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Mesenchymal Stromal Cells are Readily Recoverable from Lung Tissue, but not the Alveolar Space, in Healthy Humans

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ABSTRACT

Stromal support is critical for lung homeostasis and the maintenance of an effective epithelial barrier. Despite this, previous studies have found a positive association between the number of mesenchymal stromal cells (MSCs) isolated from the alveolar compartment and human lung diseases associated with epithelial dysfunction. We hypothesised that bronchoalveolar lavage derived MSCs (BAL-MSCs) are dysfunctional and distinct from resident lung tissue MSCs (LT-MSCs). In this study, we comprehensively interrogated the phenotype and transcriptome of human BAL-MSCs and LT-MSCs. We found that MSCs were rarely recoverable from the alveolar space in healthy humans, but could be readily isolated from lung transplant recipients by bronchoalveolar lavage. BAL-MSCs exhibited a $CD90^{Hi}$, $CD73^{Hi}$, $CD45^{Neg}$, $CD105^{Lo}$ immunophenotype and were bipotent, lacking adipogenic potential. In contrast, MSCs were readily recoverable from healthy human lung tissue and were $CD90^{Hi\ or\ Lo}$, $CD73^{Hi}$, $CD45^{Neg}$, $CD105^{Int}$ and had full tri-lineage potential. Transcriptional profiling of the two populations confirmed their status as bona fide MSCs and revealed a high degree of similarity between each other and the archetypal bone-marrow MSC. 105 genes were differentially expressed; 76 of which were increased in BAL-MSCs including genes involved in fibroblast activation, extracellular matrix deposition and tissue remodelling. Finally, we found the fibroblast markers collagen 1A1 and α-smooth muscle actin were increased in BAL-MSCs. Our data suggests that in healthy humans, lung MSCs reside within the tissue, but in disease can differentiate to acquire a profibrotic phenotype and migrate from their in-tissue niche into the alveolar space. STEM CELLS *2016;34:2548–2558*

SIGNIFICANCE STATEMENT

This investigation comprehensively characterizes and contrasts human lung derived mesenchymal stromal cells (MSCs) from tissue and bronchoalveolar lavage. MSCs isolated by bronchoalveolar lavage are rare in healthy individuals and express multiple hallmarks of fibrotic differentiation.

INTRODUCTION

Complex organisms possess a remarkable capacity for extensive and sustained tissue renewal which is mediated by reservoirs of self-renewing somatic tissue stem cells. These stem cells do not function in isolation, but rather reside in specialized niches; dependant on inputs provided by multiple cells, soluble factors and the surrounding matrix. Among the most important of these inputs are those provided by mesenchymal stromal cells (MSCs). While the best characterized stem cell niche is that found in the bone marrow; the hematopoietic stem cell niche [1], an analogous niche also exists in lung. In the pulmonary stem cell niche, type 2 pneumocytes function as stem cells where their proliferation and differentiation is coordinated by cues from lipofibroblasts and other mesenchymal cells [2]. MSCs increase the proliferative potential of a key epithelial progenitor cell—the bronchoalveolar stem cell [3] and, remarkably, restore bioenergetics in damaged lung epithelium and induce repair programmes through mitochondrial donation [4, 5]. Underscoring their critical role in organ homeostasis, the loss of tissue resident MSCs contributes to the pathology of murine bleomycin induced pulmonary fibrosis [6]. Together, these studies suggest that epithelial progenitors and MSCs work hand-in-hand to achieve epithelial repair

following injury and so maintain lung homeostasis. Identifying the phenotype and function of MSCs in human lung is therefore of increasing interest to researchers studying lung diseases characterized by aberrant epithelial repair such as; asthma, chronic obstructive pulmonary disease, post-transplant obliterative bronchiolitis and pulmonary fibrosis.

The International Society for Cellular Therapy has developed a set of three criteria to determine if a cell is an MSC. Cells must be adherent to plastic when seeded in vitro; express the surface proteins CD105, CD90, CD73 and lack the expression of CD45 and finally, be able to differentiate into each of the mesenchymal lineages; bone, fat, and cartilage [7]. In the human lung, cells which fulfil this criterion have been isolated from two sources; digested parenchymal lung tissue (LT-MSCs) [8, 9] and bronchoalveolar lavage fluid from lung transplant recipients (BAL-MSCs) [10]. It remains to be determined if these cells represent one population of endogenous lung MSCs or unique populations with specialized functions specific for distinct pulmonary stem cell niches. BAL-MSCs are derived from the donor allograft [10] and possess multiple characteristics which may be beneficial to a lung allograft such as growth factor secretion [11] and the capacity to supress T lymphocyte proliferation [12]. Despite this, BAL-MSCs can be found within the fibrotic lesions which characterize post-transplant obliterative bronchiolitis, which introduces the idea that BAL-MSCs are myofibroblast progenitors. These findings are further complicated by recent reports which suggest the criteria developed by The International Society for Cellular Therapy lack the specificity to distinguish between MSCs and closely related populations such as fibroblasts [13, 14]. Fortunately, transcriptional profiling and bioinformatics analysis is emerging as an improved and specific means for categorising mesenchymal populations [15].

The aim of this study was to, in humans, compare the immunophenotype, differentiation potential and gene expression of MSCs isolated from BAL fluid to those isolated from parenchymal lung tissue digests. In order to confirm the identity of our putative MSC populations, we then compared the transcriptional profile of the BAL-MSCs and LT-MSCs to an online repository of the profiles of true MSCs isolated from non-pulmonary tissues. We hypothesised that both BAL-MSCs and LT-MSCs would be true MSCs, but that they would be phenotypically and transcriptionally distinct.

Materials and Methods

Patient Groups and MSC Isolation

BAL-MSCs were isolated from bronchoalveolar lavage fluid. BAL fluid was obtained from healthy controls undergoing bronchoscopy for the investigation of chronic cough, where no abnormality was found, and from lung transplant recipients as part of their standard post-operative care (Supplementary Table 1). Samples collected from lung transplant patients were sorted into three groups. Lung transplant patients within the first 12 post-operative months were categorized as early post-transplant. Lung transplant patients past the first 12 post-operative months were categorized as late post-transplant and this group was further subdivided based on the presence or absence of chronic lung allograft dysfunction. The criteria for chronic lung allograft dysfunction was defined as per The International Society for the Heart and Lung Transplantation guidelines[16].

BAL was performed by wedging the bronchoscope in the middle lobe or lingula, infusing 100 ml of 0.9% saline via the working channel, and aspirating the effluent. BAL fluid was transported to the laboratory on ice immediately after collection, centrifuged, resuspended in Dulbecco's Modified Eagle Medium (DMEM, Carlsbad, California) supplemented with 10% fetal calf serum (FCS, Carlsbad, California), penicillin/streptomycin (Gibco) and seeded at a density of 0.3–0.4 $\times$ 10^6 cells/well in six well culture plates. Cells such as macrophages, neutrophils, and eosinophils could be observed in early primary BAL cultures, however these cells lost viability, adherence and were washed off within the first week of culture.

