

Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs

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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. 1B and Fig. S1). These vestigial pancreata consisted of loose connective tissue dotted with ductal structures and small islands of epithelial cells (Fig. 1C). No cells showed acinar differentiation; such cells were observed in the pancreas of a normal fetus (Fig. 1C) 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. 1D 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,

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The authors declare no conflict of interest.

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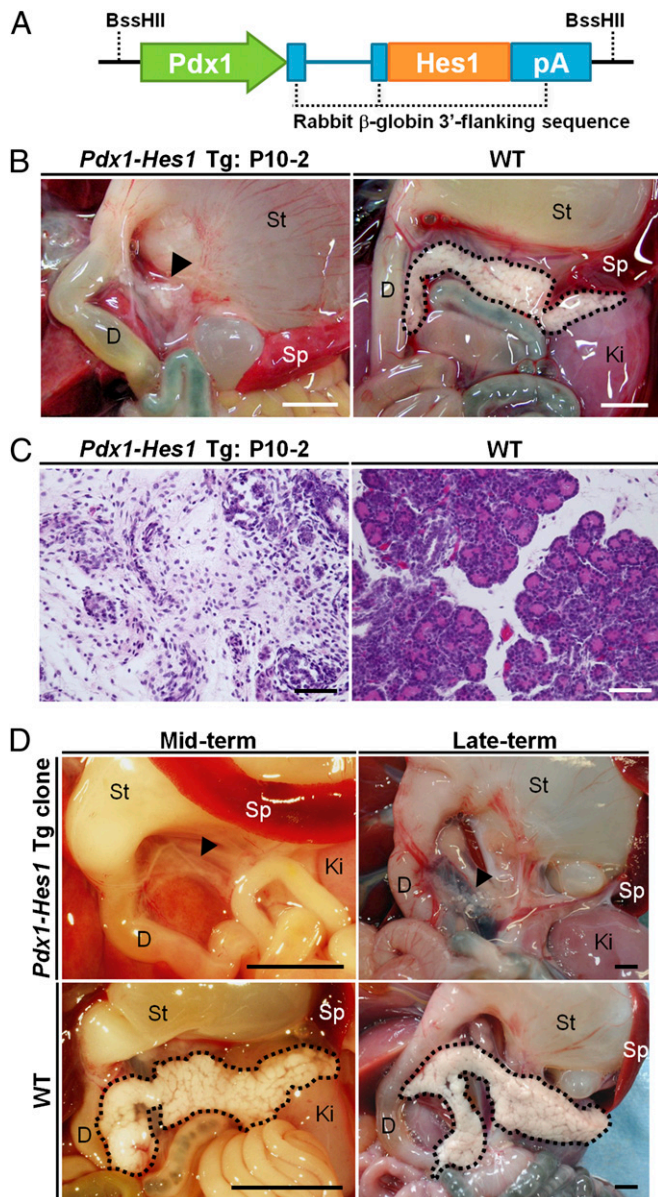


Fig. 1. Generation of pancreatogenesis-disabled pigs. (A) Construction of a *Pdx1-Hes1* expression vector consisting of the mouse *Pdx1* promoter, mouse *Hes1* cDNA, and rabbit β -globin 3' flanking sequence, including the polyadenylation signal (pA). (B and C) Macroscopic and microscopic appearances 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 μ m.)

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. 3A). Chimeric blastocysts ($n = 96$) obtained after culture for 1 or 2 d were transferred to the uteri of two estrus-synchronized recipient gilts (Fig. 3B). We obtained 14 late-term fetuses (day 110 or 111) in which we assessed pancreas formation (Fig. 3C–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. 3C, 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. 3C, *Hes1*) and 4 female fetuses derived from *huKO* cells and expressing orange fluorescence (Fig. 3C, *huKO*), were not chimeric, as substantiated by PCR analysis.

Formation of morphologically normal pancreata in the chimeric fetuses was confirmed by necropsy (Fig. 3D). Orange fluorescence of the donor cells was evident throughout the generated pancreata (Fig. 3D). 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. 3E). Almost all pancreatic tissues, including islets, acinar tissue, and ducts, stained with anti-*huKO* antibody (Fig. 3E). 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. S2A). Immunostaining of skin, lung, and kidney of four chimeric fetuses revealed that ~ 40 –60% of each sample by area expressed *huKO* (Fig. S2B).

