Generation of Kidney from Pluripotent Stem Cells via Blastocyst Complementation

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Because a shortage of donor organs has been a major obstacle to the expansion of organ transplantation programs, the generation of transplantable organs is among the ultimate goals of regenerative medicine. However, the complex cellular interactions among and within tissues that are required for organogenesis are difficult to recapitulate *in vitro*. As an alternative, we used blastocyst complementation to generate pluripotent stem cell (PSC)-derived donor organs *in vivo*. We hypothesized that if we injected PSCs into blastocysts obtained from mutant mice in which the development of a certain organ was precluded by genetic manipulation, thereby leaving a niche for organ development, the PSC-derived cells would developmentally compensate for the defect and form the missing organ. In our previous work, we showed proof-of-principle findings of pancreas generation by injection of PSCs into pancreas-deficient *Pdx1*^{-/-} mouse blastocysts. In this study, we have extended this technique to kidney generation using *Sall1*^{-/-} mouse blastocysts. As a result, the defective cells were totally replaced, and the kidneys were entirely formed by the injected mouse PSC-derived cells, except for structures not under the influence of *Sall1* expression (ie, collecting ducts and microvasculature). These findings indicate that blastocyst complementation can be extended to generate PSC-derived kidneys. This system may therefore provide novel insights into renal organogenesis. (Am J Pathol 2012; 180:2417–2426; <http://dx.doi.org/10.1016/j.ajpath.2012.03.007>)

At present, most patients with end-stage renal failure are treated by dialysis. Some patients eventually undergo kidney transplantation, but this option is limited by a shortage of donor organs. Therefore, the number of patients undergoing dialysis continues to grow, with complications, poor quality of life, and increasing medical costs. Moreover, the shortage of donor organs has led to other social problems, such as organ trafficking, transplant tourism, and transplant commercialism.¹ Under these circumstances, to transplant donor organs derived from pluripotent stem cells would be a much welcomed alternative. Induced-PSC (iPSC) technology^{2,3} has recently enabled the generation of individual- or patient-derived PSCs, with studies of disease-targeting stem cell replacement therapy. However, the generation of an organ from iPSCs is considered impractical because it remains difficult to replicate *in vitro* the complex interactions among cells and tissues during organogenesis. To overcome this obstacle, we attempted to generate organs *in vivo* using the blastocyst complementation technique originally reported by Chen et al⁴ in analyses of genes involved in lymphocyte development. We reported the successful application of this technique to generate PSC-derived mouse and rat pancreas in the *Pdx1*^{-/-} mouse.⁵ In the PSC-generated pancreas, defective cells were totally replaced, and the pancreas was formed almost entirely by the injected mouse and rat PSC-derived cells. The mouse and rat PSC-derived pancreas produced a variety of hormones, including insulin, and the transplantation of PSC-derived pancreas islets improved hyperglycemia in a diabetic mouse model. The premise driving this work is that a niche for organogenesis can be created in postblastocyst mutant mouse embryos that are genetically precluded from developing a particular or-

Supported by grants from the Japan Science Technology Agency (Ministry of Education, Culture, Sport, Science, and Technology, Japan).

Accepted for publication March 1, 2012.

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gan. Injected PSC-derived cells will colonize this developmental niche and compensate for the developmental defect to form a donor-induced organ *in vivo*.

Here we report our extended application of blastocyst complementation to generate kidneys from PSCs using *Sal1*^{-/-} mice. *Sal1* is the mammalian orthologue of the *Drosophila* region-specific homeotic gene *spalt* (*sal*). *Sal1* expression in the organism is restricted temporally to the period of nephrogenesis and disappears thereafter. *Sal1* expression in the embryonic and newborn kidney is restricted histologically to metanephric mesenchyme and renal stroma. In embryonic kidney development, *Sal1* is essential for ureteric bud attraction toward the mesenchyme. Mice deficient in *Sal1* therefore die soon after birth because of kidney agenesis or severe dysgenesis, whereas structures formed independently of *Sal1* such as the ureters and bladder are present.⁶ This study was performed to establish whether *Sal1*^{-/-} blastocysts offer a developmental niche for kidney tissue and could therefore be used to generate PSC-derived kidneys via blastocyst complementation.

Materials and Methods

Animals

C57BL6/NCrSlc, BDF1, and ICR mice were purchased from SLC Japan (Shizuoka, Japan). 129/OlaHsd (129/Ola) mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Sal1* mutant-EGFP knock-in heterozygous mice were used for the production of ESC-derived chimeras⁷ or *Sal1* knock-out heterozygous mice were used for the production of iPSC-derived chimeras.⁶ They were crossed with C57BL/6 or BDF1 strain mice. In the *Sal1* mutant-EGFP knock-in heterozygous or homozygous mouse, EGFP fluorescence was limited to *Sal1*-expressing cells. All experiments were performed in accordance with the animal care and use guidelines of the Institute of Medical Science at the University of Tokyo.

Culture of Embryonic Stem Cells/iPSCs

Undifferentiated mouse embryonic stem cells (ESCs; EB3-DsRed) were maintained on gelatin-coated dishes without feeder cells in Glasgow's modified Eagle's medium (GMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan), 0.1 mmol/L 2-mercaptoethanol (Invitrogen, San Diego, CA), 0.1 mmol/L nonessential amino acids (Invitrogen), 1 mmol/L sodium pyruvate (Invitrogen), 1% L-Glutamine Penicillin Streptomycin (Sigma-Aldrich), and 1000 U/mL of leukemia inhibitory factor (LIF; Millipore, Bedford, MA). The EB3-DsRed cells, gifts from Dr. Hitoshi Niwa (CDB RIKEN), were derived from EB3 ES cells and carried the DsRed.T4 gene under the control of the CAG expression unit. The EB3 cells were a subline derived from E14tg2a ES cells⁸ and were generated by targeted integration of the *Oct3/4*-IRES-BSD-pA vector into the *Oct3/4* allele.⁹

