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Original Article

Induction of functional islet-like cells from human iPS cells by suspension culture

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ABSTRACT

Introduction: To complement islet transplantation for type1 diabetic patients, cell-based therapy using pluripotent stem cells such as ES cells and iPS cells is promising. Many papers have already reported the induction of pancreatic β cells from these cell types, but a suspension culture system has not usually been employed. The aim of this study is to establish a suspension culture method for inducing functional islet-like cells from human iPS cells.

Methods: We used 30 ml spinner type culture vessels for human iPS cells throughout the differentiation process. Differentiated cells were analyzed by immunostaining and C-peptide secretion. Cell transplantation experiments were performed with STZ-induced diabetic NOD/SCID mice. Blood human C-peptide and glucagon levels were measured serially in mice, and grafts were analyzed histologically.

Results: We obtained spherical pancreatic beta-like cells from human iPS cells and detected verifiable amounts of C-peptide secretion *in vitro*. We demonstrated reversal of hyperglycemia in diabetic model mice after transplantation of these cells, maintaining non-fasting blood glucose levels along with the human glycemic set point. We confirmed the secretion of human insulin and glucagon dependent on the blood glucose level *in vivo*. Immunohistological analysis revealed that grafted cells became α, β and δ cells *in vivo*.

Conclusions: These results suggest that differentiated cells derived from human iPS cells grown in suspension culture mature and function like pancreatic islets *in vivo*.

1. Introduction

Type1 diabetes is an autoimmune disease in which pancreatic β cells are selectively destroyed. Type1 diabetic patients need frequent insulin injections and have a potential risk of hypoglycemia. Islet transplantation is a very effective treatment for these patients. However, it is not common in Japan because of a donor shortage. To solve the problem, cell-based therapy using pluripotent stem cells such as ES cells and iPS cells is considered to be promising [1–3] because they have a large potential of cell growth. D'amour et al. first reported methods for differentiating human ES cells into pancreatic β cells, and many others have reported the induction of insulin secreting cells with both kinds of cells [4–13]. Among them, Rezania et al. and Pagliuca et al. showed that induced pancreatic β cells from ES cells functioned *in vivo* by ameliorating hyperglycemia in diabetic mice [8,9]. Many small molecules were reported to improve the efficacy of differentiation towards pancreatic β cells from human pluripotent cells [11–13]. On the other hand, since ES cells and iPS cells have different characteristics, optimal conditions for differentiation protocols also differ and therefore should be adjusted for each cell line to mimic the developmental process of fetal pancreas. We previously reported our original methods employing 6 stages for inducing functional pancreatic β cells from human iPS cells [14]. At that time, we adopted an adherent culture system until the fifth stage and were able to improve cellular function by forming 3D spheroids at the last stage. We realized the importance of 3D structure for functional

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Abbreviations

DMEM	Dulbecco's modified Eagle's medium
KSR	knockout serum replacement
NEAA	non-essential amino acids
2-ME	2-mercaptethanol
BSA	bovine serum albumin
PS	penicillin and streptomycin
ITS-X	insulin, transferrin, selenium, ethanolamine solution
FGF	fibroblast growth factor
BMP4	bone morphogenetic protein 4
EGF	epidermal growth factor
HGF	hepatocyte growth factor
IGF-1	insulin-like growth factor 1
ILV	indolactam V
ROCK inhibitor	Rho-associated kinase inhibitor
NOD)/SCID mouse	non-obese diabetic/severe combined immunodeficiency mouse
iPS cells	induced pluripotent stem cells
ES cells	embryonic stem cells
STZ	streptozotocin

β cells, as reported by Takeuchi et al. [15]. Toyoda et al. also reported the positive effects of cell aggregation cultures on the differentiation of pancreatic bud cells [6].

For clinical applications of cell therapy, large numbers of cells, more than 10^9, will be required, based on data from islet transplantation, which usually uses 5000 IEQ/kg as a minimal requirement. For that purpose, an adherent culture system involving manually handling of many dishes is not practical for large-scale culture, so we decided to use a suspension culture system in which scaling up is relatively easy. Rungarunlert et al. reported the benefits of bioreactors for embryo body formation from ES and iPS cells [16]. Olmer et al. reported that they succeeded in generating aggregate formation of undifferentiated hiPS cells up to 2×10^8 cells by using the 100 ml stirred bioreactors. They also indicated that the inoculation density, impeller type and rotation speed were important to form uniform aggregates [17]. They increased the agitation speed to 60 rpm to achieve homogenous mixing. Matsuura et al. reported that they developed a bioreactor to expand and induce cardiac differentiation of human iPS cells by using 100 ml vessels [18]. Their agitation rate was 40 rpm. We paid attention to the rotation speed of the suspension culture system because we thought the faster the rotation speed becomes, the stronger the shear stress to the cells becomes. We selected the 30 ml spinner type vessels commercially available and their impellers were the same shape as Matsuura used.

