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G. 知的財産権の出願・登録取得状況 (予定を含む)

1. 特許取得

松尾清一:

1) 低血清培養法を用いた脂肪由来幹細胞の分離・培養法: 特許出願済み
2) 脂肪由来多分化能幹細胞を含有する細胞製剤: 特許出願済み

槇野博史:

1) 特願 2003-071029: 腎臓幹細胞前駆細胞、腎臓幹細胞前駆細胞の分離方法, および腎疾患の治療法(出願日平成 15 年 3 月 14 日) (平成 20 年 1 月 18 日特許査定完了)
2) 特願 2005-058494: 糖尿病性腎症の治療用医薬組成物 (出願日平成 17 年 3 月 3 日)
3) 特願 2005-084218: 糖尿病性腎症の治療用医薬組成物 (出願日平成 17 年 3 月 23 日)

東京歯科大　(株)バイオリンク:

1) PCT/JP2007/065375　: 腎臓幹/前駆細胞の分離方法および腎臓幹/前駆細胞、並びに腎疾患治療薬 (出願日平成 18 年 8 月 6 日)

2．実用新案登録

なし

3．その他

なし

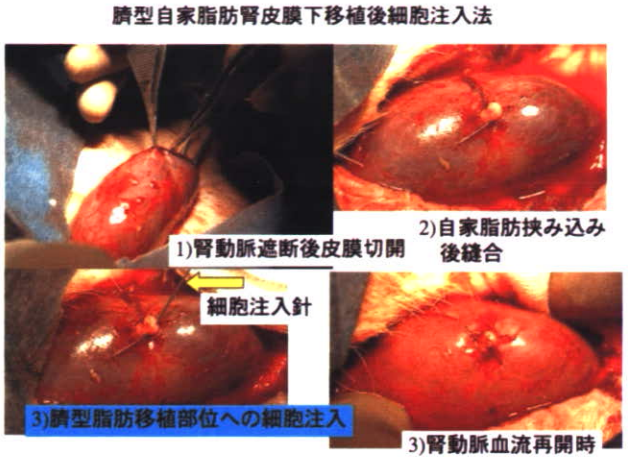
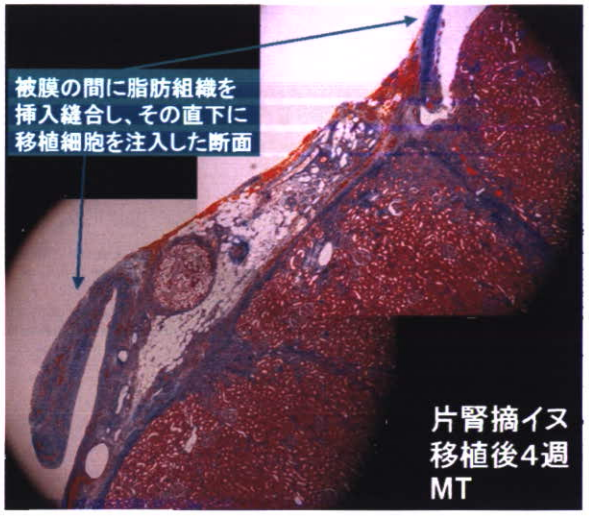


図 2-1



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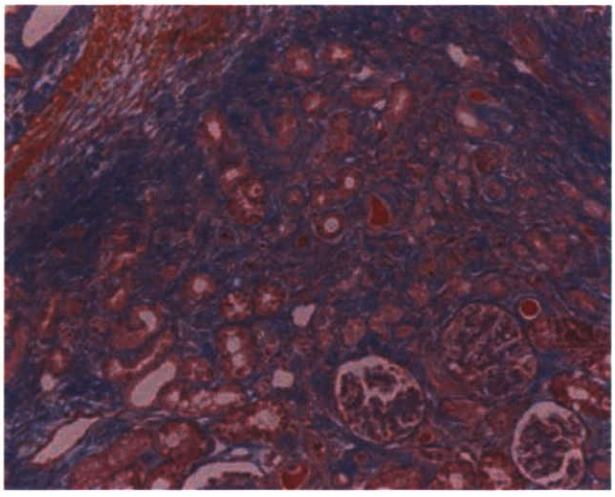

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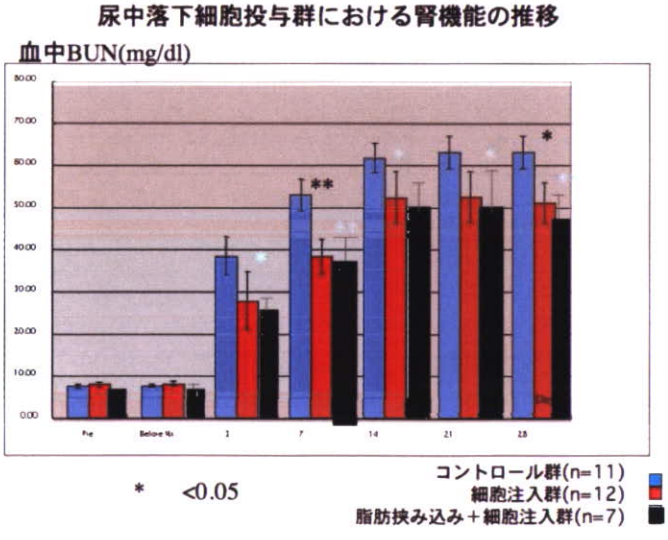

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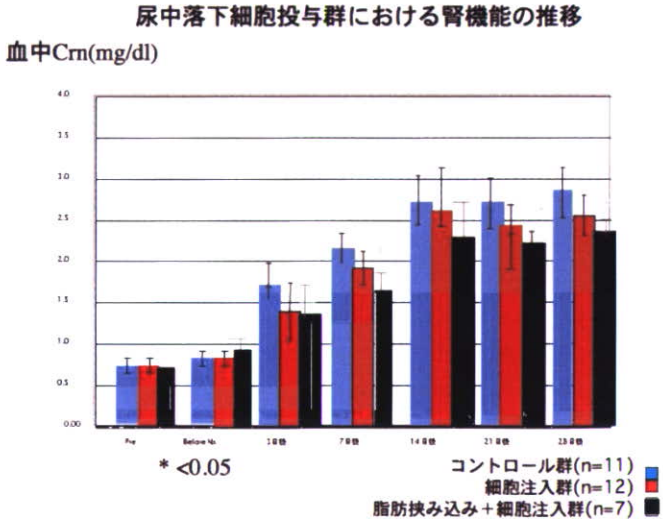


図 2-5

図1 Processes of SVF preparation

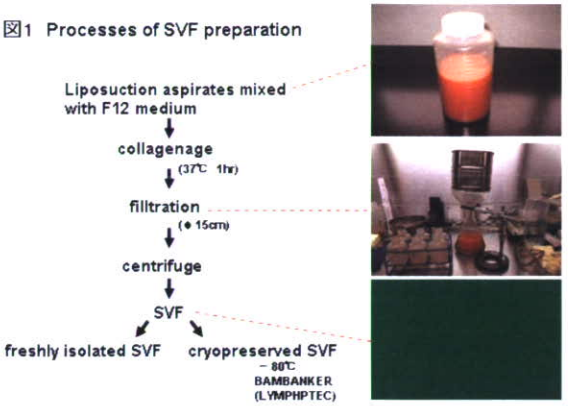
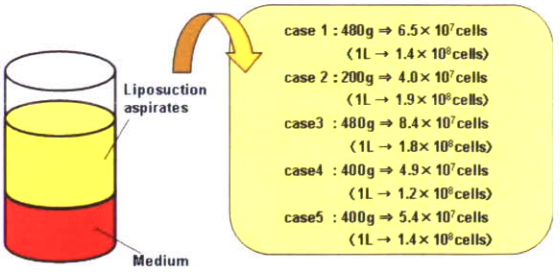


図 4 - 1

図2 *Number of cells retrieved from liposuction aspirates*

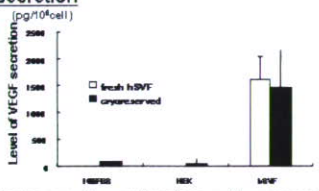


The number of nucleate cells in SVF were 1.63 ± 0.40× 10^8 per 1L of adipose potion.

図 4 - 2

図3 *Viability of freshly isolated SVF and cryopreserved SVF:*

(2) VEGF secretion



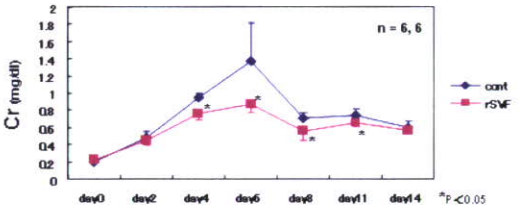
Growth factor secretion and surface antigen expression in freshly isolated SVF and cryopreserved SVF were examined. Growth factor secretion was determined by ELISA test.

(3) Surface antigen expression

FACS analysis showed that the rates of CD34 positive cells were 15~45% in fresh isolated SVF whereas the rate was 15% in cryoreserved SVF.

図 4 - 3

図4 *The creatinine levels of cisplatin nephropathy*



SVF cells protected cisplatin-treated rat from a deterioration in renal function. SVF cells were purified from the adipose tissue of a male F344 rat and injected under the renal subcapsule(1X10^6 cells) of a male F344 rat 2days after cisplatin (7 mg/kg subcutaneously). Blood creatinin (Cr) levels were measured at different time intervals. Data are mean± SD.