Undifferentiated induced pluripotent stem cells (iPSCs; GT3.2) from mice were maintained on mitomycin-C-treated

mouse embryonic fibroblast (MEF) cells in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 15% knockout serum replacement (KSR; Invitrogen), 0.1 mmol/L 2-mercaptoethanol, 0.1 mmol/L nonessential amino acids, 1 mmol/L HEPES buffer solution (Invitrogen), 1% L-Glutamine Penicillin Streptomycin, and 1000 U/mL of LIF. The GT3.2 cells were generated from tail-tip fibroblasts of a male GFP transgenic mouse, kindly provided by Dr. Masaru Okabe (Osaka University) by the introduction of three factors (*Klf4*, *Sox2*, *Oct3/4*) in retroviral vectors. Viruses were prepared as described.¹⁰ That GT3.2 cells ubiquitously express EGFP under the control of the CAG expression unit had been established.⁵

Embryo Manipulation

Preparation of *Sal1* heterozygous intercrossing embryos was performed as described.¹¹ In brief, eight cell/morula-stage embryos were collected in M2 medium (Millipore) from the oviducts and uteri of 2.5-day postcoital (dpc) *Sal1* heterozygous mice. These embryos were transferred into KSOM-AA medium (Millipore) droplets and cultured for 24 hours until development to the blastocyst stage.

For micromanipulation of the embryos, blastocysts were transferred into M2 medium droplets and the ESCs/iPSCs were trypsinized and suspended in the culture medium droplets. For each blastocyst, after careful drilling of the zona pellucida and trophectoderm under microscopy using a piezo-driven micromanipulator (Prime Tech, Tokyo, Japan), 10 to 15 ESCs/iPSCs were introduced into the blastocyst cavity near the inner cell mass. DsRed-marked ESCs were injected into blastocysts obtained by intercrossing of *Sal1*^{+/-EGFP} mice; EGFP-marked iPSCs were injected into blastocysts obtained by intercrossing of *Sal1*^{+/-} mice. After injection, the embryos were cultured in KSOM-AA medium for 1 to 2 hours and were thereafter transferred into the uteri of 2.5-dpc pseudopregnant recipient ICR mice.

Flow Cytometry and Genotyping Analysis of ESC-Derived Chimeras

To identify the genotypes of neonatal *Sal1*-EGFP knock-in mice, EGFP-positive cells from the brain and kidney were sorted by fluorescence-activated cell sorting and genotyped by genomic PCR. The analysis and sorting were performed by Mo-flo (Beckman Coulter, Fullerton, CA) or using a FACS Vantage SE system (BD Bioscience Pharmingen, Franklin Lakes, NJ). DNA was extracted from collected cells using a QIAamp DNA Mini Kit (Qiagen, Germantown, MD) and was used to confirm mouse genotypes. The PCR primers used for the amplification of the wild-type *Sal1* allele and the mutated allele were as follows: 5'-AGCTAAAGCTGCCAGAGTGC-3', 5'-CAACT-TGCGATTGCCATAAA-3', and 5'-GCGTTGGCTAC-CCGTGATAT-3' (288 bp for the wild-type *Sal1* allele and 350 bp for the mutated allele).⁷ Nested PCR primers were as follows: 5'-AGAATGTGCCCCGAGGTTG-3', 5'-TACAGCAAGCTAGGAGCAC-3', and 5'-AAGAGCTTG-GCGGCGAATG-3' (237 bp for the wild-type *Sal1* allele

and 302 bp for the mutated allele). PCR amplification was performed as described elsewhere.^{6,7}

Genotyping of iPSC-Derived Chimeras Using a Single EGFP-KSL Cell-Derived Colony or Splenocytes

To identify the genotypes of neonatal mice among the *Sal1* knock-out mice, single hematopoietic stem cells (HSCs) were assayed. Bone marrow cells were obtained from neonatal mice and suspended in PBS containing 3% FCS. Cells were stained with phycoerythrin (PE)-conjugated anti-Sca-1 monoclonal antibody (mAb; BD Bioscience Pharmingen), allophycocyanin (APC)-conjugated anti-c-Kit mAb, and a biotinylated anti-lineage antibody cocktail containing anti-Gr-1, anti-Mac-1, anti-B220, anti-CD4, anti-CD8, and anti-Ter-119 mAbs (e-Bioscience, Kyoto, Japan). The biotinylated antibodies were developed with APC-Cy7-conjugated streptavidin (SA; e-Bioscience). Single EGFP-negative KSL cells were collected in individual wells of round-bottomed, 96-well plates and cultured 10 to 14 days under conditions like those described,⁵ but with one modification (erythropoietin was included in the culture medium instead of G-CSF, IL-6, and IL-11). EGFP-negative splenocytes were also prepared from the same mice and were collected by Mo-flo. DNA was extracted from colony-forming cells derived from single EGFP-KSL cells or splenocytes. The PCR primers used to amplify the neomycin-resistance (*neo*^r) gene inserted in the *Sal1* locus were: *neo*^r locus, forward 5'-AAGGGACTGGCTGCTATTGG-3' and reverse 5'-ATATCAGGGGTAGCCAACGC-3' (420 bp); *Sal1* wild-type locus, forward 5'-GTACACGTTTCTCTCAGGAC-3' and reverse 5'-TCTCCAGTGTGAGTTCTCTCG-3' (200 bp).⁶ PCR amplification was performed as described above.

