paraffin and sectioned, and the sections were placed on glass slides. After deparaffinization and rehydration, the sections were stained for proteoglycan with 0.1% Safranin-O or immunostained with type II collagen antibody. For immunohistochemistry, the slides were incubated with a diluted primary anti-human type II collagen antibody (F-57: Daiichi Fine Chemical, Toyama, Japan) for 16 h at 4°C, followed by incubation with the EnVision + Mouse/HRP secondary antibody (K4000: DAKO, Glostrup, Denmark) for 1 h at room temperature. Finally, the sections were stained with diaminobenzidine (K3466: DAKO) and counterstained with hematoxylin. Coverslips were mounted onto the slides and sealed with nail polish. The slides were then examined under a microscope and images were captured (Biozero BZ-8000, KEYENCE, Osaka, Japan).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 20.0 software (IBM Corporation, NY, USA). The proportional data were subjected to arcsine transformation and evaluated by one-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey's test. The level of significance was set at p values < 0.05.

Results and discussion

Maintenance of cell sheet structure and cell viability after vitrification

Ten cell sheets were vitrified using the coating method in the presence of 10% COOH-PLL (Table 1). After rewarming, all the recovered sheets (100%) showed no visible damage and had same appearance as non-vitrified cell sheets (Figure 3A, B). Eight sheets were vitrified using the coating method in the absence of COOH-PLL (Table 1); only one of the recovered sheets (12.5%) did not have visible damage, all of the remainder exhibited cracks (Figure 3C). Cell viability in the vitrified cell sheets did not differ between protocols with or without COOH-PLL (92.1% vs. 91.9%); the rates of cell viability were comparable to that of the non-vitrified control (94.6%).

These results clearly showed that vitrification by the coating method in the presence of COOH-PLL as a supplemental non-permeable CPA was capable of preserving the membranous structure of the cell sheet with a high survival rate for the constituent cells. To the best of our knowledge, our results represent the first successful vitrification of cell sheets grown in temperature-responsive dishes. In this study, we vitrified cell sheets in LN vapor rather than by direct immersion in the LN. This also had a critical influence on the maintenance of the membranous structure of the cell sheet during vitrification. In preliminary experiments, all of the cell sheets cracked, even in the presence of COOH-PLL, when they were vitrified by direct immersion in LN. Possibly, the direct immersion approach might have had a more drastic impact on membrane integrity than the vapor and, thereby, impaired cell sheet structure.

Since the coating method proved successful for vitrification of cell sheets, we examined whether cell sheets enveloped in a thin film could also be vitrified successfully. Seven cell sheets were placed into film envelopes, vitrified and rewarmed (Table 1). All the sheets (100%) were recovered without visible damage (Figure 3D). However, cell viability (86.8%) was slightly lower compared to that in the coating method ($p < 0.05$).

For use in clinical applications, it would be preferable if vitrified cell sheets could be stored and distributed in hygienic coverings. Our results demonstrated that a cell sheet enveloped in a thin film with a minimum volume of VS could be successfully vitrified. However, wrapping a cell sheet with a film might influence the optimal cooling and rewarming rates during vitrification and rewarming processes. As cooling and warming rates have a crucial influence on the viability of vitrified cells [34,35], it will be important to identify robust film materials that have high thermal conductivity and are protective against invasive pathogens, as well as improving cooling and rewarming methods.

We also examined the influence of the volume of vitrification solution on the morphology and survival of cell sheets. Seven cell sheets were vitrified using the dish method (Table 1) that involves a substantially greater

Table 1 Structural maintenance and cell viability after vitrification of triple-layered rabbit chondrocyte sheets

Vitrification method	Presence of COOH-PLL in VS*	No. of cell sheets recovered without fracture / No. of cell sheets examined (%)	Cell viability (mean ± SEM)
Non-vitrified control		8/8 (100)	94.6 ± 0.5^{a}
Coating	+	10/10 (100)	92.1 ± 0.9^{a}
	-	1/8 (12.5)	91.9 ± 0.7^{a}
Envelope	+	7/7 (100)	86.8 ± 0.7^{b}
Dish	+	7/7 (100)	77.6 ± 3.15^{c}

[a-c]Values with different superscript differ significantly ($p < 0.05$).
*VS: Vitrification Solution.

