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Expression of stem cell marker CD133 in fetal and adult human kidneys and pauci-immune crescentic glomerulonephritis

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Summary. Background: Different cell types in the kidney, including those in tubules, glomeruli, and interstitium, have been proposed as candidate adult renal stem cells, raising controversy about the very existence of such cells. In this study, we sought to clarify the location and nature of adult renal stem cells and to address their reparative role in native kidney disease. Methods: The expression of the stem cell marker CD133 was analyzed in 31 normal fetal and adult human kidneys by immunohistochemical methods. Co expression of CD133 with Ki-67 and tubule specific markers was also examined. Seventeen cases of pauci-immune glomerulonephritis were evaluated for CD133 and Ki-67 expression, and this was correlated with the patients' renal prognosis. Results: CD133 was expressed in S-shaped bodies of fetal kidneys and co-expressed with Ki-67. It was highly expressed in mature tubules of fetal and adult kidneys without Ki-67 co-expression. $CD133^+$ cells were most abundant in the S3 segment of proximal tubules and co-expressed with the distal tubule marker, suggesting multipotency. Most tubular $CD133^+$ cells in normal adult kidneys exhibited pathologic features of acute and chronic injury. In pauci-immune glomerulonephritis, tubular CD133 and Ki-67 co-expression tended to be higher in cases where renal function recovered. Conclusion: These results suggest that adult renal stem cells reside predominantly in the S3 segment of the proximal tubule, where they remain mitotically silent under normal conditions, but can be induced by cellular injury. These results also suggest a potential role for adult renal stem cells in recovery from native human kidney disease.

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Introduction

Adult stem cells, which retain proliferative and differentiation potential, generally reside in a specialized site, or niche, in the organ, which helps the cells to maintain their stem cell characteristics. For example, hematopoietic stem cells reside in the bone marrow, epidermal stem cells are found in the hair bulge, neural stem cells are localized in the subventricular zone and hippocampus, and gastrointestinal stem cells are present in the intestinal crypts (Eckfeldt et al., 2005). In the kidney, different cell types from multiple sites have been proposed as candidate adult renal stem cells; indeed, the identity and niche of adult renal stem cells remain matters of debate. The interstitial cells in the renal papilla of adult rodent kidneys have been reported to be adult renal stem cells (Oliver et al., 2004). Subsequent studies suggested that tubular cells and glomerular parietal epithelial cells (PECs) in the corticomedullary junction (CMJ) and the cortex may also serve this function (Bussolati et al., 2005; Gupta et al., 2006; Sagrinati et al., 2006; Kim et al., 2008). These disparate findings suggest that a systematic evaluation of stem cells in developing fetal and mature adult human kidneys is warranted to help clarify the identity and niche of adult renal stem cells.

Adult stem cells play a critical role in maintaining the normal homeostasis of an organ and in repair of tissue damage following injury. Thus, considerable effort has been devoted to identifying cell surface marker(s) that can be used to purify and isolate live stem cells from the kidney, with the expectation that such cells may be valuable for stem cell therapy of kidney diseases. One

such marker is the pentaspan membrane glycoprotein, CD133, initially identified as a cell surface marker of hematopoietic stem cells (Miraglia et al., 1997). Subsequent reports have demonstrated that CD133 can also be used as a marker of stem cells in various human tissues, including brain, skin, prostate, and pancreas, as well as kidney (Mizrak et al., 2008). Previous studies using CD133 as a single stem cell marker or one of multiple markers showed that $CD133^+$ renal cells possessed the stem cell characteristics of self-renewal and multipotency, differentiating not only into renal constituents, such as tubules, glomerular podocytes, and endothelial cells, but also into mesenchymal components, including osteogenic cells, adipocytes, and neurons (Bussolati et al., 2005; Sagrinati et al., 2006; Kim et al., 2008; Ronconi et al., 2009). These results support the idea that $CD133^+$ renal cells are the stem cells of the kidney.

The role of stem cells in renal repair has been demonstrated in animal models and renal allograft studies (Bao et al., 2008; Loverre et al., 2008). During the regenerative phase of post transplantation acute tubular necrosis in patients with delayed graft function, $CD133^+$ cells were increased and engaged in proliferation (Loverre et al., 2008). $CD133^+$ cell numbers were also increased in the early stage of chronic tubulointerstitial damage in an allogeneic rat transplant model (Bao et al., 2008). Furthermore, injection of $CD133^+$ cells reduced proteinuria and improved chronic renal injury in an adriamycin-induced nephropathy model in severe combined immune deficiency (SCID) mice (Ronconi et al., 2009). These findings suggest that adult renal stem cells play an important role in repairing renal damage caused by the acute and chronic injury that accompany renal dysfunction and proteinuria.

Pauci-immune crescentic glomerulonephritis is the most common cause of rapidly progressive glomerulonephritis (RPGN) in elderly patients. RPGN is an aggressive renal syndrome, characterized by rapid and progressive decline of kidney function. If untreated, death from renal failure ensues within weeks to months. The high levels of initial serum creatinine and the high percentages of crescentic and sclerotic glomeruli in renal biopsy specimens have been suggested as clinical indicators of poor prognosis in RPGN (Keller et al., 1989, 1994). However, the prognostic significance of adult renal stem cells for recovery from pauci-immune glomerulonephritis has not yet been studied.

Here, with the goal of further expanding our understanding of adult renal stem cells, we investigated the temporal and spatial expression of CD133, and evaluated CD133 participation in cell proliferation in both fetal and adult human kidneys. To identify the tubular location and nature of $CD133^+$ cells in adult human kidney, we studied the co-expression of CD133 and tubule specific markers. Finally, we analyzed the prognostic significance of glomerular and tubular CD133 expression in pauci-immune crescentic glomerulonephritis.