We modified our previous protocol to adjust for suspension culture and succeeded in obtaining functional islet-like cells from human iPS cells. We demonstrated the reversal of hyperglycemia in STZ-induced diabetic model mice after transplantation of these cells.

2. Methods

2.1. Human iPSC culture and differentiation

The human iPSC line TkDN4-M was a kind gift from Dr. M Ohtsu at The Institute of Medical Science, The University of Tokyo. Freeze-stored iPSCs were thawed and cultured as described previously [14,19] in hiPS medium (DMEM/Ham's F12; Wako) in the presence of 20% knockout serum replacement (KSR; GIBCO), 1× non-essential amino acids (NEAA; Wako), 55 μM 2-mercaptethanol (2-ME, GIBCO) and 7.5 ng/ml recombinant human fibroblast growth factor 2 (FGF2) (Peprotech) on mitomycin-C (Wako)-treated SNL feeder cells to maintain an undifferentiated state. Cultured iPSCs were dissociated with CTK solution, rinsed with D-PBS several times, and then dissociated into single cells using Accumax (Innovative Cell Technologies, San Diego, USA). Dissociated cells were seeded at a density of 1×10^6 cells/ml in a spinner type reactor (Biott) containing 30 ml of mTeSR1 (Veritas) with 10 μM ROCK inhibitor (Y-27632; Cayman Chemical) at a rotation rate of 45 rpm. Spheroids formed by cell aggregation during 2 day-culture and then were cultured in hiPS medium for 1 day before starting differentiation. Differentiation protocols are summarized in Fig. 1a.

At stage 1 (DE: definitive endoderm), spheroids were cultured for 4 days in RPMI 1640 (Wako) supplemented with 0.25% bovine serum albumin (BSA; Sigma), 0.4× penicillin and streptomycin (PS; Wako), 1 mM sodium pyruvate (Wako), 1× NEAA, 80 ng/ml recombinant human activin A (Peprotech) and 55 μM 2-ME. Fifty ng/ml FGF2, and 20 ng/ml recombinant bone morphogenetic protein 4 (BMP4; Peprotech) and 3 μM CHIR99021 (Biovision) were added for the first 2 days, and 0.5% KSR was added on Day 4.

At stage 2 (PGT: primitive gut tube), spheroids were cultured for 3 days in RPMI 1640 supplemented with 0.25% BSA, 1 mM sodium pyruvate, 1× NEAA, 0.4× PS, and 50 ng/ml recombinant human FGF7 (Peprotech), 1% B27 supplement (GIBCO) and 1:333 insulin, transferrin, selenium, ethanolamine solution (ITS-X; Gibco). The medium was changed on the third day.

At stage 3 (PFG: posterior fore gut), spheroids were cultured in DMEM (8 mM glucose) supplemented with 0.15% BSA, 0.4× PS, 1× NEAA, 50 ng/ml FGF7, 1% B27 supplement, 1:333 ITS-X, 0.5 μM EC23 (Santa Cruz Biotechnology), 0.2 μM LDN 193189 (Cayman Chemical), 0.3 μM indolactam V (ILV; Cayman Chemical), and 0.25 μM SANT1 (Cayman Chemical) for 4 days. The medium was changed every 2 days during stage 3.

At stage 4 (PP: pancreatic progenitor), spheroids were cultured in DMEM (8 mM glucose) supplemented with 0.15% BSA, 0.4× PS, 1× NEAA, 50 ng/ml recombinant human FGF10 (Peprotech), 1% B27 supplement, 1:333 ITS-X, 0.04 μM EC23, 0.2 μM LDN 193189, 0.3 μM ILV, and 0.25 μM SANT1, 10 μM Alk5 inhibitor II (Rep Sox; Biovision) and 5 μM $ZnSO_4$ (Sigma) for 3 days. The medium was changed on the third day.