LT-MSCs were isolated from patients undergoing a lung resection for recurrent pneumothorax. This tissue source is ideal for the study of adult distal lung cell populations since these individuals are generally young, healthy, non-smokers who require the resection of a congenital sub-pleural bleb to prevent pneumothorax recurrence. Tissue was also transported immediately to the laboratory on ice and mechanically digested into small pieces (1 mm $\times$ 1 mm) and seeded onto 10 cm diameter plastic cell culture dishes at a density of 1 g/plate. BAL and tissue cultures were monitored for colony formation, which were then detached from the plate surface, resuspended and allowed to clonally expand up to a confluence of 60%–80%. Cells were then cryopreserved in liquid nitrogen until use. All experiments were performed using cells between passages 2–4 [16].

Bone Marrow MSCs (BM-MSCs)

Passage 2–4 human BM-MSCs were generously gifted to us from Cell and Tissue Therapies, Royal Perth Hospital. BM-MSCs were isolated from the mononuclear fraction of aspirated bone marrow, in accordance to local standard operating procedures. BM-MSCs were then expanded in culture and preserved in liquid nitrogen. These cells fulfil the criteria for MSC classification outlined by the International Society for Cell therapy guidelines (2006) [7].

Antibody Labelling and Flow Cytometry

Single cells were blocked (20% Octagam), stained with monoclonal antibodies to CD90, CD73, CD105, CD45, CD45RO, CXCR4, CD34 (BD Biosciences, San Jose, California, http://www.bdbiosciences.com) and fixed with 4% paraformaldehyde (BD Biosciences, San Jose, California, http://www.bdbiosciences.com). Expression was measured using a FACSCanto flow cytometer (BD Biosciences, San Jose, California, http://www.bdbiosciences.com) and data was analysed using the flow cytometry software, FlowJo (Tree Star Inc, Ashland, Oregon, http://www.flowjo.com/). The mean fluorescent intensity (MFI) of the sample was determined as a ratio of the MFI of the stained sample to the MFI of its unstained control. Intracellular labelling of collagen1A1 (COL1A1, AbCam, Cambridge, UK, http://www.abcam.com/), α-smooth muscle actin (α-SMA, Invitrogen) and vimentin (AbCam, Cambridge, UK, http://www.abcam.com/) was performed after fixed cells were permeablized using Perm Buffer (BD Biosciences, San Jose, California, http://www.bdbiosciences.com). LT-MSCs were sorted using a BD FACS

Aria based on their expression of CD90. Dead cells were excluded using 7-AAD.

Differentiation Assays

Cells were induced to undergo differentiation using the StemPro adipogenesis, osteogenesis, and chrondrogenesis differentiation kits for 21 days, as per the manufacturer's instructions (Gibco, Carlsbad, California, https://www.thermofisher.com/au/en/home/brands/gibco.html). Controls consisted of matched samples, cultured in DMEM containing 2% FCS and antibiotics. Cultures were then fixed (4% paraformaldehyde, BD Bioscience, San Jose, California) and stained with either alizarin red (osteogenesis, Sigma-Aldrich, https://www.sigmaaldrich.com), oil red o (adipogenesis, Sigma-Aldrich, St. Louis, Missouri) or alcian blue (chondrogenesis, Sigma-Aldrich, St. Louis, Missouri) as per the manufacturers recommendations.

Real-Time PCR

Differentiation was quantitatively assessed by real-time qPCR. RNA was extracted using the RNEasy RNA extraction kit (Qiagen, Hilden, Germany, https://www.qiagen.com) and converted to cDNA using the Quantitech Reverse Transcription Kit (Qiagen, Hilden, Germany, https://www.qiagen.com) as per the manufacturer's protocol. QPCR was performed using commercially purchased primers (QuantiTect – Qiagen, Hilden, Germany, https://www.qiagen.com) and Syber Green Fast Chemistry master mix (Qiagen, Hilden, Germany, https://www.qiagen.com), in accordance with the manufacturer's instructions. Expression was measured using a ViiA7 PCR machine (Applied Biosystems, Foster City, California, http://www.thermofisher.com/au/en/home/brands/applied-biosystems.html). Beta-2-microglobulin was used as a house keeper and data was analysed using the standard curve method.

Colony Forming Unit (CFU) Efficiency Assay

CFU-efficiency was determined using an adaption of previously published methodology [17, 18]. Briefly, cells were seeded into culture at a density of 10.5 cells per cm^2 in triplicate. Cells were maintained under standard culture conditions for 14 days. After which, cells were fixed with 4% paraformaldehyde (BD Bioscience, San Jose, California) and stained with 2% toluidine blue (Sigma-Aldrich, St. Louis, Missouri). CFUs consisting of at least 10 directly adjacent cells were counted. Data points represent the average of three replicates (Supporting Information Fig. 2).

Microarray and Bioinformatics

RNA was collected from P4 $CD90^{Hi}$ LT-MSCs and BAL-MSCs. RNA quality was assessed using a nanodrop (ThermoScientific, Waltham, Massachusetts) and a bioanalyzer (Agilent, Santa Clara, California, http://www.agilent.com.au/home). All samples had an A280:230 ratio between 1.9–2.1 and an A260:A230 ratio between 1.9–2.2 and RIN values greater than 9.5. Samples were then shipped to the Ramaciotti centre for Genomics (University of New South Wales, Australia) where samples were assessed using the Illumina Beadchip V4.0 microarray. Non-transformed expression data was then inputted into Stemformatics and MultiExperiment Viewer for bioinformatics analysis. Two class, unpaired Significance Analysis of Microarrays testing was used to identify differentially expressed genes using 100 permutations, a median false discovery rate of 0 and no minimum fold change criterion. Stemformatics was then used for hierarchical clustering of differentially expressed genes and the Rohart MSC test [15, 19]. All microarray data is hosted and publically accessible on Stemformatics.

Validation PCR was performed as described above. For BAL-MSCs, this was performed on 15 distinct patients. For LT-MSCs, this was performed on six distinct patients, four patients whose samples were used for microarray (but different CFU used) and from healthy non-cancerous tissue of five patients who had either a pneumonectomy or lobectomy to treat cancer. Patient demographics for these patients are included in Supporting Information Table 2.

Ethics and Statistics

Approval for this study was granted by the Human Research Ethics Committee, Metro North Hospital and Health Services, The Prince Charles Hospital (HREC/13/QPCH/96) and written, informed consent was obtained prior to sample collection. Statistical analysis was performed using Stata v11 (StataCorp, College Station, Texas, http://www.stata.com/) using Chi-squared, Fisher's exact test or Mann–Whitney *U* test, as appropriate. All data is expressed as the median with interquartile range (IQR) unless otherwise stated.

Results

Patient Demographics

Healthy parenchymal lung tissue was collected from eleven patients (7 (63.6%) male) with a median age of 24.2 (22.0–27.7) years. Healthy control BAL was collected from nine patients (4 (44%) male) with a median age of 59.5 (48.8–71.9) years and from 57 lung transplant recipients (36 (63.2%) male) with a median age of 47.9 (34.2–56.0) years and a median donor age of 39 (24–48) years. Full patient demographics are shown in Supporting Information Tables 1 and 2.

Stromal Cells are Readily Recoverable from Healthy Parenchymal Lung Tissue but Only Rarely from Healthy BAL

BAL-MSCs were isolated from only one of nine (11.1%) healthy control BAL fluid samples. In contrast, LT-MSCs were abundant in primary parenchymal lung tissue cultures and isolated from 11 of 11 (100%) lung tissue samples ($p < .001$ vs. healthy control BAL, Fig. 1).