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 \times 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. 4A); these were farrowed in the same litters as the chimeric offspring (Fig. 4A 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. 4C, 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).

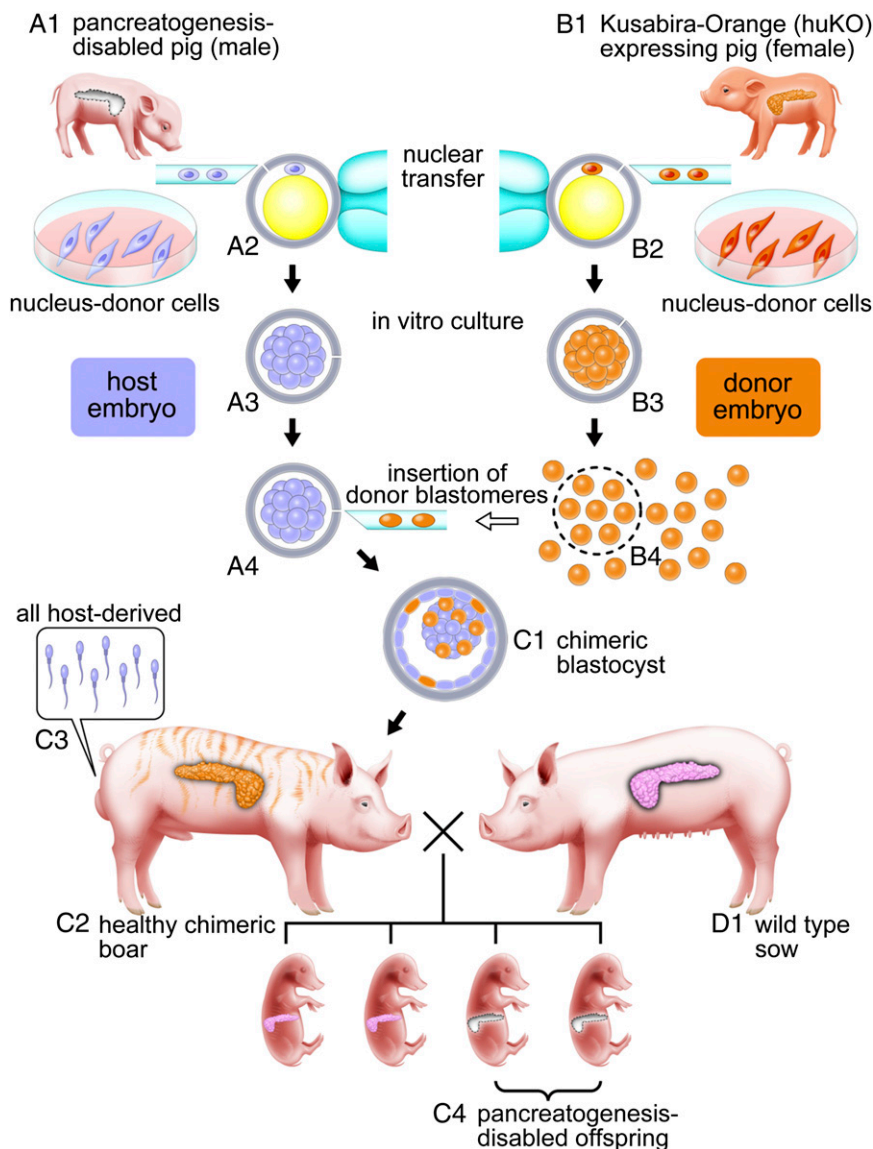


Fig. 2. Schematic representation of complementation for *Pdx1-Hes1* cloned pig embryos with a pancreatogenesis-disabled phenotype using cloned embryos expressing *huKO*. Primary fibroblast cells as nucleus donor cells for somatic cell cloning were established from a pancreatogenesis-disabled cloned pig 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. 4B and D). As shown in Fig. 4E, 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. 4F).

