Targeted cell replacement with bone marrow cells for airway epithelial regeneration

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Wong AP, Dutly AE, Sacher A, Lee H, Hwang DM, Liu M, Keshavjee S, Hu J, Waddell TK. Targeted cell replacement with bone marrow cells for airway epithelial regeneration. Am J Physiol Lung Cell Mol Physiol 293: L740–L752, 2007. First published July 6, 2007; doi:10.1152/ajplung.00050.2007.—It has been suggested that some adult bone marrow cells (BMC) can localize to the lung and develop tissue-specific characteristics including those of pulmonary epithelial cells. Here, we show that the combination of mild airway injury (naphthalene-induced) as a conditioning regimen to direct the site of BMC localization and transtracheal delivery of short-term cultured BMC enhances airway localization and adoption of an epithelial-like phenotype. Confocal analysis of airway and alveolar-localized BMC (fluorescently labeled) with epithelial markers shows expression of the pulmonary epithelial proteins, Clara cell secretory protein, and surfactant protein C. To confirm epithelial gene expression by BMC, we generated transgenic mice expressing green fluorescent protein (GFP) driven by the epithelial-specific cytokeratin-18 promoter and injected BMC from these mice transtracheally into wild-type recipients after naphthalene-induced airway injury. BMC retention in the lung was observed for at least 120 days following cell delivery with increasing GFP transgene expression over time. Some BMC cultured in vitro over time also expressed GFP transgene, suggesting epithelial transdifferentiation of the BMC. The results indicate that targeted delivery of BMC can promote airway regeneration.

bone marrow cell; lung; epithelium; airway regeneration

THE ETHICAL CONCERNS of using embryonic stem cells have prompted great interest in identifying the utility of adult tissue-specific progenitor cells and bone marrow-derived stem cells for the purpose of tissue repair or regeneration. Although controversial, some data suggest that adult stem cells can engraft and transdifferentiate into tissue-specific cell types of various organs such as the heart (29), pancreas (6), and nervous system (9). This potential plasticity suggests that bone marrow cells (BMC) could also be used as “cell replacement” therapy for pulmonary diseases, such as cystic fibrosis, a debilitating incurable disease that is characterized by abnormal salt and water transport leading to abnormal airway secretions, impaired mucociliary clearance, and chronic inflammation due to secondary bacterial infections (32).

Studies have shown that BMC delivery to the injured lung promotes lung repair and regeneration (19, 31). The common method of cell transplantation into the lung is intravenous injection by tail vein (24, 26, 31) or jugular vein (5, 44). However, intravenous injection may not be the optimal technique, and direct cell delivery via the transtracheal route has many theoretical advantages, including better efficiency of cell delivery to the airway and respiratory epithelium, reduced risk of systemic delivery, and reduced requirement for tissue injury to promote cell homing. Here, we assessed the potential of short-term cultured plastic-adherent BMC using transtracheal cell delivery in a targeted, acute, mild, and reversible naphthalene-induced airway injury known to specifically deplete airway Clara cells (36). By depleting these local progenitor cells (3, 10), we hoped to facilitate the contribution of BMC to airway regeneration. Thus, in a manner analogous to bone marrow transplantation, naphthalene was used not as a model of airway injury to be ameliorated, but as a “conditioning regimen” to create an airway-specific niche for cell incorporation.

Our results demonstrate enhanced airway and alveolar incorporation of BMC in or on the epithelium after naphthalene-induced injury with direct transtracheal delivery compared with intravenous cell delivery. Some of the retained BMC expressed the airway epithelial markers CCSP. To further demonstrate the transdifferentiation potential of these BMCs, we developed a transgenic mouse model with the human cytokeratin-18 promoter driving green fluorescent protein (GFP) expression. Using this animal as bone marrow donor, we demonstrated cytokeratin-18 promoter-driven transgene expression, which increased over time and persisted up to at least 120 days. These important and novel observations suggest that the bone marrow does indeed harbor cells with the potential to contribute to airway regeneration. Moreover, using a combination of short-term ex vivo culture, a simple and direct mode of cell delivery, and a specific mild epithelial injury, targeted incorporation of BMC to the lung epithelium can be achieved. Subsequently, these cells can differentiate to express epithelial genes.

MATERIALS AND METHODS

Animals. Adult male (4–6 wk) C57Bl/6 (Charles River Laboratories, Wilmington, MA) and transgenic K18GFP (reporter gene-enhanced GFP is driven by the cytokeratin-18 promoter; CD1 background) mice were BMC donors. Recipients were strain- and age-matched females (4–6 wk). All animal procedures were approved by the University Health Network Animal Care Committee. All mice received care in compliance with the Principles of Laboratory Animal Care of the National Institutes of Health.

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Bone marrow harvest, culture, and fluorescent labeling. BMC were harvested as previously described (24). Briefly, whole bone marrow was harvested aseptically by flushing femurs and tibiae of donor mice with cold DMEM (GIBCO, Grand Island, NY) supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin (GIBCO) using a 23-G needle. BMC were plated on standard plastic tissue culture flasks at a density of 5 × 10^6 cells/cm^2, and the medium was changed every 2–3 days. Nonadherent cells were gradually washed away with successive media changes. The remaining plastic-adherent BMC were cultured for 7 days in DMEM containing 10% FBS and 1% penicillin-streptomycin before use as BMC in vivo. Before delivery, BMC were labeled with CellTracker Orange CMTMR (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol.

