

# Isolation and Characterization of Kidney-Derived Stem Cells

Sandeep Gupta,<sup>\*†</sup> Catherine Verfaillie,<sup>\*‡</sup> David Chmielewski,<sup>\*</sup> Stefan Kren,<sup>\*</sup> Keith Eidman,<sup>\*</sup> Jeffrey Connaire,<sup>\*</sup> Yves Heremans,<sup>‡</sup> Troy Lund,<sup>‡</sup> Mark Blackstad,<sup>‡</sup> Yuehua Jiang,<sup>‡</sup> Aernout Luttun,<sup>‡</sup> and Mark E. Rosenberg<sup>\*</sup>

<sup>\*</sup>Department of Medicine and <sup>‡</sup>Stem Cell Institute, University of Minnesota, and <sup>†</sup>Veterans Affairs Medical Center, Minneapolis, Minnesota

Acute kidney injury is followed by regeneration of damaged renal tubular epithelial cells. The purpose of this study was to test the hypothesis that renal stem cells exist in the adult kidney and participate in the repair process. A unique population of cells that behave in a manner that is consistent with a renal stem cell were isolated from rat kidneys and were termed multipotent renal progenitor cells (MRPC). Features of these cells include spindle-shaped morphology; self-renewal for >200 population doublings without evidence for senescence; normal karyotype and DNA analysis; and expression of vimentin, CD90 (thy1.1), *Pax-2*, and *Oct4* but not cytokeratin, MHC class I or II, or other markers of more differentiated cells. MRPC exhibit plasticity that is demonstrated by the ability of the cells to be induced to express endothelial, hepatocyte, and neural markers by reverse transcriptase-PCR and immunohistochemistry. The cells can differentiate into renal tubules when injected under the capsule of an uninjured kidney or intra-arterially after renal ischemia-reperfusion injury. *Oct4* expression was seen in some tubular cells in the adult kidney, suggesting these cells may be candidate renal stem cells. It is proposed that MRPC participate in the regenerative response of the kidney to acute injury.

*J Am Soc Nephrol* 17: 3028–3040, 2006. doi: 10.1681/ASN.2006030275

Toxic and ischemic insults to the kidney lead to acute renal failure, most often manifest as acute tubular necrosis. After injury, the kidney undergoes a regenerative response that leads to recovery of renal function. New cells are required to replace damaged cells. Three possible sources of new tubular cells are adjacent, less damaged tubular cells; extrarenal cells presumably of bone marrow origin that home to the injured kidney; or resident renal stem cells.

There is evidence to support a role for less injured tubular cells. Recapitulating developmental paradigms, these cells dedifferentiate, proliferate, and eventually reline denuded tubules, restoring the structural and functional integrity of the kidney (1–5). Molecular events that define this renal regeneration have been characterized, and strategies to accelerate the repair process have been tested in both experimental models and in humans. Recent studies have demonstrated that the contribution of extrarenal cells to the regenerative renal response is minimal to none (6–11).

Tissue-specific stem cells have been found in many organs, including bone marrow, gastrointestinal mucosa, liver, brain, prostate, and skin (12–16). These cells participate in the normal cell turnover of these organs and are a potential source of cells after organ injury. In regard to the kidney, stem cells exist in the metanephric mesenchyme and can give rise to all of the cell

types of the adult kidney, except those that are derived from ureteric bud (17,18). Renal stem cells persist in the adult kidneys of other organisms, such as the skate and the fresh water teleost. These cells can participate in new nephron formation after partial nephrectomy (19–21). Potential candidate stem cells have been detected in the adult mammalian kidney using different identification methods (22–26).

The purpose of this study was to test the hypothesis that renal stem cells exist in the adult kidney. We used an approach and culture conditions similar to those used to isolate multipotent adult progenitor cells from bone marrow, muscle, and brain (27). We refer to progenitor cells that were isolated from the kidney as multipotent renal progenitor cells (MRPC).

## Materials and Methods

### Isolation of MRPC

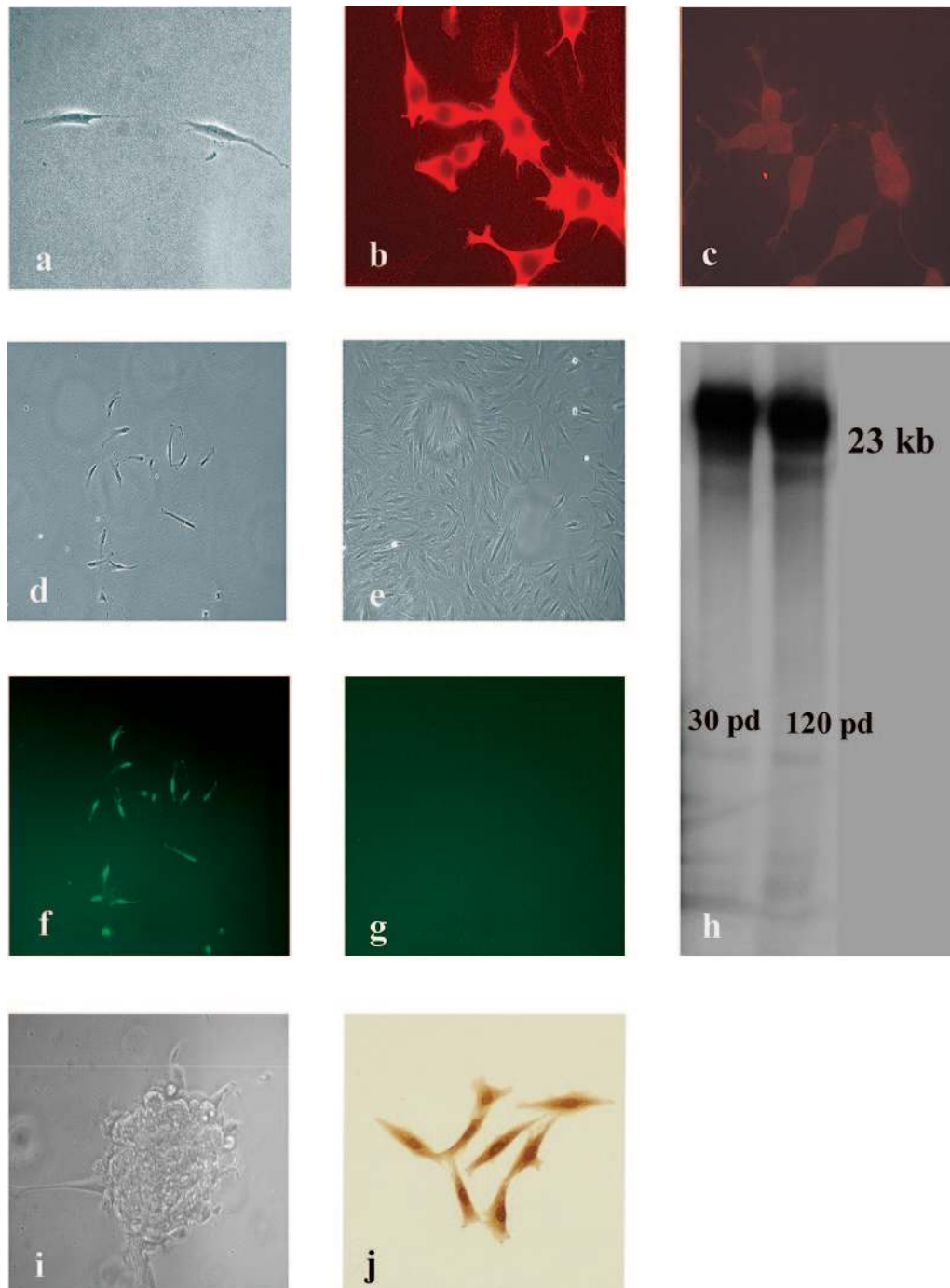
All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. MRPC were isolated from adult rat kidneys using culture conditions that were similar to those used for the culture of bone marrow-derived multipotent adult progenitor cells with the exception that we did not deplete the cells that were positive for CD45 or glycophorin A (27). The source for the rat kidneys were 2- to 4-mo-old Fisher rats, including *Oct4*  $\beta$ -Geo transgenic rats that contain a transgene that combines a neomycin-resistance gene with a lacZ reporter under the control of 3.6 kb of the mouse *Oct4* upstream sequence, including both proximal and distal enhancers (gift from Dr. Austin Smith, University of Edinburgh, Edinburgh, Scotland) (28). *Oct4* is a POU family transcription factor that is expressed in embryonic and adult stem cells and immortalized nontumorigenic cell lines and tumor cells but not in differentiated cells (27,29–31).

Kidneys were perfused *in vivo* with saline to flush the blood from the

Received March 24, 2006. Accepted August 7, 2006.

Published online ahead of print. Publication date available at [www.jasn.org](http://www.jasn.org).

