Limited Restoration of Cystic Fibrosis Lung Epithelium *In Vivo* with Adult Bone Marrow–derived Cells

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Rationale: Recent literature suggests that adult bone marrow–derived cells can localize to lung and acquire immunophenotypic characteristics of lung epithelial cells. We speculated this might be a potential therapeutic approach for correcting defective lung epithelium in cystic fibrosis.

Objective: To determine whether adult bone marrow–derived cells containing normal cystic fibrosis transmembrane conductance regulator protein (CFTR) could repopulate lung epithelium in transgenic mice deficient in that protein.

Methods: Stromal marrow cells or total marrow obtained from adult male wild-type mice were transplanted into adult female *Cftr* knockout mice. To increase marrow cell recruitment naphthalene was used to induce airway epithelial injury in recipient mice.

Measurements and Main Results: At 1 wk, 1 mo, and 3 mo after transplantation, *Cftr* mRNA was detected in lung homogenates of recipient mice by reverse transcription–polymerase chain reaction. *Cftr* mRNA was not found in either donor marrow cells or mature circulating leukocytes. *In situ* examination of recipient mouse lungs demonstrated rare (0.025%) chimeric airway epithelial cells, some of which (0.01%) expressed CFTR protein. Naphthalene-induced airway remodeling nonsignificantly increased the number of chimeric airway epithelial cells expressing *Cftr*.

Conclusions: These results demonstrate that adult marrow cells can be recruited to airway epithelium and induced to express *Cftr* in mice otherwise lacking this protein. However, the number of observed chimeric epithelial cells is small and new strategies for enhancing airway epithelial remodeling by adult bone marrow–derived cells will be necessary for correction of defective CFTR-dependent chloride transport.

Keywords: cystic fibrosis; cystic fibrosis transmembrane conductance regulator protein; epithelium; lung; stem cell

Although controversial, a growing number of studies suggest that adult bone marrow–derived stem cells exhibit plasticity and can transdifferentiate into a variety of organ-specific cells (1–4). In certain instances, fusion of hematopoietic stem cells or of differentiated marrow-derived myeloid cells with organ-specific cells such as hepatocytes and skeletal muscle fibers can also occur (5, 6). Other populations of cells isolated from adult bone marrow including mesenchymal stem cells and multipotent adult progenitor cells have been demonstrated *in vitro* and to a limited

extent *in vivo* to acquire phenotypic and in some cases functional characteristics of organ-specific cells (7, 8).

The complex structure of the lung is made up of many different cell types in which limited regenerative capacity has been attributed predominantly to endogenous lung stem or progenitor cells (9–11). Nonetheless, reports have demonstrated that adult bone marrow–derived stem cells can localize to lung and acquire phenotypic characteristics of lung epithelial, interstitial, and vascular endothelial cells (12, 13). As in other organs, injury increases marrow-derived stem cell recruitment to lung and functional participation of marrow-derived cells in lung remodeling after injury has been described (14–21). This can be deleterious, with marrow-derived cells participating in the formation of fibrotic lesions (13, 16, 20). However, amelioration of both fibrotic and inflammatory lung injury by marrow-derived cells has been demonstrated (15, 18, 19). Fusion of marrow-derived cells with lung epithelial cells, while inducible in tissue culture, does not appear to occur with significant frequency *in vivo* (22, 23).

Clinical manifestations of cystic fibrosis (CF), a devastating genetic disease for which there remains no cure, result from a defect of a multifunctional protein, the CF transmembrane conductance regulator protein (CFTR). In airway epithelium, defective CFTR contributes to alterations in airway surface fluid, mucus, and antibacterial defenses leading to chronic airway occlusion and infection (24). Since identification of the CFTR gene in 1989, CF has been a target of gene therapy efforts although no viable approach has yet proven successful (24). We therefore determined whether transplantation of adult marrow cells containing the gene for wild-type *Cftr* might result in functional *Cftr* expression in lung epithelium. To accomplish this, we transplanted two different populations of adult bone marrow–derived cells containing the wild-type *Cftr* gene into transgenic *Cftr* knockout (KO) mice: cultured stromal marrow cells and total marrow cells. We were subsequently able to detect *Cftr* mRNA and protein in the lungs of the *Cftr* KO recipient mice by reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. However, the total number of chimeric lung epithelial cells exhibiting *Cftr* expression was small and unlikely to affect overall CFTR-dependent chloride transport and other functions in airway epithelium. Nonetheless, if strategies for increasing airway epithelial repopulation with adult bone marrow–derived stem cells can be found, this approach may be a potential therapeutic option for CF lung disease.

METHODS

Animals

Adult male (6–12 wk) C57BL/6 mice and transgenic C57BL/6 mice constitutively expressing green fluorescent protein (GFP) were used as donors (25). Adult female (8–12 wk) *Cftr* KO mice [*Cftr*^{tm1Unc}-Tg(FABPCFTR)1Jaw/J] were used as recipients (26). All studies were subject to Institutional Animal Care and Use Committee review at the University of Vermont (Burlington, VT) and conformed to institutional and American Association for Accreditation of Laboratory Animal Care standards for humane treatment of laboratory animals.

