Cell, Vol. 105, 369–377, May 4, 2001, Copyright ©2001 by Cell Press

Multi-Organ, Multi-Lineage Engraftment by a Single Bone Marrow-Derived Stem Cell

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Summary

Purification of rare hematopoietic stem cell(s) (HSC) to homogeneity is required to study their self-renewal, differentiation, phenotype, and homing. Long-term repopulation (LTR) of irradiated hosts and serial transplantation to secondary hosts represent the gold standard for demonstrating self-renewal and differentiation, the defining properties of HSC. We show that rare cells that home to bone marrow can LTR primary and secondary recipients. During the homing, CD34 and SCA-1 expression increases uniquely on cells that home to marrow. These adult bone marrow cells have tremendous differentiative capacity as they can also differentiate into epithelial cells of the liver, lung, GI tract, and skin. This finding may contribute to clinical treatment of genetic disease or tissue repair.

Introduction

Several recent reports suggest that there is far more plasticity than previously believed in the developmental potential of many different adult cell types. Recently, we and others showed that a bone marrow population enriched for HSC can differentiate into mature hepatocytes in the liver of rodents (Petersen et al., 1999; Theise et al., 2000a), and this differentiation of bone marrow cells into mature cells of the liver also occurs in humans (Theise et al., 2000b; Alison et al., 2000). Other examples of this surprising plasticity include the in vivo generation of murine skeletal muscle cells from bone marrow cells (Ferrari et al., 1998) and of bone marrow from skeletal muscle cells (Jackson et al., 1999). Some of these studies have shown mesodermally derived tissue arising from ectodermally derived tissue and vice versa, such as the reconstitution of bone marrow from cultured brain (Bjornson et al., 1999) and glial cells arising from bone marrow (Eglitis and Mezey, 1997). Therefore, the boundaries determined by embryologic trilaminar origin are not maintained in the adult. The phenotype of the bone marrow subpopulation that has this increased plasticity is not yet known. Here we study whether a unique bone marrow subpopulation highly enriched for hematopoietic stem cells also has the ability to differentiate into epithelial cells previously thought to be exclusively of endodermal or ectodermal derivation.

HSC are present in mouse bone marrow at a frequency of 1 in 10^5 cells (Harrison et al., 1990). The rarity of these cells and the absence of specific markers have made the search for a pure HSC population a challenge for the past 50 years. The lack of ideal in vitro assays for HSC requires that functional assays be utilized to establish their presence. We and others have shown that LTR is possible with small numbers (1–10) of HSC (Jones et al., 1996; Spangrude et al., 1995; Osawa et al., 1996), but serial transplantation (self-renewal) of single cell reconstituted recipients serving as donors for new recipients has not yet been shown convincingly.

We test, using a two day homing protocol, whether individual marrow cells that rapidly home to the bone marrow are enriched for HSC. We use a membrane bound dye (PKH26) to track and recover cells from specific locations in vivo. This allows us to determine cell cycle activity as the dye is equally distributed to each daughter cell. We demonstrate that at least some HSC home to the bone marrow and remain quiescent for up to 48 hr following transplantation. After labeling and injection into a first female recipient, quiescent male cells that are recovered from the bone marrow 48 hr post transplantation are capable of LTR when transplanted into other female mice (Lanzkron et al., 1999). In the current study, our goals were to see if these recovered cells are enriched for a pure population of HSC with LTR ability and to examine the potential of limited numbers of these bone marrow-derived stem cells to engraft nonhematopoietic tissues.

It is not yet known which bone marrow cells are capable of epithelial differentiation. Based on previous data in which we showed that purified $CD34^+lin^-$ bone marrow cells can differentiate into hepatocytes in the liver (Theise et al., 2000a), we hypothesize that the same cells that reconstitute hematopoiesis can also differentiate into nonhematopoietic tissues. We test this by examining the nonhematopoietic tissues of animals that engraft with functionally isolated (homed) bone marrow cells.

Many different surface markers have been used to identify and isolate HSC from mouse bone marrow, and a consensus regarding which markers are consistently expressed on these cells has not yet been reached. An emerging body of work suggests that the HSC may not display CD34 (Goodell et al., 1996; Zanjani et al., 1998; Bhatia et al., 1998), as was previously thought (Krause et al., 1994; Morel et al., 1996). Osawa et al. (1996) showed that a single HSC expressing a low level of CD34 message could LTR mice, and our group has shown low expression of CD34 on HSC (Jones et al., 1996). We have subsequently demonstrated (Donnelly et al., 1999) that the HSC compartment is phenotypically heterogeneous with populations of HSC that are positive and negative for CD34 expression. It may be that expression

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Table 1. Engraftment and Self-Renewal Potential of Single HSC Transplanted into Lethally Irradiated Mice

	% Donor Cells (Peripheral Blood)		Percent Male CFU[a]	Engraftment after Serial Transplantation[b]	
	5 mo	11 mo	11 mo BM	2 mo PB	4 mo PB
Mouse 1	30	13	0.0	0	1 ± 0
Mouse 2	76.5	54.5	77.5	15 ± 04	49 ± 0.4
Mouse 3	91	75.5	95.5	18 ± 0.1	38 ± 0.2
Mouse 4	85.5	86.5	97.5	28 ± 0.10	77 ± 0.1
Mouse 5	78	12	88.0	1 ± 0	2.5 ± 0

[a] FISH was performed on pooled myeloid and erythroid progenitors harvested from each individual mouse.
[b] Peripheral blood (PB) was drawn from secondary recipients 2 and 4 months post transplant. Shown are the mean ± SE for the four recipients of donor marrow from each of the five long-term surviving mice. BM = bone marrow.

of CD34 is related to cell cycle activation (Sato et al., 1999) and may be reversible in vitro (Nakamura et al., 1999). The homing assay used herein enriches for HSC without using specific surface markers to identify the cells. We have analyzed the expression of CD34 and SCA-1 on these cells before and after they home to the marrow and spleen.