図 4 - 4

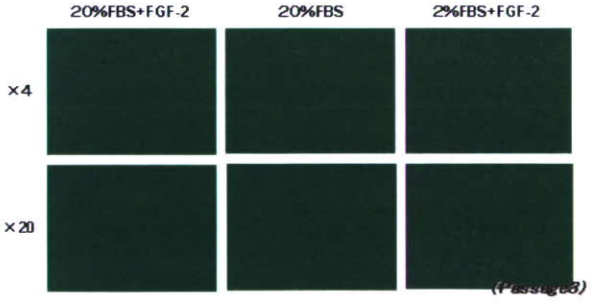


図5 高血清培養法(FGFあり・なし両方)では低血清培養法と比べて早期より紡錘状の形態変化が起こる。

図 4 - 5

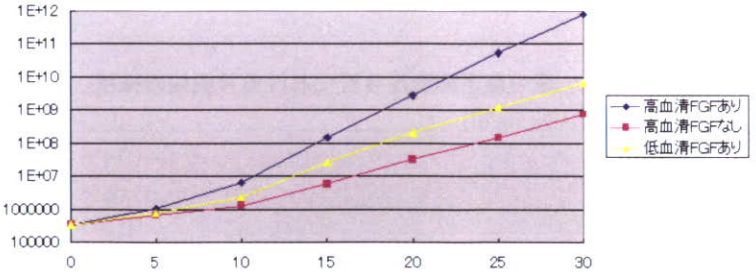


図 4 - 6

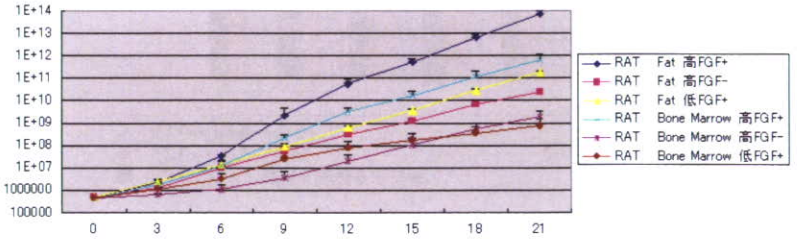


図 4 - 7

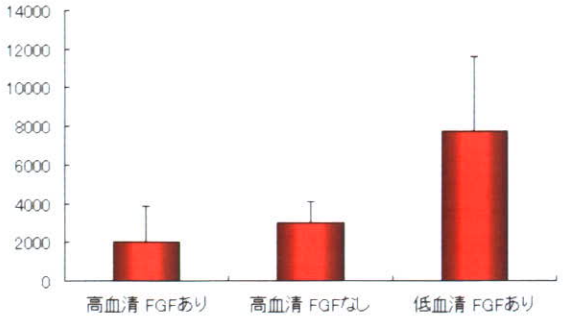


図４-８

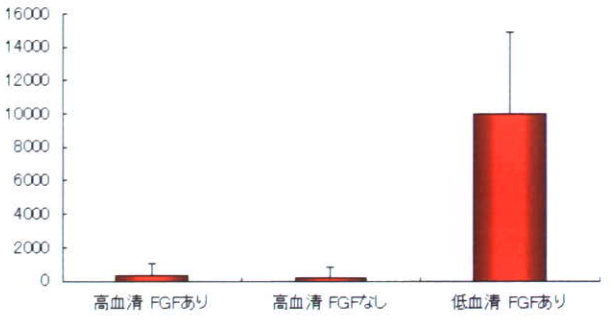


図４-９

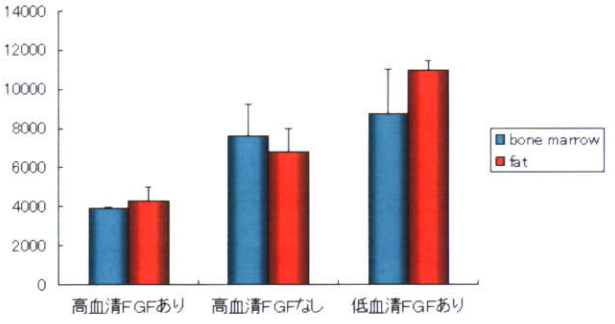


図４-１０

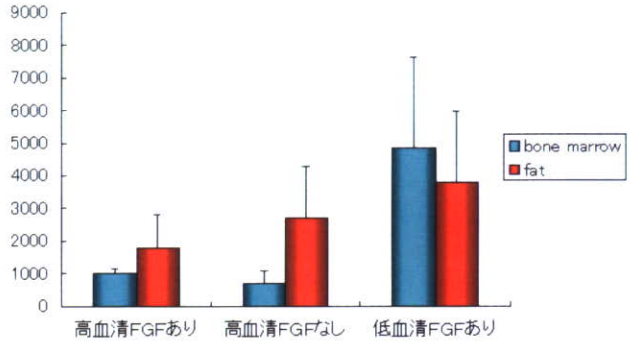


図４-１１

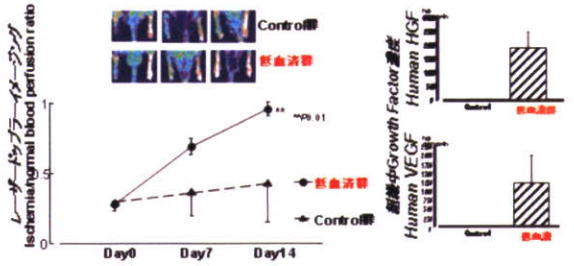


図12. 低血清培養ヒト脂肪組織由来間葉系幹細胞による下肢虚血(ASO)モデルの治療効果の検討

図４-１２

表 4-1 ***Characteristic of human adipose-derived cells***

	Present study (average)*	Yoshimura	Zuk	Gronthos	Lee	David
Passage	0	0	2	2	2	2
Antibody CD13	39.56	20	99	99	-	-
CD31	19.88	16	2.22	1	-	2
CD34	36.98	28	3.55	28	0	0
CD45	36.55	28	2.52	0	-	1.4
CD90	48.42	25	25.96	-	98.3	98.85

*** In the present study, cell surface antigens of SVF freshly isolated from nine patients were examined.**

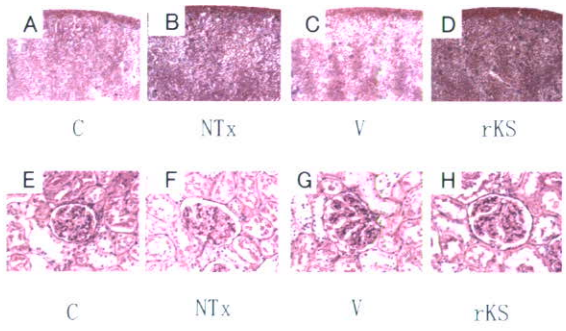


図 5-1 片腎摘＋抗 Thy-1 抗体投与モデルにおける腎障害評価と rKS56-lacZ 細胞投与における細胞生着の検討（PAS 染色）：5 週後。慢性腎不全誘導 5 週後の bluo-gal 染色と PAS 染色の二重染色像を示す。コントロール群における腎障害は認められず（A：皮質、E：糸球体部）、片腎摘群においても障害は軽度であるが（B：皮質、F：糸球体部）、片腎摘＋抗 Thy-1 抗体投与モデルにおいても皮質、糸球体でも障害の程度はあまり変化なく（C：皮質、G：糸球体部）、rKS56-lacZ 細胞投与群において、bluo-gal 陽性の細胞もあまり認められなかった（D：皮質、H：糸球体部）。

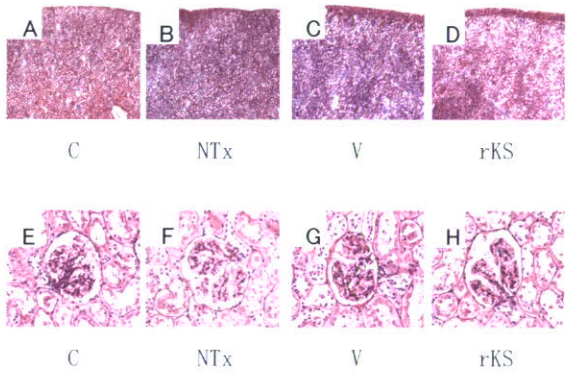


図 5-2 片腎摘＋抗 Thy-1 抗体投与モデルにおける腎障害評価と rKS56-lacZ 細胞投与における細胞生着の検討（PAS 染色）：10 週後。慢性腎不全誘導 10 週後の bluo-gal 染色と PAS 染色の二重染色像を示す。5 週後と比べ腎障害の程度はあまり変化は認められず、コントロール群における腎障害は認められず（A：皮質、E：糸球体部）、片腎摘群においても障害は軽度であるが（B：皮質、F：糸球体部）、片腎摘＋抗 Thy-1 抗体投与モデルにおいても皮質、糸球体でも障害の程度はあまり変化なく（C：皮質、G：糸球体部）、rKS56-lacZ 細胞投与群において、bluo-gal 陽性の細胞もあまり認められなかった（D：皮質、H：糸球体部）。

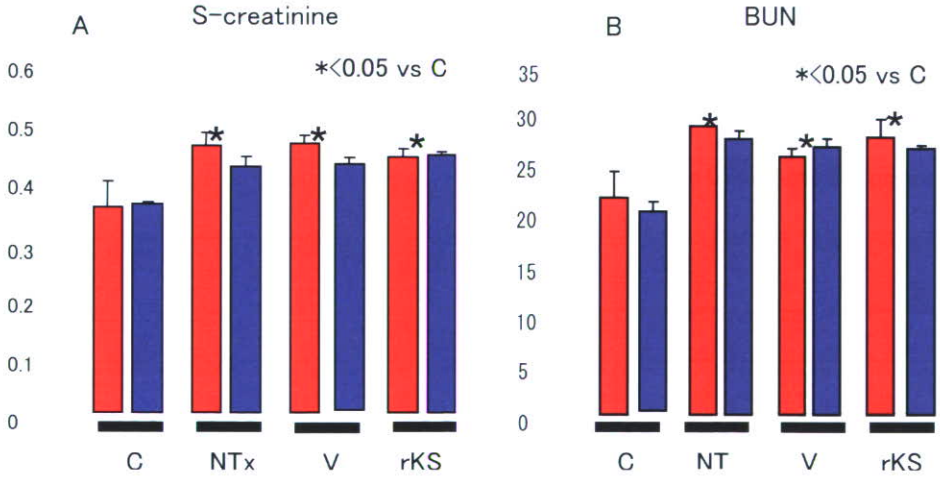


図 5-3 rKS56-LacZ 細胞治療による腎機能（BUN・Cr）の改善効果。片腎摘＋抗 Thy-1 抗体投与モデルにおいて、片腎摘群（N）で軽度腎機能の経時的低下