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Bone Marrow Harvest, Culture, and Transplantation

Total marrow was obtained from adult male GFP-expressing mice as previously described (12, 13). Plastic-adherent stromal cells isolated from C57BL/6 mice were cultured for 7 to 10 d in Dulbecco's modified Eagle's culture medium with 15% fetal bovine serum and then administered by tail vein injection to nonirradiated female *Cftr* KO mice (10^6 stromal cells/mouse) (13, 27). For transplantation of total marrow cells, as the *Cftr* KO mice are on a mixed strain background (C57BL/6, 129, FVB/NJ), CD3-positive T cells were depleted to minimize risk of early graft-versus-host disease (28). Adult female *Cftr* KO (recipient) mice underwent total body irradiation (800 rads), using a cesium-137 cell irradiator, followed by tail vein administration of CD3-depleted total bone marrow cells (20×10^6 cells/mouse).

Naphthalene-induced Lung Injury

Naphthalene (275 mg/kg body weight in sterile corn oil; Mazola; ACH Food Companies, Memphis, TN) was administered by intraperitoneal injection to (1) naive adult female *Cftr* KO mice 3 d before administration of cultured stromal cells and (2) chimeric adult female *Cftr* KO mice 1 mo after transplantation with total marrow cells (29).

Assessment of Donor-derived Cells in Recipient Lungs

Recipient female *Cftr* KO mice were killed by lethal overdose of pentobarbital at the indicated times, the lungs were gravity fixed with 4% paraformaldehyde, and 5- μ m paraffin sections were mounted on glass slides. Hematoxylin- and eosin-stained sections were assessed for inflammation and injury.

Donor-derived cells were assessed by fluorescence *in situ* hybridization for Y chromosome-positive cells followed by immunohistochemical characterization of epithelium and leukocytes, using antibodies directed against Clara cell secretory protein (CCSP), pro-surfactant protein C (pro-SPC), CFTR, cytokeratin, and CD45 (30). Sections were systematically visualized with an LSM 510 META confocal microscope (Carl Zeiss, Oberkochen, Germany) and a BX50 confocal microscope (Olympus America, Melville, NY).

Analysis of *Cftr* Expression by RT-PCR

Total RNA was isolated from donor marrow cells, mature donor neutrophils, alveolar macrophages, and splenic lymphocytes, and from homogenates of recipient mouse left lung as previously described (31–33). One microgram of total RNA was subjected to reverse transcription and *Cftr*, glyceraldehyde-3-phosphate dehydrogenase, and β -actin cDNAs were amplified (40 cycles) by PCR with *Taq* polymerase. *Cftr* was amplified with forward exon 9 primer (mCF11, 5'-CTT GTG GGA AAT CCT GTG CTG AA) and reverse exon 11 primer (mCF12, 5'-CCT TCT CCA AGA ACT GTG TTG TC) (34).

Flow Cytometry

Peripheral leukocytes obtained from chimeric mice 1 mo after transplant were assessed for percent GFP-positive cells (engraftment) with a Coulter Epics XL analyzer (Beckman Coulter, Fullerton, CA). Recipient lung homogenates were assessed for percentage of GFP- and CD45-positive and negative cells after incubation with anti-CD45 antibody (35).

Statistical Analyses

Data are expressed as means $\pm$ SD. Poisson analysis for rare events was applied to evaluate the distribution of rare donor-derived cells under the various experimental conditions (36).

See the online supplement for additional details on all methods.

RESULTS

Localization of Stromal Marrow Cells in Transplanted *Cftr* KO Mouse Lungs

Two approaches were used to determine whether donor bone marrow-derived cells could repopulate *Cftr* KO mouse lungs

(Figure 1). In the first (Figure 1A), plastic-adherent marrow stromal cells from adult male C57BL/6 mice were administered by tail vein injection to adult female *Cftr* KO mice with intact bone marrow. Mice were assessed 1 wk, 1 mo, and 3 mo after transplantation for the presence of, and to characterize, marrow-derived cells in the lung by simultaneous fluorescence *in situ* hybridization (FISH) for detection of the Y chromosome (30) and by immunohistochemical detection of hematopoietic (CD45) and lung-related (CCSP, pro-SPC, CFTR, cytokeratin) markers.

At each time point, rare donor-derived cells were observed in recipient mouse lungs (Figures 2A and 2B). The majority of donor-derived cells were CD45 negative, were located predominantly in alveolar walls, and demonstrated morphologic characteristics of alveolar epithelial and/or endothelial cells (Figure 2A). Rare donor-derived cells expressing the Clara cell secretory protein were also detectable in airways of recipient lung at all time points after administration of stromal bone marrow cells (Figure 2B). Positive and negative controls for the detection of the Y chromosome by FISH are also shown for comparison (Figures 2C and 2D).

To determine whether airway remodeling resulting from naphthalene injury would increase donor-derived stromal cell recruitment to the airways, naphthalene was administered to recipient *Cftr* KO mice 3 d before administration of stromal marrow cells (Figure 1A). Bioactivation of naphthalene by the cytochrome P-450 2F2 isoenzyme results in dose-dependent Clara cell toxicity leading to epithelial cell necrosis (29). Administration of naphthalene resulted in desquamation of airway epithelium within 3 to 5 d of administration, with subsequent epithelial regeneration noticeable at 10 to 20 d (Figure 3B). One month after naphthalene administration, nearly complete airway regeneration was achieved (Figure 3C). Complete airway regeneration was observed 3 mo after naphthalene administration (Figure 3D).