At stage 5 (EP: endocrine progenitor), spheroids were cultured in DMEM (20 mM glucose) supplemented with 0.15% BSA, 0.4× PS, 1× NEAA, 20 ng/ml recombinant human epidermal growth factor (EGF; Peprotech), 1% B27 supplement, 1:333 ITS-X, 0.02 μM EC23, 0.2 μM LDN 193189, 0.25 μM SANT1, 10 μM Rep Sox, 5 μM $ZnSO_4$, 50 ng/ml exendin-4 (Abcam), 10 μg/ml heparin (Sigma), 10 μM Y27632, 0.5 μM DBZ (Cayman Chemical) and 5 mM Nicotinamide (Sigma) for 7 days; the medium was changed every 2 days during stage 5.

At stage 6 (BETA: βcell stage), spheroids were cultured in DMEM (20 mM glucose) supplemented with 0.15% BSA, 0.4× PS, 1× NEAA, 1% B27 supplement, 1:333 ITS-X, 10 μM Rep Sox, 5 μM $ZnSO_4$, 50 ng/ml exendin-4, 10 μg/ml heparin, 5 mM Nicotinamide, 10 ng/ml BMP4, 50 ng/ml recombinant human hepatocyte growth factor (HGF; Peprotech), 50 ng/ml insulin-like growth factor 1 (IGF-1; Peprotech) and 5 μM forskolin (Wako) for 10 days. The medium was changed every 2 days during stage 6. We added 1 μM R428 (Cayman Chemical) to the medium of batch C (#5, 6, 7). In the final stage, cells were analyzed between 26 and 31 days.

2.2. Immunostaining and immunohistochemistry

Cultured spheroids were observed every day under an IX71 microscope (Olympus). Spheroids were collected during and after

A

stage	1(DE)			2(PGT)	3(PFG)	4(PP)	5(EP)	6(BETA)
day	1,2	3	4	5,6,7	8,9,10,11	12,13,14	15-21	22-31
medium	RPMI1640			RPMI1640	DMEM(8 mM glucose)		DMEM(20 mM glucose)	
supplement			KSR	B27 ITS-X				
growth factor & small molecule	Activin A			FGF7	FGF10		EGF	BMP4 HGF IGF
	FGF2 BMP4 CHIR99021			EC23 SANT1 LDN 193189				
	2-ME			ILV				
						RepSox $ZnSO_4$		
							Exendin4 heparin nicotinamide	
							Y27632 DBZ	forskolin (R428)

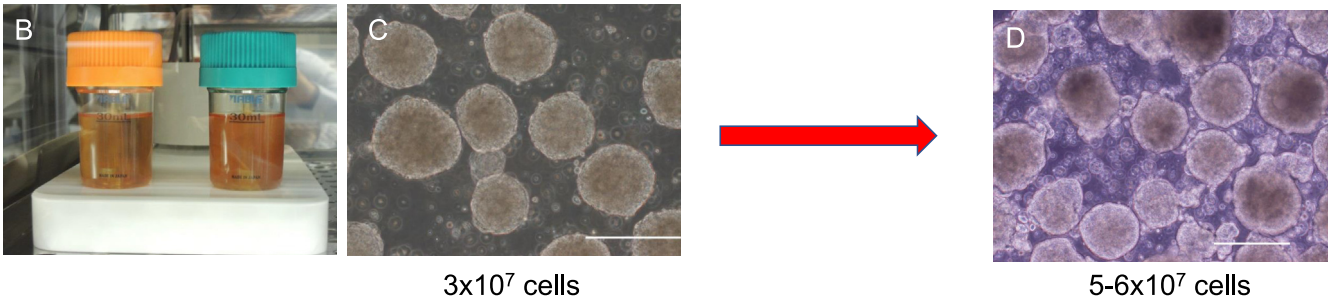

Fig. 1. Overview of the differentiation protocol of iPS cells in suspension culture. A: Summary of 6-stage differentiation protocol including supplements and additives (growth factors and small molecules). DE: definitive endoderm, PGT: primitive gut tube, PFG: posterior fore gut, PP: pancreatic progenitor, EP: endocrine progenitor, BETA: β cell stage. B: Photograph of the spinner type 30 ml reactor with magnetic stirrer in the incubator. C, D: Representative phase-contrast images of iPS cells-derived spheroids before (C) and after (D) differentiation process. Scale bar = 200 μm.