Materials and methods

Kidney tissues

This study was approved by the Asan Medical Center Institutional Review Board. Archive autopsy files between 2005 and 2007 in the Department of Pathology at Asan Medical Center were searched for normal fetal and pediatric kidneys. Of the 300 autopsy cases during this period, 19 therapeutic abortion cases with no clinical or pathologic evidence of renal abnormalities or autolysis were selected after reviewing medical and pathology records and examination of glass slide specimens. Three of the 19 cases were from the first gestational trimester, 10 were from the second gestational trimester, and three were from the third gestational trimester. One case each of postnatal 1-, 2-, and 34-month kidneys were also included. The adult kidney tissues were prepared from nine cases of surgically removed kidneys, because of neoplastic and non-neoplastic kidney diseases. A normal portion of the adult kidneys that was remote from the surgical lesion was selected after gross and microscopic examination.

The expression patterns of CD133 and Ki-67 were confirmed in kidney needle biopsy tissues, which were taken from three individuals with normal (clinically and serologically) renal function. These three cases were selected from among 2614 kidney needle biopsy cases from 1995 to 2007 in the archive files of the Department of Pathology at Asan Medical Center. Their serum levels of blood urea nitrogen and creatinine were within normal limits at the time of the biopsy and until the last follow up, at 5, 36, and 42 months, respectively. Clinicopathologic features of all cases are summarized in Table 1.

Pauci-immune crescentic glomerulonephritis was chosen as the focus of this study because of the availability of kidney tissue, the severe damage to both glomeruli and tubules, and the fact that these patients receive a standardized treatment regimen. Immunohistochemical and immunofluorescence investigations were performed on the residual renal biopsy samples after conventional light microscopic, immunofluorescence, and ultrastructural studies. Of the 25 cases of biopsy-proven pauci-immune crescentic glomerulonephritis diagnosed at Asan Medical Center from 1995 to 2007, eight were excluded for the following reasons: suboptimal amount of residual kidney tissue (3 cases), lack of relevant follow-up data (2 cases), and death during treatment (3 cases). To examine the relevance of adult renal stem cells in the renal recovery from pauci immune crescentic glomerulonephritis, the remaining 17 cases were divided into two groups: those who recovered renal function (10 cases) and those who failed to recover after treatment (7 cases). Renal recovery was defined as an absence of dialysis dependence and a decline in the glomerular filtration rate (GFR) at the last follow-up to a

value equal to or less than that at the time of treatment initiation.

Immunohistochemistry and immunofluorescence

Immunohistochemical and indirect immunofluorescence studies were performed as previously described using formalin-fixed paraffin-embedded kidney tissues (Kim et al., 2008). The avidin-biotin complex immunoperoxidase technique was used for immunohistochemistry with diaminobenzidine as the chromogen. A universal secondary antibody kit (UltraVision Plus/HRP, Lab Vision, Fremont, CA) was used according to the manufacturer's instructions. Hematoxylin and periodic acid-Schiff (PAS) were used for counterstaining, and 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei in immunofluorescence studies. Additional information about the primary antibodies and methods used in this study is summarized in Table 2. When quadruple immunostaining was performed using CD133/1 (AC133), Ki-67, CD10, and cytokeratin 7 (CK7) antibodies, tissues were first double-stained by immunohistochemical methods for CD133 and Ki 67 followed by indirect immunofluorescent staining for CD10 then CK7 using FITC- and TRITC-conjugated secondary antibodies, respectively. Concentration-matched isotype controls for respective antibodies were used as a negative control (negative control mouse IgG1 and rabbit immunoglobulin fraction, DakoCytomation, Glostrup, Denmark). Light microscopic images and fluorescence signals were detected with a fluorescence microscope (Olympus, Center Valley, PA). Because CD133 expression varied markedly within the kidney, all tubular and glomerular $CD133^+$ cells in the stained tissue were counted separately, and $CD133^+$ expression was normalized to the two-dimensional area (mm^2) of the biopsy sample, which was measured using Motic Images Advanced 3.2 program (Motic, China).

Statistical analyses

Chi-squared and Mann-Whitney U-tests were employed, as appropriate, and statistical analyses were performed using the SPSS software (ver. 12.0K; SPSS, Chicago, Illinois). Differences were regarded as

Table 1. Clinicopathologic features of 31 fetal and adult cases.

Case No.	Procedure	Age		Autopsy / pathology diagnosis
1	Autopsy	Fetus	11 wks	No pathologic abnormalities of the fetus, aborted due to maternal chronic renal failure
2	Autopsy	Fetus	12 wks	Cystic hygroma
3	Autopsy	Fetus	13 wks	Arthrogryposis multiplex
4	Autopsy	Fetus	16 wks	No pathologic abnormalities of the fetus, acute chorioamnionitis
5	Autopsy	Fetus	16 wks	Agenesis of cerebellum and corpus callosum, hydrocephalus
6	Autopsy	Fetus	19 wks	Holoprosencephaly, cerebellar hypoplasia
7	Autopsy	Fetus	20 wks	Agenesis of corpus callosum, hypoplasia of cerebellum, pulmonary hypoplasia, ASD
8	Autopsy	Fetus	21 wks	Congenital diaphragmatic hernia
9	Autopsy	Fetus	21 wks	Arnold-Chiari malformation
10	Autopsy	Fetus	24 wks	Interrupted aortic arch (type B), ASD, VSD
11	Autopsy	Fetus	25 wks	Ectromelia, axial form
12	Autopsy	Fetus	25 wks	Ventriculomegaly of bilateral lateral ventricle.
13	Autopsy	Fetus	26 wks	Tricuspid valve stenosis
14	Autopsy	Fetus	28 wks	Intraventricular hemorrhage with secondary hydrocephalus
15	Autopsy	Fetus	30 wks	Bilateral pulmonary hypoplasia
16	Autopsy	Fetus	36 wks	Absent pulmonary valve syndrome, hypoplasia of left lung,
17	Autopsy	Infant	30 D	Obstruction of right common carotid artery by organized thrombi, ASD, AS
18	Autopsy	Infant	65 D	Atelectasis and pulmonary edema, ASD, PDA
19	Autopsy	Childhood	34 Mo	Acute necrotizing encephalopathy, familial, possibly autosomal dominant
20	Radical nephrectomy	Adult	21 yrs	Clear cell renal cell carcinoma
21	Radical nephrectomy	Adult	27 yrs	Renal cell carcinoma, unclassified
22	Simple nephrectomy	Adult	30 yrs	Arteriovenous malformation
23	Radical nephrectomy	Adult	32 yrs	Clear cell renal cell carcinoma
24	Radical nephrectomy	Adult	38 yrs	Clear cell renal cell carcinoma
25	Radical nephrectomy	Adult	57 yrs	Clear cell renal cell carcinoma
26	Radical nephrectomy	Adult	62 yrs	Clear cell renal cell carcinoma
27	Radical nephrectomy	Adult	67 yrs	Clear cell renal cell carcinoma
28	Radical nephrectomy	Adult	79 yrs	Clear cell renal cell carcinoma
29	Needle biopsy	Adult	24 yrs	No diagnostic abnormalities in kidney, accidentally biopsied instead of liver
30	Needle biopsy	Adult	56 yrs	No diagnostic abnormalities in non-neoplastic kidney, angiomyolipoma
31	Needle biopsy	Adult	62 yrs	No diagnostic abnormalities, donor work-up for kidney transplantation

