Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs

Hitomi Matsunari[a,b,c,1], Hiroshi Nagashima[a,b,c,1,2], Masahito Watanabe[b,c], Kazuhiro Umeyama[b,c], Kazuaki Nakano[c], Masaki Nagaya[b,c], Toshihiro Kobayashi[a], Tomoyuki Yamaguchi[a], Ryo Sumazaki[d], Leonard A. Herzenberg[e,2], and Hiromitsu Nakauchi[a,f,2]

[a]Japan Science and Technology Agency, Exploratory Research for Advanced Technology, Nakauchi Stem Cell and Organ Regeneration Project, Chiyoda-ku 102-0075, Japan; [b]Meiji University International Institute for Bio-Resource Research, Kawasaki 214-8571, Japan; [c]Laboratory of Developmental Engineering, Department of Life Sciences, School of Agriculture, Meiji University, Kawasaki 214-8571, Japan; [d]Department of Child Health, Faculty of Medicine, University of Tsukuba, Tsukuba 305-8575, Japan; [e]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305; and [f]Division of Stem Cell Therapy, Center for Stem Cell Biology and Medicine, Institute of Medical Science, University of Tokyo, Minato-ku 108-8639, Japan

Contributed by Leonard A. Herzenberg, January 8, 2013 (sent for review December 23, 2012)

In the field of regenerative medicine, one of the ultimate goals is to generate functioning organs from pluripotent cells, such as ES cells or induced pluripotent stem cells (PSCs). We have recently generated functional pancreas and kidney from PSCs in pancreatogenesis- or nephrogenesis-disabled mice, providing proof of principle for organogenesis from PSCs in an embryo unable to form a specific organ. Key when applying the principles of in vivo generation to human organs is compensation for an empty developmental niche in large nonrodent mammals. Here, we show that the blastocyst complementation system can be applied in the pig using somatic cell cloning technology. Transgenic approaches permitted generation of porcine somatic cell cloned embryos with an apancreatic phenotype. Complementation of these embryos with allogenic blastomeres then created functioning pancreata in the vacant niches. These results clearly indicate that a missing organ can be generated from exogenous cells when functionally normal pluripotent cells chimerize a cloned dysorganogenetic embryo. The feasibility of blastocyst complementation using cloned porcine embryos allows experimentation toward the in vivo generation of functional organs from xenogenic PSCs in large animals.

apancreatic pig | organ reconstitution | transplantation | somatic cell nuclear transfer | chimera

Rapid progress in stem cell science promises novel therapeutic approaches for a number of intractable diseases. In particular, recent developments in induced pluripotent stem cell (iPSC) technology has enabled reprogramming of somatic cells that will offer new ways to treat a myriad of diseases with regenerative medicine using individual patients' own cells. Although millions of patients suffer from end-stage organ failure that can be cured only by organ transplantation, treatment options are limited due to a shortage of donor organs. Therefore, generation of organs from stem cells is one of the ultimate goals of regenerative medicine. Current stem cell therapy, however, mainly targets diseases that can be treated by transplantation of cells of a single type [e.g., dopaminergic neurons for Parkinson disease (1), oligodendrocytes for spinal cord injury (1), pigment epithelial cells for retinal degenerative diseases (2)]. This is partly because generation of organs from iPSCs has been considered impractical due to difficulty in the replication in vitro of the complex interactions between cells and tissues during organogenesis.

We recently demonstrated that functional organs can be generated from pluripotent stem cells (PSCs) in vivo by blastocyst complementation in organogenesis-disabled mouse embryos (3, 4). To apply this principle in generating human organs, large nonrodent mammals must be used. This entails overcoming several difficulties, such as making organ-deficient large animals and supplying them in large numbers to collect embryos for blastocyst complementation procedures. More fundamental, however, is whether functional organs can be generated from exogenous PSCs in nonrodent large mammals. To address this, we used pigs, a species in which somatic cell nuclear transfer (SCNT) is feasible. We have shown that the level of *Hes1* (hairy and enhancer of split 1) expression is critical for development of the biliary system (5). Proceeding from the assumption that overexpression of *Hes1* under the promoter of *Pdx1* (pancreatic and duodenal homeobox 1) inhibits pancreatic development, we have generated *Pdx1* promoter-*Hes1* transgenic pigs with an apancreatic phenotype. Here, we demonstrate that as in rodent models, donor pluripotent cell complementation of cloned blastocysts that would otherwise give rise to apancreatic animals yields pigs with pancreata of normal configuration and function that survive to adulthood. Blastocyst complementation using cloned porcine embryos thus may permit use of a large animal for the generation of functional organs from xenogenic PSCs, including human iPSCs.

Results

Creation of Pancreatogenesis-Disabled Pigs by a Transgenic Approach. We introduced a *Pdx1-Hes1* transgene construct into in vitro matured pig oocytes by intracytoplasmic sperm injection (ICSI)-mediated gene transfer (6) and produced transgenic pig fetuses by embryo transfer (Fig. 1 and Table S1). Among the five transgenic fetuses obtained, the pancreatogenesis-disabled phenotype was observed in one male fetus (day 74) and one female fetus (day 80), each of which had a vestigial pancreas (Fig. 1*B* and Fig. S1). These vestigial pancreata consisted of loose connective tissue dotted with ductal structures and small islands of epithelial cells (Fig. 1*C*). No cells showed acinar differentiation; such cells were observed in the pancreas of a normal fetus (Fig. 1*C*) at the same developmental stage.

Reproduction of Pancreatogenesis-Disabled Pigs by Somatic Cell Cloning. We established primary cultures of fibroblast cells from the male fetus with a vestigial pancreas (Fig. 1 and Fig. S1) to use as nucleus donor cells for somatic cell cloning. Using SCNT from these *Pdx1-Hes1* transgenic cells, we produced cloned fetuses. Observations in five midterm (day 59) and four late-term (day 110) cloned fetuses confirmed that the pancreatogenesis-disabled phenotype in the original male transgenic fetus was reproduced in its clones (Fig. 1*D* and Table S2). These findings demonstrate that transgenic pigs expressing *Pdx1-Hes1* displayed a pancreatogenesis-disabled phenotype and that somatic cell cloning could faithfully reproduce this phenotype. In addition,

Author contributions: H. Nagashima and H. Nakauchi designed research; H.M., H. Nagashima, M.W., K.U., K.N., T.K., and T.Y. performed research; T.K., T.Y., and R.S. contributed new reagents/analytic tools; H.M., H. Nagashima, M.W., K.U., K.N., M.N., T.K., T.Y., L.A.H., and H. Nakauchi analyzed data; and H.M., H. Nagashima, L.A.H., and H. Nakauchi wrote the paper.

The authors declare no conflict of interest.

[1]H.M. and H. Nagashima contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: nakauchi@ims.u-tokyo.ac.jp, hnagas@isc.meiji.ac.jp, or lenherz@stanford.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1222902110/-/DCSupplemental.

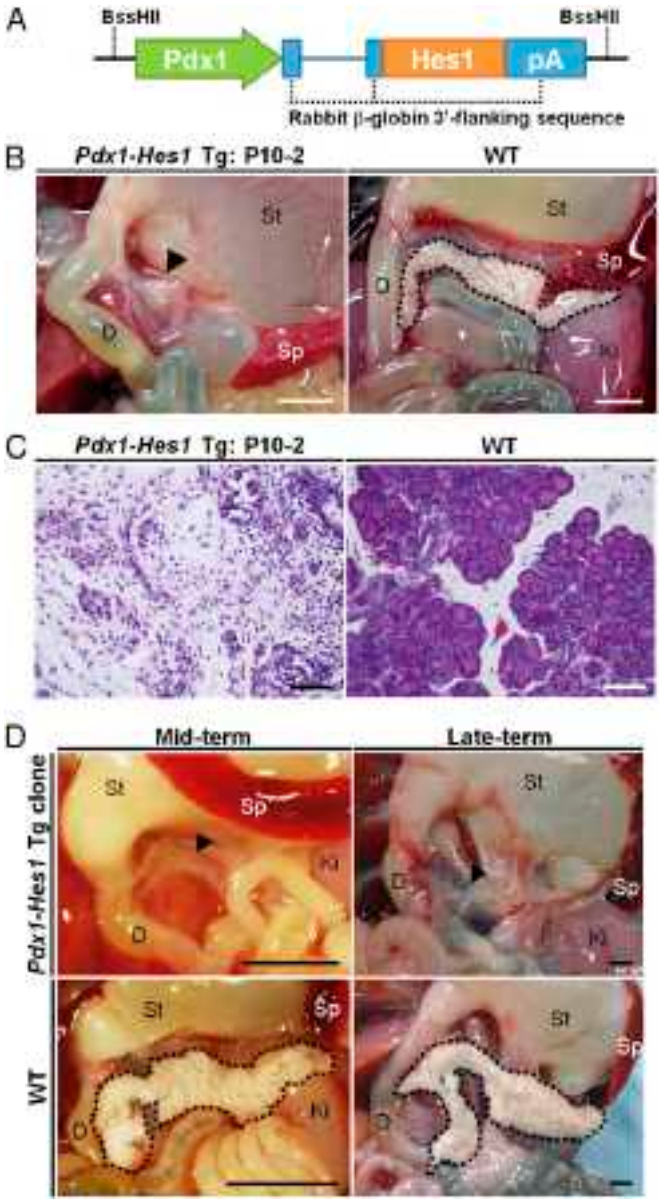


Fig. 1. Generation of pancreatogenesis-disabled pigs. (*A*) Construction of a *Pdx1-Hes1* expression vector consisting of the mouse *Pdx1* promoter, mouse *Hes1* cDNA, and rabbit β-globin 3′ flanking sequence, including the polyadenylation signal (pA). (*B* and *C*) Macroscopic and microscopic appearances of the vestigial pancreas (arrowhead) of a *Pdx1-Hes1* transgenic (Tg) male fetus and the pancreas of a WT fetus at the same gestational age. (*D*, *Upper*) Faithful reproduction of the pancreatogenesis-disabled phenotype of the *Pdx1-Hes1* Tg fetus in fetuses cloned from it. (*D*, *Lower*) Normal pancreata of age-matched WT fetuses. D, duodenum; Ki, kidney; Sp, spleen; St, stomach. (Scale bars: *B* and *D*, 5 mm; *C*, 50 μm.)

they hold out the prospect of large-scale production of such embryos via SCNT from *Pdx1-Hes1* transgenic fibroblasts.

Apancreatic Phenotype in *Pdx1-Hes1* Cloned Pigs Rescued by Blastocyst Complementation. Next, we investigated whether in pancreatogenesis-disabled pigs, as in rodents (3), blastocyst complementation could generate pancreata (Fig. 2). Using cloned embryos carrying *Pdx1-Hes1* (white coat color) as hosts and cloned embryos carrying the gene encoding orange fluorescent protein humanized Kusabira-Orange (*huKO*) as donors (7), we produced chimeric embryos (Table S3). Host embryos at the morula stage were injected with ∼10 blastomeres of morula-stage donor embryos (Fig. 3*A*). Chimeric blastocysts ($n = 96$) obtained after culture for 1 or 2 d were transferred to the uteri of two estrus-synchronized recipient gilts (Fig. 3*B*). We obtained 14 late-term fetuses (day 110 or 111) in which we assessed pancreas formation (Fig. 3 *C*–*E* and Fig. S2).

In this study, we complemented cloned embryos derived from the male pancreatogenesis-disabled fetus with blastomeres from female cloned embryos expressing huKO fluorescent protein (*huKO* transgenic embryos). We have confirmed that when male and female embryos are combined to produce a chimeric pig embryo, the chimera develops as a male (8). Fetuses with the host embryo's male sex that expressed donor cells' orange fluorescence were accordingly viewed as likely chimeric.

Of the 14 full-term fetuses, 5 male fetuses (35.7%) appeared chimeric because they systemically displayed orange fluorescence derived from donor cells (Fig. 3*C*, chimeras 1–3). Chimerism was substantiated by detection of *Pdx1-Hes1* and *huKO* sequences on PCR analysis of fetal genomic DNA. The remaining 9 fetuses, 5 male fetuses derived from *Pdx1-Hes1* cells and expressing the pancreatogenesis-disabled phenotype (Fig. 3*C*, Hes1) and 4 female fetuses derived from *huKO* cells and expressing orange fluorescence (Fig. 3*C*, huKO), were not chimeric, as substantiated by PCR analysis.

Formation of morphologically normal pancreata in the chimeric fetuses was confirmed by necropsy (Fig. 3*D*). Orange fluorescence of the donor cells was evident throughout the generated pancreata (Fig. 3*D*). The weights of the generated pancreata were 0.056–0.102% (average, 0.082%) of respective body weights, values like those for normal pancreata and cloned fetuses at the same gestational age (0.081–0.101%; average, 0.085%) (Table S4). The generated pancreatic tissue was densely cellular; acini had developed, and acinar cells were replete with zymogen granules. Additionally, many islets of Langerhans ("islets") were scattered throughout the pancreas, as in normal pigs. We concluded that the generated pancreata were histologically and cytologically normal (Fig. 3*E*). Almost all pancreatic tissues, including islets, acinar tissue, and ducts, stained with anti-huKO antibody (Fig. 3*E*). This result indicates that almost all pancreatic tissues of the chimeric fetuses were huKO-expressing cells derived from donor cells. Orange fluorescence was observed in skin over the whole body and in every internal organ of these fetuses, indicating that chimerism was present throughout the entire body (Fig. S2*A*). Immunostaining of skin, lung, and kidney of four chimeric fetuses revealed that ∼40–60% of each sample by area expressed huKO (Fig. S2*B*).

Chimeric Pigs Generated by Complementation of *Pdx1-Hes1* Embryos Have Functionally Normal Pancreata and Grow into Fertile Adults. To assess whether these chimeric pigs could survive until term, be born alive, and grow normally after birth, we again used *Pdx1-Hes1* clone embryos as hosts and *huKO* clone embryos as donors (Fig. 2). In addition, we used cloned embryos derived from a colored-coat WT Duroc × Berkshire hybrid sow as donors. Blastocyst complementation with either sort of donor gave rise to viable chimeric piglets (Fig. 4 and Tables S5 and S6). Production efficiencies for chimeric piglets were 4 (30.8%) of 13 with *huKO* and 1 (16.7%) of 6 with hybrid donor embryos. Blastocyst complementation also produced nonchimeric male cloned piglets derived from *Pdx1-Hes1* host embryos and nonchimeric female clones derived from donor embryos (Fig. 4*A*); these were farrowed in the same litters as the chimeric offspring (Fig. 4 *A* and *B* and Tables S5 and S6).

Two of the 10 nonchimeric *Pdx1-Hes1* cloned piglets produced were stillborn. These animals were confirmed at necropsy to be pancreatogenesis-disabled with vestigial pancreata, as observed in full-term *Pdx1-Hes1* cloned fetuses (Fig. 4*C*, *Upper*). Two of the 8 surviving newborn piglets had undetectably low serum insulin concentrations. Their serum glucose concentrations were 12 and 33 mg/dL; these levels suggest a hypoglycemic condition common in newborn pigs (9).