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. 4G). The pancreas (Fig. 4H), the duodenum, and the extrahepatic biliary tract lacked any histopathological abnormalities. On immunostaining for *huKO*, nearly every pancreatic cell marked for *huKO* (Fig. 4H 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)

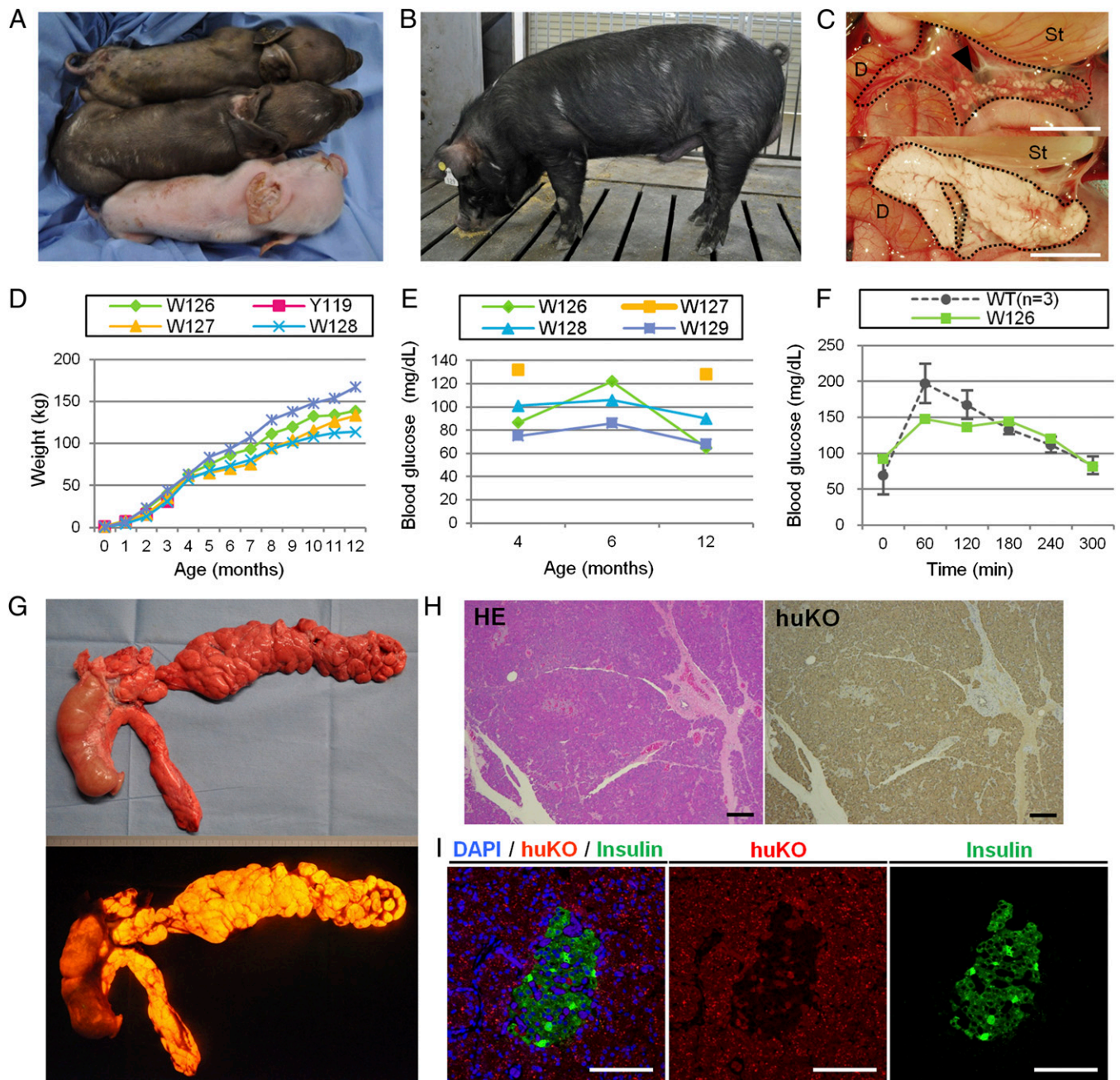


Fig. 4. Production of chimeric offspring by complementation of *Pdx1-Hes1* pancreatogenesis-disabled embryos. (A) Chimeric pig (middle pig) was obtained after complementation of host *Pdx1-Hes1* embryos with embryonic cells from a coat-colored WT donor. Sibling nonchimeric cloned pigs were derived from 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 μ m.)

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 μ L) were cocultured with 2.5 ng/ μ L 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 cytoplasm 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 pancreatic 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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ -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'-AGGTGGTCTTGAAGTGGCACTTGTG-3' for the first round of PCR and the primers 5'-

ACCTTACACAGTCTGCAGACC-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'-CAATGATGGCTCCAGGG-TAA-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.