Results

Hematopoietic Engraftment and Self-Renewal

Male donor marrow cells, first fractionated (Fr25) via elutriation, and then lineage depleted (lin^-), were labeled with PKH26 and injected intravenously into lethally irradiated female recipients as we previously described (Lanzkron et al., 1999). Two days post transplant, PKH26 bright donor cells were recovered by flow cytometric sorting of recipient bone marrow. By limiting dilution, 30 new irradiated female hosts were each transplanted with a single recovered PKH26-labeled cell. Survival and donor reconstitution were assessed for 11 months post transplant. We previously demonstrated that 10^2 cells that homed to marrow, but not 10^4 cells that homed to spleen, had LTR ability (Lanzkron et al., 1999). In our current study, as a control, 10^3 or 10^2 $PKH26^+$ $Fr25lin^-$ cells from male donors were transplanted into female recipients for LTR without first utilizing the homing procedure. The control animals did not survive past 12 weeks or had no male donor cell reconstitution prior to death (data not shown). Of the 30 mice transplanted with a single recovered PKH26 bright cell, five survived long-term. In Table 1, the percent donor cell reconstitution is shown for the surviving recipients 5 and 11 months post transplant. Because 17% of animals that received a single male cell showed long-term male reconstitution, there is a 500- to 1000-fold enrichment of LTR cells after homing of the $Fr25lin^-$ starting population.

The five long-term survivors of a single cell were sacrificed at 11 months and cells from each of their marrows were plated for hematopoietic progenitors and also used for serial transplantation. Table 1 shows that marrow from four of the five survivors had between 77.5% and 97.5% male-derived colonies. Mouse #1, which only had 13% donor peripheral blood cells, had no detectable male donor progenitor cell activity at 11 months post transplant, but mouse #5, which also had low peripheral blood donor cells (12%), had 88% donor-derived colonies. The engraftment (2 and 4 months after serial transfer of 10^6 cells from each of the five primary long-term survivors) into groups of four new female lethally irradiated recipients is shown in Table 1. Mice # 2, 3, and 4 provided marrow that engrafted recipients with male cells four months post serial transplant approaching a level of engraftment equal to that observed in the primary recipient. This represents strong evidence for HSC self-renewal.

Cell Surface Antigen Expression of HSC

We examined the frequency and absolute number of CD34 and SCA-1-positive cells labeled with PKH26 prior to and 48 hr after transplantation into lethally irradiated recipient mice. Recovered PKH26 bright (quiescent) cells from the bone marrow of the recipients had higher frequencies of $CD34^+$ and $SCA\text{-}1^+$ cells (46% and 24%, respectively) compared with the starting population (approximately 4% and 3% $CD34^+$ and $SCA\text{-}1^+$ cells, respectively, Table 2). It is not clear whether the cells that homed to the marrow underwent an upregulation of CD34 expression, or if CD34-expressing cells from the starting population homed preferentially to the marrow. If the latter possibility is true, then 29.5% and 9.3% of the $CD34^+$ and $SCA\text{-}1^+$ injected cells, respectively, home to the marrow as opposed to 12.75% of the total cell population which we reported previously (Lanzkron et al., 1999). In contrast, PKH26 bright cells recovered from the spleen after 48 hr were not enriched for $CD34^+$ and $SCA\text{-}1^+$ cells.

Stem Cell Homing in CD34 Knockout Mice

To further examine the role of CD34 in stem cell homing, we used the 2 day homing assay to assess localization of cells from CD34 knockout mice in the spleen. $PKH26^+$ $Fr25lin^-$ cells from mice with a disruption in their CD34 gene (Suzuki et al., 1996) seeded the spleen of normal recipient mice to a greater extent than did normal HSC (data not shown). This finding provides additional evidence that CD34 may be responsible in part for the directed homing of cells with LTR ability early after transplant.

Engraftment of Epithelial Tissues in the Long-term Chimeric Mice

Analysis of the epithelial tissues from the five mice that had been transplanted with single "homed" cells yielded a surprisingly extensive differentiation repertoire. Immu-

Table 2. The FR25Lin$^-$ PKH$^+$ CD34$^+$ and Sca-1$^+$ Frequency before and after Transplant and the Absolute Recovery 48 hr Post Transplantation

Organ	Phenotype	Day 0 (% +)	Day 2 (% +)	% Rec.[a]
BM	CD34	4.2 ± 0.01	45.8 ± 0.16	29.5 ± 6.33
BM	SCA-1	3.4 ± 0.02	24.0 ± 0.10	9.3 ± 4.40
SPL	CD34	N/A	9.4 ± 0.04	4.8 ± 2.10
SPL	SCA-1	N/A	7.0 ± 0.03	3.3 ± 0.63

Values represent the mean ± SEM for three experiments.