傾向が認められ、片腎摘＋抗 Thy-1 抗体投与モデルにおいても軽度腎機能の経時的低下傾向が認められたが、rKS-SC 群にて血清クレアチニン(A)と尿素窒素（B）の有意な改善効果を認めた。C:コントロール、Ntx:片腎摘群、V：片腎摘＋抗 Thy-1 抗体投与モデル＋生食投与群、rKS: 片腎摘＋抗 Thy-1 抗体投与モデル＋rKS56-LacZ 細胞・被膜下投与群。

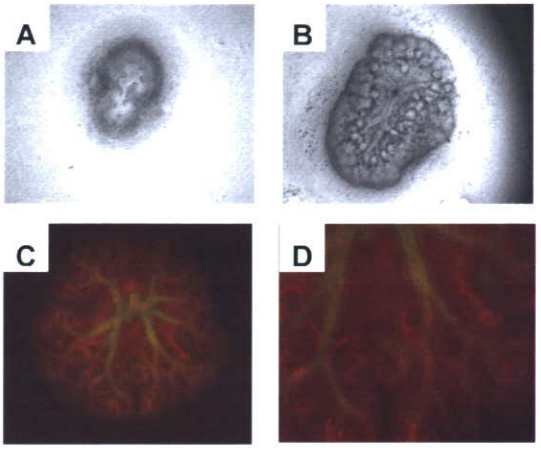


図 5-4 胎生 13 日目の腎臓を使用した organ culture。 A：胎生 13 日目の腎臓、B：transwell 上で 4 日培養した腎臓、C：培養 4 日後の腎臓の PNA、DB 染色像（赤：PNA、緑：DB）。D:強倍率像（赤：PNA、緑：DB）。

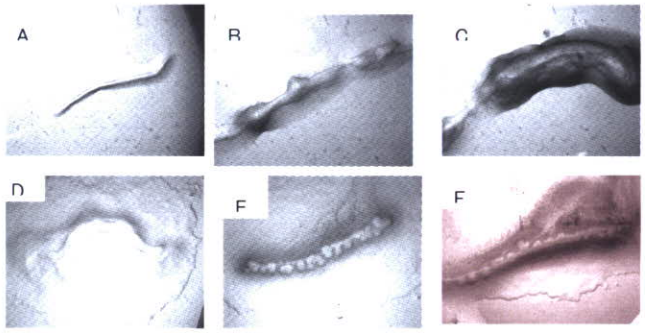


図 5-5 ウォルフィアン管（WD）の初期発芽の検討。 A:中胚葉組織を取り除いた WD、B: 中胚葉組織を軽度取り除いた WD、C：中胚葉組織を取り除いたていない WD、transwell 上培養 4 日後；D：中胚葉組織を取り除いた WD、E：中胚葉組織を軽度取り除いた WD、F：中胚葉組織を取り除いていない WD

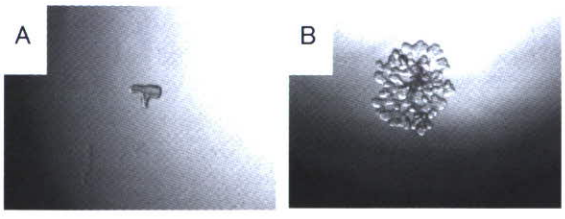


図 5-6 尿管芽（UB）培養。 A:胎生 13 日目の尿管芽、B:培養 7 日後の UB、培養条件:BSN-CM ＋ 10 % FCS+GDNF （125ng/ml） ＋ FGF- b (125ng/ml) ＋ 1 %antibiotics。

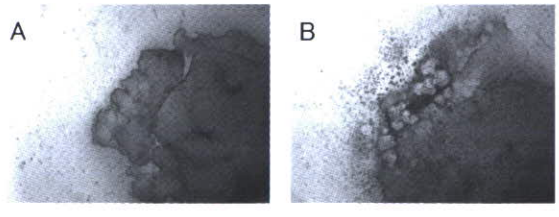


図 5-7 後腎発生系間葉系幹細胞（MM）の上皮化誘導。 A：MM と胎生 13 日目の神経管（spinal code）の共培養。B：培養 3 日目：MM の部分にコンマシェイプ・ボディが観察され、上皮化が誘導されている。

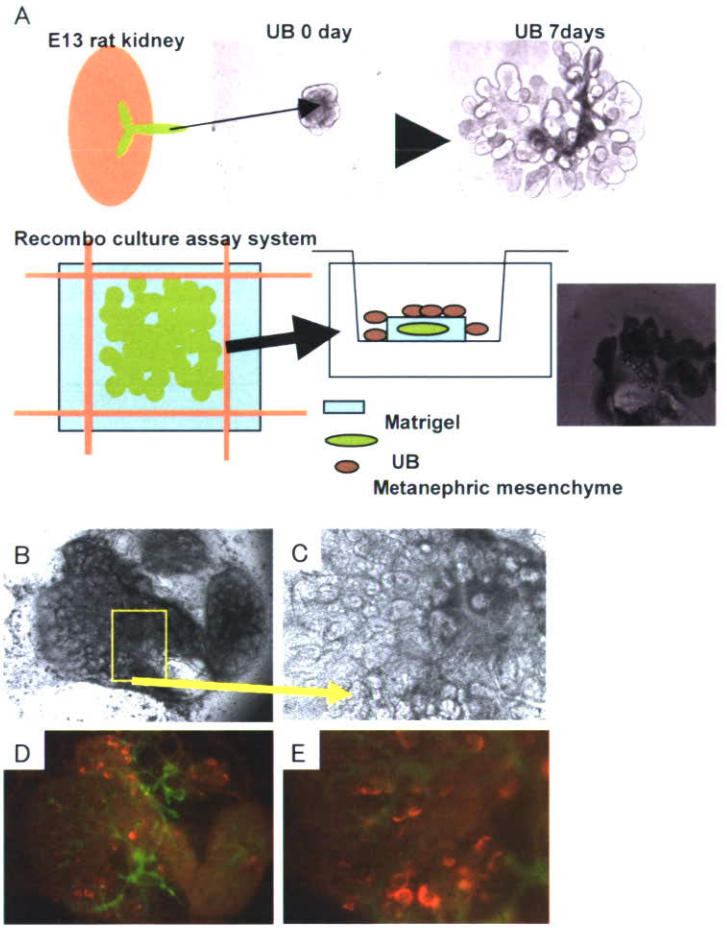


図 5-8 recombo assay system。 A：胎生期 13 日目の UB を培養し、数分枝するまで成長させ、マトリゲルから取り出し、transwell 上で成長した UB と MM 組織を共培養することにより、腎臓発生を再現した。B：培養 7 日目の人工腎臓。MM の部分にコンマシェイプ・ボディィが確認され、MM が分化誘導されている。また UB も分枝は促進し、相互作用により、腎臓形成が促進されている。（C:強倍率）D：人工腎臓の染色像。赤：PNA, 緑：DB。E:強倍率像。

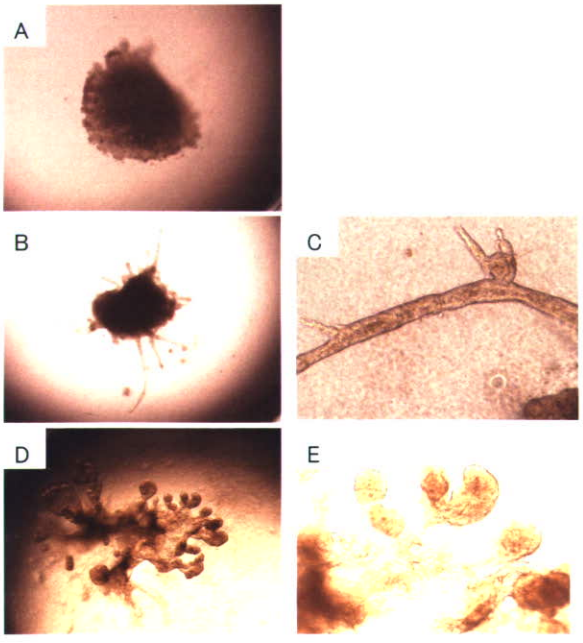


図 5-9 rKS56 細胞を使用した組織構築能（in vitro）。 A:ハンギング・ドロップ法を用いて rKS56 細胞を細胞塊にした。B:rKS56 細胞をマトリゲル内にて培養（K-1medium＋MCS supernatant）。管腔構造形成が認められる。D:強倍率、E:rKS56 細胞をマトリゲル内にて培養（K-1medium＋MCS supernatant+GDNF（250ng/ml）＋FGF-b（250ng/ml）＋HGF（250ng/ml）＋BMP-7（250ng/ml）＋1% antibiotics）。

