

An Effective New Cryopreservation Procedure for Pancreatic Islets Using Hollow Fiber Vitrification

Authors

M. Nagaya^{1,2*}, H. Matsunari^{1*}, T. Kanai³, M. Maehara³, K. Nakano^{1,3}, I. Umeki³, Y. Katsumata³, Y. Kasai³, R. Sakai³, M. Kobayashi³, M. Honda¹, N. Abe^{3,4}, M. Watanabe^{1,3}, K. Umeyama^{1,3}, H. Nagashima^{1,3}

Affiliations

Affiliation addresses are listed at the end of the article

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Correspondence

M. Nagaya, MD, PhD
Meiji University International
Institute for Bio-Resource
Research
1-1-1 Higashimita Tama-ku
Kawasaki
Kanagawa 214 8571
Japan
Tel.: +81/44/934 7824
Fax: +81/44/934 7824
m2nagaya@meiji.ac.jp

H. Nagashima, PhD

Department of Life Sciences
Laboratory of Developmental
Engineering
School of Agriculture
Meiji University
1-1-1 Higashimita Tama-ku
Kawasaki
Kanagawa 214 8571
Japan
Tel.: +81/44/934 7824
Fax: +81/44/934 7824
hnagas@isc.meiji.ac.jp

Abstract

The present study aimed at establishing a new cryopreservation method for mouse pancreatic islets by vitrification using hollow fibers as a container. A unique feature of the hollow fiber vitrification (HFV) method is that this method achieves stable vitrification using a minimum volume of cryoprotectant (CPA) solution, thereby ensuring high viability of the islets. The cytotoxicity, optimum composition, and concentration of the CPAs for vitrifying islets were examined. The viability, functional-integrity of vitrified islets were evaluated in comparison with those vitrified by conventional methods. Insulin secretion was measured in vitro by a static incubation assay and the metabolic functions was tested

after transplantation into Streptozotocin-induced diabetic mice. The combination of 15% dimethyl sulfoxide+15% ethylene glycol resulted in the best CPA solution for the HFV of islets. HFV showed the highest viability in comparison to 2 vitrification methods, open pulled straws and vitrification with EDT324 solution. The vitrified islets stably expressed β -cells markers NeuroD, Pancreatic and duodenal homeobox-1, and MafA. Transplantation of the vitrified islets achieved euglycemia of the host diabetic mice and response to an intraperitoneal glucose tolerance test to a similar extent as non-vitrified transplanted islets. The HFV method allows for efficient long-term cryopreservation of islets.

Supporting Information for this article is available online at <http://www.thieme-connect.de/products>

Abbreviations

HBSS	Hank's balanced salt solution
RPMI	Roswell Park Memorial Institute
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
EG	Ethylene glycol
EtOH	Ethanol
PROH	Propanediol
PI	Propidium iodide
HF	Hollow fiber
HFV	Hollow fiber vitrification
OPSV	Open-pulled straw virification
MVC	Minimum volume cooling
IPGTT	Intraperitoneal glucose tolerance test
SI	Stimulation index
<i>Pdx-1</i>	Pancreas duodenum homeobox-1
<i>MafA</i>	V-maf avian musculoaponeurotic fibrosarcoma oncogene homologue A

*MN and HM contributed equally to this work.

Introduction

Islet transplantation is considered as a viable option for the treatment of type 1 diabetes [1]. To achieve insulin-sufficiency by islet transplantation in diabetic patients, 5000 islet equivalents (IEQs) per kilogram of body weight are required [2]. However, at present, islet isolation is still complicated and technically difficult in some cases [3]. Therefore, islets with a low yield at isolation are not transplanted and distributed for basic research. To allow the unlimited collection of islets, islet cryopreservation methods need to be optimized. The development of islet cryopreservation methods was initiated over 20 years ago [4] and since then, many freezing methods have been investigated [5,6]. However, the existing cryopreservation methods have an islet survival rate of only about 50% [7].

Effective low temperature storage is beneficial to buy time for sterility and viability testing of the islet preparation. Endocrine function of the islets can be examined before transplantation, for example by static incubation [8]. A library of cry-

opreserved islets allows for selection based on HLA tissue type and creates more flexibility with regard to the total amount of transplantable mass [1, 2, 9].

Cryoprotectants (CPAs) are neutral solutes with a low molecular weight (allowing cell penetration), low toxicity, and high solubility in water. They are tolerated in sufficiently high concentrations and can significantly reduce the amount of ice crystals that form at any given subzero temperature [10, 11]. Dimethyl sulfoxide (DMSO) is a widely used CPA that has been reported to cryopreserve human islets [6]. Several other compounds, including ethylene glycol (EG) [12], and polyethylene glycol [13], have also been used for the cryostorage of islets. Nevertheless, current freezing methods have limitations and the formation of intracellular ice crystal is unavoidable. Ice crystals disrupt the integrity of the capsule membrane and hence impaired insulin secretion [14, 15]. If the drawbacks of cryopreservation can be overcome, banking of islets will become realistic and will greatly benefit islet transplantation treatments for diabetic patients.

An alternative approach for cryopreserving living cells was described by Rall and Fahy [16]. This process is known as vitrification [7, 17, 18] and is based on the increased viscosity of a highly concentrated aqueous solution of CPAs with decreasing temperature until an amorphous glass-like solid forms. Vitrification procedures have been developed and shown to effectively preserve a variety of cells, embryos, and tissues [19–22]. The efficacy of vitrification has been demonstrated for embryos of various mammalian species [23]. The vitrification procedure involves ultra-rapid cooling of embryos in a solution containing a high concentration of CPAs, thereby preventing ice crystal formation. Established protocols that allow for high survival of the vitrified embryos include the solid surface [24], cryoloop [25], open-pulled straw (OPS) [26], and cryotop [27] methods. These methods are commonly characterized by the minimum-volume cooling (MVC) concept [28], in which embryos are vitrified in a minimal amount of solution to maximize the cooling rate.

Recently, we have developed a new vitrification method using hollow fiber (HF) as a device for loading embryos [29, 30]. The hollow fiber vitrification (HFV) method has been demonstrated to have superior performance to cryopreserve cryosensitive embryos such as porcine in vitro-produced embryos [30]. In addition, the HFV method allows the vitrification of large numbers of embryos at the same time, while still following the MVC principle. This is a distinctive difference from the previous methods, which are suitable only to vitrify a small number of embryos in a single device. In this study, we set out to apply the improved HFV method to cryopreservation of pancreatic islets. Adapting our baseline vitrification protocols to islets led to the identification of a variety of specific technical challenges. Nonetheless, we found that cryopreservation using the HFV technique is a practical method for long-term storage of islets.

Materials and Methods



Animals

Adult 8 to 10-week-old male ICR and ICR SCID mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). All mice were bred under conventional conditions in an air-conditioned room with free access to tap water and standard pelleted chow. All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC 12-0019 and 12-0020).

