Epithelial Interactions and Local Engraftment of Lung-Resident Mesenchymal Stem Cells

Linda Badri^{1*}, Natalie M. Walker^{1*}, Takashi Ohtsuka¹, Zhuo Wang³, Mario Delmar¹, Andrew Flint², Marc Peters-Golden¹, Galen B. Toews¹, David J. Pinsky¹, Paul H. Krebsbach³, and Vibha N. Lama¹

¹Department of Internal Medicine and ²Divisions of Pulmonary and Critical Care Medicine and Cardiovascular Medicine, and Department of Pathology, University of Michigan Health System, Ann Arbor, Michigan; and ³Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, Michigan

Multipotent mesenchymal progenitor cells, termed “mesenchymal stem cells” (MSCs), have been demonstrated to reside in human adult lungs. However, there is little information regarding the associations of these local mesenchymal progenitors with other resident somatic cells and their potential for therapeutic use. Here we provide *in vivo* and *in vitro* evidence for the ability of human adult lung-resident MSCs (LR-MSCs) to interact with the local epithelial cells. The *in vivo* retention and localization of human LR-MSCs in an alveolar microenvironment was investigated by placing PKH-26 or DsRed lentivirus-labeled human LR-MSCs in the lungs of immunodeficient (SCID) mice. At 3 weeks after intratracheal administration, $19.3 \pm 3.21\%$ of LR-MSCs were recovered, compared with $3.47 \pm 0.51\%$ of control fibroblasts, as determined by flow cytometry. LR-MSCs were found to persist in murine lungs for up to 6 months and demonstrated preferential localization to the corners of the alveoli in close proximity to type II alveolar epithelial cells, the progenitor cells of the alveolar epithelium. *In vitro*, LR-MSCs established gap junction communications with lung alveolar and bronchial epithelial cells and demonstrated an ability to secrete keratinocyte growth factor, an important modulator of epithelial cell proliferation and differentiation. Gap junction communications were also demonstrable between LR-MSCs and resident murine cells *in vivo*. This study demonstrates, for the first time, an ability of tissue-specific MSCs to engraft in their organ of origin and establishes a pathway of bidirectional interaction between these mesenchymal progenitors and adult somatic epithelial cells in the lung.

Keywords: mesenchymal stem cell; engraftment; lung resident; connexin 43; gap junctions

Mesenchymal stem or stromal cells (MSCs) are adult connective tissue progenitor cells with multilineage differentiation potential (1, 2). In bone marrow, MSCs play a critical role in supporting the functions of the hematopoietic stem cell niche (3). Potent immunosuppressive and antiinflammatory properties of MSCs have also garnered significant interest in their application as vectors for tissue repair and cell therapy (4, 5). Exogenously administered MSCs have been found to ameliorate injury in various experimental animal models, an effect that has been demonstrated to be mediated

CLINICAL RELEVANCE

This study investigates the cellular crosstalk between resident mesenchymal stem cells and epithelial cells in the lung and demonstrates the ability of lung-resident mesenchymal cells to home and persist *in vivo* in murine lungs.

predominantly by the secretory function of MSCs rather than their local engraftment potential (6–9).

MSCs can also be isolated from adult, nonhematopoietic organs such as the lung, heart, and kidney (10–12). Studies of sex-mismatched human allografts have demonstrated that MSCs in these organs originate in the engrafted organ rather than in the bone marrow (10–12). Furthermore, lung-resident MSCs differ from bone marrow (BM)-derived MSCs with respect to their cytokine/chemokine gene expression profiles, confirming that these cells are distinct from those derived from the bone marrow. These resident MSCs thus represent a reservoir of endogenous organ-specific adult progenitor cells with a potential role in local tissue homeostasis and repair (13). However, little is known about their contributions because the majority of work studying MSCs in solid organs has focused on BM-derived MSCs. The ability of resident MSCs to interact with and modulate the local microenvironment remains to be investigated in solid organs. Similarly, whether exogenously administered tissue-specific MSCs demonstrate specific homing and retention in their organ of origin remains to be seen.

