Bronchioalveolar stem cells increase after mesenchymal stromal cell treatment in a mouse model of bronchopulmonary dysplasia

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Bronchioalveolar stem cells (BASCs) are an adult lung stem cell population capable of self-renewal and differentiation in culture, and BASCs proliferate in response to bronchiolar and alveolar lung injury in vivo. Systemic treatment of neonatal hyperoxia-exposed mice with MSCs or MSC-CM led to a significant increase in BASCs compared with untreated controls. Treatment of BASCs with MSC-CM in culture showed an increase in growth efficiency, indicating a direct effect of MSCs on BASCs. Lineage tracing data in bleomycin-treated adult mice showed that Clara cell secretory protein-expressing cells including BASCs are capable of contributing to alveolar repair after lung injury. MSCs and MSC-derived factors may stimulate BASCs to play a role in the repair of alveolar lung injury found in BPD and in the restoration of distal lung cell epithelia. This work highlights the potential important role of endogenous lung stem cells in the repair of chronic lung diseases.

alveolar; hyperoxia; bleomycin; alveolar type 2 cell

Bronchopulmonary dysplasia (BPD) remains a major complication of prematurity resulting in significant morbidity and mortality. BPD remains a major healthcare concern despite advances in perinatal care and decline in mortality rates among very-low-birth-weight (VLBW) infants (11, 14, 35). The increased rates of survival among VLBW infants contribute to the overall increase in incidence of BPD (38). The long-term health risks and healthcare costs are significant in survivors (22, 34), and most current therapies are palliative (3).

Recent advances have shown protective effects of mesenchymal stromal cells (MSCs) in repair of injured tissues, specifically in lung disease (33). Studies have shown that bone marrow-derived MSC therapy can prevent adult lung injury in animal models of endotoxin, bleomycin, monocrotaline, and hypoxia-induced lung injury (12, 20, 23, 25, 33, 40). Hyperoxia-induced lung injury is an established rodent model of lung disease similar to BPD with rarification and simplification of alveoli, thickened septa, and vascular damage (8, 41). MSCs delivered intravenously or intratracheally after birth protect neonatal mice and rats, respectively, from hyperoxic lung injury with decreased inflammation and improved alveolar structure (1, 37). MSC engraftment in these studies was very low, and therapeutic benefit was likely triggered by a paracrine-mediated mechanism and immunomodulation (1, 19, 37).

In support of this, the MSC-conditioned media (MSC-CM) delivered intravenously in the neonatal murine model supported the protective effects of MSCs (1). MSCs have been previously shown to stimulate cardiac stem cell proliferation and differentiation after cardiac injury (13), but no work to date has closely examined MSC effect on lung stem cells. The beneficial response to MSCs and MSC-CM may be due at least in part to the activation of endogenous lung epithelial stem cells.

Analysis of lung injury models has suggested that each epithelial niche in the lung contains its own stem or progenitor cell population (26). Stem cells are the self-renewing cells that are capable of giving rise to differentiated cell types of a tissue during development or in response to injury in adults. Multiple lung progenitor cells have been identified, and ongoing work continues to help define their distinct roles (4, 9, 16, 28, 32). Previous studies have shown that cells expressing both the AT2 cell marker, prosurfactant protein C (SPC), and the Clara cell marker, Clara cell secretory protein (CCSP), are present in the normal lung and may constitute a stem cell population in the distal lung epithelium (16). These cells named bronchioalveolar stem cells (BASCs) reside in the bronchioalveolar duct junction (BADJ) in terminal bronchioles. BASCs can be isolated using a fluorescence-activated cell sorting (FACS) methodology (16, 42). BASCs self-renew over multiple passages on mouse embryonic fibroblast (MEF) feeders or in a three-dimensional (3D) air-liquid interface suspended in the protein mixture Matrigel (16, 42; C.F. Kim, unpublished observa-

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tions). In differentiation conditions, BASCs give rise to bronchiolar and alveolar cells, and, although they are quiescent in normal lung, they proliferate in response to lung injury and may serve a role in lung repair (16). BPD involves defects in the distal lung and alveolar epithelial differentiation, and BASCs have the capacity to differentiate into bronchiolar and alveolar lineages in vitro. Thus it is conceivable that BASC function could be beneficial for BPD.

