Kidney Regeneration in Later-stage Mouse Embryos via Transplanted Renal Progenitor Cells

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Supplementary Materials

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Supplemental Table 1: Antibody Information

ANTIBODIES	SOURCE	IDENTIFIER	HOST	DILUTION
CK8 (TROMA- I)	DSHB	N/A	Rat	1:200
Six2	Proteintech	Cat.# 11562-1-AP	Rabbit	1:200
CDH1	BD	Cat.# 610181	Mouse	1:200
Nephrin	Progen	Cat.# GP-N2	Guinea pig	1:200
CD31	BD	Cat.# 557355	Rat	1:200
CD31	Abcam	Cat.# ab28364	Rabbit	1:200
GFP	MBL	Cat.# MBL598	Rabbit	1:200
GFP	Abcam	Cat.# ab13970	Chicken	1:200
WT1	Santa Cruz	Cat.# sc-192	Rabbit	1:50
Megalin	Santa Cruz	Cat# sc-16478	Goat	1:50

AQP1	Abcam	Cat# ab9566	Mouse	1:200
PDGFRb	R&D systems	Cat# AF1042	Goat	1:200
Anti-Rat IgG (H+L) (Alexa Fluor 405)	Abcam	Cat# ab175670	Donkey	1:200
Anti-Chicken IgY (H+L)(FITC)	Thermo Fisher Scientific	Cat# SA1-72000	Donkey	1:200
Anti-Rat IgG (H+L) (DyLight 550)	Thermo Fisher Scientific	Cat# SA5-10027	Donkey	1:200
Anti-Rat IgG (H+L) (DyLight 650)	Thermo Fisher Scientific	Cat# SA5-10029	Donkey	1:200
Anti-Rabbit IgG (H+L)(Alexa Fluor 555)	Thermo Fisher Scientific	Cat# A31572	Donkey	1:200
Anti-Mouse IgG (H+L)(Alexa Fluor 647)	Thermo Fisher Scientific	Cat# A31571	Donkey	1:200
Anti-Rabbit IgG (H+L) (Alexa Fluor 488)	Thermo Fisher Scientific	Cat# A21206	Donkey	1:200

Anti-Goat IgG (H+L) (Alexa Fluor 647)	Thermo Fisher Scientific	Cat# A21447	Donkey	1:200
Anti-Mouse IgG (H+L) (Alexa Fluor 546)	Thermo Fisher Scientific	Cat# A10036	Donkey	1:200
Anti-Guinea Pig IgG(H+L)(Alexa Fluor 647)	Jackson Immuno Research	Cat# 706-605-148	Donkey	1:200

CK8: Cytokeratin 8, Six2: Sine Oculis Homeobox Homolog 2, CDH1: Cadherin 1, GFP: green fluorescent protein, WT1: Wilms tumor 1, AQP1: Aquapolin 1, DSHB: Developmental Studies Hybridoma Bank, Proteintech: Proteintech Group, BD: Becton Dickinson Biosciences, Santa Cruz: Santa Cruz Biotechnology, MBL: Medical & Biological Laboratories, Cat#: Catalog number

Immunofluorescence

In section immunohistochemistry, for immunohistochemical analysis, the whole embryos or pieces of kidney were fixed in 4% paraformaldehyde in PBS for overnight, dehydrated with 20% sucrose in PBS for overnight, embedded in optimal cutting

temperature compound, and cryosectioned at 10-μm thickness. For fluorescence immunohistochemistry, the section samples were washed with PBS thrice. Sections were exposed to an antigen retrieval solution (Histo VT One; Nacalai Tesque, Kyoto, Japan) for 20 min at 85°C and washed in PBS twice. Next, the sections were blocked by incubation with blocking One Histo (Nakarai Tesque, Kyoto, Japan) for 10 min at room temperature. The sections were incubated overnight with primary antibodies at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature (details are presented in Supplemental Table 1). In whole-mount immunohistochemistry, the transwell was cut out together with the filter on which the sample was placed. The samples were washed with PBS thrice and fixed in 4% paraformaldehyde in PBS for 15 min at 4ºC in rocking condition, washed with 0.1% Triton X-100 (Sigma- Aldrich, Saint Louis, USA) in PBS (PBST) thrice, and blocked in 1% FBS w/v (Thermo Fisher Scientific), 0.2% nonfat dry milk powder w/v (Wako, Tokyo, Japan), and 0.3% Triton X-100 v/v for 1 h at room temperature. The samples were incubated overnight with primary antibodies at 4°C. The samples washed with PBST for 1 h thrice and incubated with secondary antibodies for 1 h at room temperature. Next, the samples washed thrice with

PBST for 30 min . After rinsing, the sections and whole-mount samples on the slides were coverslipped under SlowFade Diamond mounting medium (Thermo Fisher Scientific). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Roche).