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Supporting Information

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SI Materials and Methods

Fluorescence in Situ Hybridization. Fetal fibroblast cells derived from a pancreatogenesis-disabled transgenic pig were cultured in MEM α containing 15% FBS (vol/vol) for 3 d. The cells were then cultured with 30 μ g/mL BrdU for 4 h, followed by incubation with 0.02 μ g/mL colcemide for 2 h. After fixation with methanol-acetic acid (3:1 ratio), the cells were spread on slides

and air-dried. The cells were then stained with Hoechst 33258 and treated with UV light for G-banding. *Pdx1* (pancreatic and duodenal homeobox 1)-*Hes1* (hairy enhancer split 1). DNA was labeled with digoxigenin-11-dUTP as a probe and hybridized at 37 °C overnight. After stringent washing, the bound label was detected with anti-Dig-Cy3 using Leica DRAM2 and CW4000 FISH software.

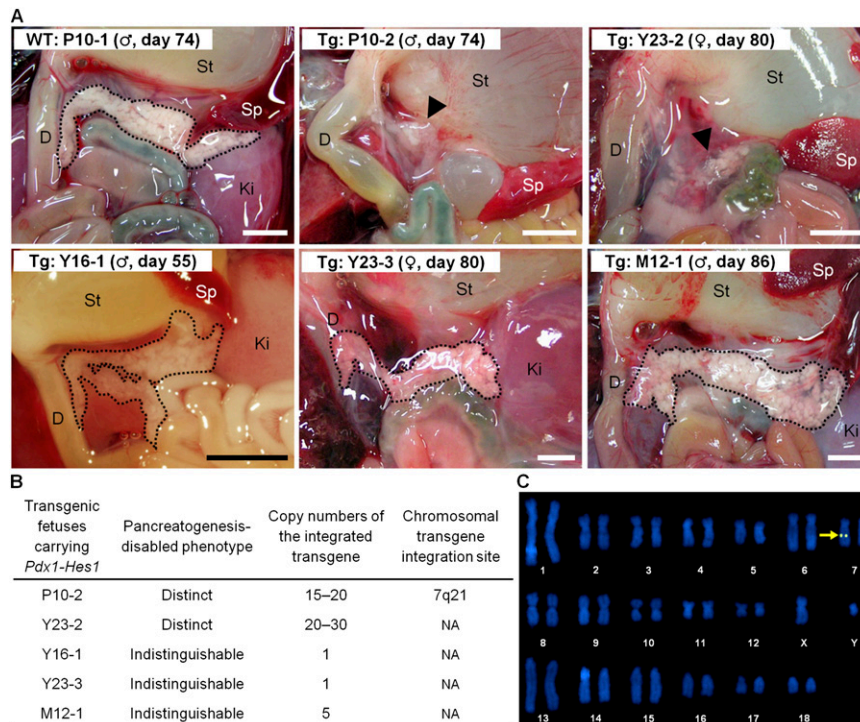


Fig. S1. Production details of pancreatogenesis-disabled *Pdx1-Hes1* transgenic pig fetuses. (A) Among the five transgenic fetuses obtained, a distinct pancreatogenesis-disabled phenotype was observed in one male (P10-2) and one female (Y23-2). These fetuses each had a vestigial pancreas (arrowhead) apparently less than 5% of normal size compared with that of a WT sibling fetus (P10-1). The pancreata of three other transgenic (Tg) fetuses (Y16-1, Y23-3, and M12-1) appeared indistinguishable from normal pancreata (P10-1). D, duodenum; Ki, kidney; Sp, spleen; St, stomach. (B) Phenotypic characteristics of the *Pdx1-Hes1* transgenic pig fetuses and integration patterns of the transgene. NA, not analyzed. (C) Karyogram for the pancreatogenesis-disabled *Pdx1-Hes1* transgenic fetus (P10-2) with fluorescent in situ hybridization, indicating single-site transgene integration (arrow). (Scale bars: A, 5 mm.)

