156 injection of 1000 IU of eCG to induce estrus. Ovulation was induced by an intramuscular injection of
157 1500 IU of hCG (Kawasaki Pharmaceutical, Kanagawa, Japan) given 66 h after the injection of eCG.
158 Sperm-injected embryos cultured for 1-3 days were surgically transferred into the oviducts of
159 recipients approximately 48 h or 72 h after hCG injection.

160 All but one of the pregnant recipients were laparotomized to recover fetuses at 47-65 days of
161 gestation, and the remaining recipient was allowed to farrow.

162

163 *PCR and Southern blot analyses*

164 Genomic DNA was extracted from tail biopsies of fetuses and newborn piglets using proteinase
165 K (Life Technologies Corporation, Carlsbad, CA, USA) and purified by the phenol-chloroform
166 method. To identify Tg pigs, DNA samples were analyzed by PCR using the following primers:
167 5'-caatgatggctccagggtaa (forward) and 5'-ctccttgaagtcgatgccctt (reverse).

168 For Southern blot analysis, genomic DNA extracted as described above was digested with the
169 *Pst*I restriction enzyme (Takara Bio), separated by gel electrophoresis, and transferred onto a nylon
170 membrane (GE Healthcare, Buckinghamshire, UK), which was then hybridized with the DIG-labeled
171 probes prepared by PCR using the following primers: 5'-caatgatggctccagggtaa (forward) and
172 5'-ggtggtgcagatcagcttca (reverse). The signal (i.e., binding of the probe) was detected by chromogenic
173 methods. The number of transgene copies integrated into the porcine genome was determined by
174 comparison of the hybridization signal with that of the copy-number control, which was diluted to
175 make a standard series (1-100 copies per diploid genome).

176

177 *Pancreas-specific fluorescence expression in Tg fetuses and G1 offspring*

178 The tails of the fetuses (day 47-65) obtained from autopsies of the sacrificed pregnant pigs were
179 used to extract genomic DNA. Tg fetuses were identified by PCR. Fetal viscera were also removed,
180 and the expression of green fluorescence in the organs was analyzed by fluorescence stereomicroscopy

(MVX10, Olympus, Tokyo, Japan; excitation wavelength of 460-480 nm; absorption filter of 495-540 nm). Pancreatic tissue samples from fetuses were fixed in 4% paraformaldehyde and used to prepare paraffin-embedded sections (hematoxylin/eosin stain). The paraffin-embedded sections were also analyzed by fluorescence microscopy (Olympus BX52; excitation wavelength of 460-480 nm; absorption filter of 495-540 nm).

A subset of the founder Tg pigs was allowed to grow to maturity and was mated with wild-type pigs. The offspring (G1) obtained were sacrificed when they reached the age of 27 days to examine pancreas-specific fluorescence expression by fluorescence stereomicroscopy.

Pancreatic tissue samples of the founder Tg pig (G0) were double-stained using anti-insulin (1:500; LS-C24686, LifeSpan BioSciences, Seattle, WA, USA) and anti-GFP (1:500-1:1000; #598, MBL Co., Ltd., Nagoya, Japan) antibodies to determine the Venus-expressing cells in the pancreatic islets. Alexa Fluor® 594 goat anti-guinea pig IgG (A11076, Life Technologies) and Alexa Fluor® 488 donkey anti-rabbit IgG (A21206, Life Technologies) were used as the secondary antibodies. The tissue sections were also double-stained for glucagon and Venus. For glucagon staining, anti-glucagon antibody (1:500; G2654) and Alexa Fluor®594 goat anti-mouse IgG (A11020, Life Technologies) were employed. After antibody treatments, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4,6 -diamidino-2-phenylindole (DAPI) for nuclear counterstaining and observed by confocal laser scanning microscopy (FV1000-D; Olympus Corporation, Tokyo, Japan).