the differentiation process, embedded in Optimal Cutting Temperature (OCT) compound (Sakura Fintek Japan) and stored at −80 °C. Then 6-μm cryosections were cut and immunostained using the following antibodies: goat anti-PDX1/IPF-1 (1: 100; R&D), mouse anti-NKX6.1 (1: 100; DSHB, University of Iowa), rat anti-C-peptide (1: 200; DSHB, University of Iowa), rabbit anti-proglucagon (1: 300; Cell Signaling Technology). The following secondary antibodies were used respectively: Alexa Fluor 488-conjugated donkey anti-goat IgG (1:400; Invitrogen), Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:400; Invitrogen), Alexa 594-conjugated goat anti-rat IgG (1:400; Invitrogen) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400; Invitrogen). Slides were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen) prior to mounting with Fluoromount (Diagnostic Biosystems). Phase-contrast or fluorescent images were taken with a charge-coupled device (CCD) camera (DP71; Olympus) and the positive rate in each image was calculated with Metamorph image analysis software (Molecular Devices) by counting more than 600 cells.

After transplantation, human iPSC-derived grafts were harvested from mice, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 3-μm sections. Hematoxylin and eosin staining was performed according to the standard protocol. For immunostaining, sections were incubated with primary antibodies (diluted in phosphate-buffered saline [PBS], 1.5% goat serum) overnight at 4 °C in a humidified chamber. The following primary antibodies were used: rat anti-C-peptide (1: 200; DSHB, University of Iowa), rabbit anti-somatostatin (1:300; Lifespan Bioscience), and rabbit anti-proglucagon (1:300; Cell Signaling Technology). Sections were washed with PBS and then incubated with a fluorescence-conjugated secondary antibody for 120 min at room temperature. The following secondary antibodies were used: Alexa 594-conjugated goat anti-rat IgG (1:400; Invitrogen) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400; Invitrogen).

2.3. Glucose-stimulated C-peptide secretion assays

Approximately 2×10^6 differentiated cells at the end of stage 6 from 3 different batches were collected and preincubated at 37 °C for 30 min with DMEM (2 mM glucose) containing 10 mM HEPES and 0.1% BSA. Cells were washed twice with DMEM (no glucose) containing 10 mM HEPES+0.1% BSA and then incubated at 37 °C for 1 h in 1 ml DMEM +2.0 mM glucose+10 mM HEPES +0.1% BSA per well. The culture medium was collected and the wells were washed twice with DMEM (no glucose)+10 mM HEPES +0.1% BSA; then, the same cells were further incubated at 37 °C for 1 h in 1 ml DMEM +20 mM glucose +10 mM HEPES +0.1% BSA. The C-peptide concentrations in culture supernatants were determined using a human C-peptide ELISA kit (Mercodia) according to the manufacturer's instructions. Double samples from each supernatant were measured for OD (optical density) by the spectrophotometer (EnVision: Perkin Elmer). After measuring OD, the number of the cells was counted by enzymatic dissociation, and the concentration was converted per 2×10^6 cells for the comparison of each lot of the experiment. We calculated the mean ± SEM for each sample. We compared the concentration of C-peptide between low glucose condition and high glucose condition with the same cells.

2.4. Animal experiments

Animal studies were conducted according to the protocols approved by the Animal Care and Use Committee in the National Center for Global Health and Medicine. To make diabetic model mice, 8-week-old male NOD/SCID mice (Japan Clea) were injected

with 130 mg/kg of streptozotocin (STZ; Sigma) intravenously. After blood glucose levels increased over 350 mg/dl, 6×10^3 spheroids including approximately 6×10^6 iPSC-derived cells in total were suspended in PBS and transplanted under the left kidney capsule of the NOD/SCID diabetic mice (n = 2–3 per batch). PBS alone was injected in the same site as control mice (n = 2). Spheroids from three different batches were examined for animal experiments.

Non-fasting blood glucose levels were examined twice during the first week and thereafter once a week via the tail vein using a glucose test kit (Glutest Neo Sensor, Sanwa Chemical). Blood samples were collected in heparin-coated capillaries from tail vein every 2 or 4 weeks and plasma were obtained after centrifugation (10 min, 4 °C, 800*g*). They were kept frozen −80 °C until use. Mice were killed 12–18 weeks after cell injection, and histological analyses of grafts were performed. In some mice (#4, #5, #6, #7), after renal vessels were ligated under anesthesia and the left kidney was resected, blood glucose levels were further examined for several days.