Although previous work has suggested that BASCs do not contribute to hyperoxic injury repair (29), the precise role of BASCs in response to hyperoxia in the neonatal model of BPD and, in particular, after treatment with MSCs or MSC-CM is unknown. Here we examined the possibility that BASCs are important in the repair of alveolar damage found in BPD and in the restoration of distal lung cell epithelia. We also further probed the potential of BASCs to give rise to alveolar cells.

**MATERIALS AND METHODS**

**Animals and hyperoxia exposure.** FVB newborn mice born in the Animal Facility at Children’s Hospital Boston from four different litters were subdivided into two groups and exposed to hyperoxia (75% O2) in a Plexiglas chamber (OxyCycler; BioSpherix, Redfield, NY) or normoxia (21% O2) within 24 h of birth. Ventilation was adjusted to remove CO2 so that it did not exceed 5,000 ppm (0.5%) (average range 1,000–3,000 ppm). Ammonia was removed by ventilation and activated charcoal filtration through an air purifier. Dams were rotated from hyperoxia to room air every 24 to 48 h to prevent excessive oxygen toxicity to the adult animals. Each litter consisted of fewer than 12 pups to control for the effect of litter size on nutrition and growth. For normoxia vs. hyperoxia comparison, mouse lung sections were obtained from Vivek Balasubramaniam’s laboratory where FVB newborn mice born in the Animal Facility at University of Colorado were exposed to hyperoxia (80% O2), achieving an alveolar Po2 of 466 mmHg at Denver altitude, or normoxia (21% O2) within 24 h of birth for 10 days. All animal procedures and protocols were reviewed and approved by the institution Animal care and Use Committee.

**MSC and MSC-CM treatment.** MSC and pulmonary artery smooth muscle cell (PASMC) confluent cultures were incubated in serum-free α-MEM media for 24 h, and conditioned media representing equal number of cells were concentrated 10-fold using Amicon Ultra Centrifugal Filter Device (Millipore, Billerica, MA) with a molecular weight cutoff of 10 kDa. A volume of 50 μl concentrated MSC-CM was injected via the superficial temporal vein on postnatal day (PND) 4 to test the potential preventative effect of treatment. PASMC-conditioned media (PASMC-CM) in the same concentration and volume served as control. A suspension of 5 × 10^4 MSCs (isolated as previously described, Ref. 1) in 50 μl PBS was injected via the superficial temporal vein on PND 4. After an additional 10 days in hyperoxia, animals were killed and lungs were perfused and embedded for histopathology (Fig. 1A).

**BASC immunofluorescence and quantification.** Paraffin-embedded mouse lung sections were stained for SPC, CCSP, and DAPI. Primary antibody staining was performed with rabbit-mouse pro-SPC (1:750; WRAB-9337, Seven Hills Bioreagents, Cincinnati, OH) and goat-mouse CCSP (1:250; sc-9773; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody staining was performed with donkey-goat 488 (1:200; Invitrogen, Carlsbad, CA), donkey-rabbit 594 (1:200; Invitrogen), and Vectastain with DAPI was used. BASCs were identified as cells that were dual positive for both CCSP and SPC.

