level was increased for only 2 days after tonsil stimulation in the mild IgAN group. The urinary M-CSF level was not changed in the chronic tonsillitis group after tonsil stimulation. These results suggest that tonsil stimulation contributes to the progression of IgAN via enhancement of glomerular production of M-CSF [47].

However, the usefulness of the tonsillar provocation test in IgAN is now doubted. Even otolaryngologists [46] who initially claimed that the tonsillar provocation test was of clinical value in patients with IgAN have already changed their opinion. Their late results showed that there was no statistically significant difference between positive and negative patients in the rate of remission of proteinuria based on any parameter of the tonsillar provocation test at any time after surgery [48, 49]. Moreover, the Japan Society of Stomato-Pharyngology officially reported the lack of value of tonsillar provocation test in determining the indications for tonsillectomy in IgAN patients [50]. We think that an increase of white blood cell count, an increase of body temperature and enhancement of erythrocyte sedimentation rate after tonsillar provocation test may not be of any clinical value in patients with IgAN, but deterioration of urinary findings after tonsillar stimulation may be significant and suggest that tonsils are related to kidneys. A questionnaire survey also showed that 51.6% of 154 medical doctors who had reported case of IgAN answered that urine protein was the most important factor in any estimation of the provocation test [51].

## TONSILLAR INFECTION AND IgAN

### Tonsillar bacterial infection and IgAN

Suzuki et al [52] reported that the antigen and antibodies of outer membranes of *Haemophilus parainfluenzae*, a common bacterium on the tonsils, were present in glomerular mesangium and sera of IgAN, respectively, suggesting that *H. parainfluenza* infection may have a role in the etiology of IgAN [52]. Further studies showed that tonsillar lymphocytes from patients with IgAN revealed a significantly higher stimulation index to *H. parainfluenza* antigens (thymidine incorporation in tonsillar lymphocytes with *H. parainfluenza*/thymidine incorporation in unstimulated tonsillar lymphocytes) than controls. The lymphocytes from patients with IgAN also showed a significantly higher level of IgA antibody and IgA1 antibody against *H. parainfluenza* antigens in culture supernatants than lymphocytes from controls [53]. In vivo study showed that mouse glomerular deposition of *H. parainfluenza* outer membrane antigens and IgA, and increases in the amount of mesangial matrix were observed after administration of *H. parainfluenza* outer membrane antigens orally or intraperitoneally, respectively. Levels of IgA antibodies against *H. parainfluenza* outer membrane antigens were significantly increased in administration groups compared with controls. That is, administration of *H. parainfluenza* outer membrane antigens to mice may induce glomerular deposition of IgA and mesangial proliferation, resembling the changes seen in IgAN, with increases in IgA antibodies against *H. parainfluenza* outer membrane antigens [54]. Furthermore, production of cytokines IL-10 and transforming growth factor-β (TGF-β) was enhanced by stimulation with *H. parainfluenza* outer membranes in tonsillar mononuclear cells from IgAN [55]. These results suggest that *H. parainfluenza* antigens stimulate tonsillar T and B lymphocytes in patients with IgAN to produce cytokines and IgA antibody and that an immune response to *H. parainfluenza* antigens may play a role in the pathogenesis of IgAN in some cases.

In additional, Rekola et al [56] reported that 38 of 187 IgAN patients had possible acute glomerulonephritis at the onset of their disease. Antistreptococcal antibodies increased in forty-three percent of the patients. Thirty-three percent of the patients had different groups of beta-hemolytic streptococci isolated from their throats. This result indicates a possible role of beta-hemolytic streptococci, a most common bacterium in tonsils or throat, in the pathogenesis of some IgAN cases [56].

### Tonsillar viral infection and IgAN

Regarding relationship between viral infection in tonsils and IgAN, there is a adult case report in which granular depositions of adeno- and herpes simplex viral antigens were detected in the glomerular mesangial areas in IgAN patients associated with episodes of recurrent tonsillitis and in the tonsillar epithelial cells by *H. parainfluenza* immunofluorescence [57]. The later study showed that the detection ratio of Epstein-Barr virus in the patients with glomerular lesions, such as IgAN and membranous nephropathy, was significantly greater than those without. However, the detection of Epstein-Barr virus was not disease specific [58]. Kunimoto et al [59] investigated viral infections in the tonsils, pharynx, and renal tissues of patients with IgAN using cell culture, polymerase chain reaction, and immunofluorescent techniques, and measured antibody titers against numerous types of viruses. As a result, no evidence was obtained that the viral infections play a significant role in the pathogenesis of IgAN [59].

## EFFECT OF TONSILLECTOMY ON IgAN

### Effect of tonsillectomy on immune system

As described above, human tonsil tissues are located at the gateway of the respiratory and alimentary tract, belong to the mucosa-associated lymphoid tissue, and play a role in the systemic immune and the local mucosal immune. What effect does tonsillectomy have on

**Table 2.** Effect of tonsillectomy on urinary findings and renal function

| Author | Year | Tonsillectomy +/– | Follow-up *months* | Urinary remission | Urinary improvement | Renal survival | Reference number |
|---|---|---|---|---|---|---|---|
| Masuda | 1988 | 16/0 | 36 | 56.3% | | | 46 |
| Tamura | 1993 | 26/0 | 24 | | 46% | | 71 |
| Sugiyama | 1993 | 28/0 | 61 | 32% | 60.7% | | 79 |
| Bene | 1993 | 34/0 | 48 | UP 3.5 g/day → | 0.9 g/day | Stable | 69 |
| Iino | 1993 | 35/15 | 36 | 54.8%/53.8% | 61%/46% | 96.8%/76.9% | 78 |
| Tomioka | 1996 | 104/0 | 12 | 38% | 94% | | 80 |
| Barta | 1996 | 35/40 | 144 | 6m UP 1.4 g/day → | 0.92 g/d | 88.6%/80.0% | 70 |
| Rasche | 1999 | 16/39 | 41 | | | NS | 74 |
| Hotta | 2001 | 250/79 | 75 | Total 48% | | | 81 |
| Xie | 2003 | 48/70 | 193 | | | 89.6%/63.7% | 17 |