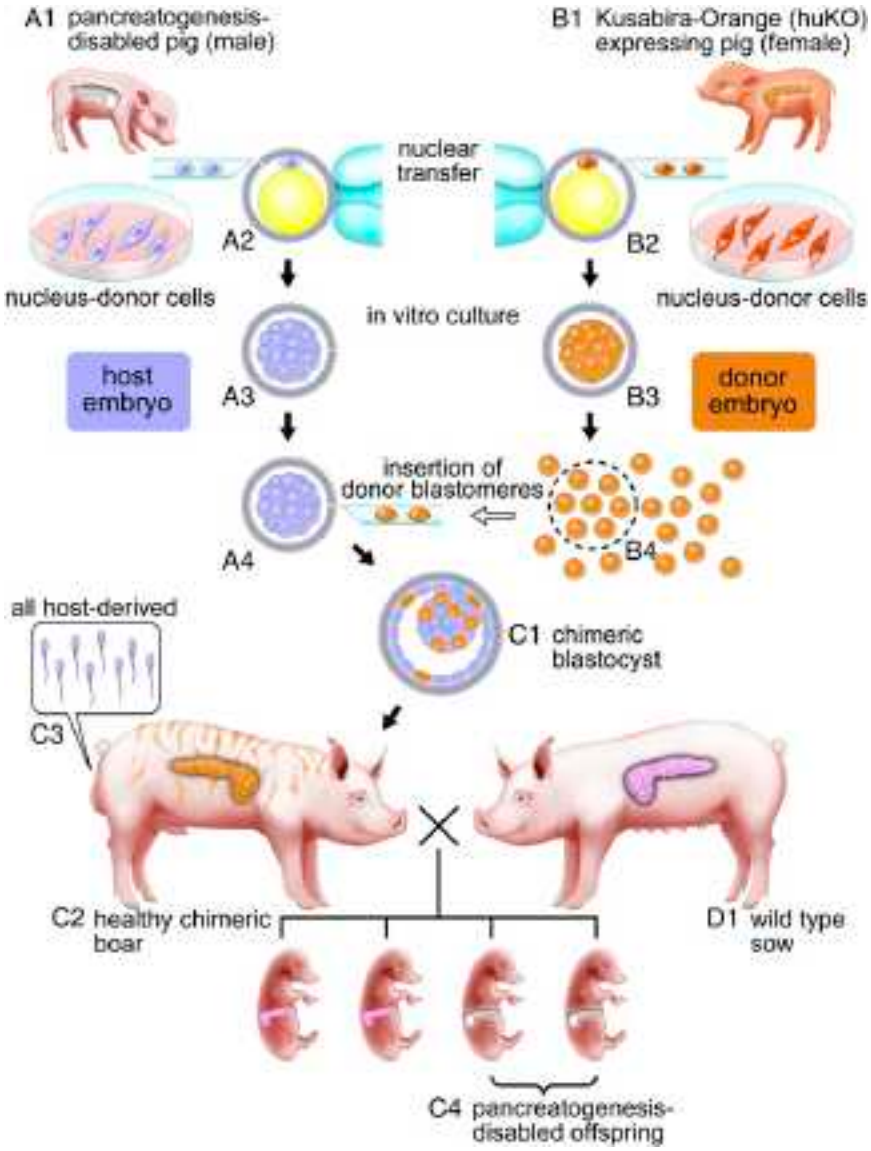


Fig. 2. Schematic representation of complementation for *Pdx1-Hes1* cloned pig embryos with a pancreatogenesis-disabled phenotype using cloned embryos expressing *huKO*. Primary fibroblast cells as nucleus donor cells for somatic cell cloning were established from a pancreatogenesis-disabled cloned pig with *Pdx1-Hes1* transgene expression (*A1*) and a cloned pig with systemic orange fluorescence conferred by *huKO* transgene expression (*B1*). (*A2–A4*) Host embryos reconstructed by nuclear transfer from male *Pdx1-Hes1* transgenic cells yielded pancreatogenesis-disabled piglets. (*B2* and *B3*) Donor embryos were reconstructed by nuclear transfer from female cells expressing *huKO*. Blastomeres isolated from donor embryos at the morula stage (*B4*) were inserted into host embryo morulae (*A4*) to produce chimeric blastocysts (*C1*) and pigs (*C2*). (*C2*) All the chimeric pigs obtained developed into fertile males as a result of intersex chimerism between the male host embryos and female donor embryos. (*C3*) Sperm of the chimeric boars theoretically originate from male host embryos carrying the *Pdx1-Hes1* transgene. After mating of chimeric boars with WT sows (*D1*), the pancreatogenesis-disabled phenotype of the *Pdx1-Hes1* host embryos was transmitted to the next generation (*C4*).

These piglets were killed after blood collection. Of the six other piglets, four died within 24 h but two survived for 3 d with serum glucose concentrations >600 mg/dL. These piglets also had undetectably low serum insulin concentrations. Their body weights fell drastically, and clinical-biochemistry findings strongly suggested severe catabolism (Table S7). The pancreatogenesis-disabled status of all *Pdx1-Hes1* cloned piglets in the litter was confirmed at necropsy.

In contrast to the pancreatogenesis-disabled piglets, the chimeric piglets developed normally (Fig. 4 *B* and *D*). As shown in Fig. 4*E*, every chimeric pig had normal serum glucose concentrations at the ages of 4, 6, and 12 mo. Clinical-biochemistry findings at the ages of 6 and 12 mo supplied no evidence for pancreatic, hepatobiliary, or renal injury, and serum electrolyte concentrations were all normal (Table S8). Oral glucose tolerance test results in one of the chimeric pigs were also normal (Fig. 4*F*).

At necropsy of a healthy-appearing 12-mo-old chimeric boar killed to examine its intestinal organs, macroscopic examination found no abnormalities. The entire pancreas distinctly fluoresced orange (Fig. 4*G*). The pancreas (Fig. 4*H*), the duodenum, and the extrahepatic biliary tract lacked any histopathological abnormalities. On immunostaining for huKO, nearly every pancreatic cell marked for huKO (Fig. 4 *H* and *I*), demonstrating that these cells were derived from donor embryos, as seen in the chimeric fetuses. All 19 organs and tissues examined, including testes and brain, contained huKO-expressing cells.

The five chimeric piglets farrowed by three recipient sows all grew normally (except for one, which died accidentally at weaning)

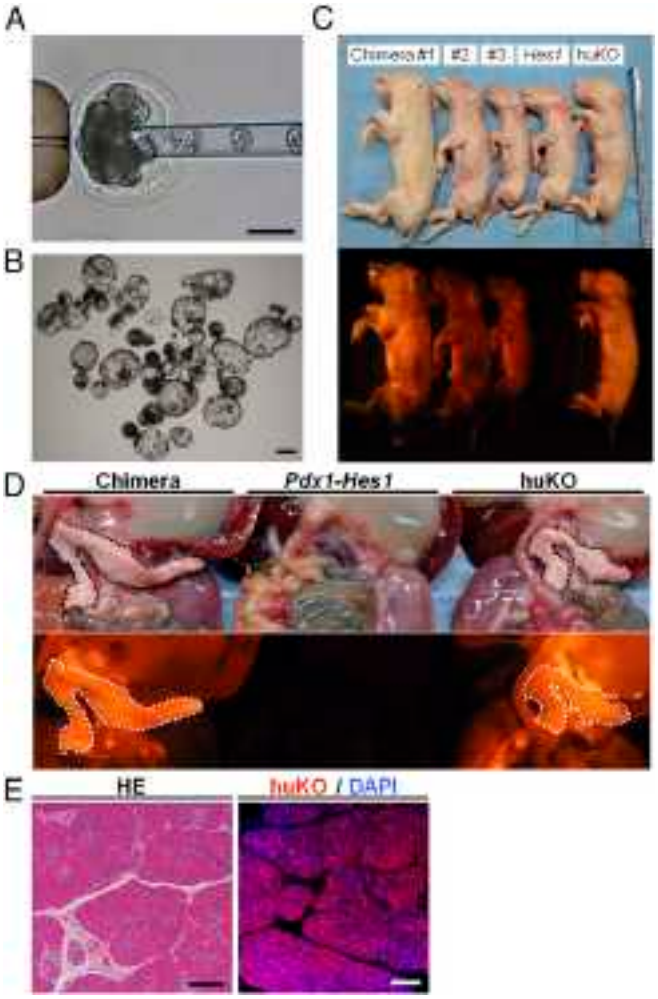


Fig. 3. Construction of chimeric embryos and fetuses from *Pdx1-Hes1* cloned and *huKO* cloned embryos. (*A*) Cloned embryo derived from the *Pdx1-Hes1* transgenic fetus via microinjection with donor morula blastomeres. (*B*) Chimeric blastocysts. (*C*) Full-term chimeric fetuses (chimeras 1–3) and sibling nonchimeric cloned fetuses derived from host (*Hes1*) or donor (*huKO*) embryos. Note that nonchimeric fetuses derived from host embryos (*Hes1*) showed no fluorescence. (*D*, *Center*) Fetuses derived from host embryos were pancreatogenesis-disabled. Pancreata of the chimeric fetuses (*Left*) appeared normal and brightly fluoresced orange throughout, as did pancreata of *huKO* clone fetuses (*Right*), indicating that pancreata of the chimeric fetuses were generated from donor embryo cells. (*E*) Almost all pancreatic tissue of chimeric fetuses stained with anti-huKO antibody. HE, H&E. (Scale bars: *A*, *B*, and *E*, 100 μm.)

and reached sexual maturity ~7–8 mo after birth. The sexually mature chimeric boars all could mate with WT sows in estrus, and all proved fertile. By examining the fetuses sired by the chimeric boars, we confirmed that the pancreatogenesis-disabled phenotype had been transmitted to their progeny (Fig. 2 and Fig. S3). Furthermore, the chimeric boars sired no fetuses derived from donor embryos (i.e., expressing *huKO*, with colored coats). Together, these results indicate that sperm of the chimeric boars were derived from host embryos (i.e., male *Pdx1-Hes1* clone embryos). They are consistent with earlier failure of intersexual chimeras produced with male and female embryos to generate sperm from cells with an XX chromosomal complement in pigs (8) and mice (10, 11).

Discussion

In this study, we demonstrated that generation of organ-deficient livestock animals is possible and that in pigs, as in rodents, a functional organ derived from exogenic pluripotent cells can be formed when organogenesis-disabled embryos are complemented by allogenic blastomeres. The pancreata derived from exogenic pluripotent cells were normal in their configuration and functions. Thus, the results clearly indicated that the principle we established in rodents also holds in large animals: If an empty developmental niche for an organ is provided, PSC-derived cellular progeny can occupy that niche, producing the appropriate organ in the vacant space. Although blastocyst complementation has been used to study development of a number of tissues and organs (3, 4, 12–14), all these studies were conducted in rodents. In this study, we demonstrate generation of a functional organ from exogenic pluripotent cells in a large animal, which is a very important step toward generation of human organs in large animals.

The chimeric pigs thus generated survived to adulthood, providing a source of sperm for large-scale expansion and production of pancreatogenesis-disabled embryos. Similarly, fibroblasts obtained from the apancreatic pig fetuses can also serve as a source of cloned embryos harboring a pancreatogenesis-disabled phenotype for complementation. SCNT technology thus has resolved initial difficulties associated with the use of large animals, such as pigs, allowing in vitro generation of viable host embryos for blastocyst complementation. It enables, in theory, limitless production of pancreatogenesis-disabled cloned embryos from cultured cells.

Now that pancreatogenesis-disabled pig embryos are available, one can investigate whether pancreata can be formed from xenogenic PSCs in the pig environment. Most nonrodent PSCs are, however, at the epiblast stage and cannot contribute to chimera formation (15). Our blastocyst complementation system thus may not work with currently available nonrodent PSCs, and it may require establishment of nonrodent PSCs with chimera-forming capability. Embryos of organogenesis-disabled pigs generated via SCNT-mediated cloning will, however, provide a simple but reliable platform for testing the potential of nonrodent PSCs for chimera formation and organ generation. Successful establishment of xenogenic organogenesis systems (cow into pig or monkey into pig) will certainly contribute to obtaining the knowledge necessary to apply this technology to humans.

Efforts to produce pancreata by complementing pig embryos with human PSCs will raise ethical questions. Generation of human germ cells in chimeras generated by xenogenic blastocyst complementation may not be acceptable, and measures to prevent human PSCs from contributing to reproductive cells in chimeric animals may be in order. Our data and those of others (8, 10, 11) suggest that formation of male germ cells from exogenic cells is suppressed when a male host embryo is complemented with female donor cells. Cell fate control (16, 17) also may permit suppression of formation of "unwanted" cells in animals generated by blastocyst complementation using human PSCs.

Cost could be another issue that needs to be considered. In the case of the pancreas, however, once a large amount of sperm is obtained from the complemented transgenic male pigs, generation of pancreatogenesis-disabled embryos is not expensive. As shown in our rodent study (3), autologous islets engrafted well without immunosuppression. This type of approach will eventually reduce medical costs and increase quality of life for those with end-stage organ failure.

These important considerations aside, the results demonstrated in this study provide the basis for potential future application of blastocyst complementation to generate transplantable human organs using livestock animals. The greatest technical challenge will be overcoming the species barrier to achieve chimerism with pig embryos as hosts.

Materials and Methods

Animal Care. All animal experiments were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC07-0005).