In this manuscript, by studying human lung-derived MSCs (LR-MSCs) *in vitro* and *in vivo*, we demonstrate their interactions with resident alveolar epithelial cells (AECs) and establish for the first time the engraftment potential of solid organ-derived tissue-resident MSCs in their native origin. Some of the results of these studies have been previously reported in the form of an abstract (14).

MATERIALS AND METHODS

Cell Isolation and Culture Conditions

MSCs were isolated from human bronchoalveolar lavage fluid of lung transplant recipients under a protocol approved by the University of Michigan Institutional Review Board as previously described (12). The MSCs used in the study were isolated from lung transplant recipients who were bronchiolitis obliterans syndrome free and had no evidence of acute rejection or infection on transbronchial biopsies and microbiological cultures, respectively. Human foreskin fibroblasts (CRL-2522), human lung fibroblasts (CCL-210), and human AECs (A549) were obtained from ATCC (Manassas, VA). Human small airway epithelial cells were obtained from Lonza (Walkersville, MD).

Labeling of Human LR-MSCs with PKH-26 or DsRed Lentivirus

Cells were labeled with the lipophilic fluorescent PKH dye PKH-26 (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions (15–17), and $97.30 \pm 0.19\%$ of LR-MSCs labeled with PKH-26

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* These authors contributed equally to this work.

Correspondence and requests for reprints should be addressed to Vibha N. Lama, M.D., M.S., Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Health System, 1500 E Medical Center Drive, 3916 Taubman Center, Ann Arbor, MI 48109-0360. E-mail: vlama@umich.edu

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demonstrated evidence of labeling by flow cytometry. DsRed lentivirus vector (DsRed pLentilox 3.7 control vector) was used as an alternate labeling method. Cells were seeded at 70% confluence and incubated with DsRed lentivirus for 48 hours.

Intratracheal Administration of Human LR-MSCs in Murine Lungs

The tracheas of female SCID mice (age 10–12 wk) (Jackson Laboratories, Bar Harbor, ME) were exposed under sterile conditions as previously described (18). Study animals received labeled LR-MSCs (5×10^5 cells/35 μ l saline) by intratracheal injection. Control animals were injected with saline (35 μ l) or labeled human control fibroblasts (5×10^5 cells/35 μ l saline). Animals from different experimental groups were killed, and lungs were harvested at 1 week, 3 weeks, 8 weeks, and 6 months after injection.

FACS Analysis

Single-cell suspensions, generated from harvested SCID lungs by collagenase digestion, were stained with propidium iodide (PI) or DAPI. Live labeled cells (PI negative, PKH-26 positive or DAPI positive, and DsRed PE positive) were analyzed and sorted using a FACS Vantage SE or a Diva FACS cell sorter. Cytospin preparations of sorted cells were stained with antihuman nuclear antibody (Millipore, Billerica, MA).

Histology and Immunofluorescence

Immunofluorescence staining was performed on frozen sections of murine lungs (5 μ M) for thyroid transcription factor (TTF)-1, surfactant protein (SP)C, CD68 (Santa Cruz Biotechnology, Santa Cruz, CA), Pancytokeratin (Invitrogen, Carlsbad, CA), E-cadherin (BD Bioscience, San Jose, CA), and CD44 (Millipore). For confocal microscopy, images were acquired with a Zeiss Axiovert 100M inverted microscope (Carl Zeiss MicroImaging, LLC Thornwood, New York, NY) using oil as immersion media and a 63 $\times$ objective. For fluorescence imaging, specimens were examined using a fluorescence microscope (TE-2000E; Nikon, Melville, NY), with image processing and analysis performed using Metamorph software (Molecular Devices, Sunnyvale, CA) and Photoshop CS3 (Adobe, San Jose, CA).

Keratinocyte Growth Factor ELISA

Keratinocyte growth factor (KGF) levels were measured in the 18-hour cell supernatant of LR-MSCs (2×10^5 /well into 6-well plates) by ELISA (R&D Systems, Minneapolis, MN) ($n = 5$).