[a]The absolute % recovery is calculated as the total number of Fr25Lin$^-$ PKH$^+$ CD34$^+$ or SCA-1$^+$ cells in the bone marrow or spleen at 48 hr divided by the number of CD34$^+$ and SCA$^+$ cells injected. The total number of marrow cells is determined by dividing the number of cells in the two hind limbs by 16%, the percentage of the total skeletal marrow that these bones represent.

$$\text{Absolute \% recovery} = \frac{\text{\# of PKH}^+ \text{ cells recovered} \times \text{\% antigen-positive cells recovered}}{\text{\# of PKH}^+ \text{ cells injected} \times \text{\% antigen-positive cells injected}}$$

The frequency measurements are the % positive cells for both PKH and CD34 or PKH and SCA-1 with a total of 10^4 cells examined.
N/A = not applicable.

nostaining for cytokeratins was used to identify epithelial cells in the tissues. The staining pattern of the cytokeratins in multiple organs is indicated in Table 3. Quantitative analysis of donor cell reconstitution was performed only for those cell types that could be definitively identified by these antibodies. Based on the data presented in Table 3, therefore, the tissues examined included lung (bronchi and alveoli), esophagus, stomach, small bowel, colon, renal tubules, biliary tree (cholangiocytes), and skin. Y chromosome-positive cells developed in the bronchi as shown in Figure 1. In this figure, the double staining approach is shown in detail. Figure 1A shows a representative low power light microscopic image. The columnar respiratory epithelium is brown due to immunoperoxidase staining with Cam5.2 antibody against cytokeratins 8, 18, and 19. A small region of this photo is reproduced larger in 1B so that single cells are apparent. FISH for the Y chromosome is shown in Figure 1C for the identical cells as in 1B. Male, donor-derived epithelial cells lining the bronchus are identified by the two arrows on the left.

Throughout the study, intraepithelial lymphocytes were excluded as a possible source of false positive identification of epithelial cells. Examination of sequential sections of liver, lung, skin, and esophagus failed to demonstrate the presence of such lymphocytes in the regions studied by FISH (data not shown). In contrast, intraepithelial lymphocytes were present in stomach, small intestine, and large intestine. Lymphocytes could be confidently excluded from our identification of epithelial cells by reliance on strict criteria for the characterization of epithelial cells. These include cell size (nuclei at least twice as large as normal lymphocytes), cytokeratin immunohistochemical staining up to the nuclear membrane, and lack of the halo indicative of lymphocyte cytoplasm (data not shown).

Table 3. Summary of Immunohistochemical Staining

	Anti-Cytokeratin Monoclonal Antibody	
Epithelial Cells of:	AE1/AE3	Cam5.2
Stomach	++	+
Esophagus	+	++
Small intestine	++	++
Large intestine	++	++
Liver		
Cholangiocytes	++	+
Hepatocytes	0	0
Kidney		
Glomeruli	0	0
Tubules	0	+
Lung		
Bronchi	++	+
Pneumocytes	0	++
Skin	+	++

Staining: 0 Absent; + variable; ++ diffuse, strong.

Macrophages were excluded as false positive cells using dual color immunohistochemical staining for the relevant cytokeratins and a macrophage-specific antibody CD11b (Figure 2A). As shown in this cross-section through a villous of the small intestine, cytokeratins (stained brown with DAB) and CD11b (stained red with fuchsin red) do not colocalize. While numerous macrophages could be identified in the lamina propria underlying the epithelia-lined surfaces, no intraepithelial macrophages were ever identified by this double staining technique. Analysis of engraftment of small bowel epithelial cells is shown in Figure 2B. In this cross-section through a villous of the small bowel that has been stained by FISH for the Y chromosome, two adjacent Y chromosome-positive epithelial cells can be seen on the right. These cells clearly are located within the columnar epithelium of the small bowel, which does not contain macrophages; they have an orange autofluorescence secondary to residual DAB from the immunohistochemistry for cytokeratins, and they have the same large oval-shaped nuclei as the other epithelial cells of the villous.

Male bone marrow donor-derived pneumocytes are shown in Figure 3A. Only the fluorescence image is shown for the tissues in Figure 3. However, the immunoperoxidase DAB staining is apparent as a red to orange to brown "pseudocolored" hue in the cell membrane and surrounding the nucleus in the cytoplasm of the epithelial cells (Pazouki et al., 1996; Theise et al., 2000b; Oosterwijk et al., 1998). In all images of Figure 3, arrows indicate Y chromosome-positive, reddish brown DAB-stained epithelial cells. Due to partial nuclear sampling, as the plane of each 3 μm section does not always cut through the Y chromosome, Y chromosomes were visualized clearly in 62% of alveolar nuclei in a male mouse (data not shown). No Y chromosome signal was observed in female mouse tissue (data not shown). In contrast, the average number of Y chromosome-positive

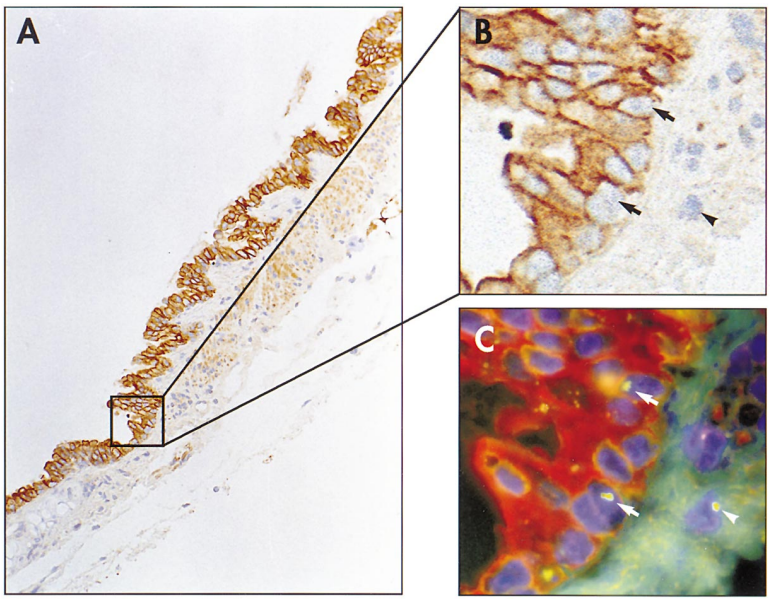

Figure 1. Immunohistochemical and FISH Analysis of Bronchus

(A and B) Light microscopic image (orig. mag. 20×) of bronchus stained by immunohistochemistry using antibody Cam5.2, specific for cytokeratins 8, 18, and 19. Epithelial cells are positive with dim cytoplasmic and dark membranous staining. Other cells are negative. Cells are counterstained with hematoxylin. The arrows indicate Y chromosome-positive epithelial cells. The arrowhead on the right indicates a Y chromosome-positive cell that does not express cytokeratins recognized by Cam5.2 and is located below the epithelium within the lamina propria; it is therefore probably either a stromal cell or a cell of hematopoietic lineage.