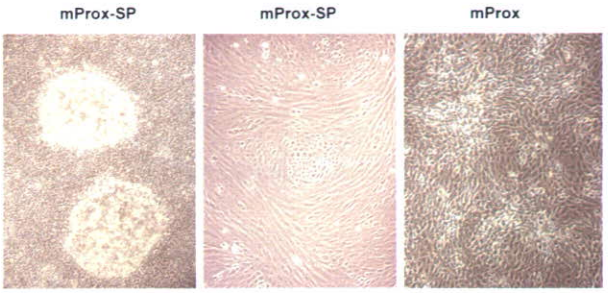


図 6-1

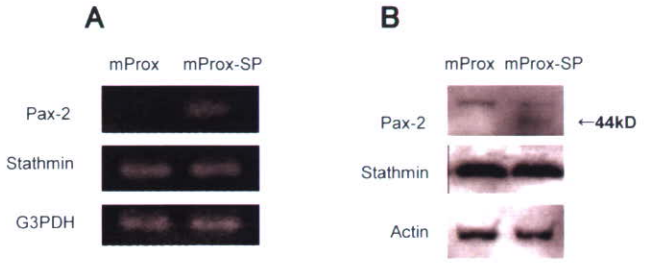
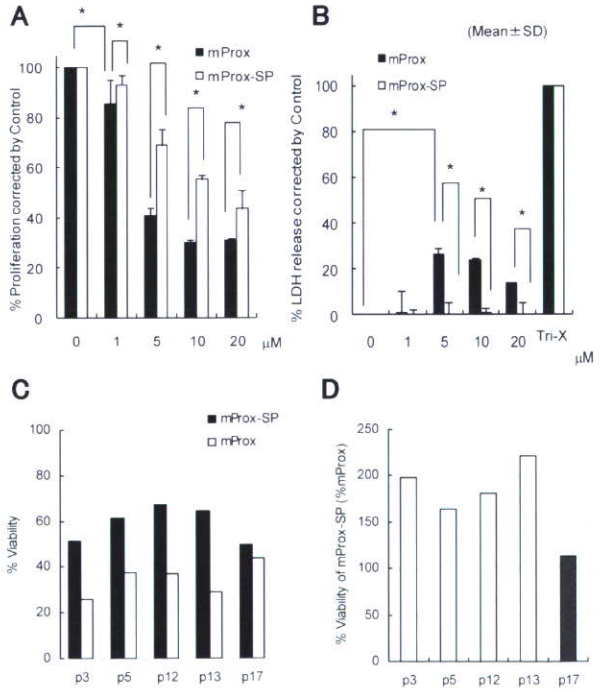


図 6-2



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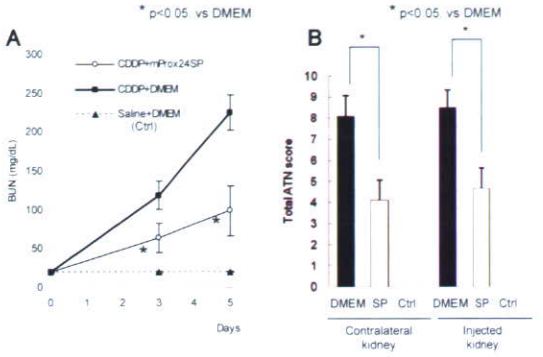
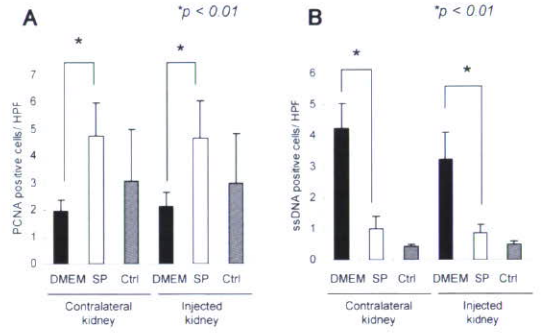


図 6-4



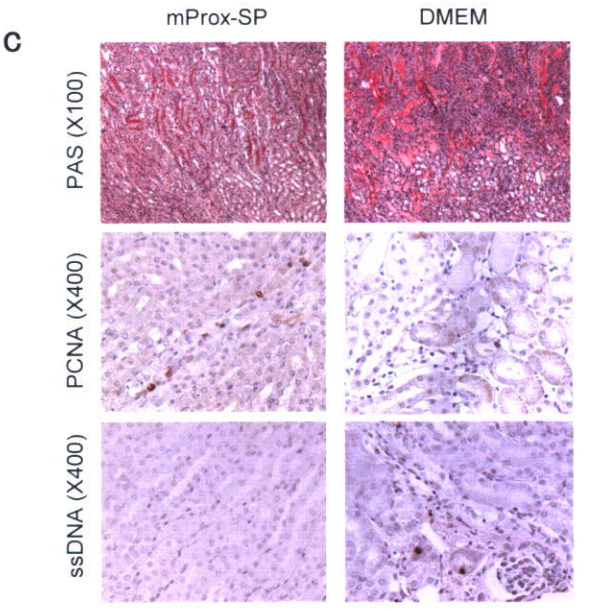


図 6-5

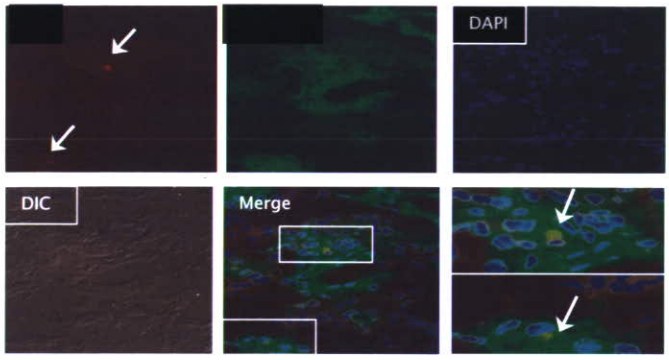
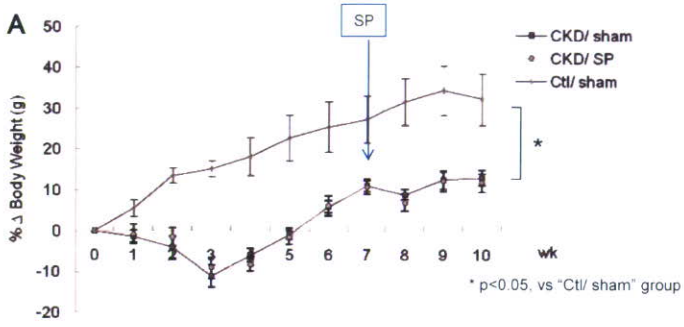
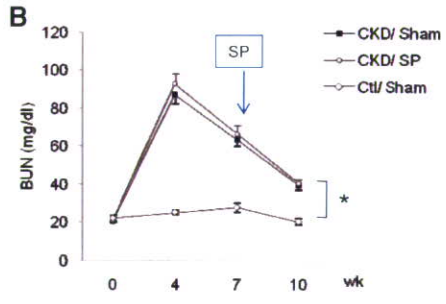


図 6-6





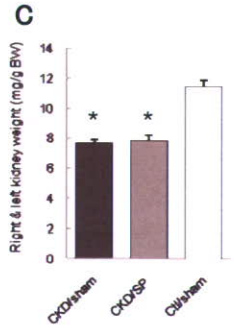


図 6-7

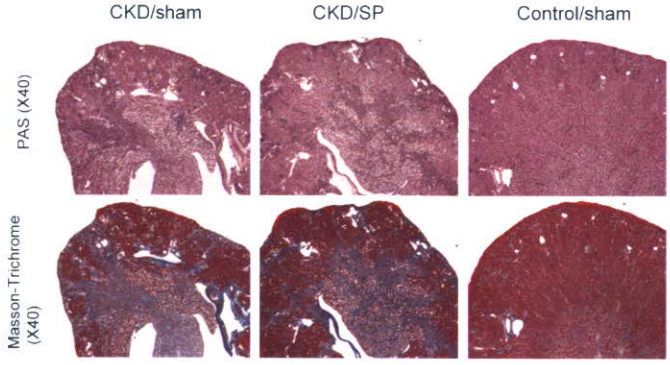


図 6-8

	Passage	BUN at day3 (t-test)	Survival Rate (Kaplan-Meier)
1st	P2-P5	p<0.05	p<0.05
2nd	P5-P8	p<0.05	N.D. (Sacrificed at day5)
3rd	P21	N.S.	N.S.

表 6-1

厚生労働科学研究費補助金（再生医療等研究事業）

研究成果の刊行物・別刷

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kitamura S, Yamasaki Y, Kinomura M, Sugaya T, Sugiyama H, Maeshima Y, Makino H	Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney	FASEB J	19	1789-1797	2005
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Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney

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ABSTRACT **Kidney is thought to be a regenerative organ in terms of repair from acute tubular injury. It is unknown whether cell population contributes to repair disordered kidney. We attempted to identify and isolate highly proliferative cells from a single cell. We dissected a single nephron from adult rat kidney. Isolated nephrons were separated into segments and cultured. Outgrowing cells were replated after limiting dilution so that each well contained a single cell. One of cell line which was the most potent to grow was designated as rKS56. rKS56 cells showed cobblestone appearance and expressed immature cell markers relating to kidney development and mature tubular cell markers. rKS56 cells grew exponentially and could be maintained for 300 days without transformation. In different culture conditions, rKS56 cells differentiated into mature tubular cells defined by aquaporin-1, 2 expression, and responsiveness to parathyroid hormone or vasopressin. Engrafted to kidney in rat ischemic reperfusion model, rKS56 cells replaced in injured tubules in part after implantation and improved renal function. These results suggest rKS56 cells possess character such as self-renewal, multi-plasticity and capability of tissue repair. rKS56 may possibly contribute to the future development of cell therapy for renal regeneration.—Kitamura, S., Yamasaki, Y., Kinomura, M., Sugaya, T., Sugiyama, H., Maeshima, Y., Makino, H. Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. *FASEB J.* 19, 1789–1797 (2005)**

Key Words: S3 segment of proximal tubules · renal tubular epithelial cells · renal progenitor-like cell.

