

Feasibility of large experimental animal models in testing novel therapeutic strategies for diabetes

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Abstract

Diabetes is among the top 10 causes of death in adults and caused approximately four million deaths worldwide in 2017. The incidence and prevalence of diabetes is predicted to increase. To alleviate this potentially severe situation, safer and more effective therapeutics are urgently required. Mice have long been the mainstay as preclinical models for basic research on diabetes, although they are not ideally suited for translating basic knowledge into clinical applications. To

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validate and optimize novel therapeutics for safe application in humans, an appropriate large animal model is needed. Large animals, especially pigs, are well suited for biomedical research and share many similarities with humans, including body size, anatomical features, physiology, and pathophysiology. Moreover, pigs already play an important role in translational studies, including clinical trials for xenotransplantation. Progress in genetic engineering over the past few decades has facilitated the development of transgenic animals, including porcine models of diabetes. This article discusses features that attest to the attractiveness of genetically modified porcine models of diabetes for testing novel treatment strategies using recent technical advances.

Key Words: Pancreatic islet; Diabetes mellitus; Pig; Transgenic; Genetic engineering; Transplantation; Xenotransplantation

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Core Tip: Safer and more effective therapeutics are urgently required for managing the diabetes epidemic. Mice have been used predominantly as preclinical models for basic research on diabetes, although murine models are not ideally suited for translating basic knowledge into clinical applications. This article discusses features that attest to the attractiveness of genetically modified porcine models of diabetes for testing novel treatment strategies using recent technical advances.

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INTRODUCTION

Diabetes is a profligate disease that is characterized by disordered glucose metabolism that results from absolute or relative deficiency of insulin. Diabetes is among the top 10 causes of death in adults and was estimated to have caused four million deaths globally in 2017^[1]. Globally, there are approximately 500 million individuals with diabetes, and this number is expected to increase by 25% and 51% by 2030 and 2045, respectively. Worldwide, diabetes imposes a large economic burden on healthcare systems, and the estimated annual global health expenditure attributable to the disease ranges from USD 612 to 1099 billion. Thus, diabetes has a major impact on the lives and well-being of individuals, families, and societies. The prevention and effective management of diabetes should be considered a public health priority to reduce the financial burden^[2].

TYPE 1 DIABETES

The three main types of diabetes are type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), and gestational diabetes mellitus. T1DM is characterized as a multifactorial autoimmune disease that occurs due to a specific immune-mediated permanent destruction of pancreatic β -cells and results in a lifelong dependence on exogenous insulin. Approximately 5%-10% of patients with diabetes have T1DM, and the incidence and prevalence of T1DM is predicted to increase^[3-5]. Worldwide, approximately 78000 youth are diagnosed with T1DM annually^[6,7]. T1DM increases the risk of unstable glycemic control, hypoglycemia unawareness, and even sudden unexplained death^[8], and induces pathological alterations including microvascular and macrovascular complications. The Diabetes Control and Complications Trial (DCCT) Research Group showed that the severity and duration of hyperglycemia exposure are

directly related to the risk of development and progression of microvascular complications such as retinopathy and diabetic kidney disease in T1DM, both in adolescents and adults^[9]. Individuals thus affected with end-stage kidney disease often require hemodialysis and, eventually, kidney transplantation to manage renal failure^[10]. Macrovascular complications, cerebrovascular disease, and peripheral vascular disease resulting from atherosclerosis are the leading causes of morbidity and mortality in adults with T1DM^[11,12]. Thus, T1DM is a serious disease that confers not only an economic burden but also psychological distress for patients over a long period.

Treatment of T1DM

Exogenous insulin therapy: Most T1DM patients require intensive insulin regimens, *via* multiple daily injections of prandial and basal insulin or continuous subcutaneous insulin infusion (CSII), to maintain euglycemia^[13,14]. The DCCT Research Group demonstrated that intensive insulin regimens achieved near-normal glycemic control and reduced the risk of the onset and progression of T1DM-related complications^[15]. Some trials of novel technological T1DM treatments have been launched and have yielded good results. The combined use of continuous glucose monitoring devices with insulin pumps has enabled the development of automated insulin delivery systems known as closed-loop devices, with predicted low-glucose suspension that has reduced the incidence of hypoglycemia in children and adolescents in clinical studies^[16-19]. Furthermore, relative to conventional and sensor-augmented insulin pump therapy, the bi-hormonal bionic pancreas was able to achieve superior glycemic regulation without the need for carbohydrate counting^[20,21]. The technology was established using a large animal diabetic porcine model^[22,23] and was subsequently applied in a clinical trial^[21]. Automated glycemic control is progressive, but it carries some risks. With closed-loop devices, T1DM patients and their families need to expend painstaking efforts to count carbohydrates, closely monitor blood glucose, and make dosing decisions for insulin—a drug with a narrow therapeutic range and a low margin for error. Hyperglycemia with ketosis due to pump infusion-set failure is another possible issue^[17]. Thus, automated insulin delivery requires patient and provider education to optimize its outcomes^[6,7,16,21,24]. Another important current limitation is that subcutaneously administered insulin, either by an CSII device (as part of a closed-loop system) or through multiple injections, will incur a lag time to initiate pharmacological action^[25-27]. In studies of bi-hormonal closed-loop devices, the results represent advances in care, but remain cumbersome, imprecise, and costly. Indeed, loss of wireless connectivity occurs up to 4% of the time^[21]. To date, no protocol has been established to eradicate exogenous insulin therapy entirely without substantial recipient risk. Thus, research needs to be continued to produce more suitable physiological therapies. An effective alternative approach is β -cell replacement therapy.

β -cell replacement therapy: Human whole pancreas or islet transplantation may constitute a life-saving therapy for T1DM patients. These therapies provide considerable advantages for diminishing the total daily insulin dose and lowering the frequency of hypoglycemic reactions^[28,29]. Pancreas transplantation has been accepted as a proven therapy, and simultaneous pancreas-kidney transplants function for an average duration of 9 years^[30]. However, organ transplantation is an insufficient treatment despite the fact that patients undergo major surgery, need lifelong immunosuppressive therapy and hold incidental risks of infection, cancer, and nephrotoxicity constitute additional challenges. Islet transplantation was performed on an experimental basis since its introduction by Shapiro *et al.*^[31] in 2000 as a feasible clinical procedure. Islet transplantation has an advantage in that it does not require major surgery. Moreover, the treatment methods have been refined substantially, including more optimal islet preparation, culture, safer transplant techniques, and more effective anti-inflammatory and immunomodulatory interventions, over the past decade^[32]. Islet transplantation therapy can be maintained for 3 years, an average, without insulin in specialized protocols^[33]. A multicenter, single-arm, phase 3 study of an investigational product containing purified human pancreatic islets was conducted at eight centers in North America^[34]. The success reported with this study has established a proof of principle for cellular transplantation, although the treatment still has several hurdles to cover to reach clinical application in real-world clinical practice (Table 1).

Table 1 Hurdles in clinical islet transplantation for type 1 diabetes mellitus

Hurdles in clinical islet transplantation for type 1 diabetes mellitus	
1	Poor access to human islets due to the scarcity of organ donors
2	Graft failure (<i>e.g.</i> , metabolic pressure, oxidative stress caused by hypoxia or inflammation)
3	Continued autoimmunity and alloimmunity
4	Immunosuppressive drug therapy
5	Behavior of the transplanted cells in the growing body for transplantations performed in adolescents/children

EXPERIMENTAL ANIMAL MODELS FOR TESTING NOVEL TREATMENT STRATEGIES

The development of therapeutic strategies for T1DM is progressing, although all of them have some imperfections. Thus, safe and potent therapeutics need to be developed to combat diabetes. Animal models are indispensable for the discovery, validation, and optimization of novel therapeutics for safe human use. Mice have long been the mainstay of preclinical research and are a widely used mammalian species in biomedical research, mainly because they are convenient and cheap to house and as methods for their genetic modification are well advanced^[35,36]. However, the translational value of murine models is limited due to their distinct anatomy and physiology^[37]. The murine models cannot replicate the complications of organ and/or cell transplantation, such as blood vessel and bile duct injuries, that might occur in human allotransplantation in T1DM patients. Experimental procedures and conditions should be accurately described to improve their reproducibility and to facilitate the translation of findings in preclinical animal models.

