Adult Kidney Tubular Cell Population Showing Phenotypic Plasticity, Tubulogenic Capacity, and Integration Capability into Developing Kidney

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Using *in vivo* bromodeoxyuridine (BrdU) labeling, a tubular cell population (label-retaining tubular cells [LRTC]) was identified recently in normal adult kidneys, which contributes actively to the regeneration process of the kidney after injury. Here, these LRTC are characterized *in vitro*. The LRTC population was isolated from BrdU-treated rat kidney by FACS. Both LRTC and non-LRTC underwent proliferation and maintained an epithelial phenotype in the presence of tubulogenic growth factors such as EGF, TGF-α, IGF-I, and hepatocyte growth factor. It is interesting that LRTC also proliferated without epithelial markers expression in the presence of soluble factors derived from an embryonic kidney metanephric mesenchyme cell line. The type of extracellular matrix strongly influenced the phenotype of LRTC. Furthermore, in three-dimensional collagen gel culture, LRTC formed tubule-like or tubulocystic structures in response to growth factors (hepatocyte growth factor and fibroblast growth factor) that are known to induce kidney cell tubulogenesis *in vitro* and/or participate in renal regeneration *in vivo*. In contrast, non-LRTC did not form these structures. When transplanted into the metanephric kidney, LRTC but not non-LRTC were integrated into epithelial components of nephron, including the proximal tubular cells and the ureteric bud. They also differentiated into fibroblast-like cells. Collectively, these findings suggest that LRTC are an adult kidney tubular cell population that shows phenotypic plasticity, tubulogenic capacity, and integration capability into the developing kidney.

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The kidney has the capacity for nearly complete regeneration after ischemia/reperfusion or toxic injury (1). After kidney ischemia, there is a marked increase in proliferation of tubular cells. Differentiated tubular cells are thought to dedifferentiate and proliferate after injury. After a surge of cell proliferation, undifferentiated regenerating cells are believed to repopulate the damaged area and then redifferentiate into mature epithelial cells to reconstruct the functional integrity of nephron. Through these steps, it is believed that most damaged tubules regain their essential functions and recover from damage (2). Understanding the cellular events involved in regeneration of renal tubules is indispensable for designing cell-based and other therapeutic strategies for acute kidney injury.

Many studies have evaluated the role of exogenously administered growth factors in enhancing renal recovery and found that several growth factors play important roles in the recovery process, probably acting as mitogens, motogens, and morphogens (3). Hepatocyte growth factor (HGF) has been shown to be a growth factor with potent renotropic action (4–9). Similarly, EGF (10–12), IGF-I (13,14), and bone morphogenetic protein-7 (BMP-7) (15) were demonstrated to be effective in promoting renal regeneration after ischemic injury in rodents. In addition, blockade of endogenous action of activin A by follistatin promoted tubular regeneration after renal ischemia (16,17). Other growth factors, including heparin-binding EGF (18,19), TGF-β (20), and PDGF (21), and leukemia inhibitory factor (LIF) (22) have been suggested to be involved in renal regeneration after renal ischemia. However, the cell population(s) that contribute to the regeneration of the kidney in response to renal injury remain unknown.

We recently provided evidence that a certain tubular cell population plays an important role in tubular regeneration after ischemic injury (23). *In vivo* bromodeoxyuridine (BrdU) labeling methods were used to detect slowly dividing cells in the kidney, because slowly dividing cells are thought to go through cell cycle infrequently to maintain the pool of cells for tissue turnover and repair. After BrdU labeling for 7 d followed by a 2-wk chase period, slowly dividing cells were identified as label-retaining tubular cells (LRTC) in normal rat kidney. In the recovery phase after ischemic injury, LRTC divided into many daughter cells, which actively proliferated and seemed to differentiate into tubular epithelial cells. These findings suggested that LRTC were the main source of regenerating cells after ischemic injury.

Here, we have successfully isolated a population of LRTC ($Hoechst^{low}$/LRTC) from adult rat kidney tubules by FACS.

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Unlike non-LRTC, in a conditioned medium that was derived from a metanephric mesenchymal cell line, LRTC proliferated without epithelial markers expression and also showed different patterns of cell shape and proliferation depending on the type of extracellular matrix (ECM). LRTC demonstrated the potential to form tubule-like structures in collagen gel. In addition, these cells integrated and/or differentiated into several different lineages of renal cells when transplanted into metanephros. These findings suggest that LRTC show phenotypic plasticity, tubulogenic capacity, and integration capability into the developing kidney. These characteristics of LRTC are suitable for acting as progenitor cells in tubular regeneration after injury.

Materials and Methods

Reagents

Recombinant human HGF, BMP-2, BMP-4, BMP-7, EGF, Activin A, TGF-α, TGF-β, glial cell line–derived neurotrophic factor, fibroblast growth factor-1 (FGF-1), FGF-2, and FGF-7 were from R&D Systems (Minneapolis, MN). Recombinant IGF-I and LIF were from Invitrogen (Carlsbad, CA) and Chemicon International, Inc. (Temecula, CA), respectively. BIOCOAT Cell Environment were purchased from Becton Dickinson (Bedford, MA). Mouse pan-cytokeratin antibody, BrdU, type I collagenase, Hoechst 33342, verapamil, propidium iodide (PI), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO). Antibodies used were mouse anti-BrdU antibody (Amersham, Piscataway, NJ), goat anti-human proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti–E-cadherin antibody (BD Biosciences Pharmingen, San Diego, CA), mouse anti-vimentin antibody (Neomarkers, Inc., Fremont, CA), and rabbit anti-zonula occludens-1 antibody (Zymed Laboratories Inc., South San Francisco, CA). Rhodamine-labeled phalloidin was from Molecular Probes (Eugene, OR). FITC-conjugated or rhodamine-conjugated dolichos biflorus (DB) lectin and FITC-conjugated lotus tetragonolobus lectin (LTL) were from Vector Laboratories (Burlingame, CA). FBS was from BioWhittaker (Walkersville, MD). 4′-Diamidino-2-phenylindole (DAPI) was from Calbiochem (San Diego, CA).

In Vivo *BrdU Labeling*

Male Sprague-Dawley rats (7 wk of age; mean weight 240 g) were purchased from Harlan (Indianapolis, IN). LRTC were detected by BrdU labeling as described previously (23) with slight modification. ALZET osmotic pumps (Durect Corp., Cupertino, CA) that contained BrdU (140 mg) were placed in the peritoneal cavity of rats. After 1 wk, osmotic pumps were removed. After the indicated chase periods, the rats were killed and kidneys were removed for histologic analysis. For the isolation of LRTC, the rats with 1 wk of BrdU labeling were killed after a 2-wk chase period. At this dose, the animals seemed healthy with normal kidney histology during the entire course of the experiments. The care and use of animals described in this study conform to the procedures of the laboratory's Animal Protocol approved by the Animal Subjects Program of the University of California, San Diego.

Ischemia/Reperfusion Injury Model

Ischemia/reperfusion injury was induced in rats with 1 wk of BrdU labeling after 2-wk chase as described previously (16). At 12 or 24 h after reperfusion, kidneys were removed and were used for histologic analysis.