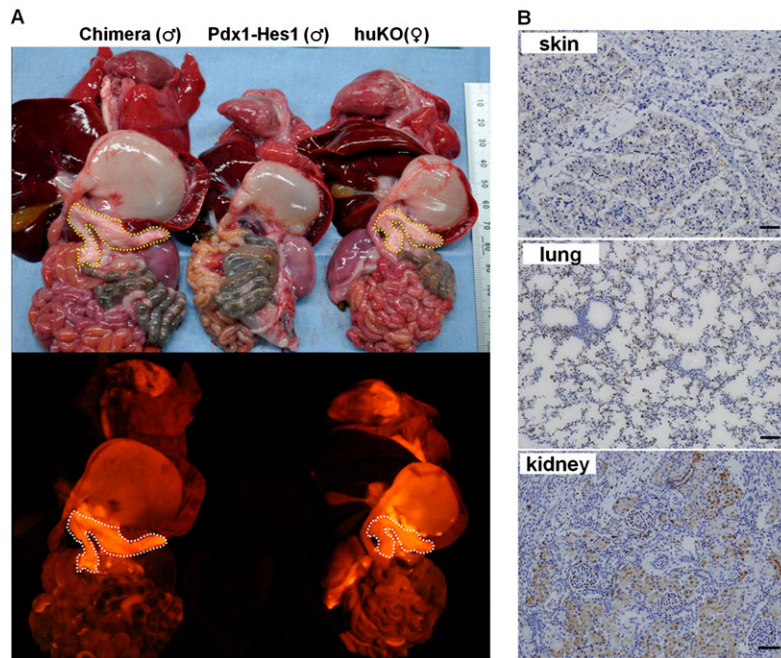


Fig. S2. Regeneration of the missing pancreata from exogenic cells in chimeric pig fetuses by complementation of *Pdx1-Hes1* clone embryos with the cloned embryos carrying the gene encoding orange fluorescent protein humanized Kusabira-Orange (*huKO*). (A) Orange fluorescence was observed systemically in chimeric fetuses, as seen in nonchimeric cloned fetuses derived from *huKO* cells. Orange fluorescence in the pancreata of the chimeric fetuses was prominent, indicating that these pancreata consisted primarily of donor cells expressing *huKO*. In contrast, a nonchimeric pancreatogenesis-disabled fetus derived from a *Pdx1-Hes1* embryo displayed no fluorescence. (B) Chimerism was confirmed histologically in all 10 of the organs/tissues observed, including skin, lung, and kidney, by immunostaining for *huKO*. (Scale bars: B, 50 μ m.)

A	Chimeric boar	No. fetuses obtained	Fetuses displaying the pancreatogenesis-disabled phenotype* (%)	Fetuses carrying the <i>huKO</i> transgene†
	W126	12	5 (41.7)	0
	W127	11	7 (63.6)	0
	W128	11	9 (81.8)	0
	W129‡	11	6 (54.5)	-

*Confirmed by PCR to contain *Pdx1-Hes1* transgene sequences.

†Confirmed by PCR.

‡Chimeric boar derived from a *Pdx1-Hes1* clone embryo and a colored-coat hybrid embryo.

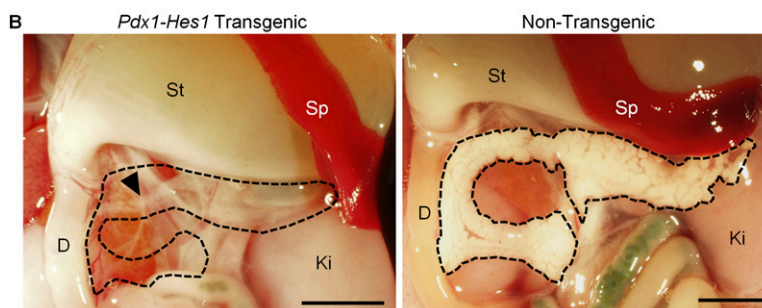


Fig. S3. Production details of chimeric pigs by blastocyst complementation and reproductive performance of the chimeric boars. (A) Transmission of the pancreatogenesis-disabled phenotype from chimeric boars to offspring. Each chimeric boar was mated with a WT sow, and fetuses between days 69 and 109 of gestation were examined. (B) Pancreatogenesis-disabled phenotype in offspring of a chimeric boar. Transgenic fetus (Left) and nontransgenic fetus (Right) littermates sired by a chimeric boar (W129) are shown at day 69 of gestation. The arrowhead (Left) and dotted area (Right) indicate a vestigial pancreas and a normally formed pancreas, respectively. D, duodenum; Ki, kidney; Sp, spleen; St, stomach. (Scale bars: B, 5 mm.)