ATTRACTIVENESS OF LARGE EXPERIMENTAL ANIMAL MODEL IN MEDICAL RESEARCH

It is preferable that such investigations are conducted on animal species that have anatomical and physiological similarities to humans. Large animals, especially pigs, are well suited for developing and refining biomedical procedures and medical equipment for biomedical research as they share many similarities with humans, including body size, anatomical features, physiology, and pathophysiology. Moreover, it is well established that the pharmacokinetics of orally or subcutaneously administered compounds in pigs are similar to those of humans^[38]. In addition, pigs are monogastric omnivores and are likely to be obese as well as dyslipoproteinemic, similar to humans. Other characteristics of pigs include the fact that they mature relatively quickly for a large species (6-7 mo), have a short gestation period (approximately 114 d) and high fecundity (8-14 offspring per litter), and their long-life cycle and high litter size make the production of genetically modified pigs less time-consuming compared to other animal species^[38,39]. Furthermore, there is wide public acceptance of the humane use of pigs in research, unlike that for other nonrodent species, such as primates. Genetically modified animals are vital for gaining a proper understanding of disease mechanisms and for developing novel therapies. The extension of genetic modification technology to pigs has greatly increased their value in biomedicine, motivating efforts to develop porcine models that replicate human diseases, including cardiovascular and neurodegenerative diseases, neoplasms, and diabetes^[35]. Pigs are now considered promising models to overcome gaps between proof-of-concept models, to bridge the gap between bench and bedside, and as precursors to clinical studies^[21-23]. The major advantages and disadvantages of pigs in medical research are listed in Table 2.

MODELING DIABETES IN PIGS

Porcine insulin differs by only one amino acid from human insulin and was widely used to treat T1DM before human insulin could be produced in large quantities by recombinant DNA technology^[40,41]. Pigs have similar pancreatic and islet structure,

Table 2 Use of pigs as biomedical research subjects

Advantages	
1	Similar body size, shape, and anatomy as that of humans
2	Human-relevant metabolic physiology and pathophysiology
3	Monogastric omnivore
4	Multiparity, short gestation, short generation interval, and long-life cycle
5	Pancreatic and islet architecture similar to that of humans
6	Can undergo the same surgical procedure as in humans
7	Tools for genetic alterations are available
8	High litter size makes the production of genetically modified pigs less time-consuming in comparison with other livestock species
Disadvantages	
1	Specialized facilities are required
2	Costly to maintain
3	Ethical issues associated with the use of pigs in biomedical research

total β -cell mass, ratio of β -cell mass to body mass, and β -cell replication capacity to that of humans^[42,43]. Thus, pigs serve as tissue and organ donors for β -cell replacement therapies in T1DM^[44-46]. Pigs are suitable for testing medical devices and surgical techniques, such as pancreatectomy and transplantation of the pancreas and islets, to develop and refine treatment methods for T1DM.

RESERCH ETHICS IN PIG MODELS

Species selection for research must be based on ethical standards and conducted on a case-by-case basis such that the benefits are assessed according to the predictability of the animal model for the specific function. In the United States, the 1966 Animal Welfare Act is the federal law that regulates how animals must be treated in research; however, it does not apply to animals raised for food^[47,48]. The European Union's (EU) Directive on the Protection of Animals Used for Scientific Purposes largely aims on eliminating or reducing the potential pain and distress of live animals included in research and also excludes the animals raised for food^[48]. The response from ethics or regulation would most certainly have been the same between the United States and Europe. We reject the general argument that pigs are completely suitable for our experiments. The decisions about choice of species are complex and has evolved with new knowledge. In a scientific sense, as mentioned previously in this article, the use of pigs for this study is based on their many similarities to humans. On the contrary, in an ethical context, the potential scientific, technical, and economic benefits, all set against the need to minimize harm. The most effective application of the principles of practical ethics to the conduct of experiments with animals, which we used in our study, is the concept of the three R's (reduction, replacement, and refinement) introduced by Russell and Burch^[49,50]. The regulated procedures state that the research must include minimum number of animals; involve animals with the lowest degree of neuro-physiological sensitivity; cause the least pain, suffering, distress, or lasting harm; and are most likely to produce satisfactory results. Legislation on the protection of vertebrate animals used for experimental and other scientific purposes has been formulated in line with the three R's. It was first presented in European Union Directive 86/609/ECC (EU Directive European Union Directive 86/609, 1986)^[51-54]. Regardless of the species involved, proper care and management are essential for the well-being of the animals, validity of the research data, and health and safety of animal care personnel. Therefore, all our animal experiments in this study were carefully inspected and approved by Meiji University's Institutional Animal Care and Use Committee (IACUC). All animals were housed and maintained in accordance with the IACUC guidelines. All animal care and experimental procedures were performed in accordance with the regulations contained in the Japanese Act on Welfare and Management of Animals. The pigs were housed in a temperature-controlled room, with free access to water and growth-stage appropriate commercial feed and observed

by an animal husbandry personnel under the supervision of an attending veterinarian on a daily basis.

METHODS TO INDUCE DIABETES IN PIG

Surgery

Diabetes can be surgically induced in pigs by pancreatectomy to ablate endogenous insulin production. The benefit of this method is the lack of toxic adverse effects on other organs. However, the prerequisites of highly specialized training, surgical equipment, confounding effects of eradicating exocrine pancreatic digestive enzymes, and other islet hormones are some of the disadvantages with this method.

Chemical induction

Chemical approaches for diabetes induction, such as use of the diabetogenic drugs streptozotocin or alloxan, target insulin-secreting β -cells. Both drugs are cytotoxic glucose analogs that have a high affinity for the glucose transporter 22 in β -cells. Diabetes can be induced by daily injection of the β -cell cytotoxin streptozotocin (0.1 mol/L at a dose of 50 mg/kg) for 3 d, resulting in a > 80% β -cell reduction, an increase in the plasma glucose to diabetic levels, and hypertriglyceridemia. Chemical induction is the standard model of diabetes in rodents, but it is often difficult to use in large animals due to reduced efficacy, relevant side effects, and an unacceptable mortality rate. Multiple injections and higher drug doses are needed for the induction of diabetes in larger animals; thus, chemical induction is an unstable method for the induction of diabetes in pigs^[38,55].

Genetic engineering

Genetic engineering of pigs is a remarkably refined approach for generating tailored porcine diabetes research models. Transgenic (Tg) pigs are an attractive model for the analysis of pancreatic development and for testing novel diabetes treatments. Several groups have generated porcine diabetic models. Renner *et al.*^[56] generated pigs with T2DM that could express a human dominant-negative incretin hormone-glucose-dependent insulinotropic polypeptide receptor mutant (GIPR^{dn}) in pancreatic islets controlled by the rat insulin promoter. A permanent neonatal diabetes pig model was established by generating Tg pigs that expressed a mutant porcine *INS* (insulin) gene (*INS* C94Y) orthologous to human *INS* C96Y^[57-60]. However, no diabetes-associated renal pathological changes were detected in these models. Diabetes has a multitude of phenotypic manifestations that are unlikely to be recapitulated in a single animal model. Pancreatic duodenum homeobox 1 (*PDX1*) expression is crucial for pancreatic organogenesis and is a key regulator of insulin gene expression^[61]. Using genome-editing technologies, *PDX1*-knockout (KO) pigs are generated; however, *PDX1*-KO is fatal^[62-64]. Nonetheless, a few of *PDX1*-modified pigs generated by the clustered regularly interspaced short palindromic repeat/clustered regularly interspaced short palindromic repeat/CRISPR-associated proteins9 (CRISPR/Cas9) system that is introduced into zygotes can survive^[65].

