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Hematopoietic and nonhematopoietic potentials of Hoechst^{low}/side population cells isolated from adult rat kidney

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Background. Although the regenerative stem cell is expected to exist in many adult tissues, the cell contributing to the regeneration of the kidney remains unknown in its type and origin.

Methods. In this study, we isolated cells that show low stain with a DNA-binding dye Hoechst 33342 (Hoechst^{low} cells) from adult rat kidney, and investigated their differentiation potentials.

Results. Hoechst^{low} cells, generally termed side population cells, existed at a frequency of 0.03% to 0.1% in the cell suspension of the digested kidney. Analysis of the kidney-derived Hoechst^{low} cells after bone marrow transplantation indicated that some of the cells were derived from bone marrow. When enhanced green fluorescent protein (EGFP)-labeled kidney-derived Hoechst^{low} cells were intravenously transplanted into wild-type adult rats, EGFP$^+$ cells were not detected in the kidney, but EGFP$^+$ skeletal muscle, EGFP$^+$ hepatocytes and EGFP$^+$ bone marrow cells were observed. Even after the induction of the experimental glomerulonephritis and gentamicin-induced nephropathy that promote the differentiation of bone marrow–derived cells into repopulating mesangial cells and tubular component cells, respectively, EGFP$^+$ mesangial or tubular cells were not observed. Neither with an in vitro system, which we established to produce mesangial-like cells from crude bone marrow culture, did Hoechst^{low} cells yield mesangial-like cells.

Conclusion. These findings implicate that Hoechst^{low} cells in the kidney may have potentials for hematopoietic and nonhematopoietic lineages, but are not stem cells for renal cells, especially mesangial and tubular cells.

Cells with developmental pluripotency and self-renewal capacity, which are referred to as stem cells, attract the intense and increasing attention because of their biologic properties and potential medical importance. One of the most significant discoveries in this field is that olfactory bulb and hippocampus of the adult brain show ongoing neurogenesis, and that neural stem cells are isolated from the spinal cord, the neural crest, and the subgranular zone in the dentate gyrus of the adult hippocampus [1, 2]. The existence of stem cells is also found in intestine [3], gonad [4], liver [5], and skin [6]. Although it is a general concept that stem cells regenerate only a subset of differentiated cell types in a given tissue, the overall developmental potentials of a stem cell seem to be much wider than expected; different environmental cues determine different repertoires of potential stem cell fates. For example, muscle-derived cells can generate blood cells [7, 8] and brain-derived cells can generate blood cells [9, 10] or muscle [11, 12].

The major part of the kidney originates from the intermediate mesoderm via the transformation from mesenchyme to epithelium. This part comprises two fundamental components: the plasma ultrafiltration unit, glomerulus, and the renal tubular epithelium. The glomerulus is damaged in glomerular diseases such as glomerulonephritis and diabetic complication. However, even chronic histologic lesions due to diabetes can be improved through normalized glucose metabolism achieved by successful pancreas transplantation [13]. Recently, we reported that bone marrow that carries mesenchymal stem cells can give rise to mesangial cells in vivo in response to acute mesangial injury [14]. We have also found that bone marrow–derived cells in culture can be converted to mesangial-like cells under the specialized conditions [15]. The renal tubular epithelium, the site susceptible for drug-related nephrotoxicity and ischemic injury, is often reversible after appropriate therapeutic strategies. Therefore, one can proceed on the assumption that a type of stem cells that possess the differentiation and self-renewal capacity contributes to the regeneration of the kidney or that differentiated cells reenter the cell

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cycle and subsequently contribute to the preponderance of regeneration. It is also likely that both systems work during the regeneration process, which seems to be analogous to the phenomenon observed in injured liver [5, 16].

The problem to pursue stem cells for the adult kidney is that little is known about markers for such cells. Recently, a fraction of cells showing low stain with a DNA-binding dye Hoechst 33342 has been closely watched as a candidate of stem cells. These cells, generally referred to as Hoechst^{low} cells or side population cells, were originally identified in murine bone marrow by the characteristics of the differential ability to efflux Hoechst 33342 [17]. The Hoechst^{low}/side population cells are mostly CD34$^-$Lin$^-$, but have hematopoietic stem cell activity [18]. Bone marrow Hoechst^{low}/side population cells are converted to cardiomyocytes in vivo [19], and Hoechst^{low}/side population cells isolated from skeletal muscle yield hematopoietic lineages and skeletal muscle [7, 20]. These results suggest that a fraction of Hoechst^{low}/side population cells includes stem cells with multipotency and that Hoechst 33342 dye efflux may be one of the universal phenotypes of stem cells.

In this study, we isolated Hoechst^{low}/side population cells from adult rat kidney, examined their property as stem cells and revealed their relationship with bone marrow.

METHODS

Animals

Sprague-Dawley rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and were maintained in a specific pathogen-free environment at the animal facility of Osaka University School of Medicine. They were allowed to get free access to standard laboratory diet and tap water. All rats weighing 150 to 200 g, anesthetized by intraperitoneal administration of pentobarbital, were subjected to the experiments. All the procedures described here were approved by the Animal Committee of Osaka University School of Medicine. Transgenic Sprague-Dawley rats carrying the enhanced green fluorescent protein (EGFP) transgene (EGFP rat) were used as described previously [14].

Preparation for cell suspension of rat kidney

Rats were anesthetized and abdominal aorta was ligated below the diaphragm. Then, rats were perfused via the abdominal aorta, first with 100 mL of solution I [Hank's balanced salt solution (HBSS) (Gibco; Invitrogen Corp., Carlsbad, CA, USA)/10 mmol/L Hepes · NaOH (pH7.5)/5 mmol/L ethylene glycol tetraacetate (EGTA)], and second with 10 mL of solution II [medium199 (Gibco)/2% fetal calf serum (FCS)/10 mmol/L Hepes · NaOH (pH 7.5)/0.05% collagenase type XI (Sigma)]. Kidneys were harvested, and the capsules were peeled off in a sterile manner. Then a small piece of the tissue was saved for the following histologic evaluations. The rest of the tissue was minced into coarse slurry with a razor blade and digested in 20 mL of the solution II for 30 minutes at 37°C. The resultant digest was passed through 70 μm of nylon mesh and centrifuged at 70*g* for 4 minutes at 4°C. Then the pellet was resuspended in 6 mL of phosphate-buffered saline (PBS)/2% FCS/2 μg/mL gentamicin, overlaid on 6 mL of Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) and centrifuged at 800*g* for 20 minutes at room temperature. A cell fraction, located at the interphase, was collected and washed three times with staining medium [phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Gibco)/2% FCS/10 mmol/L Hepes · NaOH (pH 7.5)/2 μg/mL gentamicin] at 700*g* for 10 minutes at 4°C.

Hoechst staining and cell sorting

The cells obtained above were resuspended at 1×10^6 cells/mL in the staining medium containing 5 μg/mL of Hoechst 33342 (Molecular Probes, Eugene, OR, USA), and incubated at 37°C for 90 minutes under the protection from light in the absence or the presence of 50 μmol/L of verapamil (Eisai Co., Ltd., Tokyo, Japan) [17]. Hoechst^{low}/side population cells were visualized and collected by a flow cytometer, FACS Vantage (Becton Dickinson, San Jose, CA, USA) as described previously [17] with a slight modification. As optical filters for blue fluorescence and red fluorescence, a 424/44 band pass filter and a 660/20 band pass filter were used respectively. A 610 short pass dichroic mirror was used to separate Hoechst red from Hoechst blue. Green fluorescence of EGFP was detected through a 530/30 band pass filter.

Just for the confirmation of the viability, kidney cells already stained with Hoechst 33342 were further stained with 2μg/mL of propidium iodide (Sigma).

Characterization of kidney-derived Hoechst^{low}/side population cells

Kidney cells, which were derived from wild-type rats and already stained with Hoechst 33342, were also stained with mouse anti-CD45 antibody (Chemicon International, Inc., Temecula, CA, USA) in combination with fluorescein isothiocyanate (FITC)-conjugated secondary antibody and were analyzed with FACS Vantage. Moreover, kidney-derived Hoechst^{low}/side population cells from wild-type rats were collected with FACS Vantage and their RNA was extracted with Trizol™ Reagent (Invitrogen Japan K.K., Tokyo, Japan). Then we performed reverse transcription (RT) with SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen Japan K.K.), and further performed polymerase chain reaction (PCR) with Gene Amp PCR System 9700 (Applied Biosystems Japan, Ltd., Tokyo, Japan). The pairs of primers used were

as follows: ABCG2, 5′-gccacatgattcctccacagtcccca-3′ and 5′-gctgtcctggcttcagtactttagca-3′; MDR1, 5′-ggaact ctcgctgctatcatccacggaac-3′ and 5′-acctggatgtaggcaacgat gagcacacc-3′; c-Kit, 5′-aagccgaggccactcacacgggcaaat-3′ and 5′-ccaaccaggaaaagtacggcaggatctc-3′; Sca1, 5′-cactgt ccgcagggctggattactcc-3′ and 5′-gataggtgggcgtactgcacagg agac-3′; CD45, 5′-cagcacaacattagtctcctgggctgagc-3′ and 5′-cagggccatttcgttgcaccctcccaata-3′; and Thy1, 5′-atgtccc gaggacagagggtgatcagc-3′ and 5′-cgtgcttcttcttctctcgggtca ggc-3′. The PCR conditions were as follows: all samples were heated up to 94°C for 2 minutes, and then amplified for 35 cycles consisting of 94°C for 30 seconds, a 30-second annealing step at a primer-specific annealing temperature, and 72°C for 40 seconds. All reactions were then incubated at 72°C for 3 minutes and cooled to 4°C. The annealing temperatures were 60°C as to ABCG2, c-Kit, and Sca1 and 64°C as to MDR1, CD45, and Thy1. The RT-PCR products were electrophoresed on a 2% agarose gel with GelStar Nucleic Acid Stain (Cambrex Corporation, East Rutherford, NJ, USA) or ethidium bromide, and were photographed.

