

## Radiation pneumonitis in mice: A severe injury model for pneumocyte engraftment from bone marrow

Neil D. Theise<sup>a</sup>, Octavian Henegariu<sup>b</sup>, Joanna Grove<sup>c</sup>, Jayishree Jagirdar<sup>a</sup>,  
Peter N. Kao<sup>d</sup>, James M. Crawford<sup>c</sup>, Sunil Badve<sup>c</sup>, Romil Saxena<sup>c</sup>, and Diane S. Krause<sup>c</sup>

<sup>a</sup>Department of Pathology, New York University School of Medicine, New York, NY, USA; <sup>b</sup>Departments of <sup>b</sup>Genetics, <sup>c</sup>Laboratory Medicine, and <sup>c</sup>Medicine, Yale University School of Medicine, New Haven, Conn., USA; <sup>d</sup>Department of Medicine, Stanford University School of Medicine, Stanford, Calif., USA

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**Objective.** To better understand the process by which pneumocytes can be derived from bone marrow cells, we investigated the *in vivo* kinetics of such engraftment following lethal irradiation.

**Methods.** A cohort of lethally irradiated B6D2F1 female mice received whole bone marrow transplants (BMT) from age-matched male donors and were sacrificed at days 1, 3, 5, and 7 and months 2, 4, and 6 post-BMT (n = 3 for each time point). Additionally, 2 female mice who had received 200 male fluorescence-activated cell sorter (FACS)-sorted CD34<sup>+</sup>lin<sup>-</sup> cells were sacrificed 8 months post-BMT.

**Results.** Lethal irradiation caused histologic evidence of pneumonitis including alveolar breakdown and hemorrhage beginning at day 3. To identify male-derived pneumocytes, simultaneous fluorescence in situ hybridization (FISH) for Y-chromosome and surfactant B messenger RNA was performed on lung tissue. Y<sup>+</sup> type II pneumocytes were engrafted as early as day 5 posttransplant, and eventually from 2 to 14% of the pneumocytes were donor derived in individual mice. Co-staining for epithelial-specific cytokeratins demonstrated that by 2 months, marrow-derived pneumocytes could comprise entire alveoli, suggesting that type I cells derived from type II pneumocytes.

**Conclusion.** We conclude that alveolar lining cells derive from bone marrow cells immediately after acute injury. Also, the CD34<sup>+</sup>lin<sup>-</sup> subpopulation is capable of such pulmonary engraftment. © 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.

The mechanisms of lung regrowth and repair in response to injury are not well defined. However, while there is some debate regarding the possible roles different pulmonary epithelial cells play in lung regeneration, there is consensus that surfactant-producing type II pneumocytes, among other functions, proliferate and regenerate type I pneumocytes. The latter, while representing less than 4% of alveolar lining cells, constitute greater than 80% of the alveolar surface area and provide the primary gas exchange surface of alveoli [1,2]. In response to injury from a variety of insults, in-

cluding radiation, high oxygen tension, and chemical toxins, these cells may die, with subsequent repopulation from the more numerous, though smaller, type II pneumocytes [1–5]. Support for this concept has been found both in animal models and in human tissues.

We have previously demonstrated high-level engraftment of type II pneumocytes from a single bone marrow-derived stem cell in lethally irradiated mice [6]. In that report, the levels of pneumocyte engraftment were significantly higher (up to 18.7%) than that in other epithelial cell compartments in which bone marrow engraftment could be demonstrated (0.19–3.39% in gastrointestinal lining, bile ducts, skin, and hair follicles). We hypothesized that this high level of engraftment was a response to radiation-induced pneumonitis at the time of original bone marrow transplantation, since the radiation level to which our experimental animals were

Offprint requests to: Neil Theise, M.D., New York University School of Medicine, Room 461, Dept. of Pathology, 560 First Avenue, New York, NY 10016; E-mail: Neil.Theise@med.nyu.edu

\*Dr. Crawford is now in the Department of Pathology, University of Florida School of Medicine, Gainesville, FL.

exposed (1200 cGy) was in a range where severe acute lung injury would be expected [7–10].