**Address correspondence to:** Dr. Sandeep Gupta, Division of Renal Diseases and Hypertension, Department of Medicine, University of Minnesota, 516 Delaware Street SE, Box 736, Minneapolis, MN 55455. Phone: 612-624-9444; Fax: 612-626-3840; E-mail: [gupta024@umn.edu](mailto:gupta024@umn.edu)



**Figure 1.** Characteristics of multipotent renal progenitor cells (MRPC). (a) Phase contrast microscopy of MRPC. The cells are monomorphic with a spindle-shaped morphology and contain scant cytoplasm. (b and c) Immunofluorescence microscopy of MRPC stained with an anti-vimentin antibody (b) and an anti-cytokeratin antibody (c); the cells are vimentin positive and cytokeratin negative. Phase contrast (d and e) and immunofluorescent microscopy (f and g) of rat MRPC incubated with the fluorescence  $\beta$ -galactosidase substrate (FDG). When the cells were kept in an undifferentiated state by culturing them at low density, positive fluorescence is seen (f), consistent with  $\beta$ -galactosidase and hence *Oct4* expression. When the cells were allowed to grow to confluence, they lost their undifferentiated state and FDG fluorescence, consistent with shutting off  $\beta$ -galactosidase and *Oct4* expression (g). (h) Telomere length of rat MRPC cultured for 30 population doublings (pd; lane 1) or 120 population doublings (lane 2); no change was seen during this time period. (i) MRPC formed spheres when grown at high density. (j) positive nuclear staining for Oct4 in undifferentiated MRPC.

kidney, harvested, minced, and partially digested using collagenase in the presence of soybean trypsin inhibitor. The cell suspension was washed and plated in a medium that consisted of 60% DMEM-LG (Life

Technologies-BRL, Grand Island, NY), 40% MCDB-201 (Sigma Chemical Co., St. Louis, MO),  $1 \times$  insulin-transferrin-selenium, LA-BSA 1 mg/ml (Sigma),  $0.05 \mu\text{M}$  dexamethasone (Sigma) and 0.1 mM ascorbic

acid 2-phosphate (Sigma), 100 U penicillin and 1000 U streptomycin (Life Technologies) with 2% FCS (Hyclone Laboratories, Logan, UT), 10 ng/ml EGF, 10 ng/ml PDGF-BB, and 10 ng/ml leukemia inhibitory factor (all from R&D Systems, Minneapolis, MN). The cells were plated on fibronectin coated culture flasks at low density (300 cells/cm<sup>2</sup>), to avoid cell–cell contact, and cultured at 37°C in the presence of 5% CO<sub>2</sub>. After 4 to 6 wk, most of the cell types died out and the cultures became monomorphic with spindle-shaped cells (Figure 1a). Single clones of cells were obtained by plating the cells at nontouching density and then using cloning rings to pick individual colonies of cells at the five- to 10-cell stage.

### Characterization of MRPC

**Cell Surface Marker Analysis.** All staining reactions were performed using 10<sup>5</sup> cells in 100 μl of staining buffer. Mouse embryonic stem cells for stage-specific embryonic antigen-1 (SSEA-1) or freshly isolated rat bone marrow cells (for the other markers) were used as positive control. Unstained cells and corresponding isotype antibodies were used as negative control. Primary antibodies (PE, FITC, or PerCP conjugated) were used in a dilution of 1:200. Dead cells were excluded with 7AAD, doublets were excluded on the basis of three hierarchical gates (forward/side scatter area, forward scatter height/width, and side scatter height/width). For each reaction, 5000 events were counted. Antibodies used were mouse anti-rat CD90-PerCP, CD11b-FITC, CD45-PE, CD106-PE, CD44H-FITC, RT1B-biotin, RT1A-biotin, CD31-biotin (all from Becton Dickinson, San Diego, CA), and purified anti-mouse SSEA-1 (MAB4301; Chemicon, Temecula, CA).

**Telomere Length and Telomerase Enzyme Assay.** For measurement of telomere length, DNA was prepared from cells by standard methods of proteinase K digestion followed by salt precipitation and digested overnight with Hinf III and RsaI. Fragments were run on a 0.6% agarose gel and vacuum blotted to positively charged nylon. The blot was probed overnight with a digoxigenin-labeled hexamer (TTAGGG) and then incubated with anti-digoxigenin-alkaline phosphatase-labeled antibody for 30 min. Telomere fragments were detected by chemiluminescence. The TRAP protocol adapted by Roche Applied Science (Indianapolis, IN) was used to assay for telomerase activity.

**DNA Analysis by FACS.** MRPC were fixed in ice-cold 70% ethanol for 10 min and treated with 1 mg/ml ribonuclease for 5 min at room temperature. Propidium iodide (50 μg/ml) was added to the cell suspension and analyzed using 488 nm excitation, gating out doublets

and clumps, using pulse processing and collecting fluorescence above 620 nm on a FACS Calibur (BD Bioscience, San Jose, CA). Data were analyzed using Modfit LT software (Verity Software House, Topsham, ME).

### In Vitro Differentiation

For differentiation of MRPC toward a renal cell lineage, cells were grown to confluence on fibronectin-coated four-well chamber slides and incubated with a “nephrogenic cocktail” that contained fibroblast growth factor 2 (FGF2; 50 ng/ml), TGF-β (4 ng/ml), and leukemia inhibitory factor (20 ng/ml) (32,33). All differentiation cultures were maintained for 2 wk except where stated, and medium was renewed every 48 h. For determination of whether MRPC could differentiate into cells of other germ cell layers, cells were incubated under conditions that promoted differentiation into endothelium (mesoderm), neurons (ectoderm), and hepatocytes (endoderm). Endothelial differentiation was induced by growing MRPC on fibronectin-coated wells (15,000 cells/cm<sup>2</sup>) in the presence of 10 ng/ml vascular endothelial growth factor. Neuronal differentiation was induced by growing MRPC on fibronectin-coated wells (5000 cells/cm<sup>2</sup>) in the presence of 100 ng/ml basic FGF. Hepatocyte differentiation was induced by growing MRPC on Matrigel (20,000 cells/cm<sup>2</sup>) in the presence of 10 ng/ml FGF-4 and 20 ng/ml hepatocyte growth factor. Cells were characterized by reverse transcriptase-PCR (RT-PCR) and immunofluorescence as described in the RT-PCR section. For the MRPC that were differentiated into endothelial cells, we examined LDL uptake by incubating the cells with Dil-Ac-LDL (10 μg/ml) at 37°C for 60 min. Undifferentiated MRPC were used as a control.

### In Vivo Differentiation

**Ischemia Reperfusion Experiment.** For these experiments, MRPC were transduced using a murine stem cell virus-enhanced green fluorescence protein (eGFP) retrovirus. These cells expressed eGFP and are referred to as eMRPC. Rats were anesthetized with pentobarbital (35 to 60 mg/kg intraperitoneally) and prepared, and using a midline incision, nontraumatic vascular clamps were applied across both renal pedicles for 35 min. Immediately after ischemia, 100 μl (10<sup>6</sup> cells) of an eMRPC cell suspension in PBS was injected directly into the abdominal aorta, above the renal arteries, after application of a vascular clamp to the abdominal aorta below the renal arteries to direct the flow of the

Table 1. Primers used for reverse transcriptase-PCR<sup>a</sup>

Gene	Forward Primer	Reverse Primer
<i>mFlk1</i>	TCTGTGGTTCTGCGTGGAGA	GTATCATTTCCAACCACCCT
<i>rvWF</i>	GAGGCGGATCTGTTTGAGGTT	CCCAACGGATGGCTAGGTATT
<i>rPecam</i>	GGACTGGCCCTGTCACGTT	TTGTTTCATGGTGCCAAAACACT
<i>rHNF-3B</i>	CCTCCTCGTACATCTCGTCTATCA	CGCTCAGCGTCAGCATCTT
<i>rα-F2</i>	GTCCTTTCTTCCCTCTGGAGAT	CTGTCACTGCTGATTTCTCTGG
<i>rAlb2</i>	CTGGGAGTGTGCAGATATCAGAGT	GAGAAGGTCACCAAGGTCTGTAGT
<i>rCK18</i>	GCCCTGGACTCCAGCAACT	ACTTTGCCATCCACGACCTT
<i>rHNF-1</i>	AGCTGCTCCTCCATCATCAGA	TGTTCCAAGCATTAAAGTTTTCTATTCTAA
<i>OCT4ac</i>	CTGTAACCGGCGCCAGAA	TGCATGGGAGAGCCCAGA
<i>rRex1</i>	AAAGCTTTTACAGAGAGCTCGAAACTA	GTGCGCAAGTTGAAATCCAGT
<i>rNanog</i>	GAAGACTAGCAACGGCCTGACT	GGTTTCCAGACGCGTTCATC
<i>rEras</i>	GGAACCCCTACCACAAGCAA	GTGAGCAAGGACAGCTGCAG

<sup>a</sup>For *Pax-2* primer see Materials and Methods.

injected cells. The kidneys were harvested 10 d later to examine *in vivo* differentiation of the injected cells.