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**Fig. 1.** Experimental timeline and effect of bone marrow-derived mesenchymal stromal cell-conditioned media (MSC-CM) on hyperoxic alveolar injury. A: neonatal mouse pups were exposed to hyperoxia on postnatal day (PND) 1, systemically injected with MSCs or MSC-CM on PND 4 and killed on PND 14 as previously reported (1). Control mice were injected with pulmonary artery smooth muscle cells (PASMCs) or PASMC-conditioned media (PASMC-CM). Histological specimens were prepared, and lung sections were stained with immunofluorescent (IF) markers for prosurfactant protein C (SPC) and Clara cell secretory protein (CCSP) followed by microscopic quantification. B: representative lung sections from normoxia-exposed control animals compared with hyperoxia-exposed animals treated with either PASMC-CM or MSC-CM. Treatment with MSC-CM prevented alveolar loss and improved volume density of the alveolar wall tissue compared with PASMC-CM. ×100 FITC images are shown.
The number of BASCs present per terminal bronchiole was quantified and statistically analyzed using Microsoft Excel and a two-tailed t-test. Microscopy and imaging was performed with a Nikon Eclipse 90i, X-Cite 120 Fluorescence Illumination System, and NIS Viewer software. Images were processed with NIS Viewer software and Adobe Photoshop. Investigators were blinded to experimental group identity during staining, imaging, and quantification, and code was revealed for statistical analyses.

**BASC colony growth in MSC-CM.** BASCs were isolated from WT129 4–6-wk-old mice using FACS methodology as described (16, 42). Hematopoietic lineages were excluded by selecting for CD45-negative cells, and endothelial lineages were excluded by selecting CD31-negative cells. BASCs were plated on irradiated DR4 MEF feeders in MSC-CM or PASMC-CM diluted 1:10 or 1:100 from concentrated stock or in standard BASC media containing DMEM/10% FBS/HEPES buffer/L-glutamine/penicillin-streptomycin. BASCs were plated on MEF feeders in 96-well plates with 1,000 cells per well. Plates were scored for colony growth and colony size after 7 days, and the fold change in colony formation and size difference for BASCs cultured with MSC-CM vs. standard media was determined. Additional in vitro experiments were performed to examine the effect of candidate growth factors on BASC growth.
Supplementation with VEGF (50 ng/ml) alone, hepatocyte growth factor (HGF) (50 ng/ml) alone, VEGF (50 ng/ml) and HGF (50 ng/ml), or basic fibroblast growth factor (bFGF) (50 ng/ml) and keratinocyte growth factor (KGF) (50 ng/ml) was added every other day to standard BASC media. Plates were scored for colony growth after 7 days.

In vivo lineage tracing. Knockin Cre recombinase-modified estrogen receptor fusion protein mice, CCSP-CreER; Iox-stop-lox-yellow fluorescent protein (LSL-YFP) (29) were administered tamoxifen from a 20 mg/ml stock solution dissolved in Mazola corn oil. Tamoxifen was delivered via intraperitoneal injection every other day for a total of two doses. A dose of 0.25 mg/g body wt was used. Two weeks after the final tamoxifen dose, bleomycin (30 μg of a 0.05 mg/ml solution in PBS; Sigma, St. Louis, MO) or 30 μl of PBS as a control was administered intratracheally. At time points 0 and 4 wk, animals were killed, and lungs were perfused and isolated for histopathology. Immunofluorescence was performed with four-color staining including primary antibodies of chick α-mouse GFP (1:500; 1229FP08; Aves, Tigard, OR), goat α-mouse SPC (1:100, A0609, Santa Cruz), and rabbit α-mouse CCSP (1:50; B1308; Santa Cruz). Secondary antibody staining included donkey α-chick 488 (1:100; Jackson ImmunoResearch, West Grove, PA), donkey α-goat 680 (1:200; Invitrogen), and donkey α-rabbit 594 (1:200; Invitrogen) and Vectastain with DAPI. Four-color microscopy and imaging was performed using a Nikon Eclipse 90i, X-Cite 120 Fluorescence Illumination System and NIS Viewer software. Images were processed with NIS Viewer software and Adobe Photosop.