Table S1. Production efficiency of pancreatogenesis-disabled pig fetuses by introduction of the *Pdx1-Hes1* transgene using intracytoplasmic sperm injection-mediated gene transfer

Recipient	No. of embryos transferred	Pregnancy	No. of fetuses, embryonic days	No. of transgenic fetuses	Pancreatogenesis-disabled phenotype
P9	106	—	—	—	—
P10	127	+	2 (day 74)	1	1*
P11	132	—	—	—	—
P12	95	+	Aborted	—	—
P20	136	—	—	—	—
P21	79	+	Aborted	—	—
P30	130	+	Aborted	—	—
P31	75	+	Aborted	—	—
Y16	62	+	1 (day 55)	1	0
Y17	124	—	—	—	—
Y23	70	+	3 (day 80)	2	1*
M11	53	+	Aborted	—	—
M12	93	+	5 (day 86)	1	0
Total	1,282	9	11 [0.9%, 11/1,282]	5 [0.4%, 5/1,282]	2 [0.2%, 2/1,282]

*A male fetus (P10-2) of recipient P10 and a female fetus (Y23-2) of recipient Y23 displayed the pancreatogenesis-disabled phenotype, whereas other fetuses had apparently normal pancreata.

Table S2. Reproduction efficiency of pancreatogenesis-disabled *Pdx1-Hes1* transgenic fetuses by somatic cell cloning

Recipient	No. of cloned embryos transferred	Pregnancy	No. of cloned fetuses obtained (%)	Age of the fetuses at laparotomy, d
P40	115	+	5 (4.3)	59
P41	114	+	4 (3.5)	110
Total	229	2	9 (3.9)	

Table S3. Production details of chimeric pig fetuses by complementation of *Pdx1-Hes1* clone embryos with *huKO* clone embryos

Type of SCNT embryos	<i>Pdx1-Hes1</i> transgenic [host embryos]* (%)	<i>huKO</i> transgenic [donor embryos]* (%)
In vitro production of chimeric blastocysts using cloned embryos		
SCNT embryos cultured	201	142
Normally cleaved embryos on day 2	154 (76.6%)	114 (80.3%)
Morula-stage embryos on day 4	146 (72.6%) [†]	110 (77.5%) [†]
Morulae used to produce chimeric embryos	97 (48.3%)	64 (45.1%)
Chimeric embryos produced	97 [‡]	
Chimeric blastocysts developed	96 (99.0%) [§]	
Production efficiency of chimeric fetuses after embryo transfer		
Recipient	B53 [¶]	B54 [¶]
Blastocysts transferred	45	51
Fetuses obtained	5 (11.1%)	9 (17.6%)
Chimeric fetuses	1 (20.0%) ^{**}	4 (44.4%) ^{**}
Nonchimeric fetuses derived from <i>Pdx1-Hes1</i> host embryo	3 (60.0%) ^{††}	2 (22.2%) ^{††}
Nonchimeric fetuses derived from <i>huKO</i> donor embryo	1 (20.0%) ^{**}	3 (33.3%) ^{**}

huKO, the gene encoding orange fluorescent protein humanized Kusabira-Orange; SCNT, somatic cell nuclear transfer.

**Pdx1-Hes1* ⇔ *huKO* chimera.

[†]More than 70% of the cloned embryos in both the host and donor embryo groups developed to the morula stage. These morulae, selected for blastomeres of uniform size and shape, were used to produce chimeric embryos.

[‡]Of 97 host embryos injected with ~10 donor blastomeres, 96 (99.0%)[§] developed into blastocysts after culture for 24 h. Transfer of those blastocysts on day 5 or 6 into two estrus-synchronized recipients[¶] gave rise to 14 fetuses,^{||} including 5 chimeric fetuses.^{**} Nonchimeric fetuses entirely reproducing the characteristics of the host^{††} or donor^{**} embryos were obtained in each litter.

Table S4. Comparison of features of chimeric and nonchimeric cloned fetuses (days 110 and 111)