Therefore, we examined a population of female animals that underwent bone marrow transplant with either unfractionated bone marrow or CD34<sup>+</sup>lin<sup>-</sup> selected cells from male donors. Histologic alveolar injury typical of radiation-induced pneumonitis was identified in the early days post-bone marrow transplant (BMT) with subsequent reactive changes and repair months later, though no animals expired from lung damage. Therefore, these animals represent a severe, nonlethal, acute lung injury model in which we could assay the marrow-derived cells as they differentiate into epithelial cells of the lung. We assess the kinetics of engraftment by measuring the percentage of surfactant B protein-producing cells derived from the donor bone marrow using co-fluorescent *in situ* hybridization (FISH) for surfactant B messenger RNA (mRNA) and the Y-chromosome.

## Materials and methods

### *Bone marrow transplantation*

Three- to 5-week-old, syngeneic B6D2F1 male mice served as donors for age-matched female bone marrow recipients. Bone marrow transplantation was performed as previously described [6,11]. Briefly, after whole-body irradiation with 1200 cGy, female recipients were injected via the tail vein with 20,000 male whole bone marrow cells. Archival tissues from the lungs from the same female recipients of male bone marrow from which we originally derived bone marrow–liver engraftment data were used [11]. Three recipient mice were sacrificed on days 1, 3, 5, and 7 posttransplantation, and at 2-month intervals, until 6 months posttransplantation. Coronal cross-sections of both right and left lobes of the lungs were formalin fixed and paraffin embedded. Fluorescence *in situ* hybridization (FISH) for the Y-chromosome was used to detect donor-derived cells in 2 serial sections of these tissues as described later.

Additionally, 2 female mice transplanted with 200 flow-sorted, CD34<sup>+</sup>lin<sup>-</sup> male donor cells were analyzed [12]. These mice also received 20,000 female donor-derived rotor off cells to provide short-term (30 days) reconstitution after lethal irradiation. The lineage markers used for depletion were B220, CD3, CD5, Gr1, Mac1, and Ter 119 [6,11]. These mice were sacrificed 8 months posttransplantation. The Yale University Animal Care and Use Committee approved all research involving mice.

### *Immunoperoxidase staining for cytokeratins 7 and 8*

Tissue sections were deparaffinized with xylenes and hydrated with graded alcohols. Endogenous peroxidase activity was quenched with 0.6% hydrogen peroxide for 8 minutes. Tissues were digested with 4 ug/mL pepsin (Biogenex, San Ramon, CA, USA) for 10 minutes at room temperature and washed in phosphate-buffered saline (PBS). Slides were then incubated for 30 minutes at 37°C with primary mouse monoclonal CAM5.2 antibody (Becton-Dickinson, Franklin Lakes, NJ, USA) at a dilution of 1:10. After washing, slides were incubated with secondary biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at 37°C followed by application of avidin-biotin complex for 30 minutes at room temperature. Diamin-

iobenzidine was used as the colorizing agent and slides were counterstained with Mayer's hematoxylin.

### *Fluorescence in situ hybridization for Y-chromosome*

Fluorescence *in situ* hybridization (FISH) for Y-chromosome was performed as previously described [12]. The Y-chromosome probe was generated by PCR and labeled with digoxigenin by Nick Translation (DIG-Nick Translation Mix, Boehringer-Mannheim, Indianapolis, IN, USA). Engraftment of lung epithelia was assessed by FISH on 3- $\mu$ m formalin-fixed, paraffin-embedded tissue sections after immunohistochemical staining for epithelial cytokeratins using monoclonal antibody CAM5.2. Following removal of coverslips, slides were dried, placed in methanol:acetic acid (3:1) for 30 seconds, dried, and treated with proteinase K, 10 ug/mL, for 45 minutes at 37°C. After washing, slides were incubated with probe overnight at 37°C. Detection was performed using anti-digoxigenin-rhodamine antibody (Boehringer-Mannheim, Inc.) and 4,6-diaminidino-2-phenylindole (DAPI) was used as a nuclear counterstain. With extensive washings, coarse debris, which might have been mistaken for a positive signal, were minimized. Such coarse debris, though rarely present, could be distinguished from true positive signals because the debris was visible with multiple fluorescent filters, whereas true positives were visible only with the rhodamine filter. Whole cross-sections of each organ were systematically and completely examined under medium power for positive signals. Lung tissue from normal male and female mice served as positive and negative control tissues, respectively.