Tracheal epithelial cell isolation and culture. Tracheal epithelial cells (TEC) were isolated and cultured as previously described (43). Briefly, TEC were harvested by 0.15% pronase digestion overnight at 4°C followed by Dounce homogenization on ice for 10 min. The cells were plated on plastic culture plates for 3 h to adhere fibroblasts. The nonadhered epithelial cell populations were then seeded onto collagen type IV-coated transwell membranes (Costar) and cultured in vivo. Before delivery, BMC were labeled with CellTracker Orange CMTMR (Molecular Probes, Eugene, OR) according to the manufacturer’s protocol.

Naphthalene-induced lung injury and BMC administration. Female recipient mice (4- to 6-wk-old) were given an intraperitoneal injection of naphthalene (>99% pure; Sigma Chemical, St. Louis, MO), 200 mg/kg body wt as previously described (36). The same dose was used for both C57Bl/6 and CD1 strains. Clara cell depletion was determined by immunoperoxidase staining for CCSP and blindly assessed by a pulmonary pathologist. BMC (10^6 cells/mouse) were delivered transtracheally or intravenously (via jugular vein) 2 days following injury. For transtracheal delivery, mice were anesthetized with 5% isoflurane and intubated. With the use of a pipette and sterile 100-μl volume gel-loading tips, 50 μl of cell suspension was injected directly into the trachea distal to the vocal cords. The mice were then rotated to ensure equal dispersion of the cell suspension to both lungs. For intravenous injection, mice were anesthetized (100 mg/kg ketamine and 5 mg/kg xylazine i.p.), and the left cervical area was shaved and cleaned with 70% ethanol. Using an open cut-down technique, the external jugular vein was catheterized with a 50-gauge catheter, and 50 μl of cell suspension was injected intravenously. All incisions were closed with 4-0 Prolene sutures.

Mice were killed by anesthetic drug overdose at 1, 4, 7, 14, 30, 60, or 120 days after BMC injection with at least three animals per time point. The right lungs were fixed by intratracheal instillation of 10% neutral formalin (Sigma) or freezing medium (O.C.T.; Fisher Scientific, Pittsburgh, PA) mixed in PBS (1:1) at a constant 20 cmH2O pressure for 30 min for immunohistochemistry. The heart, spleen, liver, kidney, and intestines were also harvested. Whole lungs and heart from CD1 recipient mice that received K18EGFP BMC were quickly harvested and snap-frozen in liquid nitrogen.

Lung function measurements. Mice (n ≥ 3 per time point) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) mixture. A tracheostomy was performed after dissection of the neck to expose the trachea. A metal cannula for the tracheal lumen was inserted into the trachea, and animals were connected to a volume-controlled constant flow ventilator (Inspiria Advanced Safety Ventilator, Harvard Apparatus). Anesthesia was continuously maintained with isoflurane, and body temperature was maintained at 37°C by an immersion thermostat throughout the experiment. The animals were then ventilated for 20 min with a tidal volume of 5 ml/kg, an inspired oxygen fraction of 1.0, an inspiratory/expiratory ratio of 1:2; and a frequency of 140 breaths/min. An esophageal catheter (Harvard Apparatus) was inserted for measurement of dynamic lung compliance. Airway pressures and dynamic lung compliance were continuously monitored throughout the 20-min period of mechanical ventilation with HSE-USB acquisition hardware and Pulmodyn software (H. Sachs Elektronik, March-Hugstetten, Germany).

Assessment of donor cells in recipient lungs. Recipient lungs were either fixed at constant pressure (20 cmH2O) with 10% formalin or O.C.T. or snap-frozen in liquid nitrogen. Paraffin or frozen sections were prepared for immunohistochemical characterization. Briefly, immunofluorescence staining for CCSP (cat. no. 07-623, Upstate Laboratories), pro-SP-C (cat. no. AB3428, Chemicon, Temecula, CA), cytokeratin-18 (K18: clone Ks 18.04; RDI, Flanders, NJ), cytokeratin-5/8 (K5/8: cat. no. MAB3228, Chemicon), CD11b (clone M1/70.15; Abcam, Cambridge, MA), CD3 (Clone APA1/1, Upstate), CD45 (clone OX30, Serotec), vWF (cat. no. AB7356, Abcam), and GFP (cat. no. AB6556, Abcam) was performed on nonadjacent serial frozen sections of 8-μm thickness of lung from each of the control and naphthalene-treated groups to detect Clara cells, type II pneumocytes, mature airway epithelial cells, monocytes, macrophages, T cells, and endothelial cells, respectively. Briefly, sections were fixed with icecold acetone, rinsed with wash buffer (PBS containing 0.05% Triton X-100) and blocked with 5% goat serum in PBS for 2 h at room temperature, and incubated overnight at 4°C with rabbit anti-CCSP (1:2,000) and anti-pro-SP-C (1:2,000), rat anti-CD45 (1:100), mouse anti-K18 (1:50), anti-CD11b (1:200), anti-K5/8 (1:200), anti-CD3 (1:100), anti-vWF (1:400), and anti-GFP (1:1,000). After extensive washes, the slides were then treated with Alexa Fluor 488-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:500; Molecular Probes-Invitrogen), counterstained with Hoechst (nuclear) dye (1: 5,000, Sigma), and mounted with immunofluorescence mounting medium (Vector Laboratories, Burlingame, CA). Isotype controls were used for nonspecific binding. Images were acquired using an Olympus FluoView 1000 Confocal Imaging System (Olympus, Melville, NY) with a pinhole diameter of 80 μm. Optical dissection along the z-axis was captured at 0.25- to 0.5-μm increments. Images were acquired using sequential mode to eliminate cross talk of the fluorescence with a sampling speed of 8 μs/pixel.