Kidney-derived Hoechst^{low}/side population cells were collected from wild-type rats, and attached to slide glass with Cytospin 4 (Thermo Shandon, Cheshire, England). Then they were fixed with 4% paraformaldehyde/PBS for 20 minutes and permeabilized with 0.1% NP-40/PBS for 20 minutes and further stained with either of rabbit antirat aquaporin 2 affinity-purified polyclonal antibody (Chemicon International Inc.) and anti-RECA1 antibody (Cosmobio Co., LTD., Tokyo, Japan), followed by appropriate FITC-conjugated secondary antibodies. Each procedure was followed by twice of PBS washes. As control samples, collagenase-digested whole kidney cells were also treated with the same procedures.

Bone marrow transplantation

Bone marrow transplantation was performed as described previously with EGFP rats and wild-type rats as donors and recipients, respectively [14]. Six weeks after bone marrow transplantation, the cell suspension was prepared from the kidney as described above.

Transplantation of Hoechst^{low}/side population cells and induction of experimental glomerulonephritis or tubular injury

Hoechst^{low}/side population cells which were prepared from kidneys of EGFP transgenic male rats were transplanted into wild-type female rats that had been subjected to 5 Gy of total body irradiation 1 day before the transplantation. Hoechst^{low}/side population cells were resuspended in 1 mL of the staining medium without Hoechst dye and were intravenously administered into a recipient via the tail vein. The number of Hoechst^{low}/side population cells transplanted was 3000 to 8000 cells per recipient rat. Hereafter, this rat was designated as a side population–transplanted rat. To prevent possible acute graft-versus-host disease (GVHD) or rejection, cyclosporine A (Novartis Pharma K.K., Tokyo, Japan) was subcutaneously administered to the recipient rats at a dose of 1.5 mg/kg/day for 7 days following the transplantation. To three side population–transplanted rats, anti-Thy1 antibody-mediated experimental glomerulonephritis (Thy1 nephritis) was induced 5 weeks after the transplantation as described previously [14]. To other three side population–transplanted rats, 60 mg/kg/day of gentamicin (Sigma) was subcutaneously administered for 14 consecutive days approximately 6 to 8 weeks after the transplantation to evoke gentamicin-induced nephropathy in a modified manner as reported [21].

Analysis of side population–transplanted rats

Ten weeks after the transplantation, the side population–transplanted rats were anesthetized and were perfused with 200 mL of the solution I. After one set of the tibia and femur from each rat was set aside for the following flow cytometric analysis of the bone marrow, several organs, including kidney, liver, and skeletal muscle, were taken and fixed in 10% buffered formalin for 3 hours at 4°C in the dark. Immunohistochemical analysis of frozen sections was performed as described previously [14] by the following primary antibodies: mouse anti-CD45 antibody, mouse anti-RECA1 antibody, rabbit antilaminin antibody (Monosan, Uden, The Netherlands) or purified IgG fraction of polyclonal rabbit antiserum to rat albumin (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) in combination with Texas Red–conjugated secondary antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR, USA). Sections were observed under a fluorescence microscope (Nikon Eclipse E600) (Nikon, Tokyo, Japan) with appropriate filters, and all images were captured by a digital imaging system connected to a Macintosh computer. Bone marrow cells, which were harvested by the flush of the unfixed tibia and femur with heparinized PBS/2% FCS/2 μg/mL gentamicin, were subjected to flow cytometric analysis. The kidney was evaluated both with and without the induction of Thy1 nephritis or gentamicin-induced nephropathy, but the other organs mentioned above were evaluated without the induction. The rats burdened with Thy1 nephritis and gentamicin-induced nephropathy were analyzed 5 weeks after the administration of Thy1, and 4 to 8 weeks after the last administration of gentamicin, respectively. Their kidneys were also evaluated by periodic acid-Schiff (PAS) stain.

Culture of Hoechst^{low}/side population cells

In order to test the differentiation capability of Hoechst^{low}/side population cells which were prepared from EGFP rat kidneys, an in vitro differentiation assay system which we established to convert bone

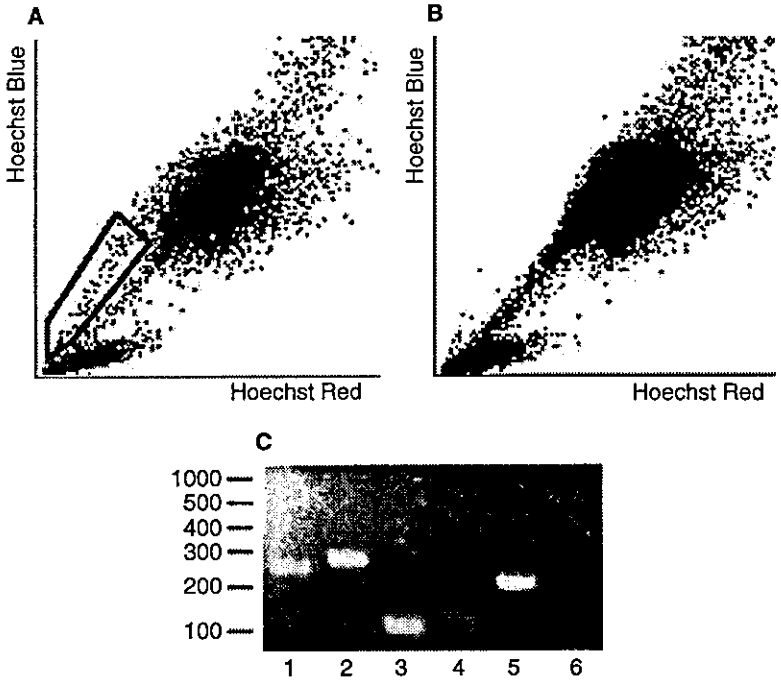

Fig. 1. Hoechst 33342 fluorescence of kidney-derived cells and reverse transcription-polymerase chain reaction (RT-PCR) analysis of kidney-derived Hoechst^{low}/side population cells. Cells prepared from wild-type adult rat kidneys were stained with Hoechst 33342 in the absence (*A*) or presence (*B*) of verapamil and were analyzed by flow cytometry. The boxed region indicates Hoechst^{low}/side population cells which are sensitive to verapamil. Signal detectors were set in the linear mode, and the voltages were adjusted to place the major population at the center of the chart. (*C*) RT-PCR analysis of kidney-derived Hoechst^{low}/side population cells. Lane 1, ABCG2; lane , MDR1; lane 3, c-Kit; lane 4, Sca1; lane 5, CD45; and lane 6, Thy1. Molecular sizes (base pairs) are indicated on the left side.

marrow cells into mesangial-like cells was employed with a slight modification [15]. In short, 1 day prior to the preparation of Hoechst^{low}/side population cells from EGFP rat kidneys, bone marrow cells were harvested from wild-type rats. The bone marrow cells were resuspended in growth medium [DMEM/10% FCS/10% horse serum (Gibco)/0.5% chick embryo extract (Gibco)/4% penicillin-streptomycin (ICN, Aurora, OH, USA)], and were placed on collagen type I-coated dishes at 37°C in 5% CO_2 for 24 hours. Nonadherent cells were collected, mixed with the Hoechst^{low}/side population cells from the EGFP rat kidneys, and were cultured on collagen type IV-coated dishes at 37°C in 5% CO_2. After 2 days, the medium was changed to differentiation medium [DMEM/2% horse serum (Gibco)/4% penicillin-streptomycin (ICN)/1 μmol/L of all-*trans* retinoic acid (Sigma)/200 ng/mL of platelet-derived growth factor-BB (PDGF-BB) (R&D Systems, Minneapolis, MN, USA)]. After 6 days, the cells were fixed for 5 minutes in ice-cold methanol and then washed with PBS three times. They were stained with mouse anti-Thy1 antibody or rabbit antidesmin antibody (Bio-Science Products AG, Emmenbruecke, Switzerland) separately, which was followed by the staining with Texas Red–conjugated secondary antibodies.

RESULTS

Existence of Hoechst^{low}/side population cells in the adult rat kidney

Hoechst^{low}/side population cells were reported to be a small yet distinct population in murine whole bone marrow by the observation of Hoechst 33342 dye fluorescence at two emission wavelengths, red and blue [17]. A similar scattergram was also reported from cell suspension prepared from murine skeletal muscle [7]. In our experiment, when cell suspension prepared from the adult rat kidney was stained with Hoechst 33342 according to the protocol for murine bone marrow Hoechst^{low}/side population cells, an almost identical pattern was seen (Fig. 1A). It is known that the low Hoechst fluorescence of bone marrow–derived or skeletal muscle–derived Hoechst^{low}/side population cells was due to high multidrug resistance protein (*mdr*) or *mdr*-like activity that actively pumps the dye out of cells [17]. Consistently, the Hoechst^{low}/side population region in Fig. 1A mostly disappeared when the staining was performed in the presence of an inhibitor of *mdr*, verapamil (Fig. 1B). The frequency of Hoechst^{low}/side population cells obtained from the adult rat kidney was 0.03% to 0.1% in our preparation. Collagenase treatment was essential to obtain Hoechst^{low}/side population cells from the kidney, and Hoechst^{low}/side population cells were always recovered from the 70*g* precipitate, not from the 70*g* supernatant (data not shown). When the kidney Hoechst^{low}/side population cells were stained with propidium iodide, 80% to 90% of them were negative, indicating that most of them were healthy and alive. When analyzed by RT-PCR, kidney-derived Hoechst^{low}/side population cells from wild-type rats were ABCG2 (+), MDR1 (+), c-Kit (+), Sca1 (+), CD45 (+), and Thy1 (−) (Fig. 1C). Because of technical limitations, we could not visualize the existence of cells which did not express ABCG2, MDR1, c-Kit, Sca1, or CD45. When

kidney-derived Hoechst^{low}/side population cells were stained with rabbit antirat aquaporin 2 affinity-purified polyclonal antibody, the positive rate was 4.3%, while that of the collagenase-digested whole kidney cells was 2.9%. When kidney-derived Hoechst^{low}/side population cells were stained with anti-RECA1 antibody, the positive rate was 6.7%, while that of the collagenase-digested whole kidney cells was 8.2%.