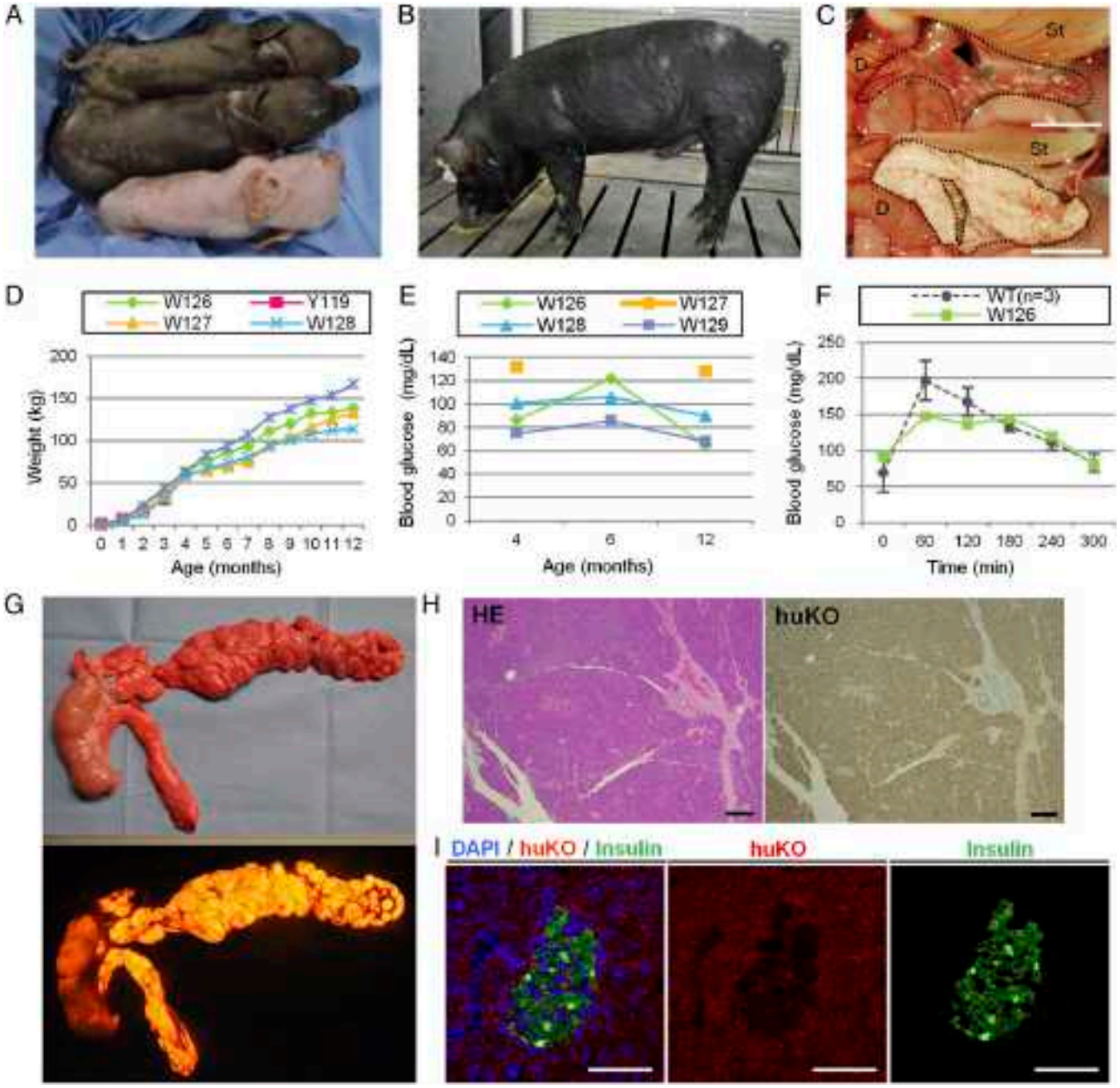


Fig. 4. Production of chimeric offspring by complementation of *Pdx1-Hes1* pancreatogenesis-disabled embryos. (*A*) Chimeric pig (middle pig) was obtained after complementation of host *Pdx1-Hes1* embryos with embryonic cells from a coat-colored WT donor. Sibling nonchimeric cloned pigs were derived from donor (top pig, brown coat) and host (bottom pig, white coat) embryos. (*B*) Mature chimeric boar exhibits WT (donor) coat-color chimerism. (*C*) Vestigial pancreas (*Upper*, arrowhead) of a host embryo-derived cloned piglet and normal pancreas (*Lower*) of a donor-embryo derived piglet. D, duodenum; St, stomach. (*D* and *E*) Normal growth and serum glucose concentrations were observed in chimeric pigs. Pig W127 did not undergo blood sampling at the age of 6 mo due to a leg injury. (*F*) Serum glucose concentrations in a chimeric pig (W126) and WT pig during oral glucose tolerance testing. (*G*) Normally formed pancreas generated in a chimeric pig (W128) exhibits orange fluorescence derived from the donor *huKO* embryo. (*H*) Histological appearance of pancreas generated in a chimeric pig, with staining throughout by anti-huKO antibody. HE, H&E. (*I*) Islet of Langerhans in the pancreas generated in a chimeric pig marks immunohistochemically for insulin. (Scale bars: *C*, 1 cm; *H* and *I*, 100 μm.)

Chemicals. All chemicals were obtained from Sigma–Aldrich unless otherwise indicated.

ICSI-Mediated Gene Transfer. A *Pdx1-Hes1* transgene construct (8.8 kb) was introduced into in vitro-matured porcine oocytes using ICSI-mediated gene transfer (6). Freeze-thawed sperm (3.75×10^5 sperm per 10 μL) were coincubated with 2.5 ng/μL DNA for 5 min at room temperature (RT). An isolated sperm head was then injected into each oocyte using a piezo-actuated microinjection unit (PMM-150FU; Primtech) and micromanipulators (MO-102; Narishige). Oocytes were electrically activated 30 min before sperm head injection. Sperm-injected oocytes were cultured in vitro for 1 or 2 d until transfer to the oviducts of estrus-synchronized gilts. Transgenic fetuses were recovered by laparotomy from pregnant recipients.

SCNT. SCNT was performed using in vitro-matured oocytes as recipient cytoplasts (7). Cultured primary cells from an apancreatic fetus, a *huKO*-expressing

fetus, and a colored-coat WT pig were used as nucleus donor cells after cell cycle synchronization by serum starvation for 48 h. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. Membrane fusion between the donor cell and recipient cytoplast was induced electrically. The reconstructed embryos were then electrically activated, followed by in vitro culture for 1–6 d and subsequent transfer to estrus-synchronized recipient gilts. To generate apancreatic cloned fetuses and piglets, SCNT embryos at the single-cell or early cleavage stage were transferred into recipients' oviducts. Chimeric pigs were produced by transferring cloned embryos at the blastocyst stage into recipients' uteri.

Embryo Manipulation for Blastocyst Complementation. Embryos for blastocyst complementation were prepared using somatic cell cloning technology (discussed above). Fibroblast cells derived from a male *Pdx1-Hes1* transgenic fetus were used for nuclear transfer to produce cloned host embryos. Cloned donor embryos were produced by nuclear transfer using female fibroblasts carrying *huKO* or female fibroblasts isolated from a colored-coat WT pig.

Donor embryos at the morula stage (day 4) were decompacted with 0.1 mM EDTA-2Na (in Ca^{2+}/Mg^{2+}-free PBS supplemented with 0.01% polyvinyl alcohol) for 20 min, followed by removal of zonae pellucidae by digestion with 0.25% pronase solution (in PBS). Blastomeres were isolated from embryos by gentle pipetting using a finely drawn glass capillary. Host embryos at the morula stage were decompacted similarly. Approximately 10 donor embryo blastomeres were injected into the center of each host embryo using micromanipulation. Injected embryos were cultured in vitro for 24 or 48 h to obtain chimeric blastocysts. Developing blastocysts were surgically transferred to uteri of estrus-synchronized recipients on day 5 or 6 (45–80 blastocysts per recipient).

Detection of Chimerism and Genotyping by PCR. Genomic DNA was extracted from tail biopsy specimens of fetuses and newborn piglets using the DNeasy Blood and Tissue Kit (QIAGEN). To detect chimerism in pigs by genotyping, DNA samples were analyzed using PCR with specific primers for *huKO* (donor embryo-derived) and *Pdx1-Hes1* (host embryo-derived) transgene sequences. Nested PCR analysis to detect donor embryo-derived DNA was performed using the primers 5′-AGCACGAAGTCTGGAGACCTCTG-3′ and 5′-AGGTGGTCTTGAACTGGCACTTGTG-3′ for the first round of PCR and the primers 5′-ACCTTACACAGTCCTGCAGACC-3′ and 5′-GCCAGCTTCAGGAACATGGT-3′ for the second round of PCR. PCR conditions were 95 °C for 60 s, followed by 25 cycles of 95 °C for 30 s, 68 °C for 30 s, and 72 °C for 60 s. PCR primers used to amplify *Pdx1-Hes1* transgene sequences were 5′-CAATGATGGCTCCAGGGTAA-3′ and 5′-TGACTTTCTGTGCTCAGAGG-3′. PCR conditions were 95 °C for 60 s, followed by 30 cycles of 95 °C for 30 s, 60 °C for 5 s, and 72 °C for 60 s.

Histological Analysis. Tissue samples from normally formed pancreata of chimeric pigs and from vestigial pancreata of *Pdx1-Hes1* transgenic pigs were fixed in paraformaldehyde, embedded in paraffin, sectioned, stained, and immunostained as described by Kobayashi et al. (3). Sections stained with H&E and immunostained (anti-insulin and anti-huKO) were examined by light microscopy to detect insulin production by pancreatic islets and *huKO* expression. Each section was incubated with primary antibody for 0.5–3 h at RT, followed by incubation with secondary antibody for 1 h at RT. Primary polyclonal antibodies against huKO (1:300, PM051; Medical and Biological Laboratories) and insulin (1:500, LS-C24686; LifeSpan BioSciences) were used. The secondary antibodies used were Alexa Fluor 488 goat anti-mouse IgG (1:200; Life Technologies Corporation) and Alexa Fluor 594 donkey anti-rabbit IgG (1:200; Life Technologies Corporation). After antibody treatments, sections were mounted in Vectashield mounting medium (Vector Laboratories) containing DAPI for nuclear counterstaining and observed by confocal laser scanning microscopy (FV1000-D; Olympus Corporation). The distribution of donor-derived cells expressing *huKO* in chimeric pigs' tissues was determined in immunostained materials with peroxidase chromogen development (Histofine; Nichirei Biosciences) after hematoxylin counterstaining.

ACKNOWLEDGMENTS. We thank H. Kadoi, T. Kanai, and T. Matsuda for their help with maintenance of pigs and E. Haruyama, S. Takayanagi, T. Fujiwara, Y. Ikezawa, S. Saito, Y. Takeuchi, K. Honda, and M. Maehara for experimental/technical assistance. We also thank A. S. Knisely for critically reading the manuscript. This work was supported by the Japan Science and Technology Agency, Exploratory Research for Advanced Technology, Nakauchi Stem Cell and Organ Regeneration Project, Tokyo, and Meiji University International Institute for Bio-Resource Research.

1. Lindvall O, Kokaia Z (2006) Stem cells for the treatment of neurological disorders. *Nature* 441(7097):1094–1096.
2. MacLaren RE, et al. (2006) Retinal repair by transplantation of photoreceptor precursors. *Nature* 444(7116):203–207.
3. Kobayashi T, et al. (2010) Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. *Cell* 142(5):787–799.
4. Usui JI, et al. (2012) Generation of kidney from pluripotent stem cells via blastocyst complementation. *Am J Pathol* 180(6):2417–2426.
5. Sumazaki R, et al. (2004) Conversion of biliary system to pancreatic tissue in Hes1-deficient mice. *Nat Genet* 36(1):83–87.
6. Kurome M, Ueda H, Tomii R, Naruse K, Nagashima H (2006) Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. *Transgenic Res* 15(2):229–240.
7. Matsunari H, et al. (2008) Transgenic-cloned pigs systemically expressing red fluorescent protein, Kusabira-Orange. *Cloning Stem Cells* 10(3):313–323.
8. Nagashima H, Giannakis C, Ashman RJ, Nottle MB (2004) Sex differentiation and germ cell production in chimeric pigs produced by inner cell mass injection into blastocysts. *Biol Reprod* 70(3):702–707.
9. Jackson PGG, Cockcroft PD (2007) *Handbook of Pig Medicine* (Elsevier, Philadelphia), pp 128–142.
10. McLaren A (1975) Sex chimaerism and germ cell distribution in a series of chimaeric mice. *J Embryol Exp Morphol* 33(1):205–216.
11. Mystkowska ET, Tarkowski AK (1968) Observations on CBA-p-CBA-T6T6 mouse chimeras. *J Embryol Exp Morphol* 20(1):33–52.
12. Müller SM, et al. (2005) Gene targeting of VEGF-A in thymus epithelium disrupts thymus blood vessel architecture. *Proc Natl Acad Sci USA* 102(30):10587–10592.
13. Stanger BZ, Tanaka AJ, Melton DA (2007) Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. *Nature* 445(7130):886–891.
14. Ueno H, Weissman IL (2006) Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. *Dev Cell* 11(4):519–533.
15. Tachibana M, et al. (2012) Generation of chimeric rhesus monkeys. *Cell* 148(1-2): 285–295.
16. Kawamata M, Nishimori K (2006) Mice deficient in Dmrt7 show infertility with spermatogenic arrest at pachytene stage. *FEBS Lett* 580(27):6442–6446.
17. Mesnil M, Yamasaki H (2000) Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: Role of gap-junctional intercellular communication. *Cancer Res* 60(15):3989–3999.

Urine excretion strategy for stem cell-generated embryonic kidneys

Shinya Yokote[a,1], Hitomi Matsunari[b,c,1], Satomi Iwai[d,1], Shuichiro Yamanaka[a,e], Ayuko Uchikura[c], Eisuke Fujimoto[e,f], Kei Matsumoto[a], Hiroshi Nagashima[b,c], Eiji Kobayashi[g], and Takashi Yokoo[a,2]

[a]Division of Nephrology and Hypertension, Department of Internal Medicine, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan; [b]Meiji University International Institute for Bio-Resource Research, Kawasaki 214-8571, Japan; [c]Laboratory of Developmental Engineering, Department of Life Sciences, School of Agriculture, Meiji University, Kawasaki 214-8571, Japan; [d]Laboratory of Small Animal Surgery I, School of Veterinary Medicine, Kitasato University, Towada 034-8628, Japan; [e]Division of Regenerative Medicine, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan; [f]Department of Urology, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan; and [g]Department of Organ Fabrication, Keio University School of Medicine, Shinjuku-ku, Tokyo 160-8582, Japan

Edited by R. Michael Roberts, University of Missouri-Columbia, Columbia, MO, and approved August 26, 2015 (received for review April 24, 2015)

There have been several recent attempts to generate, de novo, a functional whole kidney from stem cells using the organogenic niche or blastocyst complementation methods. However, none of these attempts succeeded in constructing a urinary excretion pathway for the stem cell-generated embryonic kidney. First, we transplanted metanephroi from cloned pig fetuses into gilts; the metanephroi grew to about 3 cm and produced urine, although hydronephrosis eventually was observed because of the lack of an excretion pathway. Second, we demonstrated the construction of urine excretion pathways in rats. Rat metanephroi or metanephroi with bladders (developed from cloacas) were transplanted into host rats. Histopathologic analysis showed that tubular lumina dilation and interstitial fibrosis were reduced in kidneys developed from cloacal transplants compared with metanephroi transplantation. Then we connected the host animal's ureter to the cloacal-developed bladder, a technique we called the "stepwise peristaltic ureter" (SWPU) system. The application of the SWPU system avoided hydronephrosis and permitted the cloacas to differentiate well, with cloacal urine being excreted persistently through the recipient ureter. Finally, we demonstrated a viable preclinical application of the SWPU system in cloned pigs. The SWPU system also inhibited hydronephrosis in the pig study. To our knowledge, this is the first report showing that the SWPU system may resolve two important problems in the generation of kidneys from stem cells: construction of a urine excretion pathway and continued growth of the newly generated kidney.

cloned pig | kidney generation | metanephros | somatic cell nuclear transfer | transplantation

In recent years, the number of patients with chronic kidney disease has been increasing worldwide. Because of organ donor shortages, the number of patients with end-stage renal disease (ESRD) requiring renal replacement therapy is increasing also, and these patients are at increased risk of cardiovascular disease and death (1, 2). Thus, ESRD is a major clinical problem. Recently, remarkable advances have been made in stem cell-based therapies for organ generation, and many studies have demonstrated the possibility of using stem cells to generate neo-kidneys. Nevertheless, the kidney remains one of the most difficult organs to reconstruct de novo because of its delicate and complicated architecture.

We recently generated a functional kidney de novo using the organogenic niche method (3–5). This method involved microinjecting human mesenchymal stem cells (hMSCs) into the budding region of a rat embryo. Histologically, the injected hMSCs formed a mature kidney structure, including glomerular podocytes and tubular epithelial cells (3). Histologic examination of differentiated metanephroi after transplantation into rat omenta showed they consisted of human nephrons invaded by the vascular system of the recipient. This observation indicated that the glomerular endothelial cells had originated from the recipient (4). The neo-kidney produced urine, erythropoietin in the presence of anemia (6), and renin in the presence of hypotension (7). However, the nascent kidney ultimately developed hydronephrosis and did not grow in size because it lacked a urine excretion channel (4).

In other studies, we (8), along with another group (9) generated a whole organ de novo from exogenic pluripotent cells by using the blastocyst complementation method, which is one of the most promising methods in this field of research. However, the generated organ's vascular system was a chimeric tissue derived from both recipient animal cells and injected exogenic cells. A vascular system originating from the exogenic cells might become a target of the host's immune response. To avoid this problem, an embryonic kidney that has not yet developed a vascular system must be transplanted into a host.

If these strategies are to be applied to generating human kidneys from stem cells, the generated kidneys must have urine excretion channels. Here, we demonstrate the generation of such a channel in syngeneically transplanted embryonic pig metanephroi using the stepwise peristaltic ureter (SWPU) system. Briefly, we transplanted metanephroi along with the cloaca (from which the bladder developed) into host animals and then connected the host animal's ureters to the developed bladder at an appropriate time (Fig. S1*A*). Allowing the kidney to grow large is another important issue; therefore, we used the pig, a relatively large animal, to provide a better test of the method's feasibility for clinical application (10).