Abbreviations are: UP, urine protein; NS, non-statistic difference.

the systemic and local mucosal immune? Studies demonstrated that tonsillectomy decreased the levels of serum IgA and salivary secretory IgA, especially in children, several months or years after operation [60–62]. However, these changes do not cause significant immune deficiency and are clinically insignificant. Moreover, these alterations do not increase incidence of immunomodulated diseases, such as infections of the upper respiratory tract [63]. These may be because the concentrations of serum IgA and salivary secretory IgA are higher before operation in some tonsillectomy cases than that of nontonsillectomy controls. Tonsillectomy decreased significantly the IgA levels compared with preoperation, but there is no significant difference compared with normal nonoperative controls [27]. Recent study demonstrated children with chronic tonsillitis have increased levels of $CD19^+$ B lymphocytes compared to healthy controls in the pre-operative period. The percentage of B lymphocytes bearing CD23 was found to be significantly higher in patients, most likely representing in vivo B lymphocyte activation due to chronic antigenic stimulation. After the tonsillectomy, despite ongoing B lymphocyte activation, $CD8^+$ T lymphocyte levels increased and B cell levels returned to normal [64].

Tonsillectomy may also lead to certain changes in the cellular immune systems in some boys, including slightly increased percentages of $CD21^+$ cells, raised counts of $CD4^+$ cells, absolute and relative increases in $DR^+$ cells and a raised $CD4^+DR$ count [63]. Peripheral blood $CD8^+$ cells, $CD45RA^+CD4^+$ cells, and $CD8^+CD11b^-$ cells increase significantly after tonsillectomy, compared with their preoperative values in patients with IgAN accompanied by chronic tonsillitis. In some cases, the preoperative serum tumor necrosis factor-alpha (TNF-α), and INF-γ levels were higher than normal before surgery, but decreased after surgery. These results suggest that tonsillectomy suppresses a decrease in suppressor T cells in patients with IgAN and corrects abnormal cell-mediated immune responses in these patients [65].

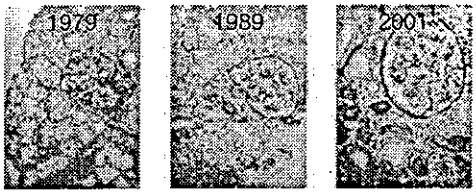
In additional, tonsillectomy has no effect on complement and saliva-derived nonimmunoglobulin host defense factors, such as lysozyme, salivary peroxidases, thiocyanate, hypothiocyanite, and agglutinins, except lactoferrin, which declined significantly [66]. The effects of tonsillectomy on serum and salivary secretory IgG, IgM, and IgE remain still controversial [67, 68].

### Effect of tonsillectomy on urinary finding and renal function

Studies demonstrated that tonsillectomy can improve the urinary finding and keep stable renal function in some patients with IgAN (Table 2). Bene et al [69] followed up the evolution of urinary protein and serum creatinine in 34 patients with IgAN, and Barta et al [70] followed up 35 IgAN patients after tonsillectomy. The urinary protein and microhematuria decreased significantly from 6 months after tonsillectomy than that before operation, and no significant variation was observed in the levels of creatininemia [69]. Furthermore, tonsillectomy stopped gross hematuria in more than two thirds of patients [70]. Tamura et al [71] reported that 46% IgAN patients with chronic tonsillitis showed distinct improvement in urinary findings after the tonsillectomy. Akagi et al [48] followed up 24 patients with IgAN for more than 2 years after tonsillectomy. Remission of proteinuria was observed in 41.7% of the patients 6 months after surgery and in 50.0% 2 years after surgery [48]. The clinical remission rate of urinary finding and the stable renal function rate in tonsillectomy patients with IgAN were significantly higher than that in nontonsillectomy patients [72].

### Effect of tonsillectomy on renal histologic findings

A repeat renal biopsy study for 35 patients demonstrated that renal histologic finding improved distinctly after the combined therapy of methylprednisolone pulse, prednisolone, antiplatelet, and tonsillectomy in IgAN patients [73]. The interval between the first and second biopsy was 18 to 138 months (mean, 77.1 months) in that study. Mesangial proliferation and interstitial mononuclear cell infiltration were significantly reduced in second biopsy specimens. Acute inflammatory glomerular lesions, such as endocapillary proliferations, glomerular tuft necrosis, and cellular crescents, were present in 32




| | | | |
|---|---|---|---|
| BP (mm Hg) | 132/88 | 120/80 | 120/80 |
| UP (g/d) | 0.4 | 0.4 | 0.5 |
| U-RBC (/hpf) | 2.3 | 2 | 2 |
| Scr (mg/dL) | 1.0 | 1.1 | 1.4 |
| Ccr (mL/min) | 113 | 78.9 | 56 |

**Fig. 3. Effect of tonsillectomy on renal histological findings.** This patient was born in 1952, diagnosed with IgA nephropathy (IgAN) by the first renal biopsy in 1979, received tonsillectomy in 1982, discovered hypertension in 1985, and received antihypertensive therapy. The patient underwent the second biopsy in 1989 and the third biopsy in 2001. Renal specimen was performed by periodic acid-Schiff (PAS) staining (original magnification ×50).

patients in first biopsy specimens, whereas these were no longer present in any of the second biopsy specimens. Although there was no significant difference in percentage of globally sclerotic glomeruli between the first and second biopsy specimens, the percentage of segmentally sclerotic glomeruli was significantly lower in second biopsy specimens. The distribution of IgA mesangial deposits had diminished in most patients, and no IgA deposits were seen in second biopsy specimens from eight patients. Impact of isolated tonsillectomy on renal histologic findings was unknown. We followed up a repeated biopsy patient with IgAN and tonsillectomy. He did not receive other drug therapy except for antihypertension. The first renal biopsy showed the marked mesangial proliferation, marked IgA deposit in glomerular mesangium, and almost normal renal tubules and blood vessel. After 10 years, the second biopsy showed the moderate mesangial proliferation, moderate IgA deposit, mild-to-moderate renal tubular atrophy, and mild arteriole sclerosis. The third biopsy after 22 years showed the enlarged glomeruli, mild mesangial proliferation, negative IgA deposit, marked tubular atrophy, and moderate arteriole sclerosis. The results of this patient demonstrated that tonsillectomy can improve IgA deposit and mesangial proliferation and cannot impact renal damage induced by other causes such as hypertension (Fig. 3).