To quantify the number of CMTMR-labeled BMC in the lungs, four random fields from each of eight to ten sections were selected per right lung. CMTMR-labeled BMC and all double-positive (CMTMR+ and marker-positive cells) were counted and normalized per 100 nuclei or 100 CMTMR+ cells, respectively. Two methods of counting were performed: 1) manual count, all nuclei (blue), CMTMR-positive (colocalization of red CMTMR and blue nuclei) cells, double-positive (colocalization of red CMTMR, green antibody stain, and blue nuclei) cells; 2) count by threshold intensity using an imaging program (Image J). The counts were similar with an average 5% variation. The level of threshold intensity of a stain was set by positive and isotype controls. The final count is an average of both methods of cell counting.

Assessment of phagocytosis and/or cell fusion. Fluorescence in situ hybridization (FISH) for the Y chromosome (1189-YMF-01, Cambio) was performed according to the manufacturer’s protocol (Cambio, Cambridge, UK) to assess phagocytosis or fusion of BMC using serial sections of male mice injected with CMTMR-labeled female BMC after 4 days of cell delivery. Images were acquired using the confocal imaging system. A total of 190 CMTMR-labeled cells were counted from three randomly selected sections of three recipient mice. The percentage of Y chromosome-positive cells was calculated by normalizing the number of CMTMR+ cells containing the Y chromosome to the total number of CMTMR+ cells counted.

Generation and characterization of K18EGFP transgenic mice. The K18mTE-EGFP construct was from the K18mTELacZ expression construct (7). It contains 3.4-kb regulatory elements consisting of the 2.5-kb 5’-flanking sequence of human K18, including the native promoter, followed by K18 intron 1, and the alfalfa mosaic virus translational enhancer (TE) sequence (20). The coding sequence of pEGFP-N1 (Clontech) was inserted immediately after TE sequence. At the 3’ end of the EGFP coding sequence, the 1.8-kb 3’-untranslated...
region (UTR) of human K18 was inserted to replace the simian virus 40 (SV40) small T antigen intron and SV40 early polyA signal of the K18mTElacZ. The K18mTE-EGFP cassette was purified as a 6.1-kb fragment, including the regulatory elements, EGFP coding sequence, and the 3′-UTR from K18. Transgenic mice were generated with CD1 mice by standard microinjection procedures. Founder mice were identified by PCR and Southern blot analysis. F1 animals were obtained by breeding founder mice with wild-type CD1 mice.

Localization of cytokeratin-18 promoter-driven GFP expression in the tissues was enhanced by staining with anti-GFP (1:500; Abcam) and visualized with Alexa Fluor 488 secondary immunoglobulin. TEC and BMC from K18GFP and wild-type mice were seeded and fixed with 4% paraformaldehyde (PFA) on slide chambers for immunostaining with anti-GFP antibody using the method described above. Nonspecific binding was determined with isotype controls.

**Real-time PCR analysis.** To quantify BMC in the lungs, excised female lungs were spliced with 104, 105, 5 × 105, and 106 male cells, and a standard curve was generated using real-time qPCR for the SRY gene (forward 5′-GGGATGCAAGTGGAAAACG-3′ and reverse primer 5′-TGTGTCCGTCGTGGATCTGA-3′) from genomic DNA. The number of BMC in the experimental mice was calculated by comparing the crosspoint threshold (CT) amplification value to the standard curve.

To detect donor-derived BMC in recipient mice and upregulation of K18GFP transgene expression, genomic DNA and total RNA were prepared from the lungs and hearts from recipient animals receiving K18GFP BMC using the DNeasy and RNasy kits, respectively (Qiagen, Valencia, CA). Lung tissues from K18GFP mice were used as controls, and detection of transgenic DNA or messenger RNA was assessed as relative expression to control transgenic lung after normalization to housekeeping gene expression. Total RNA (0.5 μg) was reverse transcribed into cDNA and K18GFP human K18 (forward primer 5′-CAAGAGCTGTAGCTCGTCTC-3′, and reverse primer 5′-TGTGTCCGTCGTGGATCTGA-3′) or GAPDH (forward primer 5′-TGTGGTCCGTCTGGATCTGA-3′ and reverse primer 5′-GATGCCGTCGTCACCATC-3′) primer sets were used to amplify respective genes by real-time PCR (SYBR green detection method and ABI7900HT robot and analyzed with SDS 2.0 software). The K18GFP primer pair spans the first intron of K18 (see Fig. 5). Genomic DNA was analyzed using primers designed so that the forward primers recognized sequences within the human K18 intron-1 (forward primer 5′-CAGCGTGCAGCCTCCTCTCC-3′), and the reverse primer 5′-CTGCACAGCCCGTCTCCTAC-3′) recognized sequences in the EGFP transgene. No reverse transcribed (RT) samples and DNA from wild-type mice treated with wild-type cells were used as negative controls.

Total RNA was prepared from TEC, fibroblasts, and BMC (n = 3 sets of BMC from different animals) from K18GFP mice using the RNasy kit (Qiagen). One microgram of RNA was reverse transcribed for first-strand cDNA using Superscript II (Sigma) according to the manufacturer’s protocol. Real-time PCR (SYBR green detection method; Applied Biosystems, Foster City, CA) was performed for amplification of K18GFP expression using the above primers.