The relationship between bone marrow and kidney

In murine bone marrow, almost all hematopoietic stem cells are enriched in the Hoechst^{low}/side population region [17]. Hoechst^{low}/side population cells that are obtained from skeletal muscle show hematopoietic activity in irradiated mice [7]. Therefore, we investigated whether Hoechst^{low}/side population cells in the kidney were linked with bone marrow. Blood cells in the vasculature of the kidney were completely washed out by the perfusion prior to the collagenase treatment, which was confirmed by histologic analysis (data not shown). As shown in Figure 2A to C, the Hoechst^{low}/side population cells prepared from rats into which bone marrow of EGFP rats was transplanted in advance contained both EGFP$^+$ cells and EGFP$^-$ cells. The EGFP$^+$ cells were approximately 10% to the total Hoechst^{low}/side population cells. The EGFP$^+$ cells broadly scattered within the Hoechst^{low}/side population region (Fig. 2A and B) yet were confined in the area with small forward scatter (FSC) and side scatter (SSC) values, both of which reflect the size and the complexity of a cell, respectively (Fig. 2C and D). In contrast, the rest of Hoechst^{low}/side population cells that are EGFP$^-$ comprised more heterogeneous population as long as evaluated by FSC and SSC (Fig. 2E). Non-Hoechst^{low}/side population cells in the preparation also contained EGFP$^+$ cells with various values of FSC and SSC (Fig. 2F and G). The CD45 positive rate of Hoechst^{low}/side population cells prepared from wild-type kidneys was approximately 1% to 3%. These results indicate that a part of Hoechst^{low}/side population cells in the kidney is derived from bone marrow.

Transplantation of kidney-derived Hoechst^{low}/side population cells

To investigate the differentiation ability of kidney-derived Hoechst^{low}/side population cells in vivo, we prepared EGFP$^+$ Hoechst^{low}/side population cells from EGFP rat kidneys and transplanted the cells to wild-type rats that were irradiated at a dose of 5Gy prior to the transplantation. Then, transplanted cells were tracked by EGFP as a tag. On average, two adult rats were used to prepare the cell suspension sufficient for sorting, for approximately 7 to 8 hours, 8000 Hoechst^{low}/side population cells at a flow rate of 1000/sec. The collected cells were combined and transplanted to a single recipient.

In the side population–transplanted rats, approximately 0.03% of the bone marrow cells expressed EGFP (Fig. 3A). The signal intensity of EGFP was almost similar to that obtained from the bone marrow of EGFP rats (Fig. 3B). When bone marrow cells with the strongest expression of EGFP were analyzed in FSC-SSC charts, side population–transplanted rats and EGFP rats showed similar distribution (Fig. 3C and D). These results suggest that kidney-derived Hoechst^{low}/side population cells were engrafted in the recipient's bone marrow and produced their progeny, even though their developmental stages and lineages were not analyzed.

Some of the skeletal muscle fibers of side population–transplanted rats clearly expressed EGFP (Fig. 4A). The expression of EGFP is restricted within the basal lamina that is visualized with laminin staining. In addition, some of hepatocytes of side population–transplanted rats expressed EGFP in a cytoplasmic pattern (Fig. 4C, arrow). The EGFP$^+$ cells were also positive to albumin, a marker for the hepatocyte (Fig. 4E to G). These results indicate that kidney-derived Hoechst^{low}/side population cells contributed to the formation of skeletal muscle and liver in vivo in the absence of apparent tissue injuries.

In the kidney of side population–transplanted rats, proximal tubules showed green fluorescence that was brighter than autofluorescence observed in wild-type rats and than fluorescence observed in distal tubules or collecting ducts of side population–transplanted rats (Fig. 5A and B). The green fluorescence in proximal tubular epithelial cells of side population–transplanted rats was not in a cytoplasmic pattern but in a granular pattern (Fig. 5C). No other cells expressing EGFP could be observed either in intraglomerular region or in interstitial region. In our previous experiments, we demonstrated that bone marrow can give rise to mesangial cells in vivo in response to mesangiolysis [14]. We examined whether transplanted Hoechst^{low}/side population cells were capable of differentiating into mesangial cells in vivo, because Hoechst^{low}/side population cells in the kidney might be the origin of bone marrow–derived mesangial cells. However, transplanted Hoechst^{low}/side population cells expressing EGFP were rarely observed in glomeruli regardless of the induction of Thy1 nephritis (Fig. 5D and E).

By using another disease model, we examined whether transplanted Hoechst^{low}/side population cells were capable of differentiating into tubular epithelial cells in vivo. In the gentamicin nephrotoxicity model following bone marrow transplantation, bone marrow–derived EGFP$^+$ cells were occasionally yet clearly observed as a tubular component. These cells were observed in Tamm-Horsfall protein–negative segments (Fig. 5H, arrow). The frequency of the engraftment to tubular component cells was 0.42% in cortex, 0.13% in corticomedullary junction, and

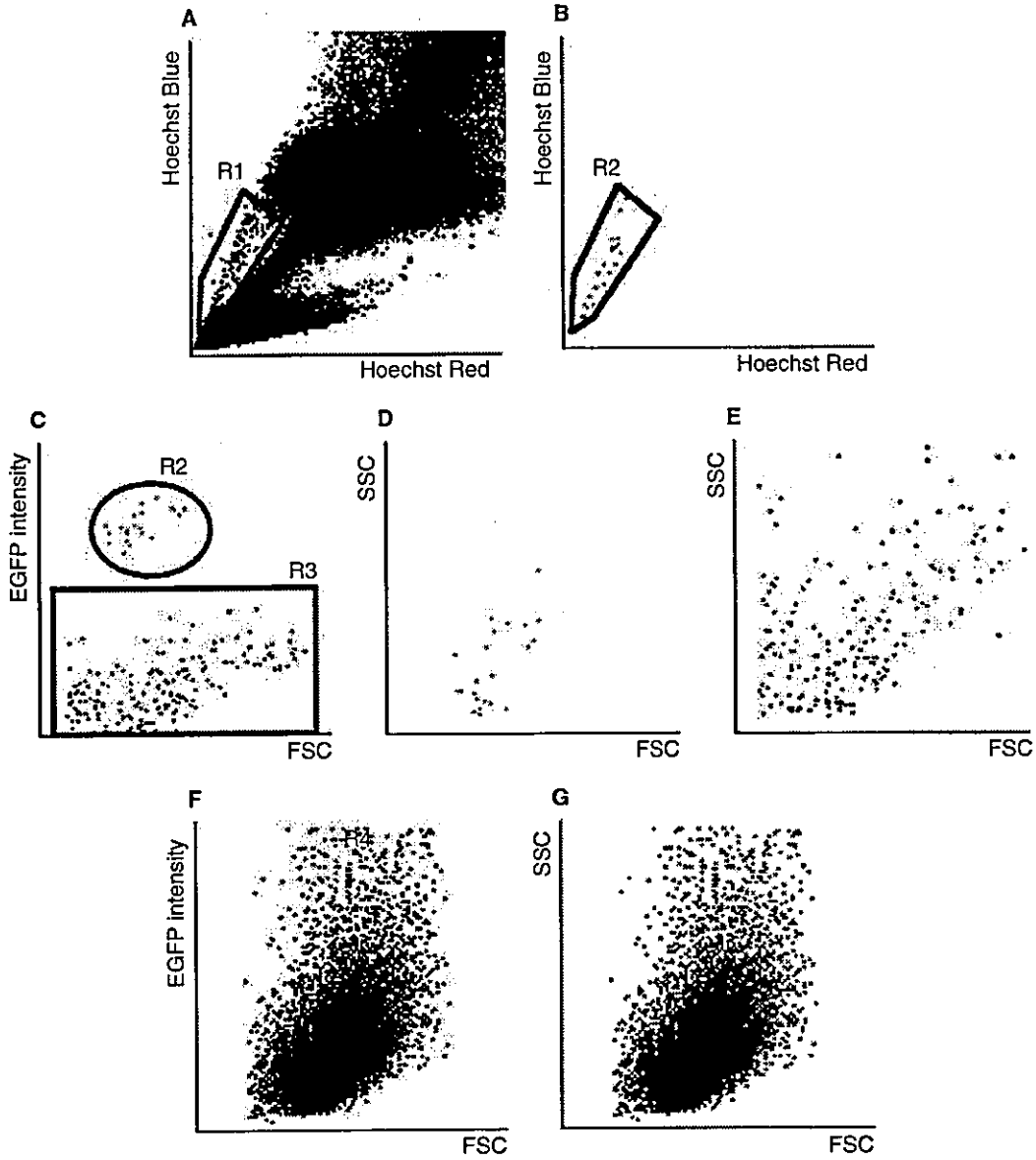

Fig. 2. Flow cytometric analysis of kidney-derived cells that are prepared from bone marrow–transplanted adult rats. (*A*) Hoechst fluorescence of whole kidney-derived cells is shown. Hoechst^{low}/side population fraction is indicated in the boxed region, R1. (*B*) Enhanced green fluorescent protein (EGFP)$^{+}$ cells in the R1 region are shown in red, which are shown in (A) also in red. These cells are also shown in the R2 region of (*C*) in red. (C) FSC-EGFP chart of cells in the R1 region. The R1 region contains EGFP^{+} cells (R2) and EGFP^{-} cells (R3). (*D*) FSC-SSC chart of cells in the R2 region. (*E*) FSC-SSC chart of cells in the R3 region. (*F*) FSC-EGFP chart of whole cells other than R1 region. Cells expressing EGFP are boxed in the R4 region. (*G*) FSC-SSC chart of cells in the R4 region. FSC, forward scatter; SSC, side scatter.