Histological Analysis

Kidneys were fixed with 4% paraformaldehyde and embedded in paraffin or, for frozen sections, in Optimal Cutting Temperature compound (Sakura Finetek, Japan). Paraffin sections were deparaffinized with xylene and hydrated with graded ethanols. A microwave oven was used for antigen retrieval. Paraffin sections were stained with hematoxylin and eosin (H&E) or periodic acid-methenamine silver (PAM) for light microscopy. Frozen sections were stained immunohistochemically. Briefly, each section was incubated with primary antibody overnight at 4°C and with secondary antibody for 1 hour at room temperature. Primary antibodies were anti-EGFP polyclonal antibody (pAb) for EGFP signal amplification (rabbit IgG, 1:500 dilution; Invitrogen), anti-DsRed pAb (rabbit IgG, 1:50 dilution; Clontech Laboratories, Mountain View, CA), anti-platelet endothelial cell adhesion molecule-1 (Pecam1) (mAb; rat IgG, 1:100 dilution; BD Bioscience Pharmingen), anti-aquaporin 1 (Aqp1) (pAb; rabbit IgG, 1:200 dilution; Chemicon International, Temecula, CA), anti-Liv2 (mAb; rat IgG, 1:200 dilution; Medical & Biological Laboratories, Nagoya, Japan), and anti-neuron class III β -tubulin (Tuj1) (mAb; rabbit IgG, 1:100

dilution; Covance, Emeryville, CA). Rhodamine- and biotin-labeled *Dolichos biflorus* agglutinin (DBA)-lectin, *Lotus tetragonolobus* (LT)-lectin, and peanut agglutinin (PNA)-lectin (Vector Laboratories, Burlingame, CA) staining was also performed. The secondary antibodies and fluorescence-conjugated streptavidin used for immunofluorescence studies were as follows: Alexa488-conjugated, Alexa546-conjugated, and Alexa647-conjugated goat anti-rabbit IgG (Invitrogen), Alexa546-conjugated and Alexa647-conjugated goat anti-rat IgG (Invitrogen), and Alexa546-conjugated and Alexa647-conjugated streptavidin (Invitrogen). After treatment with the appropriate antibodies, the sections were mounted with Vectashield mounting medium (Vector Laboratories) containing DAPI for nuclear staining and were observed by fluorescence microscopy or confocal laser scanning microscopy.

For ultrastructural analysis of glomeruli, chimeric kidneys were cut into tissue blocks (approximately 0.5 mm³) and immersed in 2.5% phosphate-buffered glutaraldehyde overnight at 4°C. The tissue blocks were postfixed in 1% osmium tetroxide for 2 hours at 4°C, dehydrated in graded ethanols, and embedded in epoxy resin (Poly/Bed 812 Embedding Media, Polysciences, Warrington, PA). Ultra-thin sections cut on a Leica Ultracut S microtome (Leica, Vienna, Austria) were stained with uranyl acetate and lead citrate. Sections were observed by transmission electron microscopy (TEM) at 75kV using a H-7000 instrument (Hitachi, Tokyo, Japan).

Results

Generation of Kidney from Embryonic Stem Cells

We conducted blastocyst complementation using ESC and *Sal1*^{EGFP/EGFP} blastocysts that would provide a niche for kidney development. As a result of blastocyst complementation (Figure 1, A and B), kidneys in *Sal1*^{+/+} chimeric neonatal mice were red (data not shown). Kidneys in *Sal1*^{+/EGFP} chimeric mice were yellow, reflecting a merger of host-derived EGFP-expressing cells and injected DsRed-marked ESC-derived cells, as in whole-body chimerism (*Sal1*^{+/EGFP} in Figure 1B). On flow cytometry analysis, the brains and kidneys of *Sal1*^{+/+} chimeric mice contained EGFP-negative and DsRed-expressing cells (Figure 2, C and D). The brains and kidneys of *Sal1*^{+/EGFP} chimeric mice contained both EGFP-expressing and DsRed-negative cells and EGFP-negative and DsRed-expressing cells (Figure 2, E and F). The genotype of each fraction of sorted cells was confirmed by PCR (data not shown). The brains and kidneys of *Sal1*^{+/EGFP} mice lacking ESC chimerism contained EGFP-expressing and DsRed-negative cells (Figure 2, A and B). The kidneys of *Sal1*^{+/EGFP} mice intrinsically contained a few EGFP-expressing and DsRed-expressing cells (Figure 2B). In contrast, the kidneys of *Sal1*^{EGFP/EGFP} chimeric neonatal mice were red throughout, indicating that the kidneys were entirely composed of DsRed-marked ESC-derived cells (*Sal1*^{EGFP/EGFP} in Figure 1B), and were of normal size. The brains of *Sal1*^{EGFP/EGFP} chimeric mice contained both EGFP-expressing and DsRed-negative

Figure 1. ESC-derived kidneys generated by blastocyst complementation. **A:** Scheme for the experimental design for PSC-derived kidney generation. Embryos obtained from *Sal1* knock-out mice for kidney generation are injected with ESCs (EB3-DsRed) or iPSCs (GT3.2). Genotyping by PCR is performed; mice with entirely ESC- or iPSC-derived kidneys are generated. **B:** To identify kidneys clearly as being ESC derived or host derived, embryos obtained from intercrosses of *Sal1* EGFP knock-in mice (*Sal1*^{+/EGFP}) are injected with DsRed-marked ESCs. The kidneys of *Sal1*^{EGFP/EGFP} mice complemented with ESCs are entirely positive for DsRed, indicating their ESC origin (*Sal1*^{EGFP/EGFP} + ESCs in **B**). The kidneys of *Sal1*^{+/EGFP} mice complemented with ESCs are positive for both DsRed and EGFP, indicating a mosaic of both ESC-derived and host-derived cells (*Sal1*^{+/EGFP} + ESCs in **B**). **C:** Representative kidney almost entirely composed of DsRed-positive cells. **D:** Result of embryo manipulation and the genotype of *Sal1* mutant.