GAPDH was determined to be the most stable housekeeping gene by geNorm program. Therefore, it was used as the housekeeping gene to normalize gene expression levels using REST-384 (relative expression software tool, available at www.Gene-Quantification.com) program. The real-time PCR conditions and instrumentation were as follows: 50°C for 2 min; 95°C for 10 min; 35 cycles at 94°C for 15 s; 60°C for 1 min followed by dissociation using the ABI7900HT robot and analyzed with SDS 2.0 software.

**Flow cytometry.** Freshly isolated BMC and plastic-adherent BMC (cultured for 7 days, 5 × 105 cells/sample) were fixed with 4% PFA and stained with mouse anti-CD34-PE (1:100; BD Biosciences, Mississauga, ON, Canada), biotin-labeled mouse anti-CD44 (1:100), anti-CD34 (1:100), anti-vimentin (1:100, Dako), or anti-mouse-CD45-PE (1:100, BD Biosciences), anti-CD106 (1:100, BD Biosciences), rat anti-mouse CD105 (1:100, BD Biosciences), or anti-CD90 (1:100, BD Biosciences) in FACS buffer (2% BSA, 0.05% sodium azide in PBS) for 30 min on ice. For vimentin staining, the cell membrane was permeabilized with FACS buffer containing 0.5% saponin followed by primary antibody treatment and incubated with Alexa Fluor 488 secondary IgG (1:500, Molecular Probes) or streptavidin-conjugated Alexa Fluor 488 (1:500). Relative expression was compared with isotype controls for all staining. A FACSCalibur flow cytometer and Expo32 Software (Becton-Dickinson, San Jose, CA) were used to assess expression of these markers.

**Statistical analysis.** Data are presented as means ± SE. Statistical comparisons between time points were done by one-way ANOVA followed by Tukey’s post hoc tests (Prism 4.0). Paired t-tests were performed to compare fresh and cultured BMC and transtracheal and intravenous delivery. For all tests, statistical significance was defined as P < 0.05.

**RESULTS**

To determine whether short-term plastic-adherent BMC could repopulate the airway epithelium, we chose to assess the potential of BMC cultured on plastic for 7 days. The phenotype of these BMC was characterized using flow cytometric analysis with a panel of BMC markers including CD34, CD44, CD45, CD90, CD105, CD106, type IV collagen, vimentin, and Sca-1 (Fig. 1). A large percentage of the BMC were positive for the stem cell marker Sca-1 (50.37 ± 0.63%). Small populations of CD34-positive (1.23 ± 0.26%) and CD45-positive (15.45 ± 3.38%) cells were also found. A larger percentage of BMC were positive for CD44 (31.6 ± 1.27%) and type IV collagen (33.93 ± 0.84%). Fewer were positive for CD105 (10.73 ± 0.9%), a marker of mesenchymal stromal cells, but were essentially negative for other mesenchymal cell markers, CD90 (0.47 ± 0.23%), CD106 (0.57 ± 0.12%), and vimentin (0%).

**Mild airway injury promotes BMC deposition.** To replace cells in the airway epithelium, we reasoned that injury to the native airway would be required. Naphthalene has been reported to selectively injure the Clara cells of the bronchiolar epithelium, in a reversible fashion (36). In our studies, naphthalene specifically but transiently depleted the airway Clara cell population from as early as 2 days following injury up to 20 days (Fig. S1). This mild and reversible injury did not significantly alter lung function (Table S2). All mice recovered without mortality. Supplemental material for this article is available online at the AJP-Lung web site.

We chose to administer cells 2 days following naphthalene injection. Fluorescently labeled BMC were delivered transtracheally into naphthalene-injured and vehicle-treated (uninjured) C57Bl/6 mice. CMTMR-labeled BMC were easily detected in the distal airways and alveoli of naphthalene-injured lungs (Fig. 2, A and B). In the absence of injury, no donor BMC were detected (Fig. 2, C and D), confirming that injury is required for BMC retention. We observed no difference in donor BMC levels in the lungs of mice that received BMC 2 or 5 days after naphthalene injection (data not shown).

CMTMR-labeled BMC were observed in or on the injured epithelium as early as 1 day following cell delivery, and these cells persisted for at least 14 days thereafter. Quantification of CMTMR-labeled BMC in the lungs showed up to 4 CMTMR-positive cells per 100 nuclei in the central sections of the lung as early as 1 day following cell delivery, which declined to ~2.5 cells per 100 nuclei by day 14 (P < 0.001 compared with day 1; Fig. 2E). Real-time PCR assessment for the SRY gene detected up
to $7.5 \times 10^6$ (~75%) of the $10^6$ male BMC in both lungs after 1 day of cell delivery and decreased to $10^5$ cells by 14 days (Fig. 2F).

**Transtracheal cell delivery enhances BMC deposition.** The lung is a unique organ in which therapeutic reagents can be delivered directly to the organ through the airway for targeted therapy. To determine if the airway might be a more effective route for cell delivery, we compared transtracheal to intravenous injections of BMC. Two days following naphthalene injury, $10^6$ CMTMR-labeled BMC were injected transtracheally or intravascularly into the jugular vein of naphthalene-injured mice. One day following cell delivery, fluorescent microscopic examination demonstrated significantly more cells in the lungs following direct transtracheal delivery than following intravenous delivery (Fig. 2G). The number of cells detected in lungs after transtracheal delivery was fourfold greater than intravenous delivery (Fig. 2H, $P < 0.001$). Following transtracheal delivery, BMC could be found in the airway, whereas following intravenous administration, the majority of the cells were seen in the lung parenchyma itself. In addition, transtracheal delivery proved to be more specific to the lung. Real-time PCR assessment for the SRY gene detected up to $5 \times 10^4$ male BMC in the right lung after 14 days of transtracheal cell delivery (Fig. 2J). Intravenous delivery yielded 10-fold fewer cells compared with transtracheal. Donor BMC were observed in the recipient liver, spleen, and bone marrow in animals that received intravenous delivery, but no cells were seen in these sites after transtracheal delivery of the cells (data not shown).