Fluorescence in situ hybridization

Peripheral blood cells derived from the two Tg founder pigs (male and female) were cultured in RPMI1640 containing 20% (v/v) FBS for 3 days. The cells were then cultured with 30 μg/ml BrdU for 5 h, followed by incubation with 0.02 μg/ml colcemide for 1 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

206 33258 and treated with UV light for G-banding. *Pdx1-Venus* DNA was labeled with Cy3 as a probe
207 and hybridized at 37 C overnight. After stringent washing, the bound label was detected with
208 anti–Dig-Cy3 using Leica DRAM2 and CW4000 FISH software.
209
210 *Tracing of pancreatic islets by fluorescence after ectopic transplantation*
211 Pancreatic islets were isolated from a 4.5-month-old Tg pig using a conventional method. The
212 pancreas collected from a Tg pig was distended by infusion with Liberase DL (Roche Diagnostics,
213 Indianapolis, IN, USA) suspended in Hank's balanced salt solution (HBSS; Life Technologies),
214 followed by a static incubation in an empty 125 ml storage bottle for 30 min at 37 C. Then the
215 digesting pancreatic tissue was gently shaken with 7 mm Teflon® beads in RPMI 1640 (Life
216 Technologies). Digestion was terminated by the addition of cold HBSS containing 10% (v/v) FBS,
217 100 IU/ml of penicillin, 100 mg/ml of streptomycin, and 2.5 μg/ml amphotericin B. The digested
218 tissue was passed through a 500 μm stainless steel mesh screen. The tissue effluent was collected in 50
219 ml conical tubes and centrifuged for 2 min at 155 ×*g* at 4 C. The islets were purified using a
220 Histopaque®-1.077 gradient with RPMI 1640. Following centrifugation at 1700 ×g for 17 min at 4 C,
221 the islets were collected from the interface between the RPMI 1640 and Histopaque®-1.077. Purified
222 islets were washed by centrifugation at 155 ×g for 2 min at 4 C in RPMI 1640 supplemented with 10%
223 (v/v) FBS. The purity of the isolated islets was confirmed to be over 90% by microscopic inspection
224 after Dithizone (5 mg/ml, in DPBS) staining.
225 Fluorescence in the isolated islets was observed by fluorescence stereoscopic microscopy
226 (MVX10, Olympus). Isolated islets were then transplanted under the renal capsules of anesthetized
227 NOD/SCID mice (CLEA Japan, Inc., Tokyo, Japan). Kidneys were removed either immediately or at
228 one month after transplantation and analyzed by fluorescence stereomicroscopy (MVX10, Olympus)
229 to determine whether the islets could be traced using Venus fluorescence as an indicator.
230

231 **Results**

232 *Efficiency of production of Pdx1-Venus Tg pigs by ICSI-MGT*

233 The ICSI-MGT method was selected for creating *Pdx1-Venus* Tg pigs. In total, 370
234 sperm-injected embryos were transferred into four recipients, all of which became pregnant.

235 Three of the recipient pigs were autopsied at 47-65 days of gestation, and 16 fetuses were
236 recovered for analysis (Table 1). The production efficiency of fetuses was between 4 and 8%, as each
237 recipient received approximately 80 embryos. Seven of the 16 fetuses were Tg (43.8%), including
238 approximately 30% of the fetuses in two of the recipients and all three fetuses in one recipient. Overall,
239 2.4-3.7% of the transferred embryos produced Tg fetuses.

240 The fourth pregnant pig, which received 127 embryos, was allowed to farrow and produced six
241 (4.7%) piglets, two of which were Tg (one female and one male).

242

243 *Pancreas-specific expression of Venus in Tg fetuses and offspring*

244 The viscera of the seven Tg fetuses obtained were examined by fluorescence stereomicroscopy,
245 and we found that all the fetuses had pancreas-specific expression of Venus fluorescence (Fig. 2A, B).
246 The Southern blot analysis of genomic DNAs indicated an integration of 5 to 100 copies of the gene.
247 Although the fluorescence intensity tended to be greater in fetuses with higher copy numbers ($\geq$15),
248 except for a female fetus (W8-1) harboring 30 copies of the gene, pancreas-specific expression was
249 clear in all fetuses regardless of the copy number (Table 2).