### *Double FISH for Y-chromosome and surfactant-B mRNA*

Tissues were processed as reported [11]. Slides containing 3- $\mu$ m tissue sections were deparaffinized in xylene and pretreated 10 minutes with 100 ug/mL proteinase K with 0.05% SDS at 45°C. Genomic DNA probe was prepared based on mRNA sequence for mouse surfactant protein B (SPB, accession: S78114). Primer pairs were synthesized at positions 3758–3781/4064–4041, 8020–8043/8500–8478, 3141–3164/3801–3778, 7849–7871/8500–8478 in the SPB sequence. Genomic PCR products were labeled by incorporation of digoxigenin-dUTP. Mouse Y-chromosome probe was labeled by PCR amplification using biotin-dUTP. PCR products were then partially digested with DNase I (1.5–2.2 ug/mL final concentration) for 15 minutes at 21°C, followed by heating to 95°C for 2 to 4 minutes. For each slide, 20 ng dig-labeled surfactant probe and 10 ng biotin-labeled Y-chromosome probe were precipitated together with mouse Cot1 DNA (GibcoBRL, Life Technologies, Frederick, MD, USA), resuspended in 10 uL hybridization buffer (50% formamide) and denatured 5 minutes at 75°C. Slides were denatured 8 minutes at 86°C and hybridized overnight at 37°C. Posthybridization washes were done at 37°C, followed by antibody detection, using 1:100 dilutions of 1 mg/mL stock protein solutions in 4 $\times$ SSC. The first detection step included mouse antidigoxigenin and equal amounts of avidin-FITC mixed with avidin Cy5; the second step included sheep anti-mouse rhodamine. After washing, slides were mounted in DAPI anti-fade. The FITC signal enabled visualization of the Y-chromosome signals whereas the Cy5 signal (infrared) was used to provide better signal-to-noise ratios during image capturing (tissue autofluorescence is higher through the FITC filter than the Cy5 filter). Signal for Y-chromosome with this FISH double staining protocol yielded a smaller, dot-like signal in the nucleus, compared to the broader signal obtained with the single Y-chromosome labeling described above. This smaller signal was comparable in

scale (or smaller) to the surfactant protein mRNA transcription centers in the same nuclei [13].

#### Image analysis

Images were taken using an Olympus Provis (Tokyo, Japan) microscope equipped with a cooled CCD camera (Quantix Corp., Cambridge, MA, USA) and specialized software (PSI Inc, League City, TX, USA). Confocal microscopy was performed using a Bio-rad MRC1024 scanning confocal microscope (Biorad, Hercules, CA, USA) with a krypton/argon laser. Autofluorescence was excited at 488 nm, and emission was collected above 515 nm. The rhodamine signal was excited at 568 nm and emission collected above 585 nm. Images were pseudocolored using image processing software (Adobe Photoshop, San Jose, CA, USA).

#### Quantitation of bone marrow-derived cells

Cell counts for bone marrow were obtained using Y-chromosome FISH as described above after combining 60 $\times$  images of DAPI-stained nuclei, FITC autofluorescence, and Cy3.5 for Y-chromosome signals. Fields were selected randomly, focused, captured under a DAPI filter, then captured using the other filters, without changing focus, thus assuring the same plane of focus. Cell counts of type II pneumocytes were similarly obtained by capturing images of twenty random 100 $\times$  power fields, selected and focused with the DAPI filter, then merging the images of other filters: rhodamine for surfactant B mRNA, Cy5 infrared for Y-chromosome. To compensate for undercounting of Y<sup>+</sup> nuclei due to partial nuclear sampling in tissue sections, cell counts were normalized to the percentage of Y<sup>+</sup> cells seen in the normal male tissue.

#### Confirmation of Y-chromosome in pneumocyte nuclei

We confirmed that the presence of the Y-chromosome was not an artifact of sectioning of an overlapping lymphocyte or other hematopoietic cell by using whole-cell mounts. "Touch prep" slides of lung tissue from engrafted mice were made according to standard methods yielding single-cell deposition onto the slides [14]. These slides were air dried and stored at room temperature until ready for FISH analysis for the Y-chromosome. FISH was performed as described above for paraffin sections, except no proteinase K digestion was necessary. Immunofluorescent staining for cytokeratin was performed after a 40-second digestion of the FISH-stained slides with pepsin.

