Proliferation of Bone Marrow-Derived Cells Contributes to Regeneration after Folic Acid-Induced Acute Tubular Injury

Te-Chao Fang,†‡ Malcolm R. Alison,†‡ H. Terence Cook,§ Rosemary Jeffery,† Nicholas A. Wright,† and Richard Poulsom†‡

†Histopathology Unit, Cancer Research UK, London, United Kingdom; ‡Institute of Cell and Molecular Science, Queen Mary’s School of Medicine and Dentistry, University of London, United Kingdom; §Division of Nephrology, Tzu Chi General Hospital, Hualien, Taiwan; and §Division of Investigative Science, Imperial College, University of London, London, United Kingdom

Studies of tissue from recipients of bone marrow transplantation or organ allograft suggest that bone marrow-derived cells (BMDC) may differentiate into a variety of nonhematologic tissues, including renal tubular epithelium. The aims of this study were to examine whether BMDC contribute to recovery after acute renal injury and to assess the effects of cytokine mobilization on regeneration. Female mice (6 wk old) were lethally irradiated and transplanted with male bone marrow (BM) cells and later assigned into control, folic acid–treatment, and folic acid–treatment with granulocyte-colony stimulating factor (G-CSF), and control with G-CSF. Tritiated thymidine was given 1 h before death. Kidney sections were stained for a tubular epithelial marker, Y chromosome (in situ hybridization), periodic acid-Schiff staining, and subjected to autoradiography. Renal tubular epithelial cells in S-phase were scored as female (indigenous) or male (BM-derived). This is the first report to show that BMDC can respond by engrafting the renal tubules and undergo DNA synthesis after acute renal injury. BMDC contributed to the renal tubular epithelial cell population, although most (90%) renal tubular regeneration came from female indigenous cells. Some evidence was found for cell fusion between indigenous renal tubular cells and BMDC, but this was infrequent and the significance and consequences of cell fusion in the kidney are unresolved. G-CSF treatment nearly doubled the frequency of thymidine-labeled BM-derived tubular cells and might facilitate the recovery of renal tubular epithelium.


Acute tubular necrosis (ATN), resulting from prolonged renal hypo-perfusion and renal ischemia or nephrotoxic substances, is a common form of acute renal failure (ARF). Pathophysiologically, ATN is associated with tubular cell death and shedding into the tubular lumen, resulting in tubular blockage, further reducing glomerular filtration. Despite major advances in intensive care and renal replacement therapy, no specific treatment is currently available. Consequently, the overall mortality rate of patients with ARF is still high, about 50% in a recent series (1–3), and has changed little over the past 30 yr. Therefore, a more powerful therapeutic intervention for ARF to decrease mortality is imperative.

Traditionally, stem cells were believed to be lineage-restricted and organ-specific. However, a growing number of studies based on bone marrow transplant (BMT) protocols have claimed adult bone marrow (BM) cells can differentiate into a variety of nonhematologic tissues in rodents and humans (4–7). Some observers have questioned stem cell plasticity and find it is really the result of the fusion of BM cells with differentiated cells in the engrafted organ; this includes hepatocytes (8–10), Purkinje cells (10,11), cardiomyocytes (10) and skeletal muscle cells (12,13).

In the kidney, several studies have demonstrated that BM-derived cells (BMDC) play a role in normal turnover and remodeling of renal cells, including renal vessels (14–18) and interstitial myofibroblastic cells (18), glomerular mesangium (19–21), podocytes (22) and tubular epithelium (22–26).

Previously we (22) demonstrated that BMDC contribute to both normal turnover of renal epithelium in mice where the level of engraftment in renal tubular cells was 3 to 8%, and regeneration after damage in humans where the level of engraftment in renal tubular cells was 1.8 to 20%. However, Krause et al. (27) found no donor-derived renal tubule epithelial cells in any of five mice transplanted with a single highly-selected hematopoietic stem cells, perhaps because of the use of a sorted hematopoietic stem cells rather than whole BM. Gupta et al. (23,24) reported that 1% of tubules had male epithelial cells in two male patients with female kidney allografts and ATN; no tubular engraftment was noted in two cases without ATN. The potential of stem cell therapy for ARF was apparent from two recent studies in mice, showing that sorted BMDC can contribute to functional regeneration of renal proximal tubules after ischemia/reperfusion (I/R) injury and BM ablation with radiation (26,28).
The adult tubular epithelium has the potential to regenerate after damage. During ATN, normally quiescent cells undergo morphologic dedifferentiation and are able to re-enter the cell cycle (29,30). Folic acid (FA) has been recognized as an essential nutrient in humans; however, a high dose of FA causes acute toxicity in some strains of mice, inducing ATN (31). We now show that BMDC contribute to the regeneration process after FA injury.