Significance

Worldwide, the number of patients with end-stage renal disease requiring renal replacement therapy is increasing because of the shortage of donor organs. We have successfully generated functional kidneys from human stem cells using the organogenic niche method. However, for these kidneys to have clinical application, a urinary excretion pathway is necessary. Using pigs, we demonstrated our stepwise peristaltic ureter system, showing that it resolves important problems regarding the construction of the urine excretion pathway and the long-term growth of the stem cell-generated embryonic kidneys.

Author contributions: S. Yokote, H.M., S.I., H.N., E.K., and T.Y. designed research; S. Yokote, H.M., S.I., S. Yamanaka, A.U., E.F., K.M., H.N., E.K., and T.Y. performed research; S. Yokote, H.M., S.I., H.N., E.K., and T.Y. analyzed data; and S. Yokote, H.N., and T.Y. wrote the paper.

Conflict of interest statement: E.K. received research funding from Otsuka Pharmaceutical Factory, Inc.

This article is a PNAS Direct Submission.

[1]S. Yokote, H.M., and S.I. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: tyokoo@jikei.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1507803112/-/DCSupplemental.

Results

Syngeneic Transplantation of Pig Metanephroi. Cloned pig fetuses at embryonic day (E) 30 were recovered from an excised uterus (Fig. 1*A* and Fig. S2). Primordial pig metanephroi were dissected under a stereomicroscope (Fig. 1*B*), and some were cryopreserved. A preliminary experiment confirmed the absence of significant differences in the growth of vitrified and nonvitrified pig metanephroi transplanted into mice (Fig. S3). We then implanted pig metanephroi in the omenta of anesthetized syngeneic host pigs (Fig. 1*C*). All transplanted metanephroi differentiated successfully into mature kidneys, growing to about 5–7 mm in length 3 wk after transplantation (Fig. 1 *D* and *E*). Histopathologic examination of the transplant-grown metanephroi showed mature glomeruli and renal tubules (Fig. 1 *F* and *G*). Five weeks after transplantation, the implanted metanephroi had grown to more than 1 cm in length and had developed ureters, which retained urine produced by the metanephroi (Fig. 1 *H* and *I*). Some recipient vessels were integrated into the metanephroi, and the differentiated metanephroi maintained their glomeruli and renal tubules, with little interstitial tissue hemorrhage (Fig. 1 *J* and *K*). Eight weeks posttransplantation, the metanephroi had grown to about 3 cm in length; however, urine production had led to dilated ureters, resulting in hydronephrosis (Fig. 1 *L* and *M*). Histologic analysis indicated thinning of the cortex, interstitial fibrosis (Fig. 1*N*), and well-differentiated metanephroi ureters including both muscular and epithelial layers (Fig. 1*O*). These results suggest that a urine excretion channel is indispensable for the development of implanted metanephroi.

Comparison Between Metanephros and Cloacal Transplantation In Rats. In rats, metanephroi developed in the same way, regardless of whether the cells had come from the cloaca, permitting development of a bladder (the MNB group), or from metanephric primordia (the MN group) (Fig. 2 and Fig. S4). In both groups, the organ weights increased but peaked at 4 wk posttransplantation; there were no significant differences between the MN and MNB groups in the weights of the developed organs (Fig. 2*B*). However, renal tubular dilation was observed at 3 wk posttransplantation in the MN group

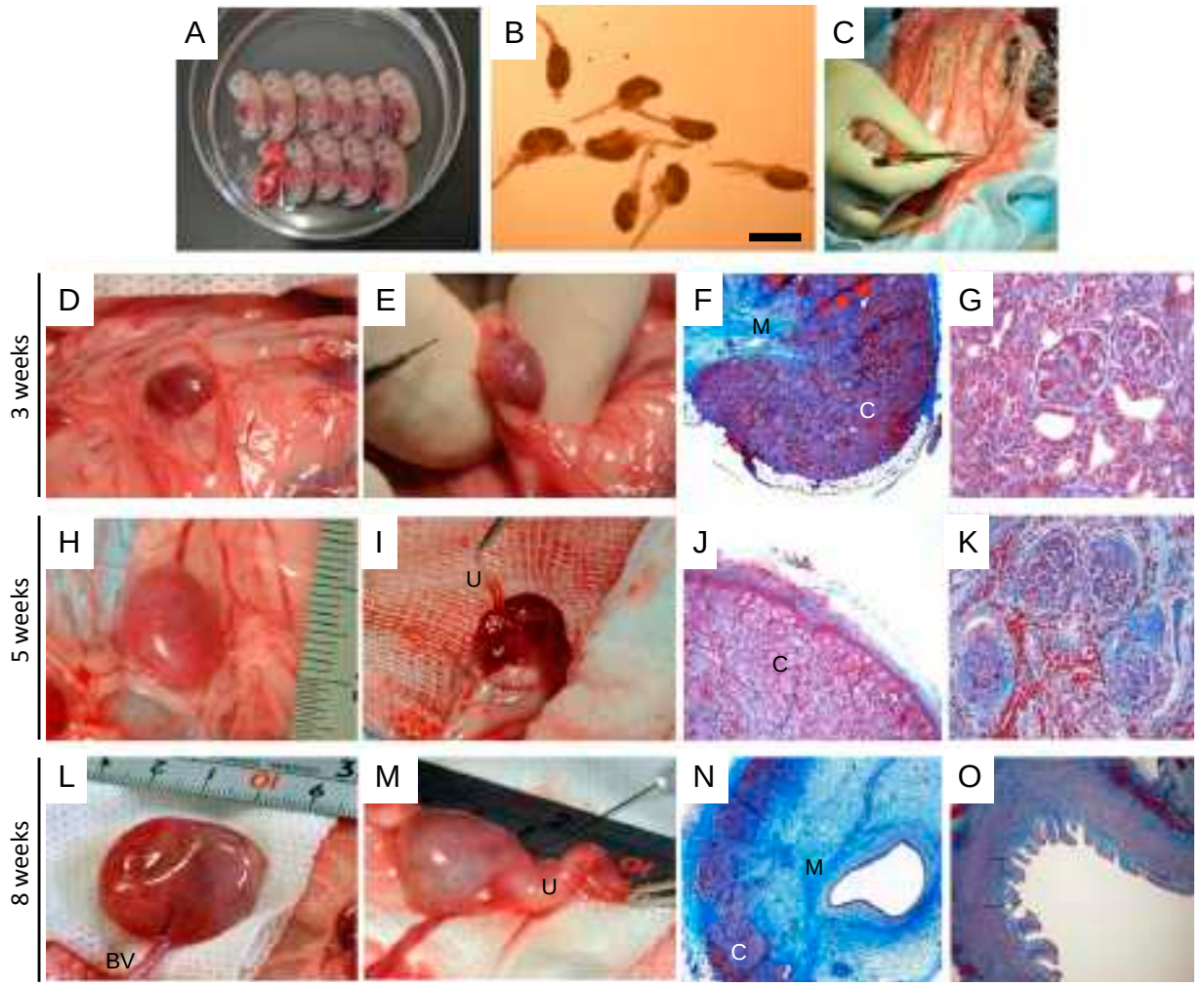


Fig. 1. Syngeneic transplantation of metanephric primordia in pigs. (*A*) Cloned pig embryos at E30. (*B*) Cloned pig metanephroi removed from the embryos. (Scale bar: 2 mm.) (*C*) Metanephroi are syngeneically transplanted into the omenta of the recipient pigs. (*D* and *E*) Three weeks after transplantation, transplanted metanephroi are 5 mm in size. (*F* and *G*) Histopathologic analysis with Masson's trichrome staining reveals that at 3 wk metanephroi have cortex tissue that consists of glomeruli and renal tubules. (*H* and *I*) Five weeks after transplantation, transplanted metanephroi are more than 1 cm long (*H*) and exhibit ureters (*I*). (*J* and *K*) Masson's trichrome staining shows that at 5 wk transplanted metanephroi maintain glomerular structure and have little hemorrhage in the interstitial tissue. (*L* and *M*) Eight weeks after transplantation, blood vessels from the omentum are integrated into the metanephros, which is about 3 cm long, but the metanephros developed hydronephrosis. (*N* and *O*) Thinning of the cortex and interstitial fibrosis of metanephroi (*N*) and good differentiation of the metanephroi ureters, including muscular and epithelial layers (*O*) are observed in sections stained with Masson's trichrome. BV, blood vessel from recipient's omentum; C, cortex; M, medulla; U, ureter from transplanted metanephros.

($P < 0.05$) (Fig. 2 *A* and *C*) and had progressed at 4 wk after transplantation ($P < 0.0005$) (Fig. 2*C*). Conversely, renal tubular dilation was not observed in the MNB group. Histopathologic examination showed that at the time the animals were killed the tubular lumina dilation, interstitial fibrosis, and reduction of glomerular numbers were more pronounced in the MN group than in the MNB group (Fig. 2 *A* and *C–E*); the vesicoureteral junction was better differentiated in the MNB group (Fig. S5); urine volume produced from the metanephroi was significantly larger in the MNB group than in the MN group ($P < 0.05$) (Fig. 2*G*); and urine levels of urea nitrogen (UN) and creatinine (Cr) were higher in the MNB group than in the MN group ($P < 0.05$) (Fig. 2 *H* and *I*). These findings indicate that MNB transplantation is superior to MN transplantation with regard to both kidney development and urine production.

SWPU System. We created a urinary excretion channel in rats using the SWPU system (Fig. S2*A*). E15 rat cloacas were transplanted into the para-aortic area of anesthetized rats. Four weeks after transplantation, we connected each rat's left ureter to the newly developed bladder under stereomicroscopic guidance (Fig. S6). Seven to eight weeks after cloacal transplantation (3–4 wk after ureter–bladder anastomosis), urine from the bladder grown from the cloacal transplant was discharged continuously from the connected recipient ureter (Movie S1). I.v. urography showed that contrast medium appeared in the transplant-grown bladder after injection and then passed to the recipient ureter and bladder over time (Fig. 3*A*). UN and Cr levels were much higher in the urine from the transplant-grown bladder than in the sera of recipient rats (Fig. 3 *F* and *G*), suggesting that a bladder developed from a transplanted cloaca has the potential to collect urine within 8 wk after transplantation. Histopathologic analysis revealed successful anastomosis between the transplant-grown bladder and the recipient ureter (Fig. 3 *B–D*); even 8 wk after transplantation, the cloaca maintained mature renal structures, such as glomeruli and renal tubules (Fig. 3*E*). On the other hand, metanephroi that underwent anastomosis between the host and metanephroi ureters showed severe renal pelvis dilation and thinning of the cortex, resulting in hydronephrosis (Fig. S7). Furthermore, the SWPU system significantly prolonged the lifespan of anephric rats compared

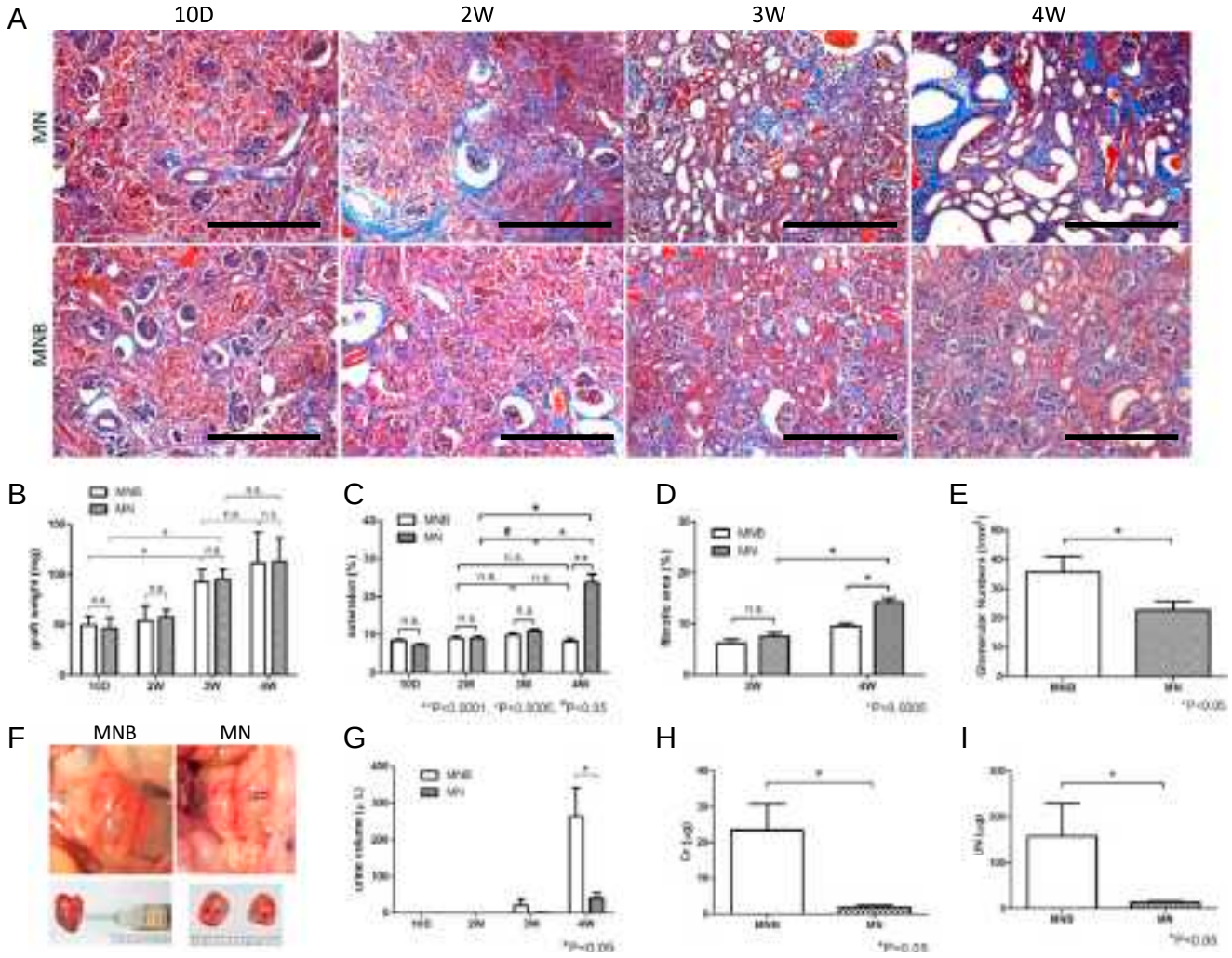


Fig. 2. Comparison of MN and MNB transplantation in rats. (*A*) Histopathologic analysis of differentiated metanephroi using Masson's trichrome staining. Renal tubular dilation and interstitial fibrosis are more prominent in the MN group than in the MNB group, and the glomerulus count is lower. (Scale bars: 400 μm.) (*B*) There are no significant differences in the weights of either the metanephroi or bladders between the MN and MNB groups. (*C*) Renal tubular dilation is observed, beginning at 3 wk after transplantation, in the MN group and progressed by 4 wk after transplantation. However, renal tubular dilation is not observed in the MNB group at 4 wk after transplantation. (*D*) Interstitial fibrosis of metanephroi at sacrifice is more severe in the MN group than in the MNB group ($P < 0.0005$). (*E*) The glomerulus count of metanephroi at sacrifice is higher in the MNB group than in the MN group ($P < 0.05$). (*F*) Differentiated metanephroi in the MN and MNB groups 4 wk after transplantation. (*G*) Urine volume at sacrifice is significantly larger in the MNB group than in the MN group ($P < 0.05$). (*H* and *I*) Urea Cr (*H*) and UN (*I*) excretion is higher in the MNB group than in the MN group ($P < 0.05$).