Stromal Cells are Readily Recoverable from Lung Transplant BAL Fluid

As the isolation rate of BAL-MSCs from healthy controls was low, we decided to study BAL-MSCs collected from lung transplant recipients, as previously reported [10]. For early post-transplant patients, we were able to isolate BAL-MSCs from 17 of 18 samples (94.4%, $p < .001$ vs. healthy BAL fluid), from which, the total cell count from BAL-fluid was 2.0×10^6 ($1.0 \times 10^6 - 2.0 \times 10^6$) and the median number of CFU present in each culture was 3.0 (IQR = 2.0–3.0). In contrast, for patients past their first post-operative year without chronic lung allograft rejection, BAL-MSCs were only isolatable from 11 of 21 samples (52.4%, $p < .001$ vs. healthy control BAL and early transplant BAL), from which, the total cell count from

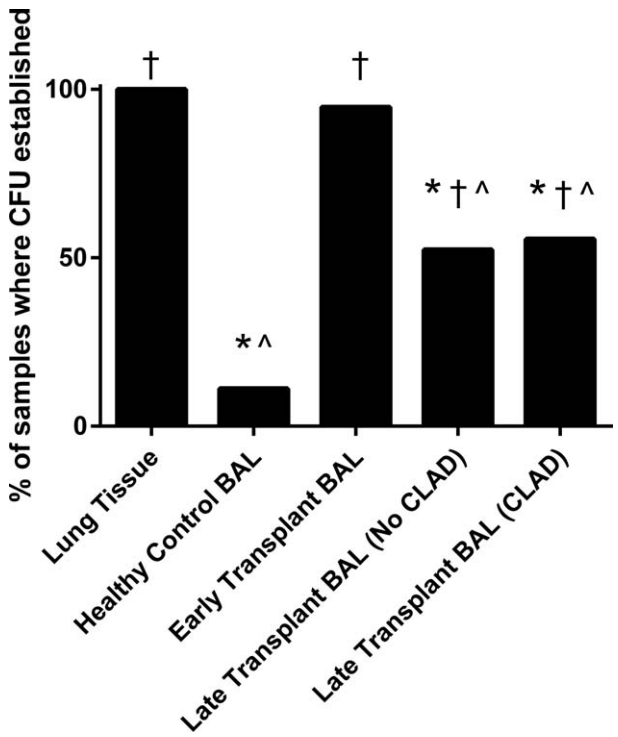

Figure 1. Isolation rate (%) of mesenchymal stromal cells (MSCs) from healthy lung tissue ($n = 11$) and BAL fluid from healthy controls ($n = 9$) and lung transplant recipients. Transplant patient BAL was divided into three groups; early transplant (<12 months post-transplant, $n = 18$) and late transplant (>12 months post-transplant) with ($n = 18$) or without ($n = 21$) CLAD. MSC were abundant in healthy lung tissue, whereas their isolation was rare from BAL fluid from healthy controls. Despite this, MSC were also abundant in BAL fluid collected from lung allografts early post-transplant, however, the rate of isolation declined after the first post-operate year, irrespective of CLAD. CLAD was defined as a >20% decrease in forced expiratory volume in 1 second. $p < .001$ compared to (*) lung tissue, (†) healthy BAL and (^) early transplant. Groups were compared using Fisher's Exact Test. Abbreviations: BAL, bronchoalveolar lavage fluid; CLAD, chronic lung allograft dysfunction.

BAL-fluid was 2.0×10^6 ($1.1 \times 10^6 - 3.0 \times 10^6$) and the median number of CFU present in each culture was 1.0 (IQR = 1.0–2.0). Similarly, for patients past their first post-operative year with chronic lung allograft dysfunction, BAL-MSCs were observed in 10 of 18 samples (55.6%, $p < .001$ vs. healthy control BAL and early transplant BAL), from which, the total cell count from BAL-fluid was 1.6×10^6 ($2.5 \times 10^6 - 4.4 \times 10^6$) and the median number of CFU present in each culture was 1.0 (IQR = 1.0–2.0). Despite the higher isolation rate of BAL-MSCs within the first post-operative year, we found that all BAL-MSCs expressed a similar immunophenotype, differentiation potential, colony forming unit-efficiency, myofibroblast marker expression pattern and transcriptome profile and hence are combined and treated as one group for the remaining analyses.

LT-MSCs and BAL-MSCs Have Reduced CD105 Expression Compared to BM-MSCs

Human MSCs are typically defined as being $CD73^{Hi}$, $CD45^{Neg}$, $CD105^{Hi}$, and $CD90^{Hi}$. BM-MSCs, LT-MSCs, and BAL-MSCs all strongly expressed CD73 and lacked CD45 (Fig. 2). Both LT-MSCs and BAL-MSCs expressed lower levels of CD105 compared to BM-MSCs, with BAL-MSCs expressing the lowest level (Fig. 2 and Supporting Information Fig. 1). LT-MSCs and BAL-MSCs also strongly expressed CD90, however, we also observed a distinct subset of $CD90^{Lo}$ stromal cells in primary lung tissue cultures (Fig. 2). $CD90^{Lo}$ stromal cells were morphologically indistinguishable from $CD90^{Hi}$ cells and comprised 26.5% (18.6–31.0, $n = 6$) of LT-MSCs. As such, we FACS-sorted all samples isolated from lung tissue based on CD90 expression. We found reseeded $CD90^{Lo}$ stromal cells transitioned to $CD90^{Hi}$ cells with sub-passaging (data not shown). For the remaining experiments, only $CD90^{Hi}$ FACs sorted cells were used. Finally, we also assessed the possibility that BAL-MSCs are derived from fibrocytes. We found LT-MSCs and BAL-MSCs did not express the fibrocyte markers CD34, CD45RO and CXCR4 (data not shown).

LT-MSCs Have Tri-Lineage Potential While BAL-MSCs Only Show Dual-Lineage Potential

Classic MSCs are defined by their capacity for osteogenic, chondrogenic and adipogenic differentiation. In assessing this potential in LT-MSCs and BAL-MSCs, we found that both populations could be induced to undergo osteogenesis and chondrogenesis (Fig. 3), as indicated by strong labelling with alizarin red (calcium deposition) and alcian blue (proteoglycan synthesis). In contrast, LT-MSCs, but not BAL-MSCs could undergo adipogenesis. Adipogenesis in LT-MSCs was characterized by the marked morphological transition from a long spindle shaped cell body to a cobblestone shape cell body and the simultaneous accumulation of large dense vacuoles which stained strongly with oil red o, indicative of lipids (Fig. 3B). In contrast, BAL-MSCs retained their spindle shaped morphology and only developed small sized lipid vacuoles (Fig. 3C).