250 A histological analysis of pancreatic tissues of four Tg fetuses showed that Venus fluorescence
251 was present in cells determined to be acinar cells based on their appearance. This expression pattern
252 was consistent among all fetuses analyzed (Fig. 2C, D).

253 The two founder (G0; male and female) Tg pigs grew normally to adulthood and were crossed
254 with wild-type pigs to produce G1 offspring of six litters. Of the 22 G1 pigs obtained from the male
255 founder and the 28 G1 pigs derived from the female founder, the transgene was transmitted to ten

256 (45.5%) and 16 pigs (57.1%), respectively, indicating that the transgene was transmitted in the
257 Mendelian fashion. It was found that 10 and 30 transgene copies were integrated into the genomes of
258 the male and female founder pigs, respectively. FISH analysis of these founder Tg pigs revealed that
259 concatemerized transgenes were integrated into a single site on the chromosomes (Suppl. Fig. 1).
260 Four 27-day-old G1 piglets (Tg female and male, non-Tg female and male) were autopsied to
261 examine fluorescence expression in their viscera. The pancreas, duodenum, small intestine, liver,
262 spleen, kidneys, skin, heart, lungs, and stomach were observed under a fluorescence stereomicroscope.
263 This analysis confirmed the retention of pancreas-specific fluorescence expression (Fig. 3A and Suppl.
264 Fig. 2) as in the founder Tg fetuses. Green fluorescence was not detected in the viscera of non-Tg pigs.
265 The pancreatic tissue of the G1 Tg pigs showed green fluorescent spots throughout (Fig. 3A),
266 indicating *Pdx1-Venus* expression in islets. Venus expression was found to be confined to β-cells in the
267 pancreatic tissue after double staining with anti-insulin and anti-GFP antibodies (Fig. 3B).
268
269 *Tracing of the fluorescence expression of pancreatic islets*
270 To further examine the potential of *Pdx1-Venus* Tg pigs for future use in pancreatic islet
271 research, we investigated the traceability of the pancreatic islets using their fluorescence as an
272 indicator. As shown in Fig. 4, Venus fluorescence expression patterns were clearly observed under a
273 fluorescence stereomicroscope, which confirmed clear fluorescence spots in the islets (Fig. 4A, A').
274 The isolated islets were transplanted under the renal capsules of NOD/SCID mice, and the
275 transplanted islets could clearly be identified by their fluorescence. The fluorescence of the
276 transplanted pancreatic islets was still clear at 30 days after transplantation (Fig. 4C, C').
277

Discussion

This report describes the production of the first *Pdx1-Venus* Tg pig expressing green fluorescent protein specifically in the pancreas, particularly in β-cells. Pdx1 is a key molecule with an important role in pancreatic stem cell differentiation into β-cells [12, 13, 22, 23]. In fact, *Pdx1* knockout mice reportedly suffer impaired pancreatic development [12, 24]. The identification and separation of *Pdx1*-positive cells is therefore expected to stimulate new developments in research on islet architecture during the ontogeny and differentiation of β-cells from precursors [13, 25, 26]. Research on pancreas development and β-cell differentiation is also expected to lead to the pathophysiological analysis of diabetes and the development of new therapeutic methods [27]. In particular, the neogenesis of β-cells has been a recent focus in diabetes research [28-31].

In research using laboratory rodents, $Pdx1^{GFP/w}$ mice [32] and mouse insulin I gene promoter (MIP)-GFP Tg mice [33] have been created and used to conduct research on pancreatic development and differentiation. However, in research using pigs, a Tg model that is useful for the study of β-cell biology, including the identification of progenitor cells, has not been available. Considering that the importance of pigs, as a large laboratory animal with several similarities to humans, in translational research is now recognized and that research is being undertaken on the clinical applications of porcine islet transplantation [34], the *Pdx1-Venus* pig we have produced has strong potential for use as an effective research tool. The Expression pattern of the *Pdx1-Venus* in the islet of our transgenic pigs was similar to that reported previously in the $Pdx1^{GFP/w}$ mice [32].