THE THERAPEUTIC POTENTIAL of stem/progenitor cells has been the focus of much recent research (1–3). Stem cells possess three characteristic features such as self-renewal, multipotent differentiation (4) and ability to regenerate after organ injury (3, 5). The existence of organ-specific stem/progenitor cells in adult organs has been demonstrated (6). In particular, bone marrow-derived somatic stem/progenitor cells were shown to differentiate into endothelial cells (7), muscles (2, 8), hepatocytes (9, 10), epithelia of gastrointestinal tract (11), neurons (12), and mesangial cells (13, 14). Endothelial progenitor cells present in bone marrow have been used for treating patients with ischemic heart disease and arterial sclerosis obliterans (15).

Meanwhile, in spite of advances in modern clinical nephrology, progressive renal diseases remain incurable disorders lacking specific therapeutic approaches. Patients with ESRD (end-stage renal disease) require renal replacement therapy such as hemodialysis, peritoneal dialysis or kidney transplantation. However, hemodialysis or peritoneal dialysis does not fully compensate for the loss of kidney function. Long-term hemodialysis and peritoneal dialysis often accompany cardiovascular complications and kidney transplantation suffers from the shortage of donor kidneys, and kidney transplants recipient may undergo some complications of immunosuppressive reagents.

The common histological features of ESRD, irrespective of various pathogenic renal disorders, are glomerulosclerosis and tubulointerstitial fibrosis (16–18). Tubulointerstitial fibrosis, rather than a glomerular lesion, is related to exacerbate prognosis of renal function in progressive renal disorders (16), which lead to the requirement for effective therapeutic approaches to regenerate tubular epithelial cells. In contrast to terminally differentiated epithelial cells with limited doubling-cycles, stem/progenitor cells generally can survive and proliferate for a long time period.

A single metanephric mesenchymal cell can differentiate into various segments of nephron except the collecting duct after interaction with the ureteric bud. It is suggested that embryonic kidney contains epithe-

We declare that the establishment and the therapeutic application of rKS56 cells for renal disorders have been filed and submitted to the Japan Patent Office Issue No. 2003-071029 in March, 2003.

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lial stem cells (19). Recently, Dekel et al. (20) reported the usefulness of fetal or embryonic kidney fragments as the source of kidney precursor cells to generate mature kidneys by transplantation. Although there are several reports demonstrating that bone marrow-derived cells can repopulate and differentiate into glomerular mesangial cells (13, 14), renal stem/progenitor cells derived from adult kidney have not been reported yet. Thus, we attempted to establish renal proliferative cells modifying neurosphere methods (21), which could obtain neural stem cells.

In developing kidney, there are two major distinct areas of cell proliferation: the nephrogenic zone in the outer cortex below the renal capsule and the area in the corticomedullary junction corresponding to the primitive S3 segment of the proximal tubule (22). In the present study, we attempted to identify and establish renal highly proliferative cells existing among S3 segments of nephron in the adult rat kidney. The cells may be close to stem/progenitor cell strain and the usage of these cells contributes to the development of therapies for ESRD.

MATERIALS AND METHODS

Microdissection and culture conditions

Single nephrons were obtained by microdissection technique from kidney of male Sprague-Dawley (SD) rats that weighed 100 to 150 g (Clea JAPAN Inc., Tokyo). The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University Graduate School of Medicine and Dentistry. In Brief, animals were anesthetized with pentobarbital 50 mg/kg wt, IP. The abdominal aorta was ligated at two sites, under the right renal artery and 2 cm below the left renal artery. A catheter was then inserted in the abdominal aorta for the perfusion of the left kidney. Perfusion was made first with 10 mL ice-cold solution A, then with 10 mL ice-cold solution B. Solution A contained (mmol/L) NaCl 130, KCl 5.0, NaH_2PO_4 1.0, $MgSO_4$ 1.0, Ca lactate 1.0, Na acetate 2.0, glucose 5.5, HEPES 10, pH 7.4. Solution B was made from solution A by addition of 300 U/mg collagenase type I and 1 g/L BSA. The left kidney was decapsulated, removed, and cut in thin 1 mm transverse sections. The sections were transferred into tubes containing 3 mL of solution B with 10 mmol/L vanadyl ribonucleotide complex (VRC) (Sigma, Saint Louis, MO, USA) and incubated with bubbling oxygen at 37°C under constant agitation. After 20 min of incubation, the slices were rinsed with solution A, then transferred to a microdissection dish containing 10 mL of solution A with 10 mmol/L VRC. Microdissection to nephron segments was performed using dissecting needles under a dissection microscope with a cooling plate at 4°C. Superficial and juxtamedullary glomeruli were extracted separately. Identification of tubule segments was based on previously reported criteria (23). The following segments were microdissected: glomeruli (G), proximal convoluted tubule (S1/PCT), proximal straight tubule (S2, S3), medullary thick ascending limb of Henle's loop (MAL) and collecting duct (CD) (**Fig. 1*A, B***). Microdissected segments were transferred to a clean wash dish containing solution A using pipettes coated with 0.1% BSA. After a washing for VRC and binding debris, cleaned microdissected segments were separately transferred into wells of 96-well plates precoated with type IV collagen (BD

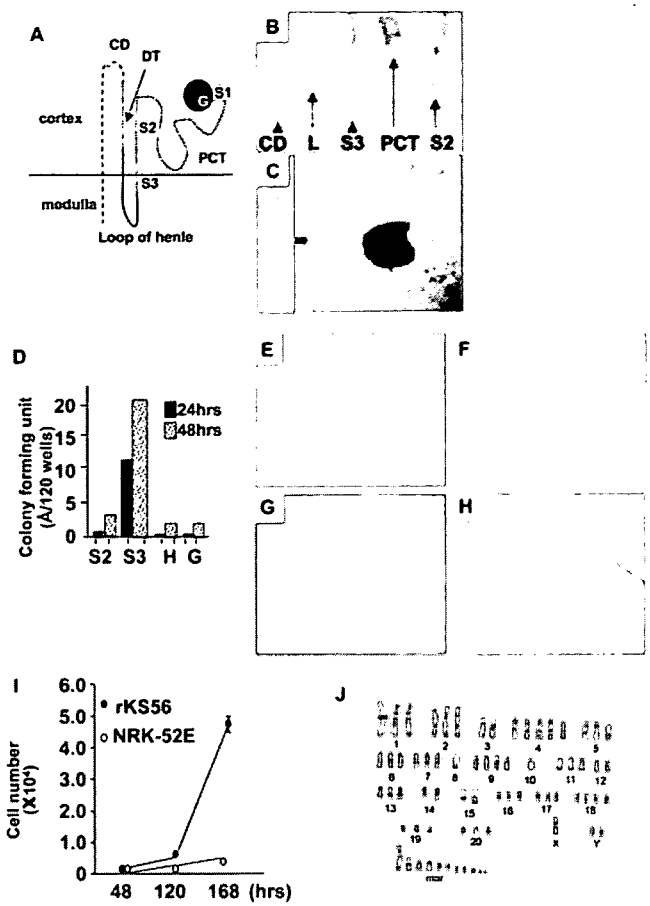

Figure 1. Establishment of rKS56 cell line. *A)* Components of nephron segments. G: glomeruli, S1-S3: proximal tubules, DT: distal tubules, CD: collecting ducts PCT: proximal convoluted tubules. *B)* Segments of nephron. CD: collecting tubule, L:loop of henle, S3:S3 segment, PCT: proximal convoluted tubule, S2:S2 segment. *C)* Outgrowing cells from S3 segment. *D)* Colony forming capacity of cells derived from different nephron segments. The number of colonies (>3 cells/colony)/120 wells (96-well plates) is shown as colony forming units (CFU). Solid bars: 24 h, dotted bars: 48 h. *E–H)* A cell clone derived from the S3 segment possessed potent mitogenic capacity and was designated as rat kidney stem/progenitor (rKS56) cells. After 1 day (*E*), after 3 days (*F*), after 5 days (*G*), after 7 days (*H*). *I)* Cell proliferation curve: rKS56 cells proliferated rapidly with exponential growth, whereas NRK-52E proliferated linearly. *J)* Karyotype of a representative line of rKS56 cells (after 250 days of subculture) detected by G-banding stain. rKS56 cells exhibited near triploid type.

Bioscience, Bedford, MA, USA) and were cultured in a 1:1 mixture of the culture supernatant which are Dulbecco's modified eagle medium [DMEM] (Sigma) containing 10% FCS (Gibco, Grand Island, NY, USA) of mouse mesenchymal cells supernatant (MCS) and modified K1 medium (1:1 mixture of DMEM and Ham's F12 medium (Gibco), supplemented with 10% FCS, 5 μg/mL insulin, 2.75 μg/mL transferrin, 3.35 ng/mL sodium selenious acid (ITS-X: Gibco), 50 nM hydrocortisone (Sigma), 25 ng/mL hepatocyte growth factor (Sigma), 2.5 mM nicotinamide (Sigma) for 24–48 h. Outgrowing cells were observed from all segments under these conditions (Fig. 1*C*). Primary cells were maintained and the cells were replated onto each well of 96-well plates to expand the cell population. After the third passage, the cells

were maintained in a 1:1 mixture of MCS and modified K1 medium, and screened for their mitogenic capacity by plating a single cell into each well of 96-well plates.

For differentiation studies, the cells were cultured in maintaining media without MCS or in the presence of 5 ng/mL of leukemia inhibitory factor (Sigma). NRK-52E cell line was purchased from Riken (Wako, Japan) as a renal epithelial cell line originally harvested from normal rat kidney. The cell line was cultured in DMEM containing 10% FCS. We used the cells between 8 to 10 passages.