**BMC found in the lung express lung epithelial markers.** To determine whether these BMC can adopt a phenotype similar to lung epithelium, we assessed for colocalization of CMTMR-labeled BMC with airway epithelial marker CCSP. Immunofluorescence microscopy without any green fluorophore showed absolutely no nonspecific imaging in the green channel due to cross over of the CMTMR label (Fig. 3A). Similarly, negative control images with an isotype control primary antibody showed no colocalization signal (Fig. 3B). Immunofluorescence imaging of an uninjured lung without BMC treatment shows airway localization of CCSP (Fig. 3C) with no nonspecific staining of the airway epithelium in the isotype control. However, some CMTMR-positive cells that also expressed the pulmonary epithelial marker CCSP were observed in the injured, cell-treated animals (Fig. 3D). To rule out overlapping cells causing apparent colocalization, confocal microscopy was used to provide optical dissection of all possible planar combinations (X-Y, X-Z, and Y-Z). Colocalization of the red and green fluorescence in all planar views was counted as a double-positive BMC. Some BMC appeared to express the airway epithelial Clara cell marker, CCSP (Fig. 3E). Morphologically, in terms of size, orientation, and location, the double-positive BMC resembled that of airway Clara cells and persisted in the lungs 14 days after cell delivery (Fig. 3F, $P < 0.001$ compared with days 1, 4, and 7). As an alternative to the images provided in Fig. 3E, another way to visualize confocal microscopy data is as a z-series stack. Optically dissected images of the section along the z-axis clearly demonstrate colocalization of the CMTMR label with the CCSP stain in one cell (Fig. S2A), ruling out superimposition of a CMTMR+ BMC with a lung cell. Additional representative confocal photomicrographs of other airway-localized CMTMR+ BMC expressing CCSP are provided in Supplementary Material (Fig. S2B).
CMTMR-labeled BMC were also seen in the alveoli and expressed the alveolar type II epithelial cell marker, pro-SP-C (Fig. S3, A and B). Few K18- (Fig. S3, C and D) or K5/8- (Fig. S3, E and F) positive BMC were found in the lungs. Approximately 50% of the CMTMR⁺ cells were positive for CD45 (Fig. S3, G and H) as early as 1 day following cell injection, whereas only 15% of the administered BMC population was CD45⁺ in ex vivo cultures suggesting these cells may either

Fig. 2. BMC persist in the injured lungs after transtracheal delivery. BMC were found in the distal airway (A) and alveolar (B) epithelium after acute airway injury 1 day after cell delivery. No donor BMC were found in the uninjured (vehicle-treated) airway (C) or alveoli (D). BMC were labeled with CMTMR and delivered transtracheally 2 days following naphthalene or vehicle treatment. Quantification of BMC in the lungs up to 14 days after delivery show between 2 and 4 CMTMR⁺ BMC could be detected per 100 nuclei counted (E). Real-time PCR assessment for the SRY gene detected up to 7.5 × 10⁶ (~75%) of the 10⁶ male BMC in both lungs after 1 day of cell delivery and decreased to 10⁵ cells by 14 days (F). *P < 0.001 compared with day 1; †P < 0.05 compared with day 1; ‡P < 0.05 compared with day 7. Greater CMTMR⁺ BMC were found in the airway epithelium when delivered transtracheally compared with intravenous delivery (G). Greater BMC were detected in the lungs (4-fold) after transtracheal delivery (H). Real-time PCR assessment for the SRY gene detected ~5 × 10⁵ male BMC in the right lung after 14 days of transtracheal (TT) cell delivery (I). Intravenous delivery yielded 10-fold fewer cells compared with the transtracheal route. *P < 0.001 compared with intravenous delivery. Means ± SE, n = 4 animals/time point. Nuclei were stained with Hoechst dye. In C, A marks an airway (scale bars, 100 μm).
Fig. 3. BMC in the airway epithelium express CCSP. Representative images of naphthalene-injured, cell-treated lungs show no fluorescent signal of the CMTMR label in the green channel (A) and no nonspecific binding of antibody (B). Representative photomicrograph of uninjured lung sections (C) stained with anti-CCSP (left) or isotype control (right). BMC in the airway epithelium express CCSP (D). Scale bar = 100 μm. Insets: magnified image of CCSP⁺ cells. Arrow points to a second BMC. Confocal 3-plane (X-Y, X-Z, Y-Z) view of a BMC in the airway epithelium that expresses CCSP 14 days after cell delivery (E). Asterisk marks CMTMR⁺ CCSP⁺ BMC. Arrow points to CMTMR⁺ CCSP⁺ epithelial cells. A marks the lumen of an airway. Scale bar represents 10 μm. F: the number of CCSP⁺ CMTMR⁺ double-positive BMC increases significantly by 14 days after cell delivery (*P < 0.001). Double-positive cells are expressed/100 CMTMR⁺ BMC. Means ± SE, n = 4 animals/time point with 10 non-sequential sections from each mouse.
have selective growth advantage in the lungs or CD45+ lung cells might have phagocytosed BMC. Very rare BMC expressed CD11b or CD3 (data not shown). There were no significant differences between all time points in expression of all other markers except CCSP. None of the BMC expressed the endothelial marker vWF (data not shown), although some cells were found in close proximity to blood vessels.