In Vitro and In Vivo Transmembrane Communication Assay

Fluorescence dye (Calcein AM) transfer assay was used to investigate gap junction intercellular communication *in vitro* between LR-MSCs and epithelial cells and *in vivo* between LR-MSCs and resident murine cells in the absence and presence of the gap junction intercellular communication inhibitor carbenoxolone (CBX) (Sigma, Saint Louis, MO). Details are provided in the on-line supplement.

RESULTS

Intrapulmonary Retention of Human Lung-Resident MSCs in Uninjured Murine Lungs

To permit tracking of human LR-MSCs in murine lungs, cells were labeled with red fluorescent dye PKH-26 (Figure 1A) or transfected with the control lentivirus pLentilox 3.7 vector, which contains DsRed sequences in its backbone (Figure 1B). Labeled human LR-MSCs were injected intratracheally into the lungs of immunodeficient SCID mice. Single-cell suspensions were prepared from murine lungs harvested at various time points (1, 3, and 8 wk) after injection, and live labeled MSCs were enumerated by propidium iodide staining and flow cytometry. A distinct population of live fluorescent cells was noted in the lungs of animals that were injected with PKH-26-labeled MSCs but not in those injected with normal saline (Figure 2A). The number of PKH-labeled cells isolated from the lungs varied from 8 to 25% of the injected population (mean $\pm$ SE: $13.16 \pm 1.31\%$ at 1 wk, $17.80 \pm 8.52\%$ at 3 wk, and $14.21 \pm 2.99\%$ at 8 wk). The

intensity of the PKH-26 fluorescence in the sorted labeled cells (327.56 ± 10.66) was unchanged from the baseline fluorescence at the time of labeling (439.05 ± 92.16), suggesting a lack of significant proliferation *in vivo* ($n = 4$ separate experiments). At 6 months after intratracheal administration, 7.71% of the injected cells could be recovered by flow cytometry, and the fluorescence intensity of these cells was similar to that noted at 3 weeks ($P = 0.44$).

To investigate whether the PKH-26-positive cells isolated from murine lungs were the exogenously administered human LR-MSCs, expression of human MHC class 1 expression on these cells was analyzed by flow cytometry; $96 \pm 2.1\%$ of sorted cells demonstrated human MHC 1 expression (Figure 2B). Control murine cells (mouse lung fibroblasts) were uniformly human MHC 1 negative. Furthermore, cytopsin preparations of sorted cells demonstrated staining with antihuman nuclei monoclonal antibody (Figure 2C). These data confirmed that the cells sorted from murine lungs by PKH-26 or DsRed positivity are in fact the human intratracheally administered LR-MSCs.

To investigate if this intrapulmonary retention of LR-MSCs is a cell-specific phenomenon, intrapulmonary retention of human LR-MSCs was compared with that of human foreskin fibroblasts (CRL-2522, ATCC) and human lung fibroblasts (CCL-210, ATCC). At 3 weeks after intratracheal administration, $3.47 \pm 0.51\%$ of injected DsRed-labeled human foreskin fibroblasts, compared with $19.3 \pm 3.21\%$ of injected DsRed-labeled human LR-MSCs, were recovered from lung minces (Figure 3). Similarly, only $4.82 \pm 0.80\%$ of injected DsRed-labeled human lung fibroblasts were recovered from murine lung at 3 weeks after intratracheal administration (Figure 3).

These data demonstrate for the first time that intratracheally administered lung-specific MSCs demonstrate significant retention in their organ of origin even in the absence of injury.