Human C-peptide and glucagon concentrations in mouse plasma were determined using human ultrasensitive C-peptide ELISA kits (Mercodia) and glucagon ELISA kits (Mercodia).

2.5. Oral glucose tolerance test

Twelve weeks after transplantation, #3 and #4 mice were fasted for 4 h, and blood glucose was measured 15, 30, 45, 60, and 120min after the oral administration of a glucose solution (2.0 g/kg). Blood samples were collected in heparin-coated capillaries before glucose load (pre) and 30 and 120 min after glucose load to measure human C-peptide and glucagon concentrations in mouse plasma. We also examined a STZ-induced DM control NOD/SCID mouse and a non-DM control NOD/SCID mouse for OGTT. Mouse C-peptide and glucagon concentrations in mouse plasma were measured using mouse C-peptide ELISA kits (Morinaga) and glucagon ELISA kits (Mercodia).

2.6. Statistical analyses

Data are expressed as the mean ± SEM. For comparisons of discrete data sets, unpaired Student's t-tests were used. $P < 0.05$ was considered significant.

3. Results

3.1. Spheroid formation

The human iPSC line TkDN4-M cells were maintained an undifferentiated state on mitomycin-C-treated SNL feeder cells. After cultured iPSCs were dissociated into single cells with CTK solution and Accumax, they were seeded at a density of 1×10^6 cells/ml in a spinner type reactor containing 30 ml mTeSR1 with 10 μM ROCK inhibitor (Y-27632). They were cultured for 2 days; then they formed spheroids by aggregating themselves (Fig. 1B and C). We examined several rotary speeds of the reactors and finally adjusted them to 45 rpm to form spheroids whose diameter became 200 ± 50 μm.

3.2. Pancreatic β cell induction

After these spheroids were cultured in hiPS medium for 1 day, the differentiation process was initiated with modifying the previously reported protocol(14), mimicking developmental process of fetal pancreas (summarized in Fig. 1A). To keep the spheroids floating and avoid adhesion to each other, rotary speeds of reactors were kept at 55 rpm from stages 2–4 and at 60 rpm from stages 5–6. We started differentiation with 3×10^7 cells/vessel and we usually obtained $5–6x10^7$ cells after differentiation.

To examine the characteristics of spheroids, immunostaining was performed between 26 and 31 days. We provided three sets of differentiated cells, batch A, B and C for evaluation of immunostaining. We took fluorescent images for PDX1, NKX6.1, C-peptide and Glucagon together with nuclear DAPI stain and the rate of positive cells in each image was calculated by counting more than 600 cells. The percent of positive cells are as follows (mean ± SEM); PDX1 (92.1 ± 4.5%), NKX6.1 (55.5 ± 2.1%), C-peptide (33.7 ± 5.5%), Glucagon (6.0 ± 2.1%). Representative images were shown in Fig. 2A.

To confirm the function of these induced pancreatic β cells, glucose-stimulated C-peptide secretion assays measured human C-peptide concentration in response to changing the glucose concentration. As shown in Fig. 2B, all three sets of spheroids from different batches secreted a certain amount of C-peptide (more than 2000 pM) at low glucose concentration, but did not significantly increase c-peptide production at high glucose concentration.