**Materials and Methods**

**Experimental Animals and Protocols**

The procedures for the animal experiments were carried out under British Home Office procedural and ethical guidelines. Six-week-old female recipient mice (FVB/N) underwent whole-body gamma irradiation with 12 Gray in a divided dose 3 h apart to ablate their bone marrow, followed immediately by tail vein injection of male whole BM cells (10^7 cells). Mice were housed in sterile conditions for 6 wk, then assigned to one of 4 groups (n = 18 in each group, see Figure 1): group CON mice received an intraperitoneal injection of only vehicle (0.2 ml sterile 150 mM NaHCO3 (S-6014; Sigma)); group FA mice received an intraperitoneal injection of FA (250 mg/kg body wt, F-7876; Sigma) in vehicle; group FA-GCSF mice were treated with FA (250 mg/kg body wt, intraperitoneal) in vehicle and also received G-CSF 250 μg/kg per d (0530-E08; Amgen), once per day for up to 5 d subcutaneously; group GCSF mice were treated with vehicle then G-CSF 250 μg/kg per d once a day for up to 5 d subcutaneously. The day of FA administration was designated as FA day 0. Mice were sacrificed either before FA administration (day 0), or FA day 3 or day 7, with 6 mice killed by overdose of pentobarbitone (Sagatal; Rhône Mérieux, 200 mg/kg, intraperitoneally), at each time point. Tritiated thymidine (3HTdR) (TRK120, American Biosciences) at a dose of 1 μCi/g body weight (intraperitoneally) was injected 1 h before the mice were killed. Terminal blood samples (0.7 ml) were taken by cardiac puncture into an EDTA-tube. Kidneys were removed, bisected down the longitudinal axis and fixed in neutral buffered formalin before being embedded in paraffin wax for later histology.

**Serum Urea Nitrogen Measurements**

Blood samples were centrifuged at 5000 × g for 10 min at 4°C and the supernatant stored at −70°C for later determination of serum urea nitrogen (SUN) using an Olympus AU600 autoanalyzer (Olympus Diagnostics, London, UK) (32). The level of SUN was expressed in milligrams per 100 ml.

**Histology and Semiquantification of Morphometric Analysis**

For histologic examination of renal tissue, 4 μm sections were stained with periodic acid-Schiff (PAS). Tubular injury was assessed at 400× overall magnification using 20 consecutive and nonoverlapping fields (10 in cortex and 10 in medulla) of PAS-stained specimens. Tubular damage, defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, necrotic tubules, and desquamation, was scored semiquantitatively. Tubular injury was categorized into one of five scores using the following criteria: 0, normal; 1, 2, 3 and 4 based upon the report by Nangaku et al. (33). The mean tubular injury scores (TIS) for each mouse represented the average score of the 20 fields examined.

**Combination of Histochemical or Immunohistochemical Staining, In Situ Hybridization, PAS, and Subsequent Autoradiography**

To evaluate whether BMDC proliferate after injury, sections were sequentially stained for an epithelial marker (lectin staining or aquaporin 2 [AQP2] by an immunohistochemical method) to identify cells with specific renal tubular cell phenotypes, followed by Y chromosome in situ hybridization (ISH) to identify BMDC, then subjected to autoradiography (3HTdR) to identify cells in S-phase. Two lectins (Phaseolus vulgaris leucoagglutinin [PHA-L] and peanut agglutinin [PNA]) and one antibody (AQP2) were used. PHA-L binds to proximal convoluted tubules, PNA binds to the distal convoluted tubule and the β-type intercalated cells of collecting tubules (34), while AQP2 expression is confined to the principal cells of the collecting tubules of the kidney (35).