cells and EGFP-negative and DsRed-expressing cells (Figure 2G). By contrast, the kidney contained EGFP-negative and DsRed-expressing ESC-derived cells, but not EGFP-expressing and DsRed-negative host cells (Figure 2H). In ESC-derived *Sal1*^{EGFP/EGFP} chimeric kidneys, host cells of collecting systems that did not express *Sal1* were included in the EGFP-negative and DsRed-negative fraction (Figure 2H). These results indicate that ESC-derived kidney was formed in *Sal1*^{EGFP/EGFP} mice by blastocyst complementation. A photograph of isolated kidneys, ureters, and bladder demonstrates the different densities of ESC-derived cells; although the kidneys were derived entirely from ESCs, the ESC contribution to ureters and bladder was minimal (Figure 1C). As expected, in both *Sal1*^{EGFP/EGFP} and *Sal1*^{+/EGFP} mice injected with DsRed-ESCs, ESC-derived cells contributed to all of the non-kidney tissues of the body, including adrenal gland, ureter, bladder, muscle, and adipose tissue (data not shown). The extent of the contribution varied from tissue to tissue and also from mouse to mouse depending on the individual degree of chimerism. Retrospective genotyping in a small population revealed that mice with

Sal1^{EGFP/EGFP}, *Sal1*^{+/EGFP}, and *Sal1*^{+/+} genotypes were born with Mendelian frequency (Figure 1D). These macroscopic and flow cytometry results substantiated that kidney had been formed in *Sal1*^{EGFP/EGFP} mice via blastocyst complementation. However, no complemented mouse pup survived to adulthood (Figure 1D). By contrast, ESC-derived *Sal1*^{+/EGFP} and *Sal1*^{+/+} chimeric mice grew into adulthood (Figure 3, A and B). Moreover, ESC-derived *Sal1*^{+/EGFP} chimeric mice and *Sal1*^{+/+} chimeric mice were fertile (Figure 3C). An ESC-marking gene,

Figure 2. Flow cytometry analysis and genotyping in ESC-derived brains and kidneys. In *Sal1*^{+/EGFP} mice without ESC-chimerism, the brain and kidneys (**A** and **B**, respectively) contained EGFP-expressing and DsRed-negative cells. Moreover, the kidneys contained a few EGFP-expressing and DsRed-expressing cells that showed autofluorescence. In *Sal1*^{+/+} chimeric mice, the brain and kidneys (**C** and **D**, respectively) contained EGFP-negative & DsRed-expressing cells that were derived from ESCs. In *Sal1*^{+/EGFP} chimeric mice, the brain and kidneys (**E** and **F**, respectively) contained both EGFP-expressing and DsRed-negative host cells and EGFP-negative and DsRed-expressing ESC-derived cells. **G:** In *Sal1*^{EGFP/EGFP} chimeric mice, the brain contained both EGFP-expressing and DsRed-negative host cells and EGFP-negative and DsRed-expressing ESC-derived cells, but not EGFP-expressing and DsRed-negative cells. **H:** Kidneys contained EGFP-negative and DsRed-expressing ESC-derived cells, but not EGFP-expressing and DsRed-negative cells. **I:** Genotyping PCR was performed on each fraction of sorted cells, as shown by boxed areas in **G**.

Figure 3. ESC-derived *Sall1*^{+/EGFP} chimeric mice in adulthood. **A:** Kidney, muscle, and adipose tissue contained various contributions of ESC-derived cells. **B:** In ESC-derived chimeric kidneys fragmented with sieve mesh, some glomeruli and some tubules consisted of both ESC-derived cells and host-derived cells. **C:** ESC-derived *Sall1*^{+/EGFP} chimeric mice were fertile. An ESC-marking gene, DsRed, was transmitted to progeny whose organs all exhibited DsRed fluorescence (circle in left panel, square in right panel).

DsRed, was transmitted to progeny whose organs all exhibited DsRed fluorescence. Figure 4 shows representative sections (H&E and PAM staining) of ESC-derived kidneys from pups of complemented *Sall1*^{EGFP/EGFP} hosts. The glomerular capillary loops and basement membrane showed normal development, with blood cells in the glomerular capillary lumina (Figure 4, B and C). The glomerular podocytes and epithelium of Bowman's capsule were distinct, with open periglomerular urinary spaces (consistent with urine production). That is, the kidneys of these mice were grossly and histologically normal. Moreover, we examined these kidney tissues further for contributions of donor ESCs to different kidney lineages. On immunohistochemical study of kidneys of pups developed from embryos complemented with *Sall1*^{EGFP/EGFP} blastomeres, the nephron epithelia (including the condensed mesenchyme, pre-tubular aggregate, comma-shaped bodies, s-shaped bodies, glomerular podocytes, Bowman's epithelia, proximal tubules, and distal tubules) and the renal stroma (including the cortical stroma and medullary stroma) consisted entirely of DsRed-positive cells (podocytes: Figure 4F; proximal tubules: Figure 4G; other data not shown). By contrast, both DsRed-positive cells and DsRed-negative cells were seen in collecting ducts (Figure 4H). Therefore, the tissues seen in this case were of ESC origin, with the exception of those in collecting-tubule epithelia. These data strongly indicate that we could generate ESC-derived kidneys by blastocyst complementation.

Generation of Kidney from iPSCs

We next addressed whether we could generate kidney from iPSCs instead of from ESCs. We used blastocysts derived from another *Sall1* knock-out mice, in which *EGFP* was not inserted, as injected iPSCs were marked with EGFP (Figure 1A). As expected, on macroscopy the kidneys of *Sall1*^{+/-} or *Sall1*^{+/+} chimeric mice were a mixture of host-derived cells and EGFP-marked iPSC-derived cells, as in whole-body chimerism (*Sall1*^{+/-} in Figure 5A). In contrast, the kidneys of *Sall1*^{-/-} chimeric mice were almost entirely composed of EGFP-marked iPSC-derived cells (*Sall1*^{-/-} in Figure 5, A and B). These iPSC-derived kidneys of *Sall1*^{-/-} chimeric mice were grossly normal in shape and size. As iPSC-derived kidneys had urine-filled bladders, those kidneys were inferred to communicate with ureters and to function well. In previous reports, the presence of bladders full of urine was shown to reflect functionality of the renal and urological systems in newborn mice.^{6,12} Although we expect that the produced