Differentiation was validated by measuring the upregulation of fatty acid binding protein-4 (FABP-4, adipogenesis), secreted phosphoprotein 1 (SPP1, osteogenesis) and aggrecan (ACAN, chrondrogenesis). FABP-4 expression was strongly upregulated in BM-MSCs (874.4-fold increase, 243.4–2578.7, $n = 8$) and LT-MSCs (1048.1-fold increase, 774.4–2362.0, $n = 6$). In contrast, FABP-4 expression in induced BAL-MSCs was only moderately increased (7.8-fold increase, 1.9–17.8, $n = 18$). This was significantly less compared to both BM-MSCs ($p < .001$) and LT-MSCs ($p < .001$, Fig. 4A). Changes in SPP1 (Fig. 4B) and ACAN (Fig. 4C) expression after osteogenic and chondrogenic induction was similar between groups.

BAL-MSCs Have Lower Capacity to Generate CFU After Reseeding Compared to LT-MSCs and BM-MSCs

The efficiency with which samples generated CFU after reseeding, expressed as number of CFU/well, was lower in BAL-MSCs (median = 5.2, IQR = 1.3–10.8, $n = 10$) compared to LT-MSCs (median = 22.8, IQR = 16.6–25.0, $n = 10$, $p < 0.005$) and BM-MSCs (median = 19, IQR = 16.2–23.7, $n = 7$, $p < .005$, Supporting Information Fig. 2). LT-MSCs and BM-MSCs formed CFU with similar efficiency.

Transcriptome Profiling Indicates LT-MSCs and BAL-MSCs are True MSCs

Of 29,377 genes, LT-MSCs and BAL-MSCs expressed 11,547 genes above the detection threshold. Of the expressed genes,

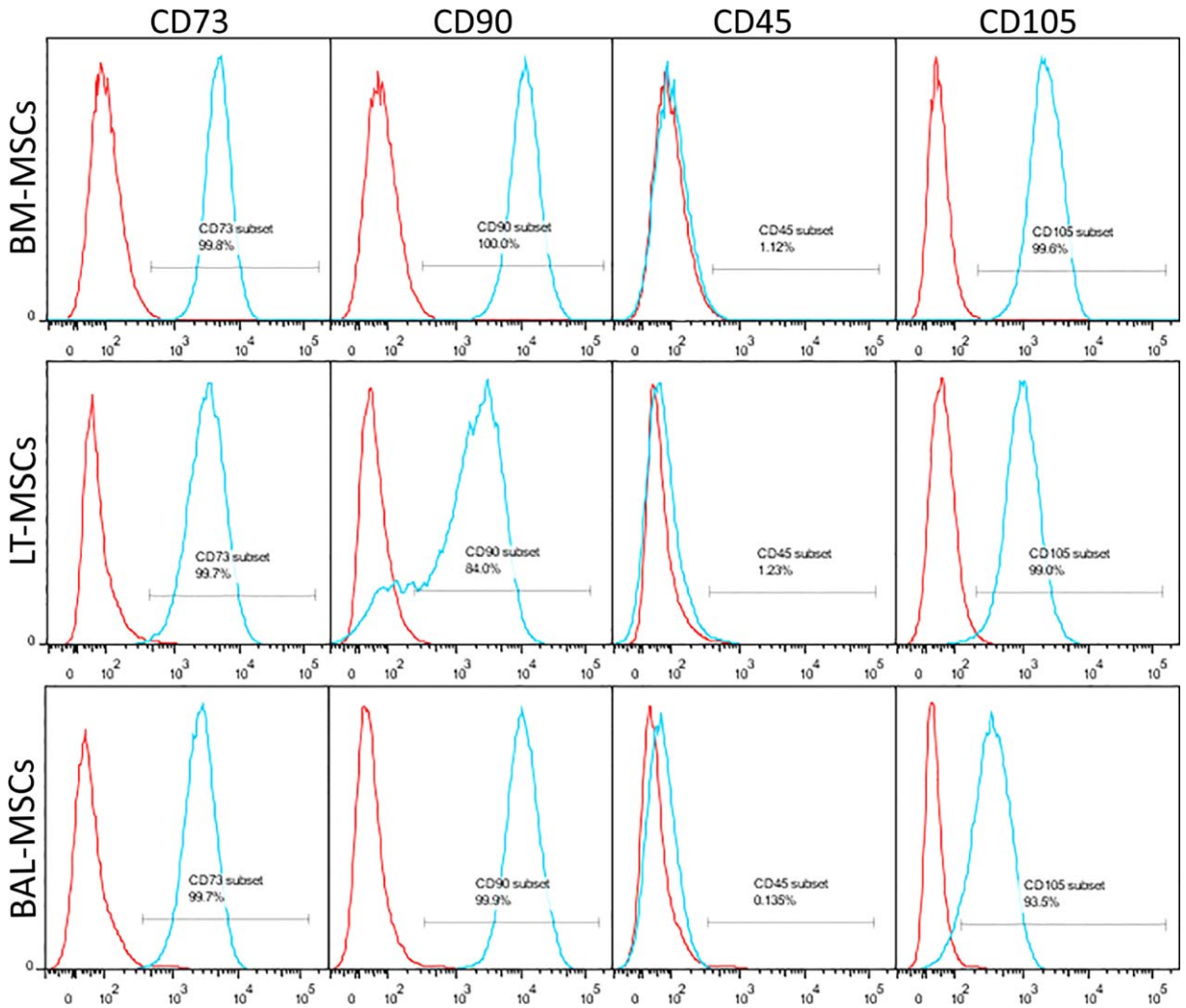

Figure 2. Representative flow cytometry plots comparing MSCs isolated from BM-MSCs, LT-MSCs, and BAL-MSC after labelling with with common MSCs markers. All populations were $CD73^{Hi}$ and $CD45^{Neg}$, however LT-MSCs were heterogeneously $CD90^{Hi\ or\ Lo}$, whereas BM-MSCs and BAL-MSCs were homogenously $CD90^{Hi}$. In addition, CD105 expression was strongest in BM-MSCs followed by LT-MSCs and finally, BAL-MSCs. Blue lines represent labelled samples and red lines represent unstained controls. Abbreviations: BAL-MSC, bronchoalveolar lavage mesenchymal stromal cell; BM-MSCs, bone marrow mesenchymal stromal cell; LT-MSCs, lung tissue mesenchymal stromal cell; MSCs, mesenchymal stromal cell.

the magnitude of expression was highly correlated between groups ($r^2 = 0.99$, data not shown).

The most stringent definition of MSC immunophenotype outlined by the International Society of Cellular Therapy is positive expression of CD105, CD73 and CD90 and negative expression of CD45, CD34, CD14, CD11b, CD79α, CD19, and HLA-DR [7]. Consistent with our flow cytometry data, CD105, CD73, and CD90 were expressed by both populations and CD34, CD14, CD11b, CD79α, CD19, HLA-DRα, and HLA-DRβ (together make HLA-DR) transcripts were below the detection threshold.

Despite this, there is a growing body of evidence to suggest that the traditional criteria for defining MSCs is only specific within the context of the bone marrow. As such, we also compared the transcriptional profiles of our stromal populations against those for MSCs listed in public data banks using the Rohart MSC test in Stemformatics [19]. We found 23/24 LT-MSCs samples (comprised of four CFU from six individuals) and 10/12 BAL-MSCs samples (1 CFU from 12 individuals) strongly qualify as MSCs. The remaining samples fell into the region of ambiguity (Fig. 5).