## Results

In order to determine the kinetics of damage and repair to the bone marrow and lung, histologic evaluation was performed 1, 3, 5, and 7 days posttransplantation, and again at 8 weeks after transplantation of 20,000 whole bone marrow cells. The bone marrow of recipient animals was depleted by day 3 postirradiation. FISH for Y-chromosome in these smears demonstrated donor-derived cells accounting for 97 to 100% of bone marrow cells in experimental animals from day 5 onward. These results are summarized in Table 1.

Histologic signs of radiation injury to the lungs were first seen on day 3, with breakdown of capillaries within alveolar septa and extravasation of erythrocytes into the alveolar spaces. The lungs also appeared hypocellular on day 3, with an absence of white cells mirroring the paucity of cells seen in the bone marrow. Capillary breakdown and erythrocyte

extravasation into alveoli reached a peak on day 5. By day 7, alveolar septal integrity was reestablished. Cellularity also increased, with leukocytes again being visible in the lung tissue as well as alveolar lining cells with reactive features suggesting proliferation and repair. Examination of lung tissue at 2, 4, 6, and 8 months postirradiation demonstrated relative hypercellularity of the alveolar septa, as has been reported [8–10].

Bone marrow donor engraftment of lung cells in the transplanted animals was assayed using two different approaches to identify pneumocytes. The first was immunohistochemistry for cytokeratin 7 and 8 followed by Y-chromosome FISH (Fig. 1). The positive signal for Y-chromosomes as seen in a male control (Fig. 1A) was a large, often irregular signal, usually at the edge of a DAPI counter-stained nucleus. Normal lung tissue from female mice was entirely negative for Y-chromosome (Fig. 1B). Beginning at day 5, Y<sup>+</sup>, cytokeratin<sup>+</sup> alveolar lining cells were present. These cells appeared scattered and isolated, often at the corners of alveoli in day-5 and day-7 lung tissues. By 2 months, and increasingly thereafter, cytokeratin<sup>+</sup> pneumocytes were present in clusters, sometimes lining entire alveoli (Fig. 1C). Y<sup>+</sup> pneumocytes were not uniformly distributed in the lung. Because of focal collapse of lung tissue during sampling and processing of tissue, individual cytokeratin-expressing cells were not always distinguishable and precise cell counts (% Y<sup>+</sup>, cytokeratin<sup>+</sup> cells) therefore could not be obtained. Thus, results from these data remain qualitative, rather than quantitative.

The second assay employed co-FISH for Y-chromosome (FITC) and surfactant B mRNA (rhodamine). This method results in co-localization in the nucleus of fluorescent signals for the Y-chromosome and for the transcription sites of the surfactant B gene [6]. Because each positive cell can be identified by its nucleus alone, precise quantification of positive cells could be confidently obtained. Double FISH for Y-chromosome and surfactant B mRNA in normal male lung tissue indicated that 70/113 (62%) of Y-chromosomes could be visualized in type II pneumocytes with this technique (Fig. 2, left panel). This divergence from 100% is due to partial sampling of nuclei in the 3- $\mu$ m sections examined. As with cytokeratin identification, cells that were stained for both Y-chromosome and surfactant B messenger RNA were first identified in day-5 lung tissue and were present in lung tissues at all time points thereafter (Fig. 2, right panel; Table 1). The presence of high levels of mRNA for the type II pneumocyte-specific protein surfactant B provides further confirmation that these Y<sup>+</sup> donor-derived, cytokeratin-expressing cells are type II pneumocytes, and suggests that these cells function normally in the lung. The Y<sup>+</sup>, surfactant mRNA<sup>+</sup> cells were quantified and adjusted values were calculated, based on the percentage of type II pneumocytes counted in normal male control tissue (Table 1). Values ranged from 0.7% at day 5 to as high as 14% of type II pneumocytes being male donor derived at 6 months.