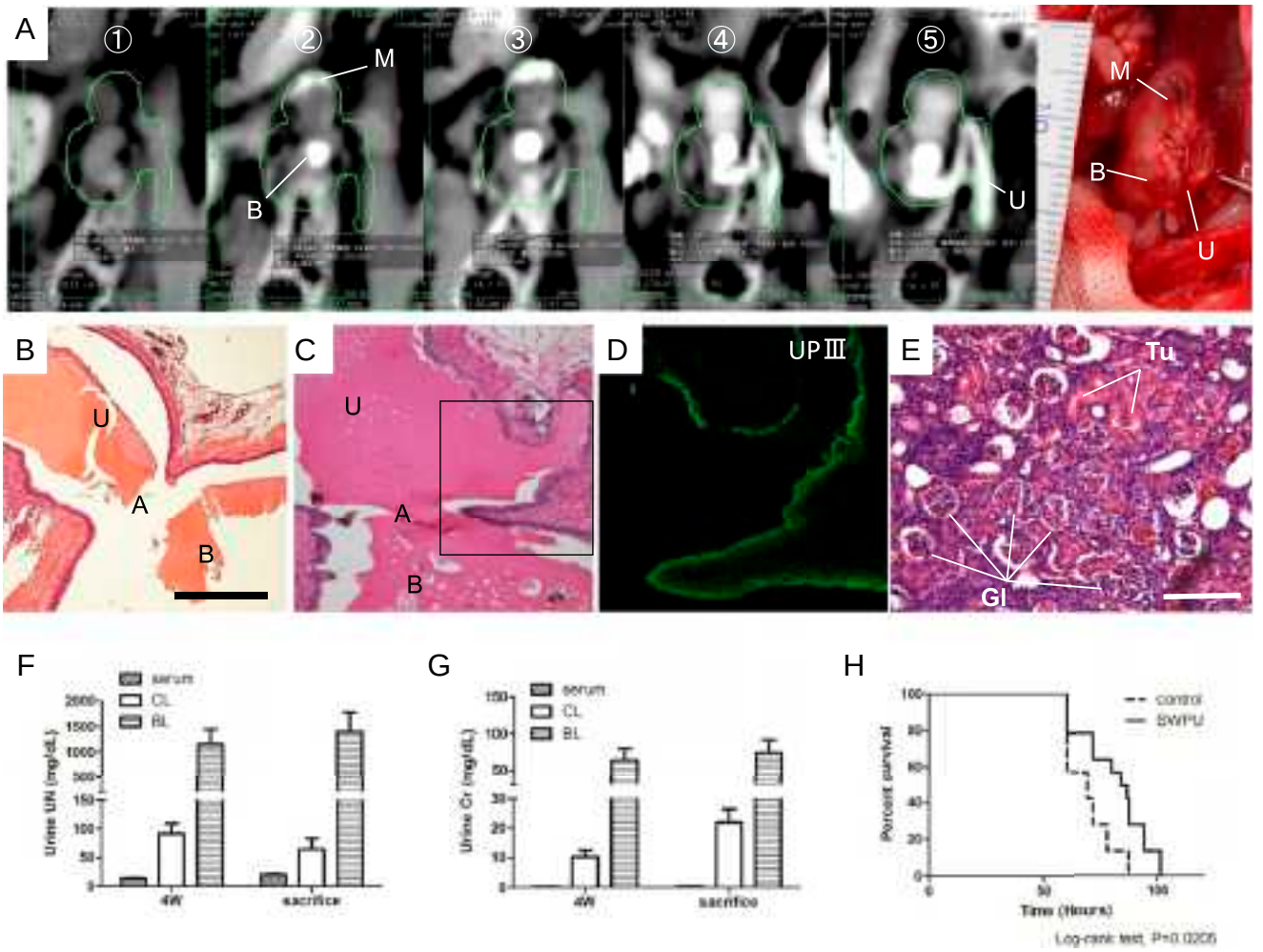


Fig. 3. SWPU system. (*A*) I.v. urography, using CT, shows that contrast medium appears in the cloaca-grown bladder after injection and then passes into the recipient ureter over time. (*B* and *C*) Histopathologic analysis using H&E staining shows successful anastomosis between the cloaca-grown bladder and recipient ureter. (*D*) Uroplakin III staining reveals the continuity of the transitional epithelium at the point of anastomosis. (*E*) The cloaca-grown kidney exhibits mature renal structures such as glomerular and renal tubules 8 wk after transplantation. (*F* and *G*) UN (*F*) and Cr (*G*) levels are much higher in the urine produced from the cloaca-grown kidney than in the sera of recipient rats. (*H*) Kaplan–Meier survival curve for rats with SWPU (continuous line, $n = 14$) and anephric control rats (dotted line, $n = 7$). Median survival of control rats with no native renal mass is 69.50 h. Animals transplanted with the SWPU system (median survival 85.38 h) survived longer than control animals ($P < 0.05$). A, anastomosis between cloaca-grown bladder and recipient ureter; B, cloaca-grown bladder; BL, urine in recipient bladder; CL, urine in cloaca-grown bladder; Gl, glomerulus; M, metanephros; Tu, renal tubules; U, recipient ureter. (Scale bars: 400 μm in *B*; 200 μm in *E*.)

with controls (Fig. 3*H* and Fig. S8). The system allowed continuous discharge of urine from a transplant-grown bladder into a recipient bladder via the recipient ureter, thus providing a urinary excretion channel for the generated kidney; such discharge is difficult to achieve conventionally.

Syngeneic Cloacal Transplantation in Pigs Using the SWPU System. First, we transplanted pig cloacas into syngeneic cloned pigs (Fig. 4*A*). Cloacas transplanted into omenta continued to develop in the same way as in rats, producing urine 3 wk after transplantation (Fig. 4*B*). Produced urine was retained in the transplant-grown bladder at 5 wk after transplantation (Fig. 4*C*). Next, we created a urinary excretion channel in cloned pigs using the SWPU system (Fig. S1*A*). E30 pig cloacas were transplanted into the parasplenic artery area of anesthetized pigs. Four weeks later, we connected the left ureter to the transplant-grown bladder (Fig. 4 *D*–*F* and Fig. S9). During this period, the levels of UN, Cr, and potassium (K) were much higher in the urine from the transplant-grown cloacas than in the sera of recipient pigs (Fig. S10). Histopathologic examination of the transplant-grown cloacas showed mature glomeruli and renal tubules (Fig. 4*G*). Cloned pigs were killed 8 wk after cloacal transplantation (4 wk after ureter–bladder anastomosis). At that time the metanephroi differentiated from cloacas maintained nephron structures similar to the structures seen at 4 wk posttransplantation (Fig. 4 *G*–*I*). At 8 wk posttransplantation the dilation of the tubular lumina and interstitial fibrosis was lower in the metanephroi of pigs in which the SWPU system had been applied than in those in which it was not applied (Fig. 1 *N* and *O*). These results suggest that the creation of a urinary excretion channel, using the SWPU system, permitted the transplanted cloacas to continue to develop for a long time. Thus, at least in pigs, the system is useful for creating urinary excretion channels and for increasing the size of the stem cell-generated embryonic kidneys.

Discussion

This report describes the construction of a urine excretion pathway for stem cell-generated embryonic kidneys that involved connecting the recipient ureter with a bladder grown from a transplanted embryonic cloaca. After cloacal transplantation, several vessels from the recipient animal were integrated into the transplanted cloaca. Thereafter, the metanephroi of the cloaca

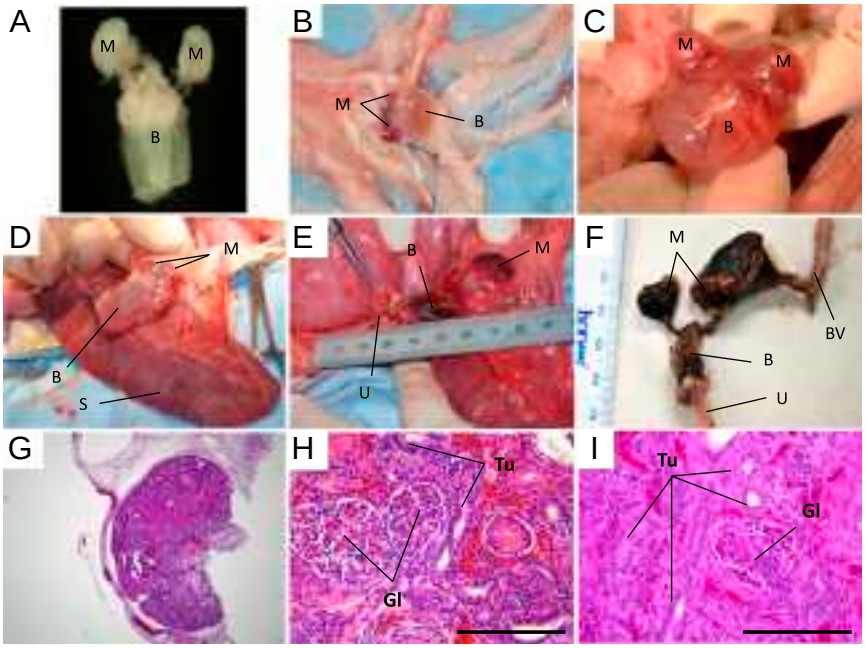


Fig. 4. Syngeneic transplantation of pig cloaca using the SWPU system. (*A*) Metanephroi (M) with bladders (CLs) from an E30 pig embryo. (*B*) Pig cloaca syngeneically transplanted into pig omentum, 3 wk after transplantation. Liquid is retained in the bladder grown from the transplanted cloaca. (*C*) Pig cloaca transplanted into pig omentum, 5 wk after transplantation, shows two metanephroi and a liquid-filled bladder. (*D* and *E*) Anastomosis between a bladder grown from a cloacal implant and a recipient ureter. Four weeks after cloacal transplantation, we connected the recipient's left ureter to the bladder grown from the cloacal implant. (*F*) One day after ureter–bladder anastomosis, blood vessels from the recipient's splenic artery are integrated into the structures grown from the cloacal implant. (*G* and *H*) H&E staining of a differentiated cloaca 4 wk after transplantation. (*I*) H&E staining of a differentiated cloaca 8 wk after transplantation. The kidneys exhibit structures such as glomeruli and renal tubules. B, bladder grown from a cloacal transplant; BV, blood vessel from recipient's splenic artery; Gl, glomerulus; M, metanephros; S, spleen of recipient pig; Tu, renal tubules; U, recipient's ureter. (Scale bars: 200 μm.)

continued to develop and started to produce urine, as previously reported (3–7, 11). The produced urine was excreted into the cloacal bladder, via the cloaca's ureter, by peristalsis. In both pigs and rats, urine collected in transplant-grown bladders and was discharged continuously by the peristaltic movements of the recipient ureter, preventing the transplant-grown cloaca from developing hydronephrosis. Eight weeks after transplantation, the concentration of UN and Cr were still much higher in the cloacal urine than in the sera of recipient rats (Fig. 3 *F* and *G*), suggesting that the SWPU system permits the transplanted cloaca to enlarge and replace kidney function in the recipient animals, an ability that, to our knowledge, has never before been demonstrated.

Previous studies described the direct connection of a recipient ureter with the ureter of a transplant-grown metanephros (uretero–ureterostomy) to create a urine excretion channel for a neokidney (Fig. S1*B*) (11–13), prolonging the short-term survival of anephric rats (12, 13). However, our SWPU system is more efficient than previous methods in many ways. First, cloacal transplantation is superior to metanephroi transplantation in preventing hydronephrosis. Our study indicated that transplanted metanephroi began urine production 3 wk post-transplantation and tended to develop hydronephrosis 4 wk after transplantation, suggesting that this method leads to renal insufficiency (Fig. 2*A*). Persistent urine discharge into the transplant-grown bladder seems to extend the time before hydronephrosis develops. Second, the SWPU system is more effective than previous methods in allowing sustainable growth and maturation of the kidney. During kidney development, sustained excretion of urine from the metanephroi is caused by peristalsis of the ureter primordia; ureter primordia obstruction has been suggested to result in dysplastic metanephroi (14). This study actually shows that the number of glomeruli and urine volume are larger following cloacal transplantation than after metanephroi transplantation. Third, the SWPU system can join two metanephroi at one time, whereas connecting the recipient ureter to two metanephroi ureters is difficult. Because previous studies showed that the total mass of the developed metanephros correlated with the duration of anephric rat survival (13), the SWPU system is thought to be more effective than conventional uretero–ureterostomies in improving survival time. Fourth, connecting the recipient ureter with a bladder grown from a cloacal transplant is easier than uretero–ureterostomy because the recipient ureter is very large compared with the metanephroi ureters, and the urine-expanded cloacal-grown bladder is either larger than or similar in size to the recipient ureter.

In future studies, we will regenerate cloacas from human stem cells in pig embryos using the organogenic niche method or blastocyst complementation method and then will transplant the cloaca into a human. The cloaca is expected to develop in the human, creating its own human-derived vascular system (15, 16). Then we will construct a urinary excretion channel using the SWPU system. However, for these strategies to work, kidney- and ureter-deficient pigs are required to avoid forming chimeric tissues. Mouse embryos lacking both *Pax2* and *Pax8* are unable to form metanephroi and ureters (17). Therefore, we plan to establish similar kidney- and ureter-deficient pigs to ensure that the ureter of the neo-kidney originates from injected human stem cells.

We have demonstrated that the SWPU system may provide the means to construct a urinary excretion pathway for stem cell-generated embryonic kidneys. The creation of such a pathway is one of the most important problems to be overcome in the de novo generation of whole kidneys, and the solution of this problem represents a significant advance in the field.

Materials and Methods

Animals. Adult male Lewis rats were purchased from Sankyo Lab Service Corporation and CLEA Japan. Pairs of animals were kept in cages and allowed free access to food and water. Crossbred gilt pigs (Hypor Japan) were used as recipients of somatic cell nuclear transfer (SCNT) embryos for producing cloned pigs. The pigs were maintained in a semi-windowless facility with a controlled temperature (15–30 °C) and received a standard porcine diet twice daily and water ad libitum. All experimental procedures were approved by the committees for animal experiments and the ethics committees of Jikei University, Meiji University, and Kitasato University.

Experimental Protocols.

Experiment 1. We generated cloned E30 pig fetuses from a line of female fetal fibroblast cells using somatic cell cloning technology, as described previously (8). Metanephroi were dissected from the cloned fetuses under a stereomicroscope (Fig. 1*B*) and were implanted in the omenta of syngeneic adult cloned gilts (Fig. 1*C*). Three, five, and eight weeks later, the recipient pigs were killed under general anesthesia induced using injected pentobarbital and inhaled isoflurane, and the transplanted metanephroi were recovered (Fig. S2).

Experiment 2. Ten-week-old Lewis rats were divided into eight groups (Fig. S4). Rats in MNB groups 1–4 ($n = 20$) were implanted with cloacas in the para-aortic area. Rats in MN groups 1–4 ($n = 16$) were implanted with metanephroi, along with the

bladders, after the metanephroi ureters in the para-aortic area were cut. Rats in both the MNB and MN groups were killed 10 d (group 1), 2 wk (group 2), 3 wk (group 3), and 4 wk (group 4) after transplantation. All of the developed metanephroi and bladders were removed at the time the animals were killed. Any metanephroi-produced urine that had collected in the bladders or ureters was extracted using a microneedle, and the volumes were measured.