Figure 4. Histology and immunofluorescence of ESC-derived kidneys at the neonatal stage. **A:** Normal morphology in sections of kidney from *Sall1*^{EGFP/EGFP} chimera. Mature glomeruli (G) were found. **B** and **C:** Glomeruli were normal in appearance in the *Sall1*^{EGFP/EGFP} chimeric kidney. **D** and **E:** Glomerular features were normal in a wild-type 129/Ola strain from which ESCs had been established. H&E staining: **A**, **B**, and **D**; PAM staining: **C**, **E**, and **F-H**. Sections were stained with various lectins and anti-DsRed antibody; DAPI was used for nuclear counterstaining. **F:** PNA-lectin-positive glomerular podocytes were DsRed positive and EGFP negative. **G:** LT-lectin-positive proximal tubular epithelium was DsRed positive and EGFP negative. Most of the LT-lectin-negative tubular epithelia, including that of the distal tubules, were also DsRed-positive and EGFP-negative. **H:** DBA-lectin-positive collecting tubule epithelia were composed of both DsRed-positive ESC-derived cells and DsRed-negative host cells. Scale bar = 50 μ m.

Figure 5. iPSC-derived kidneys generated by blastocyst complementation. **A:** iPSC-derived kidneys at the neonatal stage. In *Sal1*^{-/-} mice complemented with iPSCs, the kidneys were normal in gross appearance and almost entirely positive for EGFP, indicating that they were of iPSC origin (**left panels**). The bladders of these mice were filled with urine. The kidneys in *Sal1*^{+/-} mice complemented with iPSCs were only partially positive for EGFP, indicating the presence of a mosaic of both iPSC-derived and host-derived cells (**middle panels**). The absence of kidneys is shown in a non-chimeric *Sal1*^{-/-} mouse at the same developmental stage (**right panels**). **B:** A representative kidney is almost entirely composed of EGFP-expressing cells. All of the non-kidney organs were mosaics of cells exhibiting fluorescence and of cells failing to fluoresce. **C:** Detection of *Sal1* mutant genotyping using genomic DNA extracted from colony-forming cells derived after single-cell sorting of EGFP-KSL cells from neonatal or postnatal bone marrow. **D:** Results of embryo manipulation and genotyping of *Sal1* mutant. **E:** In one *Sal1*^{-/-} mouse complemented with iPSCs, the left kidney was hydronephrotic.

urine in newborns is more dilute than that in adults, we were unable to measure its osmolarity because of technical problems. As expected, in both *Sal1*^{-/-} and *Sal1*^{+/-} mice injected with EGFP-iPSCs, iPSC-derived cells contributed to all of the non-kidney tissues of the body, including adrenal gland, ureter, bladder, muscle, and adipose tissue (data not shown). The extent of the contribution varied from tissue to tissue and also from mouse to mouse depending on the individual degree of chimerism. Single HSC colony assays yielded material for genotyping PCR (Figure 5C). Retrospective genotyping revealed that mice with *Sal1*^{+/+}, *Sal1*^{+/-}, and *Sal1*^{-/-} genotypes were born with Mendelian frequency (Figure 5D). In all *Sal1*^{-/-} mice examined, regardless of donor chimerism in other tissues such as peripheral blood (range, 29.1% to 92%), kidneys formed bilaterally. In one mouse, one iPSC-derived kidney was normal and the other was hydronephrotic (Figure 5E). Whether this outcome was due to incomplete iPSC complementation or to other causes (eg, abnormally functioning iPSC-derived cells, *Sal1*^{-/-} status) remains unclear. These gross findings suggest that iPSC-derived kidneys developed in *Sal1*^{-/-} mice as a result of blastocyst complementation.

Next, we assessed histological findings in iPSC-derived kidneys. Figure 6 illustrates representative sections (H&E and PAM staining) of the kidney in pups of complemented *Sal1*^{-/-} and *Sal1*^{+/-} host blastocysts. On light microscopy of tissues from complemented *Sal1*^{-/-} mice, like those from complemented *Sal1*^{+/-} mice, the architecture of renal cortex and medulla was normal (Figure 6A). On immunofluorescence microscopy at low magnification, almost all tissues excluding DB-lectin-positive ureteric buds were EGFP positive. For signal amplification, we assessed the fluorescence of a chromophore coupled with anti-EGFP antibody. The glomerular capillary loops and basement membrane had formed normally, with blood cells in the glomerular capillary lumina (Figure 6B). The glomerular corpuscles were distinct, with open periglomerular urinary spaces consistent with urine production. On ultrastructural study, the glomerular three-layer architecture in complemented *Sal1*^{-/-} mice was normal, with epithelial foot processes, basement membrane, and fenestrated capillary-loop endothelium (Figure 6C). At high magnification, slit diaphragms were seen (Figure 6D). The kidneys of these mice were grossly and histologically normal. These results clearly indicate that blastocyst complementation could generate both ESC- and iPSC-derived kidneys.

Polyclonal Origin of Single Glomerulus or Nephron in Mouse Chimeras

To buttress our conclusion that donor iPSCs contributed to different kidney lineages, we analyzed and compared immunohistochemical profiles. In this process, we incidentally confirmed a developmental phenomenon of chimeric mice. Immunohistochemical study of kidney in pups of *Sal1*^{+/-} or *Sal1*^{+/+} iPSC-derived chimeras found tissues of metanephric mesenchymal lineage, including glomerular podocytes in every glomerulus, to contain both EGFP-positive cells and EGFP-negative cells (Figure 6A, Figure 7A–D). Throughout nephrogenesis, the nephron progenitors and epithelia of metanephric mesenchymal lineage (ie, including the condensed mesenchyme, pretubular aggregate, comma-shaped bodies, s-shaped bodies, mature glomeruli, proximal and distal tubules) were similar. Among glomeruli, 28 had mixed EGFP-positive and EGFP-negative podocytes, two had only EGFP-positive podocytes, and one had only EGFP-negative podocytes. Moreover, other metanephric mesenchymal components (including the epithelia of Bowman's capsule and of the proximal and distal tubules) also showed a mixture of EGFP-expressing and non-EGFP-expressing cells (Figure 7D). In summary, individual metanephric mesenchymal components were composed of cells of polyclonal origin in mouse chimeras.