### Effect of tonsillectomy on long-term renal survival

Rasche, Schwarz, and Keller [74] reported that there was no significant correlation between tonsillectomy and ESRD by observing 16 IgAN patients with tonsillectomy and 39 patients without tonsillectomy, and introduced that tonsillectomy does not reduce the risk of developing renal failure [74]. The mean observation time after renal biopsy was relatively short (3.4 ± 4 years) in that study. Another study demonstrated that ESRD was detected in four of 35 IgAN patients after 10 years after tonsillectomy, in eight patients of 40 nontonsillectomy controls [70]. We [17] followed up 118 patients with idiopathic IgAN patients, including 48 tonsillectomy patients and 70 nontonsillectomy patients, for 192.9 ± 74.8 months (48~326 months). In that study, we used three different statistical methods, including the chi-squared test, Kaplan-Meier method with log-rank test, and Cox regression proportional hazards model in order to establish the efficacy of tonsillectomy in IgAN patients. Baseline characteristics at the time of renal biopsy, pathologic finding, and therapy during observation were not significant different between tonsillectomy and nontonsillectomy patients. A mean 15 years after diagnostic biopsy, only five (10.4%) of 48 tonsillectomy patients entered dialysis, whereas 18 (25.7%) of 70 nontonsillectomy patients required dialysis, by chi-squared test, $P = 0.0393$. Kaplan-Meier analysis showed the renal survival rates of tonsillectomy patients were statistically higher than those of non-tonsillectomy (log-rank test, $P = 0.0329$). For example, renal survival rates were 89.6% and 63.7% in the patients with and without tonsillectomy, respectively, at 240 months after renal biopsy. Cox regression analysis showed that the relative risk for terminal renal failure in patients with tonsillectomy was lower compared to nontonsillectomy patients (hazard ratio 0.22, 95% CI 0.06 to 0.76, $P = 0.0164$). The results of these three statistical analyses were consistent. All revealed that tonsillectomy had a favorable effect on long-term renal survival in patients with IgAN [17].

### Indications and limitations of tonsillectomy

Tonsillectomy and adenoidectomy procedures are among the oldest surgical procedures still performed today. Otolaryngologically, the two main indications for tonsillectomy are upper airway obstruction due to tonsillar hypertrophy and recurrent acute or chronic tonsillitis. Adenoid hypertrophy with upper airway or eustachian tube obstruction and recurrent acute or chronic adenoiditis or otitis media are main indications to perform an adenoidectomy [75]. Nephrologically, indications for tonsillectomy are to date still unclear. In fact, many factors have effect on the efficacy of tonsillectomy in patients with IgAN, such as urinary finding and grades of renal damage. In general, the efficacy of tonsillectomy in patients with hemoturia type IgAN, especially presenting hemoturia after tonsil infection, is good [76]. We have showed that with a mild renal damage condition, in which the amount of urine protein excretion was less than 1.0 g/24 hours and global glomerular sclerosis less than 25%, none of 26 patients with tonsillectomy needed dialysis, whereas five (13.2%) of 38 patients without tonsillectomy required dialysis [17]. The percentage entering

dialysis in the tonsillectomy patients with moderate renal damage, such as urinary protein was more than 1.0 g/24 hours, but global glomerular sclerosis was less than 25% of total, was less than half of that in the nontonsillectomy patients [17]. On the other hand, the patients with a marked renal damage, in whom both the amount of urine protein excretion was more than 1.0 g/24 hours and global glomerular sclerosis was more than 25% of total or crescent formation was more than 25% of total might develop renal failure even if tonsillectomy was performed, that is, tonsillectomy is mainly indicated for patients with mild or moderate IgAN [17, 77, 78]. Rupture of the glomerular basement membrane occurred more frequently in the noneffective tonsillectomy than in the effective tonsillectomy group [79, 80]. Hotta et al [81] conducted a retrospective investigation of the renal outcome in IgAN patients with a median observation period of 75 months after tonsillectomy and steroid pulse therapy. Their results showed that there were no significant differences between the tonsillectomy and nontonsillectomy groups regarding the incidence of progressive renal functional loss defined as a 50% increase in baseline serum creatinine, but a combination of tonsillectomy and steroid pulse therapy had a significant impact on clinical remission by multivariate Cox regression analysis [81]. Sato et al [82] retrospectively investigated 70 patients with advanced IgAN (serum creatinine $\geq$1.5 mg/dL) classified into three groups according to their treatment regimens, that is, steroid pulse with tonsillectomy (30 patients), conventional steroid (25 patients), and supportive therapy (15 patients). During the mean follow-up period of 70.3 (12 to 137) months, 41.4% of patients reached ESRD (13.3% vs. 56.0% vs. 73.3%). The incidence of ESRD in the patients treated by steroid pulse with tonsillectomy was significantly lower than that in the patients treated by conventional steroid and supportive therapy at a baseline creatinine level of 1.5 to 2 mg/dL, but no statistical difference was observed at a level of >2 mg/dL [82]. These results suggest tonsillectomy combined with steroid pulse therapy may be effective in the IgAN patients with a baseline creatinine level of $\leq$2 mg/dL, whereas when serum creatinine >2 mg/dL, tonsillectomy may not change renal outcome even if that combines steroid therapy.