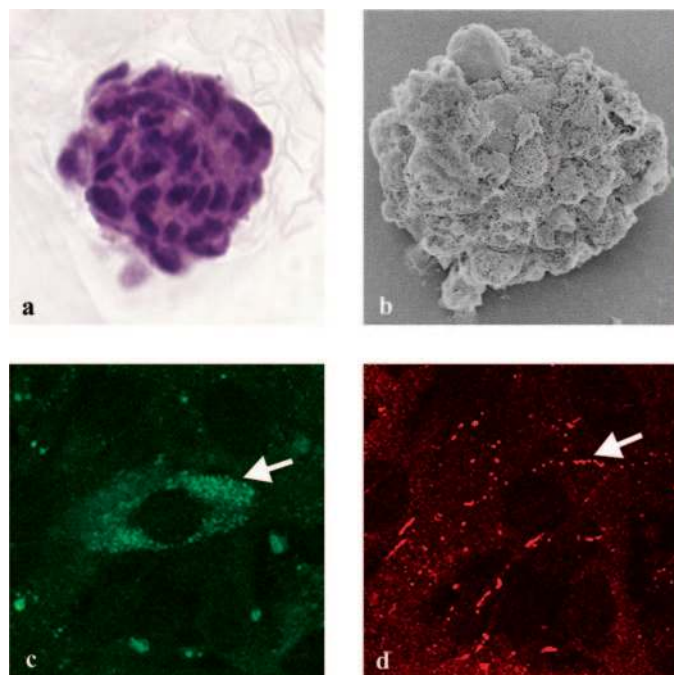
**Subcapsular Injection Experiment.** Rats were anesthetized, the kidneys exposed, and eMRPC ( $10^6$  cells) were injected under the renal capsule. Rats were killed 3 wk later, and kidneys were harvested for tissue analysis.

### Effect of MRPC on Renal Function after Ischemia-Reperfusion

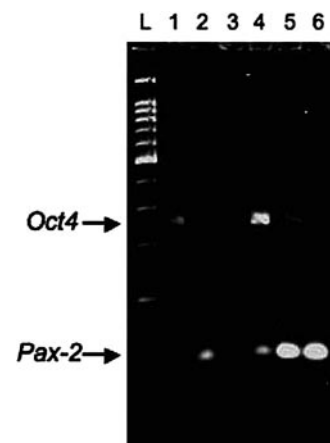
For determination of whether MRPC injection facilitated renal functional recovery, Fisher rats underwent 30 min of ischemia induced by bilateral renal artery clamps followed immediately by injection of MRPC as described above. As controls, rats were treated identically except that they received either the saline vehicle or an MRPC cell suspension ( $10^6$  cells) that had been preincubated for 12 h with actinomycin D ( $1 \mu\text{g}/\text{ml}$ ) to block transcription in the injected cells. For determination of whether injected MRPC had a deleterious effect on renal function, experiments were performed injecting saline vehicle ( $n = 2$ ) or an MRPC cell suspension ( $10^6$  cells;  $n = 2$ ) after sham operation. Renal function was assessed by serial measurement of serum creatinine and 24-h creatinine clearance.

### RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA was DNase 1 treated, and cDNA was synthesized



**Figure 2.** *In vitro* differentiation. (a and b) Light microscopy of hematoxylin- and eosin-stained section (a) and electron micrograph (b) of MRPC that were incubated with a “nephrogenic cocktail” that contained fibroblast growth factor 2 (FGF2), TGF- $\beta$ 2, and leukemia inhibitory factor. The cells changed from single-spindle shaped cells to cell aggregates as shown. (c and d) Immunofluorescence of MRPC that were incubated with the nephrogenic cocktail and stained with an anti-cytokeratin antibody (c) or anti-zona occludens-1 (anti-ZO-1; d) antibody demonstrating positive staining for both, consistent with transition to an epithelial cell phenotype.



**Figure 3.** *Oct4* and *Pax-2* expression in MRPC. L, ladder; lane 1, rat testes mRNA was a positive control for *Oct4*; lane 2, *Pax-2* positive control; lane 3, immortalized rat proximal tubular cell line termed IRPTC was a negative control for *Oct4* and *Pax-2*; lane 4, undifferentiated MRPC were positive for *Oct4* and *Pax-2*; lanes 5 and 6, MRPC from two different experiments incubated for 24 h with nephrogenic cocktail demonstrating that *Oct4* is shut off and persistent expression of *Pax-2*.

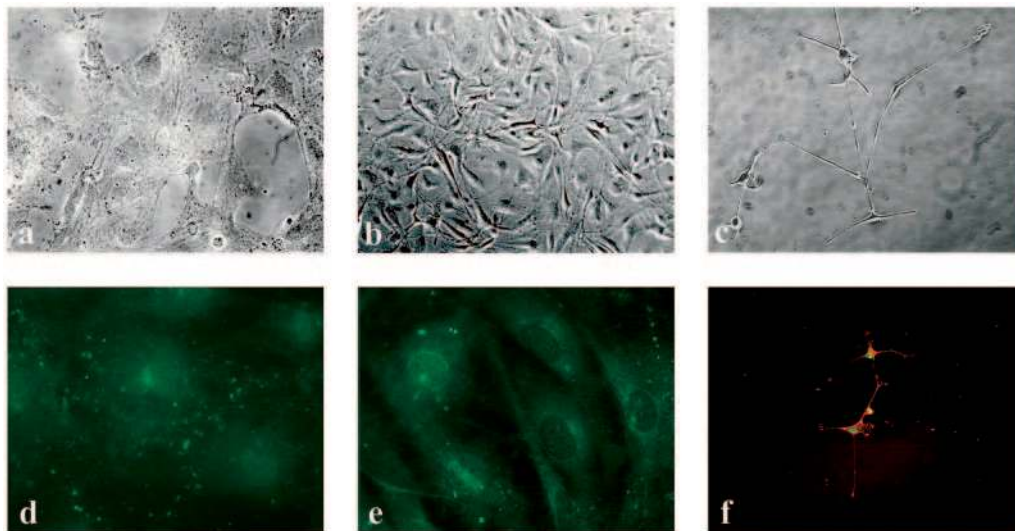
using the Taqman Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The forward and reverse primers used are listed in Table 1. For *Pax2*, we used the RT<sup>2</sup> PCR primer set for rat (LOC293992; Superarray Bioscience Corp., Frederick, MD). The BD rat universal reference total RNA was used as a positive control for this reaction (BD Biosciences).

Quantitative real-time PCR was performed on an ABI PRISM 7700 Sequence Detector, using the ABI PRISM 7700 Sequence Detector Software 1.7 (Applied Biosystems). Reaction conditions for amplification were as follows: 40 cycles of a two-step PCR (95°C for 15 s and 60°C for 60 s) after initial denaturation (95°C for 10 min) with  $1 \mu\text{l}$  of a cDNA reaction in  $1\times$  SYBR Green PCR Master Mix (Applied Biosystems).

### Immunohistochemistry

Kidney tissue sections were fixed in 4% paraformaldehyde and permeabilized with Triton X-100. After blocking with 1% BSA/PBS for 1 h, sections were incubated with primary antibodies diluted in 0.3% BSA/PBS overnight at 4°C. Slides subsequently were washed in PBS and incubated with secondary fluorochrome-conjugated antibodies for 45 min. The following antibodies were used in 1:100 dilution: Anti-von Willebrand factor (anft-vWF; F-3220; Sigma), anti-albumin (55442; ICN/Cappel, Costa Mesa, CA), FITC-conjugated anti-pan cytokeratin (F0397; Sigma), anti-neurofilament 200 (N0142; Sigma), Texas red-conjugated anti-GFP (600-109-215; Rockland, Gilbertsville, PA), anti-zona occludens-1 (anti-ZO-1; 61-7300; Zymed, San Francisco, CA), anti-MHC I (12-5321-81; eBioscience, San Diego, CA), anti-MHC II (12-5999-81; eBioscience), TRITC-conjugated anti-PCNA (SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA), anti-THP (CL-1032-A; Cedarlane, Burlington, NC), and anti-vimentin (V4630; Sigma). The following lectins were used in 1:500 dilutions for 45 min at room temperature: Rhodamine Peanut Agglutinin (RL-1072; Vector Laboratories, Burlingame, CA) and Rhodamine Phaseolus Vulgaris Erythroagglutinin (RL-1122; Vector Laboratories).

For detection of *Oct4*, 8- $\mu\text{m}$ -thick formalin-fixed, paraffin-embedded sections of rat kidney were deparaffinized in xylene for 10 min, followed by hydration through graded ethanol. Endogenous peroxidase



**Figure 4.** *In vitro* differentiation. Phase contrast microscopy (a through c) and immunofluorescence (d through f) of MRPC that were incubated under culture conditions that promoted differentiation into cells of all three germ cell layers. MRPC that were cultured on fibronectin in the presence of vascular endothelial growth factor developed endothelial morphology (a) and stained for von Willebrand factor (d). MRPC that were cultured on Matrigel in the presence of FGF-4 and hepatocyte growth factor developed an epithelial morphology (b) and stained for albumin (e). MRPC that were cultured on fibronectin in the presence of basic FGF and in the absence of PDGF-BB and EGF developed neuronal morphology (c) and stained for neurofilament-200 (f).

activity was blocked in 0.3% hydrogen peroxide solution in methanol at room temperature for 30 min. Antigens were retrieved by Antigen Unmasking Solution (Vector Laboratory, H-3300) as per the manufacturer's protocol. Sections were incubated overnight with anti-Oct4 antibody (Santa Cruz Biotechnology sc-8629). Primary antibody was detected, and signal amplified using Vectastain Elite ABC kit (PK-6105; Vector Laboratories). Diaminobenzidine was used as peroxidase substrate (SK-4100; Vector Laboratories).

**X-Gal Staining.** Staining was done using Invitrogen Kit per manufacturer's protocol at pH 7.4 using 5- to 10- $\mu$ m cryosections that were fixed for 10 min in 20% formaldehyde and 2% glutaraldehyde. Kidneys from ROSA26 mice or Fisher rats were used as positive and negative controls, respectively.