Type of fetuses	Fetus	Body length, cm	Body weight, g	Sex	Weight of pancreas or vestigial pancreas, g	Pancreas weight per body weight, %
Chimera	B53-1	30	1,392	♂	1.416	0.102
	B54-1	25	802	♂	0.448	0.056
	B54-5	22.5	452	♂	0.344	0.076
	B54-8	19	288	♂	0.262	0.091
	B54-9	18	216	♂	0.183	0.085
Average		22.9 ± 2.2	630 ± 215.7		0.531 ± 0.4513	0.082 ± 0.016
<i>huKO</i> clone*	B53-2	27	842	♀	0.678	0.081
	B54-3	23	702	♀	0.521	0.074
	B54-4	24.5	688	♀	0.588	0.085
	B54-7	20	378	♀	0.383	0.101
Average		23.6 ± 1.5	652.5 ± 97.9		0.543 ± 0.1076	0.085 ± 0.010
<i>Pdx1-Hes1</i> clone [†]	B53-3	23.5	680	♂	0.008	0.001
	B53-4	23	650	♂	0.041	0.006
	B53-5	21	458	♂	0 [‡]	0
	B54-2	23	596	♂	0 [‡]	0
	B54-6	22.5	490	♂	0.014	0.003
Average		22.6 ± 0.4	574.8 ± 43.6		0.013 ± 0.0076	0.002 ± 0.002

huKO, the gene encoding orange fluorescent protein humanized Kusabira-Orange.

*Nonchimeric cloned fetuses derived from donor embryos.

[†]Nonchimeric cloned fetuses derived from host embryos.

[‡]No vestigial pancreatic tissue was collectable.

Table S5. Production efficiency of chimeric embryos and offspring using somatic cell nuclear transfer embryos: *Pdx1-Hes1* ⇌ *huKO* chimera

Type of SCNT embryos	<i>Pdx1-Hes1</i> (host embryos)	<i>huKO</i> (donor embryos)
In vitro production of chimeric blastocysts using SCNT embryos		
SCNT embryos cultured	199	148
Normally cleaved embryos on day 2	174 (87.4%)	106 (71.6%)
Morula-stage embryos on day 4	145 (72.9%)	92 (62.2%)
Morulae used to produce chimeric embryos	113 (56.8%)	76 (71.6%)
Chimeric embryos produced		113
Chimeric blastocysts developed		109 (96.5%)
Production efficiency of chimeric pigs after embryo transfer		
Recipient	B77	B78
Blastocysts transferred	58 (day 6)	51 (day 5)
Piglets obtained	6 (10.3%)	7 (13.7%)
Chimeric piglets	3 (50.0%)	1 (14.3%)
Nonchimeric piglets derived from <i>Pdx1-Hes1</i> host embryo	3 (50.0%)	5 (71.4%)
Nonchimeric piglets derived from <i>huKO</i> donor embryo	0 (0%)	1 (14.3%)

huKO, the gene encoding orange fluorescent protein humanized Kusabira-Orange; SCNT, somatic cell nuclear transfer.

Table S6. Production efficiency of chimeric embryos and offspring using somatic cell nuclear transfer embryos: *Pdx1-Hes1* ⇌ WT chimera

Type of SCNT embryos	<i>Pdx1-Hes1</i> (host embryos)	WT* (donor embryos)
In vitro production of chimeric blastocysts using SCNT embryos		
SCNT embryos cultured	103	74
Normally cleaved embryos on day 2	81 (78.6%)	56 (75.6%)
Morula-stage embryos on day 4	66 (64.1%)	56 (75.6%)
Morulae used to produce chimeric embryos	92 (96.1%)	70 (94.6%)
Chimeric embryos produced		92
Chimeric blastocysts developed		80 (87.0%)
Production efficiency of chimeric pigs after embryo transfer		
Recipient		B83
Blastocysts transferred		80 (day 6)
Piglets obtained		6 (7.5%)
Chimeric piglets		1 (16.7%)
Nonchimeric piglets derived from <i>Pdx1-Hes1</i> host embryo		2 (33.3%)
Nonchimeric piglets derived from WT donor embryo		3 (50.0%)

SCNT, somatic cell nuclear transfer.

*Cloned embryos derived from a colored-coat WT sow (Duroc × Berkshire hybrid) were used as donor embryos.