Human Lung-Resident MSCs Demonstrate Long-Term Engraftment and Alveolar Localization in Murine Lungs

To determine the *in situ* phenotype and localization of injected LR-MSCs in the murine lung, histological sections of lungs at various time points after intratracheal administration of labeled LR-MSCs (1, 3, and 8 wk and 6 mo) were analyzed by fluorescent microscopy. Injected LR-MSCs, recognized as red fluorescent cells, were identified at all time points examined. Cytokeratin staining and confocal microscopy were used to better characterize the sites of engraftment of these cells in the lungs. PKH-26-labeled cells were seen predominantly near the corners of the alveoli embedded in the interstitium at the alveolar junctions surrounded by epithelial cells or lying attached to or embedded in the alveolar septa (Figures 4A and 4B). Very few cells were seen lying freely in the alveolar spaces. Z stack imaging confirmed that LR-MSCs in the alveolar corners were positioned deep in the interstitium surrounded by murine lung cells and were not just lying in the alveolar space superimposed on alveolar corners (Figure 4C). Quantitative analysis of 100 cells in the murine lungs 6 months after intratracheal administration demonstrated 51 cells embedded in the alveolar corners, 39 cells attached to the alveolar septa, and 10 cells lying freely in the alveolar space. ZO-1 staining demonstrated the presence of tight junctions between LR-MSCs and the surrounding cells, suggesting that this is not passive lodging of the cells (Figure 4D). To determine if this unique localization of injected cells was specific to LR-MSCs, we studied histological sections from murine lungs injected with labeled human foreskin fibroblasts. Evaluation of the lungs at 3 weeks after intratracheal administration of labeled human foreskin fibroblasts demonstrated very few recognizable fluorescent cells, and denucleated clumps of fluorescent material were noted in the airways or alveolar spaces (data not shown).

Figure 1. Labeling of human lung-resident mesenchymal stem (LR-MSCs) for *in vivo* tracking. (A) PKH-26 labeling of human lung-derived mesenchymal stem cells. Fluorescence microscopy ($100\times$ oil immersion) of cytospin of PKH-26-labeled LR-MSCs demonstrates homogeneous red fluorescent membrane staining at Day 3 after labeling. A heterogeneous cytoplasmic distribution of PKH-26 was present by Day 7, consistent with internalization of labeled membrane. (B) DsRed labeling of human lung-derived mesenchymal stem cells. Histogram of human LR-MSCs transduced with control lentivirus, the pLentilox 3.7 vector containing DsRed sequences in its backbone, is shown. The transduction efficiency was $96.3 \pm 2.9\%$, and cells demonstrated stable immunofluorescence with passaging.

The presence and unique localization of human LR-MSCs in murine lungs as long as 6 months after intratracheal administration demonstrates that tissue-resident MSCs home to specific

anatomic sites and demonstrate long-term engraftment in the lung.

Engrafted Human Lung-Resident MSCs Reside in Close Proximity to Type II AECs

The *in situ* phenotype of engrafted LR-MSCs was further studied by immunofluorescence staining. Staining with markers of type II AECs, SPC, and TTF-1 was performed to determine if mesenchymal cells undergo epithelial transformation. LR-MSCs did not express surfactant protein C but were identified in close contact with cells expressing SPC (Figure 5). Engrafted MSCs were also negative for expression of TTF-1, confirming that MSCs undergoing engraftment in uninjured alveoli do not undergo epithelial transformation. However, LR-MSCs were noted in close proximity to TTF-1-staining cells (Figure 5). LR-MSCs in the tissue continued to be negative for pan leukocyte marker CD45 and were positive for mesenchymal marker CD44 (Figure 5). Engrafted cells were also negative for the myofibroblast differentiation marker α -smooth muscle actin (data not shown). These data indicate that human LR-MSCs placed in an alveolar microenvironment demonstrate preferential localization and are seen lying in close association with AECs, specifically type II AECs.