Islet isolation

Mice were anesthetized by a mixture of ketamine (Fujita Pharmaceutical Co., Ltd, Tokyo, Japan) and xylazine (Bayer Yakuin, Ltd, Osaka, Japan). After laparotomy, the pancreas was distended with 2 ml of cold HBSS (Life Technologies, Carlsbad, CA) containing 1000 U/ml of Collagenase-Yakult (Yakult Pharmaceutical Industry Co., Ltd, Tokyo, Japan) through the common bile duct, and subsequently excised and incubated in a stationary bath at 37°C. Islets were separated by density gradients (Histopaque-1077; Sigma, St. Louis, MO, USA) and handpicked under a stereomicroscope. The isolated islets were cultured 24–36 h in RPMI1640 medium (Life Technologies, Carlsbad, CA, USA) with 10% inactivated fetal bovine serum (FBS, Life Technologies) and antibiotics in an Ultra-Low Attachment flask (Corning Incorporated, New York, NY, USA) at 37°C in a humidified air atmosphere containing 5% CO₂. The islets were used for further experiments and some of them were assigned to the non-vitrified group.

Evaluation of islet viability

The viability of the islets was determined by staining with propidium iodide (PI; Molecular Probes, Eugene, OR). PI-positive islets cells were defined as dead cells and counted by visual inspection of PI staining under a fluorescent microscope equipped with a CCD camera (Olympus Optical, Tokyo, Japan). Islets were scored mainly by evaluating the ratio of PI positive cells (◉ Fig. 1a). The area of PI positive cells in each islets was measured by Image J software (<https://imagej.nih.gov/ij/>). According to the ratio of PI positive cells, scores from 10 to 0 were assigned to each islet: score 10 for less than 10%, score 8 for less than 30%, score 6 for less than 50%, and scores 4–0 for over 50%. In determining the scores 4, 2, and 0, morphological feature of the islets, i.e., round shape, collapsing, or disrupted, was considered in addition to the PI positive ratio (> 50%).

Thus, the ratio of PI-positive cells and the morphological changes of the islets were included in our original index to evaluate viability (◉ Fig. 1a). To evaluate the score of islets, more than 10 islets were randomly picked for each evaluation and more than 2 examiners judged the score.

Cryopreservation of the islets

We first examined the sensitivity of mouse islets to various CPAs and then determined the optimum CPA composition and concentration for vitrification. For each series of experiments, the viability and/or function of frozen/vitrified islets were evaluated 3 h after thawing and compared to non-vitrified controls. Hepes (20 mM)-buffered tissue culture medium 199 (Nissui Pharmaceutical) supplemented with 20% calf serum was used as the basal solution (BS) to prepare equilibration solution (ES), vitrification solution (VS), thawing solution (TS), and dilution solutions (DS). BS was also used as washing solution (WS).

Experiment 1: Exploration of CPAs for the vitrification of mouse islets

A pilot study was undertaken to examine the toxicity of different CPAs towards mouse islets under hypothermic conditions. Various permeating CPAs, including DMSO (Nacalai Tesque, Inc., Kyoto, Japan), EG (Nacalai Tesque), propanediol (PROH), or glycerol, were tested at a concentration of 10% (v/v). A group of 12 islets was exposed for 30 min on ice to BS containing each of the CPA. Subsequently, islets were loaded with the CPA solution (30 µl) in a 0.25 ml plastic straw (IMV Technologies, L'Aigle), followed by programmed cooling at a rate of $-0.5^{\circ}\text{C}/\text{min}$ to -20°C