Karyotype of rKS56 cells

To check the karyotype of rKS56 cells, we checked the karyotype of rKS56 cells after numerous passages (250 days). The cells were studied in Nihon Gene Research Labs Inc. (Sendai, Japan). Twenty rKS56 cells were analyzed by G-banding stain.

rKS56 cells generated from a single cell and expanded itself

To trace, rKS56 cells were labeled by Vybrant™ DiO cell-labeling solution (Molecular Probes, Eugene, OR, USA). Di-O could mark cell cytoplasm in distinctive fluorescent colors. rKS56 cells were incubated for 10 min with serum free medium containing Di-O solution, and were washed twice with serum free medium. In first culture, these rKS56-Di-O cells were plated. In secondary culture, a single rKS56 cell-Di-O was cloned into each well of 96-plate precoated with type IV collagen by using limiting dilution method. After several cycles of cell division, single rKS56 cell-Di-O was plated in each well of 96-well plate.

Immunofluorescent studies

Cells were cultured on 16-well chamber slides (Nunc-Immuno Plate, Dako, Denmark) precoated with type IV collagen for several days, then fixed with ice-cold acetone for 5 min. The chamber slides or frozen tissue sections were subjected to immunofluorescence staining using primary rabbit antibodies raised to E-cadherin (Santa Cruz, Santa Cruz, CA, USA), c-met (Santa Cruz), AQP-1 (Chemicon, Segundo, CA, USA), AQP-2 (Chemicon), Musashi-1 (Chemicon) and Tamm-horsfall glycoprotein (THP, Biomedical Technologies Inc.) or mouse antibodies raised against pan-cytokeratin (Santa Cruz), Vimentin (Sigma), and secondary antibodies conjugated with FITC (goat IgG; Zymed Laboratories, San Francisco, CA, USA) or rhodamine (sheep IgG; Chemicon) as described previously (18). Images were recorded using confocal fluorescence microscopy. Formalin-fixed and paraffin-embedded tissue sections (4 μm), were processed for immunohistochemistry using an avidin-biotin peroxidase technique (Vectastain; Vector Laboratories, Burlingame, CA, USA) as described (18).

Reverse transcription-PCR

Total RNA was extracted from culture cells by using RNA extraction kit (QIAGEN, Valencia, CA, USA) following the manufacturer's manual. One microgram of total RNA was reverse-transcribed into cDNA and amplified using the One-step RNA PCR kit(AMV, (TAKARA Bio Inc., Shiga, Japan) and specific primer pairs for c-kit, Sca-1, Pax-2, WT-1, GDNF, Wnt-4, AQP-1, ClC, NaCl transporter, AQP-2, and GAPDH, subjected to agarose gel electrophoresis, and visualized as described (18).

Electrophoresis and immunoblotting

Sodium dodecyl sulfate-PAGE (SDS-PAGE) and immunoblotting were performed as described (24). Primary rabbit antibodies raised to Musashi-1 (Chemicon) and actin (Sigma) were used. Secondary goat anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase was purchased from Sigma.

Measurement of cAMP accumulation in response to PTH or AVP

The amount of cAMP was measured by using cAMP EIA kit (Amersham, Piscataway NJ, USA) following the manufacturer's instruction. Briefly, rKS56 cells grew to confluence in 96-well culture plates precoated with type IV collagen, rinsed twice with DMEM and incubated with media containing 10^{-4} M of IBMX and 10^{-7} M of parathyroid hormone (PTH, Sigma) or 1U of arginine vasopressin (AVP, Sigma) for 10 min at 37°C. Control wells were treated with saline. Then, cellular protein was extracted by the addition of ice-cold ethanol. After incubation for 30 min on ice, the supernatant was obtained, then dried under a vacuum. The dried extracts were dissolved in 500 μL of assay buffer and the concentration of cAMP was measured by enzymeimmunoassay (EIA). The amount of protein was determined by the modified Coomassie blue G dye binding assay with BSA as standard.

Cell implantation study for evaluating the plasticity of rKS56 cells

rKS56 cells were implanted into liver, spleen, kidney, skeletal muscle, or subdermis (5×10^4 cells/organ with black ink to trace the injected site) of anesthesized SD rats (Clea JAPAN Inc.). On day 28 after implantation, the rats were killed and tissues were obtained and processed for formalin fixation followed by paraffin-embedding or frozen section.

Induction of acute renal failure by ischemic reperfusion and cell therapy into injured kidney

Male SD rats (Clea JAPAN Inc.) weighing 100–150 g were used. Acute renal failure was induced by clamp of right renal artery for 40 min after removal of left kidney (ischemic reperfusion: I/R). We preliminary confirmed that acute tubular necrosis occurred mainly in outer medulla at peak of 3–4 days and renal function and morphological changes returned to normal at 7 days. rKS56 cells (1.6×10^7 cells) resuspended in 0.4 mL of PBS with black ink were implanted into subcapsule of each kidney at 4 sites on both abdominal and dorsal side using 20-gauge needle after 40 min of ischemia. Rats of I/R without cell implantation received vehicle buffer (PBS). SD rats were divided into the following subgroups: *1)* 0 day for control (n=6), *2)* I/R treatment group(n=7), *3)* after I/R with rKS56 cells implantation treatment (n=6). At the end of each experimental period, individual 24 h urine sample collections were performed. The SD rats were killed after 4 and 7 days, and kidneys were obtained and processed for formalin fixation followed by paraffin embedding or frozen section. Blood samples were drawn from the vena cava inferior under anesthesia. We measured creatinine, blood urea nitrogen, and urinary N-acetyl-β-D-glucosainidase (U-NAG) as tubulointerstitial injury maker. To trace the mobilization of rKS56 cells, cells were labeled by Vybrant™ DiO cell-labeling solution prior to cell implantation.

Statistical analysis

Data were expressed as mean ± SE. Comparisons between groups were evaluated by Student's t test. A P value of <0.05 denoted the presence of a statistically significant difference.

RESULTS

Organ culture of dissected nephron segments and establishment of rKS56

Nephron are consisted of several segments like glomeruli (G), proximal convoluted tubule (S1/PCT), proximal straight tubule (S2,S3), medullary thick ascending limb of Henle's loop (MAL) and collecting duct (CD) (Fig. 1*A*). We used an organ culture system for microdissected nephron segments (Fig. 1*B*) to define the origin of proliferating cells. Initial outgrowing cells (Fig. 1*C*) from nephron segments were observed in more than half of the wells in maintaining culture condition and there was no difference among segments on the rate of outgrowth (data not shown). After the limiting dilution, however, only a few cells survived and grew to form cell colony (Fig. 1*D*). Origin of surviving cells was distributed mainly to the S3 segment. Out of 180 single cells derived from S3 segments, we finally obtained 2 clones that survived through limiting dilution repeatedly. One of the clones with more potent growth was designated as rKS56.

Rapid proliferation rate of rKS56

rKS56 proliferated at a rapid rate even when plated at very low cell density (Fig. 1*E–H*). To evaluate the proliferative profile of rKS56, we counted cell number at several time points after replating 100 cells/well in comparison with a renal epithelial cell line, NRK-52E. As shown in Fig. 1*I*, cell number of rKS56 increased dramatically from 120 h to 168 h, and the growth curve indicated exponential growth, while NRK-52E grew steadily and the growth curve indicated linear growth. Based on the growth curve, doubling time of rKS56 was calculated as 16–24 h, which is comparable to those in stem/progenitor cell population (25).

Karyotype of rKS56 cells

One might argue that rKS56 cells are transformed cells similar to tumor cells. In regard to karyotype, we checked the karyotype of rKS56 cells after numerous passages (250 days). rKS56 cells consisted of 66–71 chromosomes and thus considered to be nearly triploid (Fig. 1*J*). However, we could not find out at which point rKS56 cells had converted the karyotype from diploid to triploid. In another sets of experiments, we implanted 1.6×10^7 of rKS56 cells (after 200 days of passages) subcutaneously to nude mice and observed for 13 months. We could not find formation of any tumor like mass in these mice (data not shown). Therefore, we consider that rKS56 cells did not give rise to tumorigenesis and so could not be considered to possess tumor cell phenotype.

rKS56 cells originated from a single cell and expanded itself

In the first culture, rKS56-Di-O cells were detected like mosaicism pattern (**Fig. 2*A, B***). In the secondary culture, a single rKS56 cell-Di-O formed colony (Fig. 2*C–E*). After several cycles of cell division, mosaicism of labeled and unlabeled progeny cells could be observed after two passages (Fig. 2*F–H*). Neural stem cells obtained by neurosphere methods could be generated from a single primary sphere. rKS56 cells possess the same characteristics as neural stem cells. These results may suggest that rKS56 cells have the potential for self-renewal.