Experiment 3. E15 rat cloacas were removed and transplanted in the para-aortic area of 9-wk-old, anesthetized recipient rats. Four weeks after transplantation, we removed the left native kidney and connected the left ureter to the bladder of the transplanted cloaca, under microscopic guidance (Fig. S6). Three or four weeks after this surgery (7 or 8 wk after transplantation), the rats were subjected to computed tomography (CT) scans, and the developed cloacas were removed. To analyze the life span of SWPU system-treated rats, E15 rat cloacas also were implanted into the para-aortic areas of 9-wk-old Lewis rats (n = 21). Four weeks after transplantation, the SWPU group (n = 14) underwent left nephrectomies and received anastomoses between the bladders grown from cloacal transplants and the recipient left ureters. Rats in the control group (n = 7) underwent left nephrectomy only. Eight weeks after transplantation, all rats underwent right nephrectomy. We measured the life spans of the rats from the time of right nephrectomy (Fig. S8).

Experiment 4. E30 cloacas dissected from cloned pig fetuses were implanted in the omenta of syngeneic, adult cloned pigs (Fig. S9). Four weeks after implantation, we removed each host animal's native left kidney and connected the left ureter to the implanted cloaca bladder. One day or four weeks after this surgery (4 or 8 wk after cloacal transplantation), the developed cloacas were removed for examination.

SCNT. SCNT was conducted as described previously (8, 18), using in vitro-matured oocytes as the recipient cytoplasts. Primary culture cells of porcine fetal fibroblasts (female) were prepared as nuclear donors after cell-cycle synchronization, which was accomplished using serum starvation (FBS, 0.5% vol/vol) for 48 h. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. Membrane fusion of the donor cells and recipient cytoplasts was induced electrically. The reconstructed embryos then were activated electrically, followed by in vitro culture for 1–6 d and subsequent transfer to the reproductive tracts of estrus-synchronized recipient gilts.

Metanephroi and Cloaca Transplantation. Pregnant sows were killed under general anesthesia. E15 rat metanephroi or cloacas and E30 pig metanephroi or cloacas were dissected from fetuses under stereomicroscope guidance. Metanephroi and cloacas were implanted in the omenta of anesthetized host animals (Fig. S5) as previously reported (10, 11, 19).

Cryopreservation of Embryonic Metanephroi. Pig metanephroi were cryopreserved using the previously reported vitrification method (8, 20), with slight modifications. Briefly, metanephroi were initially equilibrated with 7.5% (vol/vol) ethylene glycol (EG) and 7.5% (vol/vol) DMSO in handling medium [HM; 20 mM Hepes-buffered tissue culture medium 199 + 20% (vol/vol) calf serum] for 25 min, followed by a second equilibration in vitrification solution (VS), consisting of 15% (vol/vol) EG and 15% (vol/vol) DMSO in HM, for 20–50 min, on ice. Two metanephroi per device were placed on a Cryotop device (Kitazato BioPharma) with a minimum volume of VS and were plunged directly into liquid nitrogen for storage (3–36 mo). For warming, the Cryotop was immersed directly in HM containing 1 M sucrose for 1 min at 38.5 °C, followed by stepwise dilution of the cryoprotectants at room temperature. The metanephroi were transferred into HM with 0.5 M sucrose for 3 min and were washed twice in HM for 5 min before transplantation.

SWPU System. E15 rat cloacas and E30 porcine cloacas were implanted in the para-aortic or parasplenic artery areas of anesthetized host animals. Four weeks after implantation, the left native kidneys were removed, and the left ureters were connected to the bladders developed from the implanted cloacas (Fig. S1*A*).

Histochemical Analysis. For histologic analysis, tissues grown from implanted metanephroi or cloacas were placed into 4% (wt/vol) paraformaldehyde in phosphate buffer. The fixed tissues were embedded in paraffin and cut into 5-μm sections. Masson's trichrome and H&E staining were performed, as described elsewhere (7). The dimensions of the tubular lumen and the degree of interstitial fibrosis were analyzed quantitatively in 10 high-power fields of the cortical area per section, using MetaValue software (Molecular Devices) to determine the fibrotic areas in the sections stained with Masson's trichrome (blue) and in the areas of renal tubules showing dilation. The maximal 2D areas of the developed metanephroi were determined from 10 serial sections, before and after the presumed largest section, and the glomerular numbers were determined by calculating the number of glomeruli per total renal cortical area of the maximum section, measured using MetaValue software. For immunohistochemical staining of transitional epithelium cells, goat anti-Uroplakin III polyclonal antibody (sc-15182; Santa Cruz Biotechnology) was used.

Blood and Urine Biochemistry. Blood and urine samples for biochemical analyses were obtained from the inferior vena cava and bladder, respectively, of isoflurane-anesthetized rats. Serum and urine UN and Cr levels were analyzed according to the manufacturer's instructions (SRL), as reported previously (7).

I.v. Urography. To analyze whether urine from a bladder grown from a cloacal transplant could be discharged into a recipient bladder via the recipient ureter, CT scans using an Activion 16 CT system (Toshiba Medical Systems) and Omnipaque contrast medium (Daiichi-Sankyo) were performed after rats were killed.

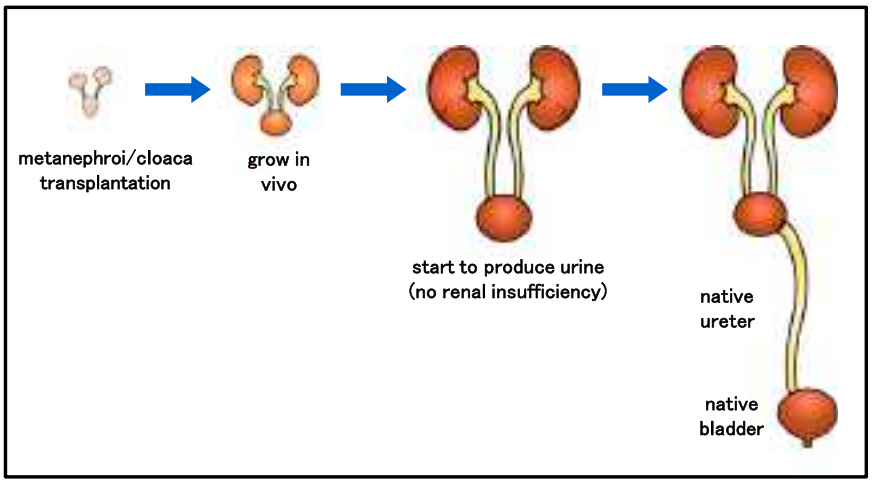
Statistical Analysis. Data are presented as means ± SEs of measurement. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). The significance of the differences between two mean values was determined using an unpaired t test. Multiple comparisons involving more than three groups were performed by ANOVA. Survival curves were created using Kaplan–Meier survival analyses.

ACKNOWLEDGMENTS. We thank H. Kadoi, K. Nakano, Y. Asano, T. Takeishi, H. Gotoh, and M. Ishida for experimental/technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Otsuka Pharmaceutical Factory. This work also was supported technically by Jikei University; Meiji University International Institute for Bio-Resource Research; Kitasato University; and Keio University School of Medicine.

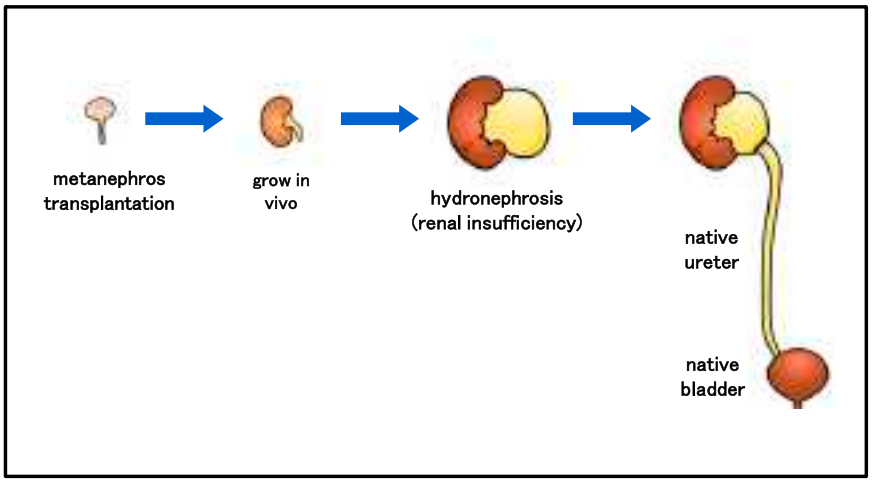
1. Lozano R, et al. (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380(9859):2095–2128.
2. Couser WG, Remuzzi G, Mendis S, Tonelli M (2011) The contribution of chronic kidney disease to the global burden of major noncommunicable diseases. *Kidney Int* 80(12):1258–1270.
3. Yokoo T, et al. (2005) Human mesenchymal stem cells in rodent whole-embryo culture are reprogrammed to contribute to kidney tissues. *Proc Natl Acad Sci USA* 102(9):3296–3300.
4. Yokoo T, et al. (2006) Xenobiotic kidney organogenesis from human mesenchymal stem cells using a growing rodent embryo. *J Am Soc Nephrol* 17(4):1026–1034.
5. Yokoo T, et al. (2008) Generation of a transplantable erythropoietin-producer derived from human mesenchymal stem cells. *Transplantation* 85(11):1654–1658.
6. Yokoo T (2014) Kidney regeneration with stem cells: An overview. *Nephron, Exp Nephrol* 126(2):54–58.
7. Yokote S, et al. (2012) The effect of metanephros transplantation on blood pressure in anephric rats with induced acute hypotension. *Nephrol Dial Transplant* 27(9):3449–3455.
8. Matsunari H, et al. (2013) Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs. *Proc Natl Acad Sci USA* 110(12):4557–4562.
9. Usui J, et al. (2012) Generation of kidney from pluripotent stem cells via blastocyst complementation. *Am J Pathol* 180(6):2417–2426.
10. Hammerman MR (2004) Renal organogenesis from transplanted metanephric primordia. *J Am Soc Nephrol* 15(5):1126–1132.
11. Rogers SA, Lowell JA, Hammerman NA, Hammerman MR (1998) Transplantation of developing metanephroi into adult rats. *Kidney Int* 54(1):27–37.
12. Rogers SA, Hammerman MR (2004) Prolongation of life in anephric rats following de novo renal organogenesis. *Organogenesis* 1(1):22–25.
13. Marshall D, Dilworth MR, Clancy M, Bravery CA, Ashton N (2007) Increasing renal mass improves survival in anephric rats following metanephros transplantation. *Exp Physiol* 92(1):263–271.
14. Ichikawa I, Kuwayama F, Pope JC, 4th, Stephens FD, Miyazaki Y (2002) Paradigm shift from classic anatomic theories to contemporary cell biological views of CAKUT. *Kidney Int* 61(3):889–898.
15. Rogers SA, Hammerman MR (2001) Transplantation of rat metanephroi into mice. *Am J Physiol Regul Integr Comp Physiol* 280(6):R1865–R1869.
16. Takeda S, Rogers SA, Hammerman MR (2006) Differential origin for endothelial and mesangial cells after transplantation of pig fetal renal primordia into rats. *Transpl Immunol* 15(3):211–215.
17. Bouchard M, Souabni A, Mandler M, Neubüser A, Busslinger M (2002) Nephric lineage specification by *Pax2* and *Pax8*. *Genes Dev* 16(22):2958–2970.
18. Matsunari H, et al. (2008) Transgenic-cloned pigs systemically expressing red fluorescent protein, Kusabira-Orange. *Cloning Stem Cells* 10(3):313–323.
19. Dekel B, et al. (2003) Human and porcine early kidney precursors as a new source for transplantation. *Nat Med* 9(1):53–60.
20. Matsumoto K, et al. (2012) Xenotransplanted embryonic kidney provides a niche for endogenous mesenchymal stem cell differentiation into erythropoietin-producing tissue. *Stem Cells* 30(6):1228–1235.

Supporting Information

Yokote et al. 10.1073/pnas.1507803112



(A) Step-wise peristaltic ureter (SWPU) system



(B) Conventional method

Fig. S1. A urine excretion strategy for neo-kidneys. (*A*) SWPU system. Metanephroi with bladders grown from cloacas were transplanted into host animals. After transplantation, we removed the left native kidney and connected the left native ureter to the cloaca-grown bladder. (*B*) Conventional connection to the recipient ureter. After matanephroi transplantation, rats with transplanted metanephroi underwent a single end-to-end anastomosis to connect the ureter of the metanephros to the host ureter.

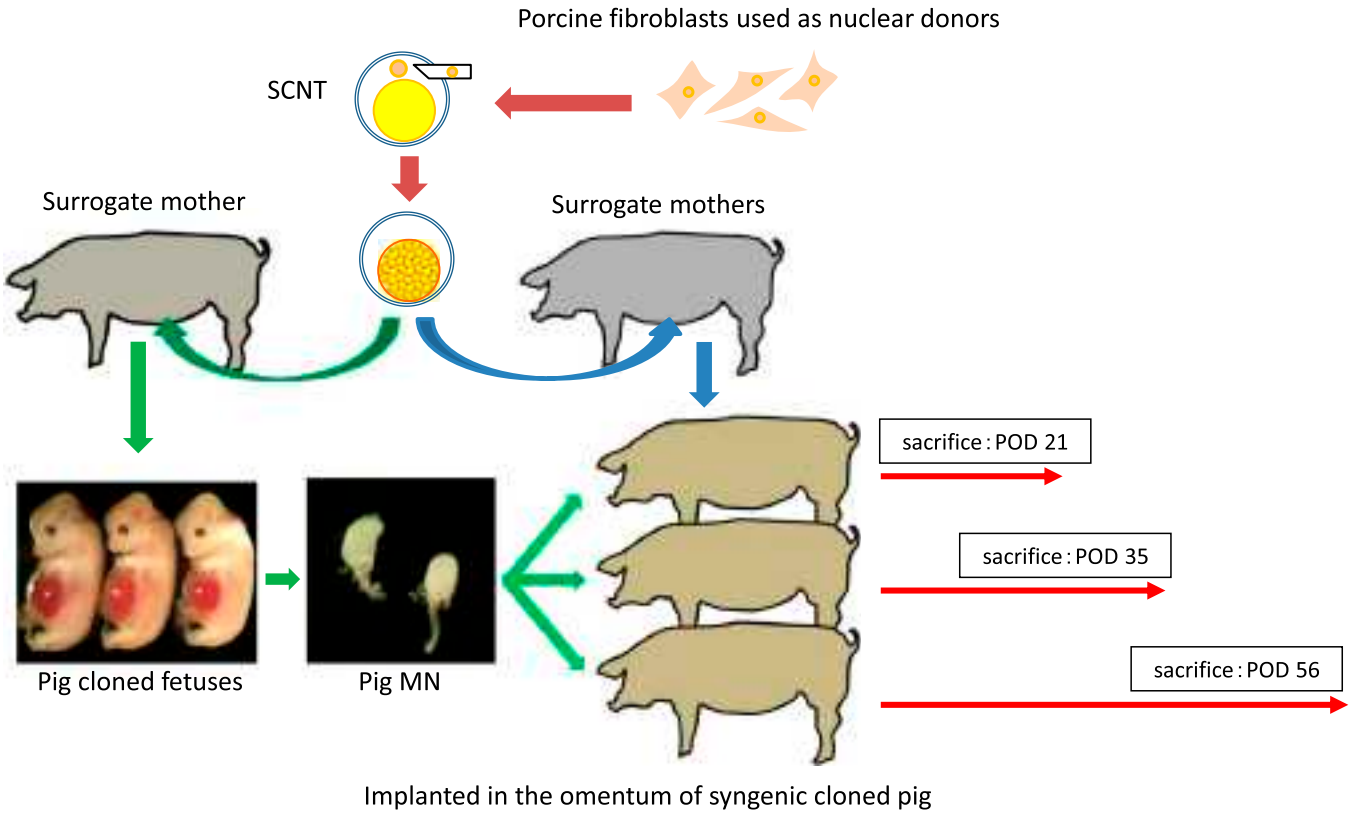


Fig. S2. Syngeneic transplantation of pig metanephroi. Cloned E30 pig metanephroi are dissected and implanted in the omenta of syngeneic, adult cloned gilts. Three, five, and eight weeks later, the recipient pigs are killed. POD, postoperative day.