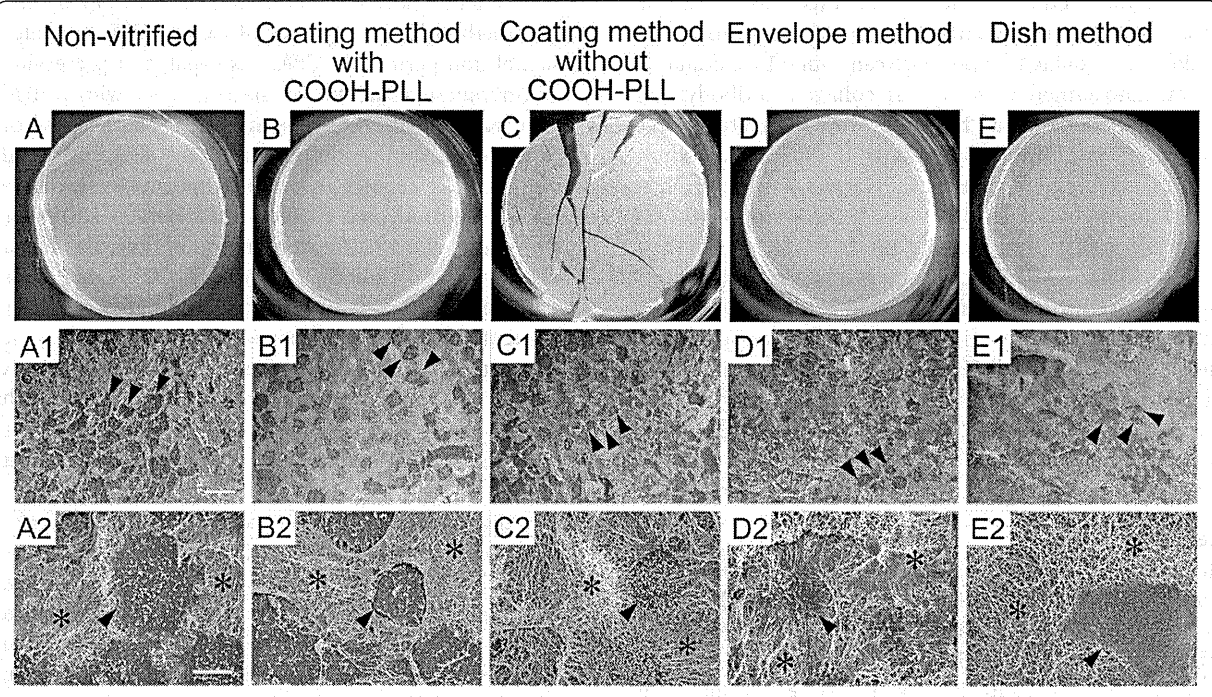

Figure 3 The macro- and microstructures of triple-layered rabbit chondrocyte sheets after vitrification. A-E: Morphological appearance of chondrocyte sheets after vitrification and rewarming. **A1-E2**: Scanning electron microscopic images of the surfaces of cell sheets recovered after vitrification and rewarming. **A1-E1**: ×300, Scale bar = 50 μm; **A2-E2**: ×2,000, Scale bar = 10 μm. The surfaces **(A1-E2)** are irregular, featuring pavement-like cell populations (arrowheads in **A1-E1** indicate representative three cells) and well-developed extracellular matrices with a dense fibrous structure (*). The microstructures of cell sheets vitrified by any of the methods described in this report were similar to those of the non-vitrified control sample.

volume of VS than the coating and envelope methods. VS containing COOH-PLL was used because a preliminary experiment revealed that its presence was essential to ensure crack-free vitrification using the dish method (Additional file 1: Figure S1). All of the 7 sheets (100%) that were vitrified were recovered without any cracks (Figure 3E). However, cell viability after vitrification was 77.6%, which was significantly lower than that observed following vitrification with the coating or envelope methods ($p < 0.05$).

High levels of viability can be achieved by minimizing the volume of the solution used for vitrification of mammalian embryos [31]. In contrast, the vitreous state becomes unstable when larger solution volumes are employed: more cracks tend to occur during the solidification of the solution and more ice crystals form upon rewarming. Our results demonstrated that addition of COOH-PLL as a non-permeable CPA was effective in stabilizing the vitrified state of the solution. However, even when the VS contained COOH-PLL, cell viability was reduced slightly with the dish method. The decrease might be attributed to the slower cooling and warming rates and/or ice crystal formation during rewarming. We observed that the VS appeared opaque for a moment during the rewarming process in the dish method, suggesting the occurrence of ice crystal formation.

Scanning electron microscopic images of the surface of vitrified cell sheets

The microstructures of the cell sheets vitrified in the four experimental groups were compared to those of the non-vitrified sample (Figure 3). Although slight differences were observed among individual sheets, overall, the cell sheets retained their basic structure of pavement-like cells (Figure 3A1-E2) distributed within well-developed extracellular matrices (Figure 3A2-E2). The sheet surfaces were irregular, featuring well-developed extracellular matrices with dense fibrous structures (Figure 3A1-E2).

The microstructures of cell sheets were maintained in the vitrified samples of all the experimental groups under the same conditions as the non-vitrified control groups. The sheets that developed cracks during vitrification with the coating method in the absence of COOH-PLL showed no microstructural abnormalities (Figure 3C1, C2), suggesting that the fracturing of the sheet structure did not affect the microstructure. The sheets vitrified by the envelope method (Figure 3D1, D2) and the dish method (Figure 3E1, E2), where cell viabilities were slightly

decreased, also exhibited no microstructural abnormalities. These results indicate that the microstructure of the vitrified cell sheet, including the extracellular matrix, were well maintained even after vitrification and rewarming under suboptimal conditions.

Histological and immunohistochemical examination of vitrified cell sheets

Cell sheets vitrified with the coating and envelope methods in the presence of COOH-PLL were histochemically and immunohistochemically examined to investigate the distribution of the major components of cartilage, i.e. proteoglycan and type II collagen. In the non-vitrified control (Figure 4A) and the vitrified cell sheet, strong Safranin-O staining was exhibited (coating method: Figure 4C, envelope method: Figure 4E). These results showed that acidic proteoglycan were, in general, densely and evenly distributed throughout the chondrocyte sheet and this distribution pattern was maintained after vitrification in the coating and envelope methods.

The vitrified samples also exhibited large amounts of type II collagen (Figure 4D, F) in a similar manner as in the non-vitrified control (Figure 4B). Overall, these data showed that the extracellular matrix of the vitrified cell sheets had been maintained in both the coating and envelope methods.

Significance of maintaining membranous structure in chondrocyte sheet cryopreservation

In the conventional slow-freezing method, cultured cell sheets are frozen in the presence of a relatively low concentration of a CPA [21]. Thus, extra- and intracellular ice crystal formation is inevitable during freezing, which destroys the cell sheet structure and decreases cell viability [21]. In contrast, with the vitrification method, a solution containing a high concentration of a CPA is rapidly cooled to achieve the transition from the liquid phase to the solid phase (amorphous) without ice crystal formation [36]. Therefore, cell sheets could be sealed in a glassy state that maintained their macro- and microstructures and also allowed high cell viability.

In cell sheet therapy, cytokines and growth factors produced by the cell sheet play an important role in healing damaged tissues [11,19,20]. We found that the formation of a chondrocyte sheet structure enhanced transforming growth factor-β secretion from the cells [37], which implies that maintaining the membranous structure after cryopreservation is a prerequisite for

