Hematopoietic Stem Cells Contribute to the Regeneration of Renal Tubules after Renal Ischemia-Reperfusion Injury in Mice

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Abstract. Ischemia-reperfusion injury (I/R injury) is a common cause of acute renal failure. Recovery from I/R injury requires renal tubular regeneration. Hematopoietic stem cells (HSC) have been shown to be capable of differentiating into hepatocytes, cardiac myocytes, gastrointestinal epithelial cells, and vascular endothelial cells during tissue repair. The current study tested the hypothesis that murine HSC can contribute to the regeneration of renal tubular epithelial cells after I/R injury. HSC isolated from male Rosa26 mice that express β-galactosidase constitutively were transplanted into female nontransgenic mice after unilateral renal I/R injury. Four weeks after HSC transplantation, β-galactosidase-positive cells were detected in renal tubules of the recipients by X-Gal staining. PCR analysis of the male-specific *Sry* gene and Y chromosome fluorescence *in situ* hybridization confirmed the presence of male-derived cells in the kidneys of female recipients. Antibody co-staining showed that β-galactosidase was primarily expressed in renal proximal tubules. This is the first report to show that HSC can differentiate into renal tubular cells after I/R injury. Because of their availability, HSC may be useful for cell replacement therapy of acute renal failure.

Hematopoietic stem cells (HSC) are lineage-uncommitted (Lin^-) bone marrow cells that are characterized by the expression of cell surface markers, Sca-1 and c-kit, and the absence of lineage-specific markers for white blood cells, red blood cells, and platelets. HSC are capable of self-renewal and can support the long-term repopulation of functional peripheral blood cells in lethally irradiated hosts. Although they are rare populations representing 0.005% to 0.01% of total bone marrow cells (1–3), HSC can be isolated by fluorescence-activated cell sorting (FACS) based on their expression of cell surface markers and their ability to efflux mitochondrial dyes (1,4), such as rhodamine-123 (Rh). We previously reported that primitive $Rh^{lo}Lin^-Sca\text{-}1^+ckit^+$ cells supported hematopoiesis for up to 6 mo in lethally irradiated mice and contributed to blood formation in secondary recipients (5). Using bioinformatics and microarray analysis, we also showed that these primitive cells exhibit distinct gene expression patterns compared with less primitive bone marrow cells, suggesting that differentially expressed genes may govern the phenotype and function of hematopoietic cells (5,6).

The pluripotency of HSC is evidenced by their capacity to differentiate into multiple lineages within the blood and immune system, as well as cells of non-hematopoietic tissues, such as hepatocytes, cardiac myocytes, gastrointestinal epithelial cells, and vascular endothelial cells (7–11). The discovery that adult HSC can cross lineage boundaries to become cells of other tissues has challenged the traditional view that somatic stem cells are lineage-restricted and organ-specific (12). One possibility is that HSC retain developmental plasticity and can be reprogrammed to express genes that are required to differentiate into the cells of the organs into which they are introduced. Another distinguishing feature of HSC is their ready availability from bone marrow, cord blood, and mobilized peripheral blood. This property makes HSC potentially useful for cell replacement therapy in regenerative medicine.

Acute renal failure (ARF) is a common disease with high morbidity and mortality. Current treatment options for acute renal failure are limited, and mortality remains 30 to 50% (13). Renal ischemia-reperfusion injury (I/R injury) is one of the most common causes of ARF. I/R injury is characterized by alterations in cell metabolism, inflammation, free radical generation, and apoptosis that result in the detachment of renal tubular cells from the basement membrane and shedding into the urine (14–16). The S3 segments of the proximal tubules located in the outer stripe of the outer medulla are particularly susceptible to ischemic injury and are primarily responsible for the pathophysiological and clinical presentations of ARF (17–

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19). Recovery from ARF requires the replacement or regeneration of lost tubular epithelial cells. This process is accompanied by complex changes in gene expression of growth modulatory molecules, such as EGF, IGF-1, and HGF (20,21). It has been generally believed that some of the surviving renal tubular cells dedifferentiate and reenter the cell cycle to produce epithelial cells that rebuild the structure and function of the renal tubules. However, the origin of the regenerating cells has not been clearly established. The ability of HSC to adopt the cell fate of the organs in which they reside suggests that HSC could potentially differentiate into kidney cells during kidney regeneration. In this report we provide evidence showing that adult murine bone marrow-derived HSC integrate into injured kidneys and differentiate into tubular epithelial cells in mice.

Materials and Methods

Isolation of HSC

HSC were isolated from 6 to 8-wk-old male Rosa26 mice (B6; 129S-GtRosa26, Jackson Laboratory, Bar Harbor, ME). The isolation method was based on immuno-depletion and positive selection with FACS as described previously (5). Briefly, bone marrow cells were collected from the femur and tibia. Lineage-negative cells (Lin^- cells) were obtained by incubation with a cocktail consisting of lineage-specific rabbit anti-mouse CD2, CD4, CD45R/B220, Gr-1, Mac-1 (CD11b), TER-119, and IL-7Rα antibodies (PharMingen, San Diego, CA) and goat anti-rabbit IgG coupled to magnetic beads (M-450; Dynal, Lake Success, NY). Unbound Lin^- cells were incubated with antibodies to Sca-1-cychrome and c-Kit-PE as well as rhodamine before isolation by FACS (FACSVantage; Becton Dickinson, San Jose, CA). Sca-1– and c-kit–positive cells exhibiting less than 15% rhodamine staining intensity (Rh^{lo}) were sorted out and designated $Rh^{lo}Lin^-Sca\text{-}1^+ckit^+$ cells. The purity of the sorted cells was confirmed by staining with an antibody to common leukocyte antigen, CD45.2 (PharMingen, San Diego, CA).

Fluorescein D-Galactopyranoside (FDG) Staining of White Blood Cells

Peripheral blood was obtained from B6-Ly5.2/Cr–recipient mice 4 to 12 wk after kidney I/R injury and transplantation with $Rh^{lo}Lin^-Sca\text{-}1^+ckit^+$ cells from Rosa26 mice. Blood was also collected from Rosa26 donor mice and untransplanted B6-Ly5.2/Cr mice as positive and negative controls, respectively. Using a published method (22), nucleated blood cells were loaded with FDG (Molecular Probes, Eugene, OR) and analyzed for fluorescence intensity by flow cytometry. The viability of the cells was confirmed by low staining with propidium iodide.

Kidney I/R Injury and HSC Transplantation

Six-week-old female B6-Ly5.2/Cr mice (National Cancer Institute, Frederick, MD) were given 11 Gy γ-irradiation 2 h before surgery. The left renal artery was separated from the vein and clamped for 15 min followed by clamp release to allow reperfusion. A group of mice also underwent right nephrectomy for evaluation of blood urea nitrogen (BUN) after left renal I/R injury. Two to four hours after surgery, $Rh^{lo}Lin^-Sca\text{-}1^+ckit^+$ cells (2×10^3 cells/mouse) isolated from male Rosa26 mice were injected via tail vein together with syngeneic Lin^- bone marrow cells (2×10^5 cells/mouse). Control mice that had irradiation and renal I/R injury received syngeneic Lin^- bone marrow cells only. Sham-operated mice underwent irradiation and syngeneic Lin^- bone marrow cell injection but did not receive kidney I/R injury. Mice were kept in a specific pathogen-free facility and were given drinking water containing baytril (0.15 mg/ml) and amoxicillin (1 mg/ml) for 2 wk. Four to twelve weeks after transplantation, peripheral blood was obtained via retro-orbital venous puncture to evaluate the engraftment. Animals were euthanized and perfused with saline to remove blood cells. Kidneys were fixed with 4% paraformaldehyde, embedded in OCT medium, frozen in isopentane, and sectioned at 5- or 10-μm-thick before mounting on Vectabond-treated slides (Vector Laboratories, Burlingame, CA). The animal protocols were approved by The Institutional Animal Care and Resource Advisory Committee.