wks, weeks; D, days; Mo, months; yrs, years; ASD, atrial septal defect; VSD, ventricular septal defect; PDA, patent ductus arteriosus; AS, aortic stenosis.

statistically significant at P values <0.05.

Results

CD133 expression in the fetal human kidney

Kidney nephrons originate from nephrogenic vesicles, which develop through interactions between the ureteric bud and the metanephric mesenchyme of the metanephros in kidney development. A nephrogenic vesicle subsequently transforms into an S-shaped body, and then its lower limb differentiates into glomerular podocytes and PECs of the Bowman's capsule of the adult kidney. The middle limb gives rise to the proximal tubule, and the upper limb develops into Henle's loop and the distal tubule (Shimazui et al., 2000).

CD133 was expressed in the apical cell membrane with a distinct spatial expression pattern (Fig. 1A-H). CD133 was strongly expressed in the S-shaped body of the nephrogenic zone (Fig. 1A). CD133 expression was strongest in the middle limb of the S-shaped body, and was weaker in the upper and lower limbs (Fig. 1A). CD133 expression was almost undetectable in the condensed mesenchyme and ureteric buds. In the deeper kidney, where glomeruli and tubules are relatively mature, CD133 was weakly expressed in glomerular PECs and focal tubules in the cortex and the medulla, including the papilla (Fig. 1B-D). Tubular CD133 expression was segmental, with strong expression in the CMJ; this was especially apparent in longitudinally sectioned tubules (Fig. 1C). This pattern of tubular and glomerular CD133 expression was observed until the early second trimester (up to the 21st gestational week). Thereafter, CD133 expression decreased in the cortex and papilla, but was relatively conserved in the CMJ.

Proliferative activity of CD133$^+$ cells in the fetal human kidney

To assess the proliferative activity of CD133$^+$ cells, we used a double-immunostaining method using antibodies against CD133, which is expressed in the apical cell membrane, and the proliferation marker, Ki-67, which is localized to the nucleus (Fig. 1E-H). In the nephrogenic zone of fetal kidneys, CD133$^+$ cells in the S-bodies co-expressed Ki-67 (Fig. 1E). In the relatively mature deeper kidney, Ki-67$^+$ cells were noted in tubules, interstitium, and blood vessels, but most CD133$^+$ cells in this region did not co-express Ki-67 (Fig. 1F-H).

CD133 expression in the adult human kidney

The fully developed adult kidney consists of the cortex and the medulla. The latter is further divided into the inner medulla, outer medulla, and papilla. The cortex is organized into the cortical labyrinth and the medullary ray. The tubular composition of the medullary ray is identical to that of the outer medulla.

CD133 expression was most prominent in tubules and less so in the other three histological renal components: glomeruli, vessels, and interstitium (Fig. 1I-L). CD133 was weakly expressed in the PECs of Bowman's capsule in focal glomeruli (Fig. 1I). In the cortical labyrinth, CD133 expression was observed in single cells and small clusters of tubular epithelial cells and frequently located at the tip of the tubular protrusion into the lumen (Fig. 1J). Similar to the expression pattern seen in fetal kidneys, tubular CD133 expression was strong in the outer medulla of the CMJ and the medullary ray of the cortex (Fig. 1J-L). In the papilla, CD133 was present in tubules of small caliber, suggesting Henle's loop (Fig. 1L). Most of the tubular CD133$^+$ cells showed pathologic features of acute tubular injury: loss of brush border with flattening and simplification of tubular epithelial cells (Fig. 1J,K,N,O). CD133 was also strongly expressed in atrophic tubules showing thick, wrinkled, lamellated basement membranes, and in tubules containing proteinaceous casts (which were observed more often in older patients

Table 2. Primary antibodies used and associated methodological details.

Antibody	Species	Source	Method	Heat pretreatment	Antibody Dilution	Reaction temperature	Reaction time	Expression
CD133/1 (AC133)	Mouse monoclonal	Miltenyi Biotech, Bergisch Gladbach, Germany	IHC	TE buffer (pH 9.0)	1:50	4°C	Overnight	Stem cell marker
CD10	Mouse monoclonal	NovoCastra, Newcastle upon Tyne, UK	IF	Citrate buffer (pH 6.0)	1:400	RT	1 h	PT
Cytokeratin 7	Mouse monoclonal	Dako, Glostrup, Denmark	IF	Citrate buffer (pH 6.0)	1:50	RT	1 h	HL, DT, CD
Keratin, Multi Ab-1	Mouse monoclonal	Lab Vision, Fremont, CA, USA	IF	Citrate buffer (pH 6.0)	1:200	RT	1 h	Tubules
CD31	Mouse monoclonal	Dako, Glostrup, Denmark	IF	Citrate buffer (pH 6.0)	1:50	RT	1 h	Blood vessels
Ki-67	Rabbit, monoclonal	Neomarkers, Fremont, CA, USA	IHC	Citrate buffer (pH 6.0)	1:100	RT	1 h	Proliferation marker

IHC, immunohistochemistry; IF, immunofluorescence; RT, room temperature; PT, proximal tubules; HL, Henle's loops; DT, distal tubules; CD, collecting ducts.

and in the CMJ; Fig. 1P). CD133 expression was not readily detectable in the interstitium or blood vessels. The same glomerular and tubular CD133 expression patterns were noted in the needle biopsied kidney tissues (Fig. 2A-D). Despite being taken from individuals with normal renal function, they also showed microscopic foci of acute and chronic tubular injuries where CD133 expression was increased.