## Results

### Isolation of MRPC

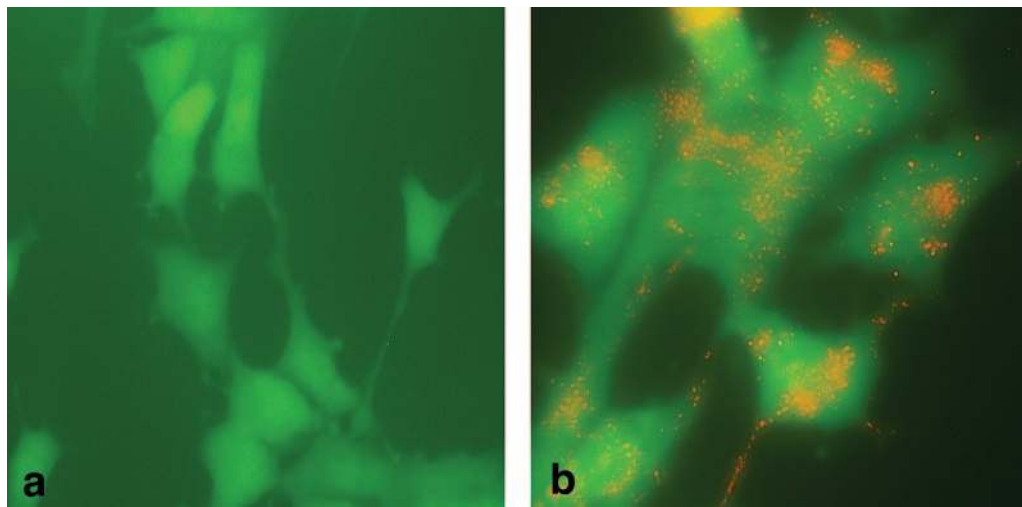
After 4 to 6 wk, most of the cell types died out and the cultures became monomorphic with spindle-shaped cells (Figure 1). These cells were 8 to 10  $\mu$ m in size, contained a large nucleus and scant cytoplasm, had a population doubling time of 24 to 36 h, formed spheres when grown at high density; and some clones have been cultured for >200 population doublings without evidence for senescence (see the Characterization of MRPC section). Successful isolation of MRPC was achieved approximately 20% of the time. Other isolations resulted in either complete cell death or more differentiated cells. Similar results were seen in cells that were isolated from either Oct4  $\beta$ -Geo transgenic rats or nontransgenic Fisher rats and with or without G418 selection. G418 selection shortened the duration of isolation but did not improve the success of the isolation procedure. We were unable to isolate MRPC from the blood of these rats despite multiple attempts.

### Characterization of MRPC

By FACS analysis, 89% of cultured MRPC were positive for CD90 (thy1.1) and 86% were positive for CD44. MRPC were negative for SSEA-1, CD-11b, CD45, CD133, CD106, MHC class I (RT1A) and class II (RT1B), CD31, and CD56 (NCAM). By immunohistochemistry, MRPC expressed vimentin but not cytokeratin (Figure 1, b and c). Incubation of undifferentiated MRPC with the  $\beta$ -galactosidase fluorescence substrate fluorescein di- $\beta$ -D-galactopyranoside resulted in cell fluorescence consistent with Oct4 expression (Figure 1, d and f). This fluorescence and hence  $\beta$ -galactosidase activity disappeared when the cells were allowed to differentiate by growing them to confluence (Figure 1, e and g). Oct4 expression was confirmed by immunostaining (Figure 1j). Average telomere length of MRPC that were cultured for 30 population doublings was 23 kb; when retested at 120 population doublings, average telomere length remained unchanged (Figure 1h). Similarly, no change in telomerase enzyme activity was observed at the two population doublings. Rat MRPC that were examined at 200 population doublings had a normal karyotype by cytogenetic analysis and normal DNA content by FACS analysis (data not shown).

### In vitro Differentiation

MRPC were incubated with a nephrogenic cocktail (see Materials and Methods) that has been shown to induce rat metanephric mesenchymes to differentiate into nephron epithelia (32,33). After 14 d, the phenotype of the cells changed from a monolayer of spindle-shaped cells to cell aggregates as shown in Figure 2, a and b. In the absence of the nephrogenic cocktail, cells grew to confluence and no cell aggregation was seen. In



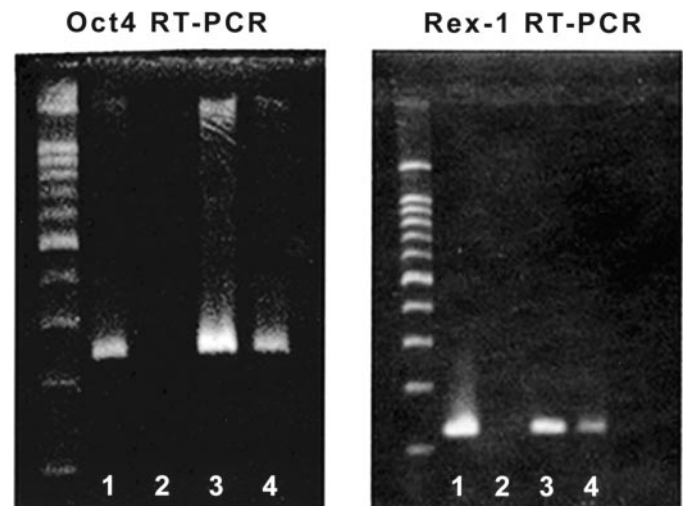
**Figure 5.** Dil-Ac-LDL uptake. (a) No uptake of Dil-Ac-LDL was seen in undifferentiated MRPC. (b) Uptake of Dil-Ac-LDL by MRPC that have been differentiated into endothelial cells. Dil-Ac-LDL appears as intracellular reddish-orange particles.

addition to changing morphology, 54% of the cells expressed the epithelial cell marker cytokeratin and 48% of the cells expressed zona occludens-1 (ZO-1; Figure 2, c and d).

*Oct4* and *Pax 2* expression in undifferentiated rat MRPC was examined by RT-PCR using rat testes mRNA as a positive control and an immortalized rat proximal tubular cell line termed IRPTC (gift of Julie Ingelfinger) as a negative control. RT-PCR for *Oct4* was positive in undifferentiated MRPC (Figure 3, lane 4) and was switched off after 24 h of culture with the nephrogenic cocktail (Figure 3, lanes 5 and 6). *Pax-2* is a transcription factor that is expressed by stem cells that are present in the metanephric mesenchyme (18) and during defined phases of nephron development, with near absent expression in the adult nephron (34). Expression of *Pax-2* was seen in undifferentiated MRPC (Figure 3). In contrast to *Oct4*, continued expression of *Pax-2* was seen after incubation of MRPC with the nephrogenic cocktail.

MRPC could be induced to express endothelial, hepatocyte, and neural markers (Figure 4). Culturing MRPC on fibronectin-coated wells in the presence of vascular endothelial growth factor resulted in an endothelial morphology with positive staining for vWF (Figure 4, a and d). The differentiated cells were positive by quantitative RT-PCR (Q-RT-PCR) for vWF, fetal liver kinase 1, and endoglin and were able to take up Dil-Ac-LDL (Figure 5). No uptake was seen in undifferentiated MRPC that were used as a control (Figure 5a).

When MRPC were grown on Matrigel in the presence FGF-4 and hepatocyte growth factor, the cells developed an epithelial morphology and stained for albumin (Figure 4, b and e). These cells were positive by Q-RT-PCR for cytokeratins 18 and 19. To induce neuronal differentiation, MRPC were grown on fibronectin in the presence of basic FGF and in the absence of PDGF-BB and EGF. The cells developed neuronal-like processes, stained positive for the neuronal marker neurofilament-200 (Figure 4, c and f), and expressed neurofilament-200 by Q-RT-PCR. All differentiation cultures were maintained for