Immunohistochemical Analysis of iPSC-Derived Kidneys

We examined these kidney tissues further for contributions of donor iPSCs to different cell lineages. On immunohistochemical study of kidneys in pups from com-

Figure 6. Histology of iPSC-derived kidneys at the neonatal stage. **A:** Normal morphology in sections of kidney from *Sall1*^{-/-} chimera. Distribution of iPSC-derived cells in sections of iPSC-derived kidney from *Sall1*^{-/-} and *Sall1*^{+/-} hosts. **Upper panels:** Paraffin sections stained with H&E. **Lower panels:** Frozen sections stained for DBA-lectin and EGFP. Most of the nephron epithelia were of iPSC origin, but this was not the case for the DBA-lectin-positive ureteric bud epithelia. The cortical stroma was of iPSC origin (**asterisk**). Scale bar = 100 μ m. **B:** Glomeruli were normal in appearance in *Sall1*^{-/-} and *Sall1*^{+/-} chimeric kidneys. Sections were stained with H&E or with PAM technique. Scale bar = 50 μ m. **C:** Transmission electron micrograph of glomerulus in iPSC-derived kidney. The triple-layer architecture of the glomerular capillary in complemented *Sall1*^{-/-} mice was normal, showing epithelial foot processes, basement membrane, and fenestrated capillary-loop endothelium. Original magnification, $\times 6000$. **D:** High-power view of **boxed region** in **C**. Foot processes and slit diaphragms (**arrows**) exhibit normal development. Original magnification, $\times 20,000$.

plemented *Sall1*^{-/-} host blastocysts, the cells of metanephric mesenchymal lineage (including the condensed mesenchyme, pretubular aggregate, comma-shaped bodies, s-shaped bodies, mature glomeruli, proximal and distal tubules) were of iPSC origin, with the exception of the collecting duct epithelium (Figure 6A, Figure 7E–G). Most of the renal stroma (including the cortical stroma, medullary stroma, and kidney capsule) was of iPSC origin (Figure 6A, Figure 8A–C). This was to be

expected, as the collecting ducts and ureters are originally derived from the ureteric bud; that is, development of these structures did not occur under the direct influence of *Sall1*.

The cortical stroma (A), in which nephron epithelia lie, medullary stroma (B), and Liv2-expressing kidney capsule (C) were replaced by EGFP-positive iPSC-derived cells. With respect to vascular systems, all of the endothelia of vascular elements (including the arteries, arterioles, glomerular endothelia, peritubular capillaries, and venules)

Figure 7. Immunofluorescence-microscopy studies of tissues of metanephric mesenchymal lineages in iPSC-derived kidneys at the neonatal stage. Polyclonal origin of a single glomerulus or tubule in mouse chimeras (using iPSC-derived kidney of *Sall1*^{+/-} chimeras). **A and B:** Immunofluorescence in the iPSC-derived kidney of *Sall1*^{+/-} chimeras. Metanephric mesenchymal lineages including condensed mesenchyme (**dashed line** in **A**), and comma-shaped bodies (**dashed line** in **B**) were mosaics of both iPSC- and host-derived cells. **C:** In the s-shaped body stage of the *Sall1*^{+/-} chimeric kidney, a glomerular crevice with Pecam1-positive endothelial cells was seen. Presumptive glomerular podocytes (**white arrow**), Bowman's epithelia (**yellow arrow**), and the upper portion of the s-shaped body (**arrowhead**) were mosaics of both iPSC- and host-derived cells. **D:** In the mature glomerulus of *Sall1*^{+/-} chimeric kidney, glomerular podocytes were hybrids of both EGFP-positive cells (**arrowheads**) and EGFP-negative cells (**arrows**). Scale bar = 50 μ m. Immunofluorescence, iPSC-complemented *Sall1*^{-/-} kidney (**E–G**). **E:** PNA-lectin-positive glomerular podocytes were totally composed of EGFP-positive iPSCs. **F:** LT-lectin-positive proximal tubular epithelia were of iPSC origin. **G:** In the medulla of the iPSC-complemented *Sall1*^{-/-} kidney, tubular epithelia of Aqp1-positive thin limbs of loops of Henle were of iPSC origin. Scale bars: 50 μ m (**A–D**); 25 μ m (**E**); 100 μ m (**F** and **G**).

Figure 8. Immunofluorescence-microscopy studies of stromal components and vasculature in iPSC-complemented *Sall1*^{-/-} kidneys at the neonatal stage. **A:** Most of the cortical stroma (asterisk) in which nephron epithelia lie was replaced by EGFP-positive iPSC-derived cells. **B:** The medullary stroma (asterisk) was of iPSC origin. **C:** Liv2-expressing kidney capsule, a layer of epithelial cells (arrows) cloaking the cortical surface, was of iPSC origin. **D:** Tuj1-expressing nerves around vessels in iPSC-derived kidney were not composed of iPSCs. **E:** Pecam1-expressing endothelium of glomerulus (arrow) and of interlobular artery (arrowhead) was chimeric, as shown by EGFP fluorescence. **F:** The Pecam1-expressing endothelium of segmental renal artery (A) and vein (V) was chimeric for EGFP. **G:** Pecam1-expressing endothelium of glomerulus, arteriole (white arrowhead), and peritubular capillary (white arrows) was chimeric for EGFP. Glomerular podocytes (yellow arrows) and Bowman's capsule epithelium (yellow arrowheads) were composed of EGFP-positive iPSC-derived cells. The glomerular mesangium (asterisk) was also of iPSC origin. **H:** In a section parallel to material shown in **E**, glomerular mesangium (asterisk) was of iPSC origin. Leiomyocytes (arrows) of interlobular artery were chimeric for EGFP. Scale bar = 50 μ m.

showed chimeric organization (Figure 8E–H). The glomerular mesangium, but not the leiomyocytes of vessel walls, was of iPSC origin (Figure 8, G and H). In addition, nearly all juxtavascular or perivascular nerves were not of iPSC origin (Figure 8D). These results indicate that in all mice, although

iPSCs supplied all kidney cell lineages, collecting duct epithelia and kidney stromal elements such as vessels and nerves were composites of host- and iPSC-derived cells.