(C) Fluorescence microscopic image (100×) of FISH for Y chromosome (pseudocolored pale yellow green), with DAPI (blue) nuclear counterstain. This image is from the same slide as in Figures 1A and 1B. Morphology of cells and persistence of DAB stain indicating cytoplasmic cytokeratins define the bronchial epithelial lining cells. Yellow Y chromosomes are identified in three such cells (arrows). The submucosal collagen autofluoresces and is pseudocolored green using a combination of filters (Cy5 for DAB, Cy3.5 for rhodamine, FITC for autofluorescence, DAPI for nuclei).

nuclei in alveoli from the transplanted mice was 12.58 ± 4% of epithelial cells (Figure 3A). After correction for sampling (62% positive in male control), the mean number of male-derived alveolar cells was 20% (Table 4).

In addition to engraftment of columnar epithelial cells in the small bowel (Figure 2), donor-derived epithelial cells were identified throughout much of the GI tract, including the lining of the esophagus, stomach, and large bowel as shown in Figures 3B–3D. In the esophagus (3B), the lamina propria is at the bottom and the lumen on the top, and the arrows indicate Y chromosome-positive keratinocytes. In Figure 3C, the branched tubular glands of the stomach are seen. The full arrow indicates a Y chromosome-positive columnar epithelial cell lining the gastric pit. The arrowheads indicate donor-derived nonepithelial cells that may be blood cells in the lamina propria. The large bowel (Figure 3D) of each animal also had donor-derived epithelial cells. In this section of colon, the donor-derived cell indicated is clearly located at the base of a gland in the mucosa of the large bowel. Importantly, additional experiments were performed in which mice were transplanted with a single visualized male bone marrow cell plus female R/O cells. These mice analyzed three months post transplant also showed both hematopoietic and multi-organ epithelial engraftment of male cells, further confirming that one cell is capable of repopulating both blood and epithelial cells.

We have shown previously that in women who were transplanted with male-derived whole bone marrow, Y chromosome-positive cells comprise 4%–38% of cholangiocytes after months to years (Theise et al., 2000b).

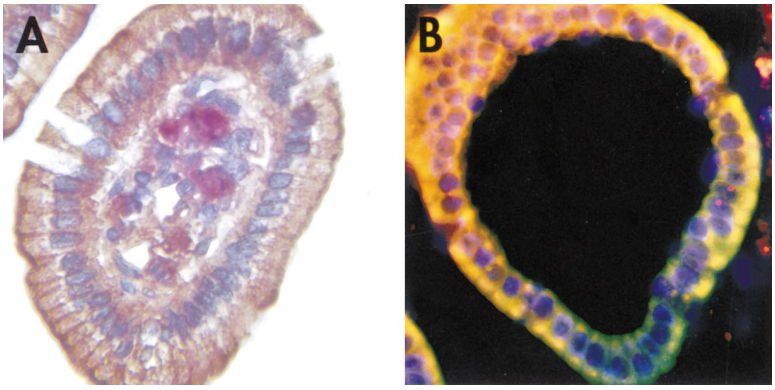

Figure 2. Immunohistochemical and FISH Analysis of Small Intestine

(A) Light microscopic image (100×) of a cross-section of a small intestinal villous showing double immunohistochemical staining with anti-cytokeratin antibody CAM5.2, specific for epithelium (brown), and with anti-CD11b antibody Mac1, specific for macrophages (red). Macrophages are confined to the lamina propria, are not found above the basement membrane within the epithelial surface, and do not coexpress cytokeratins. Cytokeratin-positive epithelial cells never coexpress CD11b. (DAB, fuchsin red, Mayer's hematoxylin.)

(B) Fluorescence microscopic image (100×) of a small intestinal villous from the same double immunostained slide after FISH for Y chromosome (red) and DAPI (blue) nuclear counterstain. Morphology of cells and persistence of DAB stain indicating cytoplasmic cytokeratins define the intestinal epithelial lining cells, two of which (right middle) display red Y chromosomes. No CD11b-positive macrophages were seen in this particular cross-section. (Filters: Cy5, Cy3.5 for rhodamine, FITC for autofluorescence, DAPI for nuclei.)