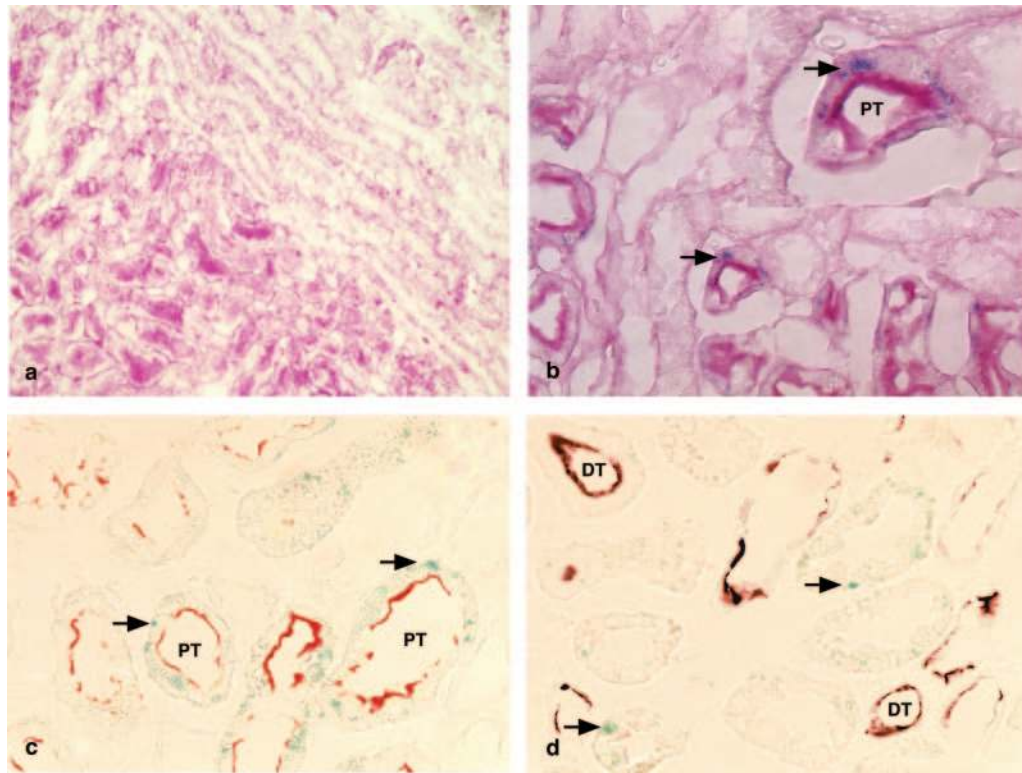


**Figure 6.** *Oct4* and *Rex-1* expression in adult kidney. Reverse transcriptase-PCR for *Oct4* is on the left, and *Rex-1* is on the right. For both gels, lanes are as follows: Lane 1, rat testes (positive control); lane 2, IRPTC (negative control); lane 3, adult Fisher rat kidney 5 d after 45 min of ischemia; lane 4, normal adult Fisher rat kidney.

14 d. In these experiments, differentiated MRPC were always compared with undifferentiated MRPC.

#### In Vivo Localization

*Oct4* was expressed by RT-PCR in both normal Fisher rat kidneys and kidneys that were harvested 5 d after 45 min of ischemia (Figure 6). *Rex-1*, a transcription factor downstream of *Oct4*, also was expressed in these kidneys (Figure 6). Kidneys from *Oct4*  $\beta$ -Geo transgenic rats also were positive by RT-PCR for *Oct4*. Taking advantage of the fact that the promoter and enhancer elements of the *Oct4* gene drive the expression of  $\beta$ -galactosidase in these rats, we stained for  $\beta$ -galactosidase protein and activity as a marker of *Oct4* expression. Control



**Figure 7.** *In vivo* localization. (a) Negative control demonstrating no X-gal staining in a nontransgenic Fisher rat. (b) X-gal staining of the kidney from a *Oct4*  $\beta$ -Geo transgenic rat demonstrating positive blue staining in cells associated with the proximal tubule (arrow) just below a peritubular capillary that contains a red blood cell. (c) X-gal-positive cells associated with the proximal tubule marker Phaseolus Vulgaris Erythroagglutinin (arrows). (d) No X-gal-positive cells were seen in the distal tubule as evidenced by the lack of co-localization with the distal tubule lectin Peanut Agglutinin (PNA; arrows indicate X-gal-positive cells).

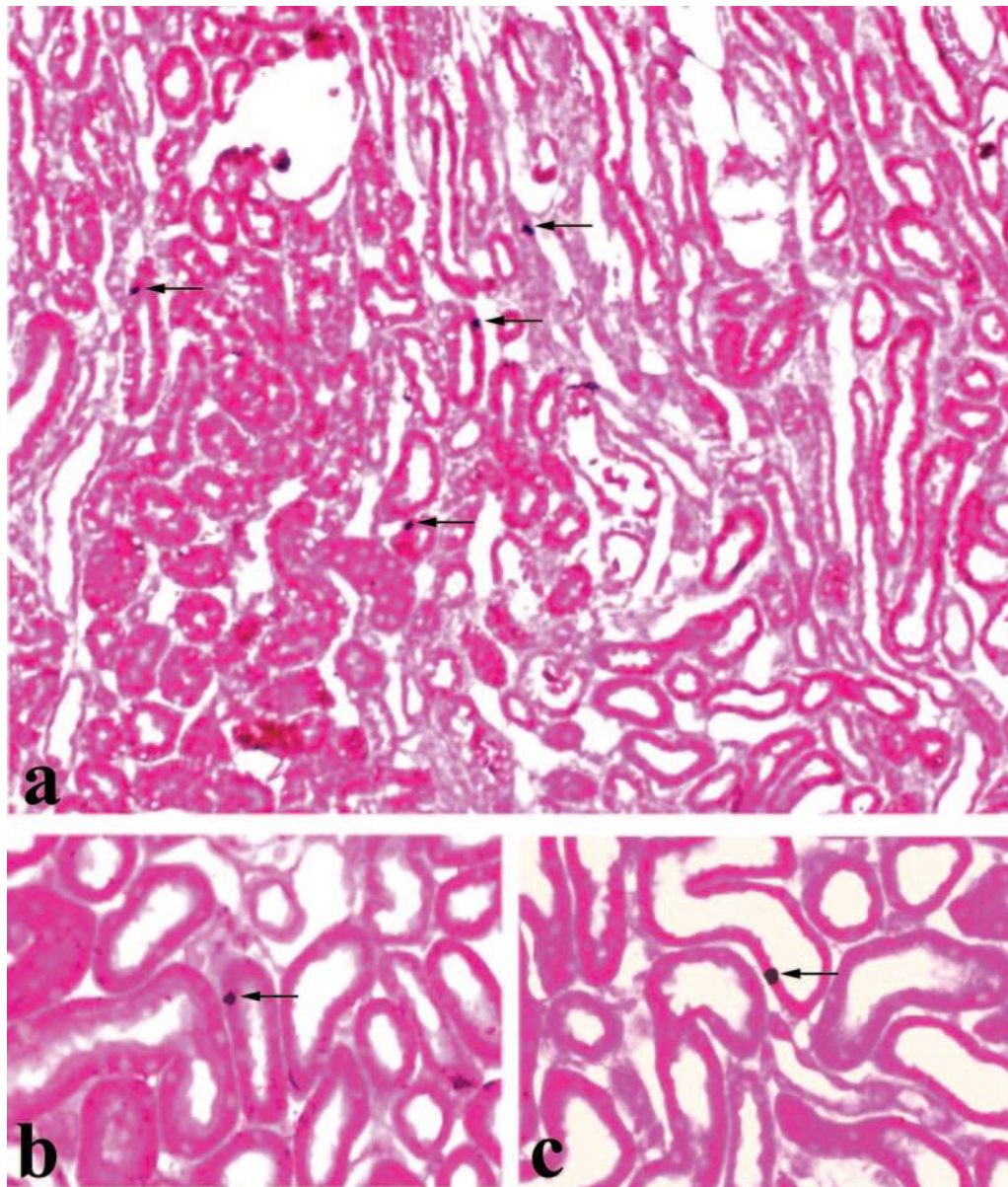
kidneys from nontransgenic rats were negative for X-gal staining (Figure 7a). Positive blue staining cells were seen primarily at the cortical medullary junction, with X-gal-positive cells being associated with the proximal tubule as demonstrated by periodic acid-Schiff staining of the brush border (Figure 7b). Very occasional cells were seen in the other parts of the cortex, and none was detected in the medulla. Positive cells co-stained with the proximal tubule marker Phaseolus Vulgaris Erythroagglutinin (Figure 7c). No positive cells were seen in the distal tubule as evidenced by the lack of co-localization with the distal tubule marker Peanut Agglutinin (Figure 7d). *Oct4* immunostaining was seen in isolated tubular cells, consistent with the pattern of X-gal staining (Figure 8). Only rare tubules expressed *Oct4*, and when present, *Oct4* immunostaining was seen only in a single tubular cell in a given tubule profile (Figure 8).

### In Vivo Differentiation

Undifferentiated eMRPC were injected into Fisher rats in two different *in vivo* models. Three weeks after injection of cells under the renal capsule, GFP-positive cellular nodules formed at the site of injection and included cyst-like structures (Figure 9a). In addition, Figure 9b demonstrates that some GFP-positive cells became incorporated into the renal tubules, frequently in groups of two to three. The injected MRPC also formed

multiple tubular-like structures as seen in Figure 10. These tubules were X-gal negative, indicating that *Oct4* was no longer expressed (data not shown), and were vimentin negative, consistent with differentiation of the MRPC because they were vimentin positive before injection.

The second model was of ischemia/reperfusion injury to the kidney. As can be seen in Figure 9, c and d, some eMRPC became lodged in the glomerulus or were found as cellular casts, both adverse consequences of the injection. Evidence for the incorporation of injected eMRPC into renal tubules was seen throughout the cortex and in the outer medulla (Figure 9, e through i). In some areas, all cells in the tubule were GFP positive, whereas in other areas, only some cells were positive. Injected eMRPC were incorporated into 5 to 10% of the renal tubules in any given kidney section. After incorporation into the renal tubules, the injected eMRPC expressed the proximal tubule marker Phaseolus Vulgaris Erythroagglutinin (Figure 9f), the distal tubule marker Peanut Agglutinin (Figure 9g), but not the loop of Henle marker Tamm-Horsfall Protein. The eMRPC stained for the proliferation marker PCNA (Figure 9h), providing evidence that the cells were capable of dividing. As can be seen in Figure 9i, GFP-positive eMRPC expressed the epithelial cell marker ZO-1 at the cell-cell junction. No incorporation was seen in the vascular or interstitial compartments in either model.