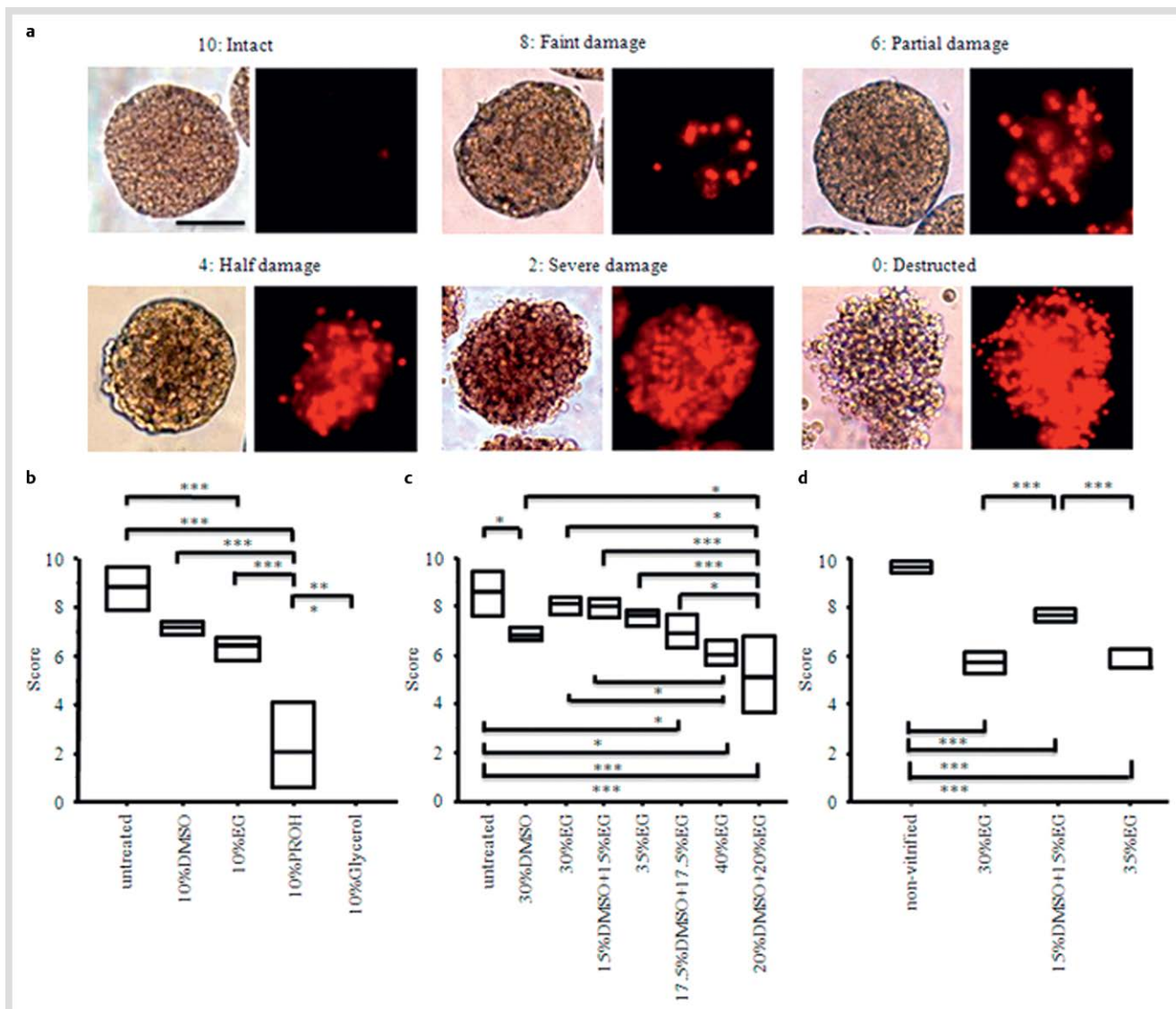


Fig. 1 Definition of the score for the viability of pancreatic islets and exploration of cryoprotectant agents (CPAs): **a** Definition of the score for the viability of islets. (left) Bright-field images of islets for each score. (right) Fluorescence microscopic image of propidium iodide (PI) staining. PI-positive nuclear staining indicates cell death. The percentage of PI-positive cells and the morphological change of islets were included in our original index to evaluate islet viability. Scale bar: 100 μ m. **b**, **c** Optimization of CPA composition for islets. The evaluation of the toxicity of different CPAs for islets was performed for various concentrations. **b** The evaluation of toxicity for islets in the CPAs by slow-rate freezing. Twelve

islets were used to test each CPA solution in 4 independent experiments. **c** The evaluation of the concentration and composition of CPAs for islets. The islets were treated with 7 types of CPA solutions to find the adequate concentration and composition of CPA solution. Ten to 12 islets were used to test each CPA solution in 5 independent experiments. **d**: Selection of CPAs for HFV of islets. The use of 15% DMSO + 15% EG for HFV showed the highest average viability score (7.7 ± 0.3), and was chosen as a novel medium for the cryopreservation of islets. All data represent means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. DMSO: Dimethyl sulfoxide; EG: Ethylene glycol; PROH: Propanediol.

using a Freeze Control CL-863 (CryoLogic Pty Ltd, Australia). Thawing was performed by exposing the straws to air (room temperature, RT) for 3 s prior to immersion of the straw directly in warm water at 37°C. Recovered islets were treated with a stepwise dilution of CPA and washed as summarized in **Table 1**. The same post-thaw procedure was used in experiments 1–3.

Experiment 2: Optimization of CPA composition and concentration for islet vitrification

The viability of islets was assessed after exposure to 7 types of VSs: 30% DMSO, 30% EG, 35% EG, 40% EG, 15% DMSO + 15% EG, 17.5% DMSO + 17.5% EG, 20% DMSO + 20% EG. Solutions with

higher DMSO concentrations (35 and 40%) were omitted as they showed evident toxicity to the islets in a preliminary test. A group of 10–12 islets was exposed on ice for 25 min to ES containing respective CPA(s) at a concentration of 15%, and then kept in VS for 2 min. When VS with $\geq 35\%$ CPA(s) was used, islets were placed in 30% CPA solution for 2 min before being exposed to the VS. Procedures for dilution and removal of CPA were the same as those in experiment 1.

Experiment 3: Comparison of 3 different vitrification methods

The open pulled straw vitrification (OPSV) method originally developed by Vajta et al. [26] has been widely used and docu-

Table 1 Composition and osmolarity of each solution, and the time and temperature of each step.

	TCM199+20% FCS	DMSO % (v/v)	EG % (v/v)	Sucrose (mol)	mOsm/l	Time (min)	Temperature
Cooling							
Equivalent solution (ES)	+	7.5	7.5	–	2938	25	on ice
Vitrification solution (VS)	+	15	15	0.5	6345	2	on ice
Thawing							
Thawing solution (TS)	+	–	–	1	2211	1	37 °C
Dilution solution (DS) 1	+	–	–	0.5	968	5	RT
DS2	+	–	–	0.25	581	5	RT
DS3	+	–	–	0.125	435	5	RT
Washing solution (WS) 1	+	–	–	–	292	5	RT
WS2	+	–	–	–	292	5	RT
Basal solution (BS)	+	–	–	–	292	–	–

FCS: Fetal calf serum; DMSO: Dimethyl sulfoxide, EG: Ethylene glycol; RT: Room temperature

mented as a feasible cryopreservation method for mammalian embryos and this method has also been used successfully for islets by Sasamoto et al. [18]. We used the OPSV and solid-surface vitrification with using EDT324 solution (SSV-EDT324) [18] as control methods against which to compare the efficacy of HFV. The solutions, the osmolarity of each solution, the time, and the temperature in each step used for both methods are shown in **Table 1S**.

OPSV protocol

OPSV was performed following the method described by Vajta et al. [18,26]. After equilibration, 15 islets were loaded into the OPS device with approximately 10 µl of vitrification solution. The OPS devices were directly plunged into liquid nitrogen. Thawing was performed by directly immersing the OPS device in TS.

SSV-EDT324 protocol

SSV-EDT324 was performed as previously described by Sasamoto et al. [18]. The CPA solution containing 15 islets was vitrified by dropping it onto an aluminum container floated on LN.

HFV protocol

Islets underwent HFV according to the methods originally developed by our groups for mammalian embryos, after some modifications as shown in **Table 1** [29,30]. Based on the result of experiment 2, we chose DMSO and EG as permeable CPAs. First, a group of 25–35 islets was placed in 4 ml of ES in a 35 mm plastic dish on ice and then aspirated in a cellulose triacetate HF (ca. 30 mm long, inner diameter 200 µm, outer diameter 230 µm; FB-150FH; Nipro Corporation, Osaka, Japan) connected to a hypodermic needle (**Fig. 2a**) (length: 5 mm, outer diameter: 0.15 mm, inner diameter: 0.1 mm; Medical Planning Corporation, Miyagi, Japan) using a 1 ml syringe and aspiration tube. Islets were loaded into the HF in a 10–15 mm column of ES flanked by air bubbles (**Fig. 2b, b', g**). The group of islets contained in a HF could be handled as a unit. The HF was detached from the needle with forceps (**Fig. 2c**) and was kept in ES. We used forceps to handle the HF containing islets in all steps. Following equilibration, the HF was transferred to 4 ml of VS (**Fig. 2d**). The HF was kept in VS, during which it was moved gently in the dish to ensure the dehydration of the islets inside. Subsequently, the HF was immersed in LN while being held vertically (**Fig. 2e**) and was kept for a brief period (5–10 min) or housed in a cryotube for longer storage (16–304 days) in an LN tank. For thawing, the HF was transferred into a tray that was filled with LN. The fiber was quickly moved and immersed in

4 mL TS in a dish. The CPAs were diluted and removed in a step-wise manner by transferring the HF from the TS to WS. In WS 2, the islets were expelled from the HF by gently squeezing the fiber from one end to the other (**Fig. 2f**).