Proliferative activity of CD133⁺ cells in the adult human kidney

In adult kidneys, Ki-67 expression was noted in a few tubular epithelial cells (Fig. 1M). Most of the

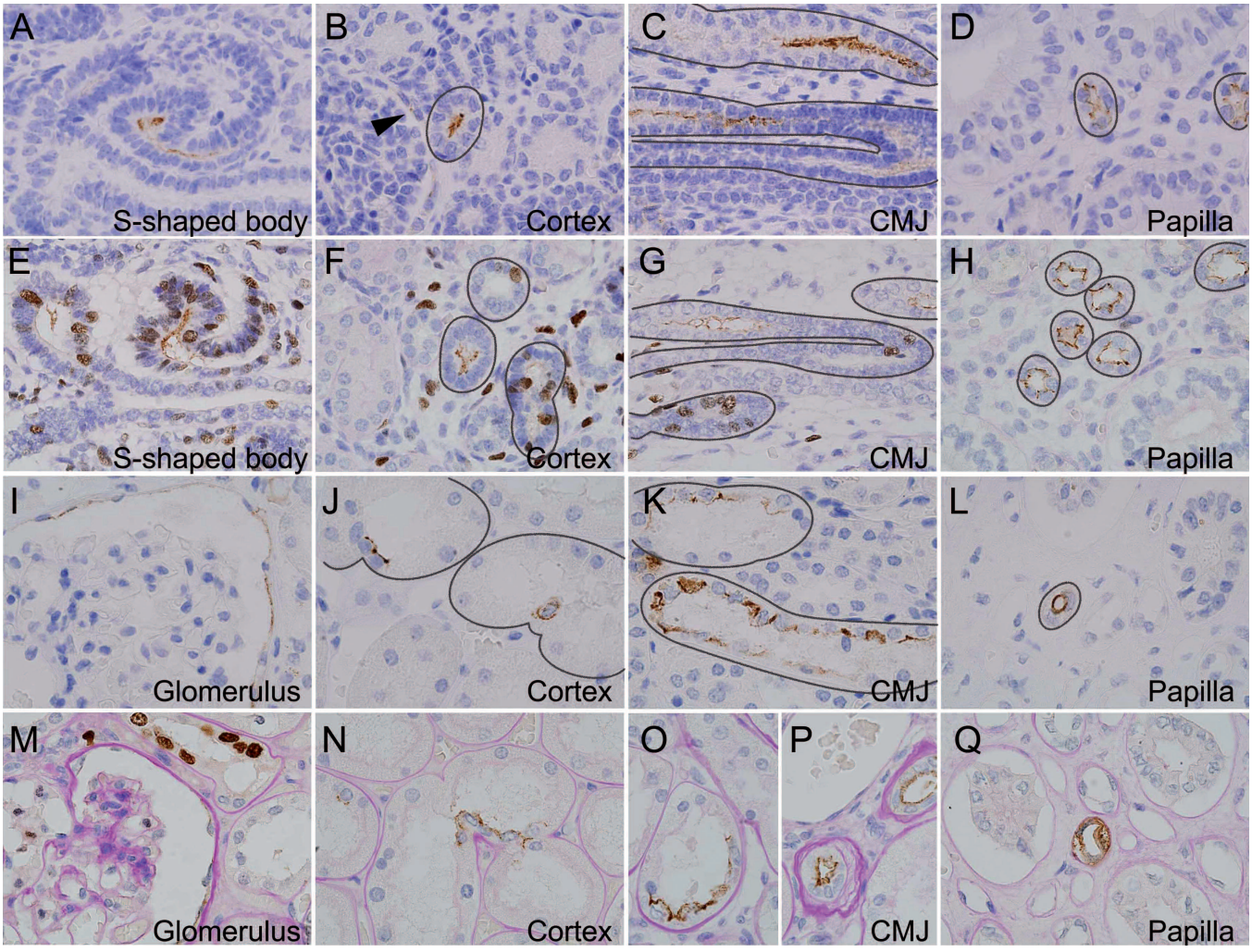

Fig. 1. CD133 expression in fetal (16-week-old) human kidneys **(A-H)** and adult (57-year-old) human kidneys **(I-Q)**, and proliferative activity of CD133⁺ cells in fetal **(E-H)** and adult **(M-Q)** kidneys. The first **(A-D)** and third **(I-L)** rows are tissues immunohistochemically stained for the stem cell marker, CD133 (positively stained at the apical cell membrane), and counterstained with hematoxylin. The second **(E-H)** and fourth **(M-Q)** rows are tissues double stained for CD133 and the proliferation marker, Ki-67 (positively stained in the nuclei), and counterstained with periodic acid-Schiff (PAS). The first column reveals S-shaped bodies in the nephrogenic zone of the fetal kidney **(A and E)** and in glomeruli of the adult kidney **(I and M)**. These images show strong CD133 expression in the middle limb of the S-shaped body **(A)**, with co-expression of Ki-67 **(E)**, and weak CD133 expression in the parietal epithelial cells of the adult glomeruli **(I)** with no Ki 67 co-expression (M). Ki-67-positive tubular cells are shown in the right upper corner in M as an internal positive control. Tubular CD133 and Ki-67 expression in the cortex, CMJ, and papilla is shown in the second, third, and fourth columns, respectively. CD133 is expressed in focal tubules in the cortex (**B, F, J, N**), CMJ (**C, G, K, O, P**), and papilla (**D, H, L, Q**), with strong expression in the CMJ. Tubular CD133 is expressed segmentally in the fetal kidney (**C, G**) and in the CMJ of the adult kidney **(K).** In the cortex of the adult kidney, tubular CD133 expression is evident in single cells or small cell clusters at the tops of tubular protrusions in the cortical tubules of the adult kidney **(J, N)**. The arrowhead in B indicates weak CD133 expression in the parietal epithelial cells of glomerular Bowman's capsule. Strong CD133 expression in atrophic tubules in the CMJ is shown **(P)**. Some tubules are outlined by solid lines. x 1000

CD133+ glomerular and tubular epithelial cells did not express Ki-67 (Fig. 1N-Q, 2A-D).