TUNEL Staining and Anti-BrdU Staining

Paraffin sections of the kidney were stained with In Situ Cell Death Detection Kit (Roche, Germany) according to the manufacturer's instruction. For BrdU incorporation, mice were injected with 50 μg/kg bromodeoxyuridine intraperitoneally 2 h before harvesting. Paraffin sections of the kidney were stained with FITC-labeled anti-BrdU antibody (BD Biosciences, San Jose, CA). Nuclei were counterstained with propidium iodide.

X-Gal Staining

Tissue sections (10-μm-thick) were fixed with 1% paraformaldehyde in PBS (150 mM NaCl, 15 mM sodium phosphate, pH 7.3) for 30 min on ice and then washed three times with PBS. The sections were incubated with staining solution (PBS containing 20 mM Tris-Cl, pH 7.3, 1.8 mM spermidine, 2 mM $MgCl_2$, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-gal) at 32°C for 16 h followed by 37°C for 4 h in dark. Stained sections were washed with PBS, and some samples were counterstained with hematoxylin and eosin (H&E) before visualization by light microscope (TE200; Nikon, Tokyo, Japan). Photographs were taken with a CCD camera coupled to a computer with analytic software.

PCR of Sry Gene

The primers used to amplify the mouse *Sry* gene were 5'-CAGCTAACACTGATCTTTTC-3' and 5'-TTACTGAGCCAGAATCATAG-3'. Genomic DNA isolated from kidney was used for amplification. The PCR conditions were incubation at 94°C for 3 min; 35 cycles of incubation at 94°C for 30 s, 50°C for 30 s, and 72°C for 2.5 min; followed by a final incubation at 72°C for 5 min. The PCR products were resolved by 1.2% agarose gel electrophoresis.

Y Chromosome Fluorescence In Situ Hybridization (FISH)

Kidney sections (5-μm-thick) were fixed with 3:1 methanol:acetic acid for 30 min at room temperature then baked at 70°C for 1.5 h. A biotin-labeled mouse Y chromosome painting probe (Applied Genetics Laboratory, Melbourne, FL) was denatured at 75°C for 10 min then immediately transferred to 37°C to allow pre-annealing for 1 h. The tissue sections were pretreated at 70°C for 2 min in 2 × SSC containing 70% formamide and immediately dehydrated through a graded ethanol series (70%, 90%, and 100%; 2 min each). Samples were air dried at room temperature and then overlaid with denatured probe (10 μl). The sections were covered with a cover slip, sealed with rubber cement, and incubated at 37°C overnight. Sections were washed twice in 2 × SSC for 10 min at 70°C and then twice in 0.1 × SSC for 10 min at 70°C. Slides were incubated in 4 × SSC containing

0.1% Tween-20 for 3 min at room temperature, then 30 μl of a 1:400 dilution of avidin-fluorescein (Vector Laboratories, Burlingame, CA) in 4 × SSC containing 1% BSA, and 0.1% Tween-20 was added. Slides were incubated at 37°C for 15 min in a humidified chamber before washing with three changes of 4 × SSC containing 0.1% Tween-20 for 3 min at room temperature. Sections were counterstained with propidium iodide before visualization by fluorescence microscopy (TE200; Nikon, Tokyo, Japan).

Immunostaining

Kidney sections were serially stained with an antibody to β-galactosidase followed by antibodies or lectins to various renal cell markers (23–27). Kidney frozen sections (10-μm-thick) were fixed with ice-cold 1:1 methanol:acetone for 10 min. After washing with PBS, the sections were blocked with both avidin/biotin Blocking Kit (Vector Laboratories) and PBS containing 1% BSA before incubation with a rabbit antibody against *Escherichia coli* β-galactosidase (Molecular Probes). The antibody was used at a dilution of 1:5000 in PBS containing 0.1% BSA, and sections were incubated at 4°C overnight. The sections were then washed and incubated with a 1:1000 dilution of biotinylated anti-rabbit IgG (Vector Laboratories) at room temperature for 1.5 h before further incubation with a 1:400 dilution of avidin-FITC (Vector Laboratories) at room temperature for 40 min. Next, the sections were stained with antibodies or lectins that recognize specific nephron segments. Proximal tubules were stained with an antibody to Fx1A at 1:100 dilution (23) or an antibody to sodium/phosphate cotransporter type 2 (NaPi-2; Dr. Heini Murer, University of Zurich-Irchel, Zurich, Switzerland) at 1:2000 dilution (24). Thick ascending limbs of loops of Henle (TALH) and distal tubules were stained with an antibody to Tamm-Horsfall Protein (THP: Dr. John Hoyer, Children's Hospital of Philadelphia, Philadelphia, PA) at 1:100 dilution (26). TALH were also stained with an antibody to the sodium/potassium/chloride co-transporter (NKCC2; Dr. Mark Knepper, NIH, Bethesda, MD) at 1:100 dilution (27). Collecting ducts were stained with TRITC-coupled *Dolichos biflorus* agglutinin (DBA; Vector Laboratories) at 1:40 dilution and an antibody to the aquaporin-2 water channel (AQP2; Dr. Mark Knepper, NIH, Bethesda, MD) at 1:2000 dilution (25). All primary antibodies were rabbit anti-mouse or rabbit anti-rat IgG except anti-Fx1A, which was biotinylated sheep anti-rat IgG. Incubation with primary antibodies was performed at room temperature for 2 h, and incubation with Alexa Fluor 594 anti-rabbit IgG (Vector Laboratories) secondary antibody at 1:400 dilution was performed at room temperature for 40 min. For anti-Fx1A staining, incubation with avidin-peroxidase at a 1:1000 dilution was performed at room temperature for 40 min followed by color development with Noval-red (Vector Laboratories).

Some kidney sections were incubated with biotinylated anti-CD45.2 (PharMingen, San Diego, CA) at 1:100 dilution at room temperature for 2 h or with rat anti-mouse macrophage, F4/80 (Caltag, Burlingame, CA) at 1:100 dilution at 4°C overnight. Avidin-FITC was used to visualize CD45.2-positive cells, and biotin anti-rat IgG (Vector Laboratories) followed by avidin-FITC was used to visualize macrophages. Sections were postfixed with 2% paraformaldehyde, rinsed with PBS, and then mounted with Vectashield (Vector Laboratories). Stained sections were photographed under epifluorescence illumination using a Zeiss Axioplan microscope, and the images were analyzed using OpenLab software.