3.3. Cell transplantation into diabetic model mice

To further investigate whether pancreatic β cells induced by our suspension culture would mature and function *in vivo*, three cohorts of cell transplantation experiments were performed using diabetic model mice. Approximately 6×10^3 spheroids, which presumably contained 6×10^6 iPSC-derived cells in total, were injected under the left kidney capsule of NOD/SCID diabetic mice that had been administered STZ (130 mg/kg) intravenously to destroy mouse pancreatic β cells. Mice whose non-fasting blood glucose levels exceeded 350 mg/dl were used for transplantation experiments. After transplantation, non-fasting blood glucose levels were examined once or twice a week. As shown in Fig. 3A, D, G, non-fasting blood glucose levels started declining about 2–4 weeks after transplantation, although some mice showed a temporary decrease soon after transplantation and returned to the previous high level again within a few days. Of note, non-fasting blood glucose levels gradually dropped below 100 mg/dl at around 10 weeks after transplantation and this level was maintained (6 out of 7 mice). We detected human C-peptide in the blood of transplanted mice 2 weeks after transplantation, although the amount of insulin was not enough to lower the blood glucose level (Fig. 3B, E, H). The blood level of human C-peptide then increased and peaked within 6 weeks in most of the transplanted mice. We also detected glucagon in the plasma of transplanted mice (Fig. 3C, F, I). Their glucagon levels increased up to 6 weeks, but then it fluctuated case by case. After we ligated renal vessels under anesthesia and resected the left kidney where the spheroids had been injected (mice #4, #5, #6, #7), blood glucose levels immediately rose (Fig. 3D, G), suggesting that the transplanted cells were the major source of insulin and that only human iPS-derived cells maintained the normal glucose level in our diabetic mice model.

Oral glucose tolerance tests were performed 12 weeks after transplantation on the #3 and #4 mice and control mice. Blood glucose levels of the transplanted mice peaked at 15 min after administration of glucose and fell below 100 mg/dl after 30 min (Fig. 4A). In contrast, blood glucose levels of a non-DM control mouse peaked at 30 min after glucose loading and gradually lowered to reach the initial level after 120 min (Fig. 4D). Blood glucose levels of a DM control mouse peaked at the upper limit (600 mg/dl) and didn't return to the starting level by 120 min (Fig. 4D). Notably, the plasma human C-peptide levels of transplanted mice peaked at 30 min, whereas the plasma glucagon level was down-regulated at 30 min (Fig. 4B, C). The plasma glucagon levels of control mice were much lower than those of the transplanted mice (Fig. 4F). These