Immunohistochemistry

Tissues (adult kidneys or cultured metanephroi) were removed, embedded in a Tissue-Tek OCT compound (Miles, Inc., Elkhart, IN), and frozen in liquid nitrogen. Frozen sections (4 μm) were cut, mounted on poly-L-lysine–coated slides, and fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. Sections then were washed in PBS, incubated in boiling water for 10 min, pretreated with 5% BSA-PBS for 1 h, and covered with primary antibodies at room temperature for 1 h. After washing in PBS, the sections were covered with a mixture of a FITC-labeled rabbit anti-goat IgG antibody and/or Cy3- or FITC-labeled rabbit anti-mouse IgG antibody and DAPI at room temperature for 1 h. Immunofluorescent images were recorded with a Spot RT Slider digital camera attached to a Nikon Eclipse 80i fluorescence microscope.

In case of BrdU staining, boiling water was used to denature DNA for BrdU antibody to gain access to the antigen instead of acid treatment. Quantitative analysis of BrdU-positive cells was performed by counting the BrdU-positive nuclei and all DAPI-positive nuclei in selected fields of cortex ($n = 3$), outer medulla ($n = 3$), and inner medulla ($n = 3$) of the kidneys. The average ($n = 9$) was calculated, and the number of BrdU-positive cells was expressed as a percentage of total cells (which includes tubular cells, glomerular cells, interstitial cells, and capillary endothelial cells) per field.

Isolation of Renal Tubular Cells

The animals were killed with CO_2. The kidneys were removed rapidly, decapsulated, and placed in ice-cold HBSS buffered with 10 mM HEPES (pH 7.4). Cortex slices were minced with a razor and incubated for 30 min at 37°C in HBSS that contained 2 mg/ml collagenase and 1 mg/ml BSA. After the minced cortex slices were passed through a 70-μm filter to remove the glomeruli, the resulting suspension that contained renal tubules was centrifuged and washed twice in HBSS that contained 1 mg/ml BSA. The final pellet was incubated in Trypsin-EDTA solution for 15 min at 37°C. After FBS was added, renal tubular cell suspension was plated and cultured in DMEM-F12 that contained 10% FBS for 48 h before FACS to remove dead cells, blood cells, and debris. Isolated cells were confirmed as renal epithelial tubular cells by immunocytochemical staining (negative for a mesenchymal marker and vimentin and positive for an epithelial marker, E-cadherin or cytokeratin).

FACS

Primary epithelial tubular cells that were isolated as described were incubated with Hoechst 33342 (10 μg/ml) in the presence or absence of verapamil (50 μM) for 1 h at 37°C. After the cells were passed through a 40-μm nylon filter, the cell suspensions were centrifuged, dissolved in PBS that contained 3% BSA and PI (5 μg/ml), and analyzed using a FACS Vantage SE flow cytometer (Becton Dickinson, San Jose, CA). The Hoechst 33342 dye was excited at 350 nm ultraviolet (UV; 100 mW), and the resultant fluorescence was measured at two wavelengths using 424/44 BP and 675 LP filters for detection of Hoechst blue and red, respectively. On the basis of Hoechst 33342 fluorescence intensity, $Hoechst^{low}$ and $Hoechst^{high}$ population were separated, sorted (6800 cells/second), and used for analysis. PI-positive cells and doublets were excluded from collected cell fractions to ensure that only single viable cells were included in the separated cell suspensions.

Immunocytochemistry

Indirect fluorescence immunostaining was performed as described previously (17,24). Briefly, cells that were cultured on coverslips were washed with PBS, fixed with 4% PFA, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS, and then incubated in 3% BSA-PBS at room

temperature for 1 h. Cells then were treated with primary antibodies at room temperature for 1 h. After washing with PBS, cells were incubated with a mixture of Cy3- or FITC-labeled rabbit anti-mouse IgG antibody or Cy3-labeled goat anti-rabbit IgG antibody and DAPI for 1 h at room temperature. Immunofluorescent images were recorded as described above. Quantitative analysis of BrdU-positive cells among the sorted cells was performed by counting the positive nuclei in approximately 100 cells of the Hoechst^{low} or Hoechst^{high} population. Values were expressed as a percentage of total cells (DAPI-positive nuclei; $n = 4$).

Cell Culture and Conditioned Medium

Inner medullary collecting duct (IMCD) cells, metanephric mesenchyme–derived cell line (BSN cells), and ureteric bud–derived cell line (UB cells) were used in this study. Cells were grown in DMEM/F12 supplemented with 10% FBS in an atmosphere of 5% CO_2 and 100% humidity at 37°C for IMCD cells and BSN cells or at 32°C for UB cells. The conditioned medium was collected from these cell lines as described previously (25). Sorted Hoechst^{low} and Hoechst^{high} populations were cultured in DMEM/F12 that contained 5% FBS with the indicated growth factors or in condition media (BSN-CM and UB-CM) that contained 5% FBS at 37°C in an atmosphere of 5% CO_2 and 100% humidity.

Measurement of Cell Proliferation

Cell proliferation was assessed by MTT assay (26). Briefly, after treatment, cells were incubated with MTT for 3 h at a final concentration of 1 mg/ml in the culture medium and then extracted with 2-propanol. MTT incorporation and metabolism to formazan dye is a measure of cell number. The concentration of formazan dye was determined spectrophotometrically at 595 nm.

Tubulogenesis Assay

Collagen gel culture was performed as described previously (27). The cultures were photographed at the indicated periods under phase contrast using a Nikon Eclipse TE300 inverted microscope with a Spot RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI).

Cell Transplantation into Cultured Metanephroi

Embryonic kidneys were isolated from the embryos of pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) at day 15 of gestation (day 0 being the day of appearance of the vaginal plug) under a stereomicroscope and were applied to the top of Transwell filters (0.4 μm pore size; Costar, Cambridge, MA) placed within individual wells of a 12-well tissue culture dish. Hoechst^{low} and Hoechst^{high} populations of cells were labeled with fluorescent dye using CellTracker Orange CMTMR [5-(and-6)-[([4-chloromethyl]benzoyl)amino]tetramethylrhodamine] mixed isomers (Molecular Probes) according to the manufacturer's instruction, counted, and transplanted into E15 rat metanephros by using a fine capillary glass tube under a stereomicroscope. Approximately 100 cells were injected at one time, and each metanephros had two different injection sites. The cell-transplanted metanephros were cultured in DMEM/F12 supplemented with 10% FBS at 37°C and 5% CO_2/100% humidity. After 5 d of culture, cultures were fixed with 4% PFA and used for histologic analysis.

Statistical Analyses

The differences between means were compared by t test, with $P < 0.05$ considered significant.

Results

Involvement of LRTC in Tubular Regeneration after Renal Ischemia

To detect slowly dividing cells in normal rat kidneys, we performed BrdU labeling as described previously (23) with slight modification. Normal rats were labeled with BrdU using an osmotic pump for 7 d, and kidneys were removed at different time points after BrdU labeling. By using this protocol, slowly dividing cells were identified as LRTC, because rapidly dividing cells would have divided many times during the chase period, and incorporated BrdU should have been diluted out. As shown in Figure 1A, there was a marked decrease in the number of BrdU-positive cells after a 2-wk chase period, followed by a slower decline thereafter. As LRTC that are detected after a 2-wk chase period seem to be depleted of rapidly cycling cells, the 2-wk period was operationally chosen as a chase period. Consistent with previous study (23), BrdU-positive cells were localized in glomerular, interstitial capillary endothelial, and tubular cells after 1 wk of labeling (data not shown). In contrast, after a 2-wk chase, BrdU-positive cells were observed only in tubules. Most BrdU-positive cells were detectable in proximal tubules (Figure 1B, left), and some were also localized in distal tubules and collecting ducts but were not detected in glomeruli and capillary vessels (data not shown).