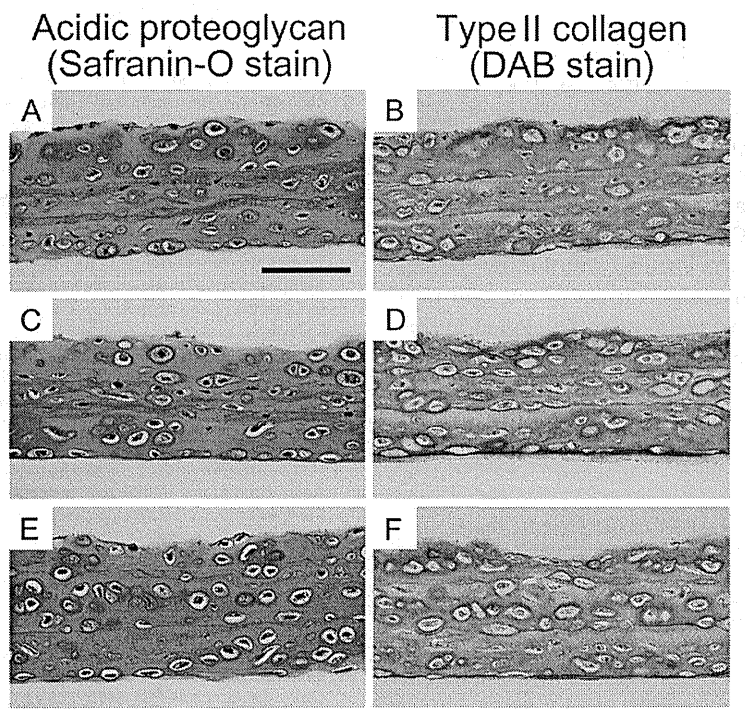

Figure 4 Histological and immunohistochemical examination of triple-layered rabbit chondrocyte sheets. Staining for proteoglycan **(A, C, E)** and type II collagen **(B, D, F)** on cross-sections of cell sheets recovered after vitrification and rewarming. **A, B**: Non-vitrified control cell sheet exhibiting large amounts of proteoglycan and type II collagen in the extracellular matrix. **C, D**: Cell sheet vitrified with the coating method using VS containing COOH-PLL; the sheet exhibits a normal extracellular matrix. **E, F**: Cell sheets vitrified by the envelope method. The extracellular matrix exhibits no difference to samples from control and coating method groups. (Scale bar = 100 μm).

function. Therefore, in the present study, we focused on both the maintenance of sheet structures and of cell viability after vitrification. Thus, our study provides the first demonstration that cryopreservation of cultured chondrocyte sheets with a fragile membranous structure can be achieved using a vitrification method developed on the basis of the MVC concept. Biochemical functions such as cytokine production by the vitrified chondrocyte sheets has yet to be analyzed. Additionally, transplantation experiments using vitrified cell sheets are under consideration.

In our preliminary study, we could successfully vitrify cell sheets with more fragile characteristics including human chondrocyte sheets. It is, therefore, likely that the vitrification method developed in the present study can be applied to different types of cell sheet other than the triple layered rabbit chondrocyte sheets. In application of the vitrification technology to human therapies, toxicity of CPAs to human cells needs to be verified.

Conclusions

In this study, we demonstrate that the vitrification method developed here facilitated the cryopreservation of a chondrocyte sheet while maintaining its macro- and microstructures and allowing a high rate of viability of the constituent cells. The coating method, where the cell sheet was vitrified with a minimum volume of VS in the presence of COOH-PLL, effectively prevented structural damage due to vitrification. Here, we propose three basic principles essential to the cryopreservation of chondrocyte sheets: (i) minimizing the volume of the vitrification solution by using the coating method, (ii) stabilizing the vitreous state via the addition of COOH-PLL as a non-permeable CPA, and (iii) preventing the occurrence of cracks in the vitrified solution by cooling samples in LN vapor instead of direct immersion into LN. The cryopreservation technology developed in this study will play a pivotal role in clinical applications of cell sheet-based therapies.

Additional file

Additional file 1: Figure S1. Protective effect of COOH-PLL against fracture of vitrified solution. COOH-PLL-free (A) and COOH-PLL-containing (B) solutions in the process of rewarming after vitrification in liquid nitrogen vapor. Note the occurrence of many cracks in the COOH-PLL-free solution (A), while the COOH-PLL-containing solution is free of cracks (B). The opacity of the solution in B indicates that ice crystals formed during the warming process.

Abbreviations

COOH-PLL: Carboxylated poly-L-lysine.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HN conceived and designed the experiments and wrote the manuscript. MM performed the experiments and wrote the manuscript together with HN. MW wrote the manuscript together with HN. TK and HM performed the experiments. MS (Michio Sato) scanned electron microscopic images. MY, MK prepared chondrocyte cell sheets. KM and HSH prepared COOH-PLL. MS (Masato Sato) and JM helped to draft the manuscript. All authors read and approved the final manuscript.

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Author details

[1]Laboratory of Developmental Engineering, School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama, Kawasaki, Japan. [2]Meiji University International Institute for Bio-Resource Research (MUIIBR), 1-1-1 Higashimita, Tama, Kawasaki, Japan. [3]Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa, Japan. [4]Laboratory of Microbial Genetics, School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama, Kawasaki, Japan. [5]School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa, Japan. [6]Center for Fiber and Textile Science, Kyoto Institute of Technology, Creation Core Kyoto Mikuruma 213, Kamigyo, Kyoto, Japan.

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ELSEVIER

Transgenic Pig Expressing the Red Fluorescent Protein Kusabira-Orange as a Novel Tool for Preclinical Studies on Hepatocyte Transplantation

T. Shigeta, H.-C. Hsu, S. Enosawa, N. Matsuno, M. Kasahara, H. Matsunari, K. Umeyama, M. Watanabe, and H. Nagashima

ABSTRACT

Introduction. Research on hepatocyte transplantation as an alternative or supplementary treatment for liver transplantation is progressing. However, to advance to clinical trials, confidence in the technique must be established and its safety must be validated by conducting experiments using animals of comparable sizes to humans, such as pigs. We used transgenic pigs expressing red fluorescence protein for investigating the distribution and survival of transplanted cells.

Materials and Methods. Donor hepatocytes were isolated from transgenic Kusabira-Orange (KO)-expressing pigs (age, 41 days; weight, 10 kg) created by in vitro fertilization using sperm from a transgenic-cloned KO pig by Matsunari et al. and ova from a domestic pig. The hepatocyte transplant recipients were the nontransgenic, KO-negative littermates. In these recipient pigs, double lumen cannulae were inserted into the supramesenteric veins to access the hepatic portal region. KO-positive donor hepatocytes from the transgenic male pig were isolated using collagenase perfusion. Hepatocytes (1×10^9 cells) were transplanted through the cannula. For estimating allogeneic immunogenicity, full-thickness skin (3×3 cm) from the same donor was grafted orthotopically on the neck region of the recipients. Immunosuppressive treatment was not implemented. The recipient pigs were humanely killed at 7 and 39 days after transplantation, and the organs were harvested, including the lungs, heart, liver, pancreas, and kidneys.

Results. Strong red fluorescence was detected in both the parenchymal and nonparenchymal hepatocytes of the transgenic male donor pig by fluorescent microscopy. Transplanted cells were detected in the liver and lung of the recipient pigs at 7 days after perfusion. Hepatocytes remained in the liver and lung of recipients on day 39, with lower numbers than that on day 7.

Conclusion. Transgenic pigs expressing the fluorescent protein KO serve as a useful model of cell transplantation in preclinical studies.