Figure 1. Schematic of experimental studies. (A) Administration of stromal marrow cells to *Cftr* knockout (KO) mice with intact bone marrow. (B) Generation of chimeric *Cftr* KO mice.

Figure 2. Transplanted male green fluorescent protein (GFP) mouse stromal marrow cells localize to lungs of female recipient *Cftr* KO mice. Donor-derived cells were detected by fluorescence *in situ* hybridization (FISH) for the Y chromosome (red). Immunostaining for leukocytes (CD45) is indicated in blue and by yellow arrows. (A) Donor-derived cell in alveolar wall (white arrow) 1 mo after transplantation; (B) donor-derived, Clara cell secretory protein (CCSP)-positive cell (green) in the airway wall 1 mo after transplantation (light blue arrow). Nuclear staining with Hoechst 33342 is shown in light blue. Insets in (A) and (B) are higher power views of the areas marked by asterisks; (C) positive FISH control (naive male *Cftr* KO mouse) highlighting the Y chromosome in airway epithelial cells (CCSP, green) and in leukocytes (CD45, blue); (D) negative FISH control (naive female *Cftr* KO mouse). Original magnification: (A, C, and D) $\times 400$; (B) $\times 1,000$.

As in non-naphthalene-exposed control mice receiving stromal marrow cells, only rare donor bone marrow-derived cells were found, mostly in alveolar walls. Most of these were CD45 negative and had the morphologic appearance of alveolar epithelial and/or endothelial cells (Figure 4A). Rare donor-derived, CCSP-expressing airway epithelial cells were detected in the lungs of naphthalene-injured mice 1 wk, 1 mo, and 3 mo after injury (Figures 4B and 4C; Figures 5A–5F). Quantitative assessment of the number of Y chromosome-positive, CD45-negative, CCSP-positive cells for each experimental condition is depicted in Table 1. Airway remodeling after naphthalene injury approxi-

Figure 4. Effect of naphthalene injury on localization of donor-derived adult stromal marrow cells in female recipient *Cftr* KO mouse lungs. (A) A Y chromosome-positive (red) donor-derived cell located in the alveolar wall 10 d after naphthalene administration (white arrow). Two CD45-positive leukocytes are indicated by yellow arrows. (B) A donor-derived, CCSP (green)-positive cell (light blue arrow) located in the airway epithelium and a donor-derived cell located in the alveolar wall (white arrow) 1 mo after naphthalene treatment are shown. Yellow arrows indicate CD45-positive leukocytes. Inset represents a higher-power view of the area marked by the blue asterisk. Nuclear staining Hoechst 33342 is shown in light blue color. (C) Donor-derived, CD45-negative, CCSP-positive cells (light blue arrows) identified in airways of transplanted *Cftr* KO lungs 3 mo after treatment with naphthalene; x-z confocal imaging is also shown. Original magnification: (A–C) $\times 1,000$.

Figure 3. Airway remodeling in recipient female *Cftr* KO lungs after acute administration of naphthalene. (A) Hematoxylin- and eosin-stained mouse lung 1 wk after administration of stromal cells (no injury). Arrow indicates uninjured epithelium. (B) Mouse lung 10 d after naphthalene administration, demonstrating denuded large airway epithelium (arrow, inset). Nearly complete (C) and complete (D) airway regeneration (arrows, insets) was observed 1 and 3 mo, respectively, after injury. Insets are higher power views of the areas marked by asterisks. Original magnification: (A–D) $\times 100$.

mately doubled the number of chimeric airway epithelial cells found at each time point, although the increase was not statistically significant. Rare events analysis by Poisson distribution revealed clustering of Y chromosome-positive, CD45-negative,

Figure 5. Localization of donor-derived adult stromal cells in lungs of recipient *Cftr* KO mice after injury with naphthalene. (C and F) Donor-derived CCSP-expressing (green) and cytokeratin-expressing (blue) cells (light blue arrows) located in the airway epithelium 3 mo after treatment with naphthalene. Images from two separate recipient mouse lungs are depicted. Y chromosome probe is shown in red. Nuclear staining with Hoechst 33342 is indicated in light blue. The same photomicrographs are shown in A and D and B and E after subtraction of the blue and the green channel, respectively, to illustrate colocalization of CCSP and cytokeratin. Insets in C and F are higher power views of the areas marked by asterisks. Original magnification: (A–F) $\times 1,000$.

CCSP-positive cells in the condition corresponding to the 3-mo time point after naphthalene treatment ($p < 0.05$), with $0.025 \pm 0.01\%$ of CCSP-positive cells being of donor origin. Approximately 20,000 CCSP⁺ cells were scored for each lung.