To determine whether colocalization of donor BMC with lung markers could be due to phagocytosis of or fusion with lung cells, a reverse strategy was employed in which female BMC were injected into the lungs of injured male mice and examined for the presence of the Y chromosome in CMTMR+ cells. Of 190 CMTMR+ BMC counted, ~1% of the cells contained a Y chromosome in the nucleus, whereas ~18% contained what appeared to be cytoplasmic Y chromosome (C). Y chromosome (green), nuclei (blue), CMTMR label (red). Scale bar = 10 μm. Asterisk marks CMTMR+CCSP+ (yellow) BMC incorporated in the airway epithelium, n = 3 animals/group.

Fig. 4. BMC fusion with lung cells is a rare event. A: positive and negative tissue control for Y chromosome fluorescence in situ hybridization of male and female lung sections. B: most of the donor BMC did not have Y chromosome in the nuclei or cytoplasm. Cytoplasmic Y chromosome was determined as green dot in CMTMR+ cells. Of 190 CMTMR+ BMC counted, ~1% of the cells contained a Y chromosome in the nucleus, whereas ~18% contained what appeared to be cytoplasmic Y chromosome (C). Y chromosome (green), nuclei (blue), CMTMR label (red). Scale bar = 10 μm. Asterisk marks CMTMR+CCSP+ (yellow) BMC incorporated in the airway epithelium, n = 3 animals/group.

Female to Male

Donor cells
Fusion
Phagocytosis

CMTMR

% CMTMR-positive cells

No Y
Nuclear Y
Cytoplasmic Y

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increments from the top of the section down along the z-axis shows a green dot that appears the same time as CMTMR fluorescence suggesting cytoplasmic localization of the Y chromosome (Fig. S5). On rare occasions (3 out of 190 total CMTMR+ cells counted), a Y chromosome was found in the nucleus of a CMTMR+ BMC (Fig. 4B and Fig. S6). Since ~1% of the total CMTMR+ cells contained a Y chromosome (Fig. 4C), which implies cell fusion, this may be an underestimation since the efficiency of detecting Y chromosome in the nuclei of normal male tissue was only ~60%. Female mice injected with male BMC showed similar frequency of detecting two Y chromosomes in the nuclei of a CMTMR+ cell (data not shown).

**BMC found in the lungs express cytokeratin-18 promoter-driven transgene mRNA.** Colocalization of fluorescent signals, even with confocal microscopy, is subject to multiple difficulties in interpretation. Therefore, we developed a cell lineage-specific transgenic mouse reporter system to evaluate donor-derived BMC by real-time PCR. Transgenic mice that express GFP under the regulatory control of the human K18 promoter (K18GFP) were created and used as a source of BMC. Cytokeratin-18-driven GFP expression was localized to the surface epithelium of the large (data not shown) and small airways of the lungs (Fig. 5A) and in isolated TEC (Fig. 5C) of K18GFP transgenic mice, but not in wild-type lungs (Fig. 5B) and BMC from K18GFP mice (Fig. 5D). To confirm epithelial specificity of GFP expression in the transgenic donor mouse, transgene expression was assessed using anti-GFP staining of various tissues. In addition to the pulmonary expression, GFP was detected in the intestinal epithelium but not in the heart or liver of K18GFP mice (Fig. 5E). In addition, real-time PCR was used to assess transgene expression using intron-spanning primers (Fig. 5F) in isolated TEC, tracheal fibroblasts, and fresh and seven-day cultured BMC of K18GFP mice. No transgene expression was detected in any cells except for TEC (Fig. 5G).

These K18GFP BMC were injected into the trachea of wild-type naphthalene-treated animals. The presence of donor-derived BMC was confirmed by analyzing genomic DNA using a primer set designed to initiate amplification within the K18 intron-1 region and end within the GFP transgene region (Fig. 5F). No donor DNA was detected in the tracheal or lung tissue isolated from wild-type mice. In contrast, low but detectable donor-derived DNA was found in the lungs (Fig. 6A) and trachea (data not shown) of wild-type recipient mice receiving K18GFP BMC. Genomic DNA decreased over time. Donor-derived DNA was also detected in the heart of recipient mice (Fig. S7A).

To examine K18-driven GFP expression, reverse-transcribed total RNA was assessed using the intron-spanning primer pair. Expression of donor-derived K18GFP mRNA increased over time in the lungs (Fig. 6B) and trachea (data not shown). K18GFP mRNA expression was not observed in the heart even though levels of genomic DNA was detected (Fig. S7B).

Anti-GFP staining of recipient lung sections shows presence of GFP-positive cells in the airways of mice at all time points (Fig. 6C). These data suggested that the plastic-adherent BMC population can initiate transcription of genes generally considered to be specific to epithelium, although in vivo several mechanisms, including fusion, might explain this finding. Thus, we analyzed freshly isolated and cultured BMC from K18GFP mice for a panel of epithelial cytokeratin proteins by flow cytometry. As expected, freshly isolated BMC from K18GFP mice were negative for all cytokeratins (Fig. 6D). Anti-GFP staining was negative in the freshly isolated cells. After 7 days in culture, no cytokeratin or GFP-positive cells were found. However, by 28 days in culture, a significant percentage of the BMC were positive for all cytokeratins and GFP (Fig. 6D).