HEPATOCYTE transplantation (HCT) is considered to be an alternative or supplementary treatment for liver transplantation (LT) in urea cycle disorders (UCD), including ornithine transcarbamylase deficiency and carbamyl phosphate synthetase 1 deficiency, in addition to acute liver failure (ALF).[1,2] HCT has many advantages compared with LT. For instance, it is less invasive, feasible as a partial or temporal liver support for metabolic disease or ALF, could be supplied from a marginal graft, and could be ready-to-use on demand.[1–4] However, HCT research is at the pioneering stage, despite increasing numbers of case reports for clinical HCT.[1,5–10] Moreover, because UCD is

From the Transplantation Center (T.S., M.K.), Clinical Research Center (H.-C.H., S.E., N.M.), National Center for Child Health and Development, Tokyo, Japan, and International Institute for Bio-Resource Research (H.M., K.U., M.W., H.N.), Meiji University, Tokyo, Japan.

Address reprint requests to Shin Enosawa, PhD, Division for Advanced Medical Sciences, Clinical Research Center, National Center for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan. E-mail: enosawa-s@ncchd.go.jp

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most common in neonatal patients, size mismatches of liver grafts represent a major problem at the time of LT. Therefore, these patients must wait until reaching about 7 kg.[10–12] During the waiting time for LT, patients are at risk of hyperammonemia, developing severe neurological impairments, or lethal coma.[2,12,13] Thus, HCT can be performed to prevent such problems in the treatment of UCD and ALF as a bridge to LT. To advance to clinical trials, confidence in the technique must be established and its safety must be validated by conducting experiments using animals of comparable sizes to humans, such as pigs. This study shows the use of transgenic pigs expressing red fluorescence to investigate the distribution and survival of transplanted cells.

MATERIALS AND METHODS

Donor hepatocytes were isolated from transgenic Kusabira-Orange (KO)-expressing pigs (age, 41 days; weight, 10 kg) created by in vitro fertilization using the sperm from a transgenic-cloned KO male pig by Matsunari et al. and ova of a domestic pig.[14]

KO-positive donor hepatocytes were isolated from the male transgenic pig by collagenase (032-10534 Wako Pure Chemicals, Osaka, Japan) perfusion; furthermore, the parenchymal hepatocytes were obtained through low-speed centrifugation (50 *g*, 1 minute, 3 times).[15,16]

Two offspring (recipients) that were nontransgenic, KO-negative littermates, were used in this study. Under isoflurane anesthesia, minimal abdominal midline incisions were made in both of the recipient pigs. Then, double lumen cannulae (18 gauge; Medicut LCV-UK; Tyco Healthcare, NJ, United States) were inserted into the supramesenteric veins of both the pigs, to access the portal vein trunk. The proximal end was used for portal pressure assessment, whereas the distal end was used for HCT. Hepatocytes (1×10^9 suspended in 100 mL saline containing 5 U/mL heparin) were transplanted once though the cannulae. To estimate allogeneic immunogenicity, full-thickness skin (3×3 cm) from the same male donor was grafted orthotopically onto the neck region of the nontransgenic recipient pigs. Immunosuppressive treatment was not implemented. The 2 recipient pigs were humanely killed at 7 (recipient 1) and 39 (recipient 2) days after transplantation, and the organs were harvested for examination, including the lungs, heart, liver, pancreas, and kidneys. Isolated organ specimens were sliced into 5- to 10-mm pieces, and observed using fluorescent microscopy.

RESULTS

Strong red fluorescence was detected in both the parenchymal and nonparenchymal hepatocytes of the male transgenic donor pig using fluorescent microscopy (Fig 1). Multiple transplanted cells were detected in the liver and lung of recipient 1, whereas the skin graft was rejected within 7 days. Hepatocytes remained in the lung and liver of recipient 2 for 39 days, with a lower number than that in recipient 1. While the skin graft was acutely rejected, the hepatocytes remained for up to 39 days. KO-positive hepatocytes were not detected in the heart, pancreas, or kidney, and no obvious ischemic change was observed on the liver surface of either recipient.

DISCUSSION

To our knowledge, this report shows the first record of using KO-transgenic hepatocytes for HCT experiments. Matsunari et al produced transgenic-cloned pigs carrying a humanized *KO* gene, which is a newly developed red fluorescent protein.[14] KO was cloned from the mushroom coral *Fungia concinna,* which yields orange-red fluorescence in its dimeric form and has 558 and 583 nm excitation and emission maxima, respectively.[17] In addition to KO transgenic pigs, transgenic pigs expressing enhanced green fluorescent protein (EGFP) were studied.[18] As a major feature, KO showed minor background autofluorescence in the liver and lung of humanized KO transgenic pigs compared with EGFP transgenic animals, in which the organs are known to exhibit autofluorescence.[19,20] Furthermore, the clear red fluorescence of the humanized KO protein is maintained even in paraffin-embedded tissue sections.[14]

In our study, lung distribution was identified, although there was no obvious presentation of pulmonary embolization. Interestingly, only a few studies have documented the possibility of pulmonary embolization after HCT.[1,8,21] Muraca et al showed that hepatocytes remained in the lung sinusoids for up to 48 hours after infusion in all pigs;

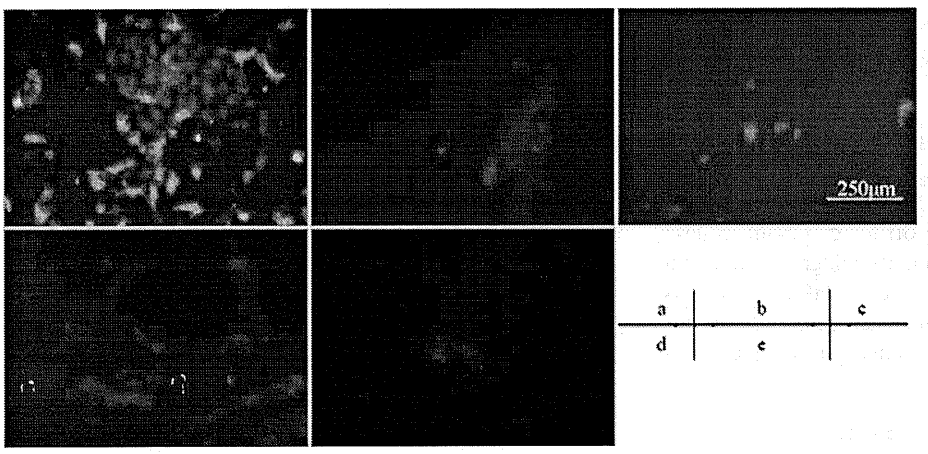

Fig 1. **(a)** Fluorescent microscopy images showing strong red fluorescence in both parenchymal and nonparenchymal hepatocytes of the transgenic donor pig. Transplanted KO-expressing transgenic hepatocytes were detected 1 week after hepatocyte transplantation **(b)** in the liver and **(c)** lung. Transplanted KO-expressing transgenic hepatocytes were detected about 1 month after hepatocyte transplantation **(d)** in the liver and **(e)** lung.