Human Lung-Resident MSCs Secrete KGF and Establish Gap Junction Communication with AECs *In Vitro*

MSCs are important secretory cells and can modulate the local microenvironment via soluble mediators (19). Because LR-MSCs in the present study were shown to engraft in close

Figure 2. Retention of exogenously administered LR-MSCs in murine lungs. (A) Live PKH-26-positive cells can be identified in the murine lungs after exogenous administration of PKH-26-labeled human LR-MSCs. Flow cytometric analysis of single-cell suspension of lungs of SCID immunodeficient mice 3 weeks after intratracheal injection of saline or PKH-26-labeled human LR-MSCs is shown. Dot plot of propidium iodide-negative cells demonstrates a distinct live PKH26-positive population in lungs injected with labeled LR-MSCs (*center panel*). This is in contrast to cells from lungs of control saline-injected animals (*left panel*). A histogram of PKH-26-positive cells sorted from murine lungs is shown on the *right*. Control unlabeled LR-MSCs are shown in *purple*. (B and C) PKH-26-positive cells sorted from murine lungs are human in origin. (B) Dot plot (*left panel*) and histogram (*center panel*) demonstrating MHC class I expression on PKH-26-positive cells sorted by flow cytometry from murine lungs. The *right panel* demonstrates the absence of human MHC I expression on murine cells (mouse lung fibroblasts). *Purple peaks* represent isotype control. (C) Cytospin preparation of PKH-26-positive cells sorted from murine lungs demonstrates the presence of human nuclei on immunofluorescence staining using mouse antihuman nuclei monoclonal antibody.

Figure 3. LR-MSCs demonstrate superior retention in a murine lung. Dot plot of single-cell suspensions of murine lungs harvested 3 weeks after intratracheal administration of saline, DsRed-labeled LR-MSCs, and control cells (DsRed-labeled human foreskin and lung fibroblasts) are shown. A distinct population of DsRed-positive cells was noted in lungs injected with labeled human LR-MSCs as compared with the saline group. This population was significantly lower in lungs injected with labeled human foreskin or lung fibroblasts. Three weeks after intratracheal administration, $19.3 \pm 3.21\%$ of injected LR-MSCs, compared with $3.47 \pm 0.51\%$ of injected control foreskin fibroblasts and $4.82 \pm 0.80\%$ of injected control lung fibroblasts, were recovered at ($n = 3$ separate experiments of three animals each).

proximity to type II AECs, we investigated the ability of LR-MSCs to secrete KGF, an important epithelial growth factor (20) that promotes proliferation and differentiation of type II pneumocytes in the normal adult lung (21). LR-MSCs demonstrated KGF secretion at baseline (405 ± 89 pg/ml), which increased in response to serum (816 ± 102 pg/ml).

Local cell-to-cell communication via gap junctions has been demonstrated to be essential in the maintenance of cellular niches and regulation of cellular proliferation, migration, and differentiation. Our finding that engrafted LR-MSCs establish close contact with AECs led us to examine gap junction-mediated communication between LR-MSCs and AECs. The Calcein transfer assay was used to assess functional coupling between LR-MSCs and AECs *in vitro*. LR-MSCs labeled with the intracellular dye Calcein AM were added to a monolayer of transformed human AECs (A549) labeled with cell membrane marker DiI at a ratio of 1:10. Transfer of Calcein from LR-MSCs to AECs was demonstrated by the emergence of a population of Calcein- and DiI-positive cells, consistent with the establishment of gap junction communications between the two populations (Figures 6A and 6B). This transfer was significantly inhibited in the presence of CBX (Sigma), a gap junction inhibitor, demonstrating that the dye transfer occurs via gap junctions. Similar results were noted with primary human small AECs (data not shown).

Connexin 43 was found to be the predominant connexin protein in LR-MSCs on Affymetrix analysis. In cocultures of LR-MSCs and AECs, connexin 43 was demonstrable at the junctions of LR-MSCs and AECs by immunofluorescence (Figure 6C).