In order to investigate the "stemness" of LT-MSCs and BAL-MSCs, we assessed their expression of the genes listed in the pluripotency network (PluriNet) [20] and also genes associated with telomere maintenance. We found LT-MSCs and BAL-MSCs both expressed 211 of the 314 (67.2%) genes listed in the PluriNet. Additionally, we found genes POT1, TERF1, TERF2, RAP1, TPP1, and TIN2, whose proteins together form the telomere protection complex, Shelterin (telosome) [21] were expressed at high levels. Despite this, LT-MSCs and BAL-MSCs did not express telomerase reverse transcriptase and Telomerase RNA Component was marginally above the detection threshold for LT-MSCs and marginally below for BAL-MSCs.

Previous studies have suggested that pericytes and MSCs are closely related populations [22]. As such, we assessed the expression of common pericyte markers and found both LT-MSCs and BAL-MSCs expressed high levels of Neuron-gial

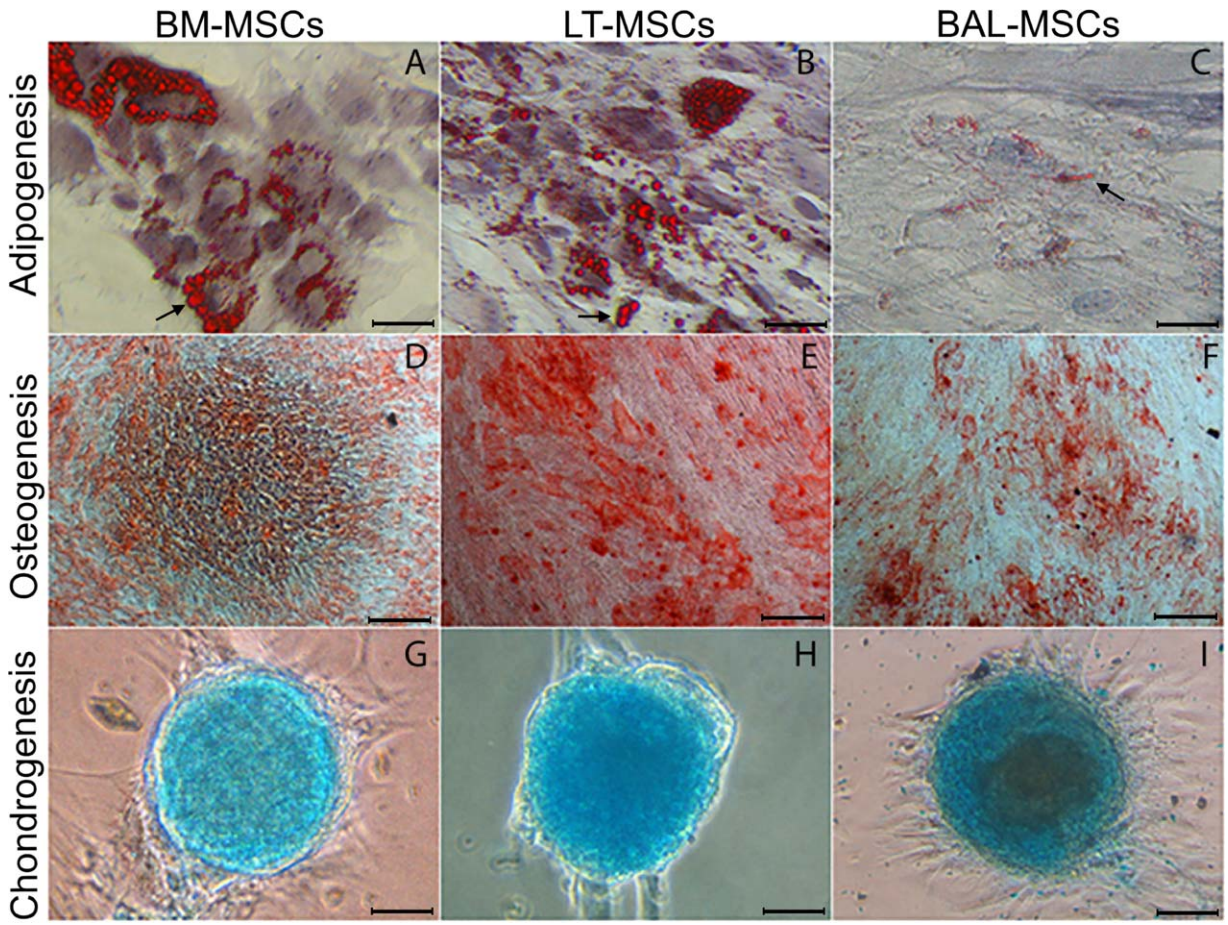

Figure 3. Panels **(A–C)**: Oil red o staining with red staining indicating the presence of lipid vacuoles indicative of adipocyte differentiation. Cells were counterstained with haematoxylin (purple, 200× magnification, scale bar represents 100μm). Panels **(D–F)**: Alizarin red staining with red staining indicating calcium deposition, typical of osteoblasts (40× magnification, scale bar represents 500 μm). Panels **(G–I)**: Alcian blue staining with blue staining indicating the presence of proteoglycans produced by chondrocytes (200x magnification, scale bar represents 100 μm). Mesenchymal stromal cells isolated from BM-MSCs and LT-MSCs have a full capacity to differentiate into each mesenchymal lineage. In contrast, BAL-MSCs are bipotent and lack adipogenic potential as indicated by their retention of a spindle shaped cell body and inability to develop large lipid vacuoles (black arrows, panels A–C). Abbreviations: BAL-MSC, bronchoalveolar lavage mesenchymal stromal cell; BM-MSCs, bone marrow mesenchymal stromal cell; LT-MSCs, lung tissue mesenchymal stromal cell.

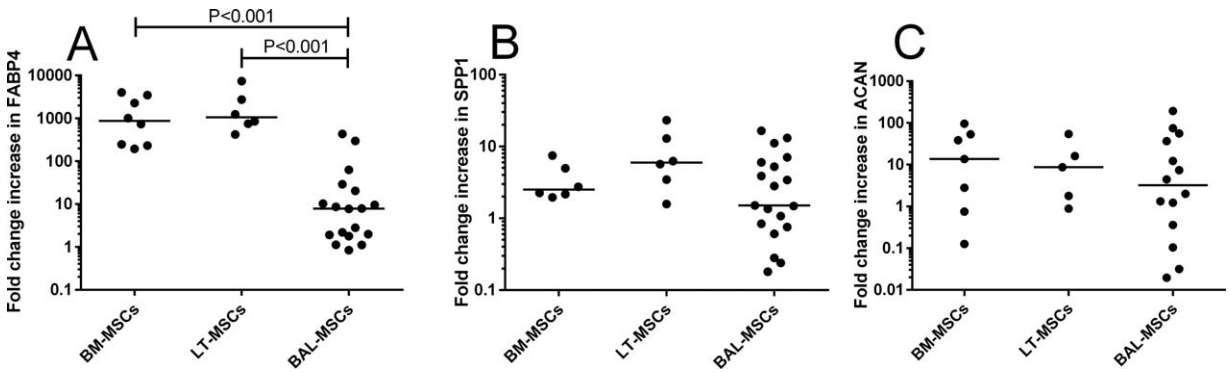

Figure 4. The differentiation potential of BM-MSCs ($n = 6–8$), LT-MSCs ($n = 5–6$) or BAL-MSCs ($n = 13–18$) was quantitated by real time-PCR for **(A)** FABP4 (adipogenesis), **(B)** SPPI (osteogenesis) and **(C)** ACAN (chrondrogenesis). Horizontal line represents median. All MSC populations have a similar capacity for osteogenesis (Panel B) and chondrogenesis (Panel C). In contrast, BM-MSCs and LT-MSCs underwent strong adipocyte differentiation, indicated by the strong upregulation of FABP4, whereas BAL-MSCs lacked adipogenic potential. Groups were compared using the Mann–Whitney *U* test. Abbreviations: ACAN, aggrecan; BAL-MSC, bronchoalveolar lavage mesenchymal stromal cell; BM-MSCs, bone marrow mesenchymal stromal cell; FABP4, fatty acid binding protein-4; LT-MSCs, lung tissue mesenchymal stromal cell; MSCs, mesenchymal stromal cell; SPPI, secreted phosphoprotein-1.