furthermore, a minor basal pulmonary infarction was identified in one of the pigs.[21] In addition, they showed that the number of infused cells was directly correlated with increased portal pressure, but not increased pulmonary artery pressure. They suggested that local vasoconstriction might contribute to the observed increase in pulmonary artery pressure after HCT. Bilir et al reported that intraportal HCT in patients with ALF was followed by hypoxia and pulmonary infiltrates in chest cardiographs, both of which improved after 24 hours.[1] In clinical HCT, hepatocytes were infused several times; hence, portal vein complication represents a serious complication.[5,8,21] In addition, there is a risk of pulmonary embolism due to the repeated infusion of hepatocytes, despite only a small number of hepatocytes passing through the liver. There was no evidence of the intrapulmonary shunt causing a problem to the experimental pigs. However, the safety of intraportal HCT requires further improvement, along with further investigations about the risk of pulmonary embolization. KO hepatocytes remained in the liver and lung for about 1 month. However, our study had a limitation in that we could not identify whether these hepatocytes were functional. Therefore, experiments using animal disease models should be considered in future studies.

Analysis of antigenicity of the donor-recipient combinations in the current study indicated that KO was xenogeneic, whereas somatic cells were allogeneic. Because KO is an intracellular protein, xenogeneic antigenicity might not accelerate normal allogeneic rejection. Immunosuppressive treatment might induce extended survival. One major problem in the clinical application of HCT is the low survival rate of donor cells. It has been hypothesized that the quick disappearance of HCT cells is due to a nonspecific inflammatory response or instant blood-mediated inflammatory reaction (IBMIR); however, the precise mechanisms require clarification.[22] The transgenic pigs used here might serve as a conclusive research tool to overcome the deficit in information, as well as toward improving the cell transplantation procedure. In addition, identical clones of nontransgenic and transgenic littermates obtained by the nuclear transfer methodology might help create a more simple research model for IBMIR research focus.[23] In conclusion, the transgenic pig expressing fluorescent protein KO provides a useful model in cell transplantation preclinical studies.

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Production of Diabetic Offspring Using Cryopreserved Epididymal Sperm by *In Vitro* Fertilization and Intrafallopian Insemination Techniques in Transgenic Pigs

Kazuhiro UMEYAMA[1,2], Kasumi HONDA[1], Hitomi MATSUNARI[1,2], Kazuaki NAKANO[1], Tatsuro HIDAKA[1], Keito SEKIGUCHI[1], Hironori MOCHIZUKI[1], Yasuhiro TAKEUCHI[1], Tsukasa FUJIWARA[1], Masahito WATANABE[1,2], Masaki NAGAYA[2] and Hiroshi NAGASHIMA[1,2]

[1]*Laboratory of Developmental Engineering, Department of Life Sciences, School of Agriculture, Meiji University, Kanagawa 214-8571, Japan*

[2]*Meiji University International Institute for Bio-Resource Research, Kanagawa 214-8571, Japan*

Abstract. Somatic cell nuclear transfer (SCNT) is a useful technique for creating pig strains that model human diseases. However, production of numerous cloned disease model pigs by SCNT for large-scale experiments is impractical due to its complexity and inefficiency. In the present study, we aimed to establish an efficient procedure for proliferating the diabetes model pig carrying the mutant human hepatocyte nuclear factor-1α gene. A founder diabetes transgenic cloned pig was generated by SCNT and treated with insulin to allow for normal growth to maturity, at which point epididymal sperm could be collected for cryopreservation. *In vitro* fertilization and intrafallopian insemination using the cryopreserved epididymal sperm resulted in diabetes model transgenic offspring. These results suggest that artificial reproductive technology using cryopreserved epididymal sperm could be a practical option for proliferation of genetically modified disease model pigs.

Key words: Cryopreserved epididymal sperm, Diabetes model pigs, Intrafallopian insemination, *In vitro* fertilization, Transgenic offspring

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The physiology and anatomy of pigs closely resemble those of humans; for this reason, pigs are regarded as a large animal model that can produce experimental data that are easily extrapolated to humans [1]. In recent years, genetic modification techniques have been used to develop pig strains that model several human diseases [2, 3].

We created genetically modified (GM) pigs that exhibit the symptoms of diabetes similar to maturity onset diabetes of the young 3 (MODY3). The diabetic symptoms of the GM pigs were induced by the dominant-negative effect of a transgene, human mutant hepatocyte nuclear factor 1 alpha (HNF-1α) [4]. In applying GM pigs to translational research, it must be ensured that enough animals can be supplied for experiments. Therefore, we aimed to establish an efficient procedure for proliferating our diabetes model pig by means of artificial reproductive technologies. In this study, the founder transgenic (Tg) cloned pig was generated by somatic cell nuclear transfer (SCNT) and treated with insulin to allow for normal growth to maturity, at which point epididymal sperm could be collected for cryopreservation.

Here, we report that *in vitro* fertilization (IVF) and intrafallopian insemination using cryopreserved epididymal sperm are useful options for reproducing the diabetes model pig. This reproductive system can theoretically be applied to other GM pigs, thereby enhancing the utility of disease model GM pigs.

Porcine fetal fibroblast cells carrying mutant human HNF-1α [4] were used to generate the founder Tg pigs by SCNT. SCNT was performed as described elsewhere using *in vitro* matured (IVM) oocytes as recipient cytoplasts [5]. In total, 333 SCNT embryos were generated and transferred to three estrus-synchronized gilts on day 1 or day 2; each recipient received 116, 109 or 108 embryos. The gilt that had received 116 embryos became pregnant and farrowed four Tg-cloned pigs. Three of the four piglets died within two days of birth for unidentified reasons or from being crushed by the recipient sow. The surviving piglet developed hyperglycemia (non-fasting blood glucose level: 374 mg/dl) at two weeks of age.