**DISCUSSION**

In this study, we present a novel concept, selective acute destruction of a cell of interest combined with carefully timed, targeted cell delivery, a concept we have termed “cell replacement therapy.” Although the level of donor cell retention was low, this study provides proof of principle that targeted delivery of short-term cultured BMC into a specific but mild and reversible airway injury milieu may favor BMC engraftment. Transtracheal delivery was more efficacious in enhancing BMC retention in the lung than the intravenous route, which not only resulted in fewer cells in the lung, but large numbers of these cells in the spleen, liver, and bone marrow. After transtracheal injection into uninjured animals, few BMC remain and are probably coughed out by mucociliary clearance, although a small fraction may home to the bone marrow. After transtracheal injection in injured animals, some BMC remain in the airway and alveolar epithelium and acquire phenotypic characteristics of the injured cell type. Importantly, we have shown that short-term cultured BMC contain a population of cells that can potentially engraft in the airways. These cells may play a prominent role in lung cell therapy. It remains unclear whether the small population of short-term cultured BMC that remain in the airway epithelium are stem/progenitor cells or just populations of mature cells that could participate in lung remodeling after injury. Gomperts et al. (13) recently demonstrated a CK5+CD45+ epithelial progenitor cell population in the bone marrow and peripheral blood that facilitates airway regeneration after injury. We are currently pursuing further identification of the BMC subpopulation with the greatest lung engraftment and regenerative potential.

To date, the concept of BMC differentiation into lung-specific cells remains highly controversial. Early reports (24, 25) prompted widespread interest in assessing the role of bone marrow-derived stem cells for cell therapy in lung repair and challenged the view that tissue-specific stem cells are the sole cell types involved in tissue repair. Various human studies and animal models of disease have shown BMC engraftment and chimera in various lung injuries such as lung transplantation (4, 22), bone marrow transplantation (1, 37), bleomycin-induced fibrosis (15, 31), radiation pneumonitis (16, 38), hypoxia-induced pulmonary hypertension (8), and elastase-induced emphysema (19). The results have been conflicting; different groups report different frequencies of engraftment and different cell types formed following lung injury. This may be, in part, due to the anatomical and functional complexities of the lung, which, depending on the specific type and degree of injury, could stimulate BMC differentiation along certain lung cell lineages and not others. Another obvious possibility is the difference in BMC subpopulations generated through different selection and culture techniques.
Nevertheless, we and many other investigators believe that, although the natural importance of BMC for lung repair and regeneration following injury is uncertain, they may be manipulated for therapeutic benefit (19, 42). Rojas et al. (35) recently showed that bleomycin injury is worsened by busulfan-induced myelosuppression and was significantly improved by exogenously delivered BMC engraftment in the lungs. They suggested that bone marrow may play an active role in limiting the extent of lung injury. Thus, despite the controversies, the therapeutic potential of BMC engraftment as epithelial cells is enticing. Correction of only 5–10% of cells can restore normal chloride secretion in a cystic fibrosis (CF) epithelial sheet (21) and improve the CF phenotype (33). Since studies show a range of 0.025% to 4% BMC airway engraftment (25, 26), strategies need to be developed to increase the percentage of engraftment, but it is clear that significant functional benefit may be achievable with far less than 100% epithelial replacement.

Fig. 5. K18GFP transgenic mice characterization. A: immunofluorescence images of cytokeratin-18 (K18)-driven green fluorescent protein (GFP) expression on the surface airway epithelium of lung of K18GFP transgenic mice after anti-GFP or isotype control antibody staining. B: no green fluorescence was observed in strain-matched wild-type lungs. Scale bar represents 100 μm on low-power images and 20 μm on inset image. Cytokeratin-18 promoter-driven GFP expression was found on isolated tracheal epithelial cells (TEC, C) but not in plastic-adherent BMC (D). Scale bars in C and D = 10 μm. Anti-GFP staining of other tissues shows no transgene expression in the heart or liver except for the epithelium of the intestine (E). Scale bar = 50 μm. F: schematic map of primer designs for real-time PCR. G: real-time RT-PCR of total RNA isolated TEC, tracheal fibroblasts, and freshly isolated and cultured BMC from K18GFP transgenic mice using the intron-spanning primer. N = 3 animals/group.
Other reports have suggested that intravenously delivered plastic-adherent BMC can transdifferentiate into lung epithelium using immunofluorescent colocalization of Y chromosome or GFP and epithelial markers to assess donor BMC transdifferentiation. Wang et al. (40) showed mesenchymal stem cells (MSC) can transdifferentiate into pan-cytokeratin-expressing, CFTR\(^+\) epithelial-like cells when cocultured with respiratory epithelium in vitro. Loi et al. (26) also showed intravenous delivery of plastic-adherent BMC cultured for 7 days can engraft in the airway and express cytokeratins and CFTR in a similar naphthalene-induced lung injury model. The frequency of engrafted BMC was less than in our study, perhaps due to our use of transtracheal delivery. Kotton et al. (24) showed intravenous delivery of plastic-adherent BMC can
express type I pneumocyte marker in vitro and engrafted as pneumocytes in vivo in an animal model of pulmonary fibrosis. In these studies, BMC engraftment levels were rare to low. Other studies using another lineage-specific reporter (transgenic SP-C-EGFP mice in which the fluorescent reporter gene was regulated by the SP-C promoter) reported low or undetectable levels of BMC engraftment in the lung (23). These discrepancies may reflect different models of lung injury, differences between the K18 and SP-C promoters and in methods of BMC delivery, or differences in populations of BMC used.