Results

$Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$ Cells Define a Rare Population of Hematopoietic Stem Cells

A combined method of immuno-depletion and positive selection with FACS was used to isolate $Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$ cells. As shown in Figure 1A, after depletion of lineage-differentiated blood cells, most Lin^{-} cells were distributed in a defined region (R1) as assayed by forward and side scattering. $Lin^{-}Sca\text{-}1^{+}ckit^{+}$ cells were identified as double-positive for Sca-1-cychrome and c-kit-PE as shown in Figure 1B. The efflux of the mitochondrial dye, rhodamine, can be used to separate HSC into less primitive HSC with high-rhodamine staining and more primitive HSC with low-rhodamine staining (4,28); low-rhodamine staining cells (representing <15% of total staining) were therefore selected for further study (Figure 1C). Because the bone marrow also contains mesenchymal cells, a post-sorting analysis was performed by staining the isolated cells with anti-CD45.2, which only labels hematopoietic cells. Figure 1D shows that more than 99% of the sorted cells were CD45.2-positive, indicating that contamination with bone marrow mesenchymal cells or other nonhematopoietic cells was minimal. An average of 5×10^3 cells were obtained from each Rosa26 donor (0.01% of total bone marrow cells). This yield is comparable to the number of cells isolated from C57BL/6Ncr mice (5) and is in agreement with the reported numbers of murine hematopoietic stem cells with long-term repopulating ability (1–3).

HSC Supported Repopulation of Peripheral Blood Cells

We reported that transplantation of $Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$ cells isolated from C57BL/6NCr (CD45.2) mice into congenic B6-Ly5.2/Cr (CD45.1) mice resulted in radioprotection in lethally irradiated mice (5). Using the same method, $Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$ cells were isolated from male Rosa26 mice and transplanted into female B6-Ly5.2/Cr mice that were subjected to renal I/R injury. Lin^{-} syngeneic bone marrow cells were co-injected into the host to provide radioprotection until blood cells had repopulated from Rosa26 mice-derived $Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$ cells. To verify the engraftment, nucleated peripheral blood cells from the recipients were stained with FDG. Hydrolysis of FDG by β-galactosidase produces fluorescein that can be detected by flow cytometry analysis. The results showed that 35% and 39% of the nucleated blood cells were able to hydrolyze FDG to fluorescein at 4 wk and 12 wk after HSC transplantation, respectively (Figure 2). The generation of fluorescein in the recipient nucleated blood cells indicated repopulation of peripheral blood cells by HSC of Rosa26 mice.

I/R Injury Results in Tubular Cell Death and Regeneration

Renal I/R injury is a well-established experimental model of ARF that results in cell necrosis and apoptosis. Depending on the degree of injury, renal tubules regenerate with varying degrees of functional and structural recovery (13). Fifteen-

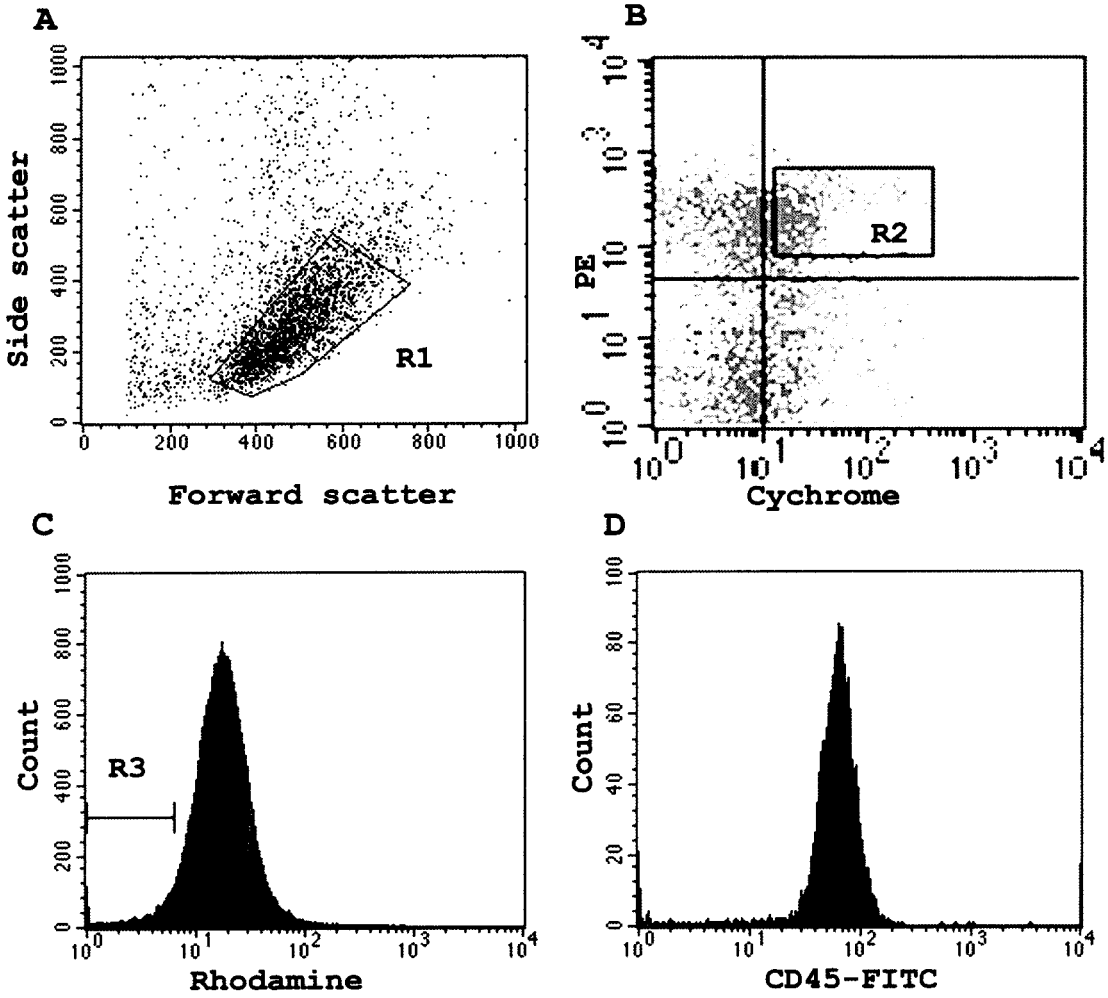

Figure 1. Isolation of $Rho^{lo}Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ cells and post-sorting analysis. (A) Lin^{-} cell distribution by forward and side scattering. Window R1 was selected for sorting. (B) Window R2 defines $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ cells. c-kit was labeled with PE, and Sca-1 was labeled with cychrome. (C) Cells were further sorted to obtain hematopoietic stem cells (HSC) with low rhodamine staining intensity (<15%) in window R3. (D) For post sorting analysis, Lin^{-} cells were incubated with FITC-CD45.2, PE-ckit, and cychrome-Sca-1. Sorted $c\text{-}kit^{+}$ and $Sca\text{-}1^{+}$ cells were reanalyzed for FITC-CD45.2 staining. This analysis showed that 99% of the isolated $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ cells were CD45.2 positive.

minute clamp of renal artery followed by reperfusion caused tubular injury and regeneration as shown in Figure 3. Three days after I/R injury, periodic acid-Schiff (PAS) staining (Figure 3B) showed that tubules were lined with a flattened epithelium. Some epithelial cells were detached from basement membrane, and some nuclei were stained dark and dense. Thinning and absence of PAS-positive brush border were also observed in the proximal tubules. Apoptotic cells were observed with TUNEL stain (Figure 3D). Tubular cell death was accompanied by incorporation of BrdU (Figure 3F), indicative of cell proliferation. Tubules were intact in sham-operated mice (Figure 3A, PAS staining; Figure 3C, TUNEL staining; Figure 3E, anti-BrdU staining). In mice with right nephrectomy and 15-min left renal artery clamp, BUN levels rose from baseline 18.6 ± 1.2 mg/dl to 90.3 ± 26.4 mg/dl at 24 h after injury and then recovered toward baseline to 24.4 ± 1.7 mg/dl (mean ± SEM, $n = 5$) at 5 d. There were no significant changes of BUN levels in sham-operated mice. These results indicate that 15 min of renal artery clamp is sufficient to cause tubular injury and regeneration in mice with lethal irradiation.