RESULTS

Systemic injection of MSCs or MSC-CM increased BASC numbers in vivo. Neonatal mouse pups exposed to either hyperoxia (75%) or normoxia (21%) were injected on PND 4 with MSCs or MSC-CM and killed on PND 14. Single-dose treatment was administered on PND 4, the start of alveolarization in mouse lung development. This treatment strategy is highly clinically relevant as a model that could be used as injury prophylaxis in a high-risk neonatal population. Immunofluorescence staining of the lung sections and quantification of BASCs were performed (Fig. 1A). Histological sections of lung tissue after hyperoxia exposure confirmed the well-described injury phenotype of alveolar simplification and decreased alveolar surface area. As previously reported (1), this parenchymal injury was ameliorated in response to MSC treatment and prevented with MSC-CM treatment (Fig. 1B). BASCs were identified as previously described (16) as cells located at the terminal bronchioles staining positive for both the Clara cell marker, CCSP, and alveolar type 2 cell marker, SPC (Fig. 2A). Consistent with previous work (29), no difference was seen in number of BASCs present in normoxia controls vs. non-MSC-treated hyperoxia-exposed animals (Fig. 2B), indicating that injury alone did not stimulate BASC proliferation. The average number of BASCs present per terminal bronchiole was significantly increased in response to MSC treatment by 1.4-fold (P = 0.03) compared with hyperoxia control (Fig. 2C) and also further increased in response to MSC-CM by 2.1-fold (P = 0.002) compared with hyperoxia controls (Fig. 2D). More terminal bronchioles had at least one BASC after MSC or MSC-CM delivery. There was also an increase in BASC number with exposure to PASMC-CM, which may indicate that PASMCs are also able to contribute in the microenvironmental control and injury response of BASCs (Fig. 2D). The percentage of terminal bronchioles with greater than one BASC was also increased in the MSC-CM-treated group compared with the MSC-treated group and other control groups (Fig. 2E). Together, these data show an increased number of BASCs present in the hyperoxia-exposed experimental states that exhibited repaired alveolar structures. The observed increase in BASCs could indicate that BASCs are important in the repair of hyperoxia-induced alveolar injury occurring after treatment with MSCs or MSC-CM. The greater increase in BASC numbers seen in the MSC-CM-treated group supports the hypothesis that paracrine factors are at least partially responsible for inducing the BASC response.

Confirming a role for paracrine-mediated mechanisms of MSC treatment in vivo, BASCs exhibited increased growth efficiency when stimulated with MSC-CM compared with standard culture media. BASCs were isolated by FACS (16, 42) and plated on an embryonic mouse fibroblast feeder layer in either MSC-CM or standard media (Fig. 3A). The colony-forming ability of BASCs exposed to 1:10 MSC-CM was increased by 1.9-fold compared with standard media (P = 0.01) and by 1.7-fold compared with PASMC-CM (P = 0.05) (Fig. 3, B and C). This effect was found with 1:10 MSC dilution but not with 1:100 dilution, suggesting a dose-dependent effect (Fig. 3D). No difference in colony formation was seen with PASMC-CM in vitro (Fig. 3C), suggesting that the PASMC-CM effect on BASCs in vivo (Fig. 2D) was unlikely to be attributable to a direct effect.