**Treatment of Sections**

Four-micrometer sections were dewaxed and incubated with 3% hydrogen peroxide in methanol, then rehydrated through a descending series of ethanol in water (100–95–70%) to 37°C for 15 min, then washed in PBS. All sections were incubated for 5 min in 20% acetic acid in methanol to block endogenous alkaline phosphatase.

**Histochemical and Immunohistochemical Methods**

For detection of lectin binding sites, slides were incubated with the biotinylated lectin for 45 min at room temperature at the following dilutions in PBS: biotinylated PHA-L 1/100 (B-1115, Vector Laboratories) or biotinylated PNA 1/800 (B-1075, Vector Laboratories). Sections

**Figure 1.** A schematic outline of the experimental procedures. BMT, bone marrow (BM) transplantation; FA, folic acid; G-CSF, granulocyte-colony stimulating factor.
were then washed in PBS and incubated with streptavidin-alkaline phosphatase (D0396; DAKO) for 30 min at room temperature. Following washes in PBS, Vector Red Substrate (SK 5100; Vector Laboratories) was applied for 8 min at room temperature and sections were again washed in PBS before the ISH protocol.

For detection of AQP2, slides were preincubated in normal swine serum (X0901; DAKO) at 1/25 for 10 min. Rabbit anti-mouse AQP2 (550649; BD Biosciences) was applied to sections at a dilution of 1/500 for 35 min at room temperature. The secondary layer was biotinylated swine anti-rabbit (EU353; DAKO) diluted to 1/500, for 35 min at room temperature. The third layer was streptavidin-alkaline phosphatase (D0396; DAKO) at 1/50 for 30 min at room temperature. Following washes in PBS, Vector Red Substrate was applied for 8 min at room temperature and sections were again washed in PBS before the ISH protocol.

In Situ Hybridization
Sections were incubated in 1 M sodium thiocyanate (S7757; Sigma) for 10 min at 80°C to improve access of probe to DNA, washed in PBS, and then digested in 0.4% w/v pepsin (P6887; Sigma) in 0.1 M HCl for 10 min at 37°C to further improve access. The probe was quenched in 0.2% glycine (G4392; Sigma) in double concentration PBS and sections were then rinsed in PBS, postfixed in 4% paraformaldehyde (P6148; Sigma) in PBS, dehydrated through graded ethanol, and air dried. A FITC-labeled Y chromosome paint (1189-YMF-01; Cambio) was used in the supplier’s hybridization mix. The probe mixture was added to the sections, sealed under glass with rubber cement, heated to 60°C for 10 min, and then incubated overnight at 37°C. The next day, all slides were washed in 50% formamide (284226P; BDH Laboratory Supplies)/2x SSC at 37°C, then washed with 2x SSC, and then incubated in 4x SSC/0.05% Tween-20 for 10 min at 37°C. To visualize the Y signals, slides were washed with PBS and incubated with 1/250 peroxidase-conjugated antifluorescein antibody (1426346; Boehringer Mannheim) for 60 min at room temperature before being developed in DAB (D5637; Sigma) plus 0.3% hydrogen peroxide, washed with PBS and subjected to periodic acid-Schiff staining.

Autoradiography for 3HTdR
After PAS staining, slides were dehydrated through graded ethanol and air-dried before dipping in ILFORD nuclear track emulsion (L4; ILFORD) (diluted 3:5 with Q water) at 45°C in a dark room using a safe light (902 filter). When dry, slides were stored in the dark at 4°C. After a standard exposure of 1 wk, slides were developed for 4 min at 20°C in Kodak D19 (Cat No. 1464593; Eastman Kodak Company), stopped in 1% acetic acid (10001CU; BDH Laboratory Supplies) for 30 s, washed in tap water for 30 s, and fixed in 30% w/v sodium thiosulfate (S-8503; Sigma) for 8 min. Slides were then rinsed in gently running, cold tap water for 60 min to ensure that all traces of sodium thiosulfate were removed. Finally, sections were counterstained lightly with hematoxylin, dehydrated, cleared and mounted in DePeX (36125-2B; BDH).