In the present study, we employed the mouse *Pdx1* promoter to drive the *Venus* expression in the transgenic pigs. However the transgene was expressed in a highly tissue-specific manner. In fact, *Pdx1-Venus* expression was confined to the pancreas during the early fetal stage (day 47) and at the adult stage. *Pdx1* is also known to be expressed in the duodenum at the fetal stage [13]. Further studies need to be undertaken to examine the expression of the *Pdx1-Venus* in the early stages of pancreatogenesis in the transgenic pig fetuses.

303 Concerning *Pdx1-Venus* expression in the islets, we observed that cells that were Venus positive
304 were also insulin-positive cells. This pig is, accordingly, very useful for tracking the behavior of
305 pancreatic progenitor cells and β-cells.
306 *Pdx1-Venus* is also useful as a cell marker following islet transplantation. The clinical
307 application of islet transplantation using human islets has been hampered, as is the case with other
308 transplants, by the shortage of donor organs. However, if xenogeneic pancreas transplantation—more
309 specifically, the transplantation of pig islets to humans—becomes possible, substantial advances will
310 be made in treatments for diabetes patients [35]. Xenogeneic transplantation will require further basic
311 studies, including a long-term follow-up of islets transplanted to animals. *Pdx1-Venus* Tg pig islets
312 will serve as a very useful tool in such research. For example, production of insulin or C-peptide from
313 the transplanted islets may be correlated with the *Pdx1-Venus* expression that indicates the viability of
314 β-cells. We have already produced diabetic model Tg pigs by mutant hepatocyte nuclear factor-1α
315 gene transfer [2]. Transplanting islets from *Pdx1-Venus* Tg pigs using such diabetic models should
316 provide knowledge that can be extrapolated from large animals to humans.
317 *Pdx1-Venus* Tg pigs were observed to show a high level of green fluorescence expression in the
318 pancreas (β-cells) with normal pancreas function. This finding was confirmed by the pigs'
319 physiological characteristics, including growth, casual blood glucose levels, postprandial blood
320 glucose and insulin levels, and blood biochemical parameters, which were measured during the period
321 from the postweaning through the growth stages (Suppl. Text, Suppl. Fig. 3, and Suppl. Table 1).
322 Based on these results, we hypothesize that *Pdx1-Venus* Tg pigs may also be suitable as donor animals
323 in studies of islet transplantation.
324 In this study, we introduced transgenes using the ICSI-MGT method. We previously reported
325 that the application of ICSI-MGT is highly effective for introducing exogenous genes to porcine IVM
326 oocytes [2, 17]. In this study, approximately 30-100% of the fetuses/piglets obtained in each litter
327 were Tg, once more demonstrating the high efficiency of the ICSI-MGT method. The production

328 efficiency of Tg fetuses or piglets obtained in this study was equal or rather higher compared with our
329 previous studies, probably due to lower detrimental effect of the transgene expression [2, 36, 37]. *In*
330 *vitro* maturation of pig oocytes is now an established method, and the combination of IVM oocytes
331 and the ICSI-MGT method can accordingly be considered a practical method for generating Tg pigs.

332 Our previous research confirmed that transgenes introduced by the ICSI-MGT method
333 generally insert into a single site on the host genome as concatemers [17, 38]. In the founder Tg pigs
334 used for generating G1 offspring in this study, it was shown that the transgenes did concatamerize and
335 integrated into a single site of the chromosome as shown in our previous studies [17, 38]. No
336 significant differences in growth were observed in fetuses with transgene copy numbers between 5 and
337 100. The level of transgene expression is considered to be more readily influenced by the integration
338 site on the chromosome than by the integrated copy number [39, 40]. Even so, in the case of Tg
339 individuals with an exceptionally high number of integrated transgenes, it is possible that high-level
340 transgene expression may influence normality in piglets and affect their long-term survival. Because
341 the copy number of the integrated genes is affected by various factors related to the binding of DNA to
342 sperm [38, 41, 42], the preliminary optimization of the transgene-sperm co-incubation will be critical
343 for the efficient production of Tg pigs using the ICSI-MGT method.