**Figure 8.** *Oct4* immunostaining in the adult kidney. Kidneys from Fisher rats were examined for *Oct4* immunostaining. (a) Positive *Oct4* staining in isolated cells from some tubular profiles (arrows). (b and c) Higher power views of positive nuclear staining.

#### *Effect of MRPC on Renal Injury*

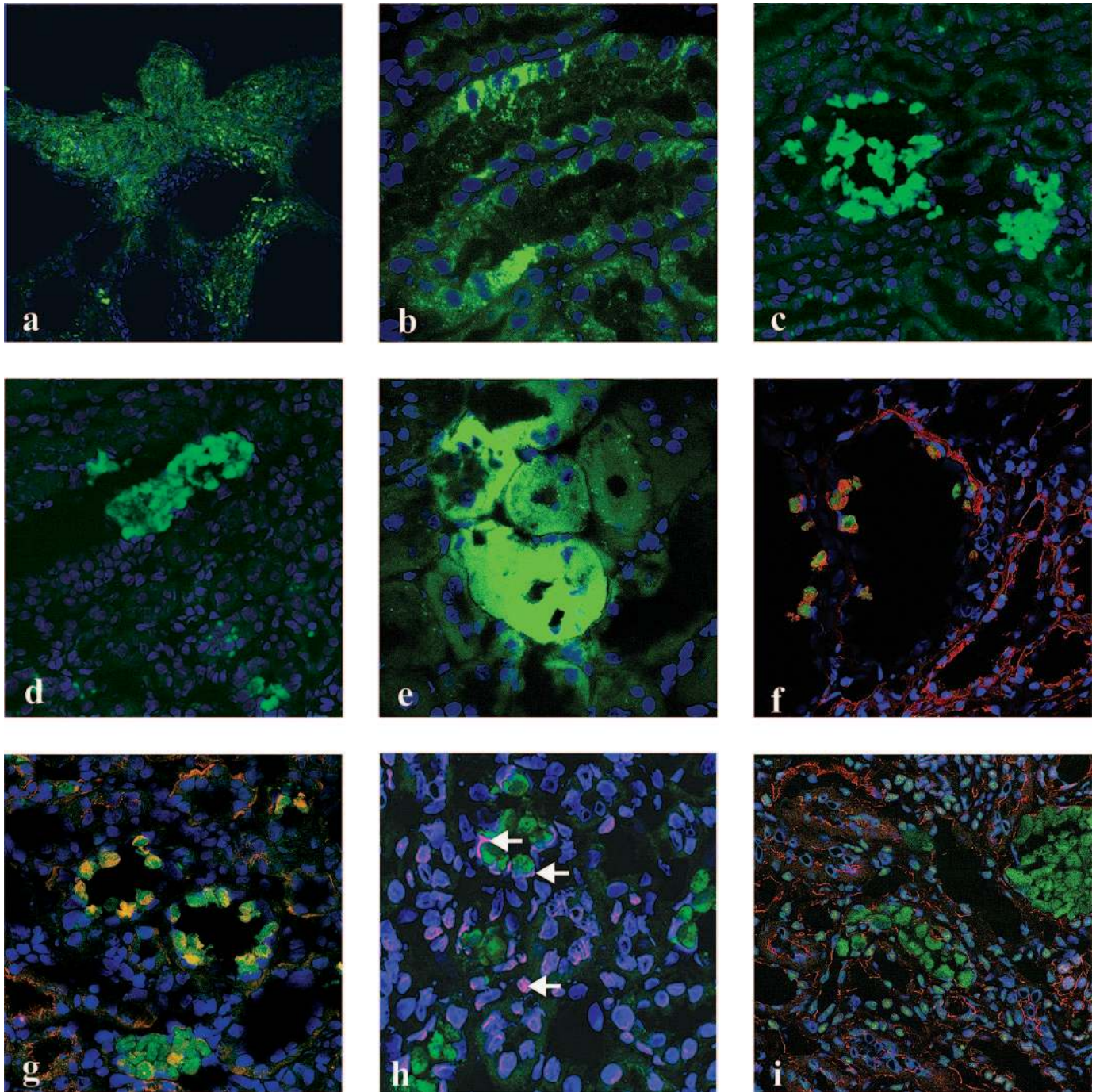
For determination of whether injected MRPC altered the course of kidney injury after ischemia-reperfusion, renal function was assessed by serial measurement of serum creatinine and 24-h creatinine clearance. Rats received either untreated MRPC or MRPC that had been preincubated for 12 h with actinomycin D to block transcription (Figure 11). As can be seen in Figure 9a, the time course and the severity of renal injury were similar between the two groups. A separate group of rats were studied to compare stem cell injection ( $10^6$  cells;  $n = 6$ ) with a different control, that of the saline vehicle. No differences in serum creatinine were observed between these two groups. We also studied the effects of stem cell injection in sham-operated rats. After sham operation, serum creatinine and creatinine clearance remained normal with

no difference being seen between saline-treated and MRPC-injected rats (Table 2).

#### **Discussion**

We have isolated unique cells from adult rat kidneys that behave in a manner that is consistent with a renal stem cell. Features of these cells include spindle-shaped morphology; self-renewal for >200 population doublings without evidence for senescence; normal karyotype and DNA content; and expression of vimentin, CD90 (thy1.1), *Pax-2*, and *Oct4* but not cytokeratin, MHC class I or II, or other markers of more differentiated cells. MRPC exhibit plasticity, demonstrated by the ability of the cells to differentiate toward cells of all three germ cell layers.

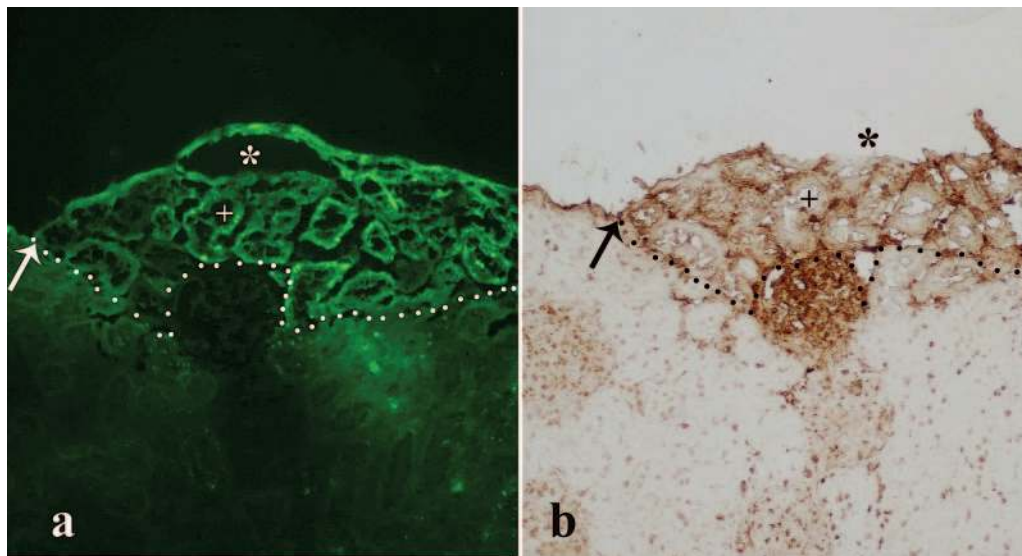




**Figure 9.** *In vivo* differentiation. (a and b) Immunofluorescence of the kidney 3 wk after injection of eMRPC under the renal capsule, demonstrating green fluorescence protein (GFP)-positive cellular nodules and cyst-like structures (a) under the capsule at the site of injection and some GFP-positive cells became incorporated into tubules (b). (c through i) Immunofluorescence of the kidney 10 d after ischemia-reperfusion injury followed by injection of eMRPC. (c) Injected cells lodged in the glomerulus. (d) eMRPC found in a cellular cast. (e) Positive tubule demonstrating incorporation of injected cells. (f) Section stained with the proximal tubule marker Phaseolus Vulgaris Erythroagglutinin demonstrating positive (red) staining in GFP-positive cells; nuclei are stained blue. (g) eMRPC were positive for the distal tubule lectin PNA (yellow staining). (h) eMRPC stained for the proliferation marker PCNA (arrow), providing evidence the cells were capable of dividing. (i) eMRPC expressed the epithelial cell marker ZO-1 as seen by the red staining and marked by arrows.

Our *in vitro* findings suggest that MRPC can be induced to a renal phenotype, although definitive tubule formation has not been demonstrated. Incubation of MRPC in a nephrogenic me-

dia that is known to induce tubulogenesis in isolated metanephric mesenchyme resulted in aggregation of cells and transition from mesenchymal-like cells that expressed vimentin and



**Figure 10.** Subcapsular injection of MRPC. (a) Immunofluorescence of the kidney 3 wk after injection of eMRPC under the renal capsule demonstrating GFP-positive tubules at the site of injection. (b) Vimentin immunostaining demonstrating that tubules that were derived from injected MRPC were vimentin negative; positive mesangial and vascular smooth muscle staining for vimentin is seen in the normal parts of the kidney. The dotted line demarcates normal kidney from that derived from the eMRPC; \* indicates same area from both sections; + indicates same tubule in both sections.