To examine the involvement of LRTC in tubular regeneration, ischemia/reperfusion injury was induced in these rats after a 1-wk BrdU labeling followed by a 2-wk chase period. There was no significant difference in the number of LRTC between normal and sham-operated kidneys (data not shown). Many LRTC were found in clusters of two cells at 12 h after reperfusion (Figure 1B, right). Double-staining was also performed using anti-BrdU antibody and anti-PCNA antibody (Figure 1C). At 24 h after reperfusion, 75.1 ± 5.2% (mean ± SE) of LRTC were positive for PCNA. These BrdU$^+$/PCNA$^+$ cells were mainly localized in proximal tubules. In contrast, 14.4 ± 1.6% (mean ± SE) of PCNA-positive cells that lacked BrdU labeling were observed, suggesting that proliferating cells in the kidney after ischemia are largely derived from LRTC.

Isolation of LRTC from BrdU-Treated Rats by FACS

The fluorescence intensity of the membrane-permeable Hoechst 33342 dye is reduced by the incorporation of BrdU into DNA, because the newly synthesized BrdU-DNA is not stainable by the Hoechst dye, which is highly specific for thymidine. This allows one to separate viable BrdU-positive cells from viable BrdU-negative cells (28). We first tested the effect of BrdU incorporation on the fluorescence intensity of Hoechst dye using a renal tubular cell line, IMCD cells. IMCD cells were cultured in DMEM/F12 media that contained 10% FBS in the absence or presence of BrdU (100 μM) for 48 h and then incubated in the presence of Hoechst dye (10 μg/ml) for 1 h before FACS. As shown in Figure 2A, fluorescence intensity of Hoechst dye in BrdU-treated cells was lower than that in BrdU-untreated cells, indicating that BrdU-positive cells can be selectively sorted as a Hoechst^{low} population by FACS. We then collected LRTC selectively from renal tubules of BrdU-treated rats. Isolated renal tubular cells that were cultured in DMEM-

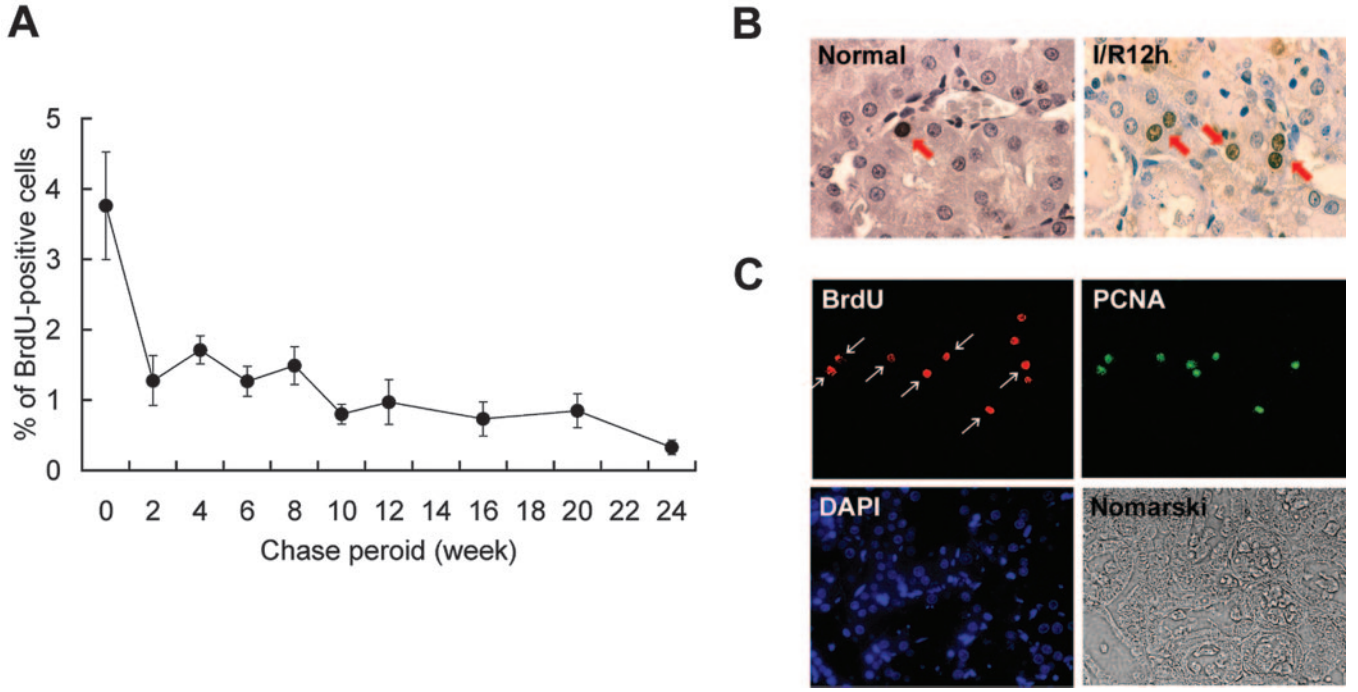


Figure 1. Involvement of label-retaining tubular cells (LRTC) in kidney regeneration after renal ischemia. (A) Quantitative analysis of bromodeoxyuridine (BrdU)-positive cells after different chase periods. Rats were killed at 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 wk after 1 wk of labeling with BrdU. Quantitative analysis of BrdU-positive cells was performed by counting the BrdU-positive nuclei and total nuclei (4′-diamidino-2-phenylindole positive) in the selected fields of cortex ($n = 3$), outer medulla ($n = 3$), and inner medulla ($n = 3$) of the kidneys. The average ($n = 9$) was calculated, and the number of BrdU-positive cells was expressed as a percentage of total cells per field. (B) Localization of LRTC in normal (left) and ischemic (right) kidneys. After 1 wk of BrdU labeling and a 2-wk chase period, ischemia/reperfusion (I/R) injury was induced in these rats, and the kidney was removed at 12 h after reperfusion. BrdU staining was performed followed by counterstaining with hematoxylin. Arrows indicate BrdU-positive cells. (C) Localization of BrdU-positive and proliferating cell nuclear antigen (PCNA)-positive cells in ischemic kidneys. After 1 wk of BrdU labeling and a 2-wk chase period, I/R injury was induced in these rats, and the kidney was removed at 24 h after reperfusion. Double immunostaining was performed using anti-BrdU antibody and anti-PCNA antibody. Arrows indicate BrdU-positive nuclei that are also positive for PCNA. Note that most LRTC were positive for PCNA at the early phase of tubular regeneration. Magnification, ×600 in B; ×400 in C.