**Table 1.** Number of Y-chromosome<sup>+</sup> nuclei in female mice transplanted with male bone marrow cells

Mouse	Time Post-BMT	Transplanted Cells	Bone Marrow Cell Count (%)	Type II Pneumocytes	
				Cell Count (%)	Adjusted percent %
Normal Male	—	none	198/200 (99%)	70/113 (62%)	100
1	1 d	wbm	n.d.	0	0
2	1 d	wbm	5/223 (2.2%)	0	0
3	1 d	wbm	3/219 (1.4%)	0	0
4	3 d	wbm	n.d.	0	0
5	3 d	wbm	scant cells	0	0
6	3 d	wbm	scant cells	0	0
7	5 d	wbm	n.d.	1/138 (0.72%)	1.2
8	5 d	wbm	203/204 (100%)	1/126 (0.79%)	0.7
9	5 d	wbm	211/213 (99%)	1/142 (0.70%)	0.9
10	7 d	wbm	n.d.	1/147 (0.68%)	1.1
11	7 d	wbm	207/207 (100%)	2/139 (1.4%)	2.1
12	7 d	wbm	211/215 (97%)	1/151 (0.66%)	1.0
13	2 mo	wbm	223/223 (100%)	7/119 (5.8%)	9.5
14	2 mo	wbm	215/218 (99%)	6/109 (5.5%)	8.7
15	2 mo	wbm	209/210 (100%)	8/106 (7.5%)	12
16	4 mo	wbm	202/203 (100%)	8/122 (6.5%)	11
17	4 mo	wbm	202/202 (100%)	7/100 (7.0%)	12
18	4 mo	wbm	214/214 (100%)	7/107 (6.5%)	11
19	6 mo	wbm	218/220 (99%)	9/111 (8.1%)	13
20	6 mo	wbm	215/216 (99%)	10/115 (8.6%)	14
21	6 mo	wbm	207/211 (98%)	9/132 (6.8%)	11
archival 1	8 mo	CD34 <sup>+</sup> lin <sup>-</sup>	552/552 (100%)	2/127 (1.6%)	2.5
archival 2	8 mo	CD34 <sup>+</sup> lin <sup>-</sup>	112/623 (18%)	1/153 (0.6%)	1.1

BMT, Bone marrow transplant; wbm, whole bone marrow; adjusted %, adjusted cell count compensating for partial sampling of nuclei in 3- $\mu$ m sections; n.d., not done.

In order to rule out the possibility that the appearance of Y-chromosomes in pneumocytes was due to an artifact of cell overlay in the tissue sections, single-cell staining was also performed. Epithelial cells can be identified on touch-prep slides by immunofluorescence for cytokeratin. Co-staining of lung touch preps for Y-chromosome and for cytokeratin confirmed that Y-chromosomes were present in pneumocytes (Fig. 3). In Figure 3A can be seen 2 Y-chromosome<sup>+</sup>, cytokeratin<sup>+</sup> cells and one Y-chromosome<sup>+</sup> nonepithelial cell. In Figure 3B can be seen one Y-chromosome<sup>+</sup> nonepithelial cell and 1 Y-chromosome<sup>-</sup> cytokeratin<sup>+</sup> epithelial cell. In addition to the isolated cells pictured, the touch-prep slides had small clumps of cytokeratin<sup>+</sup> cells. Some clumps contained all Y-chromosome<sup>+</sup> epithelial cells, and others contained only Y-chromosome<sup>-</sup> epithelial cells.

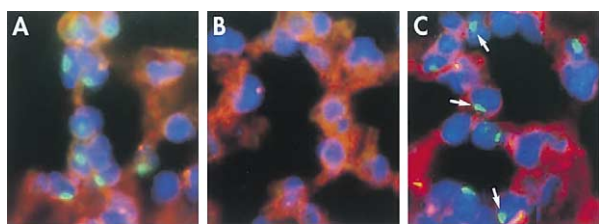
In the two mice that were sacrificed 8 months after lethal irradiation and transplantation with 200 CD34<sup>+</sup>lin<sup>-</sup> cells, there was 100% and 18% donor bone marrow engraftment. These mice also demonstrated Y-chromosome<sup>+</sup> donor-derived pneumocytes with approximately 2.5% and 1.0% type II pneumocyte engraftment, respectively.