Discussion

We have taken an innovative approach, that of blastocyst complementation, to organ regeneration and have demonstrated that this technique can be used to generate donor PSCs–derived functional kidney. If an empty developmental niche for an organ is provided (as with the *Pdx1*^{-/-} mouse and the pancreatic niche⁵), PSCs-derived cellular progeny can occupy that niche and developmentally produce a solid organ in the vacant space. Several groups have used the same technique to study development of thymic epithelia¹³ and compensation for cardiac defects,¹⁴ to determine clonal origins in yolk sac hematopoiesis and germ cell development,^{15,16} or to define developmental organ sizes of liver and pancreas.¹⁷ Here, we applied this technique to the generation of kidneys.

Our present findings demonstrated that epithelial cellular lineages originating from the metanephric mesenchyme were entirely replaced by the progeny of injected PSCs in *Sall1*^{-/-} chimeric mice (Figure 4, Figure 7). *Sall1* is expressed in the metanephric mesenchyme–derived structures in the developing kidney, and is essential not only for the initial interactions between the mesenchyme and the ureteric buds but also for the growth and development of the metanephric mesenchyme after those interactions. Indeed, *in vitro* assays indicated that the *Sall1*-expressing cell population in the metanephric mesenchyme contains multipotent nephron progenitors, and that colony formation after plating was impaired in EGFP-positive cells from *Sall1*^{EGFP/EGFP} mouse kidneys.¹⁸ During nephrogenesis in the setting of blastocyst complementation, host embryo-derived cells in *Sall1*-deficient mice could not compete with injected normal ESC- or iPSC-derived cells. In addition, all renal stromal elements (ie, cortical stroma, medullary stroma, and kidney capsule) were composed of PSC-derived cells, suggesting that these tissues' development also depends on *Sall1* expression. It is worth noting that glomerular mesangial cells, but not leiomyocytes of vessel walls, were entirely composed of PSC-derived cells, although the cellular phenotype of mesangial cells reportedly resembles that of vascular leiomyocytes.¹⁹ Our data therefore strongly indicate that *Sall1* exerts cell-autonomous functions in various lineages, including that of the metanephric mesenchyme, and that *Sall1* mutant mice could be useful as harboring a developmental niche in which to regenerate the major parts of the kidney by blastocyst complementation.

We consider that the mechanism for the rescue of tissues of renal stromal lineage including the renal capsule was related to cell-autonomous function during organ development. *Sall1* expression is reported not only in metanephric mesenchyme but also in *Foxd1*-expressing stromal components, although in the latter *Sall1* expression is relatively weak.¹⁸ Our finding that the stroma is

also replaced by PSC descendants may suggest that *Sal1* is also required for this lineage cell-autonomously, although lineage-specific *Sal1* deletion would be needed to test this hypothesis.

By contrast, collecting ducts derived from ureteric buds, microvascular endothelial cells including the glomerular endothelia, and leiomyocytes were not entirely composed of PSC-derived cells (Figure 8). These findings are consistent with the fact that *Sal1* is not expressed in the renal lineages mentioned above at kidney development. Therefore mutant mice lacking these lineages should be combined with *Sal1* knock-out mice if kidneys wholly composed of PSC descendants are to be generated.

In this study, none of the mouse pups with complemented kidneys survived to adulthood. Some chimeric mouse models with unexpectedly lethal phenotypes have been reported.^{20,21} This suggests that chimerism does not always rescue mice from embryonic lethality, perhaps partly due to the insufficient multipotency of ESCs or to low proportional chimerism. *Sal1*^{-/-} mice not only exhibited renal agenesis but also nursed poorly, as inferred from the absence of intragastric milk visible through the abdominal wall.⁶ This was also true for blastocyst-complemented *Sal1*^{-/-} pups. Because *Sal1* is expressed in the brain, it may mediate the development of nerve pathways necessary for suckling function. Four *Sal* family genes were previously identified, *Sal1* to *Sal4*, and were demonstrated to act synergistically as transcription factors.^{22,23} Therefore, each *Sal* family is possible to resemble in cellular function, and the phenotypes of the mutant mice may be similar each other. Among them, *Sal1* expression co-localizes with other *Sal* family expression, at sites such as the brain, kidney, limb buds, and heart. Indeed, *Sal3* mutant mice show anatomical defects in the brain,²⁴ permitting the inference that *Sal1*-deficient mice, without such defects, may have neurological functional abnormalities. Moreover, suckling ability can be adversely affected by other causes, including seeking the nipple, recognizing the nipple, orally grasping the nipple, sucking, and transport of oral contents to the back of the mouth, with swallowing. As expected, in PSC-injected *Sal1*^{-/-} mice, both PSC-derived cells and host cells contributed to all non-kidney tissues. Therefore, neuromuscular and endocrine organs necessary for proper suckling were composites of both cell types, and physiological function may have been imperfectly recovered in these organs despite PSC-derived complementation. This perhaps illustrates an important aspect of blastocyst complementation, in that exogenous PSC-derived cells may not compensate for cell-intrinsic developmental defects that are not occasioned by cell loss. Alternatively, chimerism achieved by exogenous cells may not be sufficient to compensate fully for *Sal1* deficiency at some sites, and functional defects caused by *Sal1* deficiency may persist. Because of the lack of progression to adulthood, it was not possible to observe functioning of PSC-derived kidneys in adult mice. However, the observation of urine accumulation in the bladders of neonatal *Sal1*^{-/-} mice derived from iPSC-complemented

blastocysts strongly suggests that iPSC-derived kidneys were functional.