Table S7. Serum biochemical profile in *Pdx1-Hes1* cloned piglets

Parameters	<i>Pdx1-Hes1</i> cloned offspring		Control [†] (n = 5)
	No. 1	No. 2	
	3-d-old	4-d-old	4- to 5-d-old*
TP, g/dL	6.7	5.4	5.0 ± 0.39
BUN, mg/dL	130.6	47.9	9.16 ± 2.76
CRE, mg/dL	1.8	0.9	0.36 ± 0.05
Na, mEq/L	157	130	136.4 ± 1.50
K, mEq/L	7.8	4.8	4.62 ± 0.65
Cl, mEq/L	121	97	94.8 ± 2.86
Ca, mg/dL	6.82	6.22	6.04 ± 0.43
IP, mg/dL	>4.84 [‡]	4.38	2.57 ± 0.79
AST, U/L	220	27	78.6 ± 61.93
ALT, U/L	95	108	44.2 ± 17.46
TCHO, mg/dL	143	121	139.2 ± 66.17
TG, mg/dL	109	33	106.6 ± 17.23
GLU, mg/dL	>600 [‡]	>600 [‡]	140.6 ± 36.64
Insulin, ng/mL	ND	ND	2.003 ± 0.99

ALT, alanine aminotransaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ca, calcium; Cl, chloride; CRE, creatinine; GLU, glucose; IP, inorganic phosphate; K, potassium; Na, sodium; ND, nondetectable; TCHO, total cholesterol; TG, triglyceride; TP, total protein.

*Mean ± SD values are provided.

[†]WT piglets of the same strain and age were used as controls.

[‡]Above the highest detectable level.

Table S8. Serum biochemical profile in chimeric pigs

Parameters	Chimera		Control	
	6-mo-old (n = 3)	12-mo-old (n = 4)	6-mo-old (n = 5)	12-mo-old (n = 4)
TP, g/dL	7.6 ± 0.33	8.1 ± 0.19	7.58 ± 0.40	7.0 ± 0.72
ALB, g/dL	3.6 ± 0.26	3.8 ± 0.54	3.28 ± 0.35	3.7 ± 0.26
A/G	0.9 ± 0.26	1.0 ± 0.26	0.8 ± 0.20	1.2 ± 0.08
BUN, mg/dL	9.9 ± 0.96	11.7 ± 2.32	13.22 ± 2.80	10.4 ± 2.14
CRE, mg/dL	1.02 ± 0.08	1.63 ± 0.15	1.294 ± 0.13	1.74 ± 0.19
Na, mEq/L	144 ± 2.05	141 ± 2.28	141.8 ± 1.47	140.5 ± 1.50
K, mEq/L	4.4 ± 0.12	4.8 ± 0.11	5.22 ± 0.65	4.5 ± 0.33
Cl, mEq/L	98 ± 4.78	99 ± 2.86	99.2 ± 3.19	101.0 ± 3.74
Ca, mg/dL	10.0 ± 0.08	9.8 ± 0.19	10.2 ± 0.36	9.4 ± 0.55
IP, mg/dL	7.2 ± 0.17	7.0 ± 0.58	7.7 ± 1.03	5.5 ± 0.53
AST, IU/L	59 ± 32.22	35 ± 3.90	32.6 ± 7.42	27 ± 5.24
ALT, IU/L	44 ± 7.41	39 ± 15.35	29.4 ± 3.26	38 ± 4.50
ALP, IU/L	335 ± 24.57	206 ± 61.64	504 ± 210.34	256 ± 75.72
γ-GT, IU/L	34 ± 16.58	53 ± 30.41	24.8 ± 11.05	48 ± 9.41
LIP, IU/L	10 ± 1.25	6.5 ± 1.50	10.6 ± 1.74	6.8 ± 1.30
TCHO, mg/dL	68 ± 7.12	60 ± 9.01	85.4 ± 8.28	58 ± 10.62
TG, mg/dL	30 ± 5.66	39 ± 10.71	44 ± 13.84	29 ± 16.32
HDL-C, mg/dL	28 ± 0.47	25 ± 5.36	39.6 ± 5.61	25 ± 2.92
TBIL, mg/dL	0.07 ± 0.03	0.06 ± 0.04	0.04 ± 0.01	0.07 ± 0.03
GLU, mg/dL	105 ± 14.73	88 ± 25.16	110 ± 37.22	86 ± 9.94
GA, %	4.5 ± 1.14	5.2 ± 1.12	3.96 ± 0.84	6.0 ± 0.31
Insulin, ng/mL	1.851 ± 0.88	1.360 ± 0.803	3.220 ± 0.85	1.907 ± 2.44

Mean ± SD values are provided. A/G, albumin-globulin ratio; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ca, calcium; Cl, chloride; CRE, creatinine; GA, glycoalbumin; GLU, glucose; γ-GT, γ-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; IP, inorganic phosphate; K, potassium; LIP, lipase; Na, sodium; TBIL, total bilirubin; TCHO, total cholesterol; TG, triglyceride; TP, total protein.