## Discussion

We demonstrate that there is early and sustained engraftment of type II pneumocytes from marrow-derived cells in

response to radiation-induced pneumonitis, eventually leading to some alveoli being lined entirely by marrow-derived epithelia. Thus, the marrow can serve as an extra-pulmonary source of some alveolar stem cells. The lung responds to irradiation with breakdown of alveolar capillaries, focal hemorrhage, and pneumocyte loss with maximal damage apparent 3 days postirradiation [8–10]. By day 5, healing is apparent and day 5 was the first time point at which cytokeratin<sup>+</sup>, marrow-derived pneumocytes made their appearance as isolated cells, usually at the corners of alveoli, typical of type II pneumocytes. The percentage of marrow-derived type II pneumocytes increased over time from an average of 0.9% at day 5 to 11–14% at month 6, demonstrating either progressive expansion of the engrafted population or continuous engraftment of circulating marrow-derived cells. In animals sacrificed at months 2, 4, and 6, engraftment yielded isolated clusters of pneumocytes, which sometimes comprised entire alveolar surfaces. This pattern of engraftment supports the concept that type I pneumocytes derive from cells that had initially engrafted as type II pneumocytes.

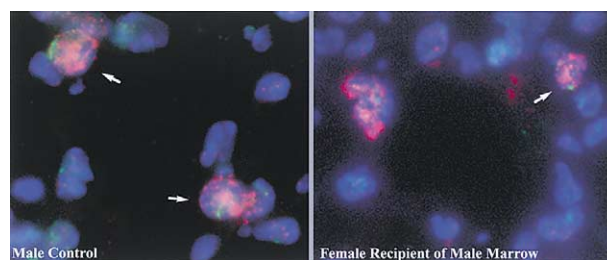
It is not clear why the 2 mice that received 200 CD34<sup>+</sup>lin<sup>-</sup> cells showed a lower level of pulmonary engraftment than the animals receiving whole bone marrow as all mice had the same radiation exposure. Possibly, this difference relates to the use of 20,000 whole bone marrow



**Figure 1.** Fluorescence in situ hybridization for murine Y-chromosome and immunostaining for cytokeratins. (A) Normal male mouse lung stained with DAPI (blue) for nuclear chromatin and rhodamine for Y-chromosome (rhodamine-labeled probe, pseudocolored to green/yellow with multiple filter acquisition). Pneumocyte cytoplasm, positive for cytokeratins, fluoresces in multiple filters, yielding a gold-red pseudocolor when images are merged. The Y-chromosomes, seen as bright comma-shaped bodies in nuclei, are identifiable in approximately half of the nuclei as the nuclei are only partially sampled in a 3- $\mu$ m tissue section. (Original magnification 60 $\times$ .) (B) Normal female mouse lung stained as in Figure 1A. No Y-chromosomes are identified. (Original magnification 60 $\times$ .) (C) Lung from a female mouse sacrificed 6 months after lethal irradiation and transplantation with male whole bone marrow, stained as in Figure 1A. Pneumocyte nuclei contain a Y-chromosome (arrows show 3 good examples), indicative of bone marrow cell origin. Note that a majority of pneumocytes in this region are positive; when adjustment for thin sectioning is taken into account, there appears to be near-complete reconstitution of pneumocytes in these alveoli from bone marrow-derived cells. (Original magnification 60 $\times$ .)

cells compared to only 200 CD34<sup>+</sup>lin<sup>-</sup>. With engraftment from an extra-organ source beginning in as little as 5 days post-BMT, this small number of cells may not have had time to expand and then engraft as type II pneumocytes throughout the lung to the extent possible with the larger donor population of whole marrow cells. Alternatively, it is possible that donor-derived pneumocyte engraftment decreases after 6 months and/or that the CD34<sup>+</sup>lin<sup>-</sup> subpopulation may not be as efficient as other marrow populations in producing pneumocytes.