CD90 (thy1.1) to epithelial cells that expressed cytokeratin and ZO-1. Undifferentiated MRPC expressed *Pax-2*, a transcription factor that is expressed by stem cells that are present in the metanephric mesenchyme and by other stem cells that were isolated from adult kidneys (18,22).

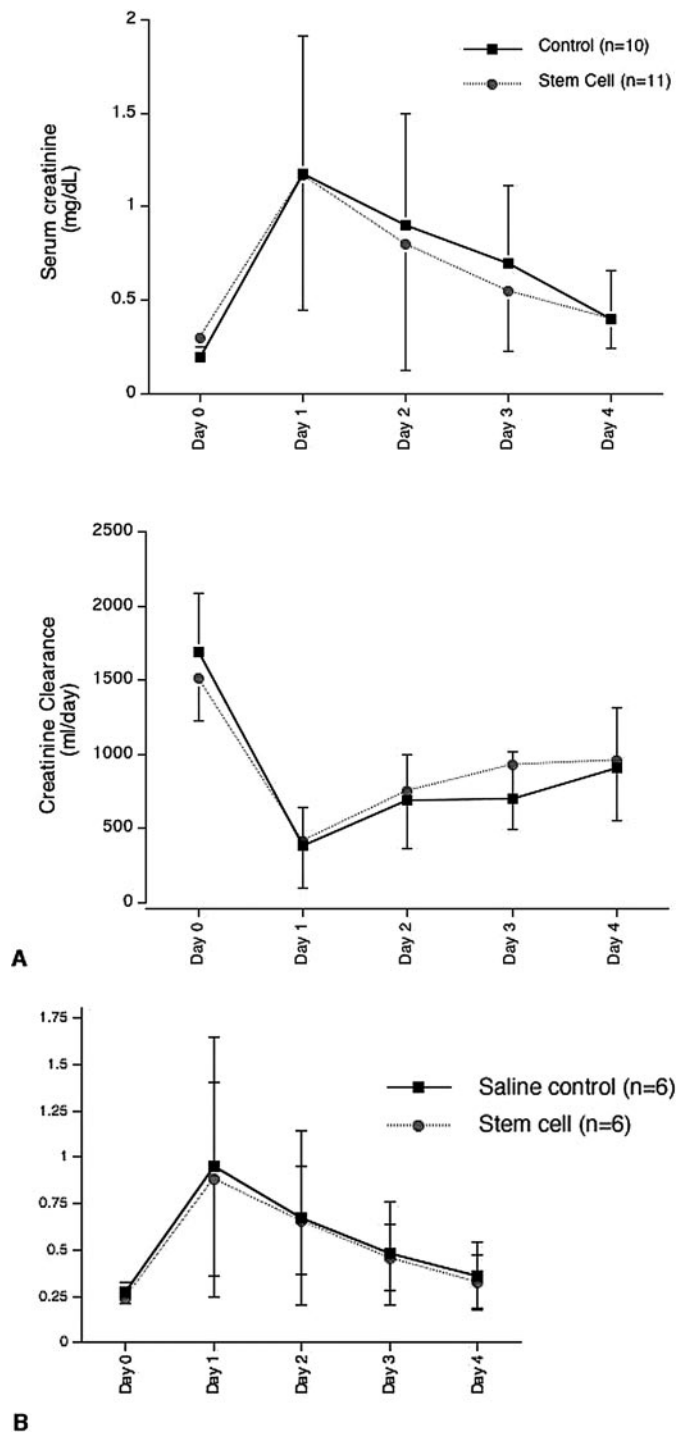
MRPC can be transduced easily with murine stem cell virus-eGFP, allowing the cells to be tracked *in vivo*. This enabled us to inject the cells either under the renal capsule or into the aorta after ischemia-reperfusion injury and to track their differentiation. After subcapsular injection, the cells not only formed tubules at the site of injection but also migrated and became incorporated into renal tubules that were more distant from the injection site. This finding in a noninjury model suggest that the MRPC can be induced to undergo tubulogenesis and can participate in the normal cell turnover of the kidney. We cannot exclude release of GFP by dead cells and uptake by proximal tubular cells. However, we believe that this is less likely given the pattern of GFP fluorescence that was seen in tubular cells with intense staining in groups of adjacent cells and no staining in other neighboring cells.

MRPC also participated in the regenerative response after renal injury. The injected cells became incorporated into renal tubules and showed evidence of proliferation and differentiation. Intra-arterial injection of the cells also resulted in some cells being lodged in the glomerulus and others forming tubular casts. Finding cells in these locations is a potential adverse consequence of the exogenous cell administration, although no adverse effects were seen after cell injection in sham-operated rats. In addition, we preincubated MRPC with the transcription inhibitor actinomycin D as a cellular control. We reasoned that these cells, although viable, would not be able to participate in the regenerative response but would be of similar size and

morphology as the untreated cells. The lack of a difference in injury between the untreated and the actinomycin D-treated MRPC suggests that no therapeutic benefit at the dosage and timing selected. However, we cannot exclude the possibility that the actinomycin D-treated cells had beneficial paracrine effects, even though they could not proliferate or synthesize new RNA (35,36). No incorporation of the actinomycin D-treated MRPC was seen in the injured kidney (data not shown).

The mechanism of how some of the injected cells become incorporated into tubules is intriguing with a number of potential possibilities. The cells could have passed through the glomerulus into the tubule lumen and attached to sites of denuded tubular basement membrane. The finding of tubular casts that were made up of injected MRPC support the feasibility of such a mechanism. Alternatively, the cells may migrate out from peritubular capillaries and cross the tubular basement membrane in a process that is the reverse of epithelial-mesenchymal transformation. Whatever the mechanism, strategies to enhance delivery of cells to the injured kidney that maximize incorporation into tubules and minimize ischemic or obstructive injury is an important area of investigation.

We propose that the kidney contains stem cells that are localized to the renal tubule. We base this proposal on the expression of the POU family transcription factor *Oct4* in a rare population of tubular cells. *Oct4* controls the differentiation potential of cells and has a limited range of expression being confined to embryonic and adult stem cells, immortalized nontumorigenic cell lines and tumor cells, but not differentiated cells (27,29–31). Expression of *Oct4* was demonstrated by RT-PCR and immunostaining and was confirmed by X-gal staining in the *Oct4*  $\beta$ -Geo transgenic rats. The identification of stem



**Figure 11.** Recovery from ischemia-reperfusion. (a) Serial serum creatinine and 24-h creatinine clearance after ischemia-reperfusion. Rats received either untreated MRPC (circles) or MRPC that were preincubated with actinomycin D ( $1 \mu\text{g}/\text{ml}$ ) to block transcription in the injected cells (squares). (b) Serial serum creatinine after ischemia-reperfusion. Rats received either untreated MRPC (circles) or saline without cells (squares).

cells that were associated with the tubule is consistent with the localization of label-retaining cells by Maeshima *et al.* (23) and the tubular expression of *Oct4* and *Rex-1* in human kidneys

demonstrated by Raman *et al.* (37). MRPC can be cultured from adult rat kidneys and are likely the *in vitro* correlate of the *Oct4*-expressing cells that were seen *in vivo*. These cells express *Oct4*, can undergo trilineage differentiation, and can be induced *in vitro* to develop a renal phenotype. Most important, MRPC can form tubules when injected under the renal capsule.

The existence of a renal stem cell in the adult kidney that is capable of self-renewal and differentiation into various cell types of the kidney is consistent with the finding of tissue-specific stem cells in other locations, such as the skin, brain, and gastrointestinal tract (12–16). Other studies have attempted to isolate renal stem cells. For example, Oliver *et al.* (24) isolated from the renal papilla of young mice and rats slow-cycling cells that have characteristics of renal stem cells. When grown in culture, these papillary cells express epithelial and mesenchymal markers, form cellular spheres, and display some evidence of plasticity with differentiation into neurons under appropriate culture conditions. Bussolati *et al.* (22) isolated and cultured a population of cells from adult human kidney using CD133 as a selection marker. These cells could be differentiated *in vitro* and *in vivo* into epithelial and endothelial cells, could form tubules and vessels, and expressed early and late nephron markers. These cells differed from MRPC in that they had limited self-renewal and differentiation potential and expressed different markers. Taking advantage of the slow cycling of stem cells, Maeshima *et al.* (23) identified a population of cells scattered among renal tubular cells in the adult rat kidney. These cells were identified as label-retaining cells and were found predominantly in proximal tubules. The cells, which subsequently were isolated, demonstrate plasticity and can be integrated into the developing kidney (25). Kitamura *et al.* (26) isolated a population of rapidly proliferating cells from microdissected proximal tubules that expressed the stem cell markers *Sca-1* and *Musashi-1* as well as early nephron markers. The cells could be differentiated into mature tubular cells in culture. These cells had a triploid karyotype but did not undergo tumor formation in nude mice. Differences in the cells that were isolated in these studies may be due to different selection markers, species, age of the kidneys, and culture conditions.