0.70% in medulla. Importantly, the cells displayed green fluorescence as a brilliant cytoplasmic signal (Fig. 5I and J, arrow), which was quite different from the granular pattern in Figure 5C. This result indicated that EGFP was produced in the cell, not derived from endocytosed protein. On the other hand, EGFP^{+} cells were not observed in the tubular epithelium of the side population–transplanted rats even after the induction of gentamicin nephrotoxicity (Fig. 5G).

In our previous experiments, we demonstrated that bone marrow can give rise to mesangial-like cells in vitro in response to PDGF-BB [15]. In order to examine whether or not the kidney-derived Hoechst^{low}/side population cells are capable of differentiating into mesangial-like cells in vitro, they were placed in a bone marrow culture system which we established to convert bone marrow–derived cells into mesangial-like cells [15]. Since Hoechst^{low}/side population cells alone did not easily stay

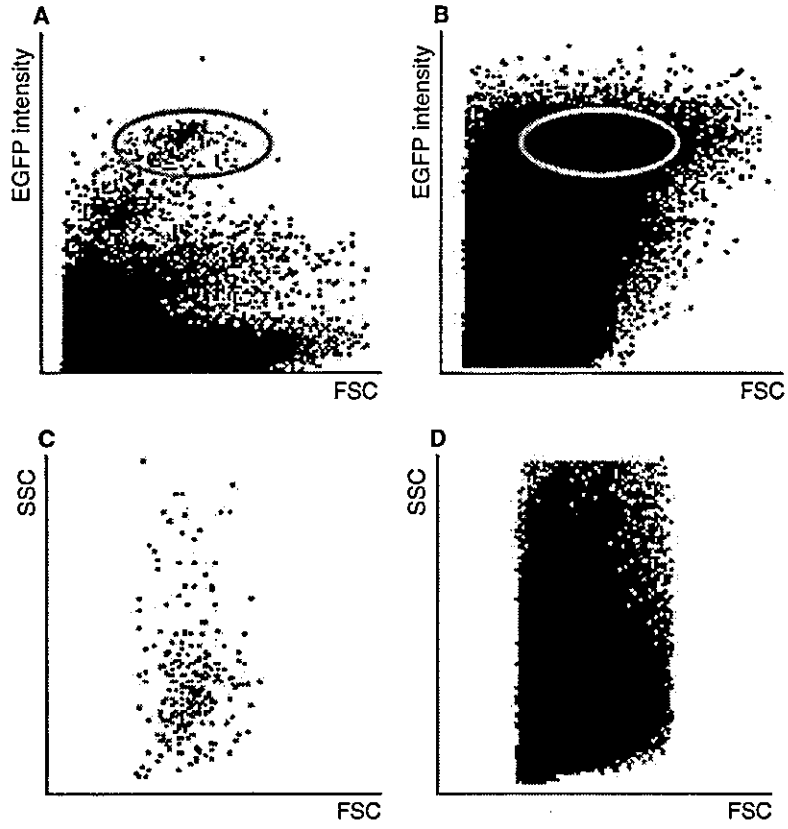

Fig. 3. Flow cytometric analysis of bone marrow of side population–transplanted rats. The whole bone marrow taken from a side population–transplanted rat or an enhanced green fluorescent protein (EGFP) rat was subjected to flow cytometric analysis. (*A* and *C*) Bone marrow from a side population–transplanted rat. (*B* and *D*) Bone marrow from an EGFP rat. (A and B) FSC-EGFP charts of bone marrow. (C and D) FSC and SSC of the circled regions in (A) and (B) were shown in (C) and (D), respectively. Bone marrow preparation from EGFP rats also provides $EGFP^-$ signals, which may be due to enucleated mature erythrocytes and/or accidental loss of EGFP fluorescence during the procedure. FSC, forward scatter; SSC, side scatter.

on collagen type IV–coated dishes, wild-type bone marrow cells that did not attach to collagen type I but attached to collagen type IV were cocultured to support $EGFP^+$ $Hoechst^{low}$/side population cells. In our previous experience, the bone marrow cells that do not attach to collagen type I but attach to collagen type IV yield $Thy1^{++}$ $desmin^+$-stellate cells in vitro. The $Thy1^{++}$ $desmin^+$-stellate cells could be considered as mesangial-like cells because they contracted in response to angiotensin II [15]. Consistently, also in this study, $Thy1^+$- or $desmin^+$-stellate cells were obtained in the mixed culture but they were $EGFP^-$ (Fig. 6). Therefore, it is unlikely that $EGFP^+$ $Hoechst^{low}$/side population cells contributed to the formation of $Thy1^+$- or $desmin^+$-stellate cells in vitro.

DISCUSSION

First, we have identified the existence of $Hoechst^{low}$/side population cells in adult rat kidney, and most of them were healthy in that they were propidium iodide negative. These cells expressed ABCG2 or MDR1, which were thought to be fundamental for $Hoechst^{low}$/side population phenotype [22, 23]. When these cells were analyzed with immunocytochemistry, RECA1-positive cells were not enriched. As to aquaporin 2, however, more number of cells were positively stained in $Hoechst^{low}$/side population cells than in the collagenase-digested whole kidney cells. But its enrichment ratio is very slight and the positively stained $Hoechst^{low}$/side population cells counted is inevitably small in number; therefore, it is difficult to state that aquaporin 2–positive cells are significantly enriched in the kidney-derived $Hoechst^{low}$/side population cells. Since RECA1 is expressed in endothelial cells and aquaporin 2 is expressed in collecting duct of the kidney [24], kidney-derived $Hoechst^{low}$/side population cell is not an enriched population of endothelial specific cells or renal specific cells.

Second, bone marrow cells were apparently included in the $Hoechst^{low}$/side population fraction of the kidney. However, more number of the $Hoechst^{low}$/side population cells in the kidney might be derived from bone marrow than the number estimated from the scattergram in Figure 2 (10%), because the bone marrow of the recipient wild-type rats into which $EGFP^+$ bone marrow cells were transplanted still carries approximately 20% of EGFP-negative cells [14]. The turnover of tissue-resident bone marrow–derived cells takes time that depends on types of tissues [25], which might also make us underestimate the contribution of $EGFP^+$ bone marrow cells to the $Hoechst^{low}$/side population cells in the kidney. It

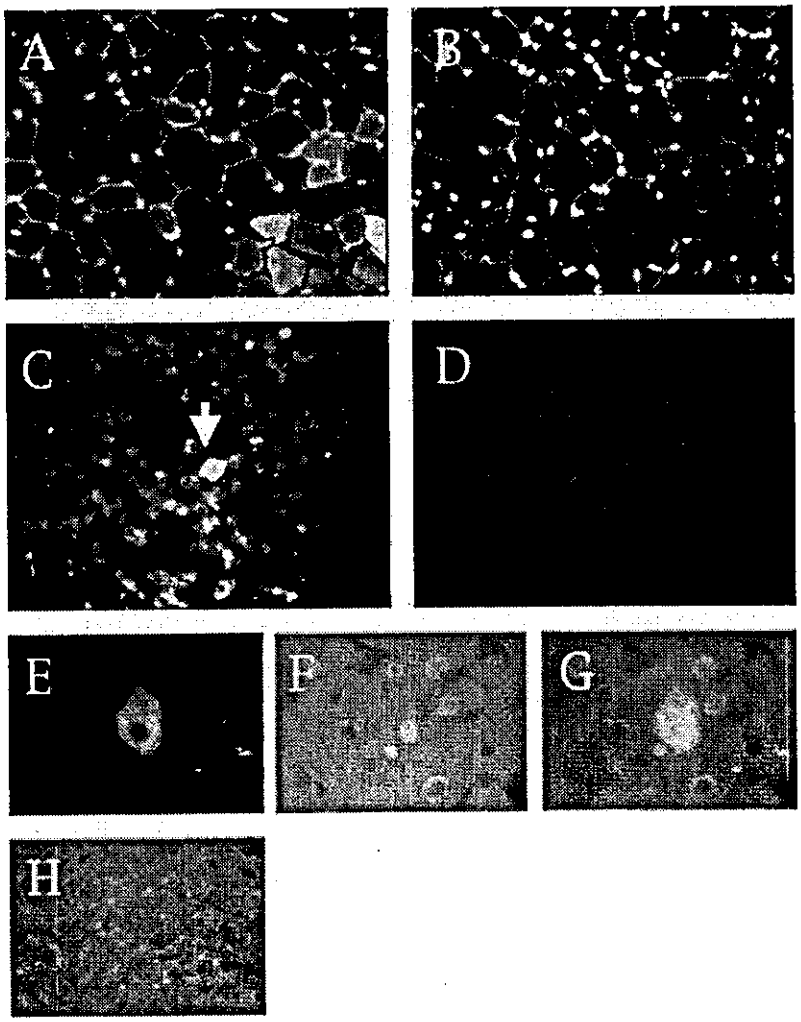

Fig. 4. Immunohistochemical analysis of skeletal muscle and liver of side population–transplanted rats. Merged fluorescence images are shown (×400, original magnification). Enhanced green fluorescent protein (EGFP) provides bright green. (*A*) Skeletal muscle from a side population–transplanted rat. (*B*) Skeletal muscle from a wild-type rat. Skeletal muscle is stained with antilaminin antibody followed by Texas Red–conjugated secondary antibody (red). (*C, E, F,* and *G*) Liver from a side population–transplanted rat. (*D* and *H*) Liver from a wild-type rat. In (C) and (D), liver was stained with anti-CD45 antibody followed by Texas Red–conjugated secondary antibody (red). Arrow in (C) indicates a cell which expresses EGFP in a cytoplasmic pattern. (E) EGFP fluorescence is observed (green). (F) Liver was stained with purified IgG fraction of polyclonal rabbit antiserum to rat albumin followed by Texas Red–conjugated secondary antibody (red). (G) Merged image of (E) and (F). (H) Wild-type rat's image equivalent to (G). Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) except in (E).

is not that simple to estimate the contribution of bone marrow cells to the Hoechst^{low}/side population cells in the kidney through CD45 staining, because surface markers such as CD45 are fragile to protease treatment. It is noteworthy that EGFP$^+$ cells that are derived from the transplanted bone marrow were also included in the non-Hoechst^{low}/side population region, indicating that bone marrow provides kidney with various types of cells such as non-side population cells.