Determination of the 3H-Labeling Index (3H-LI) of Renal Tubular Epithelial Cells, Both of Indigenous Origin and BM-Derived Tubular Cells
To estimate the separate contributions of indigenous renal tubular cells (recognized as female) and BM-derived tubular cells (BMDTC) (recognized as male) to regeneration one thousand consecutively observed renal tubular epithelial cells per mouse were scored using a light- and dark-field microscope (Nikon Eclipse ME600). Each nucleus that was Y chromosome-positive and had more than 5 overlying auto-radiographic silver grains was considered to be a BMDTC in DNA synthesis if also positive for a specific epithelial marker.

Laser Scanning Confocal Microscopy and Direct Fluorescence In Situ Hybridization for X- and Y-Chromosomes to Look for Evidence of Cell Fusion
To examine the possibility that cell fusion caused the apparent plasticity of BMDTC, a combination of Cy3-labeled mouse chromosome X paint (1200-XMCy3; Cambio) and FITC-labeled mouse chromosome Y paint (1189-YMF-01; Cambio) was used together for fluorescence in situ hybridization (FISH) to score the number of X and Y chromosomes in renal tubular cells.

Sections were treated as described for indirect FISH except that the probe mixture contained both Cy3-labeled mouse chromosome X paint and FITC-labeled mouse chromosome Y paint and heated to 80°C for 10 min before overnight incubation. After washing sections were cover-slipped with Vectashield Hard Set mounting medium with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) (H-1500; Vector Laboratories). The processed sections were observed under a confocal laser scanning microscope (Zeiss Laser Scanning Microscope LSM 510, Oberkochen, Germany). Signals from the Y chromosome paint were excited at 488 nm and detected with a 505 to 530 nm band pass filter, and colored green by the software package. Signals from the X chromosome were excited at 543 nm, detected above 585 nm, and colored red. To help visualize the renal tubular epithelium, autofluorescence was excited at 633 nm and emitted light collected above 660 nm, represented in blue; a variety of structural elements autofluoresced, with the cell nuclei usually remaining dark. While setting up the microscope, Y-FISH-positive cells could easily be found by direct observation among renal tubular epithelial cells, by their morphology, at medium power magnification (10 × 63). Several of these Y-FISH-positive cells were then scanned at a higher magnification (10 × 63 × 3) with a scan dimension of 1024 × 1024 pixels on average 8 times for each of 10 to 20 “optical sections” of 0.4 μm in the Z axis, and archived using the Zeiss LSM 510 software package. The sequential images were captured and presented as Z-projections, then exported as either QuicktimeTM movie files (see supplementary material, available online at www.asn-online.org) or TIFF images of single planes.

Statistical Analyses
SUN, 3H-LI (%), and TIS were analyzed by two-way ANOVA for repeated measures (the first factor being treatment group and the second factor being time period) for comparisons between groups. When a significant effect was detected by ANOVA, the Newman-Keuls test was used to establish which differences between means reached statistical significance (P < 0.05). The results are presented as mean ± SEM.

Results
Renal Histologic Examination, TIS, and SUN
Figure 2 shows examples of damage and regenerative changes induced in the kidney by FA administration. These changes were apparent 3 d after administration and were still present at 7 d. Changes in TIS and SUN are shown in Table 1. Neither vehicle alone nor G-CSF alone affected these parameters. FA treatment significantly increased TIS, and this coincided with a 4- to 5-fold increase in SUN. Interestingly, G-CSF treatment significantly lowered the TIS after FA and likewise the SUN.
Proportion of Y Chromosome–Positive Cells within the PHA-L–Stained Cell Population

No significant differences in the proportion of Y chromosome–positive PHA-L–stained cells were present at time zero among the four groups (Figure 3A). A significant increase in Y-positive cells was seen after FA administration, and concomitant G-CSF caused a further small but significant increase at both time points.

\[ ^{3} \text{H-LI of Proximal Tubular Cells (PHA-L Stained)} \]

Changes in the \(^{3}\text{H-LI of PHA-L–stained cells are shown in Figure 3B. Examples of }^{3}\text{H-thymidine labeling of PHA-L–stained cells are illustrated in Figure 3C to 3H. After FA, the overall LI increased sharply and was still above control values at day 7 (Figure 3B). The bulk of this S-phase labeling was observed in tubular cells of indigenous origin, rather than in BMDC (Figure 3B).} \]

Proportion of Y Chromosome–Positive Cells within the PNA-Stained Cell Population

There were no significant differences in the percentage of Y chromosome–positive PNA-stained cells in the control period among the four groups (Figure 4A). A significant increase was observed at day 3 after FA, further augmented by concurrent G-CSF treatment. These levels of engraftment were unchanged at day 7.