Administration of insulin started at three weeks of age, and the pig was raised for 15 months as the founder Tg-cloned boar. The non-fasting blood glucose level of the founder Tg-cloned boar was maintained at approximately 200 mg/dl by administration of a combination of regular insulin (Novolin R, Novo Nordisk, Bagsvaerd, Denmark) and long-acting insulin glargine (Lantus, Sanofi, Paris, France) (Table 1). The target blood glucose level was set higher than the normal blood glucose level for pigs (85–150 mg/dl) to avoid the risk of hypoglycemic coma.

The level of 1,5-anhydroglucitol (1,5-AG) in the founder Tg-cloned boar was lower than that in healthy pigs (Table 1), indicating that the animal developed hyperglycemia. By contrast, the levels of blood urea nitrogen (BUN) and of triglyceride (TG) in the blood during the insulin administration period were similar to those of healthy

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Correspondence: H Nagashima (e-mail: hnagas@isc.meiji.ac.jp)

Table 1. Biochemical parameters in the plasma of the diabetes model founder transgenic-cloned pig

		Control (n = 10)	Founder			
		Mean ± SD	4 months[a]	8 months[a]	12 months[a]	13 months[b]
ALT	(U/l)	28.2 ± 8.1	40.0	37.0	32.0	32.0
AST	(U/l)	24.8 ± 10.4	26.0	27.0	24.0	16.0
TP	(g/dl)	7.4 ± 0.7	6.7	6.5	6.1	6.5
TG	(mg/dl)	32.7 ± 27.1	48.0	28.0	41.0	74.0
T-CHO	(mg/dl)	70.7 ± 17.9	124.0	59.0	54.0	75.0
GLU	(mg/dl)	109.6 ± 26.1	246.0	230.0	195.0	536.0
1,5-AG	(mg/dl)	5.1 ± 1.8	-	1.2	1.1	1.0
BUN	(mg/dl)	9.9 ± 1.8	15.6	10.1	12.8	27.1
CRE	(mg/dl)	1.6 ± 0.5	0.8	1.5	1.4	1.5
Na	(mEq/l)	140.8 ± 2.9	136.0	134.0	143.0	135.0
K	(mEq/l)	4.9 ± 1.3	3.8	4.4	3.4	4.3
Cl	(mEq/l)	101.6 ± 2.4	99.0	94.0	97.0	94.0

[a] Treated with insulin. [b] Not treated with insulin. 1,5-AG, 1,5-anhydroglucitol; AST, aspirate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; Cl, chloride; CRE, creatinine; GLU, glucose; K, potassium; Na, sodium; T-CHO, total cholesterol; TG, triglyceride; TP, total protein.

pigs (Table 1).

The founder Tg-cloned boar grew steadily and reached a peak weight of 169 kg at 12 months. The growth rate during the period between 3.5 months and 11 months was 3.7 kg/week, which was consistent with that of healthy wild-type (WT) pigs (3.6 kg/week). When insulin administration was temporarily stopped at 13 months, the founder pig started to lose weight, non-fasting blood glucose levels reached 536 mg/dl and BUN and TG levels worsened (Table 1). Therefore, these analyses demonstrated that the founder Tg-cloned boar exhibited typical diabetic symptoms that could be controlled by insulin administration.

Following the successful rearing of this founder Tg-cloned boar, we removed one testis at 8 months and the other at 12 months. Epididymal sperm was collected from each testis and cryopreserved using the method described by Kikuchi *et al.* [6] with a slight modification. After thawing, sperm samples were used for IVF and intrafallopian insemination.

The fertilization capacities of the Tg-cloned boar sperm at 8 months (DM-8m) and 12 months (DM-12m) of age were compared with those of WT boar sperm via IVF using IVM oocytes. IVF was performed using previously reported methods [7, 8] with slight modifications. A preliminary experiment revealed the optimal insemination concentration was 1.0×10^6 cells/ml for IVF with the WT boar sperm. Therefore, we adopted this condition for IVF with the Tg-cloned boar sperm.

The fertilization rate with the Tg-cloned boar sperm was significantly ($P<0.05$) lower than that with WT boar sperm: WT, 54.6%; DM-8m, 26.7%; DM-12m, 24.0%. IVF with the DM-8m sperm yielded rates of normal cleavage and blastocyst formation equivalent to IVF with WT sperm (Table 2). However, use of DM-12m sperm for IVF significantly reduced the rate of embryonic development (Table 2).

The blood glucose level of the founder Tg-cloned boar was maintained at slightly higher levels than normal to avoid hypoglycemic coma. The fact that the Tg boar was maintained under suboptimal conditions for more than 6 months may have had a negative influence on sperm quality. Reduced sperm quality in male diabetic patients has been reported by several groups [9, 10]. Paasch *et al.* (2011) [11] reported increased accumulation of eppin (epididymal proteinase inhibitor) protein complex components, such as clusterin, lactotransferrin and semenogelin-1, in the sperm plasma membranes of diabetic patients. They also noted a possible influence of this eppin accumulation on the fertilization capacity of the sperm. A morphological study by Baccetti *et al.* (2002) [12] of diabetic patients' sperm indicated an increased incidence of acrosomal abnormalities and aberrations of the sperm nuclei and tails. Together, these results suggest that the sperm quality of the Tg-cloned boar was adversely affected by diabetic symptoms.

The fertilization rate of porcine IVF has been reported to increase with sperm concentration [13, 14]. Thus, we examined whether elevated Tg-cloned boar sperm concentrations, above the level optimal for WT boar sperm (1.0×10^6), could improve the outcome of IVF. We found that embryo cleavage rates and blastocyst formation rates were highest at 2.5×10^6 cells/ml and 5×10^6 cells/ml for the DM-8m and DM-12m sperm, respectively (Table 3). Both of these concentrations are far above the optimal sperm concentration for WT boars in our experimental settings. These results may be coincident with the deteriorated fertility of the DM-12m sperm.