## CONCLUSION

First, human tonsils are lymphoid organs and play a role in production of antibodies and local mucosal immune, especially in children. Tonsillectomy decreases the levels of serum IgA and salivary secretory IgA, but these changes do not cause significant immune deficiency and do not increase incidence of the upper respiratory tract infections. Second, tonsils are closely related to IgAN and polymeric IgA1 deposited in glomerular mesangium is at least in part of tonsillar origin. However, it is unclear that why do IgA-producing cells be predominant in tonsils with IgAN and how does IgA produced by tonsils deposit in mesangium. Third, tonsillectomy can improve the urinary findings, keep stable renal function, and have a favorable effect on long-tern renal survival in some IgAN patients. The indications of tonsillectomy in patients with IgAN include mainly the deterioration of urinary findings after tonsillar infection, mild or moderate renal damage. However, tonsillectomy may not be enough and may not change the prognosis in IgAN patients with marked renal damage. Unfortunately, studies regarding tonsillectomy were performed until now in a retrospective style and little information has been available about the side effect or complication of the operation in IgAN patients. In order to further clarify the clinical efficacy and security of tonsillecteomy, randomized prospective controlled trials are necessary because of the high degree of variability of IgAN.

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

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

*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

**Key words:** stem cell, side population, GFP.

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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 $1 \times 10^6$ 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

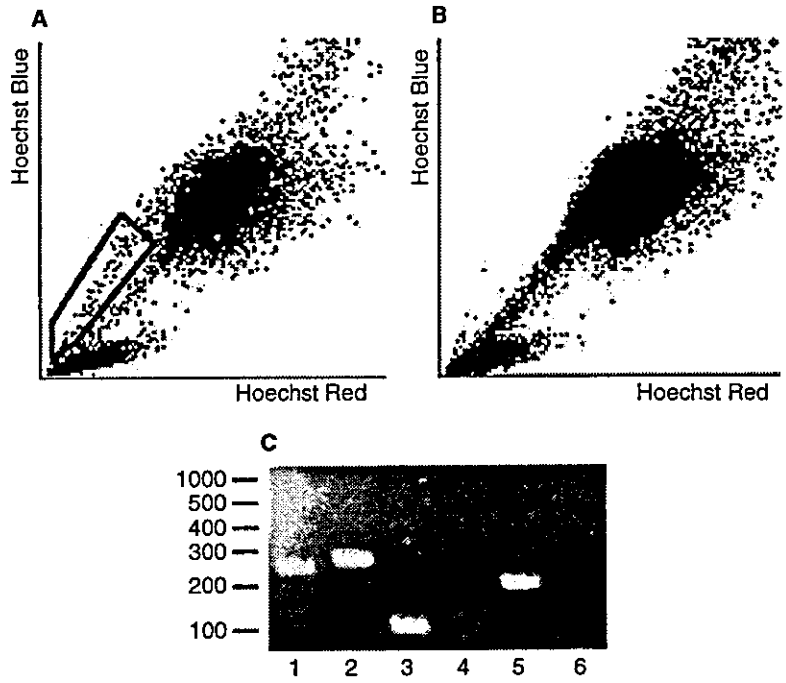


**Fig. 1. Hoechst 33342 fluorescence of kidney-derived cells and reverse transcription-polymerase chain reaction (RT-PCR) analysis of kidney-derived Hoechst$^{low}$/side population cells.** Cells prepared from wild-type adult rat kidneys were stained with Hoechst 33342 in the absence (*A*) or presence (*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