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Table 1. Effects of administration of FA alone and in combination with G-CSF on renal tubular injury score and serum urea nitrogen in female mice with male BMT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Pre-FA</th>
<th>Post-FA and G-CSF</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular injury score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group CON</td>
<td>6</td>
<td>0.12 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Group FA</td>
<td>6</td>
<td>0.11 ± 0.02</td>
<td>2.69 ± 0.04</td>
<td>1.38 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Group FA-GCSF</td>
<td>6</td>
<td>0.09 ± 0.02</td>
<td>2.34 ± 0.04</td>
<td>1.18 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Group GCSF</td>
<td>6</td>
<td>0.11 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Serum urea nitrogen (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group CON</td>
<td>6</td>
<td>15.0 ± 1.5</td>
<td>15.2 ± 1.4</td>
<td>16.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Group FA</td>
<td>6</td>
<td>16.7 ± 1.3</td>
<td>76.8 ± 2.8</td>
<td>37.2 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Group FA-GCSF</td>
<td>6</td>
<td>15.5 ± 1.2</td>
<td>60.7 ± 1.3</td>
<td>27.5 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Group GCSF</td>
<td>6</td>
<td>16.3 ± 1.1</td>
<td>16.2 ± 2.1</td>
<td>16.7 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

\( ^{a} \text{Values are means} ± \text{ SEM. FA, folic acid; G-CSF, granulocyte colony-stimulating factor; BMT, bone marrow transplant.} \)

\( ^{b} \text{P} < 0.05 \text{ versus the same group at pre-FA period.} \)

\( ^{c} \text{P} < 0.05 \text{ versus group CON at the corresponding time point.} \)

\( ^{d} \text{P} < 0.05 \text{ versus group GCSF at the corresponding time point.} \)

\( ^{e} \text{P} < 0.05 \text{ versus group FA at the corresponding time points.} \)
Figure 3. (A) Changes in the proportion of Y chromosome–positive cells within the PHA-L–stained cell population in control mice and FA-treated mice with or without G-CSF treatment (n = 6 per treatment time point). *P < 0.05 versus the same group at day 0; †P < 0.05 versus group CON at the corresponding time point; ‡P < 0.05 versus group GCSF at the corresponding time point; §P < 0.05 versus group FA at the corresponding time point. (B) Changes in the 3H-LI of PHA-L–stained proximal tubular cells of: combined indigenous and BM origin (left panel); indigenous origin (center panel); BM origin (right panel). n = 6 per treatment time point. *P < 0.05 versus the same group at day 0; †P < 0.05 versus group CON at the corresponding time point; ‡P < 0.05 versus group GCSF at the corresponding time point; §P < 0.05 versus group FA at the corresponding time point. (C to H) 3H-thymidine labeling of PHA-L–stained cells in FA-treated mice with G-CSF at day 3. (C) Black arrows indicate PHA-L–stained (red) BMDTC (brown Y-chromosome) that are also labeled with 3H-thymidine (silver grains) under bright-field illumination. Asterisks (*) indicate PHA-L–stained BMDTC not labeled with 3H-thymidine. (D) Same section as C under dark-field illumination; white arrows point out silver grains from 3H-thymidine in PHA-L–stained cells. (E) The images in C and D were combined to show silver grains over PHA-L–stained cells that are BMDTC. F, G, and H are two-fold magnifications of the boxed area in C. Original magnifications ×500.
3H-LI of Combined Renal Distal Convoluted Tubular Cells and the β-Intercalated Cells of the Collecting Duct

Figure 4B illustrates the changes in LI, and an example of thymidine labeling of a PNA-stained BMDTC is shown in Figure 4C to 4E. As seen for PHA-L–stained cells, the LI of PNA-stained cells had increased significantly at day 3 after FA and was still above control values at day 7. Again, most of the labeled cells were of indigenous rather than of BM origin (Figure 4B).