Next, we examined whether live piglets could be produced by IVF using cryopreserved Tg-cloned boar sperm. DM-12m sperm was used for IVF to verify the utility of the reduced-quality cryopreserved sperm. The insemination concentration was set at 5×10^6 cells/ml, and the resultant embryos at either the 1–8-cell stage (days 1–3) or the blastocyst stage (days 5–6) were transferred to estrus-synchronized recipients. In total, 319 embryos at the 1–8-cell stage were transferred into four recipient gilts; three became pregnant and gave birth to 22 live piglets. Seven of the live piglets (31.8%) were transgenic, carrying the human mutant HNF-1α transgene (Table 4). All of the Tg piglets exhibited hyperglycemia similar to the founder Tg-cloned boar, except for three of the Tg piglets that died before one week of

Table 2. Efficiency of fertilization and development of porcine oocytes fertilized *in vitro* with cryopreserved boar sperm

Type of sperm	Inseminated sperm concentration ($\times 10^6$ cells/ml)	No. of oocytes			No. of oocytes		
		Examined after insemination	Fertilized (%)	Normally fertilized (%)	Cultured after insemination	Normally cleaved (%)	Developed to the blastocyst stage (%)
WT	1.0	97	53 (54.6)[a]	25 (47.2)[a]	69	42 (60.9)[a]	32 (46.4)[a]
DM-8m	1.0	45	12 (26.7)[b]	10 (83.3)	37	19 (51.4)[a]	14 (37.8)[a]
DM-12m	1.0	125	30 (24.0)[b]	28 (93.3)[b]	83	23 (27.7)[b]	14 (16.9)[b]

[ab] Significant differences ($P<0.05$).

Table 3. Determination of optimal sperm concentration for *in vitro* fertilization of cryopreserved diabetic boar sperm

Type of sperm	Inseminated sperm concentration ($\times 10^6$ cells/ml)	No. of oocytes			No. of oocytes		
		Examined after insemination	Fertilized (%)	Normally fertilized (%)	Cultured after insemination	Normally cleaved (%)	Developed to the blastocyst stage (%)
DM-8m	1.0	45	12 (26.7)[a]	10 (83.3)	37	19 (51.4)	14 (37.8)
	2.5	125	86 (68.8)[b]	45 (52.3)	71	48 (67.6)[a]	41 (57.7)
	5.0	129	71 (55.0)[b]	45 (63.4)	66	31 (47.0)[b]	30 (45.5)
DM-12m	1.0	125	30 (24.0)[a]	28 (93.3)	83	23 (27.7)[a]	14 (16.9)[a]
	2.5	144	60 (41.7)[b]	51 (85.0)	95	43 (45.3)[b]	33 (34.7)[b]
	5.0	112	70 (62.5)[c]	58 (82.9)	67	38 (56.7)[b]	34 (50.7)[b]

[ab, ac, bc] Significant differences ($P<0.05$).

Table 4. Production of offspring by *in vitro* fertilization and intrafallopian insemination of cryopreserved diabetic boar sperm

Methods	Recipients	No. of embryos transferred	No. of live piglets (tg piglets included)	No. of stillborn piglets (tg piglets included)	Proportion of tg piglets/litter (%)	Average (mean ± SEM) body weight of piglets at birth (kg)
IVF-1[a]	P57	70	11 (4)	3 (0)	4/14 (28.6)	0.56 ± 0.05
	P84	112	7 (2)	0 (0)	2/7 (28.6)	1.09 ± 0.06
	P162	93	4 (1)	4 (3)	4/8 (50.0)	0.70 ± 0.13
	P58[c]	44	-	-	-	-
IVF-2[b]	P99	20	4 (1)	3 (1)	2/7 (28.6)	0.75 ± 0.10
	P135	15	5 (2)	0 (0)	2/5 (40.0)	0.84 ± 0.09
Intrafallopian insemination	B47	10[d]	6 (4)	2 (2)	6/8 (75.0)	1.07 ± 0.05
	B45[c]	10[d]	-	-	-	-
	B46[c]	8[d]	-	-	-	-
	B51[c]	14[d]	-	-	-	-

[a] Embryos were transferred at the 1–8-cell stage. [b] Embryos were transferred at the blastocyst stage. [c] Returned to estrus. [d] No. of ovulations.

age. Four of the Tg piglets developed to the weaning stage.

A total of 35 blastocysts produced by IVF were transferred into two recipients, both of which became pregnant. The gilts produced 9 live piglets, of which three (33.3%) were transgenic (Table 4). One of those developed to the weaning stage and exhibited hyperglycemia.

These results demonstrated that DM-12m sperm could be used to produce viable piglets through IVF despite reduced fertility.

Piglets were also produced by intrafallopian insemination using cryopreserved Tg-cloned boar sperm. DM-8m sperm, which exhibited better fertilization capacity in IVF, was used to minimize the risk of infertility in this *in vivo* experiment. Insemination of four ovulation-induced gilts resulted in one pregnancy, which produced four live (66.7%) Tg piglets out of six farrowed (Table 4). Three of the four Tg piglets developed to the weaning stage and exhibited hyperglycemia.

In this study, we demonstrated that IVF and intrafallopian insemination using cryopreserved epididymal sperm were effective in reproducing the diabetes model Tg pig. Production of offspring through artificial reproductive technology was possible even using reduced-quality sperm from the founder Tg boar; therefore, this approach should be applicable for other pig strains that model human diseases.

Materials and Methods

Animal care

The Institutional Animal Care and Use Committee of Meiji University approved all animal experiments in this study (IACUC 09-0006).

In vitro maturation of porcine oocytes

SCNT and IVF were performed using IVM oocytes. Porcine ovaries were collected at a local abattoir and transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS; Nissui Pharmaceutical, Tokyo, Japan) containing 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate, 2.5 μg/ml amphotericin B and 0.1% (w/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes (COCs) were collected by aspiration from ovarian antral follicles with a diameter of 3.0–6.0 mm. COCs with at least three layers of compacted cumulus cells were selected and cultured in NCSU23 medium [15] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10% (v/v) porcine follicular fluid, 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate, 10 IU/ml equine chorionic gonadotropin (eCG; ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (hCG; ASKA Pharmaceutical). The COCs were cultured for 22 h with hormones in a humidified atmosphere of 5% CO_2 and 95% air at 38.5 C, followed by additional culture for 18–20 h without hormones in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 C.

Production of Tg embryos by somatic cell cloning

Porcine fetal fibroblast cells (#111-4) carrying an expression vector for the mutant human HNF-1α cDNA (HNF-1αP291fsinsC), which had been established in our previous study [4], were used as nuclear donor cells. SCNT was performed using IVM oocytes as recipient cytoplasts as described elsewhere [5]. SCNT embryos were cultured in porcine zygote medium-5 (PZM-5; Research Institute for the Functional Peptides, Yamagata, Japan) in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 C for 1 or 2 days, and then they were transferred into an estrus-synchronized recipient.