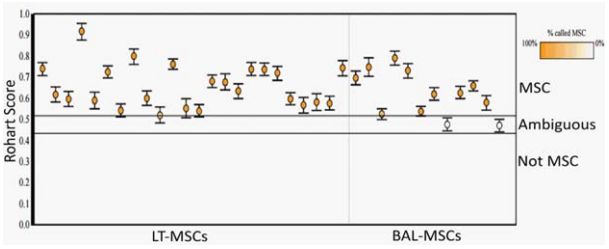

Figure 5. LT-MSCs and BAL-MSCs qualify as MSCs in accordance to the Rohart MSC test. The Rohart MSC test utilises transcriptional microarray data to determine if a cell can be classified as an MSC based on its transcriptional profile. 23/24 LT-MSC (four samples from six patients) and 10/12 BAL-MSC (1 sample from 12 patients) samples scored above the conservative threshold (top horizontal line), strongly suggesting these are MSCs. 1 LT-MSC and 2 BAL-MSC samples fall within the ambiguous region (between upper and lower horizontal boundaries). Notably, no samples score below the lower threshold, which would suggest they are not MSCs. Abbreviations: BAL-MSC, bronchoalveolar lavage mesenchymal stromal cell; LT-MSCs, lung tissue mesenchymal stromal cell; MSCs, mesenchymal stromal cell.

antigen 2 and β-type platelet-derived growth factor receptor (CD140b) but lacked CD146, low-affinity nerve growth factor receptor (CD271) and ATP-binding cassette sub-family G member 2 (ABCG2).

LT-MSCs and BAL-MSCs Can Be Distinguished by Transcriptome Profiling

Despite the high level of transcriptional congruence between LT-MSCs and BAL-MSCs, we were able to identify 105 differentially expressed genes. Hierarchical clustering of genes and samples created two primary groups consisting entirely of either LT-MSCs or BAL-MSCs (Fig. 6). Of the differentially expressed genes, 76 were expressed at higher levels in BAL-MSCs and included transcripts encoding extracellular matrix molecules (TIMP3, FBLN5, and COL7A1), ALK1/TGFβ pathway (ACVRL1, INHBE, ID1, ID3, TSPYL2), fibroblast activation (FAP and POSTN) and cellular motility (COL6A, TSPAN9, PTK7, RHOBTB1). In addition, we also noted one gene, CCL11 (eosinophil chemotractic protein) was expressed in BAL-MSCs and absent in LT-MSCs. A full list of the differentially expressed genes is listed in Supporting Information Table 3.

Twenty-nine transcripts were increased in LT-MSCs and encoded proteins related to cytoskeletal organisation (TLN2, FHOD1, WASF3), cell maintenance or were unmapped. In addition, three genes (HOPX, STAC, and TBX4) were exclusively expressed in LT-MSCs. HOPX and TBX4 each have roles in lung organogenesis [23, 24], however the role of STAC in the context of the lung or MSCs still remains to be elucidated.

Notably, when BAL-MSCs are sub-categorized into early post-transplant (<12 months post-operative), late post-transplant (>12 months post-operative) without chronic lung allograft dysfunction and late post-transplant (>12 months post-operative) with chronic lung allograft dysfunction, we were unable to identify any differentially expressed genes.

We next wanted to validate our findings and selected a set of 10 genes related to the extracellular matrix, TGFβ signalling and fibroblast activation. Using qPCR, we found TIMP3, ID3, POSTN, FAP, CCL11 were significantly upregulated in BAL-MSCs ($n = 15$) compared to LT-MSCs ($n = 15$, $p < .01$). In addition, we found TFAM expression to be significantly higher in

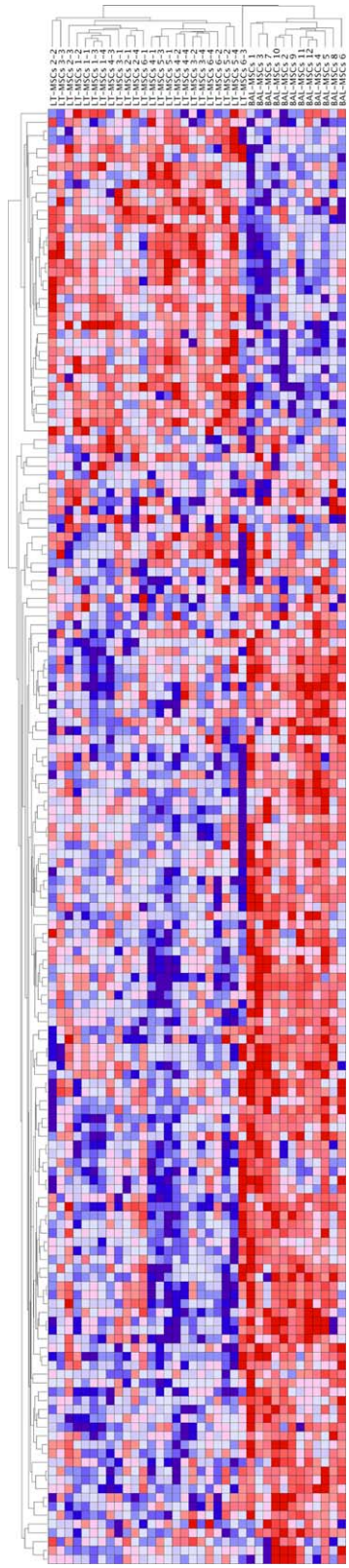

Figure 6. Hierarchical clustering of 105 differentially expressed genes detected using Significance Analysis of Microarrays. Lung tissue ($n = 24$, four samples from six patients) mesenchymal stromal cells (LT-MSCs) and bronchoalveolar lavage ($n = 12$, 1 sample from 12 patients) mesenchymal stromal cells (BAL-MSCs) segregate into two distinct groups when samples and probes are clustered using Pearson correlation.