Diabetic Tg pigs generated in our laboratory

Tg-cloned pigs with a mutant human hepatocyte nuclear factor 1 α gene: Maturity-onset diabetes of the young (MODY3) is characterized by impaired insulin secretion, with less impact on insulin action, and is commonly caused by dominant-negative mutations in the gene encoding hepatocyte nuclear factor 1 α (*HNF-1 α*)^[66,67]. MODY3 is a noninsulin-dependent type of diabetes with autosomal dominant inheritance wherein *HNF1 α* gene mutations lead to pancreatic β -cell dysfunction and impaired insulin secretion. We generated a pig model for MODY3 by expressing a mutant human *HNF1 α* gene (*HNF1 α* P291fsinsC) using intracytoplasmic sperm injection (ICSI)-mediated gene transfer (MGT) and somatic cell nuclear transfer (SCNT)^[68-70]. After we generated the Tg pigs, their sperm was frozen and frozen sperm heads that were thawed subsequently and preincubated with the gene construct were microinjected into oocytes for fertilization, resulting in the incorporation of foreign genes into the genome of the host egg. A system for the mass *in vitro* production of mature eggs with high developmental ability in pigs has been established. Therefore, we could create Tg pigs as needed. Piglets developed hyperglycemia at 2 wk, and showed glomerular nodular lesions in the kidneys, a hallmark of diabetic nephropathy (described in the section of Microvascular and Macrovascular Complications) at 19 wk

that further expanded over the 10-mo observation period^[69,70]. Furthermore, Tg pigs manifest diabetic retinopathy and cataracts, similar to those in T1DM patients^[70].

Tg cloned pigs carrying *PDX1*-Hairy and enhancer of split 1: Hairy and enhancer of split 1 (*HES1*) control tissue morphogenesis by maintaining undifferentiated cells. *HES1* encodes a basic helix loop helix (bHLH) transcriptional repressor that functionally antagonizes positive bHLH genes, such as the endocrine determination gene neurogenin-3 (*NGN3*)^[71]. We generated a new Tg pig model for diabetes through genetic engineering of the *PDX1* and *HES1* genes^[72,73]. We confirmed pancreatic agenesis in *PDX1-HES1* Tg mid-gestation fetuses and established primary fibroblast cultures from Tg fetuses for somatic cell-based cloning. The cloned fetuses and offspring showed pancreatic agenesis. For the production of Tg porcine strain, we used the blastocyst complementation technique, as described elsewhere^[74]. Subsequently, a Tg chimera pig with germ cells carrying a construct expressing *HES1* under the control of the *PDX1* promoter was mated with wild-type (WT) gilts to obtain Tg piglets. These Tg piglets had a high rate of perinatal death due to severe diabetes, although this phenotype could be rescued by insulin treatment. β -cells were not detected, even in the adult pancreas, although other endocrine cells were detected, and exocrine cells functioned normally. The pigs showed no abnormalities in any organ, except for diabetes-associated pathological alterations, such as retinopathy and renal damage. *PDX1-HES1* Tg pigs showed the induction of a stable diabetic phenotype and manifested diabetes-associated complications relatively early. The Tg pig recapitulated several phenotypic manifestations of DM. Therefore, this model seems useful for elucidating the underlying causes and for developing novel treatments for diabetic retinopathy and nephropathy as well as diabetes-related complications.

Apancreatic *PDX1*-KO phenotype generated by genome-editing: We generated diabetic Tg pigs using different strategies^[67,72,73]: Tissue-specific and developmental stage-specific hyperexpression of specific genes and KO of master regulator gene. Porcine male fetal fibroblast cells carrying transcription activator-like effector nuclease (TALEN)-induced biallelic mutations in exon 1 of *PDX1* were used for SCNT. Cloned embryos were generated from nuclear donor cells and transferred to recipient gilts. Analysis of cloned fetuses retrieved at mid-gestation (day 55) revealed that *PDX1*-KO mutations generated an apancreatic phenotype^[62].

GENERATION OF OTHER TG PIGS IN OUR LABORATORY FOR DIABETIC RESEARCH

The other Tg pig models we generated may play an important role in finding answers to the issues in β -cell replacement therapy for T1DM (Table 1).

Tg pigs with pancreas-specific expression of green fluorescent protein

Genetically modified pigs that express fluorescent proteins, such as green and red fluorescent proteins, have become indispensable in biomedical research. The *PDX1* gene promoter is conjugated to Venus, a green fluorescent protein, and then introduced into 370 *in vitro*-matured porcine oocytes by ICSI-MGT. From these Tg pigs, a Tg-chimeric boar that produced fertile sperm carrying the *PDX1-Venus* expressing vector was obtained^[74]. After confirming specific Venus expression in β -cells, the Tg-chimeric boars were mated with WT gilts to acquire Tg piglets that exhibiting green fluorescence in β -cells. These Tg pigs were used in our basic diabetic research.

Generation of two Tg cloned porcine models expressing the far-red fluorescent protein Plum and modified Plum

Monomeric Plum, a far-red fluorescent protein with photostability and photopermeability, is potentially suitable for *in vivo* imaging and detection of fluorescence in body tissues. Using the same technique applied in Tg pig to express green fluorescence in β -cells, we generated Tg cloned pigs that exhibit systemic expression of Plum using SCNT technology. Nuclear donor cells for SCNT were obtained by introducing a Plum-expression vector driven by a combination of the cytomegalovirus early enhancer and chicken β -actin promoter into porcine fetal fibroblasts (PFFs). These Tg pigs exhibited high levels of Plum fluorescence in the skin, heart, kidney, pancreas, liver, spleen, blood cells, lymphocytes, monocytes, and granulocytes^[75]. Cell or tissue

transplantation studies using fluorescent markers should be conducted to ascertain whether the xeno-antigenicity of the fluorescent proteins affects engraftment or graft survival. Therefore, we generated a Tg-cloned pig harboring a derivative of Plum modified by a single amino acid substitution in the chromophore. The cells and tissues of this Tg-cloned pig expressing the modified Plum (mPlum) did not fluoresce. However, Western blotting and immunohisto-chemical analyses clearly showed that mPlum had the same antigenicity as Plum. Thus, we have obtained primary proof of principle for creating a cloned pig that is immunologically tolerant to fluorescent protein antigens^[76,77]. Transplantation between these two pigs provides much information on aspects such as appropriate transplantation site, behavior of the transplanted organ, and/or cells without any influence of xeno-antigenicity.

Production of medical-grade pigs using the uterectomy-isolated rearing method

The domestic pig best meets the criteria for xenotransplantation. The establishment of an efficient system for the production of designated pathogen-free (DPF) pigs is a prerequisite for the clinical application of xeno-islet transplantation therapy. Therefore, we developed a feasible and economic method that consists of uterectomy of a full-term sow, recovery of fetuses from the uterus, and rearing of neonatal piglets under aseptic conditions in specially designed isolator units. Full-term sows were subjected to a midventral incision under general anesthesia. Each excised uterus was transferred to the recovery unit through a disinfecting tube, and the fetuses were recovered from the uterus. The piglets thus recovered were then transferred to the second isolator unit for artificial nursing with a γ -irradiated milk substitute. Swab samples of the body surface of the pigs and the internal surface of the rearing unit were collected weekly to test the sterility of the piglets. The samples were examined using a standard protocol that was developed and recommended by the Japanese Association for Laboratory Animal Science. At the end of the rearing period, the blood and internal organs, including the pancreas, were collected to analyze sterility and the presence of viruses. The average recovery rate of live piglets was 93.3%. The body weight of the neonatal piglets did not differ significantly from that of the control piglets that were obtained *via* natural farrowing. Blood and tissue samples obtained from the piglets after the rearing period tested negative for bacteria, fungi, and protozoa. Of the viruses currently designated in the guideline for xenotransplantation by the Japanese Ministry of Health, Labor and Welfare, 58 were proven to be absent based on the results of negative polymerase chain reaction analysis. The uterectomy-isolated rearing method has potential applicability for the practical production of donors using neonatal piglets for xeno-islet transplantation^[78].