Measurement of insulin secretory activity

Glucose-stimulated insulin release was measured in a static incubation assay. In one experiment, 20 islets from the non-vitrified and vitrified (OPSV or HFV method) groups were plated in the wells of a 48-well culture plate (SUMILON, Sumitomo Bakelite Co., Ltd, Tokyo, Japan) and were subjected to static incubation in Krebs-Ringer bicarbonate buffer (KRB; 115 mM NaCl, 5 mM KCl, 25 mM HEPES, 2.5 mM CaCl₂·2H₂O, 24 mM NaHCO₃, 1 mM MgCl₂·6H₂O) supplemented with 2.8 or 28 mM glucose and 0.1% BSA. First, islets were immersed into KRB with low glucose. They were further incubated in another well containing KRB with low glucose (2.8 mM), followed by incubation in KRB with high glucose (28 mM), each incubation was for 1 h at 37 °C. The supernatants were collected and stored at –80 °C. The insulin concentration was assessed using an enzyme-linked immunosorbent assay kit (Shibayagi, Gunma, Japan). The stimulation index (SI) was calculated by dividing the insulin level in response to 28 mM glucose by that in response to 2.8 mM glucose.

Transplantation

Diabetes was induced by intraperitoneal injection of 180 mg/kg Streptozocin (STZ, Sigma) freshly dissolved in an equivalent volume of 0.05 M citrate buffer (pH 4.5) into 7-week-old ICR SCID mice. Blood glucose was measured using a Glucocard™ G+ meter (ARKRAY, Inc., Kyoto, Japan) using whole blood samples obtained by tail puncture. Body weight was also monitored. Diabetes was confirmed by the presence of hyperglycemia with fed blood glucose levels higher than 350 mg/dl. Islet transplantation was carried out 7 days after STZ injection. Recipients were anesthetized with isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan). The left kidney was exposed through a lumbar incision. Three hundred IEQs of the non-vitrified or HFV groups were packed in a capillary tube and were transplanted under the kidney capsule of diabetic mice. After transplantation, the blood glucose levels were monitored 3 times per week. We needed at least 300 islets to achieve euglycemia even if we used non-vitrified islets. The mice were considered to be euglycemic when the blood glucose levels were below 200 mg/dl. On day 25, an intraperitoneal glucose tolerance test (IPGTT) was performed in the fasting (8 h) state, using a 10% glucose solution (2 g/kg body weight). Blood glucose levels were measured at 0, 15, 30, 60, and 120 min. On

day 29 or 30, the mice underwent nephrectomy to remove the transplant-bearing kidneys and were subsequently monitored.

Histology

The islets from the non-vitrified or HFV group were fixed in absolute ethanol. The kidney tissue was fixed in 4% paraformaldehyde, then embedded in paraffin and sliced into 4 μ m sections. After deparaffinization and blocking, diluted primary antibodies were added to the sections, which were incubated overnight at 4°C. For amplification, biotinylated anti-rabbit antibodies (Life Technologies) were used, followed by incubation with Alexa Fluor® 488 Streptavidin Conjugates (Life Technologies). The antibodies used in this study were: mouse anti-NeuroD (Abcam, Plc., Cambridge, UK), rabbit anti-Pancreatic and duodenal homeobox (pdx)-1 (Transgenic Inc., Kobe, Japan), rabbit v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (MafA) (Bethyl Laboratories, Inc. Montgomery, TX, USA), Glucose transporter, type 2 (GLUT2) (Millipore, Billerica, MA), mouse anti-glucagon (Sigma), guinea pig anti-insulin (Linco Research Immunoassay, St. Charles, MO, USA). The reason why we selected these antibodies were as follows. The 3 transcription factors, *NeuroD*, *Pdx1*, and *MafA*, bind the insulin promoter region and regulate glucose-responsiveness of insulin [31, 32]. The expression of these transcriptional factors in islets is crucial for β -cell specificity. Glucose transporter 2 (GLUT2) is a transmembrane carrier protein that enables protein-facilitated glucose movement across cell membranes [33]. Immunocytochemistry shows that GLUT2 is localized in β -cells [34]. In mice, the few glucagon-immunoreactive cells in islets are localized to the periphery of the islets [35]. Rabbit anti-C-peptide (Cell Signaling Technology, Inc., Danvers, MA) and Alexa⁴⁸⁸-conjugated and Alexa⁵⁹⁴-conjugated antibodies (Molecular Probes, Eugene, OR) were used as secondary antibodies. The methods used for immunohistochemistry and immunocytochemistry were previously described [36]. Cell nuclei were counter-stained with DAPI (VECTASHIELD Mounting Medium with DAPI) or Hoechst 33342. As a negative control, only secondary antibodies were applied. Islets and pancreas sections were stained as positive controls. Sections were imaged using a confocal microscope (FV-1000, Olympus, Tokyo, Japan).

Statistical analysis

Experimental results were expressed as the means \pm standard error of mean (SEM). Student's *t*-test, ANOVA, and Fisher's protected least significant difference test were used, and $p < 0.05$ was considered statistically significant.

Results

Cryopreservation of the islets

Experiment 1: Exploration of CPAs for the vitrification of mouse islets

It was clear that slow-rate freezing of islets with 10% PROH or 10% Glycerol resulted in a much less compact gross structure. The average score of the viability in both of these groups is significantly lower than that of the 10% DMSO and/or 10% EG groups (\bullet Fig. 1b). On the other hand, the islets in the DMSO and EG groups showed a regular periphery with a continuous layer.

Experiment 2: Optimization of CPA composition and concentration for islet vitrification

The islets in all groups retained gross structural integrity, indicating that the permeable CPAs in the compositions and concentrations tested were less toxic. Among the CPAs tested, the highest viability scores were obtained for the groups with 30% EG (8.3 \pm 0.2), 35% EG (7.8 \pm 0.3), and 15% DMSO+15% EG (8.2 \pm 0.3) (\bullet Fig. 1c). These 3 types of CPA solutions were therefore selected for subsequent evaluation of the HFV method. When the islets were vitrified using these 3 CPA solution conditions, the highest viability score was obtained in the group of 15% DMSO+15% EG (7.7 \pm 0.3, \bullet Fig. 1d).

Experiment 3: Comparison of 3 different vitrification methods

In the SSV-EDT324 group, the islets were mostly disrupted and the viability score was low (Score: 1.5 \pm 1.2, \bullet Fig. 3a). The islets in the OPSV and HFV groups retained gross structural integrity with a high proportion of the cells maintaining membrane integrity. Hence, we used OPSV as the control method to compare the efficacy of the HFV method. The viability of islets cryopreserved using the HFV method was the highest of all 3 methods.