***Cftr* Expression in Lungs of *Cftr* KO Mice after Transplantation of Stromal Marrow Cells**

At 1 wk, 1 mo, and 3 mo after transplantation, rare donor-derived airway epithelial cells expressing CFTR protein were detected by FISH immunofluorescence (Table 2; Figures 6A–6F). The *Cftr*-expressing cells also stained positively for cytokeratin and had morphologies characteristic of columnar epithelial cells. Interestingly, *Cftr*-positive donor-derived airway epithelial cells often showed a clustered pattern of engraftment, with two cells being localized in a single high-magnification field (Figures 6A–6F). See the online supplement for additional images

Figure 6. Detection of *Cftr* expression in female *Cftr* KO mouse lungs after transplantation with male GFP stromal marrow cells. (C and F) Donor-derived (Y chromosome, red), *Cftr*-positive (green), and cytokeratin-positive (blue) cells are indicated by light blue arrows in airway walls of lungs assessed 1 wk after transplantation. Images from two separate recipient mouse lungs are depicted. The same photomicrographs are shown in A and D and B and E after subtraction of the blue and the green channel, respectively, to show coexpression of *Cftr* and cytokeratin by the donor-derived cells. Insets in C and F are higher power views of the areas marked by asterisks. Original magnification: (A–F) $\times 1,000$.

of CFTR staining including positive and negative control antibody staining.

Wild-type *Cftr* mRNA was found in lung homogenates by RT-PCR at all time points after administration of stromal marrow cells to naphthalene-injured mice (Figure 7). Importantly, neither mature differentiated leukocytes nor marrow cells from the transgenic GFP donors (either cultured stromal cells or fresh isolates of total marrow cells) expressed wild-type *Cftr* mRNA. This indicates that the marrow-derived hematopoietic cells used in the transplant were not the source of the *Cftr* mRNA that was detected in recipient lungs, and suggests that a small number of marrow-derived cells were recruited to lung and acquired an epithelial-specific phenotype not found in the donor cells. Wild-type *Cftr* mRNA was not consistently found in all naphthalene-injured mice receiving stromal cells and there were noninjured mice that also exhibited wild-type *Cftr* mRNA after stromal

TABLE 1. DISTRIBUTION OF DONOR-DERIVED CLARA CELL SECRETORY PROTEIN-POSITIVE CELLS UNDER VARIOUS EXPERIMENTAL CONDITIONS

Condition/Mouse No.	No. of Y ⁺ , CD45 ⁺ , CCSP ⁺ Cells/Mouse	Mean ± SD
Noninjured, 1 wk		
1	0	
2	0	
3	1	
	1	0.33 ± 0.58
Naphthalene, 1 wk		
1	1	
2	1	
3	1	
	3	1 ± 0
Noninjured, 1 mo		
1	1	
2	0	
	1	0.5 ± 0.71
Naphthalene, 1 mo		
1	2	
2	1	
	3	1.5 ± 0.71
Noninjured, 3 mo		
1	2	
2	2	
	4	2 ± 0
Naphthalene, 3 mo		
1	3	
2	4	
3	8	
	15	5 ± 2.64

CCSP-positive cells (20,000) were examined on 8–12 nonadjacent 5- μ m sections for each lung. Poisson analysis revealed clustering of donor-derived CCSP-positive cells in the condition corresponding to treatment with naphthalene and analysis after 3 mo ($0.025 \pm 0.013\%$). $\text{Pr}(X : 5 | \mu = 1.8) < 0.05$, where μ is the mean number of observed donor-derived CCSP-positive cells per lung (33).

cell administration (Table 2). See the online supplement for a depiction of the no-RT control.

Localization of Marrow-derived Cells in Lung of Chimeric *Cftr* KO Mice

In the second transplantation approach (Figure 1B), CD3-depleted total bone marrow from adult male transgenic GFP-expressing mice was administered to adult female *Cftr* KO mice previously myeloablated by total body irradiation. As a result, *Cftr* KO mice chimeras were generated with marrow cells expressing the GFP transgene and bearing the Y chromosome. One month was allowed for chimeras to achieve marrow reconstitution. One month after transplantation recipient mice were engrafted with an average of 93% bone marrow cells of donor origin. The rate of survival of adult female *Cftr* KO mice myeloablated and transplanted with total marrow cells was 87%.

Photomicrographs of mouse lungs assessed 5 wk after transplantation demonstrated numerous donor-derived cells staining positively for the Y chromosome (Figures 8A and 8B). Most donor-derived cells were located in lung parenchyma and costained for CD45, indicating their identity as donor-derived leukocytes (Figures 8A and 8B). Y chromosome-positive, CD45-negative cells of donor origin were detectable in the interstitium just below the airway epithelium (Figure 8A). Y chromosome-positive, CD45-negative cells that stained for pro-SPC were also located in the alveolar septa, suggesting an immunophenotype consistent with type 2 alveolar epithelial cells (Figure 8B). Similar findings were observed in mouse lungs assessed 8 wk after transplantation (Figure 8C). There was no obvious difference

TABLE 2. WILD-TYPE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR PROTEIN (CFTR) EXPRESSION IN TRANSPLANTED *Cftr* KNOCKOUT MICE

Condition/Mouse No.	<i>Cftr</i> mRNA by RT-PCR	CFTR Protein by IHC (No. Y ⁺ , CD45 ⁺ , CFTR ⁺ Cells)
Noninjured, 1 wk		
1	–	0
2	+	0
3	+	0
Naphthalene, 1 wk		
1	–	0
2	+	2
3	–	2
Noninjured, 1 mo		
1	–	0
2	NA	0
Naphthalene, 1 mo		
1	++	1
2	NA	0
Noninjured, 3 mo		
1	++	1
2	+++	0
Naphthalene, 3 mo		
1	–	2
2	–	2
3	++	2

Definition of abbreviations: IHC = immunohistochemistry; NA = not assessed; RT-PCR = reverse transcription–polymerase chain reaction.