Generation of α 1,3-galactosyltransferase and cytidine monophospho-N-acetylneuraminic acid hydroxylase gene double-KO pigs

Target editing is possible through site-specific nucleases, of which the following are most commonly used: zinc finger nucleases (ZFN) and TALEN systems. Pigs can be modified to create porcine models of human disease^[35] or provide tissue and organs for xenotransplantation^[44]. Thus far, our team has knocked out α 1,3-galactosyltransferase (*α 1,3GT* or *GGTA1*) and cytidine monophospho-N-acetylneuraminic acid hydroxylase (*CMAH*) genes in pig using genome editing technology and SCNT^[79]. Porcine fibroblast cell lines were derived from the *GGTA1*-KO pigs. These cells were subjected to an additional KO of *CMAH* gene. A pair of ZFN-encoding mRNAs targeting exon 8 of the *CMAH* gene was used to generate heterozygous *CMAH*-KO cells, from which cloned pigs were produced by SCNT. One of the cloned pigs was re-cloned after additional KO of the remaining *CMAH* allele using the same ZFN-encoding mRNA to generate *GGTA1/CMAH*-double homozygous KO pigs. The use of TALEN-encoding mRNAs targeting exon 7 of the *CMAH* gene resulted in efficient generation of homozygous *CMAH*-KO cells, which were used for SCNT to produce cloned pigs that were homozygous for a double *GGTA1/CMAH* KO. The combination of TALEN-encoding mRNA, *in vitro* selection of the nuclear donor cells, and SCNT provides a robust method for generating KO pigs. ZFN and TALEN are new tools for producing gene-KO animals. In this study, we produced genetically modified pigs in which two endogenous genes were knocked out. Porcine fibroblast cell lines were derived from homozygous α 1,3-galactosyltransferase (*GalT*) KO pigs. These cells were subjected to an additional KO for the cytidine monophospho-N-acetylneuraminic acid hydroxylase (*CMAH*) gene. A pair of ZFN-encoding mRNAs targeting exon 8 of the *CMAH* gene was used to generate heterozygous *CMAH*-KO cells, from which cloned pigs were produced by SCNT. Subsequently, one of the cloned pigs was re-cloned after additional KO of the remaining *CMAH* allele using the same ZFN-encoding mRNA to

generate *GalT/CMAH*-double homozygous KO pigs. The use of TALEN-encoding mRNAs targeting exon 7 of the *CMAH* gene efficiently generated homozygous *CMAH*-KO cells that were used for SCNT to produce cloned pigs that were homozygous for a double *GalT/CMAH*-KO. These results demonstrate that the combination of TALEN-encoding mRNA, *in vitro* selection of nuclear donor cells, and SCNT constitutes a robust method for generating KO pigs.

Production of mini-pig models exhibiting phenotypes resembling those of human diabetes

We previously generated Tg cloned diabetic pigs introduced by dominant-negative mutant *HNF1a*^[67]. However, rearing pigs is expensive and technically difficult. As micro-mini pig handling is easier than managing common domestic pigs, we produced Tg pigs using gamete intrafallopian transfer (GIFT), a tool used for assisted reproductive technology against infertility in humans. In vitro fertilization and intrafallopian insemination in the micro-mini pig using the cryopreserved epididymal sperm of a Tg-cloned pig carrying a dominant-negative *HNF1a* gene resulted in a diabetes model with smaller Tg offspring compared to the original model. Thus, relative body weight of the Tg-mini-pig was markedly reduced, reaching 50% that of domestic pigs at 12 wk of age (Tg-mini-pig *vs* WT, 12 kg *vs* 24.6 ± 2.7 kg, **Figure 1**). The Tg mini-pig developed diabetes-associated complications, such as hyperglycemia and cataract, similar to those of human DM patients, and nodular lesions in the renal glomeruli, similar to that in the original Tg pigs. Thus, artificial reproductive technology using cryopreserved epididymal sperm and GIFT is a practical option for generating Tg mini-pigs.

Production of PDX1-HES1 Tg SCID-pig models receptive to human cells

So far, we have generated interleukin-2 receptor gamma (*IL2RG*) KO pigs *via* the SCNT method in a short duration^[80]. The combination of ZFN-encoding mRNAs and SCNT provides a simple and robust method for producing KO pigs without genomic integration. The *IL2RG* gene was knocked out in PFFs using ZFN-encoding mRNAs, and *IL2RG*-KO pigs were subsequently generated using these KO cells through SCNT. The resulting *IL2RG*-KO pigs completely lacked a thymus and were deficient in T and NK cells, similar to that in human patients with X-linked SCID. By mating this pig with *PDX1-HES1* Tg pig^[72,73], we generated an SCID pig with pancreatic agenesis that can greatly contribute to preclinical evaluations of stem cell transplantation and xenotransplantation.

SEEKING ANSWERS FOR β-CELL REPLACEMENT THERAPY IN THE CLINICAL SETTING

Our group focuses on diabetes treatment through β-cell replacement therapy, especially cell transplantation. The issues of β-cell replacement therapy in the clinical setting requires several factors to be clarified (**Table 1**). The issues and challenges are described as follows.

Shortage of donors: Alternative islet cell sources

The number of patients awaiting transplantation is constantly increasing. Moreover, the available organ donor supply will remain insufficient to match the potential demand if cellular replacement therapies play a greater role in the treatment of T1DM patients. The donor islets available are only adequate to treat 1%-2% of potential transplant recipients^[75]. In addition, most cases require two to three donor organs^[31]. Thus, alternative strategies, including gene therapy, stem cell transplantation, and xenotransplantation, are being explored to bridge this gap.

Gene therapy: Trans-differentiation of cells from a patient's own tissue into pancreatic β-cells could address one of the challenges of donor shortage. The advantage of this strategy is that the patient does not require immunosuppressive drugs. Transfecting non-islet cells to contain and express glucose-regulated insulin is an attractive approach, and the studies have proved challenging^[81-83]. However, the efficiency was extremely low, even in mice, and the use of viruses poses some limitations in clinical use.

Stem cell transplantation: Technologies in the field of regenerative medicine provide enormous opportunities for generating β-cells from different stem cell sources for