F12 that contained 10% FBS for 48 h were trypsinized and then sorted by FACS. Forty to 60% of the cells were viable (PI negative) at this time point. On the basis of Hoechst fluorescence intensity, the primary tubular cell suspension was separated into two populations (Hoechst^{low} and Hoechst^{high}), and both populations were sorted and used for further analysis (Figure 2B). The cell viability after sorting was approximately 70% regardless of Hoechst treatment. Immunocytochemistry for BrdU confirmed that most cells that were derived from Hoechst^{low} population were BrdU positive (Figure 2C). Quantitative analysis showed that 85.7 ± 7.2% (mean ± SE) of cells from the Hoechst^{low} population were BrdU positive, whereas the percentage of BrdU-positive cells in the Hoechst^{high} population was 2.2 ± 1.6% (mean ± SE; Figure 2D), indicating that the Hoechst^{low} population is very similar to the LRTC population *in vivo*. For eliminating the contamination of side population (SP) cells (29,30), which is defined by the property to exclude the Hoechst 33342 dye, cells were incubated with Hoechst 33342 in the presence of verapamil, an inhibitor of Hoechst 33342 transport, in several experiments. There was no significant difference in the percentage of the Hoechst^{low} population with and without verapamil treatment (8.6 ± 4.7 and 9.3 ± 3.4%, respectively; mean ± SE) or in the results between the verapamil-treated Hoechst^{low} population and the verapamil-untreated Hoechst^{low} population, suggesting that the Hoechst^{low} population in this study is different from the SP cell population, although the possibility that verapamil-nonsensitive SP cells might be included within Hoechst^{low} population cannot be excluded.

We also examined the effect of Hoechst 33342 on cell viability without sorting, because Hoechst 33342 affects the viability of several types of cells. At the concentration of 10 μg/ml, the viability of Hoechst-treated cells was 88.9 ± 3.9% (NS, *versus* Hoechst-untreated cells). In contrast, Hoechst dye significantly decreased cell viability at the concentration of >15 μg/ml. We also examined the effect of UV treatment on cell viability of Hoechst-treated cells and BrdU-treated cells, because UV exposure is known to induce DNA damage or apoptosis. The cell viability of Hoechst-treated cells with UV exposure was decreased by 20 ± 5% compared with Hoechst-treated cells without UV. The percentage of viable BrdU-treated cells with UV was 80 ± 4% compared with BrdU-treated cells without UV.

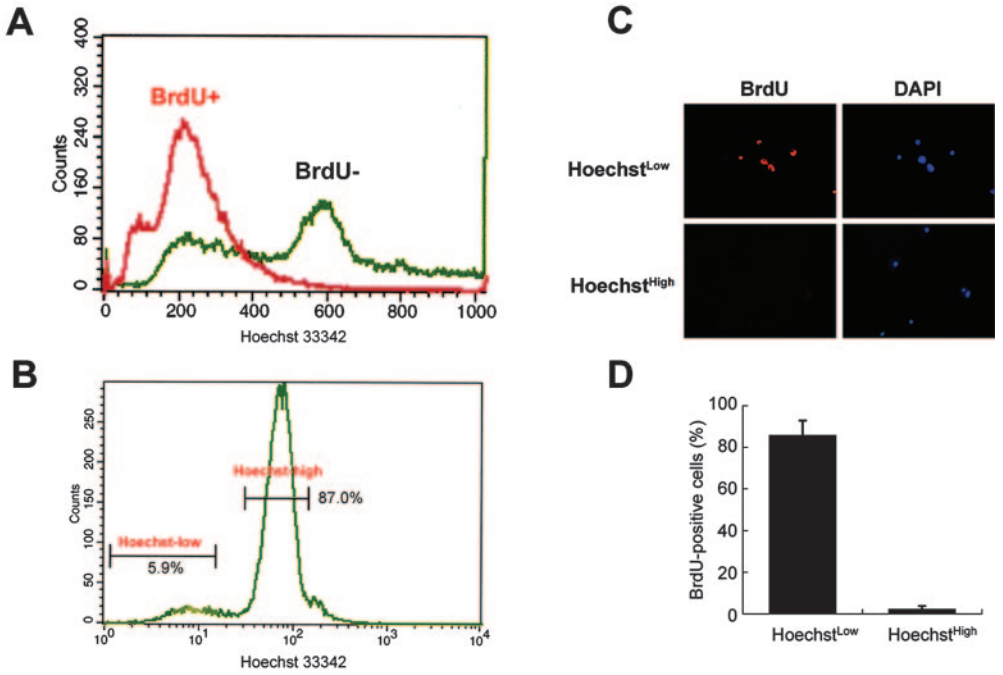


Figure 2. Isolation of LRTC from renal tubular cells of BrdU-treated rats by FACS. (A) Flow cytometric analysis of Hoechst 33342 fluorescence intensity in inner medullary collecting duct (IMCD) cells treated with or without BrdU. IMCD cells were cultured in DMEM/F12 medium that contained 10% FBS in the absence or presence of 100 μM BrdU for 48 h. Cells then were treated with Hoechst dye (10 μg/ml) for 1 h before FACS. (B) Flow cytometric analysis of Hoechst 33342 fluorescein intensity in renal tubular cells that were isolated from BrdU-treated rats. Representative data of 10 separate experiments are shown. (C) BrdU staining of Hoechst^{low} population and Hoechst^{high} population. BrdU (red), nuclei (blue). (D) Quantitative analysis of the number of BrdU-positive cells in the Hoechst^{low} population and Hoechst^{high} populations. The percentage of BrdU-positive cells in total cells is shown as the mean ± SE (n = 4). Magnification, ×400 in C.

UV laser slightly decreased cell viability in the process of FACS. However, there was no significant difference in cell viability between Hoechst-treated cells and BrdU-treated cells after UV exposure.

Proliferation and Differentiation of Isolated LRTC in Monolayer Culture

We examined the effect of various growth factors and conditioned media purified from BSN cells (BSN-CM) and UB cells (UB-CM) on cell proliferation in LRTC. As shown in Figure 3A, EGF, TGF-α, IGF-I, and HGF significantly increased the number of Hoechst^{low}/LRTC as well as Hoechst^{high}/non-LRTC. Other growth factors tested, including TGF-β (1 ng/ml), activin A (200 ng/ml), BMP-2 (200 ng/ml), BMP-4 (200 ng/ml), BMP-7 (200 ng/ml), FGF-1 (250 ng/ml), FGF-2 (250 ng/ml), FGF-7 (250 ng/ml), glial cell line–derived neurotrophic factor (200 ng/ml), and LIF (200 ng/ml), did not induce any significant change in cell number (data not shown). It is interesting that BSN-CM stimulated cell proliferation in Hoechst^{low}/LRTC but not in Hoechst^{high}/non-LRTC. Hoechst^{low}/LRTC survived for >2 mo in BSN-CM (data not shown). UB-CM did not induce significant cell proliferation in both populations (data not shown). A significant difference of growth rate between Hoechst^{low}/LRTC and Hoechst^{high}/non-LRTC was observed only in the presence of BSN-CM.

These growth factors or conditioned media also affected the morphology of Hoechst^{low}/LRTC. In control conditions (DMEM/F12 that contained 5% FBS), Hoechst^{low}/LRTC seemed viable but did not grow significantly (Figure 3Ba). In the presence of HGF, Hoechst^{low}/LRTC formed epithelial cell sheets with typical epithelial dome-like structures (Figure 3B, b and c). In BSN-CM, Hoechst^{low}/LRTC remained spherical and separated, not forming typical epithelial cell islands (Figure 3Bd). In contrast, Hoechst^{high}/non-LRTC did not proliferate in the presence of BSN-CM even though they formed epithelial sheets whenever they proliferated under other conditions (*e.g.*, HGF, TGF-α, EGF, IGF-I; data not shown).