Finally, kidney-derived Hoechst^{low}/side population cells were proved to include the cells that differentiate into hematopoietic lineage, skeletal muscle, and/or liver when injected intravenously. Therefore, multipotent stem cells both for blood cells and for organs such as skeletal muscle and liver might be able to circulate and lodge in peripheral organs. However, intravenous administration of the kidney-derived Hoechst^{low}/side population cells neither led to apparent engraftment to the kidney nor produced any renal cells in vivo. No glomerular mesangial cells were produced and induced not only in the in vivo model even after the induction of Thy1 nephritis in the side population–transplanted rats, but also in an in vitro culture system under which bone marrow cells on collagen type IV give rise to mesangial-like cells. In the proximal tubular epithelial cells of the side population–transplanted rats, bright EGFP fluorescence was observed in a granular pattern. This makes a sharp contrast to the homogeneous cytoplasmic pattern of EGFP driven by a hybrid promoter composed of chicken β-actin promoter and cytomegalovirus enhancer [14]. It is most likely that EGFP coming out of broken EGFP$^+$ cells were filtered through glomeruli, and then taken up by the tubular epithelial cells because EGFP is a hydrophilic protein with a molecular weight of 27 kD [26]. Even after the induction of gentamicin nephrotoxicity to side population–transplanted rats, no EGFP$^+$ tubular epithelial cells were observed. In other words, transplanted cells were engrafted neither as tubular

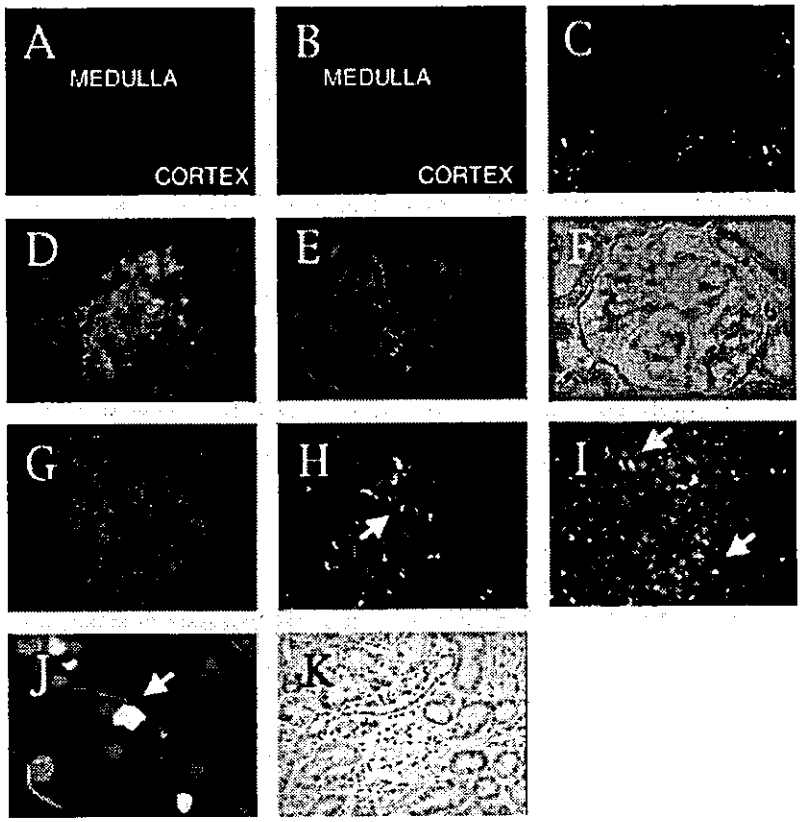

Fig. 5. Kidney of side population–transplanted rats and bone marrow–transplanted rats. Fluorescence images of kidneys that are derived from wild-type rats, side population–transplanted rats or bone marrow–transplanted rats are shown. (*A*) Wild-type rats. (*B, C, D, E,* and *G*) Side population–transplanted rats, (*H, I,* and *J*) Bone marrow–transplanted rats. (B, C, and D) Kidney from side population–transplanted rats without Thy1 nephritis or gentamicin-induced nephropathy. (E) Kidney from side population–transplanted rats 5 weeks after the induction of Thy1 nephritis. Green is enhanced green fluorescent protein (EGF), red is Thy1 and blue is 4′,6-diamidino-2-phenylindole (DAPI). (*F*) Kidney sections obtained from side population–transplanted rats 5 weeks after the induction of Thy1 nephritis were stained with periodic acid-Schiff (PAS). (G) Kidney from side population–transplanted rats 4 to 8 weeks after the last administration of gentamicin. Green is EGFP, red is laminin, and blue is DAPI. (H) Kidney from bone marrow–transplanted rats 4 weeks after the last administration of gentamicin. Green is EGFP, red is Tamm-Horsfall protein, and blue is laminin. (I) Kidney from bone marrow–transplanted rats 4 weeks after the last administration of gentamicin. Green is EGFP, red is laminin, and blue is DAPI. (J) Magnification of the lower right area of (I). Arrows in (H, I, and J) indicate cells which express EGFP in a cytoplasmic pattern. (*K*) Kidney sections obtained from side population–transplanted rats 4 to 8 weeks after the last administration of gentamicin were stained with PAS [original magnification (A and B) ×100; (C to K) ×400].

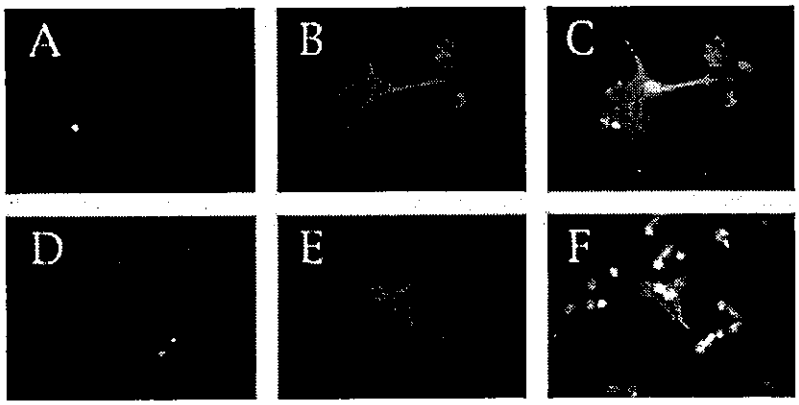

Fig. 6. Culture of kidney-derived Hoechst^{low}/side population cells. Enhanced green fluorescent protein (EGFP)$^{+}$ kidney-derived Hoechst^{low}/side population cells were cocultured with wild-type bone marrow cells that were preplated on collagen type I as described in the **Methods** section. The cells were fixed and stained as follows (×400). (*A*) EGFP fluorescence of the culture (green). (*B*) Thy1 was stained with anti-Thy1 antibody in combination with Texas Red–conjugated antibody (red). (*C*) The merged image. (*D*) EGFP fluorescence of the culture (green). (*E*) Desmin was stained with antidesmin antibody in combination with Texas Red–conjugated antibody (red). (*F*) The merged image. Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Only a trace amount of green fluorescence was sometimes observed within a cell, but it is unlikely that the signal was derived from EGFP transcribed and translated by the cell itself.

epithelial cells nor as mesangial cells. In consideration of the fact that bone marrow–derived cells can differentiate into tubular epithelial cells as we and other researchers have confirmed [27, 28], circulating precursor/stem cells for mesangial cells and renal epithelial cells seem to be distinct from the Hoechst^{low}/side population cells in the kidney. Further experiments need to be done to answer this question.

No contribution of the kidney-derived Hoechst^{low}/side population cells to the kidney might be interpreted in the following way. The first causative possibility is that the transplanted Hoechst^{low}/side population cells were small in number. In our previous bone marrow transplantation experiment where 1×10^8 bone marrow cells were transplanted to a recipient, many bone marrow–derived cells existed outside of the capillaries in the interstitium of the kidney [14, 29]. Given that bone marrow has approximately 0.05% of Hoechst^{low}/side population cells (our unpublished result), it falls that 50,000 bone marrow–derived Hoechst^{low}/side population cells were transplanted along with other types of cells. In contrast, the number of the kidney-derived Hoechst^{low}/side population cells transplanted in this experiment was approximately 3000 to 8000, which might lead to the mere detection failure in the limited number of histologic evaluations. This number of Hoechst^{low}/side population cells, however, was technically maximum because collection and transplantation of 5000 Hoechst^{low}/side population cells required about 15 hours in all. The second possibility is that collagenase treatment of the kidney might disrupt cell surface molecules that are crucial for the specific adhesion of Hoechst^{low}/side population cells to the kidney.

It was recently reported that two or more embryonic stem cells can fuse in vitro and yield one mononuclear cell that acquire a mixed phenotype derived from each single cell [30, 31]. Because skeletal muscle is physiologically composed of multinucleated cells that are formed mainly by cell fusion not by undivided proliferation [32] and because liver gets fused and multinucleated in response to a certain stimulation such as chloroprocaine, a local anesthetic [33], these two organs may be innately programmed to fuse. Therefore, the differentiation of the kidney-derived Hoechst^{low}/side population cells into skeletal muscle and liver might be accepted as an event of "cell fusion." If it is the case in our results, it is possible that the kidney-derived Hoechst^{low}/side population cells simply comprise hematopoietic stem cells. On the other hand, according to one report, hematopoietic stem cells' differentiation into glomerular mesangial cells was not due to the cell fusion [34]. In order to answer the question of cell fusion, we are trying to tag and trace recipient cells and donor cells simultaneously.