Cryopreservation of boar spermatozoa from epididymides

The cauda epididymides of the founder Tg-cloned boar and a WT boar (17 months old) were removed under general anesthesia and transferred to the laboratory at ambient temperature. Luminal fluid containing spermatozoa was extruded from the distal portion of each cauda epididymis by air pressure from a syringe, followed by perfusion with approximately 10 ml of collecting solution [6]. The recovered sperm suspension was diluted to a concentration of 2.0×10^9 cells/ml by adding collecting solution. The sperm suspension was then cooled down from 25 C to 15 C over 60 min (average cooling rate: 0.17 C/min). After incubation for 2 h at 15 C, the sperm suspension was centrifuged at 800 × *g* for 10 min at 15 C and the supernatant was discarded. Precipitated spermatozoa were resuspended in equal amounts of Niwa and Sasaki Freezing (NSF)-I extender [6]. The sperm suspension was again cooled down from 15 C to 5 C over 120 min (average cooling rate: 0.08 C/min) and was then diluted to a concentration of 2.0×10^9 cells/ml with NSF-I extender. It was finally mixed with an equal volume of NSF-II extender containing 6% (v/v) glycerol [6]. The sperm suspension (0.2–0.3 ml) was loaded in 0.5-ml plastic straws (Cryo Bio System, Paris, France). The straws were the placed in liquid nitrogen vapor (–150 C) approximately 4 cm above the surface of liquid nitrogen for 20 min and were finally plunged into liquid nitrogen.

IVF

IVF was performed using IVM oocytes with expanded cumulus cells [16]. A straw containing frozen sperm was thawed by immersion in warm water (37 C) for 30 seconds. The sperm were then suspended in 5 ml DPBS supplemented with 0.1% bovine serum albumin (Wako Pure Chemical Industries, Osaka, Japan) and washed three times by centrifugation at 1,000 × *g* for 4 min. After washing, the sperm pellets were resuspended in porcine fertilization medium (PFM; Research Institute for the Functional Peptides) at a concentration of 1×10^7 cells/ml.

For insemination, 20 COCs that had been matured *in vitro* were placed in a 100-μl drop of PFM containing spermatozoa ($1.0–5.0\times10^6$ cells/ml); the oocytes and sperm were incubated for 8 h at 38.5 C in a humidified atmosphere containing 5% CO_2, 5% O_2 and 90% N_2. After insemination, the eggs were transferred to Tyrode lactose medium containing 10 mM Hepes and 0.3% (w/v) polyvinylpyrrolidone (Hepes-TL-PVP); cumulus cells and excess sperm were removed by gentle pipetting. Eggs that showed release of one or more polar bodies with normal cytoplasmic morphology were cultured (20–30 eggs/20–30 μl drop) in PZM-5 under paraffin oil (Kanto Chemical, Tokyo, Japan) in a plastic petri dish maintained in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 C until transfer. Eggs were cultured beyond the morula stage by adding 10% (v/v) fetal calf serum to the medium.

Some eggs were fixed in 1:3 acetic acid:methanol after 12 h of culture and then stained with 1% aceto-orcein. Eggs with two pronuclei and two polar bodies were considered to have undergone normal fertilization. Other categories of fertilized eggs were identified as polyspermic: eggs with two pronuclei, two polar bodies and a swollen sperm head(s) and eggs with three or more pronuclei.

Embryo transfer

Crossbred (Large White / Landrace X Duroc) prepubertal gilts weighing 100–105 kg were used as recipients. To induce estrus in the gilts, 1000 IU of eCG was injected intramuscularly, followed by an injection of 1500 IU hCG (Kyoritsu Seiyaku Corporation, Tokyo, Japan) 66 h later.

SCNT embryos were transferred at the 1-cell (day 1) or 2–8-cell (day 2) stages into the oviducts of estrus-synchronized recipient gilts by midventral laparotomy under general anesthesia [5]. Transfer of *in vitro*-fertilized embryos was conducted at 1–8-cell (days 1–3) or blastocyst (days 5–6) stage. Embryos on days 1–2, day 3 and days 5–6 were respectively transferred to recipients at 51–54 h, 75–78 h and 147–150 h after the hCG injection. Pregnant recipients were allowed to farrow, and piglets obtained were raised with the standard diet and water *ad libitum*.

Intrafallopian insemination

The gilts used for the intrafallopian insemination experiment were induced to ovulate by an injection of 1000 IU eCG, followed 72 h later by administration of 1500 IU hCG. Intrafallopian insemination was performed by midventral laparotomy approximately 46 h after hCG injection. The ovaries of estrus-induced recipients were examined to confirm that ovulation had occurred. Frozen-thawed epididymal sperm suspended in Beltsville thawing solution [17] at a concentration of 2.7×10^7 cells/0.1 ml were injected into both ampullae of the fallopian

tubes using a 3.5 Fr catheter (Tom Cat Catheter, Tyco Healthcare Group, Mansfield, MA, USA) with a syringe. The proportion of sperm with progressive motility was 30%.

Control of blood glucose levels in the diabetic founder boar

Blood glucose levels were measured daily (morning and evening) with a glucose test meter (GLUCOCARD GT-1820, Arkray, Kyoto, Japan) using blood samples from the ear vein. The diabetic founder pig received a subcutaneous injection of insulin glargine (0.5–1.1 U/kg) at the time of feeding in the morning. Blood glucose levels were measured again 60–90 min after feeding, and a subcutaneous injection of regular insulin (0–0.2 U/kg) was given to maintain a blood glucose level of approximately 200 mg/dl.

Biochemical analysis of blood components

Venous blood samples were collected in tubes containing heparin to determine the concentrations of aspirate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total cholesterol (T-CHO), triglyceride (TG), total protein (TP), sodium (Na), potassium (K) and chloride (Cl) using a dry-chemistry analyzer (FUJI DRI-CHEM 7000, FUJIFILM Corporation, Tokyo, Japan). The concentration of 1,5-anhydroglucitol (1,5-AG) was determined using the enzymatic method (SRL, Tokyo, Japan).

Determination of transgenic offspring

Determination of transgenic offspring was performed using PCR. Genomic DNA was extracted from offspring tail tissue, purified with a DNeasy Blood & Tissue Kit (QIAGEN, Tokyo, Japan) and amplified using TaKaRa Ex Taq (Takara Bio, Shiga, Japan) with the following set of primers: 5'-AGGACCTGAGCCTGCCGAGCAAC-3' and 5'-AGGGCTCTCCATAGGCCCAGGCT-3'. The PCR conditions were as follows: 94 C for 3 min, followed by 25 cycles of 94 C for 30 s, 60 C for 15 s and 72 C for 20 s.

Statistics

Statistical analyses were performed using the SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences in the rates of fertilization and embryo development between the DM sperm and control WT sperm were analyzed using a χ^2 test.

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