We also examined cell phenotype of Hoechst^{low}/LRTC by immunofluorescent staining (Figure 3C). Some Hoechst^{low}/LRTC were positive for LTL or DB, which are the markers for proximal tubules and collecting duct, respectively. E-cadherin, cytokeratin, F-actin, and zonula occludens-1 staining were apparent in Hoechst^{low}/LRTC when they attached on a plate after sorting. In the presence of HGF, Hoechst^{low}/LRTC formed epithelial sheets and expressed these epithelial markers, indicating epithelialization and formation of intracellular junctions. While growing, they also expressed a mesenchymal marker, vimentin, although they did not express vimentin before HGF treatment. In contrast, expression of these markers was absent in Hoechst^{low}/LRTC when cultured in the presence of BSN-CM. Thus, BSN-CM has a selective proliferative effect on LRTC but, interestingly, does not preserve their epithelial phenotype.

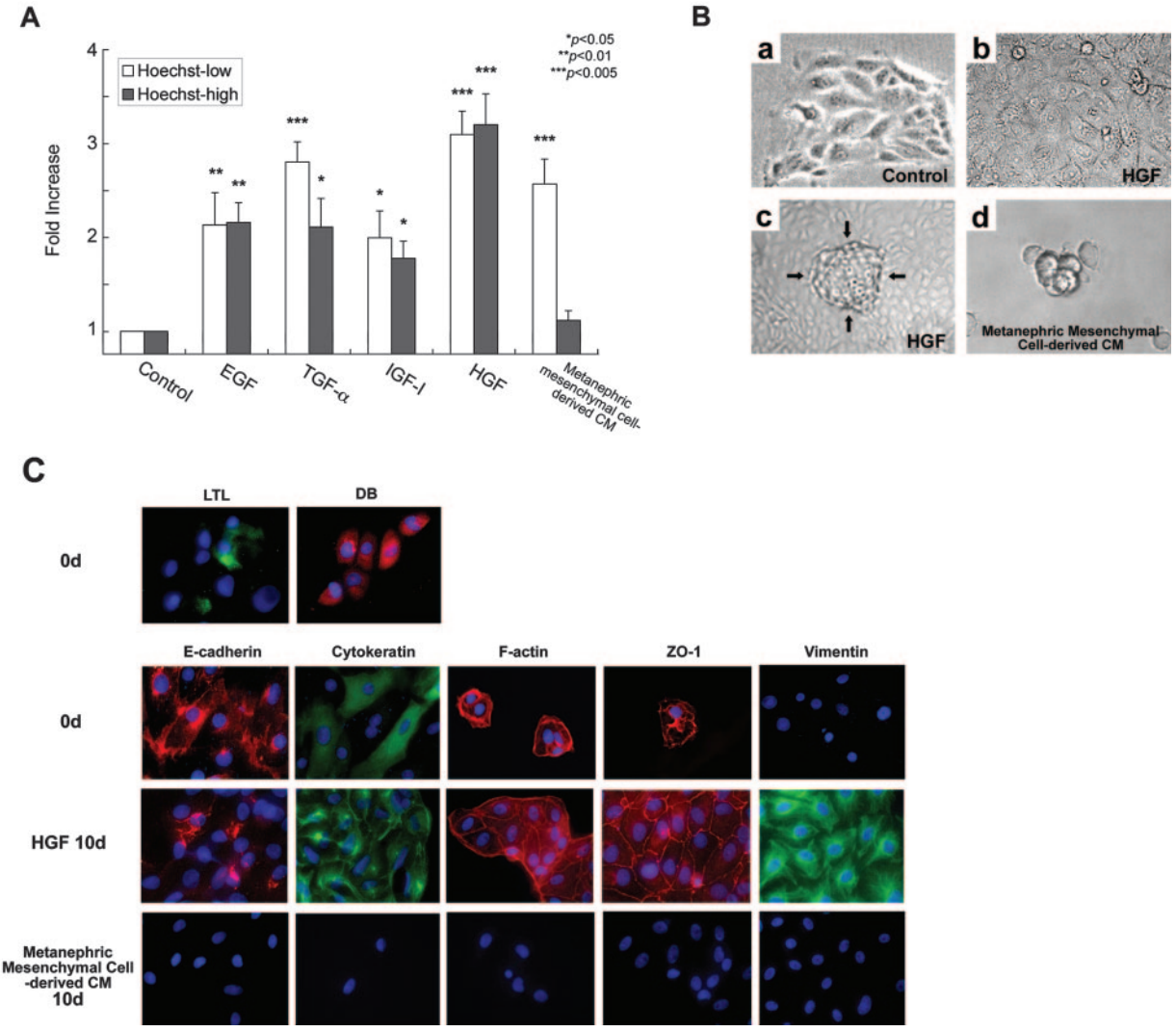


Figure 3. Effect of growth factors and conditioned media on cell proliferation in Hoechst^{low}/LRTC. (A) Quantitative analysis of the effect of growth factors and conditioned media on cell proliferation in Hoechst^{low}/LRTC and Hoechst^{high}/non-LRTC. Hoechst^{low}/LRTC and Hoechst^{high}/non-LRTC were cultured in DMEM-F12 that contained 5% FBS alone or with hepatocyte growth factor (HGF; 100 ng/ml), TGF-α (200 ng/ml), EGF (200 ng/ml), or IGF-I (200 ng/ml) or in a metanephric mesenchymal cell–derived condition medium (CM; 5% FBS) for 10 d. Cell proliferation assay was performed as described in Material and Methods. Values are mean ± SE (n = 5). (B) Morphology of Hoechst^{low}/LRTC in monolayer culture. Hoechst^{low}/LRTC were cultured for 10 d in DMEM-F12 that contained 5% FBS alone (a) or with HGF (100 ng/ml; b and c) or in a metanephric mesenchymal cell–derived CM (5% FBS; d). Note that Hoechst^{low}/LRTC formed dome-like structure (arrows) in the presence of HGF. (C) Expressions of epithelial and mesenchymal markers in Hoechst^{low}/LRTC. Hoechst^{low}/LRTC were fixed in 4% paraformaldehyde (PFA) immediately after they attached to the plate (0 d) or were cultured in DMEM-F12 that contained 5% FBS with HGF (100 ng/ml) for 10 d or in a metanephric mesenchymal cell–derived CM (5% FBS) for 10 d. The expression of E-cadherin, cytokeratin, F-actin, zonula occludens-1 (ZO-1), vimentin, lotus tetragonolobus lectin (LTL) and dolichos biflorus (DB) were examined by immunohistochemistry. E-cadherin, F-actin, ZO-1, DB (red), cytokeratin, vimentin, LTL (green), nuclei (blue). Magnification, ×600 in a, b, and d and ×100 in c in B; ×600 in D.