X-Gal Staining of the Kidney after I/R Injury and HSC Transplantation

To test if HSC can integrate into the regenerating kidney, HSC were transplanted into recipient mice after renal I/R injury. To distinguish donor-derived cells from recipient cells, HSC from male Rosa26 donors were transplanted into nontransgenic female recipients. Rosa26 mice constitutively express the *E. coli* lacZ gene (encoding β-galactosidase) in all tissues (29). Therefore, donor-derived cells can be identified by their blue color after staining with X-Gal. Figure 4 shows representative X-Gal staining of regenerating kidneys harvested from mice that were injected with $Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$ cells from male Rosa26 mice. An intense distribution of LacZ-positive cells was seen in the outer stripe of the outer medulla where the S3 segments of the proximal tubules are located

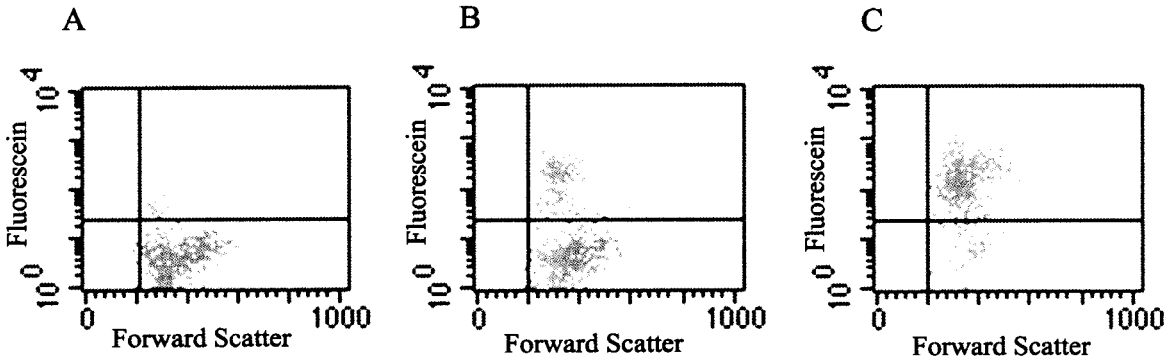

Figure 2. Expression of β-galactosidase in the recipient peripheral blood cells after HSC transplantation. Fluorescein D-galactopyranoside (FDG) staining of peripheral nucleated blood cells in mouse without HSC transplantation (A), mouse with renal ischemia/reperfusion (I/R) injury and Rosa26 mice-derived HSC transplantation (B), and Rosa26 mouse as a positive control (C). Right upper quadrants define cells that contain β-galactosidase activity and are able to hydrolyze the nonfluorescent FDG to generate fluorescein.

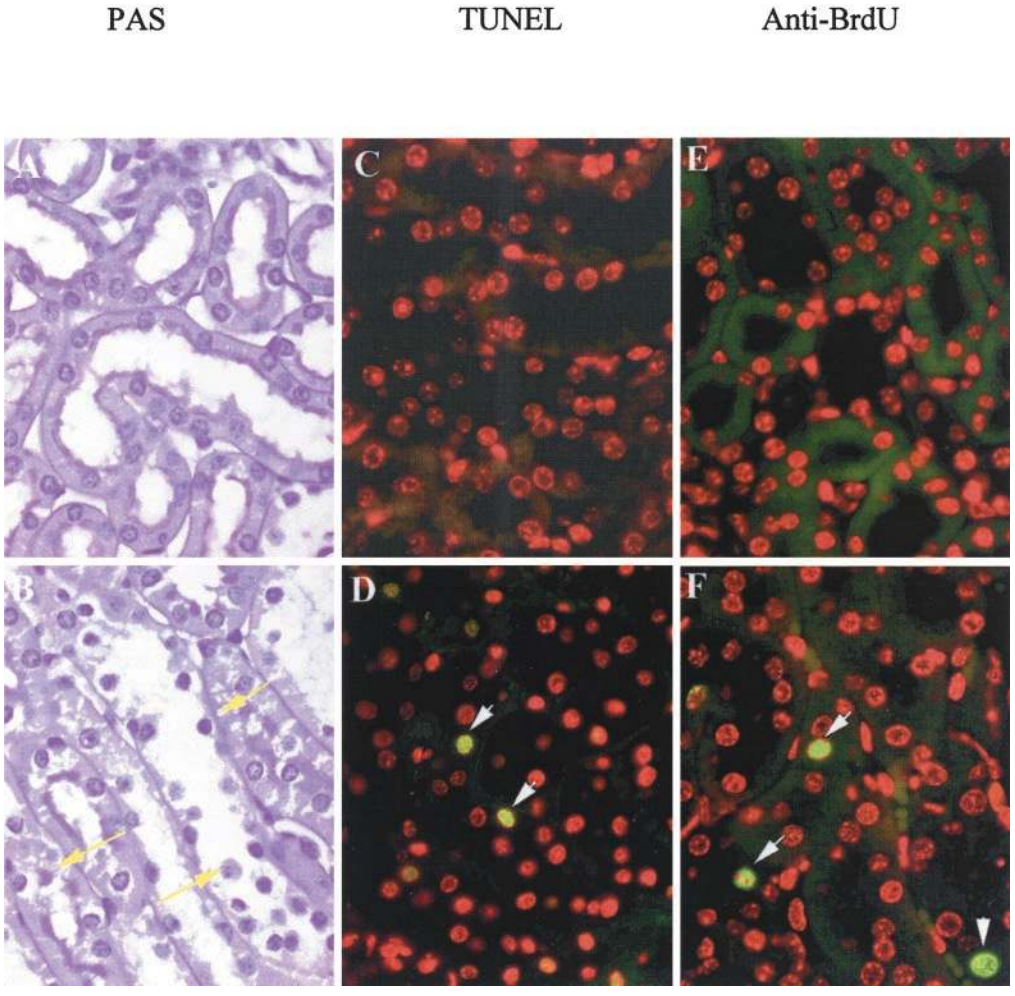

Figure 3. Renal artery clamping for 15 min and reperfusion resulted in tubular injury and regeneration. Kidneys of sham-operated mice (panels A, C, and E) and mice that had renal artery clamping for 15 min (panels B, D, and F) were harvested 3 d after I/R injury and stained with periodic acid-Schiff (PAS; panels A and B), TUNEL (panels C and D), and anti-BrdU antibody (panels E and F). Yellow arrows indicate tubules with flattened epithelial cells, cells detached from the basement membrane, and thinning or absence of the PAS-positive brush border in panel B. Some nuclei were stained dark and dense. White arrows indicate apoptotic cells in panel D or BrdU-positive cells in panel F. Original magnification, ×630.