Proportion of Y Chromosome–Positive Cells within the AQP2 Immunopositive Population

The proportion of Y chromosome–positive AQP2-expressing cells showed no significant difference among the groups at the various time points, the levels varied from 0.58 ± 0.11% to 0.76 ± 0.12%.

3H-LI of Principal Cells of the Collecting Duct

There were no changes in 3H-LI of AQP2 stained cells in any of the experimental groups, the overall 3H-LI of AQP2 stained cells of indigenous origin varying from 0.13 ± 0.07% to 0.20 ± 0.08% and for BMDTC varying from 0.017 ± 0.017% to 0.033 ± 0.021%.

Cell Fusion between BMDC and Renal Tubular Cells

Table 2 shows the distribution of karyotype patterns of Y chromosome–positive tubular cells in control (female mice after
male BMT) and FA-treated mice with or without G-CSF. Normal female cells were recognized as XX and cells were scored as fusion events if their nucleus contained XXY (possible cell fusion, see Figure 5) or XXY (definite cell fusion). Among Y chromosome-positive tubular cells, some XXY cells were found, suggesting cell fusion may be occurring.

**Discussion**

This study has demonstrated that BMDC can differentiate into renal tubular cells and have the ability to proliferate, not only in the normal wear-and-tear turnover of renal tubular cells but also after acute tubular injury. However, in our study BMDC play a relatively small role (10% of S-phase cells) in the regeneration of renal tubular epithelial cells after acute renal injury, with most (90%) renal tubular regeneration coming from indigenous tubular cells. The abundance of BMDTC was increased after FA administration and was further enhanced when G-CSF was combined with FA. We note that G-CSF nearly doubled the $^3$H-LI of BMDTC and consider that this mobilizing treatment might accelerate the recovery of renal tubular epithelium after FA because the TIS and levels of SUN were lower in the combined FA with G-CSF group than those with FA treatment alone. G-CSF had no statistically significant effect on the $^3$H-LI of indigenous tubular cells. Cell fusion between BMDC and renal tubular epithelium may occur, although we were unable to detect any cells with the XXY complement predicted for fusion. The significance of fusion in this model remains to be determined.

Our previous studies (22) and those of others (23–26,36) have found variable levels of BM cell engraftment in the kidney after sex-mismatched renal allografts or BMT. In this study we combined use of phenotypic markers with autoradiography to show specific cell types in S-phase, and this study is the first to show that tubule cells of BM origin can respond appropriately to damage by cell proliferation. An earlier study from Iwano et al. (37) reported that 27% of proliferating renal interstitial fibroblasts were derived from BM after unilateral ureteric obstruction, although this value was based on the use of proliferating cell nuclear antigen (PCNA) as a marker to indicate cell proliferation. As PCNA is not a phase-specific marker of cell proliferation (38,39) and has a half-life in excess of 20 h, PCNA may be detected in noncycling cells, e.g., those in G1 phase as well as those undergoing DNA repair.

Under normal wear-and-tear renewal, i.e., at day 0, the $^3$H-LI of tubular cells was around 0.5% and approximately 1 in 20 of these $^3$H-labeled–cells were of BM origin. Three days after injury, cells of BM origin accounted for 10 and 11% of $^3$H-labeled–cells in the proximal tubule and distal nephron, respectively. Thus, approximately 90% of the regeneration of tubular cells came from the indigenous renal tubular cells. This is compatible with surviving epithelial cells migrating to cover the exposed areas of the basement membrane, with cell de-differentiation and proliferation to restore cell numbers, followed by differentiation (40). The migration onto denuded tubular basement membrane and de-differentiation of renal epithelial cells has been described in vitro (41) and in vivo (42).

There are several hypothetical and incomplete mechanisms to explain the abundance of BM-derived renal tubular epithelial cells after induction of ATN. First is that some of the BM cells that engraft the kidney soon after transplantation could function as renal stem cells, but this seems unlikely because no patches of BMDTC were seen. A second is that there is cell fusion between a resident tubular cell and a BM cell, either near the time of transplantation or subsequently after damage. A third is that individual BM cells arrive in the kidney near the time of transplantation, transdifferentiate appropriately, and then are able to proliferate during both the period of relatively normal wear-and-tear and after tubular injury. However, this can not explain why the proportion of BMDC increases after FA damage; another factor seems necessary, such as the mobilization of additional cells from the transplanted BM in response to renal injury.