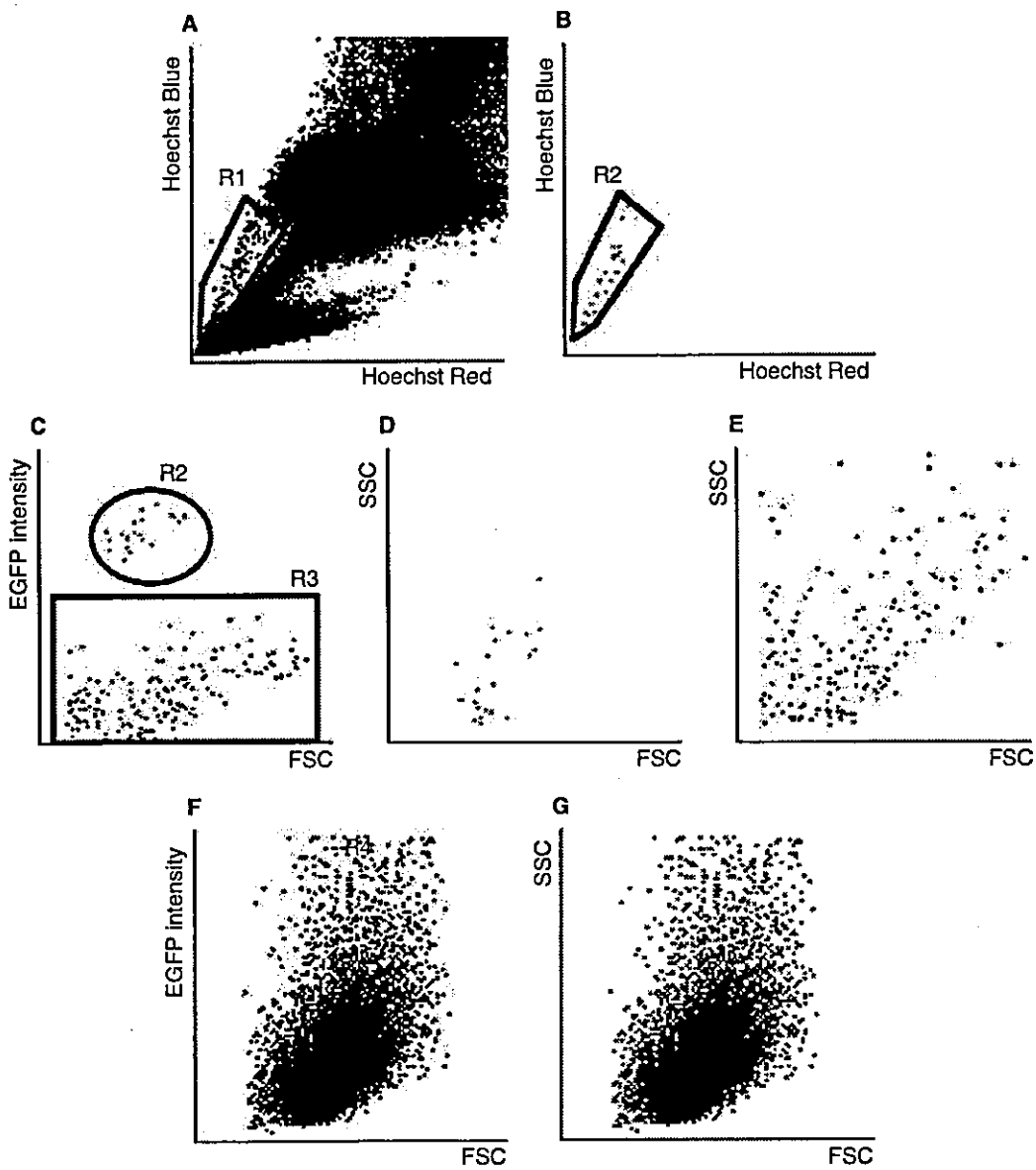


**Fig. 2. Flow cytometric analysis of kidney-derived cells that are prepared from bone marrow–transplanted adult rats.** (*A*) Hoechst fluorescence of whole kidney-derived cells is shown. Hoechst$^{low}$/side population fraction is indicated in the boxed region, R1. (*B*) Enhanced green fluorescent protein (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

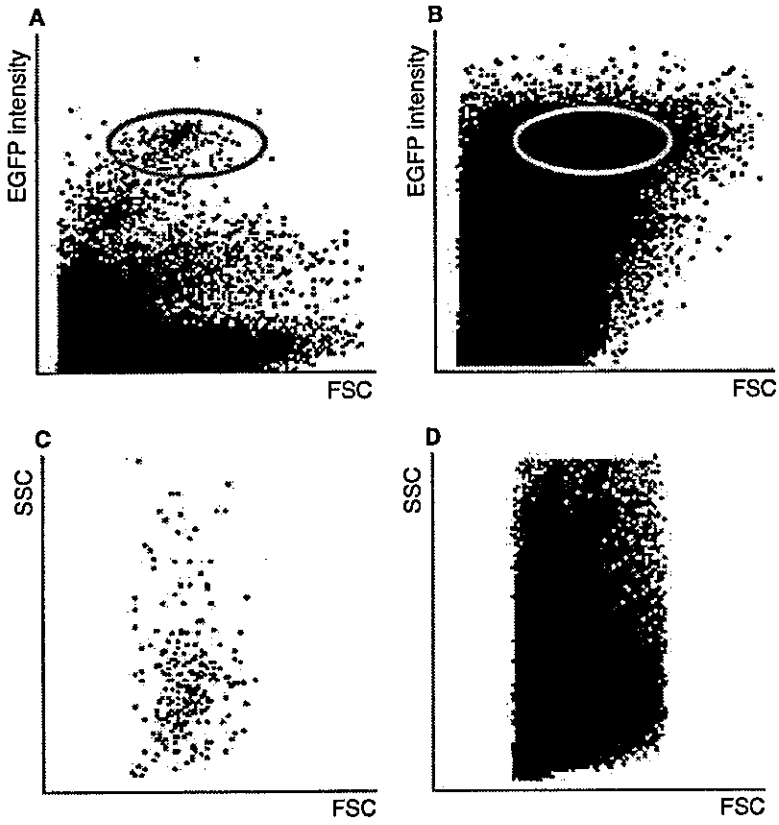


**Fig. 3. Flow cytometric analysis of bone marrow of side population–transplanted rats.** The whole bone marrow taken from a side population–transplanted rat or an enhanced green fluorescent protein (EGFP) rat was subjected to flow cytometric analysis. (*A* and *C*) Bone marrow from a side population–transplanted 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

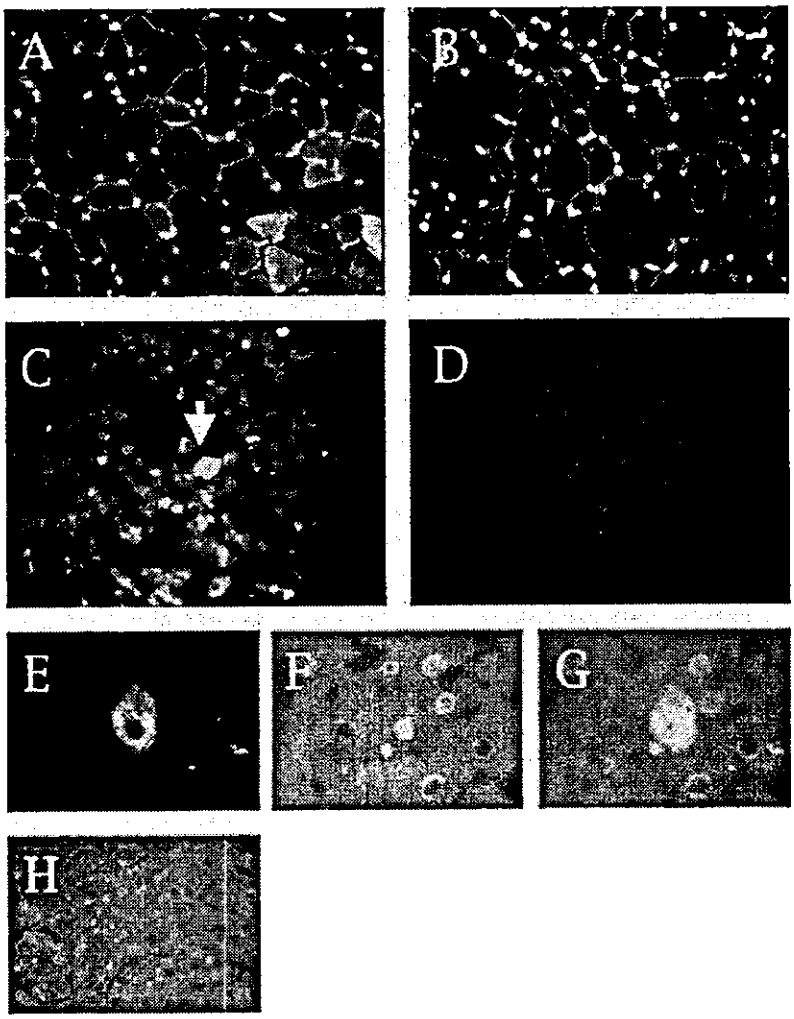


**Fig. 4. Immunohistochemical analysis of skeletal muscle and liver of side population–transplanted rats.** Merged fluorescence images are shown (×400, original magnification). Enhanced green fluorescent protein (EGFP) provides bright green. (*A*) Skeletal muscle from a side population–transplanted rat. (*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