Immature tubular epithelial-like phenotype of rKS56

Next, we attempted to characterize the rKS56 cells. rKS56 cells exhibited a cobblestone appearance and expressed pancytokeratin (**Fig. 3*A***), indicating epithelial cell phenotype. As for mature tubular epithelial cell markers, water channel aquaporin (AQP)-1 was weakly expressed in cytoplasmic pattern (Fig. 3*B*). rKS56 cells were also positive for vimentin, a mesenchymal cell marker (Fig. 3*C*), and c-met, a receptor for hepatocyte

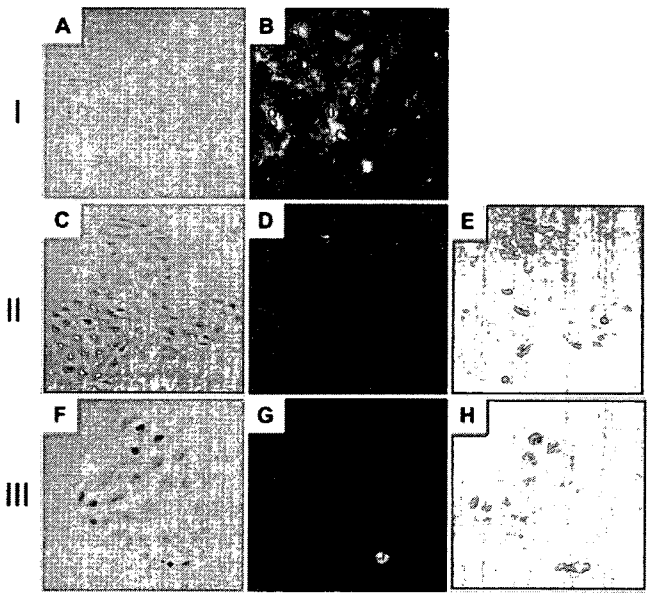

Figure 2. rKS56 cells generated from a single cell. rKS56 Cells were labeled with Di-O and plated. In the first passage, the expanded cell population was composed of Di-O(+) and Di-O(−) cells. Left: light microscopy (*A*), middle: fluorescent microscopy (*B*). Cells were harvested, and a single cell was replated and observed for further two passages. Second passage from a single Di-O positive cell: left: light microscopy (*C*), middle: fluorescent microscopy (*D*), right: merged images (*E*). Third passage from a single Di-O positive cell: left: light microscopy (*F*), middle: fluorescent microscopy (*G*), right: merged images (*H*).

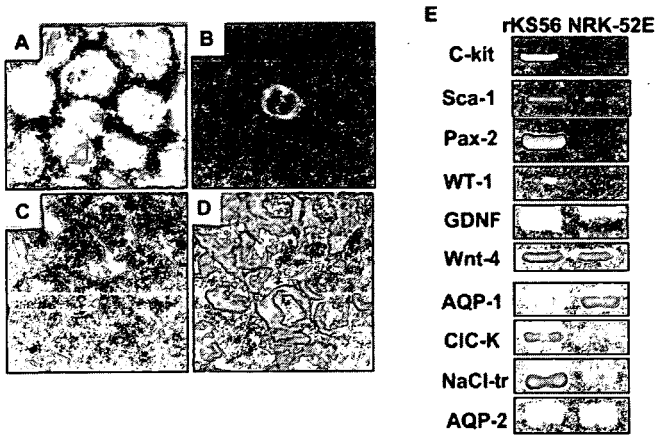

Figure 3. Characterization of rKS56 cells. rKS56 cells expressed pan-cytokeratin (A), aquaporin-1 (*B*), vimentin (*C*), and c-met (*D*) detected by immunocytochemistry (x400). *E)* Expression of development-associated genes and renal-specific genes in rKS56 cells was observed by RT-PCR. ClC-K: chloride channel-K, NaCl tr: NaCl transporter.

growth factor (Fig. 3*D*). RT-PCR revealed that rKS56 expressed various molecules relating to progenitor cell marker, nephrogenesis and mature tubular cell functions (Fig. 3*E*). rKS56 cells also expressed mRNAs for Sca-1 and c-kit, markers of progenitor cells, and expressed mRNAs for Pax-2, GDNF, WT-1 and Wnt-4 which are essential molecules involved in nephrogenesis, while NRK-52E showed limited expression. rKS56 also expressed mRNAs for AQP-1, chloride channel, NaCl transporter, AQP-2 which are functional molecules in mature tubular epithelial cells, while NRK-52E showed limited expression. These results suggest that rKS56 cells are derived from renal epithelial cells and possess relatively immature phenotype as compared with NRK-52E.

Musashi-1 expression in rKS56

Specific markers for immature renal stem/progenitor cells have not been identified yet. We, therefore, examined the expression of Musashi-1, which is a well known marker for neural stem cells. Interestingly, rKS56 expressed protein in a cytoplasmic pattern (**Fig. 4*A***) and Musashi-1 mRNA detected by RT-PCR (Fig. 4*B*). The expression of Musashi-1 in rKS56 was further confirmed by immunoblot using cell lysates. rKS56 contained Musashi-1 protein 2.5-fold higher than NRK-52E (Fig. 4*C*). These results suggest that rKS56 cells may have an immature character such as neural stem cells.

Ability of rKS56 to differentiate into mature renal tubular epithelial cells

Because rKS56 cells appear to possess immature renal epithelial phenotype, we explored whether rKS56 cells could differentiate into mature tubular epithelial cells under various culture conditions. In general, AQP-1 is expressed on renal proximal tubular epithelial cells and cells in the descending loop of Henle. AQP-2 is expressed mainly on collecting duct epithelial cells and weakly on cells in ascending loop of Henle and distal tubular epithelial cells. THP is expressed mainly in epithelial cells in the loop of Henle. When rKS56 cells were cultured with MCS, AQP-1 and AQP-2 were observed as cytosolic pattern (**Fig. 5*A, C***) and only a single cell was positive for THP (Fig. 5*B*). However, when rKS56 cells were cultured without MCS, AQP-1 was observed as membrane pattern (Fig. 5*D*), and AQP-2 was observed as membrane pattern (Fig. 5*F*). Numerous THP positive cells were observed under culture condition without MCS in maintenance culture media (Fig. 5*E*). These findings suggest that rKS56 cells may differentiate to renal component cells such as AQP-1 positive cells, AQP-2 positive cells and Tamm-Horsfall positive cells.

The phenotypic changes into more mature tubular epithelial cells were further confirmed by responses to parathyroid hormone (PTH) and arginine vasopressin (AVP). Generally, proximal tubular epithelial cells respond to PTH and distal tubular epithelial cells and collecting duct cells respond to AVP. The response to these hormones was evaluated by accumulation of cAMP. Addition of LIF to maintenance culture condition enhanced cAMP accumulation in response to PTH (**Fig. 6*A***). Furthermore, maintenance culture condition with LIF and without MCS dramatically increased cAMP accumulation in response to PTH and AVP (Fig. 6*B*). These findings suggest that rKS56 may have the capacity to differentiate into mature tubular epithelial cells.

Ectopic implantation of rKS56 cells results in tubular differentiation

To examine the plasticity of rKS56 cells in vivo, cells were transplanted into various organs including liver,

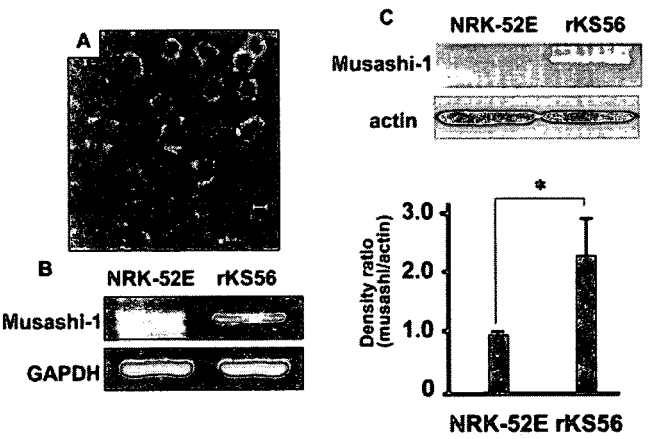

Figure 4. Musashi-1 expression in rKS56 cells. rKS56 cells expressed Musashi-1 protein detected by immunocytochemistry (×400, *A)* Similar results were observed by RT-PCR (*B*) and immunoblot (*C*). (*C*, upper panel) Each band was scanned and subjected to densitometry. Intensities of Musashi-1 protein relative to actin are shown (C, lower panel). The expression of Musashi-1 in rKS56 cells was 2.5-fold higher than NRK-52E cells. $*P < 0.05$ vs. NRK-52E. Each column consists of mean ± SE.

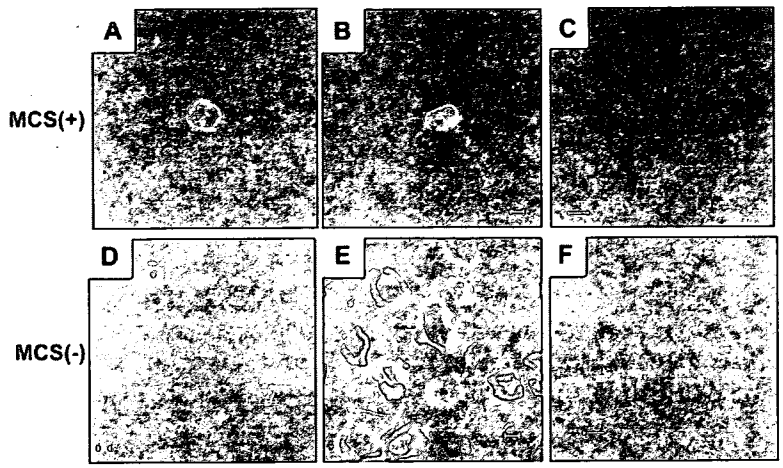

Figure 5. Differentiation of rKS56 cells. rKS56 cells exhibited weak expression of aquaporin-1 (AQP-1, *A*), Tamm-Horsfall protein (THP, *B*), and aquaporin-2 (AQP-2, *C*) under maintenance culture conditions detected by immunocytochemistry (x400). However, under maintenance culture condition without MCS, rKS56 cells exhibited differentiated renal tubular epithelial phenotype highly expressing kidney-specific proteins such as AQP-1 (*D*), THP (*E*), and AQP-2 (*F*), and intracellular localization of these proteins were altered. AQP-1 and AQP-2 were detected as plasma membrane pattern and not as cytoplasmic pattern under culture condition without MCS.

spleen, kidney, skeletal muscle and skin of SD rats. On day 28 after implantation, we could not identify any cell clusters in the liver or spleen (data not shown), whereas a small mass composed of numerous tubule-like structure was observed at the intramuscular injection sites identified by retention of black ink (**Fig. 7*A, B***). These tubule-like structures were positive for AQP-1 and AQP-2 (Fig. 7*C, D*).