Effect of ECM on Morphology of LRTC

We examined the effect of ECM on morphology of Hoechst^{low}/LRTC (Figure 4). As shown in Figure 3Bd, Hoechst^{low}/LRTC proliferated and scattered in the presence of BSN-CM on plastic. By changing the ECM coating, these cells showed different morphologies and/or growth patterns. Hoechst^{low}/LRTC became spindle-shaped fibroblastic cells when cultured on type I collagen or laminin. These cells were positive for vimentin (data not shown). On type IV collagen, their phenotype was similar to that on plastic. Fibronectin, however, facilitated clustering without changing their shape. Hoechst^{high}/non-LRTC did not exhibit these ECM responses, at least in the presence of BSN-CM. These data are summarized in Table 1.

Morphology of Isolated LRTC in Three-Dimensional Culture

To characterize further the behavior of LRTC, we performed three-dimensional (3D) tubulogenesis experiments. Hoechst^{low}/

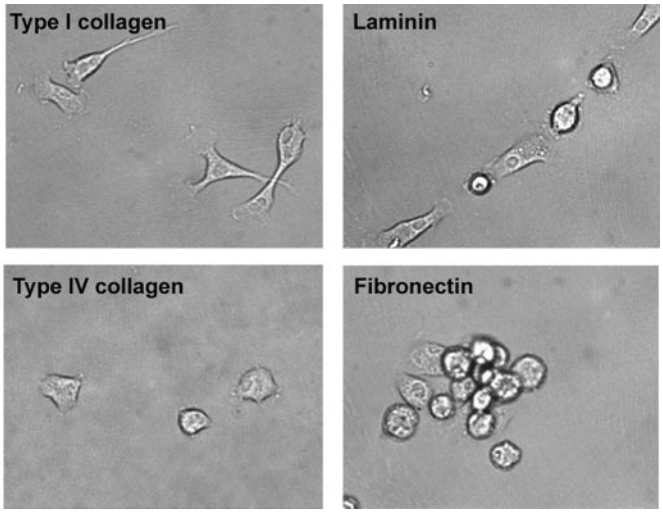


Figure 4. Morphology of Hoechst^{low}/LRTC in monolayer culture (2D) on extracellular matrix. Hoechst^{low}/LRTC were cultured on type I collagen–, laminin-, type IV collagen–, and fibronectin-coated plates for 7 d in the presence of metanephric mesenchyme–derived cell line (BSN)-CM/5% FBS. Note that Hoechst^{low}/LRTC developed in spindle shape when cultured on type I collagen or laminin. Magnification, ×600.

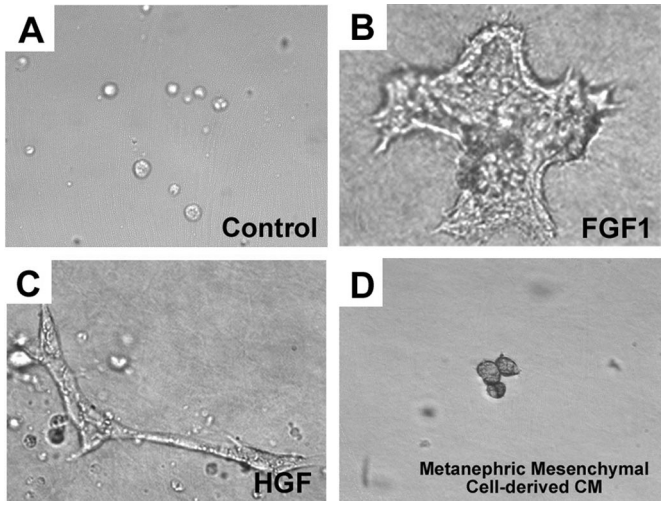


Figure 5. Morphology of Hoechst^{low}/LRTC that were cultured in type I collagen gel. Hoechst^{low}/LRTC were cultured for 7 d in type I collagen gel in DMEM-F12 that contained 5% FBS alone (A) or with fibroblast growth factor-1 (FGF-1; 250 ng/ml; B) or HGF (100 ng/ml; C) or in BSN-CM/5% FBS (D). Magnification, ×400.

LRTC and Hoechst^{high}/non-LRTC were cultured in type I collagen gels in the presence of the indicated growth factors or conditioned media. In control condition (DMEM/F12 that contained 5% FBS), Hoechst^{low}/LRTC did not show any morphologic changes after 7 d of culture (Figure 5A). In the presence of FGF-1, some Hoechst^{low}/LRTC formed cyst-like structures (Figure 5B). FGF-2 and FGF-7 had similar effects on Hoechst^{low}/LRTC as FGF-1 (data not shown). In the presence of HGF, a number of Hoechst^{low}/LRTC formed tubule-like structures with lumens (Figure 5C). UB-CM also induced tubulogenesis in Hoechst^{low}/LRTC (data not shown). In BSN-CM, Hoechst^{low}/LRTC divided several times but did not form any tubular structures (Figure 5D). In contrast, Hoechst^{high}/non-LRTC did not exhibit morphologic changes in collagen gel regardless of the growth factors included (data not shown). We also cultured approximately 2000 cells from the Hoechst-untreated whole tubular cell population in a collagen gel in the presence of HGF. However, there were no tubular structures observed (data not shown). This is probably because Hoechst^{low}/LRTC are a small percentage of whole tubular cells and only a part (not all) of Hoechst^{low}/LRTC have such a tubulogenic activity in the gel. These data are summarized in Table 2.

Integration of Transplanted LRTC into Developing Kidney

Considering that regenerative processes may recapitulate some aspects of the tubulogenic program during development, it is possible that adult kidney–derived LRTC proliferate or differentiate in response to factors that are produced by the embryonic kidney. To examine this issue, we transplanted LRTC into the metanephros. Immediately after the transplantation, fluorescence-labeled Hoechst^{low}/LRTC were found to be scattered in metanephric kidneys (Figure 6A, a and b). Three days later, fluorescence-labeled Hoechst^{low}/LRTC formed a cluster of cells (Figure 6A, c and d) and the number of clusters increased at 5 d after the transplantation (Figure 6A, e and f). Actual fluorescence intensity of Hoechst^{low}/LRTC after 5 d of culture was apparently lower than that immediately after transplantation, suggesting that transplanted Hoechst^{low}/LRTC proliferated in the metanephros (although the difference of fluorescence intensity among the images in Figure 6 is not recognizable, because the signal intensity was enhanced in each

Table 1. Effects of various types of ECM on Hoechst^{low}/LRTC phenotype[a]

	Morphology	Attachment	Separation	Clustering
Control (plastic)	Spherical	−	+	−
Type I collagen	Spherical or spindle shaped (fibroblastic)	+	+	−
laminin	Spherical or spindle shaped (fibroblastic)	+	+	−
Type IV collagen	Spherical	−	+	−
fibronectin	Spherical	−	−	+

[a]ECM, extracellular matrix; LRTC, label-retaining tubular cells.

Table 2. Effects of growth factors and conditioned media on Hoechst^{low}/LRTC and Hoechst^{high}/non-LRTC in two-dimensional or three-dimensional culture[a]

	Hoechst^{low}				Hoechst^{high}			
	Monolayer		In Collagen		Monolayer		In Collagen	
	Proliferation	Epithelial Differentiation	Tubulocystic Structure	Tubular Structure	Proliferation	Epithelial Differentiation	Tubulocystic Structure	Tubular Structure
HGF	+	+	−	+	+	+	−	−
EGF	+	+	−	−	+	+	−	−
TGF-α	+	+	−	−	+	+	−	−
IGF-I	+	+	−	−	+	+	−	−
FGF-1 or FGF-2 or FGF-7	−	−	+	−	−	−	−	−
Metanephric mesenchymal cell line–derived CM	+	−	−	−	−	−	−	−
Ureteric bud cell line–derived CM	−	−	−	+	−	−	−	−

[a]HGF, hepatocyte growth factor; FGF, fibroblast growth factor; CM, condition medium.