Genotyping primers

Genotyping was performed using the following primers: iDTR[4] (wt product ~620 bp, iDTR product ~240 bp), 5′-AAAGTCGCTCTGAGTTGTTAT-3′ (RosaFA), 5′-GGAGCGGGAGAAATGGATATG-3′ (RosaRA), and 5′-CATCAAGGAAACCCTGGACTACTG-3′ (SpliAcB)[5]; Cre (~410 bp), 5′-CTAATCGCCATCTTCCAGCAGG-3′ (CreF) and 5′ -AGGTGTAGAGAAGGCACTTAGC-3′ (CreR)[6].

RPC transwell culture

RPCs were co-cultured at the air-fluid interface of a polycarbonate filter (0.8 mm; Corning Inc., Corning, NY, USA) with mouse embryonic spinal cords taken from E13.5 embryos for six days in α-MEM supplemented with 20% fetal bovine serum (Invitrogen)

and 1% antibiotic-antimycotic (Gibco Life Technologies). Place the spinal cord on top of the membrane, and drop the cell suspension using the glass tube. Organs were cultured for 1–7 days at 37°C under 5% CO_2. The medium was changed once every two days.

Quantitative RT-PCR

"Dissociated RPC," "Cultured RPC," and "Post-Exoutero RPC" groups were analyzed using quantitative RT-PCR. "Dissociated RPC" group included dissociated single cells (DSCs) prepared from approximately 20 metanephros of GFP mouse at E13.5. "Cultured RPC" group included DSCs cultured in Transwell chambers for 6 days and collected. In the "Post-Exoutero RPC" group, GFP-DSCs were transplanted into B6 mouse E13.5 via the Exoutero method. Subsequently, the sites at which GFP was expressed in the fetus were identified using a fluorescent stereomicroscope after 6 days, and the collected samples were used for PCR. Each group comprised 6 cells. Total RNA was isolated from DSCs and the collected samples using the RNeasy Micro Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 50 ng RNA using the PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). Real-time PCR was performed using

TaqMan® Master Mix and TaqMan® Assay (Six2; Mm03003557_s1, NPHS1; Mm01176615_g1, NPHS2; Mm01292252_m1, LRP2 (Megalin); Mm01328171_m1, SLC12A1; Mm01275821_m1, KRT8 (Cytokeratin 8); Mm00835759_m1, COLA1A1; Mm00801666_g1; Thermo Fisher) with the Rotor-Gene Q (Qiagen). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize expression levels (Mm99999915_g1). The experiment was repeated twice.

Deposition of fetuses to foster mother

Mice with transplanted cells underwent cesarean section with E19.5, and the fetuses were deposited with the mother of the C3H/HeSlc (C3H) mouse immediately after birth. The C3H mouse was the mother mouse that gave birth on the same day. The C3H mouse fetuses were thinned to half for making the deposited fetuses more viable. After the cesarean section the fetuses were removed using forceps to avoid the transmission of the smell of human beings. The fetuses were observed on the heat insulation board until breathing normalized; if the breathing was shallow, the back was stimulated using a cotton swab. The mother of the C3H raised the deposited fetuses like her own fetus. As the C3H

mouse is brown in color, it can be distinguished from the black B6 mouse or Six2Cre/iDTR mouse deposited. The grown mice were dissected on day 14 and histologically analyzed.

