blotting and immunohistochemistry. Anti-human CC-10 (Santa Cruz, SC-25554), anti-human SP-C (Santa Cruz, SC-7705), anti-human AQP-5 (Santa Cruz, SC-28628), anti-human LGR6 (Santa Cruz, SC-48244), anti-human LGR5 (Santa Cruz, SC-68580), anti-human LGR5 (Sigma, HPA012530), anti-human Integrinα6 (Santa Cruz, SC-10730), anti-human C-Kit (Dako, A4502), anti-human Sox-9 (Millipore, AB5535), anti-human mitochondriald Ab (Thermo, MAS-12017), mouse anti-human nuclei Ab (Millipore, MAB1281), TTF-1 (Abcam, ab 40880), α-tubulin (Sigma, T9026), p38β (Cell Signaling, 9228), MAPKAPK2 (Cell Signaling, 3042), P-MAPKAPK2 (Cell Signaling, 3007), p-53 (Cell Signaling, 2524), p-p53 (Cell Signaling, 9287), C/EBPα (Santa Cruz, sc-61), Integrin β-1 (Santa Cruz, sc-6622).

For flow cytometry and immunofluorescence. Single-cell FACs was performed (MoFlo, Dako) following incubation with anti-human LGR6 (Santa Cruz, SC-99123), anti-e-cadherin (CD324, Biogen, 50-3249), anti-Integrinα6 (CD49f, Biogen; 12-0495), anti-α SMA) (Sigma, F3777), anti-CD31 (Biogen, 303104), anti-CD34 (Biogen, 316410), anti-C-Kit (CD117, Biogenet, Cat: 323416), anti-C-Kit (CD117, Biogenet, Cat: 313202), anti-CD73 (Abcam, ab54217), anti-CD45 (Biogenet, 304006), anti-human CD105 (Biogenet, 323204), anti-CD117 (Biogenet, 313202), and anti-human e-cadherin (AbCam, Ab53033) antibodies.

Secondary antibodies. All the corresponding secondary antibodies (mouse, rabbit, goat, rat, 488, 555, 594, 647) were Alexa Fluor from Invitrogen and PE from Santa Cruz.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Authors contribution: FO-W performed experiments and designed experiments, and analysed results. AG performed experiments and analysed results. AO performed experiments and designed experiments, analysed results and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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