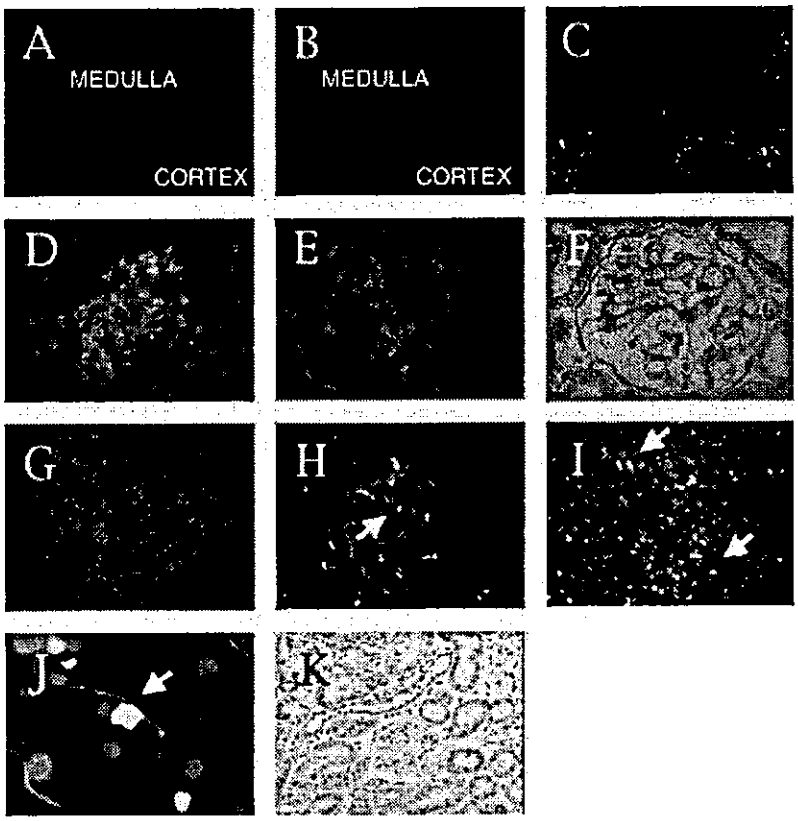


**Fig. 5. Kidney of side population–transplanted rats and bone marrow–transplanted rats.** Fluorescence images of kidneys that are derived from wild-type rats, side population–transplanted rats or bone marrow–transplanted rats are shown. (*A*) Wild-type rats. (*B, C, D, E,* and *G*) Side population–transplanted 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].

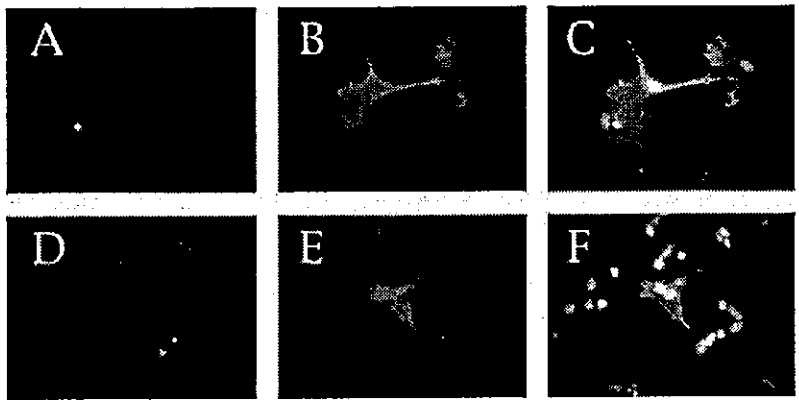


**Fig. 6. Culture of kidney-derived Hoechst$^{low}$/side population cells.** Enhanced green fluorescent protein (EGFP)$^{+}$ kidney-derived Hoechst$^{low}$/side population cells were cocultured with wild-type bone marrow cells that were preplated on collagen type I as described in the **Methods** section. 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 $1 \times 10^8$ 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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# Electroporation-mediated HGF gene transfer ameliorated cyclosporine nephrotoxicity

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**Electroporation-mediated HGF gene transfer ameliorated cyclosporine nephrotoxicity.**

*Background.* The clinical utility of cyclosporine A (CsA) has been limited by its nephrotoxicity, which is characterized by tubular atrophy, interstitial fibrosis, and progressive renal impairment. Hepatocyte growth factor (HGF) has been reported to protect and salvage from renal injury as a renotropic and antifibrotic factor. Here, we investigated protective effects of HGF gene therapy on rat CsA-induced nephrotoxicity using electroporation-mediated gene transfer.

*Method.* CsA was subcutaneously administered daily under low sodium diet, and HGF gene was transferred into skeletal muscle by electroporation on days 7 and 14. We also examined the antiapoptotic mechanism of HGF using human proximal tubular epitherial cells.

*Results.* HGF gene transfer rescued CsA-induced initial tubular injury and suppressed interstitial infiltration of ED-1–positive macrophages in CsA-induced nephrotoxicity. In addition, HGF significantly inhibited tubular cell apoptosis, and increased the number of proliferating tubular epithelial cells. In vitro studies suggest that HGF executes the antiapoptotic function by enhancing the phosphorylation of Akt and Bcl-2. Northern blot analysis demonstrated that HGF gene transfer suppressed cortical mRNA levels of transforming growth factor-β (TGF-β). Consequently, HGF gene transfer significantly reduced a striped interstitial phenotypic alteration and fibrosis.