Electron microscopy

Specimens were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde mixture in 0.1 M phosphate buffer overnight at 4°C, and then post-fixed with 1% osmium tetroxide in the same buffer at 4°C for 2 h. Dehydration was carried out using a graded ethanol series followed by propylene oxide; dehydrated specimens were then embedded in Epok 812 (Oken, Tokyo, Japan). Ultrathin sections were prepared with a diamond knife, stained with uranyl acetate and lead citrate solution, and visualized using an H-7500 electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

***In vivo* imaging using fluoro label dextran**

The P0 mouse fetuses were anesthetized with isoflurane and three ribs in the left precordium were incised. The beating apex was identified and injections were made from

the apex into the ventricle with a glass tubule. The glass tubule was fabricated by a puller (PC-100, Narishige, Tokyo, Japan). The fetus was then administered a transcardiac dose of 120 μL of 5 mg/mL tetramethylrhodamine-labeled low molecular weight dextran (3,000 molecular weight; D3308; Thermo Fisher Scientific, Waltham, MA, USA) by mouth pipetting. Fetuses were sacrificed 1 h after administration, and portions of dextran-labeled and GFP-expressing tissue were detected by fluorescence stereoscopy and removed. The collected specimens were analyzed by immunofluorescence staining.

Flow cytometry

Six2CreGFP mice express GFP under the *Six2* promoter. As Six2-positive cells express this marker, these cells were measured by detecting GFP using a flow cytometer (MACSQuant® Analyzer, Miltenyi Biotec, Bergisch Gladbach, Germany). EpCAM and PDGFRa were used as lineage-specific surface markers. An APC anti-mouse CD140a (PDGFRa) antibody (Cat.135908, Biolegend, San Diego, CA, USA; dilution 1:20), APC rat IgG2a,κ isotype ctrl antibody (Cat.400512, Biolegend; dilution 1:20), Brilliant Violet 421 anti-mouse CD326 (EpCAM) antibody (Cat.118225, Biolegend; dilution 1:80), and

Brilliant Violet 421 rat IgG2a,κ isotype ctrl antibody (Cat.400549, Biolegend; dilution 1:80) were used for staining. Data were acquired and analyzed using MACSQuantify analysis software (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell suspensions were centrifuged at 300 × *g* for 10 min and the supernatant was discarded. Then, 100 μL of each MACS buffer was added to 0.1×10^6 cells, which were placed on ice for 10 min in the dark and then washed once in 1000 μL MACS buffer; this was followed by centrifugation at 300 × *g* for 10 min. Cells were resuspended using 200 μL of MACS buffer. The cells were then used for analysis. The composition of the MACS buffer was 150 mL PBS (-) (Gibco Life Technologies), 750 mg bovine serum albumin (Nakarai Tesque, cat No. 01860-36), and 600 μL of Ultra Pure 0.5 M EDTA, pH 8.0 (Thermo Fisher Scientific, cat No. 15575-020).

Mesangium cell regeneration rate

Present the method for Figure 2F. Regenerated glomeruli expressing GFP were randomly selected (n = 5), and PDGFRb-positive cells were counted by the visual observation to determine the number of mesangium cells inside the glomeruli. Next,

PDGFRa+/GFP+ cells derived from the transplanted cells inside glomeruli were similarly counted by the visual observation, and the ratio was calculated.

Chimeric UB tip regeneration rate in the kidney-deficient mouse metanephros

Present the method for Figure 7D. The percentage of transplanted progenitor cells incorporated into host UB tip in the metanephros of kidney-deficient mice was examined. The total number of UB tips which were Six2 and Cytokeratin 8 positive was counted on a whole metanephros slice as a denominator, and the number of UB tips which were Six2+/GFP+ and Cytokeratin 8+/GFP- was counted as a numerator, and the ratio was calculated. (counted to a total of 119 tips, n = 4 slices)

Supplemental References

1. Kobayashi A, Valerius M T, Mugford J W, Carroll T J, Self M, Oliver G, et al.: Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. Cell Stem Cell 3: 169–181, 2008

2. Menon R, Otto E A, Kokoruda A, Zhou J, Zhang Z, Yoon E, et al.: Single-cell analysis of progenitor cell dynamics and lineage specification in the human fetal kidney. Development 145, 2018

3. Trzpis M, Popa E R, McLaughlin P M, van Goor H, Timmer A, Bosman G W, et al.: Spatial and temporal expression patterns of the epithelial cell adhesion molecule (EpCAM/EGP-2) in developing and adult kidneys. Nephron Exp Nephrol 107: e119–e131, 2007.