Insulin release assay

All islets of the OPSV group were disrupted during the static incubation assay. Therefore, the islets of the HFV group were compared to non-vitrified islets (\bullet Fig. 3b: left). The SIs of the non-vitrified and vitrified islets were 27.8 \pm 8.2 and 3.5 \pm 0.6, respectively ($p < 0.05$, \bullet Fig. 3b: right).

Immunostaining of islets after HFV

The expression of the markers in the appropriate locations demonstrates that the vitrification does not affect the cells. Islets were stained with indicated antibodies that recognize NeuroD (\bullet Fig. 4a, a' and f, f'), Pdx1 (\bullet Fig. 4b, b'), or MafA (\bullet Fig. 4c, c') on nuclei (green nuclear), or hormone insulin (\bullet Fig. 4a-e, a'-e') in cytoplasm (red). (DAPI; blue on the nuclei). GLUT2 expression (\bullet Fig. 4d, d') was strongly positive (green cytoplasm) in insulin-producing cells, and only a few glucagon-positive cells (\bullet Fig. 4e, e' and g, g') surrounded the insulin- or C-peptide-positive cells (\bullet Fig. 4g, g'). There were no apparent differences in the expression patterns for non-vitrified islets and islets treated by HFV.

Islet transplantation

All the mice in non-vitrified and HFV groups were euglycemic within 4–8 days after transplantation and throughout the further follow-up period (\bullet Fig. 5a', a''). On day 29 or 30, removal of the graft-bearing kidney in both groups was followed by a rapid return to hyperglycemia, indicating that the islets grafts were responsible for the euglycemic state. Results of the IPGTTs showed similar glycemic values at all-time points in both groups (\bullet Fig. 5c', c''). There were no differences in the mean blood glucose levels at 15, 30, and 60 min. Analysis of immunohistochemical staining confirmed the presence of Pdx-1, insulin-, and C-peptide-positive cells in the renal capsule space in both the non-frozen and HFV groups (\bullet Fig. 5d–l and d'–l'). Pdx1-positive cells are restricted to the insulin producing cells and c-peptide-positive cells (\bullet Fig. 5e', f' and h', i').

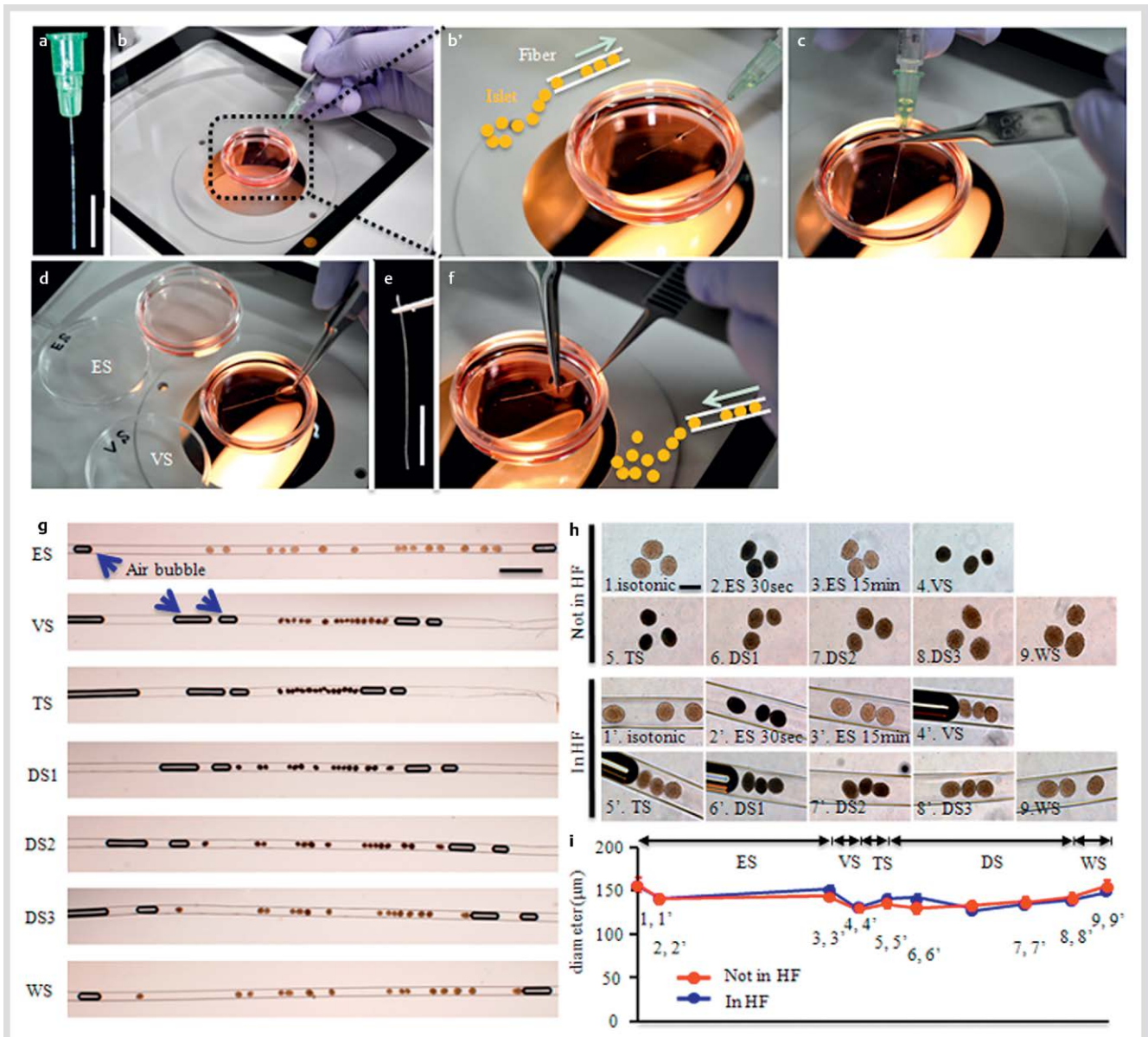


Fig. 2 Procedures for vitrification and thawing of pancreatic islets by the HFV method **a** An HFV device consisting of **b** a 30 mm long segment and a hypodermic needle. Bar = 1 cm. **b, b'** Aspiration of islets from the ES into the HFV device attached to a 1 ml syringe and an aspiration tube. **b'** Enlargement of the square shown in **b**. The cartoon shows islets are aspirated into the hollow fiber (HF). **c** Detachment of the HF holding the islets from the hypodermic needle in the ES. **d** Transfer of the HF from ES to VS using forceps. **e** Plunging of the HF into liquid nitrogen. Scale bar: 1 cm. **f** Recovery of islets from the HF in WS. The cartoon shows islets are expelled from the HF.