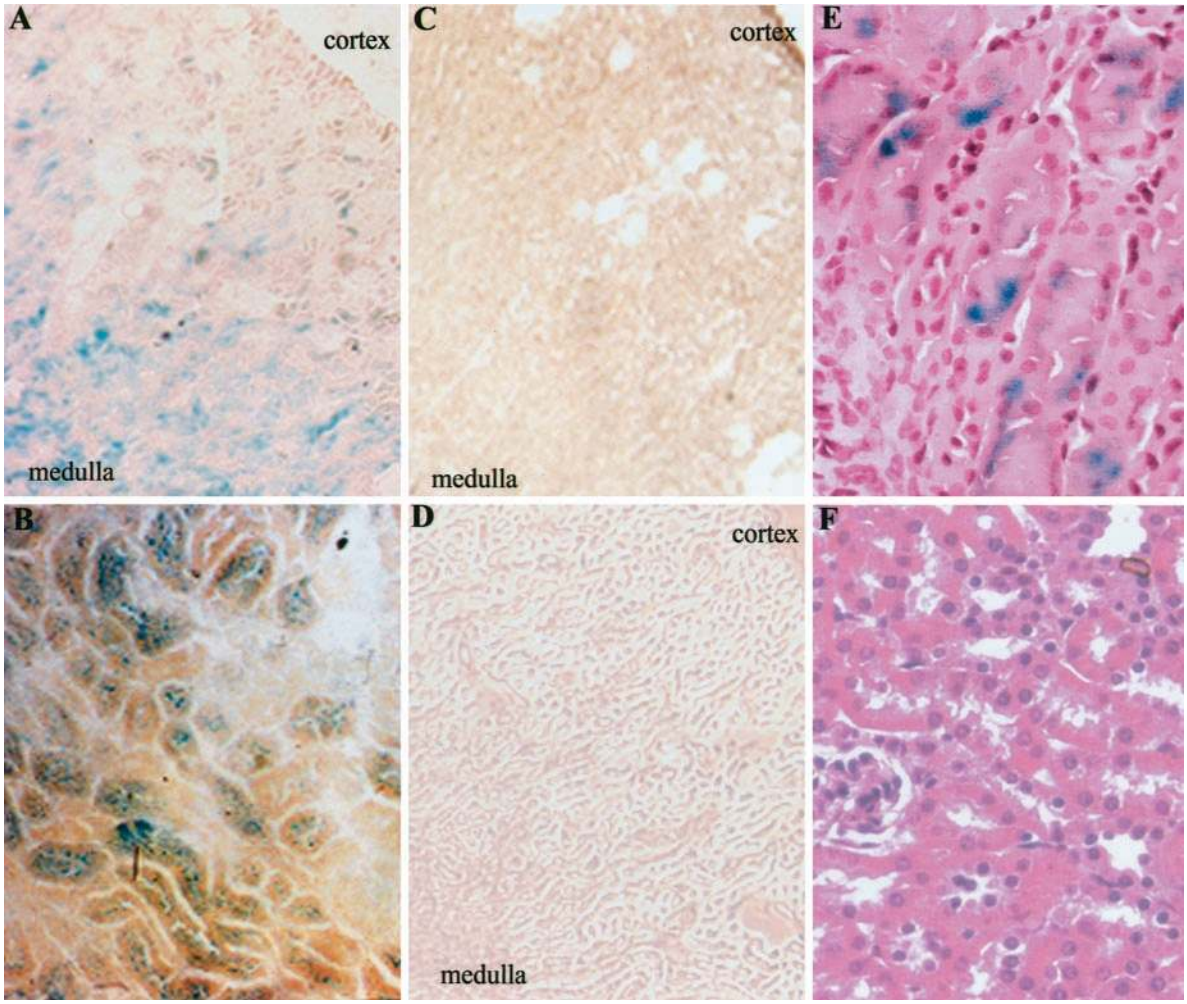

Figure 4. X-gal staining of kidneys after I/R injury and HSC transplantation. (A) X-gal staining of regenerating kidney from non-transgenic mouse that had HSC transplantation from Rosa26 donor; (B) higher power view of the same kidney as in panel A; (C) X-gal staining of regenerating kidney from mouse without HSC transplantation; (D) X-gal staining of the kidney from mouse with HSC transplantation but without I/R injury; (E) X-Gal staining followed by H&E counterstain of regenerating kidney from mouse with HSC transplantation; (F) X-Gal staining followed by H&E counterstain of the kidney from mouse with HSC transplantation but without I/R injury. Data are representative of seven independent experiments. Original magnifications: ×100 in A, C, and D; ×200 in B; ×400 in E and F.

(Figure 4A). Under higher magnification, the LacZ-positive cells were identified within the renal tubules (Figure 4, B and E). The X-Gal staining was specific because no staining was seen in normal age-matched kidneys (data not shown) or in the kidneys from mice that had renal I/R injury but without HSC transplantation (Figure 4C). Similarly, no positive staining was observed in the kidneys harvested from sham-operated mice that received Rosa26 mice-derived HSC transplants but did not undergo renal I/R injury (Figure 4, D and F). No β-galactosidase activity was detected in the renal vessels and glomeruli.

PCR of Sry Gene and Y Chromosome FISH

To provide independent evidence that HSC can differentiate into kidney cells after ischemic injury, male-specific markers were used to identify donor-derived cells in female recipients. After total body perfusion to remove circulating blood cells, genomic DNA was isolated from the kidneys of female recipients 4 wk after renal ischemic injury and HSC transplantation. DNA from normal female and male control mice were used as negative and positive controls, respectively. A pair of primers specific for the *Sry* gene was used to amplify genomic DNA from the kidneys. Figure 5A shows that the 800-bp PCR product could be amplified from the regenerating kidneys from four independent female mice. The PCR product was also detected in DNA from normal males but was absent in DNA from normal untransplanted females, demonstrating the specificity of the assay. Figure 5B shows the results of Y chromosome FISH analysis performed in the kidney frozen sections. Kidney sections were hybridized with biotin-labeled mouse Y chromosome painting probe, and avidin-fluorescein was added to reveal Y chromosome signals. No positive cells were detected in the kidneys from normal female controls (Figure 5B, panel a). About 80% of the cells in the kidney of normal male control mice showed positive signals at the periphery of nuclei