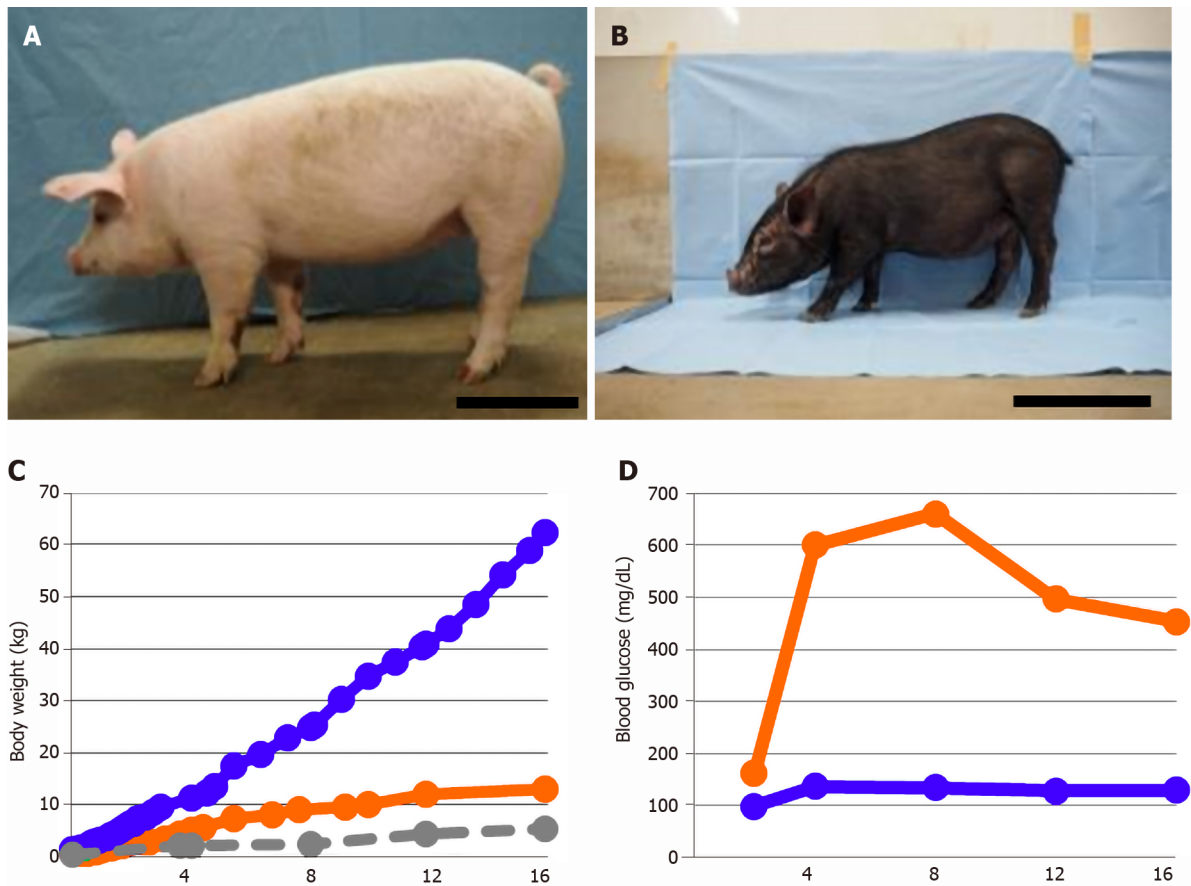


Figure 1 Generation of transgenic-mini pigs carrying a dominant-negative mutant *HNF1α* gene. A: Common domestic pig; B: Transgenic (Tg)-mini pigs carrying a dominant-negative mutant *HNF1α* gene; C: Body weights at specified times; D: Blood glucose level. Blue line: Common domestic pigs, $n = 2-4$. Orange line: Tg-mini pig, $n = 1$. Gray line: micro-mini pig, $n = 2-4$. Quantitative data are presented as means. Scale bar: 30 cm. Time after birth is indicated in days.

cellular therapy. Insulin-producing cells can be generated from a variety of stem cell types, such as pluripotent stem cells, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), although ideally, functional cells should be expanded to considerable levels by non-integrative culture techniques.

Mesenchymal stem and bone marrow cells: The ease of isolation, plasticity, and clinical translation to generate autologous cells, mesenchymal stem cells, and bone marrow cells are superior. These cells offer the hope that a personalized cell could be transplanted without the risk of alloimmunity, thereby securing sufficient supply to meet future global requirements^[84]. Mesenchymal stem cells and bone marrow cells appear attractive for cell therapy; however, they are not readily converted into functionally mature β -cells^[85].

ESCs and iPSCs: Human pluripotent stem cells, such as ESCs and iPSCs, provide unprecedented opportunities for developing cell therapies against diabetes and other diseases. Both ESCs and iPSCs have been used in clinical trials.

Human ESCs (hESCs) have been intensively investigated for their ability to differentiate into pancreatic endoderm cells^[86-89]. After the hESCs were successfully differentiated into insulin-producing cells, some clinical trials have been conducted, although without positive results^[90]. Despite marked advances in the production of insulin-producing cells from hESCs, important challenges remain. Potential ethical and religious considerations emerge when hESCs are used, as the starting cell population is derived from discarded human embryos. Moreover, hESCs need protection against both allo- and autoimmune rejection in the case of transplantation.

In 2006, Takahashi and Yamanaka^[91] developed a protocol for dedifferentiating and transdifferentiating murine fibroblast-derived iPSCs. In the subsequent year, the same group generated adult human fibroblasts-derived iPSCs by using defined factors^[92]. In diabetes research, patient-specific autologous iPSC-derived β -cell products can prevent immune rejection and be immunologically compatible. Many researchers have

attempted to generate functional β -cells from human iPSCs (hiPSCs); however, transplanting only β -cells might prove insufficient for treating T1DM. For the appropriate regulation of glucose levels, α -cells are essential^[93]. In this regard, Veres *et al.*^[94] reported significant progress in generating hiPSC-derived pancreatic cells, mainly β -like cells, α -like cells, enterochromaffin-like cells, and non-endocrine cells, using a stem cell-derived- β differentiation protocol. This recent finding can potentially inform the production of a defined and safe therapeutic product, with the removal of proliferative progenitor cells to avoid the risk of tumors^[94]. The results of the abovementioned study accelerate hiPSC-derived β -cell replacement in the clinic. Despite this promising approach, iPSC-derived β -cell replacement has several hurdles to cross. Manufacturing patient-specific products constitutes a huge advance although the costs associated with the application of good manufacturing practices in individualized stem cell therapy are immense. In other words, this therapy will require optimization of the differentiation conditions for every batch of iPSCs, and the added substantial costs and operational burden with the process could be astronomical^[95].

Pig-to-human xenotransplantation

Xenotransplantation would provide an unlimited and predictable donor source of organs and enable careful surgical planning. Furthermore, the recent progress of porcine organ graft survival in nonhuman primates may spur the possibility of successful xenotransplantation^[96]. Pigs have been considered a source of islet replacement therapy for T1DM for many years^[97]. In 1994, in a first-in-human trial, Groth *et al.*^[98] transplanted fetal pig islets encased within the human kidney capsule into T1DM patients. The study was remarkable as porcine C-peptide was detectable for more than 300 d, without any observable serious side effects, although there was no insulin independence or reduction in exogenous insulin requirement. Encapsulated pig islets, branded as Diabecell®, to treat patients with unstable T1DM have undergone clinical trials in New Zealand and Argentina, and encouraging preliminary results indicate correction of hypoglycemic unawareness and improved a modest reduction in the hemoglobin (HbA1c) levels. However, detectable porcine C-peptide was strikingly absent in these subjects^[99-103].

Response to the donor-shortage challenge – preparation of porcine pancreatic islets for transplantation to human recipients: In the current scenario for T1DM patients who are candidates for islet cell transplantation, porcine islets have the advantage of being an alternative islet cell source to resolve the issue of donor shortage. The pig may potentially be an unlimited source of organs for patients with diabetes, although the appropriate age for islet isolation is not known. As juvenile pigs are more easily reared in uncontaminated conditions, we analyzed the distribution of endocrine cell clusters by comprehensively evaluating juvenile porcine pancreatic development to propose an appropriate age cut-off for islet isolation from the juvenile porcine pancreas^[43]. We found that the isolation of porcine pancreatic islets approximately 35 d after birth may offer benefits with regard to their xenotransplantation potential. Further research into the optimal timeline for islet isolation is being conducted in DPF pigs generated at our research center.

GRAFT FAILURE DUE TO IMMUNE SYSTEM, METABOLIC PRESSURE, AND OXIDATIVE STRESS BY HYPOXIA OR INFLAMMATION

Alternative transplantation sites

The portal vein is the most frequently used site for clinical human islet transplantation, although it is far from being an ideal site. Approximately 60% of transplanted islets undergo apoptosis within the first week post-transplantation, which is attributed to poor engraftment. The engrafted islets are continuously exposed to the hepatic microenvironment, high glycemic levels, low oxygen tension, toxins, and combined innate immune attack through instant blood-mediated immune response (IBMIR), all of which are toxic to islets and lead to premature islet dysfunction/death. Several factors, such as extracellular matrix components and proinflammatory cytokines due to immune rejection, have been considered as contributory factors in graft failure^[104-106]. In addition, poor revascularization of islets combined with severe changes in the gene expression of the transplanted islets contributes to late dysfunction that, together with islet death, contribute to the poor long-term results of the demand for a large number of islets to restore glucose homeostasis. Many patients achieve insulin

independence after a portal vein islet infusion, although most eventually resume insulin injections in the long term because of the abovementioned issues^[107,108]. The protection of islets for transplantation can be achieved through the addition of anti-inflammatory agents during islet culture and systemically to the recipient after transplantation. Moreover, the administration of anticoagulants might be another way to resolve the attendant issues. Based on the identified problems with regard to the liver transplantation site, exploring alternative transplantation sites would be another possible approach. In experimental animal models, islet transplantation has been attempted at many alternative sites. To date, the subcutaneous space, the renal subcapsular space, muscle, pancreas, eye chamber, testis, and thymus can function as islet transplantation sites to reverse hyperglycemia in small animal models. Few alternative sites have the potential for clinical translation, and generally, evidence is lacking for post-transplant islet function superior to that following intraportal infusion^[109]. The omentum is one such potentially good transplantation site^[110-112]. The University of Miami is currently conducting a phase I/II clinical trial that involves laparoscopic transplantation of human allogenic islets coated in autologous plasma onto the wrapped omentum^[113].