Fig. 3. MSC-CM stimulates BASC activity in vitro. A: experimental design of coculture system to direct effect of MSC-CM on growth of BASCs on irradiated mouse embryonic fibroblast (MEF) feeder layers. Left: representative fluorescence-activated cell sorting (FACS) scatter plot displaying isolation of BASCs from adult wild-type mice. Plot shows distinct cellular populations in the lung identified by staining for Sca-1, CD45, and CD31. The percentage of total live lung cells represented by BASCs is indicated. B: representative colony images of BASCs grown in standard media, PASMC-CM, and MSC-CM. C: colony-forming ability of BASCs increased with MSC-CM compared with standard media or PASMC-CM. No difference was seen between the standard media and the PASMC-CM group. Wells were plated with 1,000 BASCs, and the number of wells with colonies was assessed after 7 days. D: maximum colony growth found with 1:10 MSC-CM dilution compared with 1:100 MSC-CM dilution. Isolation and plating as described above. BASCs were grown in standard media including supplementation with 1:10 MSC-CM or 1:100 MSC-CM dilution in standard media. No difference noted at 1:100 dilution (P = 0.58), but a significant difference was seen at 1:10 dilution (P = 0.01). E: BASC colonies grown in 1:10 MSC-CM were greater in size compared with standard media group (P = 0.01). After 7 days of growth, colonies were counted and size was noted as small (<15 cells), medium (15–30 cells), large (16–60), and extra-large (>60 cells) present in colony. Statistics performed using Cochran-Armitage test and MSTAT software. F: no difference noted in BASC colony growth with individual growth factor supplementation compared with standard media. BASCs were isolated from B-actin-green fluorescent protein (GFP) mice via FACS methodology, and 1,000 cells/well were plated on MEFs in a 96-well plate. Growth factor supplementation of 50 ng/ml was added every other day, and colonies were counted on day 7. Growth factor supplementation included keratinocyte growth factor (KGF) alone, basic fibroblast growth factor (bFGF), VEGF and hepatocyte growth factor (HGF), HGF alone, or VEGF alone. No significant difference in colony growth was noted compared with the nonsupplemented MEF alone group. G: representative pictures of GFP-positive BASC colonies from different experimental conditions illustrating similar morphology and size. No qualitative morphology difference was found in colony appearance with growth factor supplementation. *P < 0.05.
Colonies grown in MSC-CM were also larger in size compared with standard media group (Fig. 3E) ($P = 0.01$). These findings support the hypothesis that MSC-secreted components can directly stimulate BASC proliferation in vivo. When standard media was supplemented with candidate factors known to be present in MSC-CM including VEGF, KGF, bFGF, or HGF (6, 10, 17–19), no difference in BASC colony growth or morphology was detected (Fig. 3, F and G). It remains possible that a complex combination of paracrine factors that include these molecules is responsible for the observed effect on BASCs from MSCs. These findings help pave the way to identify factors that will have the ability to influence endogenous lung stem cells to participate in lung repair.

The BASC expansion we observed in response to MSCs or MSC-CM treatment in the neonatal murine BPD model is similar to the BASC proliferation previously seen in bronchiolar and alveolar injury of adult lungs after exposure to naphthalene or bleomycin (16). Some lineage-tracing data have suggested that BASCs do not contribute to repair of alveolar cells in an adult hyperoxia injury model (29). It is known that hyperoxia injury inhibits both the lung vascular...
and alveolar architecture in both adult and neonatal exposure (8, 30, 39, 41). It is possible that the vascular damage from hyperoxia in the previous lineage-tracing study (29) and in our own hyperoxia-exposed experiments without MSC-based treatment inhibited the ability of BASCs to proliferate and repair alveolar injury; BASCs may require an intact endothelial microenvironment for in vivo alveolar potential. Recent work has shown that bone marrow-derived angiogenic cells restore lung alveolar and vascular structure after neonatal hyperoxia (2), which supports our hypothesis that an influence on vascular function is necessary for alveolar repair after hyperoxic injury. Interestingly, Ding et al. (7) recently showed the importance of endothelial-derived angiocrine signals for BASC expansion and alveolarization in the setting of pneumonecrosis, supporting the importance of the endothelial microenvironment in epithelial injury repair. Other recent work has suggested that CCSP-expressing cells can participate in alveolar differentiation during development (21), further supporting the idea that BASCs can contribute to alveolar epithelia in a context-dependent fashion. It is known that angiogenesis is crucial for alveolar development and repair in BPD (36). Interestingly, MSCs and MSC-CM have been shown to prevent vascular injury in the neonatal hyperoxic model (1, 37), and paracrine factors of MSCs have been shown to recruit endothelial lineage cells (5). Putting the findings together, hyperoxic injury may limit BASC and endothelial cell functions, and MSCs, and MSC-CM may provide at least part of their beneficial effect through direct stimulation of BASC proliferation and indirectly through endothelial cells that, in turn, support BASC ability to participate in repair. Therefore, repair of the simplified alveoli found in hyperoxia-induced BPD may require coordination of endogenous lung stem cell and alveolar cell differentiation and growth as well as vasculogenesis, mimicking normal alveolar development.