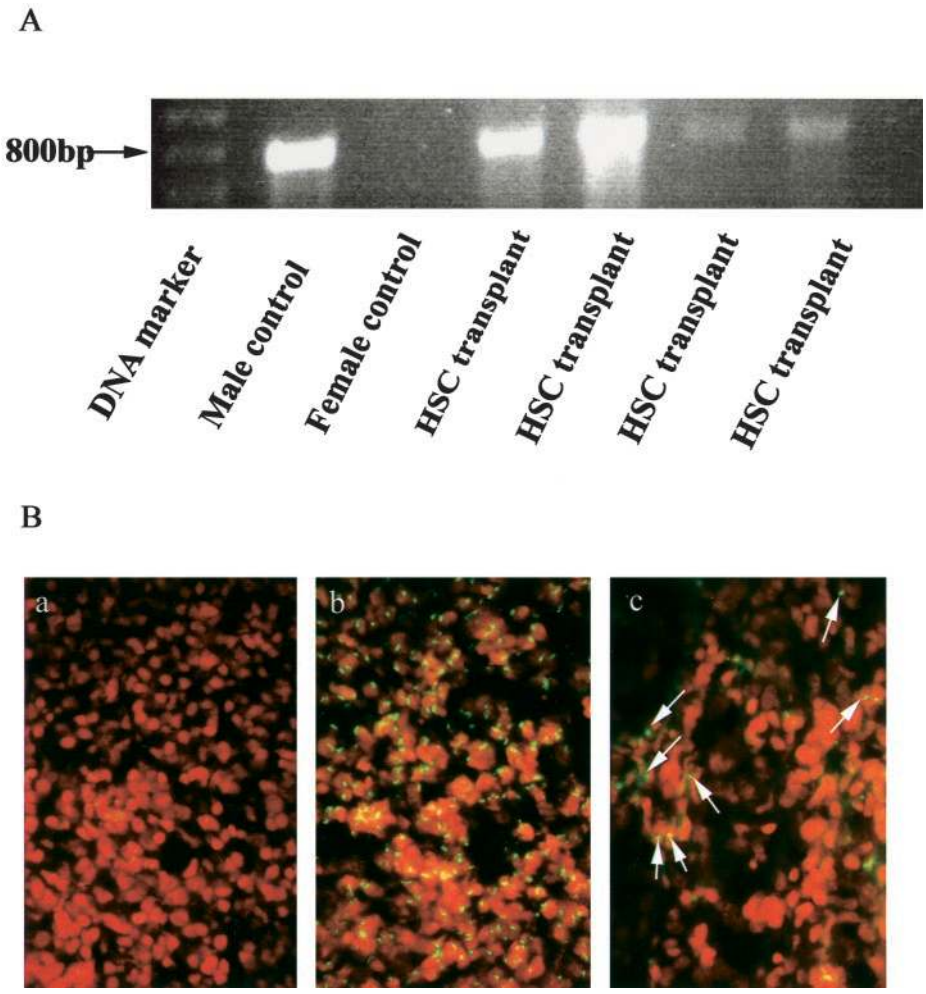

Figure 5. Presence of *Sry* gene and Y chromosome–positive cells in the female recipients. (A) PCR analysis of *Sry* gene in the kidneys 4 wk after renal I/R injury and HSC transplantation. The 800-bp band is shown in normal male kidney. No signal was detected in normal female kidney. A distinct band was observed in four HSC-transplanted female kidneys that had I/R injury. (B) Y chromosome fluorescence *in situ* hybridization (FISH) in the kidneys of normal female (a), normal male (b), and female mouse kidney 4 wk after renal I/R injury and HSC transplantation (c). Mouse Y chromosome painting probe was labeled with FITC. Propidium iodide was used to counterstain the nuclei. Arrows indicate Y chromosome–positive cells. Original magnification, ×400.

that were counterstained with propidium iodide, suggesting that this method can be used to detect male cells but that the sensitivity is not 100% (Figure 5B, panel b). In the kidneys of female mice with renal I/R injury and transplantation of HSC from male Rosa26 mice, 8.3 ± 3.2% of the cells contained Y chromosome positive signals ($n = 4$, triplicate of each kidney section; Figure 5B, panel c). These cells represented male-derived cells in the kidneys regenerated from I/R injury. The lower number of donor-derived cells detected with FISH analysis compared with X-Gal staining probably reflects a lower sensitivity of the assay.

Anti-CD45.2 and F4/80 Staining in the Kidneys

Ischemic injury causes inflammatory cell infiltration in the early phase. With renal regeneration and resolution of inflammation, inflammatory cells subside (13). To demonstrate that the number of macrophages in the kidneys did not increase, 4 wk after the injury, the kidneys were stained with H&E and with macrophage-specific antibody F4/80. Similar to normal control kidneys, H&E staining of regenerating kidneys showed rare numbers of macrophages 4 wk after injury and HSC transplantation (not shown). Staining of regenerating kidneys with F4/80 demonstrated no difference between mice with and without HSC transplantation (not shown). Furthermore, staining with an antibody to the common leukocyte antigen CD45.2 revealed only rare signals in both kidneys from mice with and without HSC transplantation (not shown). These results indicated that the majority of donor-derived cells in the kidney had differentiated into cells that were not CD45.2-expressing hematopoietic cells.

Co-Staining of Antibodies to β-Galactosidase and Markers of Renal Tubular Cells

To test if HSC-derived cells in the kidney expressed renal cell markers, the kidney sections were serially stained with antibodies to β-galactosidase and markers of specific nephron