Characterization of CD133+ tubules in the adult human kidney

The tubular component of the nephron begins at the urinary pole of the glomerulus and consists of proximal convoluted and straight tubules, Henle's loop, and distal tubule. The proximal tubules are further divided into three segments: S1, S2, and S3. The tubular components of CMJ are the S3 segment of the proximal tubule, Henle's loop, and the collecting duct.

As expected, the cortical tubules containing single

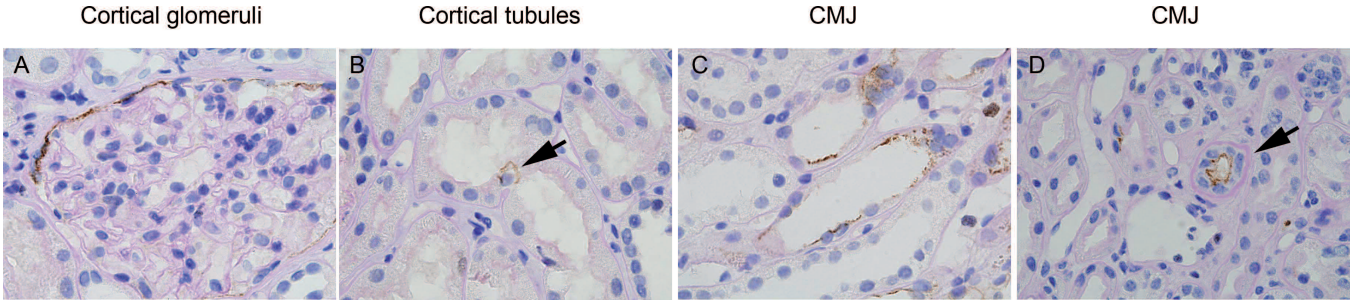

Fig. 2. Expression of CD133 and Ki-67 in adult individuals with normal (clinically and serologically) kidney function. Kidney tissues were harvested by percutaneous needle biopsy and then processed immediately for pathological examination. The tissues were double immunostained for CD133 and Ki-67 and counterstained with periodic acid-Schiff (PAS). Similar to surgically removed kidneys, CD133 is expressed in the parietal epithelial cells of the glomerular Bowman's capsule **(A)**, in single tubular cells, especially at the top of tubular protrusion in the cortex (**B**, arrow), and in CMJ tubules with microscopic foci of acute tubular injury **(C)** and chronic tubular injury (**D**, arrow). CD133+ cells do not express Ki-67. CMJ, corticomedullary junction. x 600