## Conclusion

We have isolated from rat kidneys a unique cell (MRPC) that behaves in a manner that is consistent with its being a renal stem cell. The cells can be cultured for multiple population doublings without evidence of senescence or malignant transformation. Unique features of these kidney-derived cells include expression of markers that are consistent with pluripotency such as the stem cell transcription factor *Oct4*; the expression of *Pax-2*, a marker expressed by other renal stem cells; and the ability of the cells to differentiate toward cells that are derived from all three germ cell layers. The presence of stem cells in the adult kidney has important implications to our understanding of normal cell turnover in the kidney and the source of regenerating cells after acute renal injury. The cells can differentiate into tubular cells when injected into the nor-

Table 2. Sham operation<sup>a</sup>

Day	Saline		MRPC (10 <sup>6</sup> cells)	
	SCr (mg/dl)	CrCl (ml/d)	SCr (mg/dl)	CrCl (ml/d)
0	0.5 ± 0.0	1075 ± 219	0.5 ± 0.0	1088 ± 20
1	0.5 ± 0.0	1264 ± 165	0.4 ± 0.0	1142 ± 6
2	0.5 ± 0.0	1382 ± 40	0.5 ± 0.0	1312 ± 295
3	0.5 ± 0.0	1278 ± 35	0.5 ± 0.0	1374 ± 281
4	0.5 ± 0.0	1206 ± 105	0.4 ± 0.0	1334 ± 142

<sup>a</sup>CrCl, 24-h creatinine clearance; MRPC, multipotent renal progenitor cells; SCr, serum creatinine.

mal and injured kidney. We propose that MRPC participate in the endogenous regenerative response of the kidney.

## Acknowledgments

This work was supported by National Institutes of Health grant DK68470 (M.E.R.), by a postdoctoral grant from the Juvenile Diabetes Research Foundation (Y.H.), and by an unrestricted research grant by Arthur Price.

We are thankful to Dr. Austin G. Smith (University of Edinburgh) for generously sharing with us Oct-β-Geo rats. We thank Dr. Julie Ingelfinger for the IRPTC cells and Uma Lakshmpati for technical assistance with the transfection studies.

## References

- Bacallao R, Fine LG: Molecular events in the organization of renal tubular epithelium: From nephrogenesis to regeneration. *Am J Physiol* 257: F913–F924, 1989
- Maeshima A, Maeshima K, Nojima Y, Kojima I: Involvement of Pax-2 in the action of activin A on tubular cell regeneration. *J Am Soc Nephrol* 13: 2850–2859, 2002
- Safirstein R: Renal regeneration: Reiterating a developmental paradigm. *Kidney Int* 56: 1599, 1999
- Safirstein R, Price PM, Saggi SJ, Harris RC: Changes in gene expression after temporary renal ischemia. *Kidney Int* 37: 1515–1521, 1990
- Witzgall R, Brown D, Schwartz C, Bonventre J: Localization of proliferating cell nuclear antigen, vimentin, c-Fos, and clusterin in the postischemic kidney. Evidence for a heterogeneous genetic response among nephron segments, and a large pool of mitotically active and dedifferentiated cells. *J Clin Invest* 93: 2175–2188, 1994
- Gupta S, Verfaillie C, Chmielewski D, Kim Y, Rosenberg ME: A role for extrarenal cells in the regeneration following acute renal failure. *Kidney Int* 62: 1285–1290, 2002
- Poulsom R, Forbes SJ, Hodivala-Dilke K, Ryan E, Wyles S, Navaratnasah S, Jeffery R, Hunt T, Alison M, Cook T, Pusey C, Wright NA: Bone marrow contributes to renal parenchymal turnover and regeneration. *J Pathol* 195: 229–235, 2001
- Duffield JS, Park KM, Hsiao LL, Kelley VR, Scadden DT, Ichimura T, Bonventre JV: Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells. *J Clin Invest* 115: 1743–1755, 2005
- Krause D, Cantley LG: Bone marrow plasticity revisited: Protection or differentiation in the kidney tubule? *J Clin Invest* 115: 1705–1708, 2005
- Lin F, Moran A, Igarashi P: Intrarenal cells, not bone marrow-derived cells, are the major source for regeneration in postischemic kidney. *J Clin Invest* 115: 1756–1764, 2005
- Szczycka MS, Westover AJ, Clouthier SG, Ferrara JL, Humes HD: Rare incorporation of bone marrow-derived cells into kidney after folic acid-induced injury. *Stem Cells* 23: 44–54, 2005
- Alison M, Poulsom R, Forbes S: Update on hepatic stem cells. *Liver* 21: 367–373, 2001
- Bernard-Kargar C: Endocrine pancreas plasticity under physiological and pathological conditions. *Diabetes* 50[Suppl 1]: S30–S35, 2001
- Forbes SJ, Poulsom R, Wright NA: Hepatic and renal differentiation from blood-borne stem cells. *Gene Ther* 9: 625–630, 2002
- Morrison SJ, White PM, Zock C, Anderson DJ: Prospective identification, isolation by flow cytometry and in vivo self renewal of multipotent mammalian neural crest stem cells. *Cell* 96: 737–749, 1999
- Wright NA: Epithelial cell repertoire in the gut: Clues to the origin of cell lineages, proliferative units, and cancer. *Int J Exp Pathol* 81: 117–143, 2000
- Herzlinger D, Koseki C, Mikawa T, al-Awqati Q: Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction. *Development* 114: 565–572, 1992
- Oliver JA, Barasch J, Yang J, Herzlinger D, Al-Awqati Q: Metanephric mesenchyme contains embryonic renal stem cells. *Am J Physiol Renal Physiol* 283: F799–F809, 2002
- Drummond IA, Mukhopadhyay D, Sukhatme VP: Expression of fetal kidney growth factors in a kidney tumor line: Role of FGF2 in kidney development. *Exp Nephrol* 6: 522–533, 1998
- Elger M, Hentschel H, Litteral J, Wellner M, Kirsch T, Luft FC, Haller H: Nephrogenesis is induced by partial nephrectomy in the elasmobranch *Leucoraja erinacea*. *J Am Soc Nephrol* 14: 1506–1518, 2003
- Salice CJ, Rokous JS, Kane AS, Reimschuessel R: New nephron development in goldfish (*Carassius auratus*) kidneys following repeated gentamicin-induced nephrotoxicosis. *Comp Med* 51: 56–59, 2001
- Bussolati B, Bruno S, Grange C, Buttiglieri S, Derogibus MC, Cantino D, Camussi G: Isolation of renal progenitor

- cells from adult human kidney. *Am J Pathol* 166: 545–555, 2005
23. Maeshima A, Yamashita S, Nojima Y: Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney. *J Am Soc Nephrol* 14: 3138–3146, 2003
  24. Oliver JA, Maarouf O, Cheema FH, Martens TP, Al-Awqati Q: The renal papilla is a niche for adult kidney stem cells. *J Clin Invest* 114: 795–804, 2004
  25. Maeshima A, Sakurai H, Nigam SK: Adult kidney tubular cell population showing phenotypic plasticity, tubulogenic capacity, and integration capability into developing kidney. *J Am Soc Nephrol* 17: 188–198, 2006
  26. Kitamura S, Yamasaki Y, Kinomura M, Sugaya T, Sugiyama H, Maeshima Y, Makino H: Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. *FASEB J* 19: 1789–1797, 2005
  27. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM: Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418: 41–49, 2002
  28. Buehr M, Nichols J, Stenhouse F, Mountford P, Greenhalgh CJ, Kantachuvesiri S, Brooker G, Mullins J, Smith AG: Rapid loss of oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. *Biol Reprod* 68: 222–229, 2003
  29. Byrne JA, Simonsson S, Western PS, Gurdon JB: Nuclei of adult mammalian somatic cells are directly reprogrammed to oct-4 stem cell gene expression by amphibian oocytes. *Curr Biol* 13: 1206–1213, 2003
  30. Okuda T, Tagawa K, Qi ML, Hoshio M, Ueda H, Kawano H, Kanazawa I, Muramatsu M, Okazawa H: Oct-3/4 repression accelerates differentiation of neural progenitor cells in vitro and in vivo. *Brain Res Mol Brain Res* 132: 18–30, 2004
  31. Tai MH, Chang CC, Olson LK, Trosko JE: Oct4 expression in adult human stem cells: Evidence in support of the stem cell theory of carcinogenesis. *Carcinogenesis* 26: 495–502, 2005
  32. Barasch J, Qiao J, McWilliams G, Chen D, Oliver JA, Herzlinger D: Ureteric bud cells secrete multiple factors, including bFGF, which rescue renal progenitors from apoptosis. *Am J Physiol* 273: F757–F767, 1997
  33. Barasch J, Yang J, Ware CB, Taga T, Yoshida K, Erdjument-Bromage H, Tempst P, Parravicini E, Malach S, Aranoff T, Oliver JA: Mesenchymal to epithelial conversion in rat metanephros is induced by LIF. *Cell* 99: 377–386, 1999
  34. Torres M, Gomez-Pardo E, Dressler GR, Gruss P: Pax-2 controls multiple steps of urogenital development. *Development* 121: 4057–4065, 1995
  35. Lange C, Togel F, Ittrich H, Clayton F, Nolte-Ernsting C, Zander AR, Westenfelder C: Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. *Kidney Int* 68: 1613–1617, 2005
  36. Togel F, Hu Z, Weiss K, Isaac J, Lange C, Westenfelder C: Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol* 289: F31–F42, 2005
  37. Raman JD, Mongan NP, Liu L, Tickoo SK, Nanus DM, Scherr DS, Gudas LJ: Decreased expression of the human stem cell marker, Rex-1 (zfp-42), in renal cell carcinoma. *Carcinogenesis* 27: 499–507, 2006