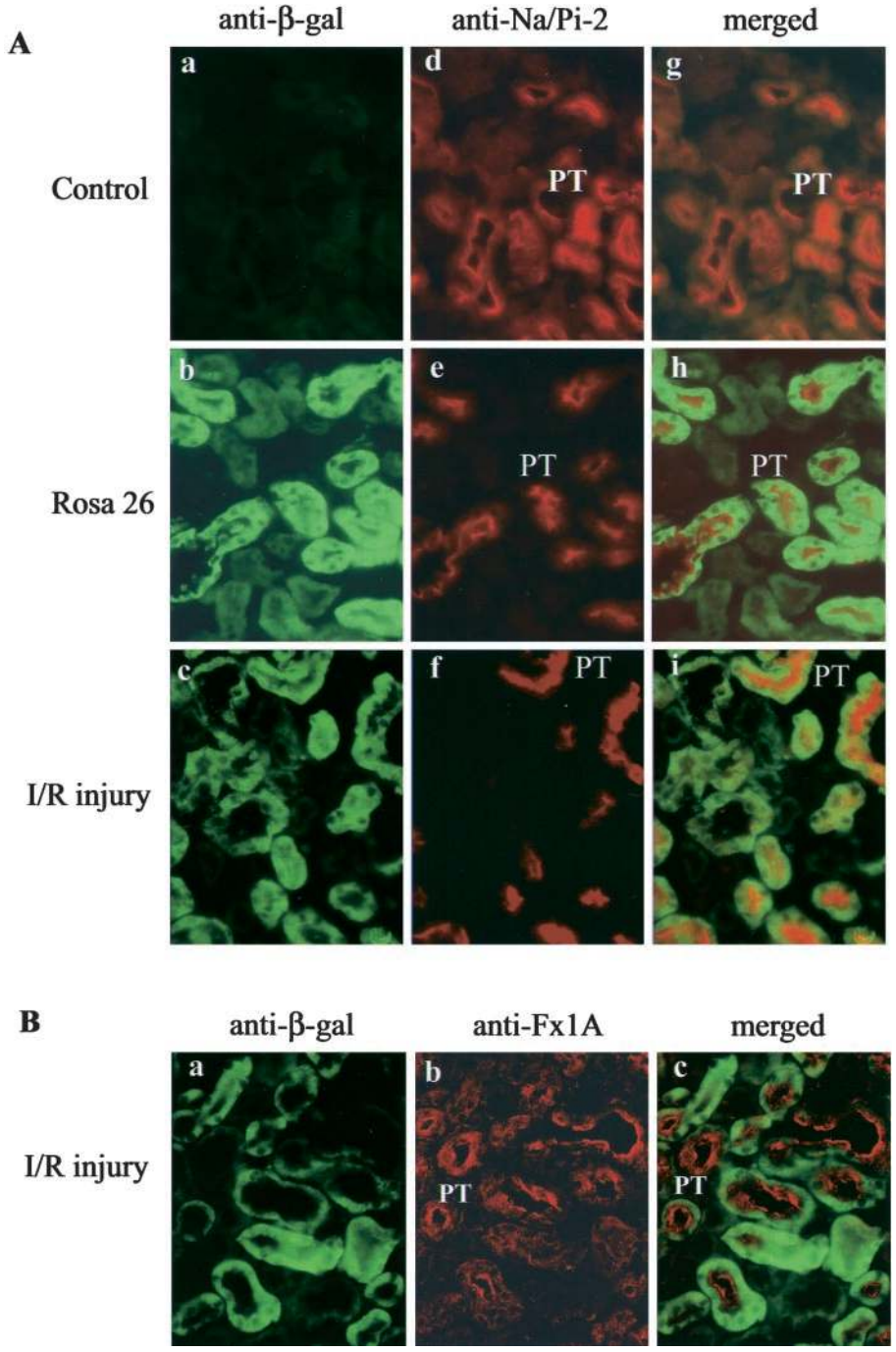

Figure 6. Co-staining of β-galactosidase with renal tubular markers in the kidneys. (A) Co-staining of kidneys with antibodies to β-galactosidase (green) and renal proximal tubular marker, Na/Pi-2 (red). Panels a through c show anti-β-galactosidase staining in kidneys of control mouse (a), Rosa26 mouse (b), and mouse with renal I/R injury and HSC transplantation (c). Panels d through f show staining with an antibody to Na/Pi-2 in kidneys of control mouse (d), Rosa26 mouse (e), and mouse with renal I/R injury and HSC transplantation (f). Panels g through i are merged images of the corresponding sections stained with antibodies to β-galactosidase and Na/Pi-2 in the kidneys of control mouse (g), Rosa26 mouse (h), and mouse with renal I/R injury and HSC transplantation (i). (B) Co-staining of kidneys of a mouse with renal I/R injury and HSC transplantation with antibodies to β-galactosidase and renal proximal tubular marker, Fx1A. Panel a, anti-β-galactosidase staining. Panel b, anti-Fx1A staining. Panel c, merged image of anti-β-galactosidase and anti-Fx1A staining. PT, proximal tubules. Original magnification, ×200.

segments. In regenerating kidneys from mice that did not receive HSC transplants, no specific signals were detected with anti-β-galactosidase staining, indicating that the immunostaining was specific (Figure 6A, panel a). In contrast, kidneys from Rosa26 control mice showed generalized cortical staining (Figure 6A, panel b). The kidneys of nontransgenic mice that were

injected with Rosa26-derived HSC showed areas containing β-galactosidase-positive cells, mostly in the outer stripe of outer medulla and the cortex (Figure 6A, panel c). Compared with the staining in the Rosa26 mouse kidney, a heterogeneous pattern of β-galactosidase staining was seen even within the same tubule, suggesting some of the tubular cells were derived from HSC of Rosa26 mice during regeneration. Panels d through f of Figure 6A show immunostaining of sodium/phosphate cotransporter type 2 (Na/Pi-2), which is located in the brush border of the renal proximal tubule. The merged image shows that most of the β-galactosidase–expressing cells also expressed Na/Pi-2 (Figure 6A, panels g through i). Similarly, co-staining with an antibody to Fx1A, a proximal tubule-specific brush border glycoprotein, showed β-galactosidase expression in Fx1A-positive proximal tubule cells (Figure 6B, panels a through c). About 80% of the proximal tubules contained some cells that were co-stained with antibodies to β-galactosidase and antibodies to Na/Pi-2 or Fx1A. These results suggest that during kidney regeneration, some of the cells in the kidney that are derived from β-galactosidase–expressing HSC have adopted a renal proximal tubular cell phenotype.

The cells of the distal nephron were also examined for the expression of β-galactosidase. Figure 7a shows that there was no co-localization of β-galactosidase and Tamm-Horsfall protein, which is expressed in the thick ascending limb of loop of Henle (TALH) and distal tubules. Likewise, there was no co-localization of β-galactosidase and NKCC2, a sodium/potassium/chloride co-transporter expressed in the TALH (Figure 7b). Figures 7c and 7d show that β-galactosidase was not expressed in collecting ducts that stained positive with DBA and AQP2.

Discussion

The results of this study demonstrate that transplanted HSC can contribute to renal tubular regeneration after I/R injury. Conventionally, it has been thought that injured tissues are repaired by proliferation of surviving parenchymal cells (12). An increasing body of evidence has revealed that somatic stem cells can be mobilized from one organ to a different organ where they can differentiate into the cells of the recipient organ and participate in structural and functional regeneration (30,31). In the kidney, the origin of the regenerating cells that appear after renal injury has not been clear. Currently, it is not known if the adult kidney contains pluripotent stem cells that