Discussion

Successful islet banking is required to optimize and modernize islet transplantation techniques. However, there are several drawbacks to preservation of the islets using conventional freezing procedures. The most critical cryo-injuries are undoubtedly caused by intra- and extra-cellular ice formation, which may irreversibly damage the cells. Taylor et al. showed that tissue and organ damage after freeze-thawing are caused by accumulating ice crystals formed in vascularized tissues that rupture the capillaries [37]. Islets have a complex cellular composition, with no less than 5 different cell types. The cells and vessels are well cross-linked to maintain polarity and the capillary vessels construct a frame of islets [38]. Therefore, the islets may not main-

tain complete structural integrity if the capillary vessels are destroyed.

Langer et al. [39] reported that mouse islets could maintain their viability after vitrification. Since then, many methods have been tested to cryopreserve islets [4–8, 12–15, 18–20]. In the study of embryo cryopreservation, vitrification has been demonstrated to be more effective than conventional freezing [30]. In particular for porcine embryos, which are known to be highly cryosensitive, vitrification has become the standard option [30, 40].

Embryos or tissues having an intrinsic cryotolerance can survive cryogenic-stress regardless of the effectiveness of the cryopreservation method used. However, an authentically effective method is required to cryopreserve tissues with high cryosensitivity or structural fragility such as islets. In this regards, the HFV method may provide a promising option for cryopreserving islets. The

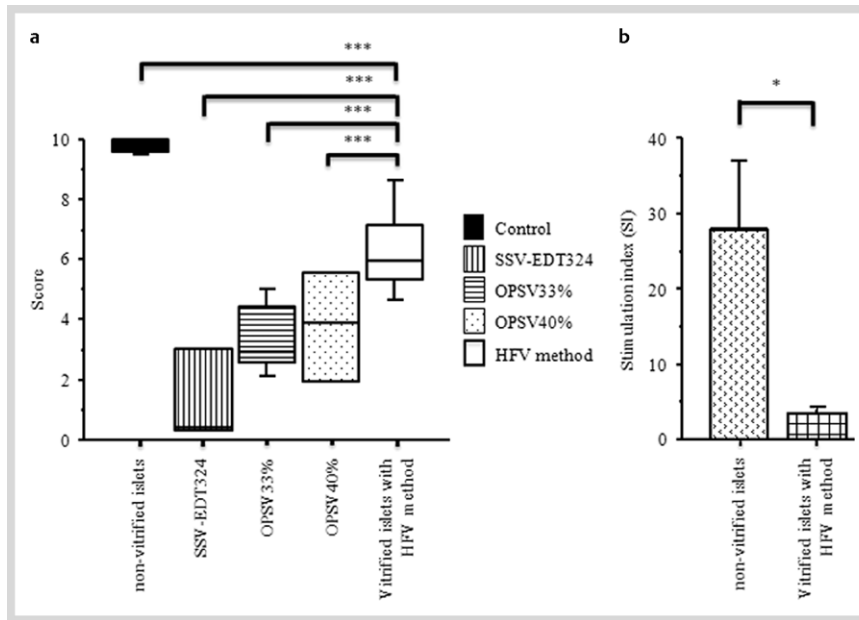


Fig. 3 Effects of different vitrification methods on the viability and insulin secretory activity of islets: **a** The viability of islets after vitrification and thawing using different methods: Solid-surface vitrification (SSV) with using EDT324 solution (SSV-EDT324), OPSV 33, 40%, and HFV. Fifteen islets were used to test each method in 3–9 independent experiments. **b** Glucose-stimulated insulin release in a static incubation assay. Three hours after thawing, 20 IEQs per sample from each group were used for the assay. Islets were incubated with low-glucose (2.8 mM) medium for 1 h followed by high-glucose (28 mM) medium for 1 h. The stimulation index was calculated as the insulin release in high-glucose medium divided by the insulin release in low-glucose medium. Data represent the means \pm SEM of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. OPSV: Open pulled straw vitrification; HFV: Hollow fiber vitrification.

superior performance of the HFV method was previously demonstrated by the efficient production of offspring from porcine vitrified embryos derived from in vitro oocyte maturation followed by in vitro fertilization (IVM/IVF) [30]. This was a significant breakthrough in the development of embryo cryopreservation technology, because none of the previously reported methods enabled cryopreserving of in vitro produced pig embryos on a practical level. The effectiveness of the HFV method proven in the embryo cryopreservation was thus extrapolated to islets.

We therefore applied the HFV method for islet cryopreservation after several modifications. For example, porcine early embryos, which are composed of only 15–40 cells, can be equilibrate with permeable CPAs after 5–7 min [29,30]. In contrast, islets contain an average of 2000 cells [41], requiring a longer equilibration time to allow the CPAs to permeate into each of the cells.

The β -cell specificity and glucose-responsiveness of insulin expression are conferred by 3 conserved enhancer elements in the insulin promoter region. These are E1, A3, and RIPE3b/C1, which, respectively, bind 3 transcription factors NeuroD, Pdx-1, and MafA [31,32]. The target genes of both *pdx-1* [42] and *mafA* [32] have been reported as they display β -cell restricted expression [33,43]. We hypothesized that the expression of those transcriptional factors would be downregulated, if the HFV method would affect the cell character and function of the islets. However, the protein expressions were well maintained after HFV (Fig. 4). The static incubation assay showed insulin secretion upon glucose challenge test (Fig. 3b), thereby demonstrating that the functions of the islets were preserved after vitrification. One of the technical advantages of the HFV method is the fact that it is so straightforward. The HF device can be handled easily using forceps through the all processes. As the cellulose-triacetate HF membrane is permeable to small molecules including CPAs, islets loaded in the HF device can rapidly respond to changes in the external solution environment, such as osmolarity. This characteristic of the HF was clearly evident in the permeability property tests performed in this study (Fig. 2).

A second beneficial characteristic of the HF membrane is its high thermal conductivity. The thin HF membrane permits ultra-rapid cooling and re-warming of the solution held inside, which is known to be critical for gaining a stable vitrified status. Gener-

ally, embryos are vitrified in a very small amount of solution to ensure an ultra-rapid cooling/re-warming rate. However, the drawback of such methods is the limited number of embryos that can be vitrified at one time. The HFV method can potentially be expanded to permit the vitrification of a large number of islets if longer HFs are used. The development of a new automatic system is currently underway.

In vitro evaluations showed apparently lower indices for HFV islets. It was therefore assumed that the islets might have lost some cells after vitrification. However, all islets of the OPSV group were disrupted during the static incubation assay. The average score of OPSV 33 and 40% was 3.3 ± 0.4 and 3.8 ± 1.4 , respectively (Fig. 3a). These scores reflected that those islets included more than 50% of dead cells. The structural integrity of those islets was assumed to be damaged, thereby became very fragile. To perform glucose-stimulated insulin release assay, islets needed to be transferred through different solutions using pipetting. During the steps of the incubation assay, the islets were disrupted one after another, so that the number of islets was remarkably reduced. In contrast, islets in HFV group showed higher score than other groups, indicating better structural integrity and functions. Sasamoto et al. [18] reported superior viability of islets after cryopreservation by the SSV-EDT324 and OPSV methods. This discrepancy from our data may be ascribed to the difference(s) in the condition of the islets used or we might have failed to reproduce special knacks that were not available in their report [18].