*Conclusion.* We demonstrated that HGF gene transfer reduced CsA-induced tubular cell apoptosis and interstitial fibrosis. HGF gene transfer could be a potential strategy for preventing renal fibrosis.

The introduction of cyclosporine (CsA) into clinical practice has resulted in marked improvement in the short-term outcome of organ transplantation, and 1-year survival of renal allografts has improved significantly [1, 2]. However, the dose-limiting adverse effect of long-term CsA administration is chronic nephrotoxicity, which remains as an unsolved problem of transplant therapy and limits clinical utility of CsA [1, 3]. Chronic CsA nephrotoxicity may progress to an irreversible renal lesion characterized by tubular atrophy, striped interstitial fibrosis, hyalinosis of the afferent arteriole, and progressive renal impairment [4, 5]. While acute CsA nephrotoxicity is thought to be a result from intrarenal vasoconstriction, the mechanism leading to the interstitial fibrosis of chronic CsA nephrotoxicity remains unclear. Previous results showed tubular epithelial cells underwent apoptosis in the course of immunosuppression with CsA [6]. In addition, CsA-induced nephrotoxicity is associated with up-regulation of transforming growth factor-β (TGF-β1), type I collagen, and TGF-β1 is considerably important to its progression [7].

Hepatocyte growth factor (HGF), a multifunctional polypeptide originally characterized as a potent mitogen for mature hepatocyte in primary culture, is a heterodimeric molecule consisting of a 69 kD α chain and a 34 kD β chain held together by a disulfide bond [8, 9]. HGF and its specific c-met receptor constitute a signaling system that plays an important role in renal development and in the maintenance of normal adult kidney structure and functions. HGF elicits potent mitogenic, motogenic, morphogenic, and antiapoptotic activities in renal tubular epitherial cells. Recent studies reports that HGF has numerous functions in diverse cell survival and tissue regeneration [10]. Administration of cisplatin caused acute renal failure in mice, while HGF administration prevented histologic destruction of renal tubules and strongly suppressed the onset of acute renal dysfunction [11]. Likewise, HGF-treated rats showed a lesser degree of histologic injury when renal ischemia was induced by arterial occlusion [12]. HGF has been also suggested to act as a cell survival factor by inhibiting apoptosis in

**Key words:** electroporation, HGF, gene therapy, cyclosporine.

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various cells. In addition to protective or regenerating effect of HGF on tubular epithelial cells [13], HGF has been reported to have antifibrotic effects [14]. Administration of exogenous HGF protein effectively inhibits the activation of matrix-producing myofibroblasts, attenuates extracellular matrix deposition and interstitial fibrosis, and by suppressing profibrogenic cytokine TGF-β1 and its type I receptor expression in vivo. These observations suggest that exogenous HGF supplementation has a therapeutic role in the development of CsA-induced nephrotoxicity.

However, it is difficult to sustain a level of exogenous HGF in vivo by intravenous repeated injection of purified protein even at very short intervals, because exogenous HGF is very unstable in blood circulation due to the rapid clearance by the liver [15]. In this point of view, repeated injections of recombinant HGF protein are both inconvenient and extremely costly. To circumvent this problem, we developed a new gene transfer system by electroporation in vivo. Recently, we demonstrated that PDGF receptor-IgG Fc chimera (PDGFR/Fc) gene transfer by electroporation to rat muscle resulted in increased plasma concentration of PDGFR/Fc molecule and thereby ameliorated the disease progression in experimental glomerulonephritis [16]. Electroporation is free from oncogenicity, immunogenicity, and cytotoxicity of viral vectors. In addition, electroporation-mediated gene transfer technique showed significantly higher transfection efficiency than hemagglutinating virus of Japan (HVJ) liposome method [17]. In the present study, we investigated the effects of HGF gene transfer into CsA-induced nephrotoxicity in rat. This gene transfer approach may provide an efficient way to investigate the therapeutic potential of HGF gene transfer for long-term survival of allograft kidney.

## METHODS

### Cell culture and treatment

Human proximal tubular epithelial HK-2 cells (American Type Culture Collection; Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) Gibco Laboratories) and piperaciline/streptomysin (Sigma Chemical Co., St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air atmosphere. Cells were seeded at $3 \times 10^5$ per 60 mm culture disk in growth medium for 24 hours before pharmacologic treatment. To examine the effect of HGF on CsA-induced apoptosis, human recombinant HGF (50 ng/mL) was added to the culture medium. One hour after HGF treatment, culture medium was replaced with 40 μmol/L of CsA dissolved in 0.1% of dimethyl sulfoxide (DMSO) and cells were incubated for 24 hours. DMSO alone was used as a control. In some experiments, cells were pretreated with 50 μmol/L of zVAD-fmk (Promega, Madison, WI, USA), a caspase inhibitor, for 1 hour before CsA treatment.

### Caspase-3 activity assay

Caspase-3 protease activity was assayed by following the manufacturer's instructions (Promega). Shortly, HK-2 cells were washed twice with phosphate-buffered saline (PBS) and carefully collected. The cell pellets were suspended in the cell lysis buffer containing 25 mmol/L HEPES (pH 7.5), 5 mmol/L $MgCl_2$, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 5 mmol/L dithiothreitol (DTT), 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL pepstatin A, and 10 μg/mL leupeptin, and the cells were lysed by freeze and thawing for three times. The homogenates were cleared by centrifugation at 15000*g*, 4°C for 20 minutes. The centrifugation step was repeated and the clear supernatants were used for the caspase assay. After determination of the protein concentration using bovine calf albumin (BCA), caspase-3 activity was measured by mixing 54 μL of equivalent extract, 32 μL of caspase assay buffer containing 312.5 mmol/L HEPES, pH 7.5, 31.25% wt/vol sucrose, 0.3125% wt/vol 3-[(3-cholamido-propyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10 μL of 100 mmol/L DTT, 2 μL DMSO, and 2 μL colorimetric substrate (10 mmol/L Ac-DEVD-pNA in DMSO). Free pNA released from the substrate upon cleavage by DEVEase, producing yellow color, was measured by a photometer at 405 nm. From the data, specific caspase-3 activities were calculated using pNA calibration curves.