In discussing the differentiation potentials especially toward hematopoietic lineages from kidney-derived Hoechst^{low}/side population cells, what we have to consider is the contamination of circulating peripheral blood cells. Donor kidneys, however, were well perfused prior to the preparation of Hoechst^{low}/side population cells. DAPI-positive roundly nucleated cells other than flatly nucleated endothelial cells were not detected within the RECA1-positive vascular lumen, and no erythrocytes or nucleated cells were observed within the vascular lumen in hematoxylin and eosin stained sections (data not shown). These indicate that peripheral blood cells were mostly cleared away. Besides, Hoechst^{low}/side population cells could not be obtained from kidney when the cell suspension was prepared in the absence of collagenase digestion following the perfusion of kidneys with PBS containing 0.9 mmol/L of Ca^{2+} and 0.33 mmol/L of Mg^{2+}, or with PBS containing 200 μg/mL of ethylenediaminetetraacetic acid (EDTA) (data not shown). Necessity of collagenase digestion strongly suggests that Hoechst^{low}/side population cells are entangled in the surrounding matrix or structures, not existing free from the surrounding structures. If one of the common features of stem cells is the side population phenotype, peripheral blood hematopoietic stem cells are likely to be also detected in the side population fraction, but nonexistence of Hoechst^{low}/side population cells when kidney cell suspension was prepared without collagenase digestion also confirmed the absence of the circulating peripheral blood hematopoietic stem cells in the transplanted kidney-derived Hoechst^{low}/side population cells. In short, there was little commitment of circulating peripheral blood cells to engraft toward bone marrow, skeletal muscle, or liver. However, as was revealed in a report that muscle-derived hematopoietic stem cells are hematopoietic in origin [8], these kidney-derived Hoechst^{low}/side population cells which are committed to hematopoietic lineage might be originally hematopoietic cells, indicating that we cannot exclude a possibility that hematopoietic stem cells constitutively lodge in the kidney as a part of it. In order to answer this question, further investigation is necessary.

CONCLUSION

The Hoechst^{low}/side population cells that show hematopoietic and mesenchymal potentials were identified in the adult rat kidney, but did not contribute to any renal components in vivo or in vitro. Supposedly, it seems that mesenchymal pluripotent cells directed to renal components such as mesangial cells and tubular epithelial cells belong to non-Hoechst^{low}/side population fraction if they reside in the kidney.

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Fetal cells in mother rats contribute to the remodeling of liver and kidney after injury

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Abstract

Fetal microchimerism indicates a mixture of cells of maternal and fetal origin seen in maternal tissues during and after pregnancy. Controversy exists about whether persistent fetal microchimerism is related with some autoimmune disorders occurring during and after pregnancy. In the current experiment, an animal model in which EGFP positive cells were taken as fetal-origin cells was designed to detect the fetal microchimerism in various maternal organs. Ethanol drinking and gentamicin injection were adopted to induce liver and kidney injury simultaneously. EGFP positive cells were engrafted not only in the maternal circulation and bone marrow, but also in the liver and kidney as hepatocytes and tubular cells, respectively. These results indicate that fetal cells are engrafted to maternal hematopoietic system without apparent injury and they also contribute to the repairing process of maternal liver and kidney.

Keywords: Fetal cells; Microchimerism; Injury; Green fluorescent protein

Microchimerism has been defined as a chimera of small number of cells from different individuals coexisting within tissues. It has been known for many years that there is a bi-directional traffic of maternal cells and fetal cells through the placenta during pregnancy. Fetal microchimerism indicates a mixture of cells of maternal and fetal origin seen in maternal tissues during and after pregnancy. It has been shown that fetal microchimerism persists in some women for more than 27 years after delivery [1,2].

The recognition that fetal cells pass into the maternal circulation and persist for many years has aroused a question of whether or not persistent fetal microchimerism may trigger a maternal immune reaction resulting in what appears to be an autoimmune disorder, because microchimeric fetal cells are semiallogenic to the maternal immune system. Therefore, many studies have focused on the association between fetal cell microchimerism and subsequent development of autoimmune diseases, especially those having features of chronic graft-versus-host disease, such as systemic sclerosis (SSc) [3–7], Sjögren's syndrome (SS) [8,9], primary biliary cirrhosis (PBC) [10], and autoimmune thyroid diseases (AITD) [11,12]. These autoimmune diseases have a predilection for women with childbearing age and often initiate or exacerbate after pregnancy. Although fetal cells are found in the local lesion of these diseases and may react with stimulation by maternal cells when separated and cultured in vitro [13], there is still a controversy about the hypothesis that fetal microchimerism may be the cause of the disease since fetal microchimeric cells are also found in tissues from patients with non-autoimmune diseases such as chronic hepatitis [14] or found in tissues from control subjects with the same frequencies as from patients with autoimmune diseases [4,7]. It is possible that the accumulation of fetal cells in the local lesion may be due to the response to tissue injury and

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therefore it may be the consequence rather than the cause of the disease.

Alternatively, there is another possibility that fetal microchimerism might have a beneficial effect on the host rather than a detrimental effect. Srivatsa et al. [11] found fully differentiated thyroid follicle formed by male fetal cells closely attached to and indistinguishable from the rest of the tissue in the thyroid specimen of one female patient with goiter. The possibility that a fetal stem cell could be transplanted and engrafted in the maternal thyroid to form mature thyroid follicles is plausible since several studies have shown that intravenously transplanted hematopoietic stem cells can migrate to sites such as muscle and liver, and be engrafted to the host organs [15–17].

As to the strategy used in the identification of fetal microchimerism in human, male cell markers have been adopted in most studies because of its simplicity. But it is important to note that fetal microchimerism is derived not only from male fetus but also from female fetus. Using male genetic markers might bring deviations to the results of the study.

Therefore, in this study, we have designed an animal model for fetal microchimerism detection where EGFP is set as the marker of fetal-origin cells in the maternal tissues. With this model, we have investigated the fetal cell residence in various maternal tissues.

Materials and methods

Animals. Male transgenic Sprague–Dawley rats carrying the enhanced green fluorescent protein (EGFP) transgene (EGFP rat) and female Sprague–Dawley (SD) rats were purchased from Japan SLC (Hamamatsu, Japan). All rats were maintained at the animal facility of Osaka University School of Medicine. They were allowed to get free access to standard laboratory diet and tap water. The female rats weighing 250–300 g were subjected to the following experiment. All the procedures described here were approved by the Animal Committee of Osaka University School of Medicine.

Animal model of microchimerism and the subsequent induction of liver and renal tubular injury. After mating with a single EGFP male rat and the subsequent delivery of babies, a single SD female rat was kept alone in a cage and burdened with simultaneous liver and renal tubular injury. Ethanol was used to induce chronic liver injury with a method modified from Carmiel-Haggai's report [18]. Gentamicin administration was adopted to evoke gentamicin-induced nephropathy [19,20]. The postpartum rats were supplied with drinking water containing 5% of ethanol, without any free access to automatic tap water. The period of ethanol drinking lasted for at least 30 days. The body weight and the amount of the ethanol-containing water drunk by each female rat were recorded everyday to calculate the ethanol taking dosage. At the same time, these postpartum rats received 60 mg/kg/day of gentamicin (Sigma, St. Louis, MO) by subcutaneous injection for 14 consecutive days after the delivery of babies. The same procedure was repeated after a 2-week interval.

Flow cytometric analysis of mononuclear cells in peripheral blood. The postpartum rats were anesthetized by intra-peritoneal administration of pentobarbital and sacrificed after two cycles of gentamicin injection. Peripheral blood was taken via abdominal aorta and kept in the tube rinsed with heparin in advance. One milliliter of the peripheral blood was set aside for the assay of blood chemistry as described later. Ficoll density separation combined with NH_4Cl-lysis method was adopted to collect mononuclear cells from the peripheral blood. In brief, 2 ml of the peripheral blood was carefully overlaid on 5 ml of Lymphoprep (Axis-shield PoC AS, Oslo, Norway) and centrifuged at 800*g* for 20 min at room temperature. After the centrifuge, a cell fraction, located at the interphase, was collected and washed twice with phosphate-buffered saline (PBS)/2% fetal calf serum (FCS). Then the pellet was resuspended with 0.5 ml PBS/2% FCS and then 7.5 ml of lysis solution [8.26 g/L NH_4Cl /1.0 g/L $KHCO_3$ /0.037 g/L $EDTANa_4 \cdot HCl$ (pH 7.4)] was added. After gentle mixing, it was incubated at room temperature for 5 min and then centrifuged at 300*g* for 5 min at room temperature. The pellet was washed with PBS/2% FCS twice and resuspended with 0.5 ml PBS/2% FCS. Then the sample was subjected to flow cytometric analysis by FACScan (Becton–Dickinson, San Jose, CA, USA). The mononuclear cells in the peripheral blood of EGFP rats and wild type rats were collected in the same manner and were set as positive and negative controls, respectively.

Flow cytometric analysis of bone marrow cells. The postpartum rats were anesthetized and sacrificed as described above. The bone marrow cells were collected by flushing the tibia and femur with ice-cold heparinized PBS/2% FCS and filtered through 70 μm nylon mesh. After the wash, the cells were resuspended with 2 ml PBS/2% FCS and subjected to flow cytometric analysis.

Measurement of plasma creatinine, BUN, ALT, and AST. Peripheral blood was taken via aorta as described above. Plasma was separated from 1 ml of whole blood by centrifuge at 300*g* for 5 min at 4 °C. Creatinine (Cr), blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by using appropriate biochemical methods in a commercial laboratory (SRL, Tokyo, Japan).