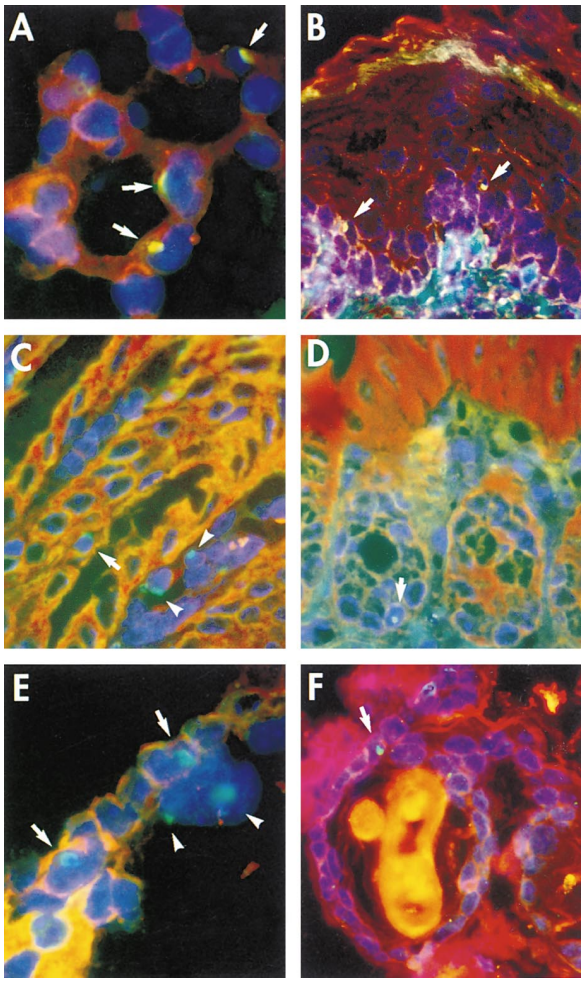

Figure 3. Epithelial Lining Cells of the Lung Alveoli, GI Tract, Cholangiocytes, and Hair Follicle Cells Show Male Marrow-Derived Derivation

Fluorescence microscopic images of (A) lung, (B) esophagus, (C) stomach, (D) colon, (E) bile duct cyst, (F) skin. (Filters as above, original magnifications of [A], [C], and [E], 100×, [B], [D], and [F], 60×.) Due to pseudocoloring of images to enhance cellular details, Y chromosomes appear yellow in (A), and blue-green in (B)–(F).

Similarly, in the mice transplanted with a Fr25lin$^-$ homed cell, male donor-derived cholangiocytes lining the bile ducts were present. In Figure 3E, two Y chromosome-positive cholangiocytes are shown lining a biliary cyst. These DAB-stained, Y chromosome-positive cholangiocytes clearly make up part of the wall of the bile cyst. Y chromosome-positive cells were also present in the skin. As shown in Figure 3F, the male donor-derived cells tended to be localized to the neck region of the hair follicles, but were also present in the epidermis (not shown). This follicular location in the neck region is a common location for the follicular "bulge," which has recently been demonstrated to be a site for skin progenitor cells (Taylor et al., 2000). No donor-derived Y chromosome-positive cells were identified amongst the cytokeratin-stained renal tubule cells of these mice.

In addition to identifying epithelial cells in the organs by cytokeratin staining, we used FISH analysis for surfactant B mRNA to confirm the identity of epithelial cells in the lung. Surfactant B is transcribed exclusively in type II pneumocytes, and it is produced to such a high degree in these cells that two large transcription centers are apparent in the nuclei using a fluorescent probe for surfactant B mRNA (Figure 4). The presence in a single nucleus of a Y chromosome and transcription centers for surfactant B identifies a male-derived type II pneumocyte. In Figure 4, simultaneous FISH analysis for surfactant B and the Y chromosome is shown in the lung. The surfactant B transcription centers are green and the Y chromosome is red, as shown schematically in Figure 4b.

These data not only confirm that the Y chromosome-positive cells are epithelial, but that they are functional cells that express tissue specific genes. Moreover, the type II pneumocyte is known to be the intraorgan stem cell in the lung parenchyma, responsible for regenerating new type II pneumocytes as well as type I pneumocytes, which account for greater than 80% of the alveolar surface. Thus, engraftment of type II pneumocytes from the marrow can explain the finding that focal alveoli were entirely lined by cytokeratin-stained marrow-derived epithelia.

Quantitative analysis of donor-derived cells in each of the organs examined is presented in Table 4. Significant engraftment occurred for all of the tissues examined except kidney. The highest percentage of donor engraftment (approximately 20%) occurred in the pneumocytes of the lung. The degree of engraftment throughout the GI tract was variable with the highest percent engraftment in the esophagus and the least in the colon. Although Y-positive cells with the morphology and au-

Table 4. Percent Donor Engraftment of Nonhematopoietic Tissues 11 Months Post Transplant

	Bronchi	Alveoli	Esoph	Stomach	Sm. Bowel	Large Bowel	Skin	Bile Duct
M 1	3.6	14.8	0	0.5	0.3	0.2	2.6	0.4
M 2	2.3	10.3	0.4	0.5	0.4	0.1	2.4	0
M 3	3.5	18.7	2.2	0	0	0	1.2	0
M 4	2.2	10.1	2.5	0.2	0.4	0.3	1.6	2.2
M 5	0	9	0.5	0.4	1.6	0	2.7	0
Mean ± SD	2.32 ± 1.45	12.58 ± 4.07	1.12 ± 1.14	0.32 ± 0.21	0.54 ± 0.61	0.12 ± 0.13	2.1 ± 0.66	0.52 ± 0.95
Corr.[a]	3.74	20.30	1.81	0.52	0.87	0.19	3.39	0.84

The numbers shown represent the percentage of cytokeratin-positive cells within each tissue that was Y chromosome positive. For each tissue, over 150 cytokeratin-positive cells were analyzed. For stomach, small bowel, and large bowel, 10^3 to 3.4×10^3 cells were counted for each mouse.

[a] The percentage of immunostained epithelial cells that are Y chromosome positive is corrected for the male control for each tissue.