Challenges in finding alternative transplantation sites: Our team has continued the search for alternative islet transplantation sites that provide a better environment for prolonged function and survival. As described above, two different cloned Tg-pig models that express the far-red fluorescent protein Plum and mPlum have been under consideration in the search for alternative islet transplantation sites^[76,77].

Continued autoimmunity and alloimmunity

In pig-to-human xenotransplantation, significant phylogenetic distance results in serious immunological problems after transplantation. Xenografts are rejected by the human immune system, and there are several current challenges. Some mechanisms of rejection have been clarified and, therefore, genetic engineering techniques have been applied to establish pig models that are suitable as donors.

Hyperacute xenograft rejection: Porcine organs that are transplanted into human recipients are immediately rejected because of the so-called hyperacute immunological reaction. Xenograft rejection is mainly caused by the Gal antigen, found on the donor's cell surface, that is synthesized by the enzyme GGTA1 and synthesizes the α 1,3-galactose (α 1,3Gal) epitopes (Gal α 1, 3Gal β 1, and 4GlcNAc-R). Humans lack both the Gal antigen and the GGTA-1 enzyme but have xenoreactive antibodies directed against the porcine Gal antigen, which leads to the activation of the enzymatic complement cascade in the recipient. An optimal solution for the problem of hyperacute rejection is the inactivation of the gene encoding the GGTA-1 enzyme responsible for the formation of the Gal antigen. In 2001, the first heterozygous GGTA1-KO pigs were produced^[114] and, 1 year later, the first piglets with two KO alleles of the GGTA1 gene were born^[115]. A series of GGTA1-KO pigs has been generated using a genome editing system. Pigs with the GGTA1/CMAH/ β -1,4-N-acetyl-galactosaminyl-transferase 2 triple gene KO were generated using the CRISPR/Cas9 system. Cells from these genetically modified animals exhibited reduced levels of human immunoglobulin (Ig) M and IgG binding, resulting in diminished porcine xenoantigenicity^[116].

Challenges to hyperacute xenograft rejection: As described in the introduction to the section on the generation of other Tg pigs in our laboratory for diabetic research, our team has knocked out GGTA1 and CMAH genes in pigs using genome editing technology and SCNT^[79]. Genetically modified pigs can potentially be used as a source of cells, tissues, and organs for transplantation into human recipients.

Porcine endogenous retroviruses: The risk of cross-species transmission of porcine endogenous retroviruses (PERV) has impeded the clinical application of this approach. Recently, CRISPR-Cas9 was used to inactivate 62 copies of the PERV pol gene in a porcine cell line and resulted in > 1000-fold reduction in PERV transmission to human cells^[117-119]. Using a combination of CRISPR-Cas9 and transposon technologies, Yue *et al.*^[120] showed that pigs with inactivated PERV can be genetically engineered to eliminate three xenoantigens and to express nine human transgenes that enhance the pigs' immunological compatibility and blood-coagulation compatibility with humans. The engineered pigs exhibited normal physiology, fertility, and germline transmission of 13 genes, and 42 alleles were edited^[120]. However, in the first phase 1/2a clinical trial of the xenotransplantation of encapsulated neonatal porcine islets into non-immunosuppressed T1DM patients in New Zealand, despite the use of non-genetically modified animals, there was no confirmation of PERV transmission. Therefore, the risk

of PERV-related complications might be considered low^[100,103,121].

Encapsulation technologies to address continued autoimmunity and alloimmunity:

The potential to protect transplanted islets or stem cells from immune attack through micro- or macro-encapsulation approaches has been explored extensively. Encapsulation selectively permits passive diffusion of glucose, insulin, oxygen, carbon dioxide, and other nutrient exchange without direct cell-cell contact with immune cells. Encapsulation technology is potentially promising, although several factors, including the site of transplantation, device configuration, materials, nutrient exchange, and their ability to promote neovascularization and biocompatibility, need to be considered when evaluating such devices^[122,123]. A small number of islet encapsulation systems have been applied currently in clinical trials; however, there are insufficient data on the efficacy of these systems in humans, their adverse effects, and the duration that the product can remain safely implanted and functional^[122,124,125]. Thus far, none of the trials have resulted in the maintenance of an insulin-independent status in T1DM patients^[125,126]. Strategies to overcome these hurdles are being investigated, and some of these interventions are in the clinical trial stage, providing hope to improve results in clinical islet transplantation^[126,127].

Challenges with the use of encapsulation technologies: Several studies have demonstrated islet cell survival within encapsulation devices and production of insulin in mice, although translation to larger animals or humans is often limited by fibroblastic overgrowth around the classical implanted device^[124]. We performed transplantation of encapsulated juvenile isolated islets in adult pigs in a preliminary experiment. The pig islets were alginate encapsulated, which is generally accepted in this research and clinical field; however, the intraabdominally transplanted capsules developed peri-device fibroblastic overgrowth and adhered to the diaphragm, hepatic surface, and peritoneum. Thus, in a clinical setting of islet xenotransplantation, juvenile isolated pig islet encapsulation with some modifications might be the best method because controlling all targets for rejection may be difficult. Our conclusion is that other biocompatible materials for encapsulation as new devices or changes in the islet transplantation methods are required.

IMMUNOSUPPRESSIVE DRUGS

Protecting against immunosuppressant-related toxicity

One of the important causes of immunosuppressant-related toxicity is that currently used immunosuppressive drugs confer a risk for allosensitization in patients and are toxic to islets. Immunosuppressive drugs effectively paralyze immune responses to alloantigens as well as increase the risk of life-threatening infections or malignancies. The most potent immunosuppressive drugs (cyclosporine, tacrolimus, and sirolimus) are directly toxic to β -cells^[128-130]. The drugs reduce mitochondrial density and function without changing apoptosis rates, resulting in decreased insulin secretion. Thus, post-transplantation diabetes is possibly induced by the drug's action on mitochondrial function^[128]. The immunosuppressive drugs used in clinical islet transplantation are uniformly used for all patients and are not tailored in individualized therapy. Therefore, the present islet transplantation approach and future stem cell therapies cannot be considered ideal until such treatments can be maintained without chronic immunosuppression.

Challenges to protecting against immunosuppressant-related toxicity: We developed a new selection system for suitable immunosuppressive drugs for islet transplantation using epigenetic analysis in a three-dimensional culture system^[131]. We first isolated islets from a Tg pig specifically expressing the green fluorescent protein in the β -cells (*PDX1-Venus Tg Pig*). The islets were cultured in one well in the presence of an immunosuppressive drug. Thereafter, epigenetic analysis of the islets was performed. Over time, we observed epigenetic changes in the insulin gene promoter of the islets in a certain group, despite no change in their configuration. This proposed system can evaluate the short-term influence of immunosuppressive drugs on pancreatic islets and can potentially be used to determine which combination of immunosuppressive drugs is specifically suitable for each human islet transplantation. The weak point of the system is that the toxicity of β -cells can be evaluated only *in vitro*. However, if T cells and/or antibodies that are influenced by β -cells from T1DM patients are extracted, the system may potentially enable the identification of new drugs for T and

B cell-depleting antibodies.

BEHAVIOR OF THE TRANSPLANTED CELLS IN THE GROWING BODY IN TRANSPLANTATION PERFORMED IN ADOLESCENTS/CHILDREN

The number of islet transplantations has been increasing in an active and flourishing manner over the past decades. Islet transplantation is seldom performed in younger T1DM patients. Much information would be needed for transplantation of islets in younger patients, such as adverse effects on sexual function or fertility. Research using pigs might provide some information about these questions because pigs grow fast.