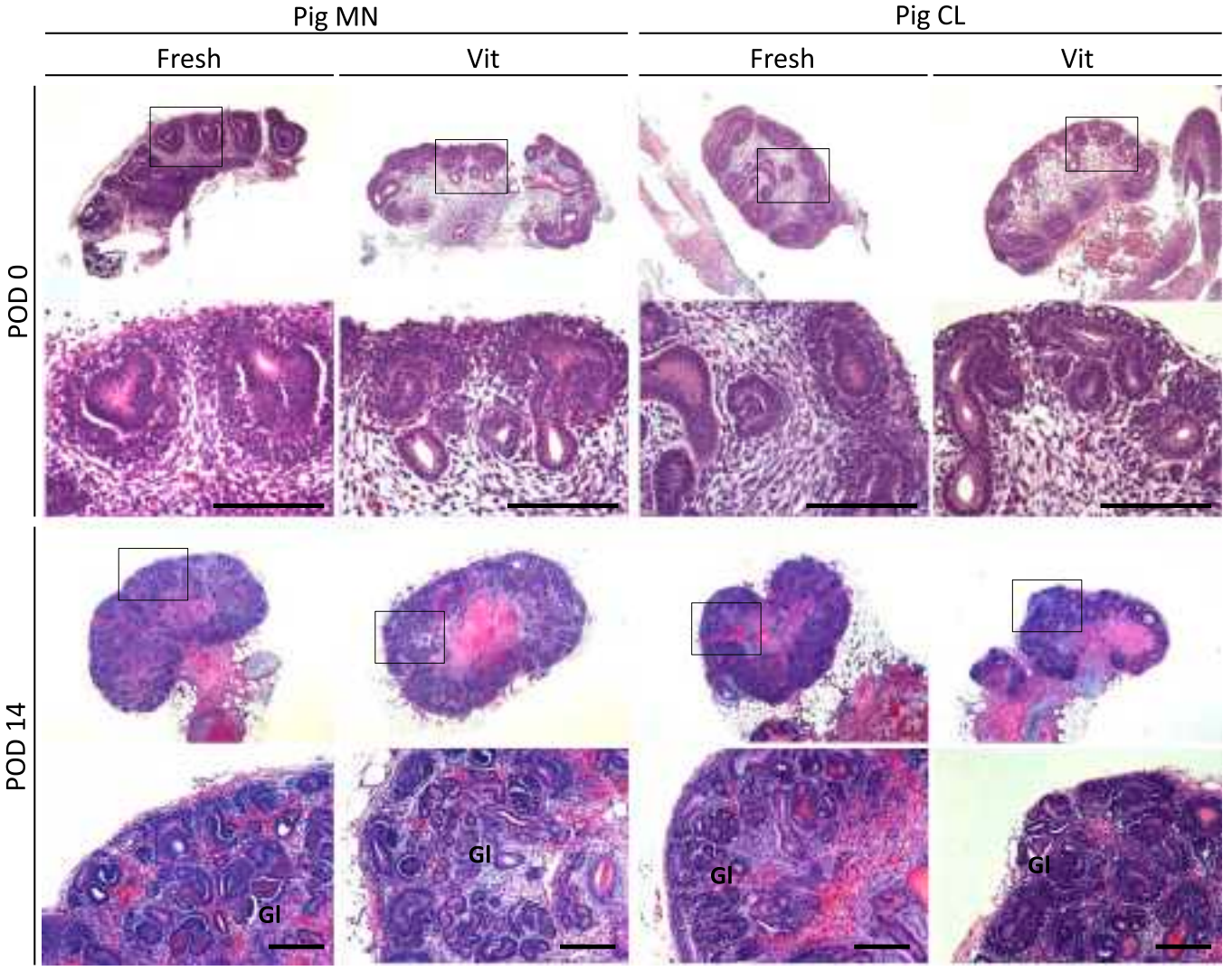


Fig. S3. Comparison of the differentiation potential of vitrified and nonvitrified pig metanephroi. Vitrified and nonvitrified pig metanephroi primordia (Pig MN) were transplanted to the omentum or abdominal wall of NOD/SCID mice (CLEA Japan). Vitrified (Vit) and nonvitrified pig metanephroi with bladders developed from cloacal transplantation (Pig CL) also were transplanted into NOD/SCID mice. Two weeks after transplantation, mice were killed. There are no significant differences in growth between vitrified and nonvitrified pig metanephroi. There also are no significant difference in growth between metanephri grown from MN transplantation and metanephroi grown from cloacal transplantation. Gl, glomerulus; Vit, vitrified. (Scale bars: 200 μm.)

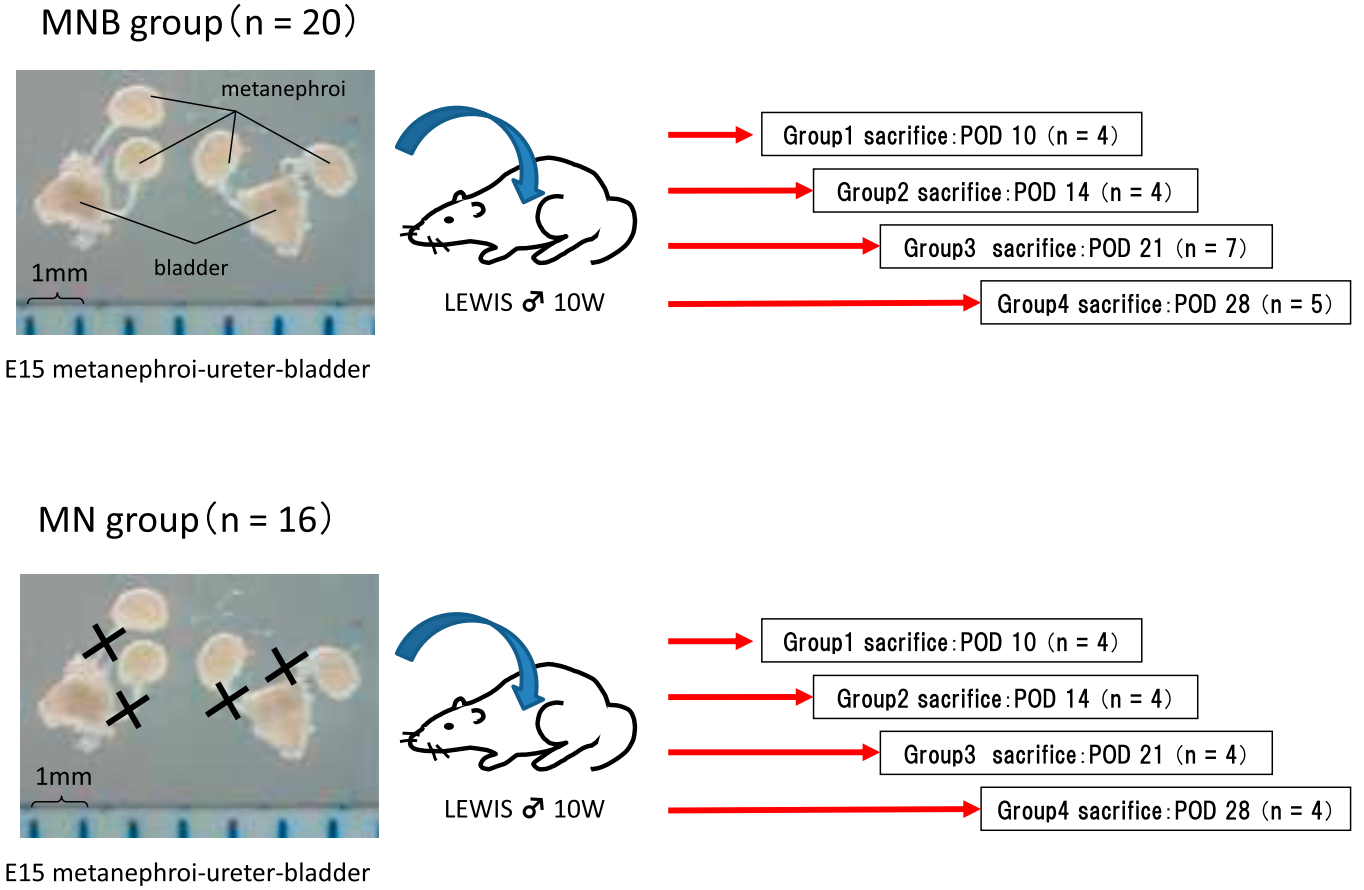


Fig. S4. E15 rat metanephroi and cloacal tissue were removed under stereomicroscopic guidance. In MNB groups 1–4 ($n = 20$), cloacas were implanted in the para-aortic area of recipient rats. In MN groups 1–4 ($n = 16$), metanephroi and bladder primordia were implanted in the para-aortic area of recipient rats after the ureter of the metanephros was cut. Ten days after surgery, rats in MN group 1 ($n = 4$) and MNB group 1 ($n = 4$) were killed. Two weeks after surgery, rats in MN group 2 ($n = 4$) and MNB group 2 ($n = 4$) were killed. Three weeks after surgery, rats in MN group 3 ($n = 4$) and MNB group 3 ($n = 7$) were killed. Four weeks after surgery, rats in MN group 4 ($n = 4$) and MNB group 4 ($n = 5$) were killed. POD, postoperative day.

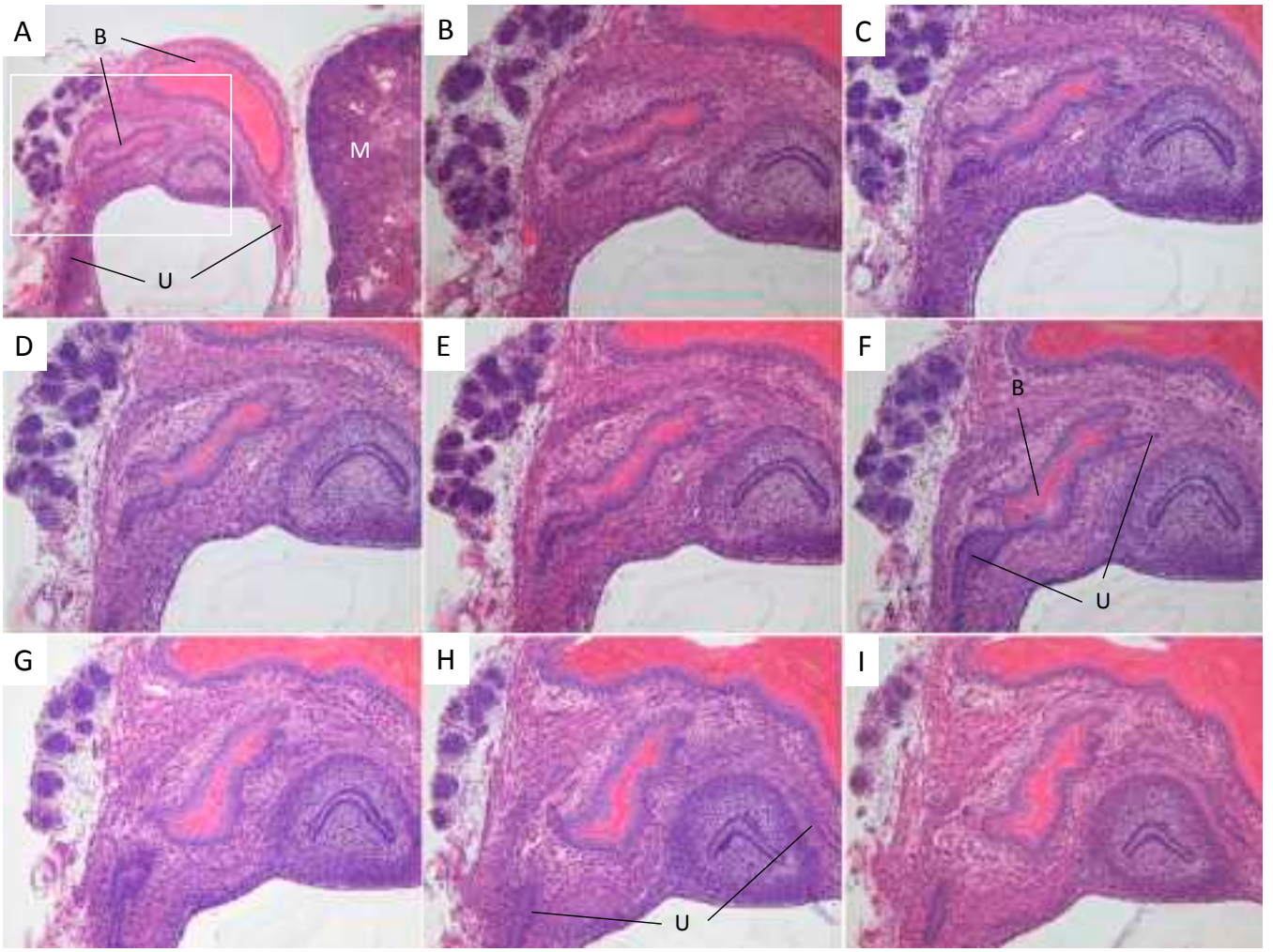


Fig. S5. Histopathologic analysis of the vesicoureteral junction in differentiated cloacas. The vesicoureteral junction is well-differentiated in the MNB group. The continuity of the cloaca-differentiated ureter and bladder is shown in serial sections (*A–I*). B, bladder; M, metanephros; U, ureter.

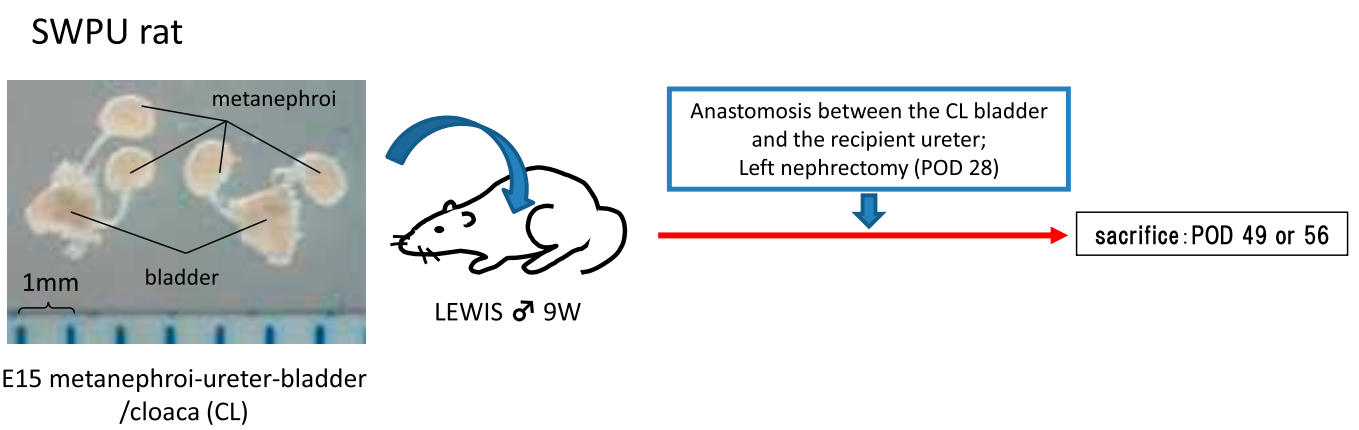


Fig. S6. Rat metanephroi with bladders developed after cloacal transplantation using the SWUP system. E15 rat cloacas were removed and transplanted into recipient rats. Four weeks later, the left native kidney was removed, and the recipient left ureter was connected to the bladder grown after cloacal transplantation. Three or four weeks after this surgery, the developed cloaca was removed. POD, postoperative day.