Correlation between RT-PCR and FISH immunofluorescence results is shown. A number of lung sections equivalent to approximately 10,000 CCSP-positive cells was examined for the presence of donor-derived CFTR⁺ cells in each lung. Clustering of donor-derived CFTR⁺ cells 3 mo after naphthalene administration was not significant according to Poisson analysis ($p > 0.1$).

in number or localization of CD45-negative donor-derived cells between lungs harvested 5 or 8 wk after transplantation. No donor-derived airway epithelial cells expressing CCSP or *Cftr* were detected by FISH immunofluorescence in airways of chimeric mouse lungs. Similarly, no wild-type *Cftr* mRNA was detectable by RT-PCR in lung homogenates at any of the time points assessed (data not shown). No histologic indication of pneumonitis was observed, suggesting that any radiation-related lung injury induced by total body irradiation at the time of transplantation had resolved by the times of lung assessment (Figure 8D).

To determine whether airway epithelial remodeling could increase donor marrow cell recruitment to the airways in chimeric mice, naphthalene was administered to recipient *Cftr* KO mice 1 mo after transplantation. Lungs from chimeric female *Cftr* KO mice were assessed 1 wk and 1 mo after naphthalene administration (corresponding to 5 and 8 wk after transplantation). At both time points lung engraftment by marrow-derived cells was similar to that observed in otherwise noninjured chimeric controls. Most of the donor-derived cells were CD45⁺ leukocytes. However, quantification of Y chromosome-positive, CD45-negative, pro-SPC-positive cells in alveolar epithelial walls (20 random $\times 60$ fields on five sections assessed for each lung) demonstrated that in both non-naphthalene-treated and naphthalene-treated chimeric mice, only approximately 0.1% of CD45-negative, pro-SPC-positive cells were of apparent donor origin (Figure 9A).

In addition, rare CD45-negative cells were observed in airway epithelial walls. Also similar to what was observed in non-naphthalene-treated chimeric mice, these appeared mostly subepithelial in localization and did not stain positively for either CCSP or CFTR (Figures 9B and 9C). As in uninjured chimeric controls, wild-type *Cftr* mRNA was not detected by RT-PCR.

Figure 7. Reverse transcription–polymerase chain reaction (RT-PCR) gels demonstrating *Cftr* signal in transplanted *Cftr* KO mouse lungs. (A) The 329-bp fragment indicated by the arrow represents wild-type *Cftr* mRNA. Each lane represents a lung homogenate from different individual mice for each indicated experimental condition. Positive (C57BL/6 mouse lung homogenate) and negative (*Cftr* KO lung homogenate) controls are indicated. *Cftr* splice variants (lower bands) are noted in the *Cftr* KO lung homogenate, as has been previously described (34). No *Cftr* signal was detected in either mature circulating leukocytes (B), cultured marrow-derived stromal cells, or in total marrow cells obtained from donor mice (C). Control PCR amplifications targeted to the constitutively expressed gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β -actin are included for each gel. Forty cycles of PCR amplification were performed for each reaction. BM = bone marrow; PMN = polymorphonuclear leukocytes.

No obvious difference was noted in the number of Y chromosome-positive, CD45-negative, pro-SPC-positive cells in naphthalene-injured or otherwise uninjured chimeric mice ($\sim 0.1\%$ of total pro-SPC-positive cells).

GFP-positive, CD45-negative cells in homogenates of recipient mouse lungs did not reach detectable levels by flow cytometric analysis for any experimental condition evaluated.

DISCUSSION

We have demonstrated that adult bone marrow-derived cells have the potential to engraft in lungs of *Cftr* KO mice and to acquire phenotypic characteristics of alveolar and airway epithelial cells, including the expression of *Cftr* mRNA and protein. However, engraftment of marrow-derived cells in airway epithelium proved to be a rare event, and therefore unlikely to contribute to correction of transepithelial CFTR-mediated chloride current by the present approach.

In addition to reports demonstrating that cells derived from bone marrow of adult mice can localize to the lung and either transdifferentiate into or fuse with airway or alveolar epithelial

cells, interstitial cells, and vascular endothelial cells, lung specimens from clinical bone marrow transplant recipients and from lung transplant recipients have demonstrated chimerism of both lung epithelial and endothelial cells (37, 38). These reports are based on *in situ* detection of a marker for the donor cells, in most cases the Y chromosome after sex-mismatched transplants. Further characterization of the donor-derived cells has been by morphologic appearance and/or by coexpression of cell-specific markers. Various reports have described up to 20% of epithelium and 37.5% of pulmonary vascular endothelium as being of donor marrow cell origin in mice and humans (14, 37). Most recently, mesenchymal stem cells obtained from bone marrow of adult patients with CF have been induced *in vitro* to acquire an epithelial phenotype including partial resumption of CFTR-mediated chloride current (39). These reports collectively suggest that repopulation of lung epithelium with adult marrow origin cells can occur although the relevance has yet to be established.