Previously we had successfully generated rat pancreas in mouse by injecting rat iPSCs into *Pdx1*^{-/-} mouse embryos.⁵ In this study, we also injected rat iPSCs into *Sal1*^{-/-} mouse blastocysts; however, to date, we have not been able to generate rat kidneys in mice (unpublished observations). If the key molecules in mice involved in the interactions of the mesenchyme and the ureteric buds do not cross-react with those in rats, to vault this hurdle it would be necessary to generate a host mouse strain lacking all of the lineages that contribute to the kidney.

In summary, we successfully generated kidneys from PSCs via blastocyst complementation. This accomplishment may open doors to insights into the cellular and molecular mechanisms of kidney development.

Acknowledgments

We thank Satoshi Yamazaki, Sanae Hamanaka, Yuji Yamazaki, Yohei Morita, Hiroko Tsukui, and Tsutomu Kohda for their technical advice and Lin Franklin for critical review of the manuscript.

References

1. Summit SCotl: Organ trafficking and transplant tourism and commercialism: the Declaration of Istanbul. *Lancet* 2008, 372:5–6
2. Takahashi K, Yamanaka S: Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006, 126:663–676
3. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S: Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007, 131:861–872
4. Chen J, Lansford R, Stewart V, Young F, Alt FW: RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development. *Proc Natl Acad Sci USA* 1993, 90:4528–4532
5. Kobayashi T, Yamaguchi T, Hamanaka S, Kato-Itoh M, Yamazaki Y, Iбата M, Sato H, Lee YS, Usui J, Knisely AS, Hirabayashi M, Nakauchi H: Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* 2010, 142:787–799
6. Nishinakamura R, Matsumoto Y, Nakao K, Nakamura K, Sato A, Copeland NG, Gilbert DJ, Jenkins NA, Scully S, Lacey DL, Katsuki M, Asashima M, Yokota T: Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. *Development* 2001, 128:3105–3115
7. Takasato M, Osafune K, Matsumoto Y, Kataoka Y, Yoshida N, Meguro H, Aburatani H, Asashima M, Nishinakamura R: Identification of kidney mesenchymal genes by a combination of microarray analysis and *Sal1*-GFP knockin mice. *Mech Dev* 2004, 121:547–557
8. Hooper M, Hardy K, Handyside A, Hunter S, Monk M: HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germLine colonization by cultured cells. *Nature* 1987, 326:292–295
9. Niwa H, Miyazaki J, Smith AG: Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 2000, 24:372–376
10. Okabe M, Otsu M, Ahn DH, Kobayashi T, Morita Y, Wakiyama Y, Onodera M, Eto K, Ema H, Nakauchi H: Definitive proof for direct reprogramming of hematopoietic cells to pluripotency. *Blood* 2009, 114:1764–1767
11. Nagy A, Gertszenstein M, Vintersten K, Behringer R: Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 2003
12. Hammes A, Guo JK, Lutsch G, Leheste JR, Landrock D, Ziegler U, Gubler MC, Schedl A: Two splice variants of the Wilms' tumor 1 gene

- have distinct functions during sex determination and nephron formation. *Cell* 2001, 106:319–329
13. Muller SM, Terszowski G, Blum C, Haller C, Anquez V, Kuschert S, Carmeliet P, Augustin HG, Rodewald HR: Gene targeting of VEGF-A in thymus epithelium disrupts thymus blood vessel architecture. *Proc Natl Acad Sci U S A* 2005, 102:10587–10592
14. Fraidtenraich D, Stillwell E, Romero E, Wilkes D, Manova K, Basson CT, Benezra R: Rescue of cardiac defects in *id* knockout embryos by injection of embryonic stem cells. *Science* 2004, 306:247–252
15. Ueno H, Weissman IL: Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. *Dev Cell* 2006, 11:519–533
16. Ueno H, Turnbull BB, Weissman IL: Two-step oligoclonal development of male germ cells. *Proc Natl Acad Sci USA* 2009, 106:175–180
17. Stanger BZ, Tanaka AJ, Melton DA: Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. *Nature* 2007, 445:886–891
18. Osafune K, Takasato M, Kispert A, Asashima M, Nishinakamura R: Identification of multipotent progenitors in the embryonic mouse kidney by a novel colony-forming assay. *Development* 2006, 133:151–161
19. Lindahl P, Hellstrom M, Kalen M, Karlsson L, Pekny M, Pekna M, Soriano P, Betsholtz C: Paracrine PDGF-B/PDGF-Rbeta signaling controls mesangial cell development in kidney glomeruli. *Development* 1998, 125:3313–3322
20. Scacheri PC, Crabtree JS, Novotny EA, Garrett-Beal L, Chen A, Edgemon KA, Marx SJ, Spiegel AM, Chandrasekharappa SC, Collins FS: Bidirectional transcriptional activity of PGK-neomycin and unexpected embryonic lethality in heterozygote chimeric knockout mice. *Genesis* 2001, 30:259–263
21. He Y, Hakvoort TB, Vermeulen JL, Lamers WH, Van Roon MA: Glutamine synthetase is essential in early mouse embryogenesis. *Dev Dynam* 2007, 236:1865–1875
22. Sakaki-Yumoto M, Kobayashi C, Sato A, Fujimura S, Matsumoto Y, Takasato M, Kodama T, Aburatani H, Asashima M, Yoshida N, Nishinakamura R: The murine homolog of SALL4, a causative gene in Okhiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with *Sall1* in anorectal, heart, brain and kidney development. *Development* 2006, 133:3005–3013
23. Kawakami Y, Uchiyama Y, Rodriguez Esteban C, Inenaga T, Koyano-Nakagawa N, Kawakami H, Marti M, Kmita M, Monaghan-Nichols P, Nishinakamura R, Izpisua Belmonte JC: *Sall* genes regulate region-specific morphogenesis in the mouse limb by modulating Hox activities. *Development* 2009, 136:585–594
24. Parrish M, Ott T, Lance-Jones C, Schuetz G, Schwaeger-Nickolenko A, Monaghan AP: Loss of the *Sall3* gene leads to palate deficiency, abnormalities in cranial nerves, and perinatal lethality. *Mol Cell Biol* 2004, 24:7102–7112