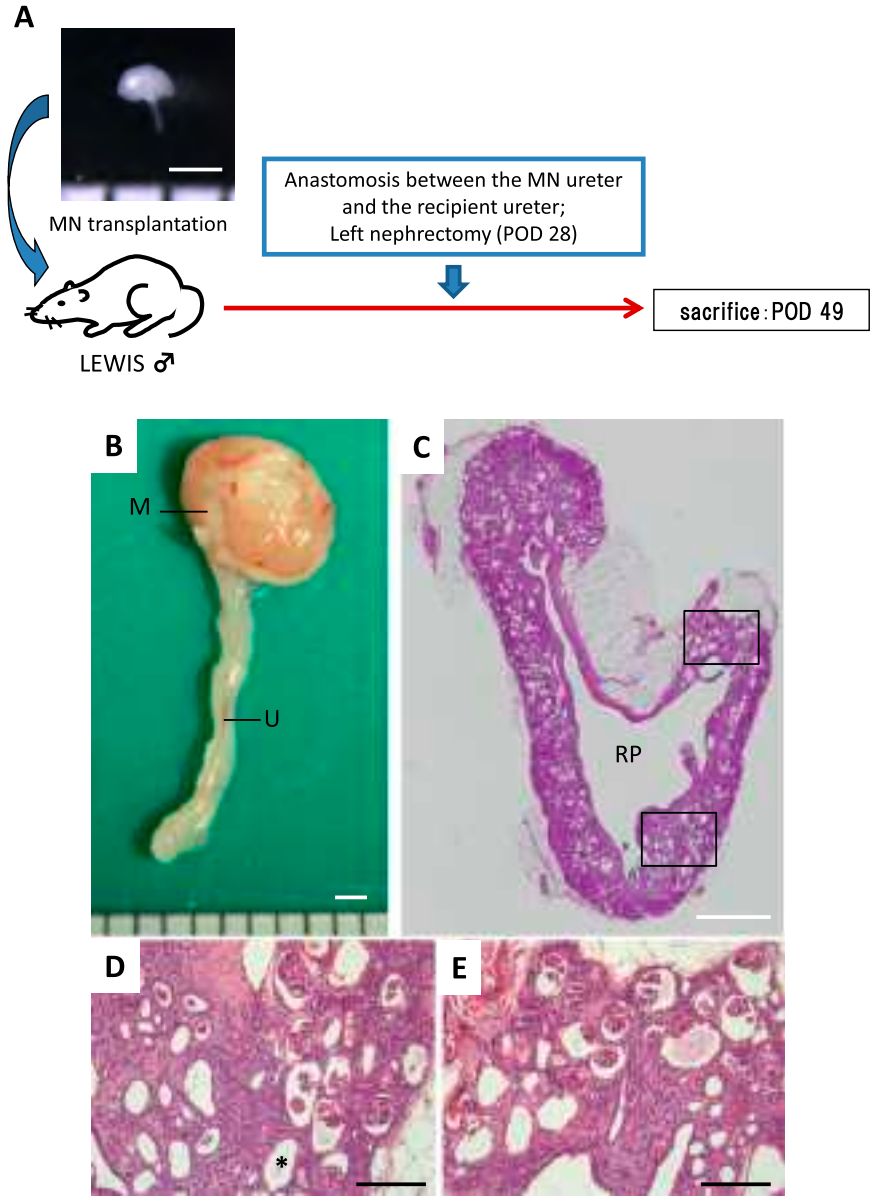


Fig. S7. Rat metanephros transplantation using uretero–ureterostomy. (*A*) E15 rat metanephroi were removed and transplanted into recipient rats. Four weeks later, the left native kidney was removed, and the recipient left ureter was connected with the metanephros-grown ureter. Three weeks after this surgery, the developed metanephros was removed. POD, postoperative day. (*B*) Seven weeks posttransplantation, the metanephros had grown to about 5 mm in length. (*C–E*) Histology of control rats undergoing uretero–ureterostomy reveals thinning of the cortex and severe dilation of renal pelvis, renal tubule, and Bowman's capsule compared with rats subjected to the SWPU system, resulting in hydronephrosis. M, metanephros; RP, renal pelvis of metanephros; U, recipient ureter. (Scale bars: 1 mm in *A–C*; 200 μm in *D* and *E*.)

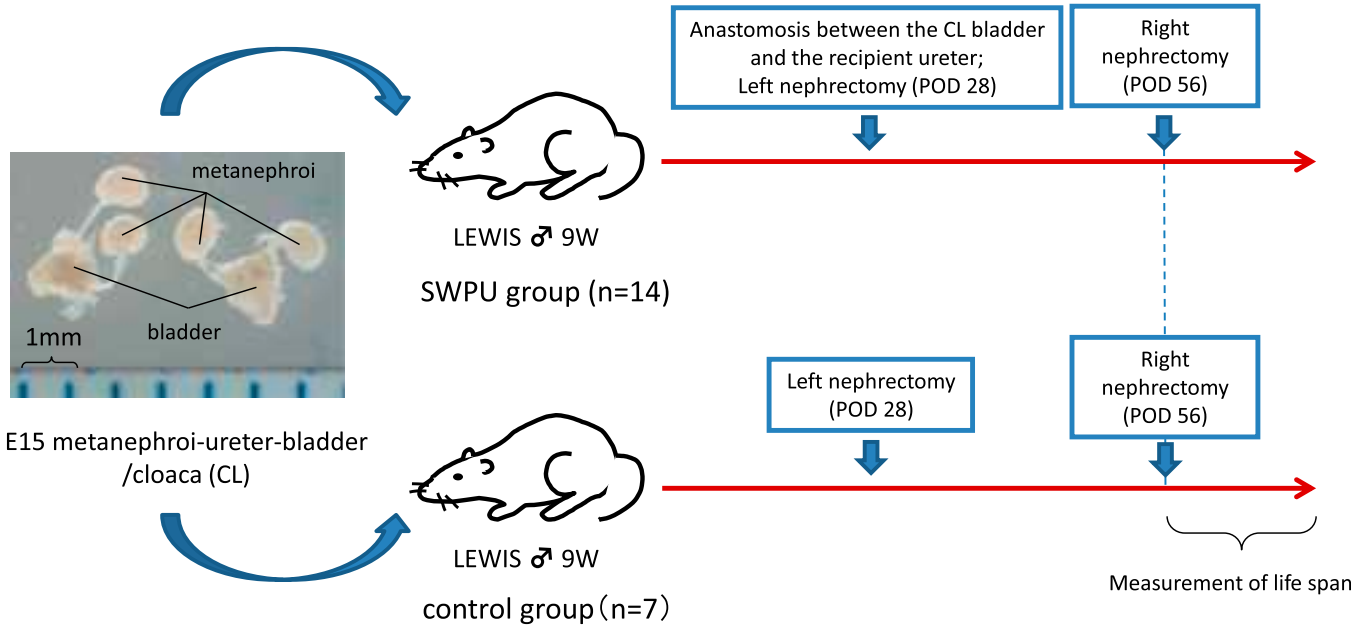


Fig. S8. Survival of rats transplanted with the SWPU system. The E15 rat metanephroi and bladder tissue developed from the cloacal transplants were removed under microscopic guidance and were implanted in the para-aortic areas of recipient rats ($n = 21$). Four weeks after transplantation, rats in the SWPU group ($n = 14$) underwent left nephrectomy and received an anastomosis between the bladder grown from the cloacal transplant and recipient's left ureter. Rats in the control group ($n = 7$) underwent left nephrectomy only. Eight weeks after transplantation, all rats underwent right nephrectomy. Rat life spans were measured from the time of right nephrectomy.

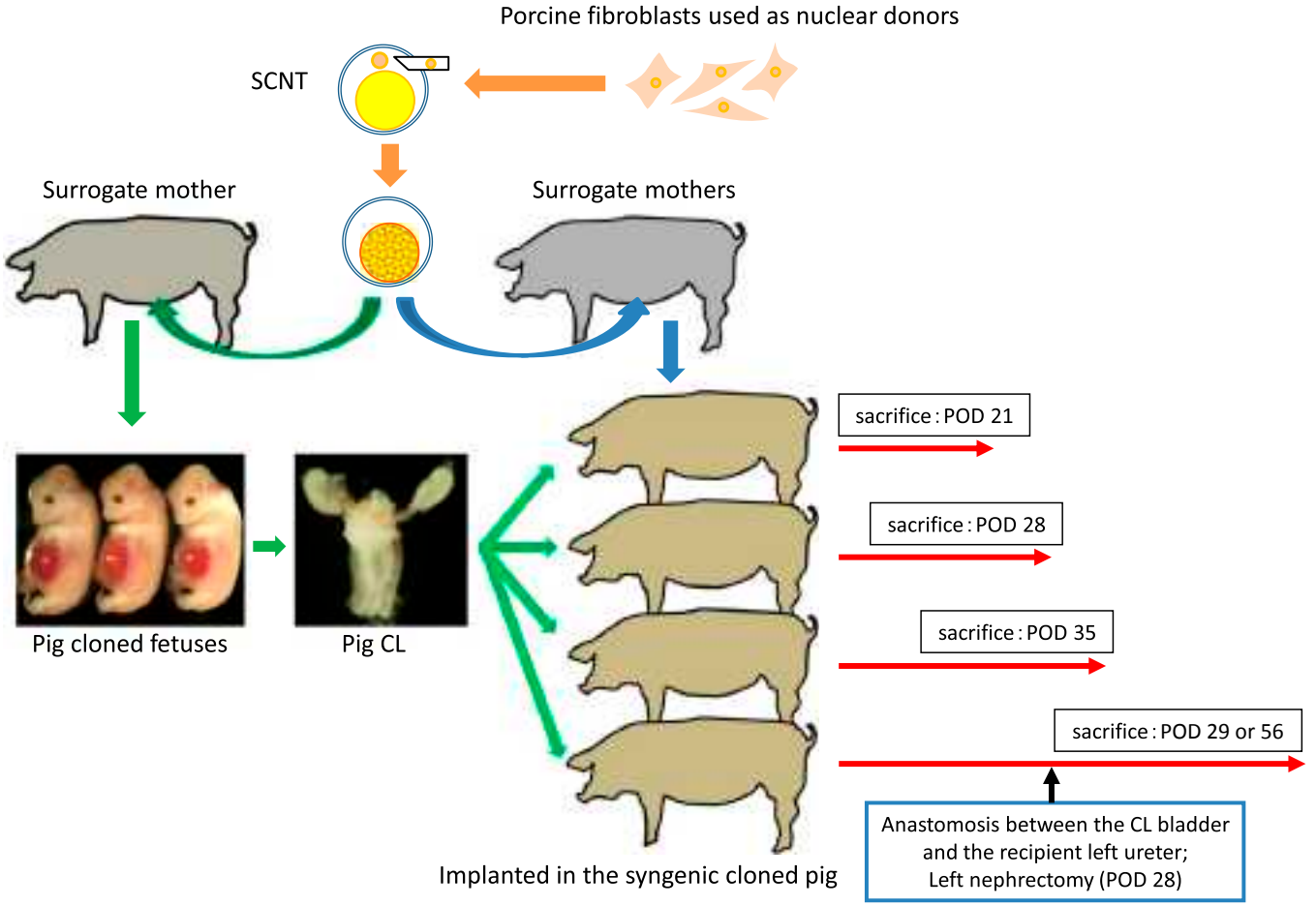


Fig. S9. Syngeneic metanephroi and bladder transplantation in pigs using the SWPU system. Cloned E30 pig cloacas were implanted into syngeneic, adult cloned pigs. Four weeks after implantation, recipient pigs underwent the SWPU system. One day or four weeks after this surgery, the developed cloacas were removed for examination.

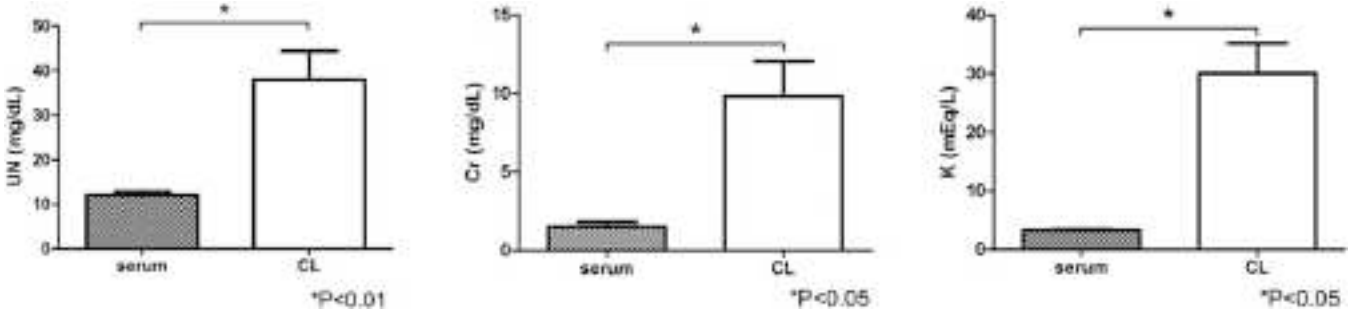
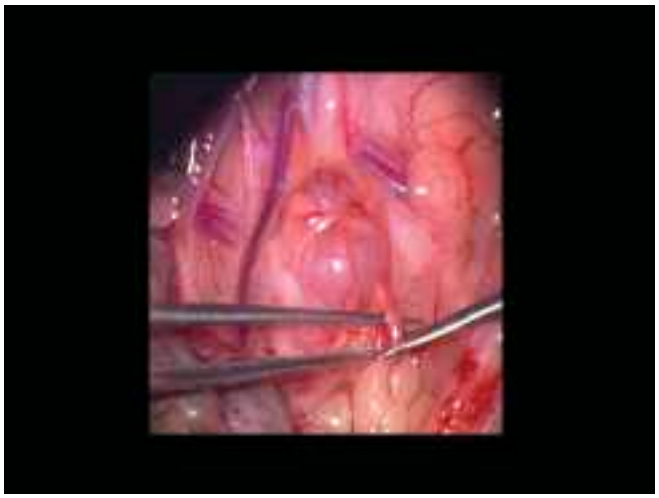


Fig. S10. Biochemical analysis of urine from metanephroi and bladders grown from cloacal transplants. Four weeks after transplantation, the levels of UN, Cr, and K were much higher in the urine from the bladders grown from cloacal transplants than in the sera of recipient pigs ($P < 0.01$, $P < 0.05$, $P < 0.05$, respectively). CL, urine in bladders grown from cloacal transplants.



Movie S1. Urine excretion from a bladder grown after a cloacal transplant. Seven to eight weeks after cloacal transplantation (3–4 wk after ureter–bladder anastomosis), urine from the bladder grown after a cloacal transplant discharged continuously from the connected recipient ureter.

Movie S1