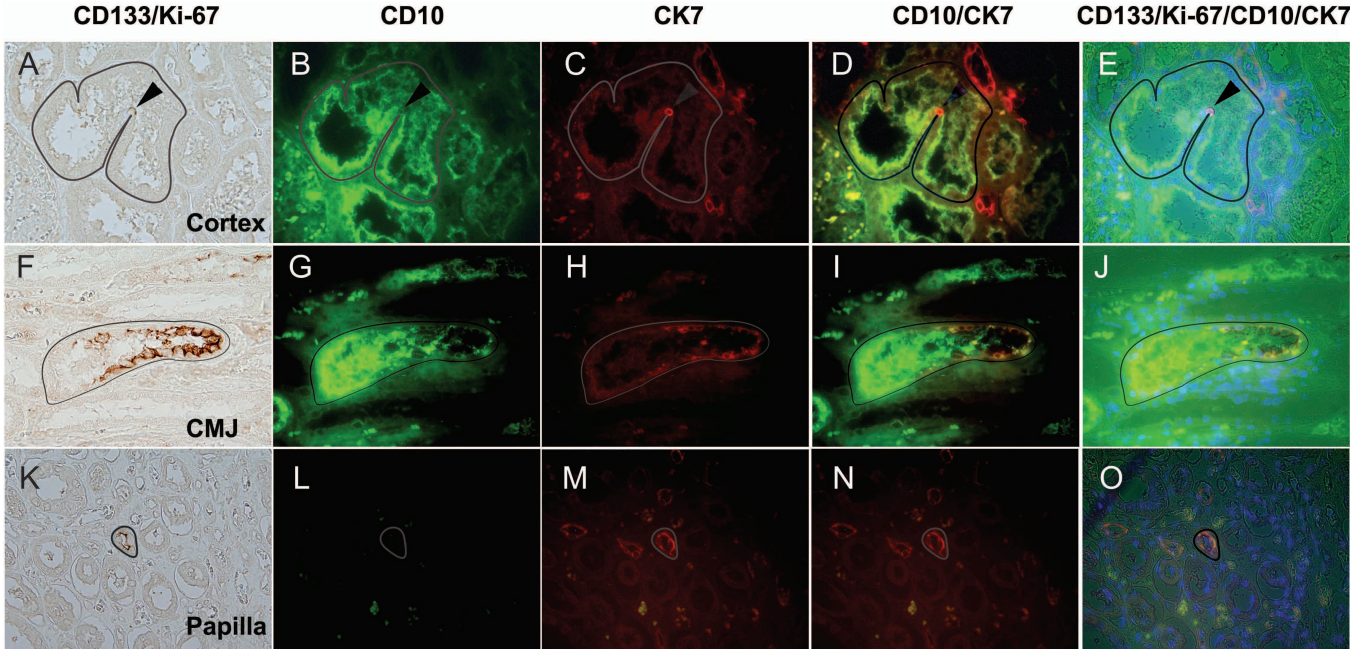

Fig. 3. Characterization of tubules in adult human kidneys that possess CD133+ cells. The cortex **(A-E),** CMJ **(F-J),** and papilla **(K-O)** of the kidney from a 30-year-old case are quadruple immunostained with CD133, Ki-67, CD10, and cytokeratin 7 (CK7). The first column shows tissue double stained for CD133 and Ki-67. The second and third columns show immunofluorescent staining for CD10 with FITC-conjugated secondary antibody, and CK7 with TRITC-conjugated secondary antibody, respectively. The fourth column shows composite images of CD10 and CK7. The fifth column shows composite images of CD133, Ki-67, CD10, and CK7 with DAPI staining for nuclei (blue). The CD133+ cells in the cortex and CMJ express CD10, corresponding to the proximal convoluted tubule and the S3 segment of proximal tubule, respectively. A single CD133+ cell in the cortex is indicated with an arrow. The CD133+ small caliber tubule in the papilla is positive for CK7 and negative for CD10, confirming its Henle's loop identity. CD133+ cells in proximal tubules in the cortex and CMJ are also positive for CK7. The CD133+ cells do not express Ki-67. Some tubules are outlined by solid lines. x 1000

CD133+ cells or small clusters of such cells in the cortical labyrinth were positive for CD10, confirming proximal convoluted tubules (Fig. 3A-E). CD133+ cells in the proximal convoluted tubules co-expressed the distal tubule marker CK7, suggesting the multipotent nature of such cells. (Fig. 3A-E). In the CMJ, CD133+ tubules were positive for CD10, consistent with such tubules corresponding to the S3 segment of the proximal tubule (Fig. 3F-J). CD133+ cells in the S3 segment, like those in the proximal convoluted tubules, were also positive for CK7 (Fig. 3F-J). In the renal papilla, CD133+ small-caliber tubules co-expressed CK7, confirming the identity of these tubules as Henle's loop (Fig. 3K-O). The CD133+ cells in Henle's loop did not co express CD10.

CD133 and Ki-67 expression in pauci-immune crescentic glomerulonephritis and its prognostic implications

To assess the role of stem cells in recovery from a native kidney disease, we studied CD133 and Ki-67 expression in 17 cases of biopsy-proven pauci-immune crescentic glomerulonephritis. These cases were divided into two groups according to renal outcome: recovery (10 patients) and no-recovery (7 patients).

As expected, the no-recovery group showed higher initial serum creatinine levels than the recovery group (p=0.014). There was no difference in age, gender, or treatment modality between the two groups. Although antineutrophil cytoplasmic antibody (ANCA)-negativity was frequent in the no-recovery group, the difference between the groups was not statistically significant. On pathological examination of the needle-biopsied tissues obtained before treatment, the percentages of crescentic or globally sclerotic glomeruli and the severity of chronic tubulointerstitial change did not differ between the recovery and no-recovery groups (Table 3).

All cases except one (94%) exhibited Ki-67-positive cells in the glomerular crescents (Fig. 4A). CD133+ cells in crescents were rare; only two cases (12%) were identified, one each in the recovery and no-recovery groups. Co-expression of CD133 and Ki-67 in crescentic

Table 3. Comparison of clinicopathologic features between recovery and no-recovery pauci-immune glomerulonephritis groups.

Variables	Recovery Group n=10	No-recovery Group n=7	p-value
Age*			
Years	60.6±8.6	52.0±11.9	0.088
Gender			
Male	5	5	0.622
Female	5	2	
ANCA serology			
MPO-ANCA	8	4	0.051
PR3-ANCA	2	0	
ANCA-Negative	0	3	
Initial serum creatinine*			
mg/dL	3.2±2.9	8.0±5.9	0.014
Last serum creatinine*			
mg/dL	1.5±0.6	7.3±4.6	0.001
Total glomeruli*			
Number	14.6±5.7	18.9±6.8	0.190
Tissue area*			
mm^2	440.9±247.2	378.4±194.2	0.590
Crescentic glomeruli*			
Percentage	50.8±28.9	54.8±29.5	0.962
Global sclerosis*			
Percentage	13.0±16.6	28.8±30.0	0.315
Chronic tubulointerstitial changes			
None / trivial (<10%)	3	4	0.546
Mild (≥10% to 25%)	4	1	
Moderate (≥25% to 50%)	1	0	
Severe (≥50%)	2	2	
Total dose of immunosuppressant*			
Prednisone (g)	6.0±2.8	6.5±3.9	0.740
Cyclophosphamide (g)	6.7±12.6	10.61±13.3	0.417
Azathioprine (g)	16.9±31.1	3.9±10.4	0.601
Tubular CD133*			
Cell number/mm^2	0.24±0.34	0.23±0.38	0.669
Tubular Ki-67*			
Cell number/mm^2	0.39±0.26	0.39±0.45	0.669
Tubular CD133 and Ki 67 co-expression			
Presence	5	1	0.307
Absence	5	6	
Follow-up*			
Months	20.7±15.5	16.0±10.8	0.669

*Values are mean ± standard deviation.