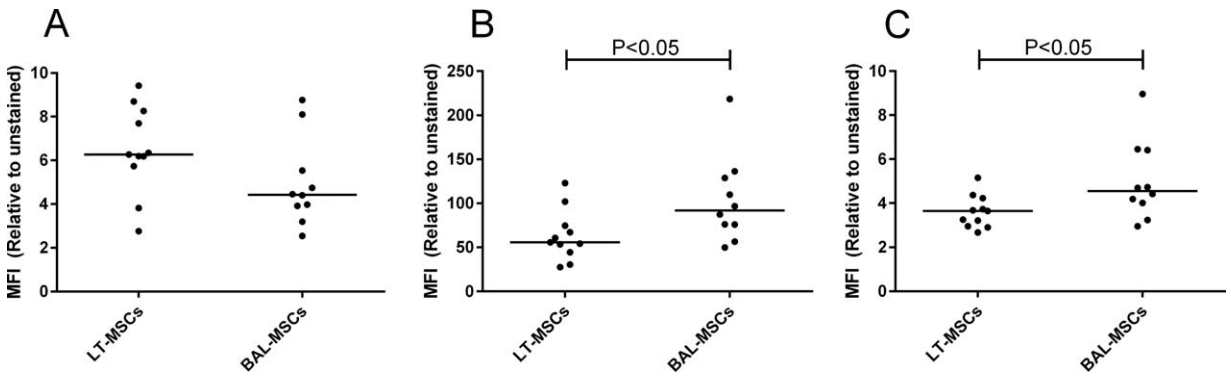

Figure 7. MFI (relative to unstained controls) of common myofibroblast markers **(A)** vimentin, **(B)** α-smooth muscle actin (α-SMA), and (c) collagen-1 on LT-MSCs and BAL-MSCs. Expression of α-SMA and collagen-1 was strongest in BAL-MSCs, whereas no significant difference was observed for vimentin. Groups were compared using the Mann–Whitney *U* test. Abbreviations: BAL-MSC, bronchoalveolar lavage mesenchymal stromal cell; LT-MSCs, lung tissue mesenchymal stromal cell; MFI, mean fluorescent intensity.

LT-MSCs ($n = 15$) compared to BAL-MSCs ($n = 15$, $p < .001$). No significant differences were observed for ID1, COL7A, FBLN5, and ACRVL1, however data trends were similar to those observed within the microarray (Supporting Information Fig. 3).

BAL-MSCs Have Increased Expression of Collagen 1A1 and α-Smooth Muscle Actin

Given that many genes associated with fibroblast differentiation and activation were identified by our microarray findings, we decided to examine the expression of common fibroblast/myofibroblast markers on LT-MSCs and BAL-MSCs. We found α-SMA expression was highest in BAL-MSCs (MFI = 92.0, 76.0–124.1, $n = 10$) compared to LT-MSCs (55.7, 48.9–71.0, n = 11, $p < .05$). Similarly, COL1A1 was also expressed the strongest in BAL-MSCs (4.6, 4.1–6.0, $n = 10$) compared to LT-MSCs (3.6, 3.1–4.0, $n = 11$, $p < 0.05$). Vimentin was similar between BAL-MSCs (4.4, 3.9–5.3, $n = 10$) and LT-MSCs (6.3, 6.0–8.0, $n = 11$, Fig. 7).

Discussion

In this study, we found that stromal cells with an immunophenotype, multipotency, transcriptome and colony forming capacity consistent with a mesenchymal stromal cell are recoverable from healthy human lung tissue. Additionally, we found MSCs were generally not recoverable from BAL fluid samples collected from healthy humans but could be isolated in samples collected from lung allografts. BAL-MSCs were abundant in samples from lung transplant patients for the first 12 post-operative months, after which, their abundance decreased irrespective of the presence of chronic lung allograft dysfunction. BAL-MSCs isolated from lung allografts expressed an atypical immunophenotype, reduced multipotency, diminished colony forming capacity, and enhanced expression of genes associated with activation by TGFβ, cellular motility and fibrosis. Importantly, time post-transplant and the presence or absence of chronic lung allograft dysfunction did not affect BAL-MSCs phenotype. These data are consistent with the idea that in healthy human lung, MSCs reside within lung tissue. In contrast, MSCs isolated from the bronchoalveolar region are likely dysfunctional and reflect disease.

BAL-MSCs failed to fulfil the traditional minimal criteria for defining an MSC as they only weakly expressed CD105 and could not differentiate into adipocytes. Despite this, BAL-MSCs expressed a typical MSC transcriptome and shared a high level of transcriptional congruence with LT-MSCs suggesting that the two populations are closely related. We found both LT-MSCs and BAL-MSCs express 67.1% of the genes listed in the Plurinet [20], which is similar to values reported for MSC populations in the kidney [25]. Additionally, LT-MSCs and BAL-MSCs also strongly express all six genes of the protein complex Shelterin (Telosome) which protects telomeres from inadvertent damage caused during DNA repair [21]. Together, this suggests these populations reside in a stem-like state with mechanisms in place that allow their continued renewal and long term persistence within tissue. It is therefore not surprising that both we and other groups have been able to isolate BAL-MSCs from patient allografts, even a decade after transplantation [10].

We and others have previously postulated that the human lung contains a mesenchymal stem cell hierarchy analogous to that of the epithelium [26, 27]. Herein, we also report the presence of a previously undescribed subset of $CD45^{Neg}$, $CD105^{Hi}$, $CD73^{Hi}$, $CD90^{Lo}$ lung stromal cells which were morphologically indistinguishable from their $CD90^{Hi}$ counterparts and importantly, were only isolated from healthy parenchymal tissue. The function of this subset, and its place in a putative hierarchical structure, remains unknown. $CD90^{Lo}$ subsets of stromal cells have previously been described in the bone marrow and are suggested to be associated with decreased immuno-suppressive capacity [28, 29]. Previous investigators have suggested that a subpopulation of pericytes, characterized by their expression of ATP-binding cassette subfamily G member 2 (ABCG2), are endogenous lung MSCs [22]. We found that BAL-MSCs and LT-MSCs are $NG2^{Pos}$ and $CD140b^{Pos}$ but also found they are $CD271^{Neg}$, $CD146^{Neg}$ and $ABCG2^{Neg}$ and are therefore, distinct populations. These findings support the notion that the human lung contains distinct, but phenotypically similar, populations of endogenous stromal cells with stem-like properties. Whether these cells exist in a community with a hierarchical structure, or whether they represent completely distinct populations, remains to be determined.

Based on our findings, we suggest that BAL-MSCs are derived from dysregulated LT-MSCs which have migrated into the alveolar space and now reside in the primary stages of

fibroblast/myofibroblast differentiation. We found BAL-MSCs had increased expression of several genes previously associated with cellular motility; which supports the notion that BAL-MSCs originate from an in tissue source. Key MSC characteristic such as CD105 expression, adipogenic potential and self-renewal capacity are reduced in BAL-MSCs, whereas common fibroblast/myofibroblast markers are increased at both RNA and protein levels. This includes the increased expression of transcripts associated with TGFβ signalling/activation such as ID3 and fibroblast activation such as fibroblast activation protein α. Notably, we found BAL-MSCs strongly express the eosinophil recruiting chemokine, CCL-11 (eosinophil chemotractic protein) which is inconsistent with the immunosuppressive characteristics described for most MSC populations. Notably, eosinophilia within the alveolar space is associated with decreased survival in lung transplant recipients [30].