APPLICATION OF PIGS FOR THE DEVELOPMENT OF NEW THERAPIES FOR DIABETES

As of 2011, according to the Organ Procurement and Transplantation Network, there were approximately 8000 deceased organ donors in the United States; however, only 1562 pancreas were recovered from donors^[132]. Many donated pancreases are unsuitable for extracting islets for transplantation because they do not meet the selection criteria. In addition, islet isolation is complicated and technically difficult in some cases. Islets are often damaged or destroyed during processing. Even in leading centers, it is difficult to recover a sufficient number of islets from a single cadaveric donor pancreas. An average islet isolation generally yields approximately 50% of the estimated number, *i.e.*, more than one million islets present in an adult human pancreas^[133,134]. Therefore, at present, islets with a low yield at isolation are not transplanted and distributed for basic research use^[135]. Thus, only a small number of islet transplantation can be performed each year. The development of an optimal islet cryopreservation method would permit the banking of unlimited islets and allow the transplantation of islets from multiple donors by a single procedure with a more flexible transplantation schedule. A library of cryopreserved islets allows for selection based on human leukocyte antigen (HLA) tissue type and creates more flexibility with regard to the total amount of transplantable mass. Therefore, pancreatic islet sheets may provide a promising option for the cryopreservation of islets. Successful cryopreservation of pancreatic islet sheet function for both pigs and humans could create a huge stock and delivery system that could alleviate islet shortage. The development of islet cryopreservation methods was initiated more than 30 years ago^[136,137]; in the early 1990s, cryopreserved islets were used to facilitate the transplantation of an adequate β -cell mass by combining fresh and cryopreserved islets from multiple donors^[138]. However, the use of cryopreserved islets has been hindered due to suboptimal recovery of islet quality and quantity^[139-142].

Cryopreservation of pancreatic islets

We have established a new cryopreservation method for pancreatic islets by vitrification using hollow fibers as containers^[139]. A unique feature of the hollow fiber vitrification (HFV) method is that it achieves stable vitrification using a minimum volume of cryoprotectant (CPA) solution, thereby ensuring high islet viability. The cytotoxicity, optimum composition, and concentration of CPA for vitrifying islets were examined in a mouse study. Insulin secretion was measured *in vitro* by a static incubation assay, and metabolic functions were tested after transplantation into streptozotocin-induced diabetic mice. The combination of 15% dimethyl sulfoxide and +15% ethylene glycol resulted in the best CPA solution for the HFV of islets. HFV showed the highest viability in comparison to the two vitrification methods, open pulled straws, and vitrification with EDT324 solution. The vitrified islets stably expressed the β -cell markers *NeuroD*, *Pdx-1*, and *V-maf musculoaponeurotic fibrosarcoma oncogene homolog A*. Transplantation of the vitrified islets achieved euglycemia in the host diabetic mice and similar responses to an intraperitoneal glucose tolerance test as in non-vitrified transplanted islets. The HFV method allows for efficient long-term cryopreservation of islets. This method is being currently tested for application in porcine islets to evaluate the possibility of developing this method for human islet transplantation.

Sheet technology

Transplantation of islet cell sheets has been considered as a feasible, safe, and therapeutically effective approach for the treatment of T1DM patients. The cryopreservation of islet sheets is another option to be considered. Islet sheet transplantation is easily feasible using laparoscopy and does not induce concerns about IBMIR. In a preliminary study, we first generated islet cell sheets with mouse islets^[143]. The cell sheets were fabricated using temperature-responsive culture dishes. The engineered islet cell sheets were stable, and the *PDX-1* promoter methylation and the expression of NeuroD, PDX-1, and glucagon proteins were similar between the sheets and islets. Moreover, in the transplantation of islet cell sheets, because of their adhesive properties, cell sheets can be easily applied to the liver and peritoneal surfaces. The mouse islet sheet did not correct serum hyperglycemia in diabetic recipient mice in our study, although other researchers have reported that multi-layered cell sheets boost glycemic stability and insulin function^[144-146]. Lee *et al.*^[147] constructed multilayered cell sheets using rat/human islets and human adipose tissue-derived stem cells. We have successfully generated islet sheets from Tg pigs with a pancreas-specific expression of green fluorescent protein (*PDX1-Venus* Tg Pig) and transplanted them onto the liver surface (Figure 2). Currently, the application of multi-layered pig islet cell sheets is being investigated, and, if feasible, they will be cryopreserved and applied in human transplantation. In addition, islet sheets from pigs with macroencapsulation may be another option.

GARGANTUAN PROJECTS

Production of human tissues and organs in living pigs

The technology used for producing cells and/or tissues of human origin in the bodies of living animals is referred to as *in vivo* bioreactors. Based on proof-of-concept studies in small animals used to produce organs^[148-154], we have challenged two different approaches to induce human pancreas in pigs by techniques involving early embryos of animals and the injection of human cells into fetuses or neonatal pigs.

Pigs with human pancreas created by blastocyst complementation as the embryonic approach: Yamaguchi *et al.*^[155] established a developmental engineering method for heterogeneous mice with rat pancreas. In this concept, a master gene of the pancreatic development *PDX1* gene in rats is knocked out. After fertilization, the blastocyst of *PDX1*-KO rats were complemented with mouse iPSCs. Therefore, the lack of the rat pancreas is complemented by the mouse pancreas. Islets taken from the chimeric rats with mouse pancreas produced an effect when transplanted into a diabetes-induced mouse model^[155]. For the application of this concept in porcine models and human studies, apancreatic pigs need to be produced. After blastocyst exchange with a healthy pig, a pancreas is formed from the exogenous cells as the apancreatic pig could not survive for a long time^[64,72,73]. Using this concept, iPSCs from T1DM patients can be injected into apancreatic pig embryos to generate human organs inside the pig's body. Once the developing embryo is implanted into a surrogate mother pig, the pig fetus has a human pancreas from the developmental stage. Thus, pigs with personalized human pancreas for T1DM patients can be produced. We have proven the complementation concept in pig-to-pig transplantation as the basic research proof of concept in large animals^[64,72], and this technology is currently under consideration to create human pancreas in pigs.

Fetal approaches via injection of human cells into fetal pig's organs: This concept involves the use of the developmental niche of pig fetuses to promote pancreatic development from exogenous pluripotent cells. To generate an organ from exogenous cells, developmental circumstances within the fetus make it an ideal *in vivo* bioreactors. Crossing the chimeric male boar carrying the *PDX1-HES1* gene (*PDX1-HES1* Tg pig, described above) with a WT female would produce the next generation of fetuses or newborns with pancreatic agenesis. Direct manipulation of *PDX1-HES1* Tg-pig fetuses to fill the organ niche with pancreatic progenitor cells from hiPSCs may possibly generate human pancreas in the niche. As several groups have already generated insulin-producing cells from hiPSCs^[93,94,156-160], we are currently conducting experiments in porcine models using pig progenitor cells to determine whether the concept is feasible. The pancreas was harvested from 40-d-old *PDX1-Venus* Tg-pig fetus, minced, and injected, under ultrasonographic guidance, through the uterine wall into the peritoneal cavity of 40-d-old pig fetus with pancreatic agenesis to track the