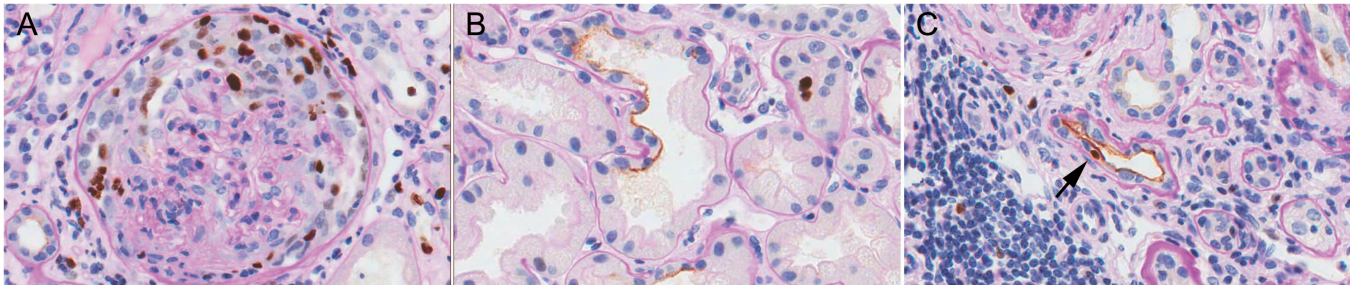

Fig. 4. CD133+ cells and their proliferative activity in pauci-immune crescentic glomerulonephritis. Representative images of tissues double-stained for CD133 and Ki-67 are shown for crescentic glomeruli **(A)**, mildly injured tubules **(B)**, and severely injured tubules **(C)** from a 62-year-old female case. A crescentic glomerulus contains many Ki-67-positive cells and lacks CD133 expression. Tubular CD133 expression is conspicuous in injured tubules (**B and C**) with foci of Ki-67 co-expression (**C**). Tubular CD133 and Ki-67 co-expression (**C**) is indicated with an arrow. x 400

glomeruli was also rare, and was observed in only two cells of one patient in the no-recovery group.

CD133 was expressed in single cells or small clusters of tubular epithelial cells showing acute or chronic injury, which was observed in both groups (Fig. 4B). The co-expression of CD133 and Ki-67 was observed in a small number of tubular cells, from 1 to 38 cells per case (0.002-0.005 cells/mm^2; Fig. 4C). The co-expression of tubular CD133 and Ki-67 was observed more often in the recovery group (5 of 10 patients) than in the no-recovery group (1 of 7 cases), although this difference was not statistically significant. Single tubular expression of neither CD133 nor Ki-67 differed between the groups.

Discussion

Previous reports have demonstrated that CD133 expression in the fetal kidney is evident only in metanephric mesenchyme-derived primordial structures, which sequentially develop from primary vesicles to the comma-shaped body, and, next, the S-shaped body (Lazzeri et al., 2007). Consistent with this, we also observed strong CD133 expression only in the S-shaped body and not in the condensed mesenchyme or ureteric buds. Lazzeri and colleagues reported that strong CD133 expression was localized to the upper and lower limbs of the S-shaped body (Lazzeri et al., 2007). In our study, CD133 expression was stronger in the middle limb, and diminished in the upper and lower limbs. Given that the middle limb gives rise to the proximal tubules of the adult kidney, the strong CD133 expression in the middle limb is compatible with the strong CD133 expression seen in the proximal tubules of the adult kidney (Shimazui et al., 2000).

Three possible sources of cells for renal tubular repair in response to injury have been proposed: (1) dedifferentiated surviving tubular cells, (2) bone marrow-derived stem cells, and (3) intrarenal stem cells. The mature tubular epithelial cells that survive renal injury may restore injured tubules after undergoing dedifferentiation and proliferation (Witzgall et al., 1994; Bonventre, 2003). Bone marrow-derived stem cells (BMSCs) are also involved in tubular regeneration (Poulsom et al., 2001). BMSCs differentiate primarily to leukocytes in the interstitium and do not appear to make a significant contribution to the repair of tubular epithelial cells (Duffield et al., 2005; Lin et al., 2005). Accumulating evidence has demonstrated that proliferating intrarenal stem cells participate in tubular regeneration in animal models and human renal allografts (Maeshima et al., 2003; Oliver et al., 2004; Bussolati et al., 2005; Gupta et al., 2006; Sagrinati et al., 2006; Ronconi et al., 2009). Together, the previous studies suggest that renal tubular repair depends primarily on intrarenal tubular cells, whether they are mature tubular cells or intrarenal stem cells.

There are various kinds of intrarenal tubular cells, but those in the S3 segment of the proximal tubule have received considerable attention, based on the observation that the S3 segment at the tubular site is the most susceptible to ischemic injury; however, this region also exhibits the highest level of proliferative activity after injury (Shanley et al., 1986; Witzgall et al., 1994). Induction of ischemic injury by clamping of unilateral renal vessels was shown to result in the development of tubular necrosis in the S3 segment, even after a short period (15-30 min) of vascular occlusion (Shanley et al., 1986). Expression of the proliferation marker PCNA is increased in the ischemic kidney, most prominently in the outer stripe of the CMJ where the S3 segment is located (Witzgall et al., 1994). On the basis of these observations, it has been suggested that S3 segment epithelial cells are renal progenitor cells (Witzgall et al., 1994; Bonventre, 2003). Consistent with this, longitudinal dissection of a single nephron along the tubular segments, and subsequent culturing of isolated cells at limiting dilution, showed that colony-forming cells (classically considered to represent stem cells) were derived primarily from the S3 segment (Kitamura et al., 2005). The S3 segment-derived cells expressed the stem cell markers, Sca-1 and Musashi-1, and demonstrated self-renewal capacity, multipotency, and renal repair capability. The stem cell-specific transcription factor, Oct4, was also found in the proximal tubule in the CMJ (Gupta et al., 2006). These results suggest that highly proliferative cells with stem cell properties reside in the S3 segment of the proximal tubule. The present study also showed that CD133 expression was most prominent in the S3 segment.

Previous studies have shown that hypoxia induces a reversible up-regulation of CD133 expression and CD133 has even been proposed as a bioenergetic stress marker, rather than a stem cell marker in glioma cells in the brain (Chen et al., 2007; Griguer et al., 2008; Soeda et al., 2009). As shown in Table 1, some autopsy cases had congenital heart anomalies, acute chorioamnionitis, or diaphragmatic hernia, which may have resulted in hypoxia, affecting systemic organs, including the kidney. The other cases had no pathologic abnormalities of the fetus or maternal diseases likely to cause systemic hypoxia. Regardless of the autopsy diagnosis, the expression patterns of CD133 were the same, suggesting that hypoxia may not have significant effects on CD133 expression in the autopsy cases.