The ability of renal ischemia and reperfusion injury to mobilize cells from BM was shown clearly by Kale and colleagues (26), and in our study we used G-CSF treatments to enhance mobilization; after this we detected an increase in BMDTC and a small improvement in SUN. In contrast, a recent study reported that hematopoietic stem cell mobilization via G-CSF had severely adverse effects (43) after preconditioning with G-CSF for 5 d, but not when G-CSF was given for 3 d. However, we gave G-CSF after inducing injury, not before, and, of course, we used a different model in which 3 d of G-CSF treatment nearly doubled (1.8-fold) the $^3$H-LI of renal tubular cells from BM. Whether the same renotrophic effect of G-CSF would occur in humans is unknown.

At this time, no study has been able to confirm or exclude cell

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**Table 2. Distribution of karyotype pattern of Y chromosome-positive tubular cells in control mice (female mice after male BMT) and FA-treated mice at 3 days with or without G-CSF**

<table>
<thead>
<tr>
<th>Karyotype Pattern</th>
<th>CON</th>
<th>FA</th>
<th>FA-GCSF</th>
<th>GCSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>XY</td>
<td>13</td>
<td>18</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>XXY</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>XXXY</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% with XXY or XXXY</td>
<td>6.7</td>
<td>9.5</td>
<td>20</td>
<td>6.3</td>
</tr>
</tbody>
</table>

*aAt least 15 Y chromosome-positive tubular cells from each group were examined using confocal laser-scanning microscopy.*
fusion as playing a role in the apparent differentiation of BMDC to renal tubular cells. Cell fusion between a marked donor cell and a resident cell could mimic what appears to be adult stem cell plasticity. Numerous studies report that cell fusion is not a major player in the transdifferentiation of BMDC into various specific cell types (6,44–46), including mesangial cells of the glomerulus; these may be generated from cloned single hematopoietic stem cells without cell fusion (44). The major shortcoming in histologic studies of fusion is that most are performed on paraffin wax-embedded tissue sections of finite thickness (4 to 6 μm). The Y chromosome is usually located at the nuclear periphery, but is only detected in 50 to 80% of male cells, dependent on the thickness of sections, proteinase digestion times, etc. Therefore, the likelihood of “missing” the extra chromosomes present in a fusion cell is very real. In this study, we found no conclusive evidence of cell fusion because the XXXY karyotype in BMDTC was not seen, although the XXY karyotype was seen, suggestive of fusion.

In conclusion, we have confirmed the existence of renal tubular cells of appropriate morphology and phenotype that bear a marker of transplanted BM origin, and additionally have shown their ability to proliferate not only during normal wear-

Figure 5. Scanning confocal fluorescence imaging of female mouse kidney after male BMT at day 3 after FA and G-CSF administration. Fluorescence in situ hybridization using a FITC-labeled Y-chromosome paint (green) and Cy3-labeled X-chromosome paint (red), with autofluorescence (blue) that allows some structure including tubular epithelial cytoplasm to be seen. The upper row shows 3 frames of a 3D panorama (see supplementary material, available online at www.asn-online.org), generated from a z-series (original magnifications approximately ×500). The middle row shows 2.8-fold magnifications of the regions marked by yellow boxes. The lower series repeats the middle row but identifies a cell within a renal tubule that contains 2 X and 1 Y chromosomes clearly within its nucleus (dashed circular line), demonstrating possible cell fusion. Vertical columns show 0°, approximately 15°, and approximately 170° rotations of the panorama.
and-tear renewal of renal tubular cells but also in response to acute tubular injury when approximately 10% of the proliferating tubular cells are BM-derived. The recovery of renal tubular epithelium after FA-induced acute renal injury was assisted by treatments with G-CSF, which nearly doubled the incidence of BM-derived tubule epithelial cells in S-phase and reduced the SUN levels.

Acknowledgments

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