### Western blot analysis

To examine the antiapoptotic mechanism of HGF on CsA cytotoxicity, cells were treated with 40 μmol/L of CsA and/or 50 ng/mL of human HGF as above. Thereafter, cells were washed with PBS and lysed in cell lysis buffer (Cell Signaling, Beverly, MA, USA). Cell lysates were centrifuged for 15 minutes at 4°C to remove debris, and the protein concentrations were determined using BCA method. The lysates (20 μg) were applied into sample buffer [58.3 mmol/L Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), and50 mmol/L DTT, melcaputoethanol, and bromophenol blue] and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE), and electrotransferred to polyvinylidene difluoride (PVDF) (Hybond P; Amersham Corp., Buckinghamshire, UK). Immunoblotting was performed with appropriate antibodies; anti-Akt antibody (Cell Signaling), anti-phospho Akt antibody (Cell Signaling), and antirat bcl-2 antibody (MBL, Watertown, MA, USA).

### Experimental design

To examine the efficiency of HGF gene transfer on CsA nephrotoxicity, 6-week-old male Sprague-Dawley rats (SLC Japan, Hamamatsu, Japan) weighing 180 to 190 g received daily subcutaneous injections of CsA at 30 mg/kg dissolved in 100 μL of sesame oil under low-salt diet (0.03% sodium) (Test Diet, Richmond, IN, USA). On day 7 or 14, rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) and tibialis anterior muscles, which were pretreated with 50 μg of bupivacaine, were exposed. We previously reported that pretreatmemt of bupivacaine combined with electroporation significantly enhanced the gene expression of the transfected muscle [16]. Thereafter, 100 μL of PBS containing pCAGGS-HGF (200 μg) (CsA/HGF group) or vehicle (CsA group) was transfected by electroporation into the bupivacaine-treated muscle as previously reported ($N = 12$ in each group) [16]. Plasmid DNA pCAGGS-HGF was constructed by inserting the *Xba*I-*Bam*HI fragment containing full length cDNA of rat HGF into pUC-CAGGS, which contains the cytomegalovirus enhancer and β-chicken promoter. Shortly, a pair of tweezers-type electrodes was inserted into the tibialis anterior muscle to encompass the DNA injection sites, and the square wave of electric pulses was delivered. Three pulses of 75 V each, lasting 100 milliseconds, and three pulses of opposite polarity were applied. On day 14 or 21, plasma samples were collected and kidneys were removed after perfusion with 20 mL of cold PBS. The cortex was carefully dissected from medulla and was then processed for evaluation by light microscopy, RNA analysis, and immunohistochemistry. Plasma was harvested immediately by centrifugation at 4°C and stored at −80°C until determined. Plasma HGF concentration was measured by enzyme immunogen assay method (Institute of Immunology, Tokyo, Japan). To investigate the effect of HGF gene transfer on normal kidney, we also transfected the HGF gene into the muscle of normal rat and harvested ($N = 3$ in each time point).

### Analysis of plasma samples

Serum creatinine and urea nitrogen levels were measured by creatinase-peroxydase method and urease-ultravioltet method, respectively (SRL, Inc., Osaka, Japan). The blood level of CsA was measured by a monoclonal radioimmunoassay for CsA (SRL, Inc.). Plasma HGF concentration was measured by enzyme immunogen assay (EIA) method (Institute of Immunology).

### Histologic examination

Tissue samples were fixed in 4% buffered paraformaldehyde for 12 hours and embedded in paraffin. Histologic sections (2 μm) were stained with periodic acid-Schiff (PAS) and Masson's trichrome. Tubulointerstitial injury was evaluated on PAS-stained sections by lesions, including tubular casts, dilation and atrophy, thickening of tubular basement membrane, and interstitial matrix expansion. The interstitial fibrotic area was stained blue in Masson's trichrome staining, and color image analyzer estimated the area quantitatively. The percentage of fibrotic areas per field of cortex was counted at ×400 magnification in the minimum of 10 fields [18].

### Immunohistochemical stainings

Renal tissues were also fixed in cold methyl Carnoy's solution for 6 hours and exchanged into cold 70% ethanol, and then embedded in paraffin in a routine fashion. Tissue sections were cut at a thickness of 4 μm and were dewaxed and stained with ED-1 antibody to identify macrophage infiltration, followed by a second reaction with biotin-labeled antirat IgG antibody (Vector, Burlingame, CA, USA). Finally, an avidin-biotin coupling (ABC) reaction was performed on the sections (Vectastain Elite) (Vector). To stain α-smooth muscle actin (α-SMA), a marker of interstitial myofibroblasts, anti-α-SMA monoclonal antibody (EPOS System) (Dako A/S, Glostrup, Denmark) was used. In order to monitoring of the proliferation of tubulointerstitial cells, we used anti Ki-67 antibody (Dako A/S).

### TUNEL assay

DNA fragmentation associated with apoptosis was detected in situ in tissue sections by the addition of fluoresceine-labeled nucleotides to free 3'hydroxyl groups in DNA by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) method using In Situ Apoptosis Detection Kit (TaKaRa Biomedicals, Tokyo, Japan). Sections were dewaxed and treated with proteinase K (400 μg/mL) for 5 minutes at room temperature and incubated with 3% $H_2O_2$ for 20 minutes at room temperature to inactivate endogenous peroxidase. Sections were rinsed with PBS three times for 5 minutes, then immersed in Labeling Safe buffer (TaKaRa Biomedicals) with TdT enzyme (TaKaRa Biomedicals) at 37°C for 60 minutes. Sections were washed by PBS and incubated with anti-fluorescein isothiocyanate (FITC) horseradish peroxidase (HRP) conjugate at 37°C for 30 minutes. Apoptotic nuclei were visualized by the addition of diaminobenzidine and counterstained with methyl green. TUNEL-positive cells were counted in the cortical tubular cells at ×200 magnification in the minimum of 10 fields.

### Semiquantitation of phenotypic alteration

The area of the fibrotic lesion of cortical interstitium was determined in sections stained by Masson's trichrome