As mentioned above, the S3 segment of the proximal tubule in the CMJ is vulnerable to hypoxia. Although we tried to examine “normal adult kidneys” in nephrectomy specimens by selecting kidney tissue remote from the tumorous lesion after gross and microscopic examination, the kidney tissues might not represent truly normal kidney, because of hypoxia induced by the ligation of renal vessels during the operation. To clarify whether operation-induced renal hypoxia increased CD133 expression, we sought truly normal kidney tissues by searching for individuals with normal renal

function, clinically and serologically, and who had undergone kidney needle biopsy. This is because during kidney needle biopsy, kidney tissues are harvested percutaneously with no renal vascular compromise and then the tissues are immediately processed for pathological examination. Similar to the surgically removed kidneys, the needle biopsy tissues also showed microscopic foci of acute and chronic tubular injuries and demonstrated the same CD133 and Ki-67 expression patterns as the surgically removed kidneys (Fig. 2). Thus, operation-induced hypoxia apparently did not have significant effects on CD133 expression in our cases. Rather, these results suggest that microscopic foci of acute and chronic renal injury exist in normal individuals and that CD133 expression is increased in those foci.

Although normal kidney would not be expected to have pathologic features of acute or chronic damage, it appears that this is not actually the case. Previous studies have shown that acute and chronic changes occur in normal human and mouse kidneys (Kaplan et al., 1975; Goyal et al., 1980; Goyal, 1982; Yabuki et al., 2003). Even while maintained in a controlled environment, normal mice showed vacuolar degeneration of the proximal tubules, one of the pathologic features of acute tubular injury (Yabuki et al., 2003). Furthermore, the acute injury became more severe with age and tubular atrophy was observed in older mice (Yabuki et al., 2003). In our study, most of the $CD133^+$ tubular cells in the outer medulla and medullary ray in both the normal kidney and in kidneys of pauci-immune crescentic glomerulonephritis patients exhibited histologic features of acute or chronic injury. $CD133^+$ cells in the cortical proximal tubules, presenting as single cells or small cell clusters, were frequently located at the tips of intraluminal protrusions of the tubular epithelial cells, which might be vulnerable to urinary flow-induced injury. These findings suggest that cellular injury may induce CD133 expression, an interpretation consistent with previous studies (Bao et al., 2008; Loverre et al., 2008). $CD133^+$/Pax-2^+ cells were increased in number and co expressed Ki 67 during the regenerative process in post transplantation acute tubular necrosis (Loverre et al., 2008). In an allogeneic rat transplantation model, $CD133^+$ cells were increased at an early stage during the exacerbation of chronic tubulointerstitial changes in a transplantation model, and co-expressed Ki-67. These studies support the hypothesis that CD133 expression is induced by acute and chronic injury. In contrast to previous reports demonstrating co-expression of Ki 67 in $CD133^+$ cells, most of the $CD133^+$ cells in the mature normal kidney in our study apparently did not co-express Ki-67. This observation may suggest that proliferation of renal stem cells is tightly regulated in normal kidneys and requires a proper microenvironment or additional stimulatory factors for proliferation initiation.

One important characteristic of stem cells is multipotency: that is, the ability to differentiate into various specialized cell types of the organ. $CD133^+$ cells in the proximal tubules expressed both the proximal tubule marker, CD10, and the distal tubule marker CK7, perhaps demonstrating the multipotent nature of such cells *in situ*. We previously showed that colonies derived from single cells of CMJ tubules had the potential to differentiate into glomerular podocytes, and expressed not only the tubular markers pan-cytokeratin and aquaporin-5, but also the glomerular podocyte marker podocin (Kim et al., 2008). The absence of CD10 co-expression with $CD133^+$ cells in Henle's loop suggests that $CD133^+$ cells may represent a heterogeneous population, consisting of multipotent and unipotent stem cells, depending on their intrarenal location.

A limitation of this study was the use of only a single stem cell marker, CD133. Previous studies have employed a combination of stem cell markers. For example, $CD133^+CD24^+$ cells have been proposed as progenitors of the embryonic kidney and of the glomerular parietal epithelium in the adult kidney (Sagrinati et al., 2006; Lazzeri et al., 2007; Ronconi et al., 2009). Bruno et al. reported that $CD133^-$ $CD146^+$ cells represented glomerular mesenchymal stem cells (Bruno et al., 2009). They used fresh frozen tissues for the demonstration of these progenitor cells in the kidney, but fresh frozen tissues of the fetal and adult cases were not available for our study. We tested three CD24 antibodies (CD24 Ab 2 SN3b from Thermo scientific, CD24 C-20 from Santa Cruz Biotechnology, and CD24 from Molecular Probes) and a CD146 antibody (CD146 antibody P1H12 from Abcam); however, specific positive signals reproducing the previous results could not be generated with our formalin-fixed paraffin-embedded tissues. CD133 expression in the fetal and adult kidneys, however, is worth evaluating because the stem cell characteristics of $CD133^+$ cells are established and CD133 is a common marker in the previous combinatorial stem cell marker studies in the kidney (Bussolati et al., 2005; Sagrinati et al., 2006; Lazzeri et al., 2007; Ronconi et al., 2009). In fact, Lazzeri et al. actually used a CD133 single marker to isolate $CD133^+CD24^+$ cells in their experiment, because $CD133^+$ cells represented a subset of all $CD24^+$ cells (Lazzeri et al., 2007).

Another potential limitation of the current study is that the greater tendency towards co-expression of tubular CD133 and Ki-67 in the recovery group of pauci-immune crescentic glomerulonephritis was not statistically significant in the 17 cases available. Thus, further studies of CD133 expression using normal kidneys harvested from animal models, and more patients with pauci-immune crescentic glomerulonephritis, will be necessary to confirm these results.

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