Engraftment and differentiation of rKS56 cells in acute tubular injury model

We next examined the capacity of rKS56 cells to repair injured tubules. rKS56 cells relabeled with Di-O were injected subcapsularly near corticomedullary lesion in the kidney after 40 min of ischemia. On the 7th day after cell implantation, localization of implanted rKS56 cells was evaluated. Most of the Di-O positive cells were observed in tubules in the corticomedullary region and were positive for cytokeratin (**Fig. 8*A–C***). These cells coexpressed AQP-1 in apical and basolateral side (Fig. 8*D–F*). Di-O positive cells were observed not only at the sites of injection but also at distinct sites such as cortex and medulla (Fig. 8*G–I*).

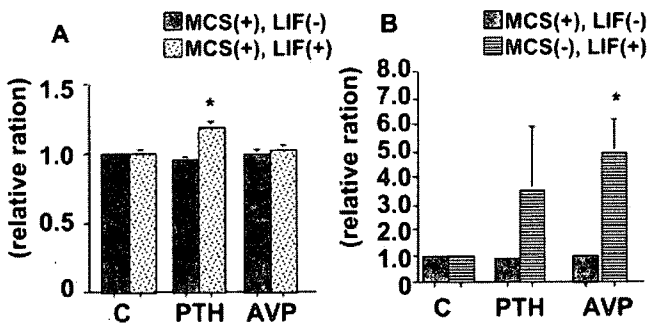

Figure 6. Hormone response of rKS56 cells. Under maintenance condition, rKS56 cells did not respond to PTH and AVP (*A, B*: filled bar). However, in culture condition containing leukemia inhibitory factor (LIF), the rKS56 cells responded only to PTH (*A*, dotted bar). In culture condition without supplementation of condition medium of mesenchymal cells (MCS) and LIF, the rKS56 cells responded to AVP more markedly than stimulation with PTH (*B*, hatched bar). *C)* Saline control, PTH: parathyroid hormone, AVP: arginine vasopressin, $*P < 0.05$ vs. saline control. Each column consists of mean ± SE.

We could not observe any significant differences in the level of serum creatinine (Cr) and blood urea nitrogen (BUN) between I/R rats treated with rKS56 cells and untreated animals (Cr of I/R rats 4 days 0.370 ± 0.017; Cr of I/R rats 7 days 0.370 ± 0.015; Cr of cell therapy and I/R rats 4 days 0.407 ± 0.052 [P=0.4866 vs. Cr of I/R rats 4 days]; Cr of cell therapy and I/R rats 7 days 0.400 ± 0.020 mg/dl [P=0.3223 vs. Cr of I/R rats 7 days], BUN of I/R rats 4 days 19.3 ± 0.704; BUN of I/R rats 7 days 18.7 ± 2.08; BUN of cell therapy rats 4 days 19.97 ± 0.977 [P=0.5924 vs. BUN of I/R rats 4 days]; BUN of cell therapy rats 7 days 21.4 ± 2.514 mg/dl [P=0.4957 vs. BUN of I/R rats 7 days]). Although we could find increase of U-NAG levels after 7 days in I/R induced/untreated group, rats receiving implantation of rKS56 cells showed a significant suppression in U-NAG levels (Fig. 8*J*). The significant suppression of U-NAG elevation suggested that damage of tubulointerstitial lesion was protected. Implanted rKS56 cells could replace injured tubular cells and differentiate into mature tubular epithelial cells, at least proximal tubular epithelial cells, in acute tubular necrosis model.

DISCUSSION

In the present study, we purified a cell population exhibiting a high proliferation rate from the S3 segment in adult rat kidney. From these cells, we established an epithelial-like cell line:rKS56, which showed three characteristic features of stem/progenitor cells: self-renewal, multiple plasticity restricted to renal epithelial cells and regenerative ability by replacing injured tubular epithelial cells in the ATN model.

Although kidney is an organ with less mitosis under normal conditions as compared with other organs such as intestine or skin, the kidney has the capacity to regenerate itself once injured or partially ablated. Acute tubular necrosis, for example, occurs by expo-

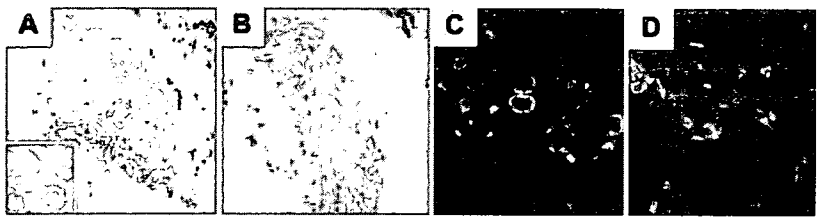

Figure 7. Ectopic implantation of rKS56 cells results in tubule-like structure formation. rKS56 cells marked with black ink were implanted into the peritoneal muscle of SD rats. Implanted cells formed tubule-like structures in peritoneal muscle after 4 weeks (hematoxylin stain, x100, *A, B*). Inset; note the tubule-like structures presumably formed by implanted rKS56 cells (*A*). The tubule-like structures partially expressed AQP-1 (*C*) and AQP-2 (*D*) detected by immunohistochemistry (×200).

sure to ischemia or reagents toxic for tubular epithelial cells. Damaged tubules are quickly repaired and regenerated after a burst of cell proliferation after the injury. However, the origin of the proliferating cells has not been determined yet. We hypothesized that the highly proliferative cell may be near the stem/progenitor cell strain and might exist in the S3 segment of proximal tubules. In this study, we used a microdissection technique and coculture system to obtain the cells possessing a high capacity of proliferation. Previous reports demonstrated the usefulness of cell-sorting technology to harvest hematopoietic, nerve and hepatic stem cells from bone marrow or adult tissue such as liver or brain, by using antibodies against cell-surface markers (26, 27). Since the detailed repertoire of unique cell surface marker proteins expressed on renal stem/progenitor cell is unknown at present, we could not apply a cell-sorting method to isolate renal stem/progenitor cells. In addition, the microdissection technique enabled us to identify the precise origin of proliferating cells. Among cells outgrowing each segment of the nephron, only the S3 segment-derived cells survived through limiting dilution, indicating limited localization of the progenitors with potential to proliferate.

The rKS56 cells expressed Sca-1 and c-kit. Sca-1 and c-kit are well known to be expressed on hematopoietic stem cells (28). The expression of Sca-1 and c-kit suggest that rKS56 cells may have potential to serve as renal stem/progenitor cells. The rKS56 cells exhibited a typical cobblestone appearance with expression of renal epithelial markers such as pan-cytokeratin, Pax-2 and Wnt-4, mesenchymal markers like vimentin and WT1. The expression of epithelial and mesenchymal markers was reported in a metanephric mesenchyme cell line, 7.1.1 cells, containing embryonic renal stem cells (19), and cell lines, mK3 and mK4, derived from SV40-LT transgenic mice (29). Coexpression of Pax-2 and vimentin in regenerative tubular epithelial cells was reported in experimental acute tubular necrosis models (30). rd, rKS56 cells appear to have molecular characteristics in common with metanephric mesenchyme and regenerative tubular cells. Tissue adult stem cells are located in a so-called "niche" where they are protected from environmental injuries (31). Although the turnover time of stem cells residing in the niche is very slow, stem cells undergo rapid multiplication when they are isolated and grown in culture. The rKS56 cells proliferated at a very rapid rate with exponential growth, while the NRK-52E cells grew linearly. The high turnover rate in the rKS56 cells was sustained over 300 days irrespective of immortalization. Neural stem cells obtained with neurosphere methods could be generated from a single primary sphere (21). rKS56 cells have the same character as neural stem cells. These results suggested that rKS56 cells have the potential for self-renewal.

Musashi-1 is a neural RNA binding protein that is specific for neural stem cells and Musashi-1 is required for asymmetric cell division of sensory organ precursor cells (32). Musashi-1 expression was believed to be restricted to neural stem cells, but ectopic expression of the Musashi family has been reported on non-neural origin stem cells, such as mouse intestinal stem and progenitor cells and human breast epithelial stem cells (33–35). We also detected Musashi-1 expression in the rKS56 cells, which is consistent with asymmetric cell division and supports the notion of rKS56 cells having a stem cell phenotype.

Multipotency is another characteristic feature of stem cell. In general, tissue stem cells were believed to have limited lineage-restricted plasticity as compared with multipotent embryonic stem cells or ES cells (36). Recent work has proposed the idea that adult stem cells can differentiate crossing lineage boundaries (5, 8, 37, 38). rKS56 cells had both epithelial and mesenchymal features based on the protein and mRNA expression patterns, and differentiated into mature proximal and distal tubular epithelial cells in vitro, and glomerular epithelial as well as proximal tubular epithelial cells in vivo, suggesting plasticity within renal epithelial cells. Transplantation experiments into extra-renal organs failed to demonstrate multipotency to cross lineage boundaries. Further studies are required to clarify the possibility of multipotency of rKS56.

Alison et al. (37) were the first to demonstrate that bone marrow cells could differentiate into renal tubular cells. Recent work also demonstrated that bone marrow-derived stem cells contributed to the regeneration of renal tubules (39, 40). It is unlikely that bone marrow-derived stem cells in the peripheral blood were contaminants during microdissection methods in our study because the kidney was perfused with saline to wash out retained blood. Even if contamination might have occurred, it was difficult for bone marrow-derived cells to differentiate into renal epithelial cells in vitro. Based on our results, rKS56 was derived from purified renal tubules and shared many characteristics with embryonic renal stem cells, rather than bone marrow-derived stem cells as described above.

We attempted cell therapy to treat acute tubular