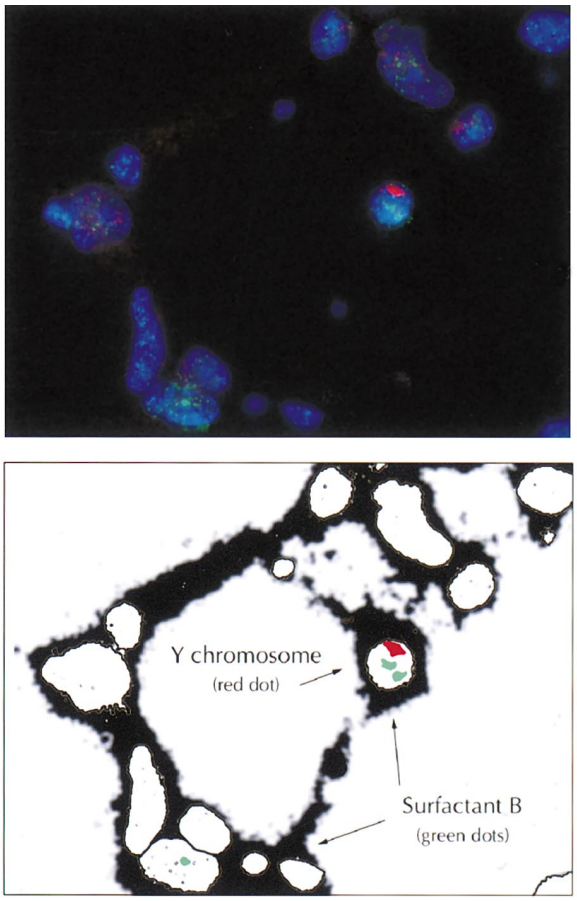

Figure 4. Double FISH for Surfactant B mRNA and Y Chromosome Confirms Identity of Donor-Derived Epithelial Cells in the Lung

The upper image was obtained by overlaying the fluorescence image obtained with the DAPI (blue nuclei), FITC (green transcription centers), and Cy3.5 (red Y chromosome) filters. The lower image was obtained by using the "find edges" command in Adobe Photoshop after increasing the gain to detect the autofluorescence of the cell bodies (shown black in this schematic).

tofluorescence of hepatocytes and cardiac and skeletal myocytes were recognized, they did not stain with the anti-cytokeratin antibodies used and therefore were excluded from formal analysis in this paper.

Discussion

Our studies show that bone marrow populations can be enriched significantly for stem cells by recovering cells that home to the bone marrow within 48 hr of transplantation. This purifies functional HSC from one in 10^3 Fr25lin$^-$ marrow cells to approximately one in six (16.6% of mice engrafted at 11 months). Significantly, single bone marrow-derived cells have nonhematopoietic differentiation potential as well. This level of enrichment may be an underestimate because, as has been suggested (Osawa et al., 1996), only 20% of recipients are likely to receive the single cell in a marrow niche (seeding efficiency), the site best suited for expansion and self-renewal of HSC. Initial experiments were performed using limiting dilution to transplant one male-derived cell per recipient. The percentage of animals that received greater than one cell calculated by Poisson statistics and viability (50% to 60% viable by propidium iodide staining) was no greater than 7%–9%. The cells were elutriated, lineage depleted by antibody treatment, labeled with PKH26, passaged in a mouse for 2 days, and passed through the cell sorter, followed by retransplantation into new recipients all contributing to this level of viability. We are confident that since 17% of the animals engrafted, at least some of them received a single cell. We have repeated these studies using direct visualization of single viable cells, rather than depending upon limiting dilution prior to injection into mice for long-term engraftment. Fifty-six percent of these mice were alive at 3 months. We observed both hematopoietic and nonhematopoietic (epithelial) engraftment in a sample of these mice, which further supports the conclusion that multi-organ, multi-lineage engraftment occurred.

The high level of engraftment of blood and marrow 11 months post transplant suggests that the expansion and differentiation of a single marrow SC to reconstitute the majority of the hematopoietic system of a lethally irradiated recipient is feasible. Three of the long-term survivors had greater than 70% donor cells in the blood at 5 months post transplant and 50% or more male cells at 11 months. This is also reflected in the large number of donor-derived progenitor cells (CFU) at 11 months (greater than 70%). Two of the mice appear to have lost most of their graft (mice #1 and #5), but only mouse #1 also lost donor type colony forming progenitors at 11 months. Even the mouse with little peripheral blood engraftment and no marrow progenitor engraftment at 11 months (mouse # 1) had nonhematopoietic cell engraftment at this time.

The variable level of engraftment following single-cell transplantation is likely due to donor HSC dilution (Jones et al., 1989) in the recovering host and the variations in successful homing to the marrow space, which is necessary for successful seeding of HSC. Adhesion molecules required for homing include VLA-4 (Craddock et al., 1997). Since CD34 may also have a role in adhesion (Healy et al., 1995), it is intriguing to speculate that upregulation of CD34 in donor cells is required for engraftment and may be related to marrow homing. Alternatively, CD34$^+$ donor cells have a homing advantage. Recent studies suggest reversible expression of CD34 both in vivo (Sato et al., 1999) and in vitro (Sato et al., 1999; Nakamura et al., 1999) due either to cytokine stimulation or cell cycle activation following 5FU administration. In contrast to these data in which changes in CD34 expression occur much later, we may be observing an increase of CD34 within the first 48 hr post transplant. At 2 days, our cells are not in cell cycle (Lanzkron et al., 1999) as would be the case with 5FU or cytokine exposure. It is possible that HSC require CD34 expression to maintain LTR potential and home to the marrow or that upregulation of CD34 expression occurs soon after cells arrive in the marrow space. In any case, the change from 4% to 45% CD34-positive cells is a reflection of an early step which may be necessary for our single HSC to LTR recipient mice.

Donor-derived epithelial cells were detected in lung, GI tract, and skin, and were distinguished from intraepithelial hematopoietic cells (i.e., lymphocytes, polymor-

phonuclear leukocytes, and macrophages) by their cytokeratin staining, morphology, and examination of parallel sections. The cytokeratins detected by the monoclonal antibodies employed here are specific for epithelial cells and are not identified in cells of any hematopoietic lineage (Moll et al., 1982; Sun et al., 1984). Moreover, when double immunohistochemistry was performed with anti-macrophage specific antibodies, single cell colocalization of the two markers never occurred, confirming that these cytokeratin-positive cells were not macrophages that had phagocytosed debris of dead epithelia.