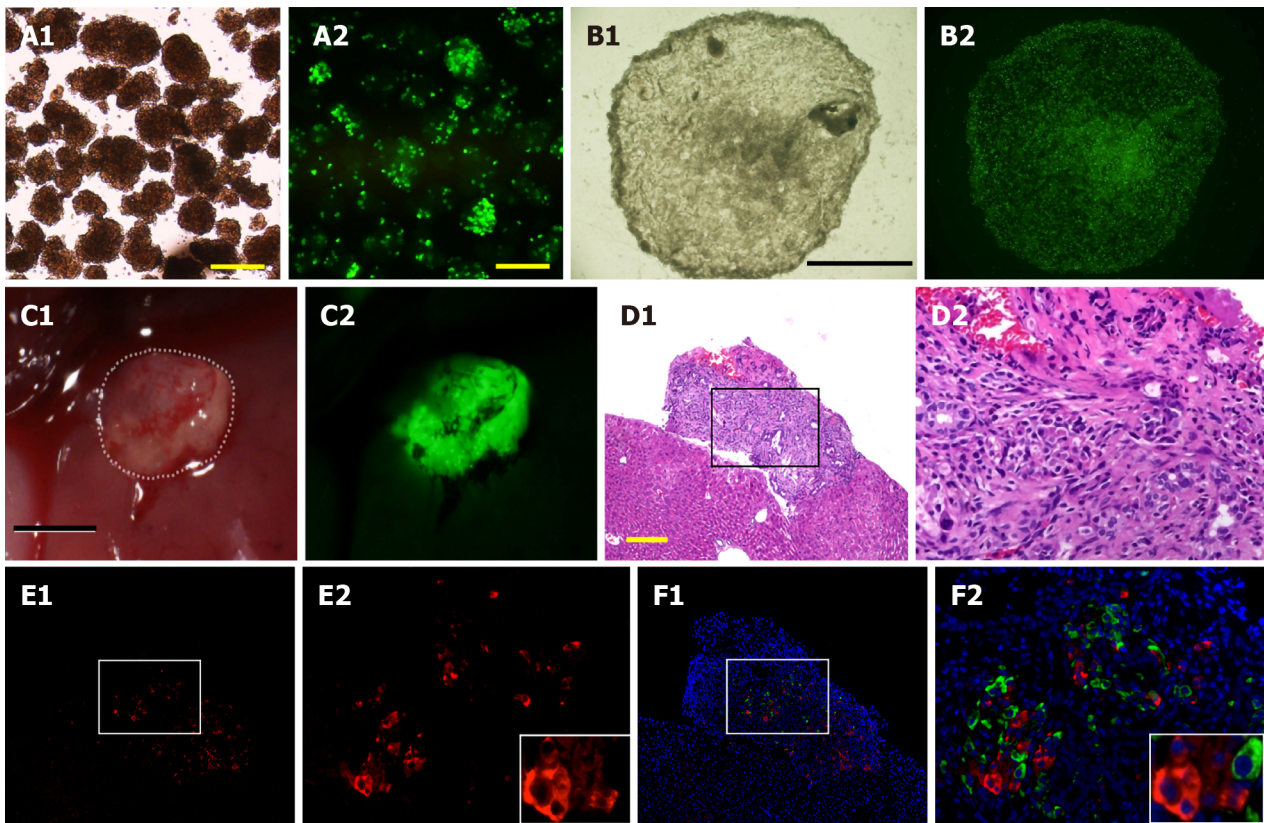


Figure 2 The engineered islet cell sheets. A: Isolated pancreatic islets from a *PDX1-Venus* transgenic pig. Bright-field image of isolated islets (A1). Fluorescence microscopic image of the islets (A2). The *PDX1-Venus* transgenic (Tg) pig expresses green fluorescent protein specifically from the β -cells. The *PDX1* gene promoter was conjugated to *Venus*, a green fluorescent protein. The isolated islets strongly emit green fluorescence in their nuclei; B: Islet cell sheets that were generated *in vitro* by seeding dispersed primary islets cells from a *PDX1-Venus* Tg pig into temperature-responsive 24-well culture plates covered with laminin. Bright-field image of the islet cell sheet (B1). Fluorescence microscopy image of the islet cell sheet (B2); C: The islet cell sheet was harvested 3 d after plating and transplanted onto the liver of streptozotocin-induced diabetic severe combined immunodeficiency mice. Bright-field microscopy. The sheet attached to the liver of diabetic mice (C1). The sheet emitted green fluorescence (C2); D-F: Immunohistochemical analysis of the transplanted islet cell sheet. Hematoxylin–eosin (HE) staining of the sheet on day 23 (D1 and D2). Immunofluorescence analysis of transplanted islet cell sheets (E1, E2, F1 and F2). Glucagon-positive cells (cytoplasm; green) and merged images (insulin-positive cells, cytoplasm; red) (F1 and F2). (D2), (E2), and (F2) present higher magnification images of the region indicated by a square in the panels (D1), (E1), and (F1), respectively. Nuclei were stained blue with DAPI (4,6-diamidino-2-phenylindole). Scale bars: yellow, 200 μ m; black, 5 mm. The time after transplantation is indicated in days.

subsequent development of pancreatic tissue (Figure 3). On day 14 after transplantation, the minced-fetal pancreas was attached to the liver and under the diaphragm. The cells survived, and histological analyses confirmed progressive development of ductal structures characteristic of the fetal pancreas^[16]. Based on this concept, a chimeric human pancreas may be obtained with a pig scaffold.

CONCLUSION

Appropriate animal models for the evaluation of the efficacy and safety of new therapeutic concepts are critical for the successful application of translational medicine. Genetically tailored porcine models have the potential to bridge the gap between proof-of-concept studies in animals and clinical studies in patients with T1DM. The translation of novel discoveries to clinical applications is a long process that is costly and often inefficient. However, these efforts definitely hold potential for the provision of appropriate treatments within a reasonable time frame. We believe that new therapeutic approaches for solving the issues with β -cell replacement therapy to treat diabetes will be imminently discovered, validated, and optimized in large animal models, which will help save patient lives.

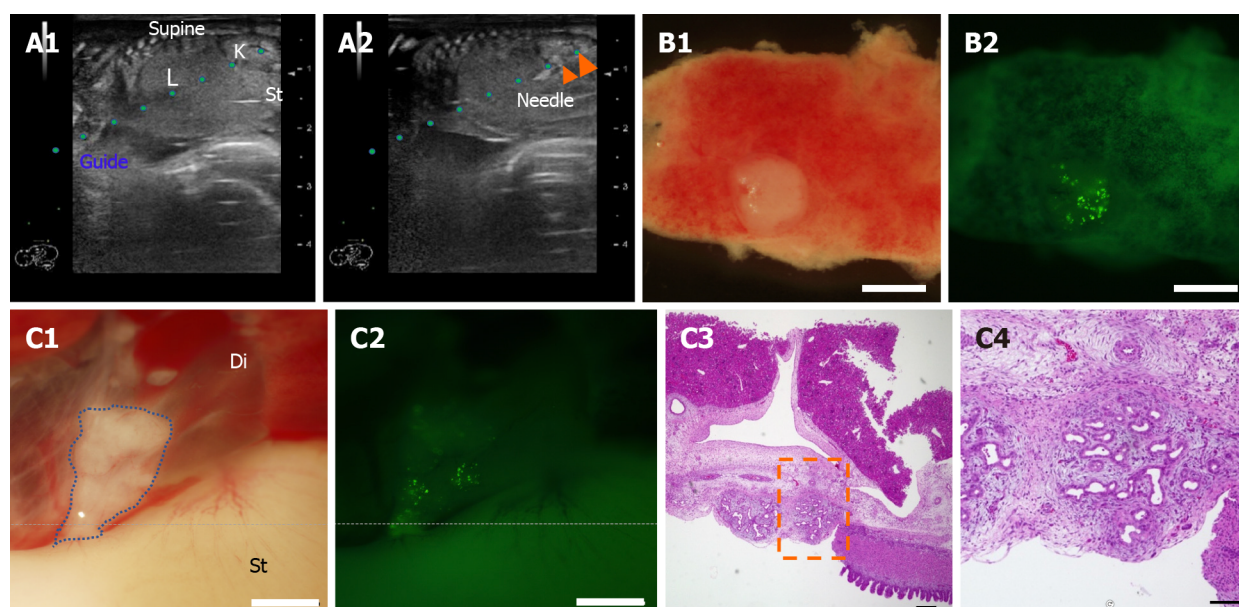


Figure 3 Ultrasound-guided fetal pancreatic tissue injection into a porcine fetus with organ niche. A: The pancreas was harvested from the 40-d-old *PDX1-Venus* transgenic-pig fetus and minced. The minced-fetal pancreas was injected, under ultrasonographic guidance, through the uterine wall into the peritoneal cavity of a 40-d-old pig fetus. Peritoneal cavity of a 40-d-old pig fetus and a guide for the needle (A1). The needle was inserted into the omental foramen, and the minced-fetal pancreas was injected (A2); B: The minced-fetal pancreas was attached to the liver on day 14 (B1) and emitted green fluorescence (B2); C: The minced-fetal pancreas was attached under the diaphragm on day 14 (C1) and emitted green fluorescence (C2). Hematoxylin–eosin staining of the pancreas on day 14 (C3 and C4). The transplanted cells survived, and histological analyses confirmed the progressive development of the pancreatic ductal structures. (C4) comprises a higher magnification image of the region indicated by a black square in panel (C3). The time after birth is indicated in days. Scale bars: white, 1 mm; black, 5 μ m. L: Liver; K: Kidney; St: Stomach; Di: Diaphragm.

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