Fibrosing lung diseases such as idiopathic pulmonary fibrosis (IPF) and post-transplant obliterative bronchiolitis are characterized by a potent pro-fibrotic response to failed epithelial repair [31]. For IPF, multiple studies now implicate telomere dysfunction in disease pathogenesis, in turn suggesting that dysfunction or depletion of cellular populations such as stem and stem-like cells, lies at the heart of pathogenesis [32]. The emerging paradigm is of senescence and/or depletion of lung-resident stem cell populations, in the setting of (usually) polygenic predisposition and suitable environmental exposure, leading to failed epithelial repair, fibrosis and respiratory failure. Understanding the phenotype, function and interactions between endogenous pulmonary stem cell populations, and how they may become senescent, depleted or dysregulated in disease is therefore highly relevant.

It still remains unclear why BAL-MSCs are more prevalent in BAL fluid within the first 12 post-operative months [33]. The isolation of BAL-MSCs in the first post-operative year is not correlated with episodes of infection or rejection [33]. It may be possible that perioperative injuries such as ischemia-reperfusion injury may lead to LT-MSCs migrating out of their niche, however, this does not explain why they remain to be isolatable many years' post-transplant (albeit at a reduced rate). It is potentially possible that LT-MSCs migrate into the alveolar space in any situation in which the epithelium is wounded and requires mesenchymal support. Evidence to support this can be found in murine studies, which have demonstrated that LT-MSC derived growth factors (e.g., fibroblast growth factor-10) drives the proliferation and propagation of epithelial progenitor cells into complex airway and alveolar structures [17]. Additionally, human BAL-MSCs administered into mice preferentially engraft proximal to alveolar progenitors—type II pneumocytes and create connexin-43 gap junctions while producing hepatocyte growth factor [11]. Recent studies demonstrate the mechanisms MSCs utilize to promote parenchymal outgrowth is far more diverse than originally thought. Notably, the intercellular export of mitochondria has been demonstrated to restabilize cellular bioenergetics and rescue wounded human bronchial and alveolar epithelial cells lines [4, 5, 34, 35]. Mitochondrial donation is mediated through ultra-fine cytoplasmic extensions termed tunnelling-nanotubes [36] but also via gap junctions such as connexin 43 [5] and exocytosed vesicles [5, 11]. MSC derived exosomes, loaded with micro-RNA, are also thought to be yet another mechanism by which MSCs provide support to local parenchymal cells [37–40]. Together, these studies provide persuasive evidence to suggest the primary role of MSCs in tissue is to provide support to the local parenchymal populations. Therefore, it is reasonable to speculate MSC dysfunction or depletion within the lung would result in the degeneration of the bronchial and alveolar epithelium.

Fibrotic lungs and lung allografts contain increased levels of TGFβ_1 [41–43], a key cytokine derived from damaged alveolar epithelium and immune cells such as alveolar macrophages. Notably, TGFβ_1 can also trigger the differentiation of MSCs into fibroblasts/myofibroblasts [44–46], potentially implicating it as a causal factor for fibrotic disease. Although previous investigators have implicated BAL-MSCs in the pathogenesis of fibrosis [33, 46], an alternate possibility, supported by our data, is that these dysregulated MSCs are bystanders which have migrated into the alveolar space and adopted a profibrotic phenotype in response to local TGFβ production.

Although the stromal cells we isolated from the alveolar space do not comply to the traditional criteria for MSC classification [7], their transcriptome profile is highly consistent with that of archetypal MSCs. Recently, it has been demonstrated that MSCs express a core set of 16 genes which is conserved in MSCs irrespective of tissue sources (e.g., bone marrow, adipose tissue, placenta), time in vitro or the consumables used to maintain them in culture [15]. This Rohart signature is demonstrably more robust than cell surface antigen staining, and less subjective for qualitative variables such as, the quality of an antibody or the expertize of the flow cytometer operator. The Rohart transcriptome-based MSC classification tool has been shown to be $>$95% accurate across hundreds of independently derived samples and is accurate enough to delineate MSCs from closely related populations such as fibroblasts and MSCs which have undergone differentiation [15]. Using this approach, we confirmed that both populations shared transcriptional properties consistent with an MSC. It is clear, however, that umbrella-classifications do not adequately capture the functional nuances that distinguish the BAL-MSC and LT-MSC populations, nor can an MSC-classification tool determine whether the BAL-MSCs are derived from a more primitive LT-MSC niche. It is noteworthy that this conundrum is not limited to lung MSC sources. Clonal bone marrow derived MSCs that share identical cell surface phenotype and closely aligned transcriptomes in culture may be functionally distinct, in vivo [47]. Surface immunophenotyping is a poor metric for tissue source, and does not distinguish bone marrow, adipose and umbilical cord MSCs from dermal fibroblasts, although transcriptional approaches have been successfully applied here [13, 14, 25]. It is therefore not surprising some researchers have argued for standardized reporting on the source, isolation and expansion methods to reconcile the inconsistencies within the literature [48]. It is likely that MSCs represent a functional spectrum that reflect their homeostatic or reparative roles, therefore a valid method of classification is to use transcriptome profiling in conjunction with functional assays which test a stromal cells' immuno-modulatory potential or ability to support parenchymal progenitor cells from their respective tissues [1, 17].

There are a number of limitations to our study which we wish to recognize. First, it could be argued that the MSCs

isolated from healthy lung tissue may have been derived from either the alveolar or the tissue compartment. We feel that it is unlikely that these MSCs were derived from the alveolar space since it was very difficult to isolate MSCs from bronchoalveolar lavage fluid obtained from healthy humans. Second, healthy control BAL, transplant BAL and parenchymal lung tissue were collected from patient groups with different ages. This potentially confounds the interpretation of subtle differences between populations. Despite this, the differences between LT-MSCs and BAL-MSCs that we have highlighted in this study, are consistent with the emerging literature of human lung MSCs and lung transplantation. Additionally, it has recently been demonstrated that MSC surface marker expression, differentiation potential and functionality are affected by ex vivo handling [17]. In order to help control for this, we used cells at the earliest feasible passage (P2-4) and passage matched our samples. Finally, we also wish to recognize the criteria we used to define a CFU in the CFU-efficiency assay differs from convention. MSC CFU are typically defined as $\geq$50 cells, whereas we defined a CFU as $\geq$10 cells. Using the standard criteria ($\geq$50 cells), it would appear as although some BAL-MSCs did not have a capacity to generate CFU when re-seeded, when in fact, cultures did contain small, slowly proliferating CFU. BM-MSC and LT-MSC CFU were almost exclusively $\geq$50 cells. We believe by adjusting the standard criteria to be more inclusive, our results more accurately reflect our observations.

Conclusion

In conclusion, lung tissue and bronchoalveolar MSCs represent closely related but distinct cellular populations, with the latter being recoverable almost exclusively from diseased humans. Our findings suggest after lung transplantation, MSCs migrate away from their in tissue niche, into the alveolar space, where they reside in the early stages of fibroblast/myofibroblast differentiation.

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Author Contributions

K.A.S.: Concept and design, Provision of study material or patients, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; S.T.Y.: Concept and design, Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; C.A.W.: Concept and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; T.C.: Collection and/or assembly of data, Data analysis and interpretation, Final approval of manuscript; P.A.H.: Provision of study material or patients, Final approval of manuscript; J.L.M.: Concept and design, Data analysis and interpretation, Final approval of manuscript; D.C.C.: Concept and design, Financial support, Provision of study material or patients, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Disclosures of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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