The epithelial engraftment was found at different frequencies in different organs. These differences may be due to (1) the degree of tissue damage induced by the transplant, (2) the residual tissue-specific stem cell capacity within each organ, and/or (3) the normal rate of cell turnover in each organ. These possibilities are supported by the variable levels reported for liver engraftment by marrow-derived cells. With injury or genetic deficiency sufficient to evoke an intrahepatic stem cell proliferation, clusters of marrow-derived hepatocytes, cholangiocytes, and oval cells form (Petersen et al., 1999; Lagasse et al., 2000). In the absence of such injury (Theise et al., 2000a; Theise et al., 2000b), isolated, scattered hepatocytes and cholangiocytes develop, suggesting that they engraft in the liver in what appears to be a random process which may bypass an intrahepatic stem cell intermediate. At the time of analysis (11 months post transplant), no histological evidence of damage was apparent in any of the tissues examined. Clusters of Y chromosome-positive cells were detected only in alveolar lining cells (Figure 3A). The high levels of donor engraftment as lung cells are analogous to those seen in severe injury models reported for the liver (Petersen et al., 1999; Lagasse et al., 2000). Lung tissue is significantly damaged by radiation yielding necrosis of alveolar lining cells, focal hemorrhage, and eventual scarring (Travis et al., 1985). Alternatively, there may be lung tissue damage due to low level viral infection in these temporarily immunosuppressed animals. In mice examined within the first week following lethal irradiation, there is focal hemorrhage and macrophage infiltration within the lung parenchyma (authors' unpublished data). Within this damaged lung tissue, surfactant B producing (type II) pneumocytes engrafting from transplanted marrow were detected as early as 5 days post transplant (unpublished data). Type II pneumocytes are thought to be the alveolar progenitor cells, giving rise to type I pneumocytes in response to injury (Magdaleno et al., 1998). Both of these pneumocyte populations can be demonstrated by immunostaining for the same panel of cytokeratins; thus the cells pictured in Figure 2 represent a mixture of type I and II alveolar lining cells. Therefore, the high percentage of Y chromosome-positive pneumocytes may reflect an early proliferative healing response to acute radiation injury and possibly to post-radiation infection.

Thus there are two patterns of epithelial engraftment of marrow-derived cells: large-scale repopulation in response to injury (as demonstrated in liver and lung) and low level engraftment as individual scattered cells in the absence of marked injury (e.g., liver, skin, and GI tract). These randomly inserted single cells may not be fully functional since they do not appear to proliferate.

The data presented herein demonstrate a high degree of plasticity with a single cell having the ability to differentiate into cells of the GI tract, lung, and skin. Although little is known about how these cells obtain this degree of differentiative potential, it is possible that the cells are "summoned" to sites of injury by factors secreted from the damaged organ. Once the cells arrive in the damaged tissue, the local environment stimulates gene expression patterns that cause a morphological change in the phenotype of the cell. Interestingly, theories regarding how cells undergo cell type-specific differentiation strongly suggest that tissue-specific transcription factors are rare. Rather, different combinations of the same transcription factors present in different ratios induce different patterns of gene expression that cause cells to differentiate down different pathways (Rosen et al., 1998; Shivdasani and Orkin, 1996; Sieweke and Graf, 1998; Zahnow et al., 1997).

We conclude that passage of a partially purified marrow SC population for two days in a lethally irradiated recipient results in enrichment of cells with the capacity to LTR mice. Single bone marrow cells can self-renew in vivo as well as differentiate into hematopoietic progenitors and mature cell types of both hematopoietic and nonhematopoietic tissues. Expression of CD34 is increased in mice shortly after transplantation in the marrow consistent with this molecule being involved in homing.

There are multiple therapeutic implications of this work. Bone marrow-derived cells that have the capacity to differentiate into mature epithelial cells could serve as target cells for gene therapy or as a source for organ reconstitution and repair. Bone marrow transplantation itself might prove useful in the treatment of some forms of tissue injury or disease. For example, gene therapy for various pulmonary disorders will require infection of a stable and renewing population of cells with expression of the desired gene product under normal physiologic control (e.g., cystic fibrosis transmembrane regulator). Pneumocytes would be an excellent target for gene therapy. One could design gene therapy vectors on which drugs that can inactivate viruses are expressed only in virus-infected cells. Populations of bone marrow stem and progenitor cells can be infected with high efficiency by retroviral vectors (Abonour et al., 2000; Ito and Kedes, 1997; Nolta et al., 1992, 1996), making the bone marrow SC a potential delivery system for hematological and epithelial gene therapy.

Experimental Procedures

Stem Cell Isolation and Transplantation

For bone marrow SC isolation, 20 male and female B6D2/F1 mice or male C57Bl/6 CD34 knockout (kind gift from Dr. Mak, Toronto, Canada) mice were killed by cervical dislocation and the hind limbs removed. Bone marrow was flushed with medium from the medullary cavities of tibias and femurs using a 25G needle. Marrow cells were elutriated as previously described (Jones et al., 1996). Male cells were collected at a flow rate of 25 ml/min (Fr25) and female cells collected after the rotor had stopped (R/O, a population enriched for progenitors and short-term repopulating cells). Fr25 cells were depleted of lineage-positive cells including T and B lymphocytes, macrophages, granulocytes, erythroid cells, and late progenitor cell