To confirm the vigorous function of the vitrified islets, we subsequently carried out in vivo evaluations. The transplantation of islet grafts vitrified using the HFV method behaved as non-vitrified islets even on IPGTTs. These results suggest that the functionality of the vitrified islets was comparable to that of fresh islets. Optimization of the in vitro insulin release assay may improve the measurement of function indices in the vitrified islets, as an increased insulin secreting ability was occasionally observed after prolonged post-thaw culture (data not shown). It is possible that the vitrified islets regain their competence under in vivo conditions following transplantation. Better criteria for the accurate in vitro evaluation of the quality of cryopreserved islets need to be developed.

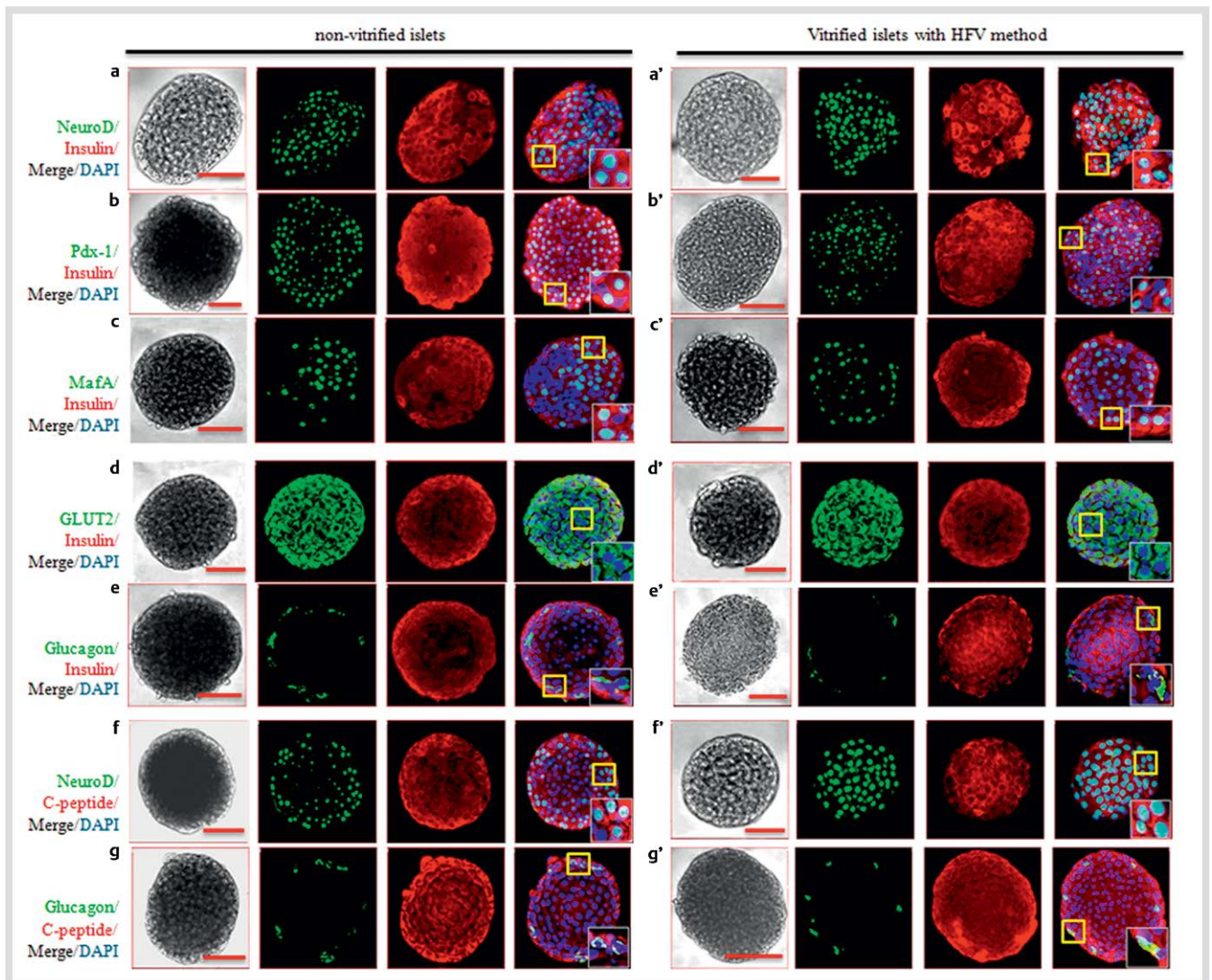


Fig. 4 Immunofluorescence analysis of the islets after HFV: (Left) Non-vitrified islets **a–g**. (Right) HFV islets cultured for 3 h after thawing **a'–g'**. **a–g, a'–g'**: The left panel in each staining shows the bright-field image of the islet. The right panel is the merged image of each staining. (**a–e** and **a'–e'**) Insulin staining (red). (**f, g** and **f', g'**) C-peptide staining (red). (**a–c, f, and a'–c', f'**) Nuclear staining (green) for NeuroD (**a, a'** and **f, f'**), Pdx-1 (**b, b'**) and Maf A (**c, c'**). Fluorescence micrographs show the expression of transcription factors NeuroD, Pdx-1, or MafA. Nuclei are stained in blue with DAPI. There was complete overlap between transcription factor ex-

pression and DAPI nuclear staining. **d, d'** and **g, g'**: Immunocytochemistry shows that GLUT2 (cytoplasm; green) is localized in insulin-producing cells (**d, d'**) and C-peptide-positive cells (**g, g'**). **e, e'** and **g, g'**: Glucagon-producing cells (cytoplasm; green) surround the insulin-producing cells (**e, e'**) and c-peptide-positive cells (**g, g'**). Inset images are higher magnifications of the region indicated by a yellow square in each merged image. There were no apparent differences in the transcription factor expression pattern between non-vitrified and HFV islets. Scale bar: 100 μ m.

The present study demonstrates the potential use of HFV for islet cryopreservation. Additional practical issues will need to be addressed while further developing this approach to a scale that allows for the processing of clinically relevant sample sizes.

Author Contributions

M.N. and H.N.: Conception and design, financial support, collection and assembly of data, data analysis, interpretation, and manuscript writing. H.M. and M.M.: Collection and assembly of data and data analysis and interpretation. T.K., K.N., I.U., Y. Katsumata, Y. Kasai, M.H., N.A., M.K., R.S., M.W., and K.U.: Collection and assembly of data. All authors approved the final manuscript.