344 In conclusion, building on our current knowledge, this study verifies that using IVM oocytes
345 and ICSI-MGT together is an effective method for producing Tg pigs. Additionally, because the
346 *Pdx1-Venus* Tg pigs produced in this study express green fluorescent protein specifically in the
347 pancreas (β-cells) and maintain normal physiological function, we can conclude that this large animal
348 model is suitable for research on pancreatic development and regeneration as well as diabetes.
349

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Table 1. Efficiency of the ICSI-MGT method for the production of Tg pig fetuses and offspring carrying the *Pdx1-Venus* gene.

	Recipient	No. of embryos transferred	Production efficiency of fetuses or offspring (%)[*1]	Production efficiency of Tg fetuses or offspring (%)[*2]
Fetus	W8	83	8.4 [7/83]	28.6 [2/7]
	W9	81	3.7 [3/81]	100 [3/3]
	W11	79	7.6 [6/79]	33.3 [2/6]
Offspring	W10	127	4.7 [6/127]	33.3 [2/6]

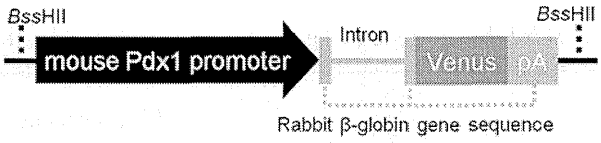
[*1] No. of fetuses or piglets / No. of embryos transferred × 100

[*2] No. of Tg fetuses or piglets / No. of fetuses or piglets obtained × 100

489 **Table 2.** Expression of the *Pdx1-Venus* gene in Tg pig fetuses produced by the ICSI-MGT method.

Fetus	Fetal age	Fetal sex	Fluorescence intensity	Transgene copy number
W8-1	Day 48	F	+	30
W8-5	Day 48	F	+	5
W9-1	Day 47	F	+	5
W9-2	Day 47	M	++	15
W9-3	Day 47	M	++	70
W11-2	Day 65	F	+	5
W11-5	Day 65	F	++	100≤

490



491

492 **Figure 1.** Structure of an expression vector for the *Pdx1-Venus* cDNA.

493 A schematic presentation of the *Pdx1-Venus* transgene used to generate transgenic pigs. The fusion

494 gene (8.4 kb) consists of 6.5 kb of the mouse Pdx1 promoter and a rabbit β-globin gene including an

495 insertion of 0.72 kb Venus cDNA in the 3rd exon and a polyadenylation signal in the 3 –flanking

496 region. Transcription and translation start site are indicated by +1 and M, respectively.