Figure 4. (A and B) Presence of exogenously administered human LR-MSCs in murine lungs at 6 months after intratracheal administration. At 6 months after intratracheal administration, LR-MSCs could still be distinctly identified in the alveolar spaces. Representative confocal microscopic images demonstrate LR-MSCs, identified by their red fluorescent PKH-26 staining. Mouse lung alveolar epithelial cells (AECs) are stained with cytokeratin (FITC, green) to better visualize the anatomy. LR-MSCs in the murine alveolar spaces demonstrated a round morphology with a prominent nucleus. LR-MSCs were seen predominantly near the corners of the alveoli embedded in the interstitium (arrow) or attached to the alveolar septa (solid arrow). Blue = DAPI (A: 20 $\times$ magnification; B: 40 $\times$ magnification). Bar = 10 μ m. (C). Z stack imaging of confocal microscopy demonstrating PKH-26-labeled LR-MSCs embedded in the interstitium surrounded by AECs. Another LR-MSC (arrow) is seen attached to the alveolar septa near the corner lying adjacent to a type II AEC. (D) ZO-1 staining demonstrates the close proximity of an engrafted LR-MSC to type II AECs, with expression of ZO-1 (green) at the margin of these adjoining cells.

Exogenously Administered Human Lung-Resident MSCs Establish Gap Junction Communication with Resident Murine Cells *In Vivo*

Connexin 43 was noted at the junctions of engrafted LR-MSCs and surrounding AECs in histological sections of murine lungs injected with labeled LR-MSCs (Figure 7A). To investigate if engrafted LR-MSCs establish functional gap junctions *in vivo*, double-labeled LR-MSCs (PKH-26⁺ and Calcein AM⁺) were injected intratracheally into SCID mice. Flow cytometric analysis of single-cell digests of murine lungs at various time points after intratracheal injection demonstrated the emergence of a Calcein AM-positive but PKH-26-negative population of acceptor cells (Figures 7B and 7C). This transfer was significantly inhibited when LR-MSCs were treated with the gap junction inhibitor CBX before intratracheal administration. This emergence of a Calcein-positive but DsRed-negative population of cells, which was attenuated in the presence of a gap junction inhibitor, demonstrates that exogenously administered LR-MSCs establish gap junction communications with resident cells, leading to Calcein transfer.

Figure 5. Exogenously administered human LR-MSCs maintain their mesenchymal phenotype but localize in close proximity to type II AECs. Immunofluorescent staining of murine lungs 6 months after intratracheal injection of PKH-26-labeled human LR-MSCs demonstrating their *in situ* phenotype in an alveolar microenvironment. PKH-26-labeled LR-MSCs (red) did not express surfactant protein (SP)C or thyroid transcription factor (TTF)-1 but were seen lying in close contact with SPC- or TTF-1-positive type II AECs. LR-MSCs resident in murine tissue continued to be negative for pan leukocyte marker CD45 and positive for cell surface glycoprotein receptor CD44. Bar = 10 μ m.

DISCUSSION

In this study of human lung-derived MSCs, we demonstrate for the first time the ability of these tissue-specific MSCs to engraft in their organ of origin and to communicate with locally resident epithelial cells via gap junction communications. Human LR-MSCs demonstrated long-term persistence in the murine lungs in which they were injected and remained engrafted in lung tissue up to 6 months after their exogenous administration. *In vitro*, LR-MSCs were capable of establishing gap junction communication with lung alveolar and bronchial epithelial cells and demonstrated an ability to secrete KGF, an important modulator of epithelial cell proliferation and differentiation. *In vivo*, LR-MSCs placed in a murine lung demonstrated preferential localization in the alveolar corners, lying in close approximation to the alveolar type II cells, the progenitor cell of alveolar epithelial type I cells in the lungs. Connexin 43 was noted at the junction of exogenously administered LR-MSCs and resident lung cells, and Calcein dye transfer assay demonstrated *in vivo* gap junction formation between these cells. In aggregate, these data suggest that lung-resident MSCs closely interact with AECs, communicating with these cells via, among other mechanisms, direct gap junction communication.