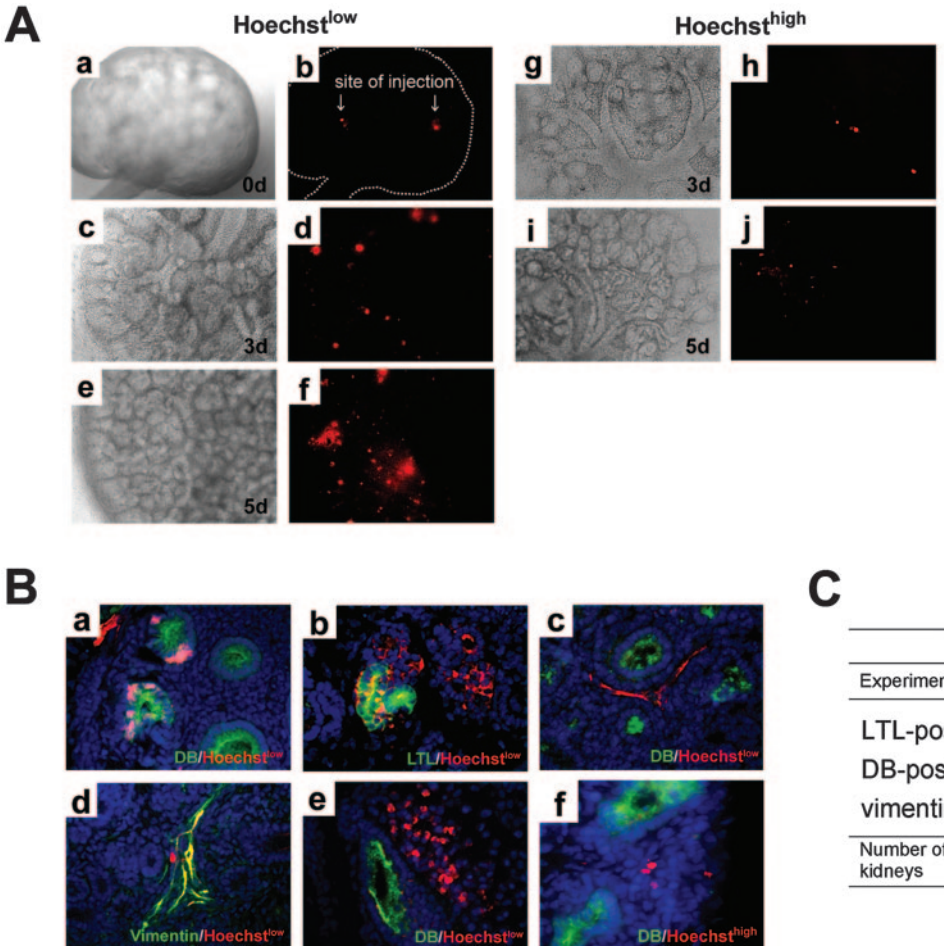
image to visualize the transplanted cells clearly). In contrast, fluorescence-labeled Hoechst^{high}/non-LRTC did not form any cluster of cells in this organ culture system (Figure 6A, g through j).

To characterize these Hoechst^{low}/LRTC-derived structures, tissue sections were stained with several markers of nephron segments (Figure 6B). Approximately 60% of transplanted Hoechst^{low}/LRTC were localized in the interstitium without apparent proliferation or differentiation (data not shown). However, the remaining transplanted cells integrated into various parts of metanephros. Some of them were integrated into ureteric bud, which was identified as a DB-positive structure (Figure 6Ba). Other Hoechst^{low}/LRTC-derived structures were positive for LTL, a marker for proximal tubules (Figure 6Bb), or became spindle shaped, incorporating to the stroma around the UB (Figure 6Bc). Some of these latter cells were vimentin positive (Figure 6Bd). In addition, many transplanted Hoechst^{low}/LRTC had a spherical appearance in the interstitium (Figure 6Be), consistent with their behavior in metanephric mesenchyme (MM) cell–derived conditioned medium. In marked contrast, no transplanted Hoechst^{high}/non-LRTC became incorporated into any tubular structures or proliferated. Most of them were localized as single cells (Figure 6Bf) or vimentin-positive spindle cells (data not shown) in the interstitium. These results indicate that LRTC possess integration capability into the developing kidney when transplanted into the embryonic kidney (Figure 6C).

Discussion

We recently reported the existence of certain renal tubular cells that participate in kidney regeneration. These cells were identified as LRTC and were shown to be a potential source of regenerating cells after renal ischemia (23). Here, we isolated LRTC from adult kidney tubules by separating a Hoechst^{low} population by FACS. These cells could be distinguished by expression of marker genes and by functional assays. We found that (*1*) LRTC proliferate with epithelial marker expression in response to certain growth factors but, unlike non-LRTC, have a unique *in vitro* proliferation and differentiation response to MM cell line–derived soluble factors; (*2*) the growth and differentiation pattern of LRTC is affected by ECM; (*3*) LRTC form cyst-like or tubule-like structures in collagen gels; and (*4*) LRTC are integrated into several types of renal cells when transplanted into embryonic kidney.

During tubular regeneration after injury, the expression of various growth factors is altered (3) and there are drastic changes in the composition of the ECM surrounding renal tubules (31,32). In response to these changes in the microenvironment, a certain tubular cell population is thought to proliferate and eventually differentiate into mature tubular cells to reconstruct a new tubular epithelium. However, it is unknown which tubular cells have an ability to respond to these external stimuli. We demonstrated here that Hoechst^{low}/LRTC respond differently to external cues. Hoechst^{low}/LRTC proliferate with epithelial markers expression in the presence of HGF. In BSN-CM, however, Hoechst^{low}/LRTC also proliferated but did not express any epithelial markers (Figure 3). Thus, some soluble factors from the MM enhance cell survival and proliferation but do not catalyze their epithelialization under these circumstances. In contrast, Hoechst^{high}/non-LRTC–formed epithelial sheets under all conditions induced their proliferation (data not shown). These results suggest that Hoechst^{low}/LRTC exhibit two different patterns of cell proliferation: (*1*) Proliferation with an epithelial phenotype and (*2*) proliferation without an epithelial phenotype. In addition, Hoechst^{low}/LRTC underwent epithelial tubulogenesis in 3D culture in the presence of HGF, but Hoechst^{high}/non-LRTC did not (Figure 5), indicating the tubulogenic capacity of the LRTC. Other growth factors induced a different type of epithelial morphogenesis in 3D culture. For example, FGF-1, FGF-2, or FGF-7 induced intermediate cyst/tubule-like structures in Hoechst^{low}/LRTC, even though these factors did not induce cell proliferation in Hoechst^{low}/LRTC in monolayer culture. In contrast, BSN-CM did not induce any epithelial structures in Hoechst^{low}/LRTC in collagen gel, although it induced cell proliferation in monolayer culture. Because