Lineage-tracing experiments after bleomycin injury supported our hypothesis that BASCs can contribute to alveolar repair after injury. Bleomycin injury, which causes isolated damage to the alveolar epithelial cells without endothelial damage (15, 24, 27), was previously shown to expand the BASC population 2 wk postinjury, at the height of tissue damage, and BASC numbers returned to baseline after injury repair (16). An inducible Cre knockin mouse (29) was used to in vivo lineage tag CCSP-expressing cells before bleomycin delivery. As previously shown, CCSP-expressing

Fig. 4. CCSP-expressing cells contribute to alveolar repair after bleomycin injury. A: experimental design of lineage-tracing study. CCSP-CreER; lox-stop-lox-yellow fluorescent protein (LSL-YFP) mouse (29) was given a total of 2 doses (0.25 mg/g body wt) of tamoxifen. Tamoxifen was delivered via intraperitoneal injection with 1 dose given every other day (qod). Two weeks after the last tamoxifen dose, bleomycin (0.025 units) or PBS was delivered intratracheally (IT). Four weeks after IT administration, mice were killed, and lungs were sent for histopathology. Lung sections were stained for 4-color IF with SPC, CCSP, YFP, and DAPI. Microscopic quantification was performed to determine the number of YFP-positive cells present in the alveolar space. B and C: representative immunofluorescence images of CCSP-CreER; LSL-YFP mouse BADI at time point 4 wk after intratracheal injection of PBS (B) or bleomycin (C). IF for CCSP (red), SPC (pink), YFP (green), and DAPI (blue). Merged 4-color image and separate dual channels pictured below in enlarged area (box). Arrows, YFP-positive, SPC-positive, CCSP-negative AT2 cells. D: increased percentage of YFP-positive lineage tagged AT2 cells found in the alveolar space after injury with bleomycin compared with control animals (P < 0.05). The percentage of all SPC-positive, CCSP-negative cells that exhibited YFP staining from 4 mice in each experimental group is shown. E: total AT2 cell numbers are unchanged 4 wk after bleomycin injury and repair. AT2 cells were identified as cells in the alveolar space as SPC positive, CCSP negative. Total number of AT2 cells present per ×20 microscopic field were counted and averaged. No difference in number of AT2 cells present between groups was found at 4 wk postinjury and repair (P = 0.15). F: BASC numbers are unchanged 4 wk after bleomycin injury and repair. BASCs were identified as SPC-positive, CCSP-positive cells present at the BADI. No difference in number of BASCs was found at 4 wk postinjury in bleomycin group compared with PBS control group, a time point where repair has largely completed (P = 0.06). Data represented is from 4 independent experiments.

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**DISCUSSION**

Bone marrow-derived MSCs have shown great promise in mitigating lung injury in animal models of disease, and recent studies have pointed to the therapeutic benefit likely attributable to soluble factors secreted by the MSCs. Identification of the active components of the MSC-CM that stimulate BASCs may lead to new therapeutic strategies for chronic lung diseases and protection of the premature neo-
natal lung. Continued work to determine the array of paracrine factors responsible for the protective effects of the MSC-CM remains crucial in understanding the mechanism of action. Future studies will need to be pursued to identify the factor(s) and pathway(s) involved in the observed MSC-CM, including approaches such as fractionation of conditioned media coupled with protein mass spectrometry of active fractions and use of antibody arrays, yet will require extensive additional work beyond this study.

This work highlights the potential of endogenous lung stem cells to be stimulated to differentiate and participate in the repair of alveolar injury and lung disease including BPD. These studies raise the possibility of selected targeting of endogenous lung stem cells in chronic lung disease states.

A J C E R ; L S L - Y F P

IntraTracheal Administration
Bleomycin 0.025 units or PBS

IP Tamoxifen
(x 2 doses qod)

Timepoint 0
Timepoint 4 weeks

Histologic lung sections
Microscopic quantification

4 color IF staining with SPC,
CCSP, DAPI, YFP

CCSP
SP-C
DAPI
YFP

YFP
DAPI
SP-C
DAPI
CCSP
DAPI

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

12. L836 MSC EFFECT ON LUNG STEM CELLS IN BPD