497

498

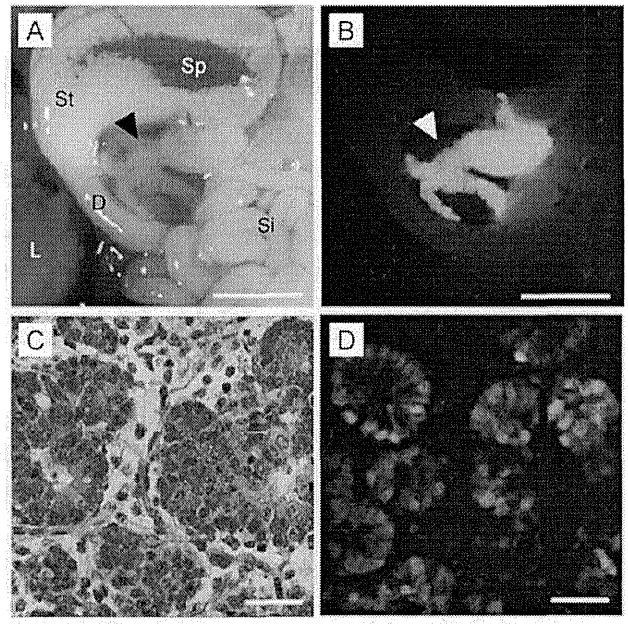
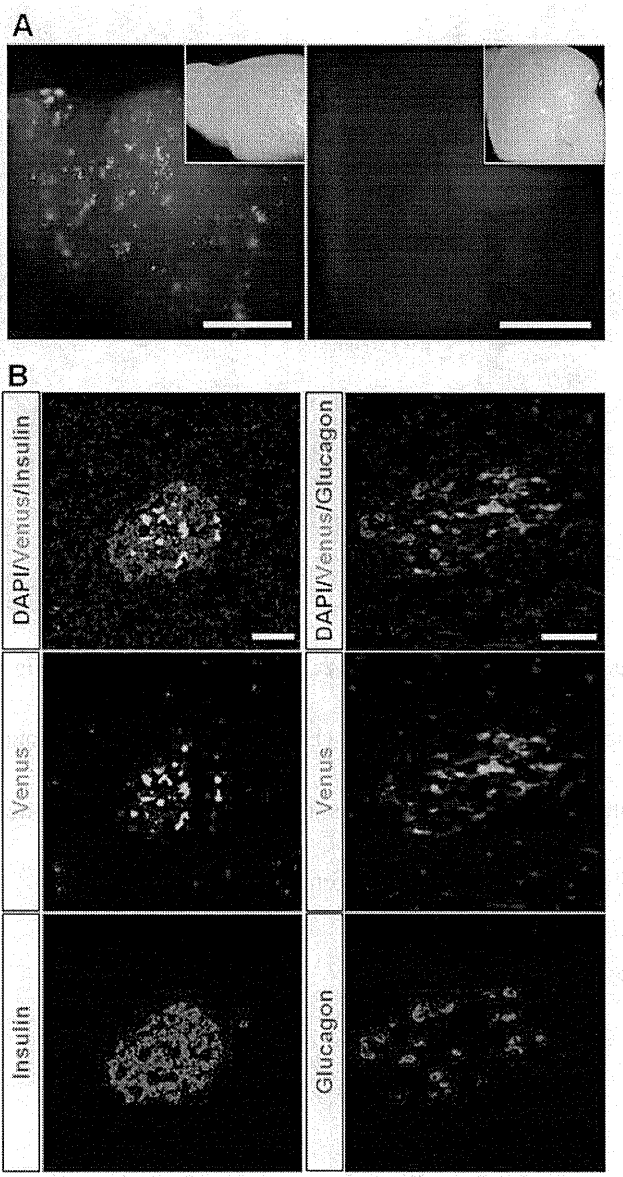


Figure 2. Pancreas-specific expression of the *Pdx1-Venus* gene in the Tg pig fetus. Bright-field (A) and fluorescence microscopic (B) observation of the pancreas (arrowheads). Acinar cells (C, HE stain) showed prominent Venus expression (D). D, duodenum; L, liver; Si, small intestine; Sp, spleen; St, stomach. Scale bars = 5 mm (A, B); 50 μm (C, D).



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507 **Figure 3.** Expression of the *Pdx1-Venus* gene in the pancreas of a Tg pig.

508 (A) Green fluorescent spots were observed by fluorescence stereomicroscopy throughout the

509 pancreatic tissue of the Tg pigs (left panel), indicating *Pdx1-Venus* expression in islets.

510 Right panel: pancreatic tissue of a control wild-type pig. The inset in each panel presents a bright-field

511 image of the tissue. Scale bars = 2.5 mm.

512 (B) Immunohistochemical staining of pancreatic islets of a *Pdx1-Venus* Tg pig. Merged images of the

513 Tg pig islet demonstrated that the expression of the *Pdx1-Venus* gene was confined to β-cells (top left),

514 whereas this gene was not expressed in glucagon-producing cells (top right). Scale bars = 50 μm.

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