Although our data indicate that at least some of the bone marrow cells of origin for these regenerative processes are CD34<sup>+</sup>lin<sup>-</sup>, this is consistent with recent data showing that specific bone marrow progenitors that are capable of reconstituting hematopoiesis can also give rise to hepatocytes [6,15]. Another recent paper demonstrated engraftment of type I pneumocytes from transplanted bone marrow cells that had been cultured as adherent cells in vitro, and expressed collagens type I and IV, laminin, and fibronectin [16]. The ability of different marrow subpopulations to engraft as epithelial cells suggests that there is no single absolute phenotype for highly plastic bone marrow-derived epithelial progenitors. It reinforces the broadened paradigm that there is greater cell plasticity than previously believed, and that interpretation of data from studies such as these must always be viewed in light of the specific isolation procedures and experimental conditions used [17,18]. Interestingly, the adherent marrow-derived cells did not engraft as type II pneumocytes in the setting of bleomycin-related

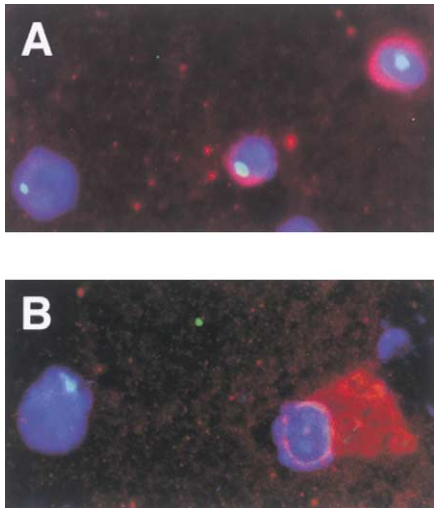


**Figure 2.** Double FISH staining for Y-chromosome and surfactant B mRNA. On the left, normal lung of a male mouse with FITC (green)-labeled Y-chromosomes. Bright red dots in two DAPI-stained nuclei indicate transcription centers for surfactant B mRNA, confirming that these are type II pneumocytes. The finely stippled, bright red staining surrounding the nuclei is cytoplasmic surfactant B mRNA. On the right, lung of a female mouse, eight months after bone marrow transplantation with CD34<sup>+</sup>lin<sup>-</sup> male bone marrow cells. Two nuclei show a red-labeled transcription centers for surfactant B mRNA, confirming that they are type II pneumocytes. One of these cells (arrow) also contains a green-labeled Y-chromosome, indicating that it is a marrow-derived, type II pneumocyte in this female mouse. (Original magnification 100 $\times$ .)

lung injury. The differences between their findings and ours could be due to the type of damage induced. Their data, however, are consistent with our previous findings that marrow-derived cells can engraft as isolated mature epithelial cells in liver, skin, and gastrointestinal tract without demonstrable prior engraftment as intermediate intra-organ stem/progenitor cells.

Because the bone marrow cells were injected intravenously, it remains unclear whether bone marrow-derived precursors normally reside in the target organ, or are capable of homing to damaged lung tissue from the bone marrow. Evidence to suggest that the latter may occur physiologically is that there are low levels of hematopoietic stem cells circulating in the peripheral blood. Furthermore, bone marrow does not need to be injected intravenously to engraft visceral organs, as was shown in men who received orthotopic liver transplants from female donors and developed donor-derived hepatocytes [19,20]. Consistent with these data, men who received kidneys and hearts from female donors also developed Y-chromosome<sup>+</sup> renal epithelial cells [21] and cardiac myocytes [22], respectively.

In summary, we confirm that CD34<sup>+</sup>lin<sup>-</sup> bone marrow-derived cells are capable of differentiation into pulmonary epithelial cells in vivo. Moreover, in response to serious injury, in this instance radiation-induced pneumonitis, circulating cells participate in visceral organ regeneration at physiologically important levels, echoing data from studies in liver and cardiac regeneration in animals [11,23] and humans [19,22]. The discovery that regenerating lung epithelium after lung injury can arise from bone marrow-derived stem cells could have important implications for understanding the pathogenesis of the often-severe lung injury after allogeneic bone marrow transplantation and solid



**Figure 3.** Double staining of “touch-preps” for Y-chromosome and cytokeratin. Isolated cells are pictured that have been stained by FISH for the Y-chromosome (aqua dot in nucleus) and by immunofluorescence for cytokeratin (red). (A) and (B) represent two different fields on the same slide. The nuclei are counterstained blue with DAPI. (Original magnification 60 $\times$ .)

organ lung transplantation in humans. Therapeutic implications of this work include using bone marrow cells as vehicles to deliver gene therapy to mature pneumocytes, or as a source for organ reconstitution and repair. Bone marrow transplantation itself might prove useful in the treatment of some forms of lung injury or disease.

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