Gene transfer studies have suggested that approximately 10 to 15% of defective airway epithelium needs to be corrected to allow resumption of normal CFTR-mediated chloride current (24). If so, available data suggest that this level of epithelial repopulation could be achievable with marrow-derived cells. Moreover, observations that preexisting lung injury increases lung repopulation by adult bone marrow-derived cells suggests additional relevance for cystic fibrosis. The CF lung epithelium is chronically injured and is also subject to repeated acute injuries from recurrent infections. This may fortuitously provide a more conducive or permissive environment for adult bone marrow cells to localize to lung epithelium. Importantly, adult bone marrow cells can be genetically manipulated *ex vivo* with subsequent expression in lung (40). This suggests that the bone marrow of a patient with CF could be removed, treated *ex vivo* to correct defective CFTR, and subsequently readministered to correct underlying lung disease.

For these reasons, we attempted to transplant wild-type adult marrow cells expressing *Cftr* in the airway epithelium of *Cftr* KO mice. However, despite the use of two different transplantation protocols and two populations of marrow-derived cells, the number of donor-derived cells that appeared to acquire lung epithelial phenotype, including *Cftr* expression, was small when compared with previous reports. Even with robust airway remodeling induced by naphthalene injury in nonirradiated mice, the number of donor-derived airway epithelial cells expressing wild-type *Cftr* was small ($\sim 0.01\%$) up to 3 mo after transplantation, and not significantly increased compared with noninjured mice. There was not always good correlation between *Cftr* RT-PCR and immunohistochemistry results demonstrating CFTR protein expression. This likely resulted from clustering of small numbers of chimeric airway epithelial cells in discrete sporadic locations in the lung. Approximately 50% of chimeric airway epithelial cells exhibited detectable *Cftr* expression whereas all chimeric airway epithelial cells stained positively for CCSP and/or cytokeratin. The reason for this is unclear as CCSP-positive Clara cells have been described to express CFTR (41, 42). The cytokeratin antibody used was an anti-pancytokeratin that stains several types of epithelial cells including both basal and differentiated airway epithelial cells. Future studies will incorporate more specific cytokeratin antibodies. Initial attempts at flow cytometry to further quantitate the number of Y chromosome-positive, CD45-negative cells in homogenates of recipient lungs were unsuccessful as there were too few cells to detect. The low number of chimeric cells also made it difficult to assess whether fusion of donor-derived cells with epithelium had occurred.

These results contrast published data in several respects. Other reports have described higher levels of chimerism despite the use of similar protocols and marrow cell populations (12–21).

Figure 8. Localization of donor-derived cells in lungs of chimeric female *Cftr* KO mice after transplantation with male GFP-expressing mouse total marrow cells. Donor-derived cells (Y chromosome, red), depicted by pink arrows, were found in the airway walls (CCSP, green) just beneath the epithelium 5 wk (A) and 8 wk (C) after transplantation. White arrows indicate donor-derived cells located in alveolar walls (not further characterized). (B) Donor-derived (Y chromosome, red), pro-surfactant protein C-positive (green) cells located in the alveolar walls 5 wk after transplantation (orange arrow). (D) Hematoxylin- and eosin-stained section of chimeric mouse 5 wk after transplantation, showing no obvious evidence of pneumonitis. Several donor-derived leukocytes (CD45, blue) are depicted by yellow arrows. Insets in A and B are higher power views of the areas marked by asterisks. Original magnification: (A and C) $\times 200$; (B) $\times 600$; (D) $\times 100$.

Figure 9. Effect of naphthalene-induced lung injury on recruitment of donor-derived marrow cells to lungs of chimeric *Cftr* KO mice. (A) One week after naphthalene administration rare donor-derived (Y chromosome, red), pro-SPC-positive (green) cells are detectable (orange arrow). (B and C) Donor-derived (Y chromosome, red) cells (pink arrows), in airway epithelial walls (CCSP, green) of chimeric mice 1 wk (B) and 4 wk (C) after naphthalene administration (5 and 8 wk after transplantation, respectively). Several donor-derived leukocytes (CD45, blue) are depicted by yellow arrows. Insets are higher power views of the areas marked by asterisks. Original magnification: (A) $\times 600$; (B and C) $\times 200$.

In part this may reflect the analytical technique used, particularly the immunohistochemical methods. We have found that the majority of donor-derived cells in lungs of chimeric mice are CD45-positive leukocytes. Overlap of leukocytes with lung cells in histologic sections may occur and can falsely elevate perceived numbers of chimeric lung cells. Furthermore, as radiation-induced lung injury can contribute to the engraftment of lung with marrow-derived cells (14, 16, 17, 21), we had attempted to minimize radiation effects when generating chimeric mice by using a lower dose than is more commonly used in studies reporting higher levels of engraftment (14, 16, 18, 19). This may also have contributed to the lower engraftment levels observed in our study. However, in parallel studies we conducted to evaluate the role of endotoxin and NO₂-induced lung injury in repopulation of mouse lung epithelium with adult bone marrow-derived cells, higher doses of radiation, although increasing overall mortality in the recipient mice, did not result in increased chimerism of airway epithelium (43). There are many other potential confounding factors including potential immunogenicity of both the Y chromosome in the recipient female mice as well as the expression of GFP. Further studies will need to be undertaken to assess the influence of these and other factors that can potentially influence cell plasticity.