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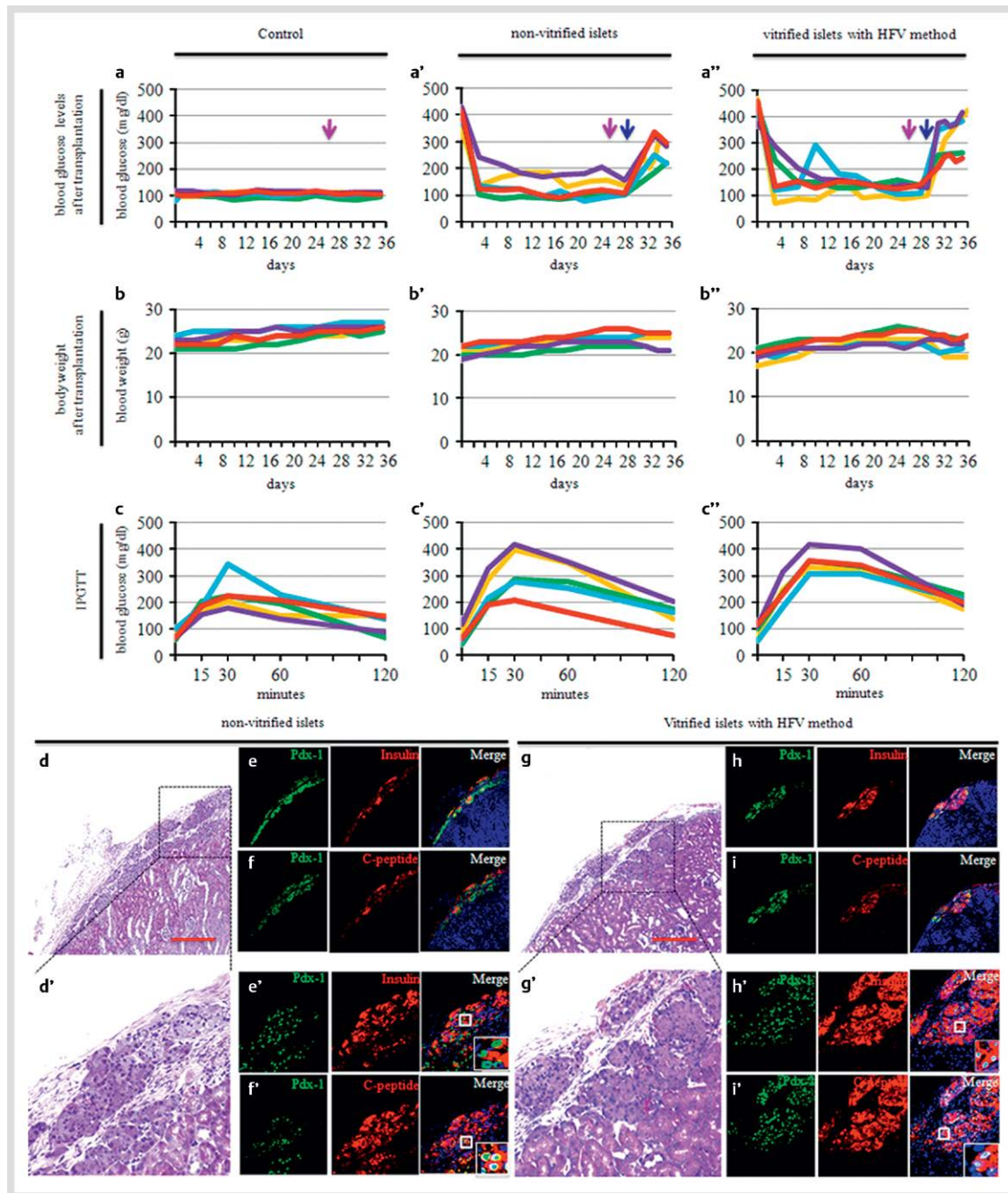


Fig. 5 Islet transplantation into the left renal subcapsular space. Islet transplantation was carried out 7 days after STZ injection. Three hundred IEQs of non-vitrified (a'–c', n=5) or HFV islets (a''–c'', n=5) were transplanted under the left kidney capsule of diabetic syngeneic mice. Control mice were prepared as well (a–c, n=5). Blood glucose levels a', a'' and body weight b', b'' of diabetic mice were measured after transplantation into the left renal subcapsular space. Mice transplanted with non-vitrified islets a' and HFV islets a'' were euglycemic within 4–8 days after transplantation. Nephrectomy was performed on day 29 or 30 after islet transplantation (blue arrows). There were no significant differences in blood glucose levels and body weight between both groups. An intraperitoneal glucose tolerance test (red arrows) was performed on day 25 after islet transplantation. Mice were subjected to IPGTTs with 10% glucose solution (2 g/kg body weight). The glycemic values of mice transplanted with HFV islets c'' were similar to those of mice with non-vitrified grafts c' at all times. However, these levels were significantly higher compared to the control group c. d–i and d'–i' On day 29 or 30, transplanted mice underwent nephrectomy of the transplant-bearing kidney for immunofluorescence analysis. The staining confirmed the presence of Pdx-1 with

insulin- and C-peptide-positive cells in the renal capsule space of HFV islet transplants. Serial sections of each islet were prepared, and immunohistochemical staining was performed. (Left) Non-vitrified islets d–f and d'–f'. (Right) Vitrified islets with HFV method g–i and g'–i'. d–i The panels of d–i are the same magnification. d'–f' and g'–i' Enlargement of the square shown in d and g, respectively. d, d' and g, g': Hematoxylin and eosin staining of a kidney section with the transplanted islets. e, f, h, i and e', f', h', i' Immunofluorescence analysis of Pdx-1 (e, f, h, i and e', f', h', i', left), insulin (e, h and e', h', middle), and c-peptide (f, i and f', i', middle) expression. The right panel is the merged image of each staining. e, f, h, i and e', f', h', i', left: Fluorescence micrographs show the expression of Pdx-1 (Nuclear; green). Nuclei are stained in blue with Hoechst 33342. There was complete overlap between transcription factor expression and nuclear staining. e', f' and h', i': Inset images are higher magnifications of the region indicated by a white square in each merged image. There were no apparent differences in the transcription factor expression pattern between non-vitrified and HFV islets. Most of the islet cells in the graft area showed strong staining for all markers. Scale bar: 100 μ m.

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Affiliations

¹ Meiji University International Institute for Bio-Resource Research, Higashimita Tama-ku Kawasaki Kanagawa, Japan

² Department of Immunology and Medicine, St. Marianna University School of Medicine, Sugao Miyamae-ku Kawasaki, Japan

³ Laboratory of Developmental Engineering, Department of Life Sciences, School of Agriculture, Meiji University, Higashimita Tama-ku Kawasaki Kanagawa, Japan

⁴ Otsuka Pharmaceutical Factory Inc., Naruto Tokushima, Japan

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