C

	Hoechst^{low}			Hoechst^{high}		
Experiment No.	1	2	3	1	2	3
LTL-positive	+	+	+	-	-	-
DB-positive	+	+	+	-	-	-
vimentin-positive	+	+	+	-	+	+
Number of embryonic kidneys	6	6	6	6	5	6

Figure 6. Transplantation of Hoechst^{low}/LRTC into metanephric kidney. (A) Fluorescence-labeled Hoechst^{low}/LRTC (a through f) or Hoechst^{high}/non-LRTC (g through j) were transplanted into E15 metanephros, and pictures were taken immediately (a and b), at 3 d (c, d, g, and h), and at 5 d (e, f, i, and j) after transplantation. (a, c, e, g, and i) Phase contrast images. The signal intensity was enhanced in some of the images to visualize the transplanted cells clearly. (B) Fluorescence-labeled Hoechst^{low}/LRTC (a through e) and Hoechst^{high}/non-LRTC (f) were transplanted into E15 rat metanephros. After 5 d of culture on Transwell filter, metanephroi were removed, fixed in 4% PFA, and processed for paraffin section. Sections were stained with FITC-labeled DB (a, c, e, and f) or LTL (b) or vimentin (d). Transplanted cells (red); nuclei (blue); DB, LTL, and vimentin (green). (C) Integration of transplanted cells into embryonic kidney in organ culture. Presence of fluorescence-labeled transplanted cells that were positive for LTL or DB or vimentin in each experiment was assessed. +, detected; −, not detected. Magnification, ×40 in c through j and ×100 in a and b in A; ×600 in B.

Hoechst^{high}/non-LRTC did not develop into 3D structures in collagen gel, these various responses to external cues might be a defining characteristic of LRTC and indicative of considerable phenotypic plasticity depending on their circumstances. In support of this view, LRTC showed different patterns of cell shape and proliferation depending on the type of ECM (Figure 4).

Various growth factors as well as transcription factors involved in kidney development act as regulators of tubular regeneration (3,17,33,34). Thus, there are parallels between tubular regeneration upon injury and kidney development. This prompted us to hypothesize that cell proliferation and differentiation of LRTC are under the control of factors that are produced by embryonic kidney. If so, then the embryonic kidney might provide a hospitable environment for inducing the LRTC to follow distinct morphogenetic pathways. To address this issue, we transplanted Hoechst^{low}/LRTC into embryonic kidney organ cultures and found that Hoechst^{low}/LRTC were integrated into UB- or MM-derived epithelial segments of the nephron or differentiated into fibroblast-like cells (Figure 6). Hoechst^{high}/non-LRTC were not integrated into specific nephron segments. Therefore, integration into different parts of the developing nephron seems to be a property of the LRTC.

In the presence of BSN-CM, Hoechst^{low}/LRTC changed into spherical shape and lost their epithelial cell phenotype but still could be propagated for >2 mo *in vitro* (data not shown). Hoescht^{high}/non-LRTC could not survive in the same condition. Therefore, it is likely that LRTC can be maintained in a relatively immature state in the presence of certain MM factors. Among the factors that are produced by metanephric mesenchyme, there might be new renotropic factors that can increase the number of renal progenitor cells *in vivo*. Considering that regenerating cells after renal injury have an immature mesen-

chymal phenotype (1,17,33,34), LRTC conceivably could be a cell population that has similar properties to the metanephric mesenchyme.

At the later stages of kidney development, whereas other tubular cells stop dividing as they mature, LRTC may continue to divide slowly, even in the adult kidney. After renal injury, there are striking changes (likened to those of kidney development) in the external environment surrounding LRTC, including the release of growth factors, changes in the ECM composition, and loss of cell–cell contact. In response to these external cues, LRTC and non-LRTC alike proliferate rapidly to replace dead cells. However, LRTC may also migrate to specific environments and undergo complex 3D morphogenesis in response to particular growth factors, eventually leading to the formation of new renal tubules. The phenotypic plasticity, tubulogenic capacity, and integration capability (into the developing nephron) of LRTC that we have observed *in vitro* is consistent with this type of participation in the regeneration process.

We isolated Hoechst^{low}/LRTC by FACS according to a method described previously (28), in which 20 μg/ml Hoechst dye was used to isolate BrdU-positive cells from muscle by FACS, and both the Hoechst^{low} and Hoechst^{high} populations were shown to be viable after sorting. However, it also was demonstrated previously that Hoechst 33342 dye has cytotoxic effects on cultured lymphocytes (35). This raises the possibility that Hoechst^{high}/non-LRTC failed to form tubular structures or integrate into the developing nephron because of Hoechst toxicity. For addressing this issue, primary tubular epithelial cells that were isolated from normal rats were incubated with BrdU and were treated with or without Hoechst dye. We found that Hoechst dye itself did not significantly affect cell viability of primary renal tubular cells at the concentration used here. We also observed that the cell viability of both BrdU-treated cells and Hoechst-treated cells were slightly reduced (10 to 20%) by the exposure of UV laser during FACS. However, there was no significant difference between the two populations, suggesting that cell viability of Hoechst^{low} (BrdU positive) cells and Hoechst^{high} (BrdU negative) cells were equally affected by cell sorting and UV laser. Therefore, it seems unlikely that Hoechst^{high}/non-LRTC population did not grow in the gel or in the embryonic kidney because of Hoechst toxicity in our experiments. In support of this view, both Hoechst^{low}/LRTC and Hoechst^{high}/non-LRTC were able to grow similarly in the presence of growth factors such as HGF (Figure 3A). Perhaps the cytotoxic effect of Hoechst dye is different depending on the cell type as reported previously (36).

Oliver *et al.* (37) recently reported the presence of label-retaining renal stem cells in normal kidney using BrdU labeling/chase method. Whereas LRTC that were defined by our protocol were localized in tubules, the label-retaining cells that were identified by Oliver *et al.* were detected in the interstitium of renal papilla. This discrepancy might be due to the difference of the experimental protocol, especially the age of animals used. Whereas, in this study, LRTC were detected in 7-wk-old rat kidneys after BrdU labeling for 1 wk and a 2-wk chase, in the other study, neonatal kidneys were labeled with BrdU for 3 d and were chased for 2 mo. Unlike adult kidneys, neonatal kidneys are still developing, and most cells that constitute the nephrogenic zone are highly proliferative at this stage (38). The localization of population of label-retaining cells may be different at each stage of kidney development.

The Hoechst^{low}/LRTC population that was defined by our protocol is heterogeneous in terms of both its origin and its purity. LRTC were localized in several different nephron segments (Figure 1), and sorted Hoechst^{low}/LRTC contained not only LTL-positive but also DB-positive cells (Figure 3C). The possibility that only a certain clonal population within the Hoechst^{low}/LRTC has the unique cell properties described above cannot be denied. For addressing this issue, culture conditions for clonal expansion of the cells that were derived from Hoechst^{low}/LRTC that we describe will need to be determined. Thus, although these cells (or a subpopulation of them) may possess certain characteristics that are said to be associated with "stemness," at present, we suggest only their potential relevance to tubular regeneration in the context operationally defined by our experimental protocol.

Acknowledgments

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