Plastic-adherent stromal marrow cells administered to naive nonirradiated mice resulted in engraftment of donor-derived airway epithelial cells, particularly after naphthalene-induced airway remodeling, although at low percentages (~0.025%). In contrast, no donor-derived airway epithelial cells were detectable in irradiated mice transplanted with CD3-depleted total marrow cells. The stromal cells used were a heterogeneous population of cells and not purified mesenchymal stem cells as has been described by others (15, 27). This suggests that the population of adult marrow cells relevant for repopulating lung epithelium may be found in the plastic-adherent stromal cell compartment. These results coincide with findings of several other groups and suggest further study with mesenchymal stem cells and other cell populations isolated from the stromal compartment.

In conclusion, the current study supports the concept that functional airway epithelium can originate from adult bone marrow stem cells. This phenomenon may provide the basis for a therapeutic approach toward cystic fibrosis through replacement of defective epithelium with gene-corrected adult bone marrow-derived stem cells expressing wild-type CFTR. However, because adult bone marrow-derived cells home to airway epithelium with a low frequency by current techniques, further investigation on the molecular mechanisms that govern marrow cell recruitment and phenotypic conversion is needed in order for this approach to be clinically feasible.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Wagers AJ, Christensen JL, Weissman IL. Cell fate determination from stem cells. *Gene Ther* 2002;9:606–612.
- Korbling M, Estrov Z. Adult stem cells for tissue repair: a new therapeutic concept. *N Engl J Med* 2003;349:570–582.
- Prockop DJ. Further proof of the plasticity of adult stem cells and their role in tissue repair. *J Cell Biol* 2003;160:807–809.
- Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cell. *Blood* 2003;103:3483–3493.
- Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. Cell fusion is the principal source of bone marrow-derived hepatocytes. *Nature* 2003;422:897–901.
- Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. *Nature* 2003;422:901–904.
- Periera R, Halford K, O'Hara M, Leeper D, Sokolov B, Pollard M, Bagasra O, Prockup D. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc Natl Acad Sci USA* 1995;92:4857–4861.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;418:41–49.
- Borthwick DW, Shahbazian M, Krantz QT, Dorin JR, Randell SH. Evidence for stem-cell niches in the tracheal epithelium. *Am J Respir Cell Mol Biol* 2001;24:662–670.
- Engelhardt JF. Stem cell niches in the mouse airway. *Am J Respir Cell Mol Biol* 2001;24:649–652.
- Hong KU, Reynolds SD, Watkins S, Fuchs E, Stripp BR. Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. *Am J Pathol* 2004;164:577–588.
- Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001;105:369–377.
- Kotton DN, Ma BY, Cardoso WV, Sanderson EA, Summer RS, Williams MC, Fine A. Bone marrow-derived cells as progenitors of lung alveolar epithelium. *Dev Suppl* 2001;128:5181–5188.
- Theise ND, Henegariu O, Grove J, Jagirdar J, Kao PN, Crawford JM, Badve S, Saxena R, Krause DS. Radiation pneumonitis in mice: a severe injury model for pneumocyte engraftment from bone marrow. *Exp Hematol* 2002;30:1333–1338.
- Ortiz LA, Gambelli F, Phinney DG. Murine mesenchymal stem cells (MSCs) engraft as epithelial type II cells in injured, but not in normal, mouse lung and after metalloproteinase (MMP) expression. *Proc Natl Acad Sci USA* 2003;100:8407–8411.
- Epperly MW, Guo H, Grettton JE, Greenberger JS. Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2003;29:213–224.
- Abe S, Lauby G, Boyer C, Rennard SI, Sharp JG. Transplanted BM and BM side population cells contribute progeny to the lung and liver in irradiated mice. *Cytherapy* 2003;5:523–533.
- Ishizawa K, Kubo H, Yamada M, Kobayashi S, Numasaki M, Ueda S, Suzuki T, Sasaki H. Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema. *FEBS Lett* 2004;556:249–252.
- Yamada M, Kubo H, Kobayashi S, Ishizawa K, Numasaki M, Ueda S, Takashi S, Sasaki H. Bone marrow-derived progenitor cells are important for lung repair after lipopolysaccharide-induced lung injury. *J Immunol* 2004;172:1266–1272.
- Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH. Bone marrow-derived progenitor cells in pulmonary fibrosis. *J Clin Invest* 2004;113:243–252.
- Abe S, Boyer C, Liu X, Wen FQ, Kobayashi T, Fang Q, Wang X, Hashimoto M, Sharp JG, Rennard SI. Cells derived from the circulation contribute to the repair of lung injury. *Am J Respir Crit Care Med* 2004;170:1158–1163.
- Spees JL, Olson SD, Ylostalo J, Lynch PJ, Smith J, Perry A, Peister A, Wang MY, Prockop DJ. Differentiation, cell fusion, and nuclear fusion during *ex vivo* repair of epithelium by human adult stem cells from bone marrow stroma. *Proc Natl Acad Sci USA* 2003;100:2397–2404.
- Harris RG, Herzog EL, Bruscia EM, Grove JE, Van Arnam JS, Krause DS. Lack of a fusion requirement for development of bone marrow-derived epithelia. *Science* 2004;305:90–93.
- Weiss DJ, Pilewski J. Current status of gene therapy for cystic fibrosis. *Semin Respir Crit Care Med* 2003;24:749–770.
- Schaefer BC, Ware MF, Marrack P, Fanger GR, Kappler JW, Johnson GL, Monks CR. Live cell fluorescence imaging of T cell MEK2: redistribution and activation in response to antigen stimulation of the T cell receptor. *Immunity* 1999;11:411–421.
- Zhou L, Dey CR, Wert SE, DuVall MD, Frizzell RA, Whitsett JA. Correction of lethal intestinal defect in a mouse model of cystic fibrosis by human CFTR. *Science* 1994;266:1705–1708.
- Phinney D, Kopen G, Isaacson RL, Prockop D. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J Cell Biochem* 1999;72:570–585.