4. Fukunaga S, Yamanaka S, Fujimoto T, Tajiri S, Uchiyama T, Matsumoto K, et al.: Optimal route of diphtheria toxin administration to eliminate native nephron progenitor cells in vivo for kidney regeneration. *Biochem Biophys Res Commun* 496**:** 1176–1182, 2018

5. Buch T, Heppner F L, Tertilt C, Heinen T J, Kremer M, Wunderlich F T, et al.: A Cre-

inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. *Nat Methods* 2**:** 419–426, 2005

6. Grgic I, Campanholle G, Bijol V, Wang C, Sabbisetti V S, Ichimura T, et al.: Targeted proximal tubule injury triggers interstitial fibrosis and glomerulosclerosis. *Kidney Int*, 82**:** 172–183, 2012

Supplemental Figure 1. Figure of entering the embryonic membrane using steel puncture needle before using injector device

A - 1) Since the amniotic membrane is slightly hard, making an initial hole with a steel needle (yellow arrow) makes it easier for it to pass through the glass needle. A-2) The glass needle inserted into the small hole opened earlier. (B) The 3-axis manipulator (YOU-1, Narishige, Tokyo, Japan) and electric motor-driven injector. (SBP-100G-LL; Takasago Electric, Aichi, Japan) C) Electric motor driven small amount injector. C-1) Control panel of injector, C-2-a, b) Injector, C-2-c) A three-way stopcock (TS-TL2K, Terumo Corporation, Tokyo, Japan) installed between the glass needle and the injector so that the cells will not overflow the glass needle when fitted with the glass needle. C-2-d) Connector to connect with a glass tube. (CI-2, Narishige, Tokyo, Japan) C-3) Photo with all the injector parts installed, C-4) Steel needle for leading puncture. (J-type No. 2 0.18 mm × 30 mm, SEIRIN Corporation, Shizuoka, Japan)

Supplemental Figure 2. Dissociated renal progenitor cells characterized using flow cytometry

Dissociated single cells (DSCs) were extracted from the metanephros of E13.5 Six2CreGFP mouse fetuses. Flow cytometry revealed that approximately 30% of the extracted DSCs contained Six2-positive NPCs.[1] In addition, PDGFRa-positive cells as stromal progenitor cells[2] and EpCUM-positive cells as UB cells[3] were present in approximately 40%.

NPC: nephron progenitor cell, UB: ureteric bud, SPC: stromal progenitor cell

Supplemental Figure 3. Integration of donor UB cells into the host kidney

When renal progenitor cells were transplanted into a wild type embryo and cells were delivered into the host kidney, a few UBs and collecting ducts also formed donor and host chimeras. Most host UBs did not show the integration of the donor UB cells.

Red lines indicate the UB structures. Yellow lines and yellow arrows indicate chimera UB structure. Blue Lines indicate UB structures constructed with only donor UB cells.

Supplemental Figure 4. Non-epithelial cells of generated cap mesenchyme (CM) in the kidney deficient model

Platelet-derived growth factor receptor-b (PDGFRb) is he stromal lineage marker[24]. In the generated CM, all PDFGRb-positive cells were GFP-negative, indicating that they were not derived from transplanted cells.

Supplemental Movie 1: Movie of fetus myometrium dissection

Legend: E13.5 fetal myometrium is dissected with scissors along the major axis of the uterus.

Supplemental Movie 2: Movie of fetus transplantation of renal progenitor cells

Legend: Small holes were made in the amniotic membrane with a steel needle, and a glass needle was inserted into the hole. A glass needle punctured the renal development area of the fetus and renal progenitor cells expressing GFP were injected with an electrically driven injector.

Supplemental Movie 3: Dissociated single renal progenitor cells

Dissociated cells were extracted from metanephros collected from E13.5 GFP mouse fetuses. A glass tube was injected into PBS with a microinjector. Individual cells and not lumps were seen.

Supplemental Movie 4: 14th day after birth of the fetus after cell transplantation

Legend: Movie of fetus after transplantation on day 14 after being raised by foster parents. Renal progenitor cells were transplanted into B6 mouse of E13.5, and they were produced by cesarean section 6 days later. The fetus was placed in the foster mother of brown C3H mice.