This study is the first investigation of interaction of human adult lung-derived MSCs and the resident epithelial cells. Mesenchymal epithelial interactions have an important role in developing and in adult lungs. During embryogenesis, lung-specific mesenchyme is crucial for epithelial differentiation and morphogenesis (22), and

in vitro coculture of embryonic stem cell with lung mesenchyme promotes their differentiation to alveolar epithelium (23). In an adult lung, bidirectional regulation of the epithelial and stromal components by soluble mediators is well documented (24–27), and loss of this mesenchymal epithelial interaction is thought to have an important role in the pathogenesis of various fibrotic lung diseases, such as idiopathic pulmonary fibrosis (28, 29). Our results demonstrating that LR-MSCs secrete KGF, an important epithelial growth factor, suggest that lung MSCs can likely modulate AECs in a paracrine fashion. MSCs have also been recently demonstrated to regulate lung fibroblast function (30), providing additional evidence that the products released by MSCs can influence lung pathobiology. Furthermore, we demonstrate that LR-MSCs establish gap junction communication with epithelial cells, thereby permitting direct intercellular transfer of molecules.

Human lung-resident MSCs expressed connexin 43 and established heterocellular gap junction communications with lung epithelial cells *in vitro*. Connexin 43 has an important role in alveolar and vascular formation during lung development, and connexin 43 gene deletion has been shown to be fatal in mice secondary to hypoplastic lungs (31). Connexin 43 is also expressed by type I and type II AECs, allowing formation of heterocellular gap junction communications between these cells (32). These gap junction communications, formed by the interaction between two connexin hemichannels on the surface of opposing cells, offer a direct passage between the cytoplasm of intercon-

Figure 6. Demonstration of *in vitro* gap junction communication between human lung-resident MSCs and AECs. (A) Calcein AM-labeled LR-MSCs were cocultured with Dil-labeled human alveolar epithelial A549 cells for various time intervals in the absence or presence of the gap junction communication inhibitor carbenoxolone (CBX). The emergence of an increasing number of double-positive populations over time (circle) demonstrates transfer of Calcein from LR-MSCs to AECs. This transfer was inhibited in the presence of CBX (100 μ M). (B) Quantitative analysis of dye transfer between LR-MSCs and AECs. Percentages of Calcein AM acceptor cells are shown over time. Each point represents the mean $\pm$ SD of three separate LR-MSC lines. * $P < 0.0001$. (C) Immunofluorescence staining of a coculture of LR-MSCs and AECs demonstrating expression of connexin 43. AECs can be identified by their cuboidal shape and cytokeratin staining (red). Connexin 43 staining is shown as green dots lining the spindle-shaped LR-MSCs. Bar = 10 μ m.

nected cells, enabling the diffusion of signaling molecules and metabolites (33). In the bronchi, gap junctions synchronize ciliary beating by enabling the propagation of calcium waves (34). Similarly, gap junctions are thought to allow the propagation of signals such as mechanical stimulation from the surface of type I cells to the type II cells sitting in alveolar corners. Although previous studies have demonstrated expression of connexin 43 and gap junctions among lung fibroblasts (35), the interaction of the mesenchymal and epithelial compartments in the lung via heterocellular gap junction communications has not been reported. The novel finding of gap junction communications between LR-MSCs and epithelial cells demonstrates another unique pathway of bidirectional interaction between these cells. How this direct communication of LR-MSCs with epithelial cells modulates the functions of these cells remains to be determined.

Engrafted LR-MSCs appeared as round cells with a prominent nucleus, in contrast to the long spindle-shaped structure of LR-MSCs in *in vitro* culture. This difference in morphology of LR-MSCs between the *in situ* and *in vitro* circumstance is reminiscent of that which has been noted when freshly isolated round BM cells acquire a spindle shape after adherence in plastic culture conditions (36–38). Our data demonstrate that spindle-shaped MSCs revert to a round morphology when introduced back in their native lung environment. Because the phenotype/morphology of MSCs *in vivo* in tissues is not well established, this finding has important relevance to studies trying to identify MSCs *in situ*.