To improve airway engraftment, the mode of BMC delivery for optimal repair and regeneration of injured tissue may be critically important. Engraftment in the airways by the intravascular route is dependent on the ability of these cells to receive and respond to the airway injury signals and migrate out of blood vessels to the injured airways. As expected, we found significantly greater donor BMC in the lungs after transtracheal delivery (10-fold greater than intravenous delivery). Most of the intravenously delivered BMC were found in the alveoli, and only rarely were airway-localized cells found. The advantages of direct airway delivery are that cells can be targeted to the airways, reduced systemic delivery of the BMC to other organs, and reduced requirement for tissue damage to induce BMC homing to the lung.

In our study, some of the airway-localized BMC expressed the airway Clara cell protein CCSP. This was confirmed with optical dissection of all possible planar combinations (X-Y, X-Z, and Y-Z) of the tissue section to rule out overlapping cells that may be misinterpreted as a BMC expressing an epithelial marker. At 14 days after cell delivery (16 days after naphthalene injury), the donor-derived CMTMR+ cells constituted 3.35 ± 0.76%, as a percentage of all CCSP+ airway cells. This represented a significant decline compared with 41.1 ± 13.0% at 1 day (data not shown). Between 1 and 10 days after naphthalene injection, there was considerable CCSP+ cell loss from the airway epithelium, which explains the apparent high contribution of donor CCSP+BMC. Between 10 and 20 days, the airway epithelium was gradually regenerated with CCSP+ cells (Fig. S1), possibly from local progenitor cells (12, 17, 18, 34). Therefore, assessment of the total contribution of BMC to CCSP+ cells in the airway at day 14 (16 days after naphthalene injection) is not an accurate estimate since the airway epithelium is still undergoing rapid cell turnover and regeneration (36). We speculate that the total contribution of BMC to the airway epithelium after transtracheal delivery would be at most 1% after complete airway epithelial regeneration. The total contribution of BMC-derived type 2 alveolar epithelial cells was 7.5 ± 1.5% with no significant changes in the number of SP-C+ CMTMR+ cells in the alveoli from day 1 up to day 14 after cell delivery (data not shown). However, since alveolar injury is also seen in this naphthalene injury model (34), we would also speculate that the total contribution of type 2 alveolar cells from BMC would be less than 6.7 ± 1.8% (at day 14) after complete regeneration of the epithelium. Overall, these data illustrate that the total contribution of BMC giving rise to CCSP and SP-C-expressing cells is markedly increased after transtracheal delivery compared with other published reports (26, 27).

Our study further validates the possibility of BMC transdifferentiation into lung epithelium by using BMC from transgenic mice that expressed transgene in epithelial lineage-committed cells. BMC from K18GFP transgenic mice was delivered into wild-type recipients, and the K18GFP transgene was progressively upregulated in lungs of recipient wild-type mice. Plastic-adherent BMC or myofibroblasts from these transgenic mice do not express K18GFP mRNA, suggesting that in vivo detection of K18GFP expression by PCR or anti-GFP immunofluorescence occurred as a result of transdifferentiation of the BMC into K18-expressing cells. K18GFP mRNA was not detected in the hearts of recipients even though transgenic DNA was present. This suggests that the lung milieu may play an important role in dictating epithelial-lineage commitment. This is consistent with recent data from MacPherson et al. (28) who also suggest that residence within the lung milieu is required for cytokeratin expression by donor BMC.

While cell phagocytosis was seen by FISH, it seems unlikely that macrophage or epithelial phagocytosis (efferocytosis) of BMC (39) could result in such prolonged transgene expression. Although expression of transgene may occur as a result of cell fusion, this is likely a rare and possibly undetectable event in vivo (14). In vitro, without coculture with any other cell type, some BMC from K18GFP mice demonstrated detectable GFP expression after longer term culture, proving that BMC-epithelial fusion is not required for expression of K18 promoter-driven gene expression.

Cell proliferation is clearly important for tissue regeneration. We did not observe an increase in donor cell number or genomic DNA over time. This suggests that substantial cell proliferation did not occur, although we speculate that a small population of donor cells may be undergoing low levels of cell division. Therefore, direct assessment of cell proliferation of the retained BMC by conventional BrdU labeling, or staining for proliferation antigens Ki67 and PCNA, was not performed. Despite the improved level of BMC retention demonstrated in this study, overall levels clearly are still not sufficient to achieve large-scale tissue regeneration. This study should encourage further efforts to optimize BMC selection and delivery methods.

The recent discoveries that a subset of BMC have immunomodulatory roles is promising for the potential therapeutic use of these cells. Fazel et al. (11) and Ayach et al. (2) showed that bone marrow stem/progenitor cells are important for cardiac repair and regeneration by promoting angiogenesis and matrix remodeling. Similarly, Ortiz et al. (30) and Xu et al. (41) showed the immunomodulatory role of MSC in protecting against bleomycin-induced fibrosis and endotoxin-induced local and systemic inflammation, respectively. In both studies, intravenous delivery of MSC provides anti-fibrotic and anti-inflammatory effects after lung injury. Detailed evaluation suggested that these cells need to home to, but not necessarily engraft in, the lung to have protective effect. Our study provides an alternative route of cell delivery targeted to the lung that will enhance cell delivery to the site of injury and possibly may further enhance the immunomodulatory role of BMC.

Transtracheally delivered short-term cultured BMC remain in the lung after an acute, mild, and reversible airway-specific injury. Importantly, some of these cells persist to at least 120 days and adopt an “epithelial-like” phenotype. With further development and enhancement of targeting strategies to improve airway engraftment, BMC-mediated cell replacement therapy may become a potential therapeutic modality to treat airway diseases such as cystic fibrosis.
REFERENCES