28. Panoskaltsis-Mortari A, Taylor PA, Yeager TM, Wangenstein OD, Bitterman PD, Ingbar DH, Vallera DA, Blazar BR. The critical early proinflammatory events associated with idiopathic pneumonia syndrome in irradiated murine allogeneic recipients are due to donor T cell infusion and potentiated by cyclophosphamide. *J Clin Invest* 1997;100:1015–1027.
29. Stripp BR, Maxson K, Mera R, Singh G. Plasticity of airway cell proliferation and gene expression following acute naphthalene injury. *Am J Physiol* 1995;269:L791–L799.
30. Trotman W, Beckett T, Goncz KK, Beatty BG, Weiss DJ. Dual Y-chromosome painting and in situ immunofluorescence staining of donor marrow cells in lung following syngeneic transplantation. *Histochem Cell Biol* 2004;121:73–79.
31. Suratt B, Young SK, Lieber J, Nick JA, Henson PM, Worthen GS. Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am J Physiol* 2001;281:L913–L921.
32. Warner RL, Paine R, Christensen PJ, Marletta MA, Richards MK, Wilcoxon SE, Ward PA. Lung sources and cytokine requirements for *in vivo* expression of inducible nitric oxide synthase. *Am J Respir Cell Mol Biol* 1995;12:649–661.
33. Levin D, Constant S, Pasqualini T, Flavell R, Bottomly K. Role of dendritic cells in the priming of CD4⁺ T lymphocytes to peptide antigen *in vivo*. *J Immunol* 1993;151:6742–6750.
34. Goncz KK, Colosimo A, Dallapiccola B, Gagne L, Hong K, Novelli G, Papahadjopoulos D, Sawa T, Schreier H, Wiener-Kronish J, *et al.* Expression of $\Delta F508$ CFTR in normal mouse lung after site-specific modification of CFTR sequences by SFHR. *Gene Ther* 2001;8:961–965.
35. Davis GS, Pfeiffer LM, Hemenway DR. Interferon- γ production by specific lung lymphocyte phenotypes in silicosis in mice. *Am J Respir Cell Mol Biol* 2000;22:491–501.
36. Zar JH. Biostatistical analysis. Englewood Cliffs, NJ: Prentice-Hall; 1974.
37. Suratt BT, Cool CD, Serls AE, Chen L, Varella-Garcia M, Shpall EJ, Brown KK, Worthen GS. Human pulmonary chimerism after hematopoietic stem cell transplantation. *Am J Respir Crit Care Med* 2003;168:318–322.
38. Kleeberger W, Versmold A, Rothamel T, Glockner S, Bredt M, Haverich A, Lehmann U, Kreipe H. Increased chimerism of bronchial and alveolar epithelium in human lung allografts undergoing chronic injury. *Am J Pathol* 2003;162:1487–1494.
39. Wang G, Bunnell BA, Painter RG, Tom S, Lanson NA, Spees JL, Bertucci D, Peister A, Weiss DJ, Valentine VG, *et al.* Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. *Proc Natl Acad Sci USA* 2005;102:186–191.
40. Grove JE, Lutzko C, Priller J, Henegariu O, Theise ND, Kohn DB, Krause DS. Marrow-derived cells as vehicles for delivery of gene therapy to pulmonary epithelium. *Am J Respir Cell Mol Biol* 2002;27:645–651.
41. Chinnet TC, Gabriel SE, Penland CM, Sato M, Stutts MJ, Boucher RC, Van Scott MR. CFTR-like chloride channels in non-ciliated bronchiolar epithelial (Clara) cells. *Biochem Biophys Res Commun* 1997;230:470–475.
42. Kulaksiz H, Schmid A, Honscheid M, Ramaswamy A, Cetin Y. Clara cell impact in air-side activation of CFTR in small pulmonary airways. *Proc Natl Acad Sci USA* 2002;99:6796–6801.
43. Beckett T, Loi R, Prenovitz R, Poynter M, Goncz KK, Suratt BT, Weiss DJ. Acute lung injury with endotoxin or NO₂ does not enhance development of airway epithelium from bone marrow. *Mol Ther* 2005;12:680–686.