Another interesting finding of this study was that when introduced back *in vivo* in an alveolar microenvironment, LR-MSCs demonstrated a propensity to localize to the corners of the alveolar spaces. Here LR-MSCs were noted adjacent to AECs, specifically type II AECs. This localization to alveolar corners is intriguing because it has been previously demonstrated that

mesenchymal cells, termed “interstitial fibroblasts,” reside in the tricellular corners of the lung alveoli with macroscopically visible intercellular communications (foot processes) extending from these cells to AECs (39). Furthermore, secondary to the role of mesenchyme-derived growth factors in epithelial regeneration, it has been suggested that recruitment of MSCs is important for epithelial repair in the lung (40). The KGF secretory function of LR-MSCs and their ability to preferentially localize in close proximity to epithelial cells when exogenously administered raises the possibility of using these lung-derived mesenchymal progenitors as vectors for therapeutic application aimed at promoting epithelial cell growth and repair (41).

In vivo, LR-MSCs were also demonstrated to establish gap junction communications with the resident murine cells within hours of their intratracheal administration. These data are supported by work demonstrating the existence of dye communication between embedded MSCs and their adjoining endothelial cells in a matrigel angiogenesis assay (42). Those studies used BM-derived murine MSCs. The acceptor cell type of Calcein in our *in vivo* study remains to be elucidated and could represent epithelial or endothelial cells.

This study is the first to document an ability of lung-derived MSCs to establish long-term residence in their organ of origin when administered exogenously. LR-MSCs persisted in the murine lungs for prolonged time periods (studied up to 6 mo) after intratracheal administration. The persistence of these cells and the demonstration of ZO-1 and connexin 43 at the junction of exogenously administered LR-MSCs with the adjacent host cells provide support for the concept that these cells are engrafted in the lung tissue. This finding is in sharp contrast to studies of MSCs derived from other sources. No significant engraftment of BM-MSCs was demonstrated in the lung in various injury models using

Figure 7. Demonstration of *in vivo* gap junction communication between exogenously administered LR-MSCs and resident lung cells. (A) Connexin 43 expression in human LR-MSCs engrafted in murine lungs. Sections from murine lungs injected with human LR-MSCs were examined for expression of connexin 43. Connexin 43 (green) is seen as punctuate dots at the periphery of the engrafted PKH-26-stained LR-MSCs (red). Bar = 10 μ m. (B) Demonstration of *in vivo* gap junction communications between LR-MSCs and tissue-resident cells. PKH-26-labeled and Calcein-labeled human LR-MSCs (1×10^6) were injected intratracheally into murine (SCID) lungs. Flow cytometric analysis of single-cell homogenates of murine lungs at various time points after intratracheal injection demonstrates the emergence of a Calcein-AM-positive but PKH-26-negative population of acceptor cells (circle). This population was significantly decreased in the presence of CBX. (C) Quantitative analysis of dye transfer between LR-MSCs and murine lung resident cells. Percentage of Calcein AM-positive, PKH26-negative acceptor cells are shown over time. * $P < 0.0001$ ($n = 4$ separate experiments).

intratracheal (6) or systemic routes of administration (7). Similarly, in a study of umbilical cord-derived MSC in immunodeficient SCID mice with or without bleomycin injury, no evidence of their presence was noted at 28 days after exogenous administration (43). The high degree of engraftment of LR-MSCs in the present study is even more notable because these cells were injected in the absence of injury. Failure of control cells (human foreskin and human lung fibroblasts) to persist or engraft in the SCID lung further confirmed that LR-MSC persistence is not nonspecific lodging but is likely a unique attribute of this tissue-specific progenitor cell. These characteristics in the context of lung injury remain to be determined.

In aggregate, the findings of this first investigation of the *in vitro* and *in vivo* interactions of lung-specific MSCs offers several novel translational directions. First, the extensive and prolonged engraftment of exogenously administered LR-MSCs in the lung suggests that tissue-specific cells might be better poised than BM-derived progenitors to engraft during organ-specific therapeutic interven-

tions. Second, the ability of LR-MSCs to negotiate across the epithelial barrier and to establish residence in close approximation to epithelial cells raises the possibility of using MSCs as a delivery vehicle. Third, our findings of gap junction communications between LR-MSCs and epithelial cells opens the field to further understanding of the role of heterocellular gap junction communications between resident progenitor cells in lung repair and disease responses.

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