Tissue preparation. After blood sample was taken via aorta, organs including kidney and liver were thoroughly perfused through the aorta with ice-cold PBS followed by 4% paraformaldehyde (PFA)/PBS before harvest. The organs were harvested and fixed in 4% PFA/PBS for 6 h at 4 °C in the dark and further in 30% sucrose/PBS overnight at 4 °C in the dark. After that, the tissues were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and kept at −80 °C for cryostat sections.

Immunohistochemistry of fixed frozen samples. Immunohistochemical analysis of frozen sections was performed as described previously [19]. In brief, for albumin staining of the liver, 4-μm fixed frozen sections were incubated with normal goat serum for 30 min first and then with purified IgG fraction of polyclonal rabbit antiserum to rat albumin (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) for 1 h at room temperature. The sections were washed with PBS three times, were incubated with Texas red-conjugated anti-rabbit IgG antibody (Vector Lab, Burlingame, CA, USA) for 30 min at room temperature, and were washed with PBS three times. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR, USA) for 3 min at room temperature and then washed with PBS three times. Finally, the sections were mounted with VECTASHIELD Mounting Medium (Vector Lab, Burlingame, CA, USA) and observed under a fluorescence microscope (Nikon Eclipse E600) (Nikon, Tokyo, Japan) with appropriate filters. All images were captured by a digital imaging system connected to a Macintosh computer. For laminin staining of the kidney, the procedure was the same except that rabbit anti-laminin antibody (Monosan, Uden, The Netherlands) was used as a primary antibody.

Results

Existence of EGFP positive cells in maternal peripheral blood

As mentioned in Materials and methods, EGFP positive cells were regarded as the fetal cell origin and were

used as the marker of fetal microchimerism in our experiment. When we analyzed the peripheral mononuclear cells from wild type rats (Figs. 1A–C) and EGFP transgenic rats (Figs. 1D–F), a distinct different pattern of distribution was observed. The majority of the cells from EGFP transgenic rats distributed over 200 on the EGFP intensity scale which formed two sharp peaks while cells from wild type rats distributed in the region less than

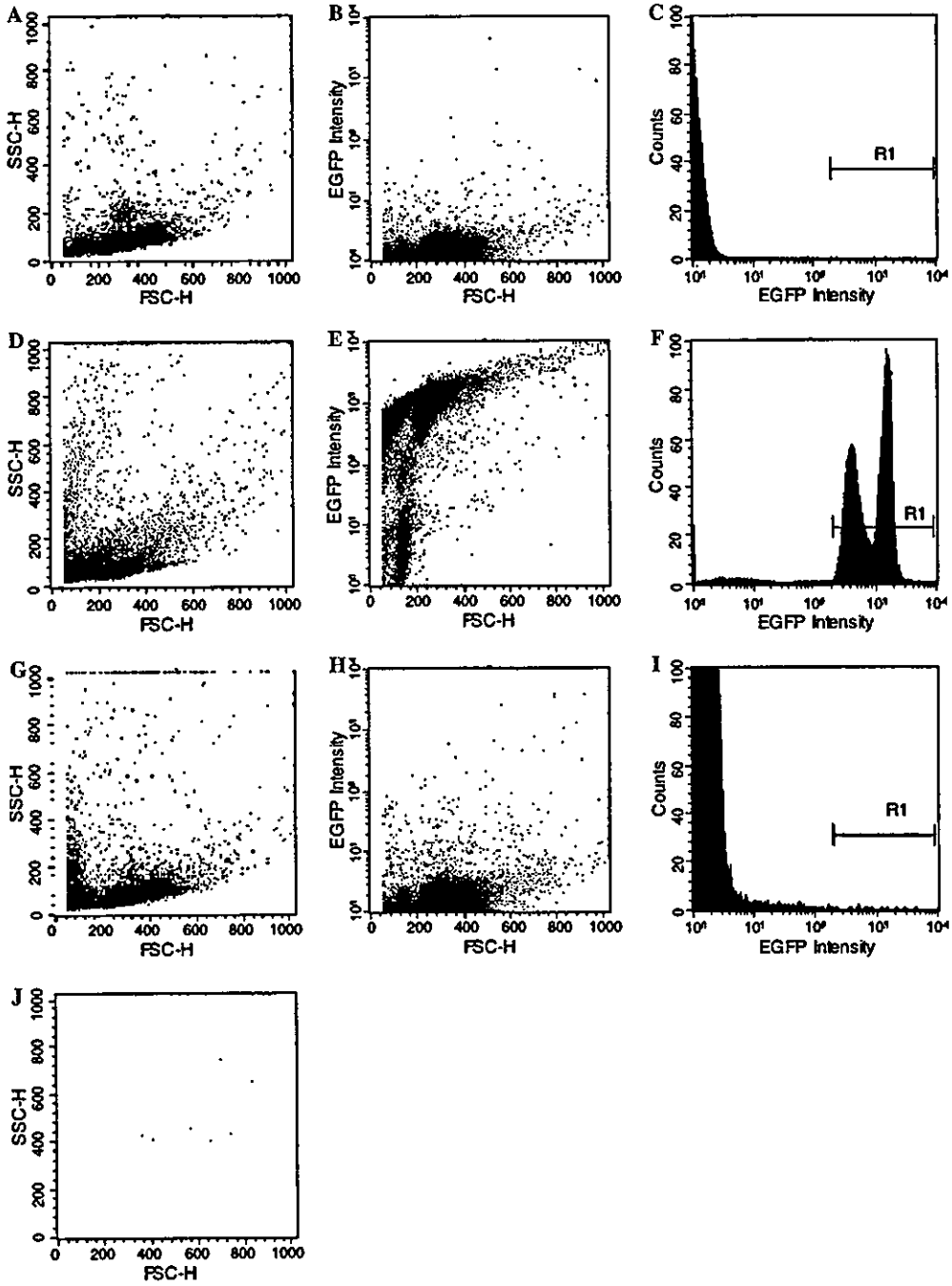

Fig. 1. Flow cytometric analysis of peripheral mononuclear cells. (A–C) Peripheral blood from wild type rats as a negative control. (D–F) Peripheral blood from EGFP transgenic rats as a positive control. (G–J) Peripheral blood from rats treated with gentamicin and ethanol. (A,D,G) The FSC-SSC chart. (B,E,H) The FSC–EGFP chart. (C,F,I) The histogram of EGFP. (J) The FSC–SSC distribution of cells in the R1 region of (I). The distribution pattern indicates that EGFP intensity 200 might be set as the cut-off point to distinguish EGFP positive cells from negative cells. Some cells in our treated rat distributed in the region over 200 on EGFP intensity scale (R1) are EGFP positive cells derived from fetus.

200 on the EGFP intensity scale. This indicates that EGFP intensity 200 might be set as the cut-off point to distinguish EGFP positive cells from negative cells. Based on this cut-off point, we found EGFP positive cells existed in the peripheral mononuclear cells of our treated rats (Figs. 1G–J).

Existence of EGFP positive cells in maternal bone marrow

Bone marrow cells prepared from the treated rats were analyzed in the same manner as that used for peripheral mononuclear cells. EGFP positive cells were also found engrafted in the maternal bone marrow (Figs. 2A–D). This indicates that fetal cells migrated from the maternal circulation and resided in the bone marrow in the absence of apparent injury to the bone marrow.

Engraftment of EGFP positive cells in liver as hepatocytes

Table 1 shows the average amount of daily ethanol consumption during the period of ethanol supply, and the results of blood biochemistry of each rat. The average consumption of ethanol for each rat is over 4 mg/kg/day which is enough to induce liver injury according to Carmiel-Haggai's report [18]. In accordance, the ALT level of all 4 rats was elevated, which supported the existence of liver injury. When the frozen liver specimens were observed under a fluorescence microscope directly,

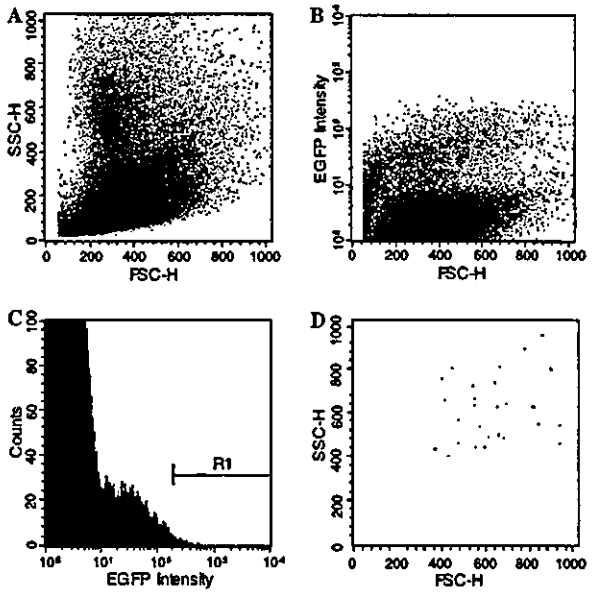

Fig. 2. Flow cytometric analysis of bone marrow cells from rats treated with gentamicin and ethanol. (A) The FSC–SSC chart. (B) The FSC–EGFP chart. (C) The histogram of EGFP. (D) The FSC–SSC chart of cells in the R1 region of (C). There are several cells distributed in the region over 200 on EGFP intensity scale (R1) which are EGFP positive cells originated from fetus.

Table 1
Ethanol consumption and blood chemistry of the rats treated with ethanol and gentamicin

No.	Average consumption of ethanol (g/kg BW/day)	ALT (IU/L)	AST (IU/L)	Cr (mg/dL)	BUN (mg/dL)
1	4.8	36	64	0.43	25.2
2	9.1	36	122	0.31	28.7
3	6.7	63	239	0.91	53.1
4	7.7	42	151	0.21	29.4
	Normal range	24 ± 6.4	68 ± 16.5	0.6 ± 0.11	15.4 ± 2.54

The average consumption of ethanol for each rat is over 4 mg/kg/day which is enough to induce liver injury. The ALT and BUN levels of the 4 rats were all elevated.