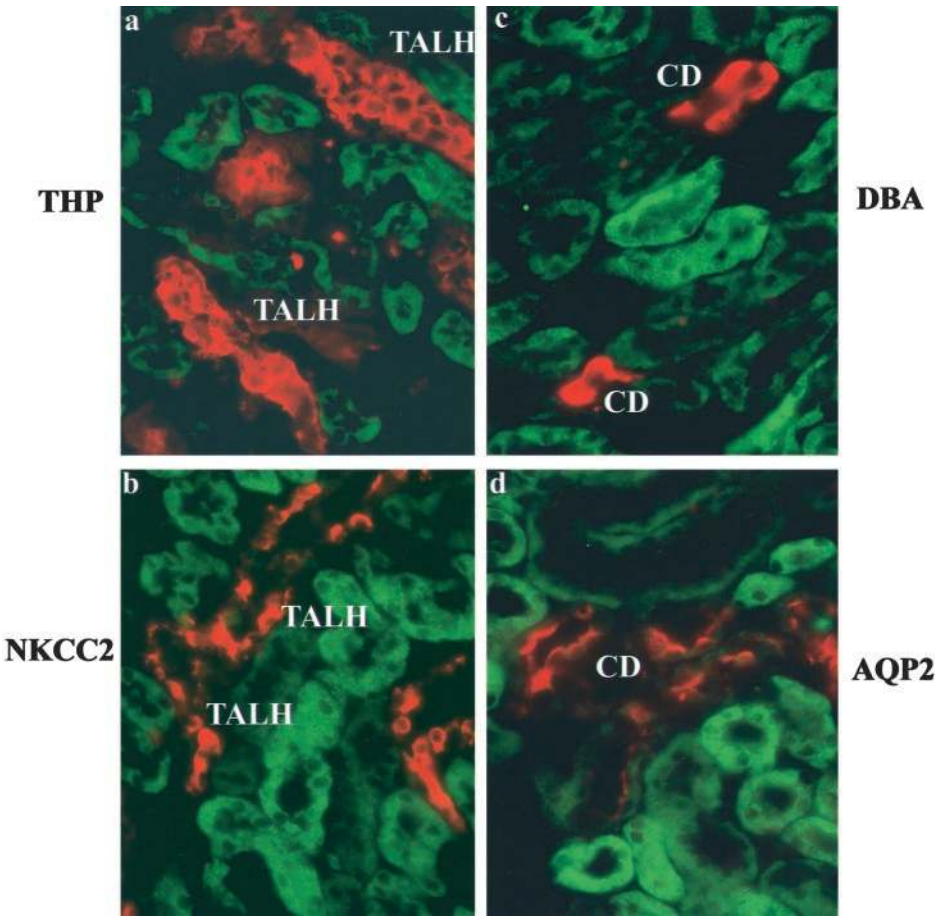

Figure 7. Co-staining of kidney with antibodies to β-galactosidase and distal nephron markers. Co-staining of the thick ascending limb of loop of Henle and distal tubules with antibodies to β-galactosidase (green) and THP (red) in panel a and NKCC2 (red) in panel b. Co-staining of the collecting duct with antibodies to β-galactosidase (green) and *Dolichos biflorus* agglutinin (DBA; red) in panel c, and aquapoin-2 (AQP2; red) in panel d. TALH, thick ascending limb; CD, collecting duct. Original magnification, ×200.

have the potential to produce renal epithelial cells (32). Murine HSC have been extensively characterized for the expression of cell surface markers and their function in hematopoiesis (1–3). The current study demonstrates that murine HSC can also integrate into regenerating kidneys after I/R injury. Transplanted HSC lose expression of the common leukocyte marker CD45.2 and gain expression of renal cell markers, indicating that HSC can differentiate into renal cells during regeneration. Adult bone marrow contains mesenchymal stem cells with multi-lineage potential (33); therefore, a highly purified fraction of hematopoietic bone marrow cells was used to reduce the possibility that bone marrow-derived mesenchymal cells were potential progenitor cells. It is also unlikely that the HSC-derived cells in the kidneys represent infiltrating inflammatory cells. Study of the kinetics of leukocytes in the kidneys sustaining severe I/R injury shows that inflammatory cell infiltration subsides within 10 d after I/R injury (13). Moreover, 4 wk after renal I/R injury, we did not observe an increased number of macrophages or CD45.2-positive hematopoietic cells in the recipient kidneys. We did not detect donor-derived cells in the renal glomeruli or large renal vessels with X-gal staining or immunostaining with anti-β-galactosidase antibody. In addition, β-galactosidase–positive cells did not stain positive for endothelial cell marker, vWF, suggesting no formation of endothelial cells from HSC in our mouse model.

Using whole bone marrow cell transplantation, Poulsom *et al.* (34) reported that bone marrow derived cells expressing epithelial cell markers were detected in histologically normal mouse kidneys. In male patients who received kidney transplant from female donors, 7.9% of the cortical epithelial cells in the injured kidneys are positive for Y chromosome and renal tubular cell markers. These results suggest that bone marrow cells contribute to renal parenchymal turnover and regeneration in mice and humans. Similarly, when kidneys from female donors were transplanted into male recipients, 1% of the kidney tubules contained Y chromosome–positive cells (35). In both studies, the exact source(s) of these male extrarenal-derived cells were not clear. Although bone marrow–derived cells have previously been identified in the kidney, the current study is the first report to show that a purified population of HSC can differentiate into renal tubular cells after I/R injury in the murine model. In our study, the percentage of Y chromosome–positive cells in the regenerating kidney was 8.3 $\pm$ 3.2%. Furthermore co-staining with antibodies to β-galactosidase and Na/Pi-2 or Fx1A showed that approximately 80% of the proximal tubules contained some cells that express both β-galactosidase and Na/Pi-2 or Fx1A. No β-galactosidase–positive cells were observed in the kidneys of mice that were transplanted from Rosa26 donors but that did not receive renal I/R injury. These results indicate that the integration of HSC-derived cells in the kidney is enhanced after I/R injury. Interestingly, we found that a small number of cells (3 to 5 cells per section) in the contralateral kidney were stained positive with X-Gal staining, suggesting that systemic factors may facilitate recruitment and differentiation of HSC in the kidney.

Differentiation of HSC to nonhematopoietic cells does not occur in every animal model. A recent report by Weissman and colleagues (36) showed very limited developmental plasticity of HSC in previously healthy mice. After irradiation, the mice were given primitive HSC isolated from GFP mice. Few GFP^+ non-hematopoietic cells were detected in the brain and liver and no HSC-derived cells were observed in the kidney, gut, skeletal muscle, heart, and lung. Except irradiation, no specific organ injury was induced in this study, which might explain why few HSC-derived cells were observed in the recipient organs.

The term "transdifferentiation" has been suggested to describe the phenotypic conversion of pluripotent somatic stem cells of one tissue type to another tissue type (12). One explanation for this phenomenon is genetic reprogramming of the stem cells once they are introduced into a new environment (12). Upon instruction by environmental signals, pluripotent stem cells exhibit developmental plasticity and transdifferentiate into alternative cell types. Two recent studies challenging this view have suggested that under selection pressure bone marrow cells or neural stem cells can fuse with embryonic stem cells (ES cells) in cultures. Terada *et al.* (37) showed that bone marrow cells could fuse with ES cells, adopt an ES cell phenotype. In this *in vitro* study mixed bone marrow cells were used, and the frequency of fusion was 2 to 11 per 10^6 bone marrow cells. The Sca^+Lin^- fraction of bone marrow cells did not increase the frequency of fusion, suggesting that HSC were unlikely to be involved in the fusion event. Similarly, neural stem cells fused with ES cells and subsequently differentiated to many cell types. The frequency of fusion was in the range of 10^{-4} to 10^{-5} per brain cell (38). Fusion is unlikely to explain our results because the number of HSC-derived cells greatly exceeds the reported frequency of spontaneous cell fusion. In addition, fusion of HSC with adult somatic cells has not been shown *in vivo* during normal development or recovery from injury. Although the concept of transdifferentiation has been challenged, transplantation of highly purified HSC into tyrosinemic mice resulted in the cure of the metabolic liver disease, demonstrating the therapeutic potential of HSC (7).

After renal I/R injury and transplantation of exogenous HSC, most of the HSC-derived cells were observed in renal proximal tubules. By X-Gal staining and immunohistochemistry study, the outer stripe of outer medulla was the most extensive site of HSC-derived cells. This result is consistent with the sensitivity of S3 segments of the proximal tubules to I/R injury. In the cortex, a few β-galactosidase–positive cells were negative for the proximal tubular marker, Na/Pi-2. This absence of Na/Pi-2 staining likely reflects the weak expression of Na/Pi-2 in the proximal convoluted tubules of superficial nephrons (39). No β-galactosidase–positive, HSC-derived cells were observed in the distal nephron or collecting ducts, which are less susceptible to ischemic injury. The expression of a proximal tubular transporter in the newly formed tubules suggests that HSC-derived tubular cells may mediate ion transport. Further studies will be required to determine whether transplanted HSC can accelerate functional recovery after bilateral ischemic renal injury. Because HSC are more readily available compared with other somatic stem cells they can potentially be utilized as a source for cell replacement therapy.

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