populations (Fr25lin$^-$) as previously described (Lanzkron et al., 1999; Jones et al., 1996). Male B6D2F1 or C57Bl/6 knockout mice Fr25lin$^-$ cells were labeled with PKH26 and 10^7 labeled cells injected into lethally irradiated female B6D2F1 recipients as described (Lanzkron et al., 1999), or in the case of the CD34 knockout experiment, recipients were irradiated (1050 to 1100 cGy from a gamma cell small animal Irradiator, Atomic Energy, Canada) wild-type female C57Bl6/J mice. At 48 hr post transplant, the female recipients were sacrificed and spleens and marrow harvested. PKH26 fluorescence intensity of single cell suspensions of spleen and marrow was measured by an Epics740 flow cytometer (Coulter Electronics, Hialeah, FL). For transplant studies, the male Fr25lin$^-$ PKH26$^+$ cells following passage in lethally irradiated female mice for 2 days were injected into additional lethally irradiated female recipients. PKH$^+$ cells were obtained at the same intensity and size as those stained before the first transplant. Viability was determined by propidium iodide. We estimate that our limiting dilution resulted in the injection of 0.5–0.6 viable cells/animal. A group of 30 lethally irradiated recipients received such a transplant along with 2×10^4 unstained female R/O cells in order to provide short-term but not long-term reconstitution (Jones et al., 1996). In additional experiments, to be absolutely certain that the mice received one male donor-derived, passaged PKH26$^+$ cell, rather than using limiting dilution, a single cell visualized under the microscope was drawn up and delivered directly to a syringe. This cell was then injected along with 2×10^4 female rotor off cells in a total volume of 500 μl.

Engraftment and FISH Analysis

At 5 and 11 months post transplant, surviving mice underwent retroorbital bleeds to assess the percent of donor cell engraftment in the blood. At 11 months, recipients were sacrificed. Colony assays were performed with bone marrow by routine methods. Colonies from primary long-term survivors or peripheral blood samples from primary and secondary (Table 1) surviving female hosts were collected and FISH for the Y chromosome was done as previously described (Jones et al., 1996; Hawkins et al., 1992). Also, at 11 months, 1×10^6 marrow cells from each host were transplanted into additional groups of four female hosts (for a total of 4×10^6 cells). FISH analysis was performed 2 and 4 months post transplant for the presence of male cells.

After formalin fixation and paraffin embedding, tissues of the five 11 month engrafted mice were analyzed for the Y chromosome. The identification of epithelial cell-specific proteins while performing FISH is difficult due to the extensive protease digestion required for FISH, which obliterates antigenic sites needed for antibody binding. Therefore, we used a two step procedure to identify specific cell types and to determine which are male-derived cells as described previously (Theise et al., 2000b). First, immunoperoxidase staining using Cam5.2, an antibody against shared epitopes of cytokeratins 8, 18, and 19, or AE1/3, a monoclonal antibody cocktail specific for high molecular weight cytokeratins, was used to label epithelial cells, specifically. After counterstaining with hematoxylin, the sections were color photographed at 20× magnification, and printed as 5×7 in hard copy pictures obtained. The second step of analysis involved FISH staining for Y chromosome. Double immunohistochemical staining for cytokeratins and CD11b, a macrophage-specific antigen, was accomplished by adding an incubation step for the biotinylated anti-CD11b rat monoclonal antibody followed by colorization with fuchsin red chromogen (DAKO Inc).

For double FISH for Y chromosome and surfactant-B (SPB) mRN*A,* slides containing 3 μm tissue sections were deparaffinized and digested with 100 μg/ml proteinase K with 0.05% SDS at 45°C. Genomic DNA probes were prepared based on mRNA sequence for mouse surfactant protein B (SPB, accession number 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 and PCR products were labeled by incorporation of digoxigenin-dUTP. Mouse Y probe was labeled by PCR using biotin-dUTP. PCR products were then partially digested with DNase I. 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), resuspended in 10 μl hybridization buffer (50% formamide), and denatured. Slides were denatured 8 min at 86°C, and hybridized overnight at 37°C. Posthybridization washes were done at 37°C, followed by antibody detection, using 10 μg/ml protein solutions in 4×SSC. The first detection step included antidigoxigenin and equal amounts of avidin-FITC mixed with avidin Cy5; the second step included sheep antimouse Cy3. 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 green filter than the Cy5 filter). After washing, slides were mounted in DAPI antifade.

Tissue Analysis and Cell Counts

Counting of Y-positive nuclei was accomplished by systematically examining the FISH-stained tissue, field by field, under 60× magnification, using an Olympus Provis (Tokyo, Japan) microscope equipped with a cooled CCD camera (Quantix Corp., Cambridge, MA) and specialized software (PSI Inc, League City, TX). 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). Cell counts were obtained by first counting all of the Y chromosome-positive cells in a defined area on the tissue, and then counting the total number of cells in that area using the 5×7 immunostained photographs. To compensate for undercounting of Y-positive nuclei due to partial nuclear sampling in tissue sections, cell counts were normalized to the percentage of Y-positive cells seen in the normal male tissue.

Acknowledgments

We thank Dr. Steven Baylin for critical reading of the manuscript, Dr. Richard Jones for helpful discussions, and Dr. Steven Piantadosi for statistical analysis. We thank Drs. Patricia Bray-Ward, David Ward, and Joseph Madri for generously allowing us to use their microscopes and image analysis software, Katherine Henderson for assistance with figure preparation, and Dr. Joanna Grove for advice and assistance. Supported by NIH grants: RO1 HL54330, RO1 DK53812, PO1 CA70970, RO1 DK53037, NIDDK P30-34989, PO1 HL63357, a Primary Biliary Cirrhosis Seed Grant from the American Liver Foundation, and funds from the Mary Lea Johnson Richards Research Institute and the Robert Krieger Memorial Fund.

Received October 26, 2000; revised April 3, 2001.

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