EGFP positive cells were found scattered in the liver (Figs. 3A–C). The EGFP positive cells were also positive to albumin (Figs. 3D–F), a marker of hepatocytes. These results indicate that fetal cells contributed to hepatocytes in the maternal injured liver.

Engraftment of EGFP positive cells in kidney as tubular epithelial cells

As shown in Table 1, BUN levels of the 4 rats were all elevated. We have already confirmed that gentamicin nephropathy is a good model for trapping circulating tubular precursor cells in the bone marrow [19]. No EGFP positive cells could be found in the glomeruli because cells expressing EGFP protein were detected within the tubular basement membrane, indicating they were tubular cells. These cells expressed EGFP in a cytoplasmic pattern, which excludes the possibility that we simply observed EGFP endocytosed from the circulation or from the tubular fluid by tubular epithelial cells (Figs. 4A–D).

Discussion

Human fetal cells can be found in the maternal circulation as early as 4–5 weeks of gestation and persist for several decades after delivery [1,2]. The results of the current animal experiment prove the existence of fetal cells in the rat maternal circulation which is in accordance with the previous reports on human, and support the notion that fetal microchimerism is a common phenomenon associated with pregnancy in nature. Furthermore, the finding of fetal cells engrafted in maternal bone marrow, liver, and kidney indicates that fetal cells do not only exist in the peripheral circulation but also migrate from the peripheral circulation and reside in multiple maternal organs.

The majority of the studies on fetal microchimerism have traced male DNA as a marker by PCR or FISH

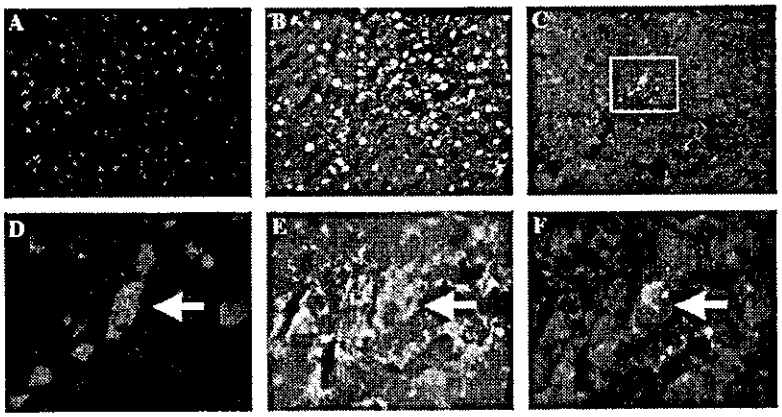

Fig. 3. Chimeric state of liver. (A) Wild type rat as a negative control; (B) EGFP rat as a positive control; (C–F) postpartum rat treated with gentamicin and ethanol; (A,B,D) liver is stained with DAPI. (DAPI, blue) (E) liver is stained with purified IgG fraction of polyclonal rabbit antiserum to rat albumin (Albumin, red) (F) merged image of (D,E) and also an electronically magnified image of rectangle in (C). (Albumin, red; DAPI, blue) arrow in (D) indicates cells which express EGFP in a cytoplasmic pattern. The same cell is pointed by arrow in (E,F). The cell pointed by arrow in (F) is positive to both EGFP and albumin, indicating fetal-derived hepatocyte. Representative images are shown (400×, original magnification).

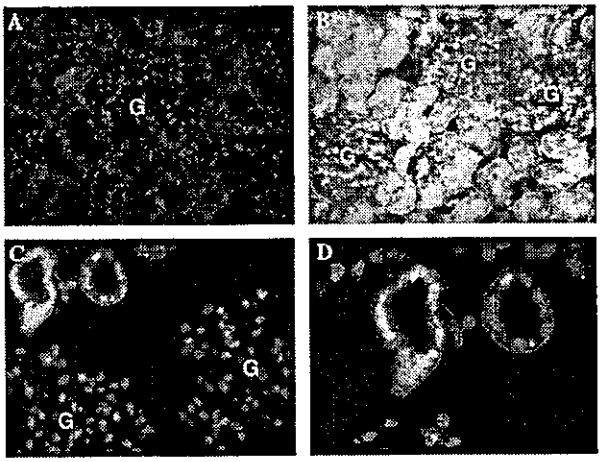

Fig. 4. Chimeric state of kidney. (A) Wild type rat kidney as a negative control; (B) EGFP rat kidney as a positive control; (C,D) kidney from postpartum rat treated with gentamicin and ethanol. A letter G indicates glomeruli. (A–D) Kidney is stained with with anti-laminin antibody and DAPI. EGFP positive cells within the tubular basement membrane in (C) are tubular cells. Laminin, red; DAPI, blue. Representative images are shown [400× for (A,B) 600× for (C) 800× for (D)].

to detect microchimerism in those female hosts who have given birth to or aborted sons. But when targeting male DNA, the particular Y-chromosome sequence can be important. Moreover, fetal cell microchimerism would also exist in mothers who have given birth to daughters but such microchimerism cannot be found out as long as by tracing Y-chromosome. In our experiment, we avoided such problems by mating wild type female rats with EGFP transgenic male rats as employed by Imaizumi's report [21]. EGFP transgenic rats are engineered to express enhanced green fluorescent protein (EGFP) in all cells except for hair and red blood cells, whose expression is driven by cytomegalovirus enhancer and chicken β-actin promoter [22]. Therefore, it is convenient to recognize fetal microchimerism as the EGFP positive cells in the wild type maternal tissues, which can be easily detected by direct observation under a fluorescence microscope regardless of the gender of baby rats.

Chimeric fetal cells are semiallogenic to the maternal immune system. The persistence of semiallogenic fetal cells in maternal solid organs has created an interest in the relationship between these fetal-origin cells and local lesions of some autoimmune diseases with postpartum onset and/or exacerbation. Most evidence comes from the studies on SSc, an autoimmune disease with a characteristic of graft-versus-host disease and with a predilection for women in childbearing age. Nelson et al. [3] found that the frequency of fetal cell microchimerism in circulation was higher in SSc patients than in healthy controls. Artlett et al. [5] reported the same results and also found fetal DNA and cells in skin lesions of SSc women. Ohtsuka et al. [23] reported the existence of significantly higher quantities of fetal DNA in their skin lesions. Similar results have been reported by studies of PBC and AITD. They suggest that fetal cells residing in maternal organs might lead to the local inflammations in such autoimmune diseases. However, Johnson et al. [4] investigated the distribution of fetal cells in various organs of SSc patients and proved that not only the affected organs but also the organs without clinical phenotypes had fetal cells. Johnson et al. [14] also reported the case of fetal microchimerism in liver of chronic hepatitis-C patient which is not an autoimmune disease. Fetal cells were also found in the specimen of non-autoimmune thyroid adenoma [11]. In our animal experiment, fetal cells

were found engrafted not only in maternal bone marrow which was kept intact throughout the experiment, but also in liver and kidney to which injury was induced by chemicals in a non-immunological manner. Thus, the migration and residence of fetal cells in various maternal organs might be a common phenomenon. What seems to be an important factor for the existence of fetal microchimerism in a certain organ could be the cell turnover rate. Bone marrow has a rapid turnover, facilitating engraftment of fetal cells without injury. In kidney or liver, cell turnover is not as rapid as bone marrow [24]. However, in a state where organs are chronically injured with whatever causes, it is likely that fetal microchimerism is established more frequently and detected more easily than in normal physiological state. Given that the accumulation of fetal cells in local lesions of autoimmune diseases might result from changed profiles of inflammatory factors and chemoattractants, inflammatory cells including fetal-origin cells from other organs such as bone marrow can be recruited to the local lesions. Another possibility is in situ proliferation of already engrafted fetal cells in the local lesions because the fetal-origin cells had great power of proliferation under local stimuli in vitro [13]. In our experiments, however, we did not observe EGFP positive cells in non-injured maternal organs (data not shown). Therefore, we assume that recruitment from the circulation to injured organs is the dominant mechanism for establishing chimeric liver and kidney.

Several types of fetal cells have been demonstrated in the maternal circulation during pregnancy, including $CD34^+$ and $CD34^+CD38^+$ hematopoietic progenitor cells [1], nucleated erythroblasts [25], trophoblasts [26], and leucocytes [5]. Persistent fetal hematologic progenitor cells have been detected in maternal peripheral blood as long as 27 years after delivery in human. Fetal leucocytes may play a role in the inflammatory process of the autoimmune diseases. However, fetal progenitor cells might bring different effects on the maternal body. We found that fetal cells join the construction of the maternal organs with the phenotypes which are indistinguishable from their maternal counterparts. Evidence from the research on stem cells of bone marrow has proved that the stem cells reside in the recipient's organs after transplantation and take part in the repair process of the organs in response to injury [15–17]. In the current study, fetal cells resided in the kidney as tubular cells when gentamicin-induced nephropathy was evoked, but no EGFP positive cells were observed in the glomerular region. Gentamicin-induced nephropathy is an animal model of renal tubular injury, and we reported that bone marrow-derived cells are engrafted in renal tubular cells of this animal model [19]. We also reported that intravenously transplanted kidney-derived side population (SP) cells contributed to non-injured liver [19]. These results provide the clue that stem/progenitor cells from fetal microchimerism may engraft liver, kidney, and/or bone marrow, and may take part in the regeneration and reconstruction of renal tubules and/or hepatocytes after injury.

In conclusion, fetal cell microchimerism is a common phenomenon observed in many females after pregnancy. The fetal cells reside not only in the maternal circulation, but also in bone marrow, and in liver and kidney after injury. These fetal cells may take part in the repair process of the maternal organ when injured or during the rest of life even without injury.

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