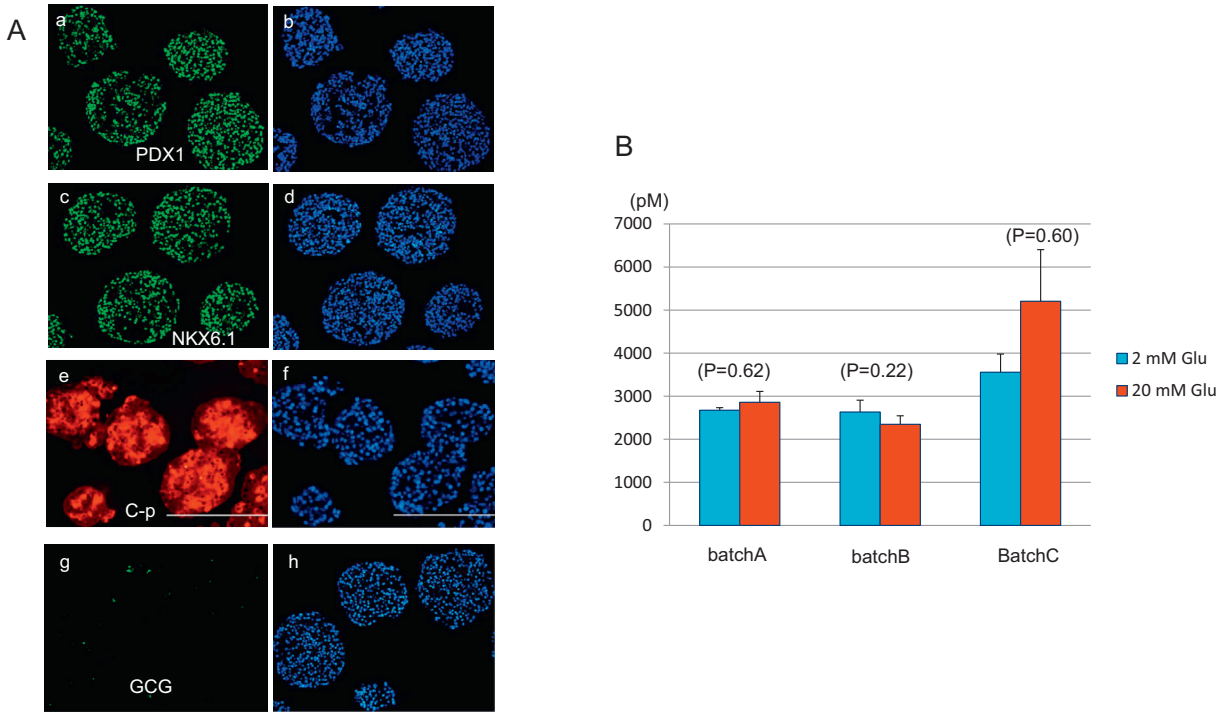

Fig. 2. Immunohistochemical analysis and C-peptide secretion assay with the final stage spheroids. A(a-h): Representative immunofluorescent staining of spheroids at the end of the final stage. PDX1 (a), NKX6.1 (c), C-p: human C-peptide (e), GCG: glucagon (g), DAPI (b, d, f, h). Scale bar = 200 μm. B: Glucose-stimulated C-peptide secretion assay was performed for each batch of spheroids (X-axis A, B, C). Y-axis: the concentration of human C-peptide secreted in the supernatants. The concentrations of human C-peptide secreted under 2 mM glucose conditions (blue bar) and under 20 mM glucose conditions (red bar) were measured with the human C-peptide ELISA kit and a spectrophotometer. We measured the concentration of C-peptide for each batch twice and calculated the mean ± SEM. P value was shown in parenthesis. $P < 0.05$ was considered significant.

results indicate that the transplanted cells responded to the concentration of blood glucose level as do normal islets. In addition we compared the gene expression profiles of differentiated cells before and after transplantation. We extracted RNA from spheroids just before transplantation and two different grafts which were collected 8 and 12 weeks after transplantation. We performed qRT-PCR analysis of several genes, NGN3, MAFA UCN3, ABCC8, SLC30A8, PCSK1 and PCSK2. The gene expression of *MAFA*, *UCN3, SLC30A8, and PCSK2* was upregulated *in vivo*, while the expression of *NGN3* was downregulated *in vivo* (Supplementary Fig. 1).

3.4. Immunohistochemical analysis

Resected left kidneys including human iPSC-derived grafts were fixed in 10% buffered formalin, embedded in paraffin, and cut into 3-μm sections. Hematoxylin and eosin staining of a #3 mouse kidney sample demonstrated that the transplanted cells had formed solid tissue where they were closely surrounded by the capillaries between the renal capsule and the parenchyma (Fig. 5A, B). Although duct-like structures were observed among the grafts, no cancerous regions were found. Immunohistochemical analysis revealed that more than 50% of the engrafted cells were human C-peptide positive and approximately 20–30% were glucagon positive (Fig. 5C). They were mutually exclusive; double positive cells were rarely detected. In addition, somatostatin positive cells were scattered within the grafts and comprised less than 10% of the engrafted cells (Fig. 5D).

4. Discussion

In this study, we focused on establishing a suspension culture of iPS cells for inducingβcells and also on the *in vivo* effects of these induced iPS-derivedβcells after transplantation into diabetic model mice. We succeeded in producing spherical islet-like cells from human iPS cells using our suspension culture method and clearly demonstrated that they ameliorated hyperglycemia in STZ-induced diabetic NOD/SCID mice. Although many papers have reported various methods for differentiating pancreatic β cells from ES cells or iPS cells, only a few have used a suspension culture system throughout the differentiation process. Even if these induced pancreatic β cells were shown to have glucose responsive insulin secretion *in vitro*, they didn't always function *in vivo* [14]. Pagliuca et al. reported that they started culture with 3×10^8 cells in a 500 ml spinner flask and generated functional human pancreatic β cells from ES cells [6]. They also reported that fasting blood glucose levels of NRG-Akita mice were maintained between 100 and 200 mg/dl after transplantation. Recently Mihara et al. reported the production of pancreatic progenitor cells from human iPS cells in a 3-D suspension culture [7]. They finally obtained 1.6×10^8 progenitor cells with 100 ml stirred bioreactor from 3.7×10^7 cells of undifferentiated state, but they did not report any *in vivo* data. We used the same culture method as Mihara's and we started with 3×10^7 cells in a 30 ml spinner reactor, and finally obtained $5–6x10^7$ cells. We also calculated the adequate cell number for transplantation and estimated the scale of the suspension culture in the clinical setting. According to our results, 6×10^6 cells were sufficient for reversal of hyperglycemia in mice whose weight are 25–30 g. Considering the weight of human, 2000 times more cells will be needed for one patient. We estimate the adequate cell number required for transplantation in human is approximately 1.2×10^{10}. If we can obtain 5×10^7 cells after the differentiation by the 30 ml culture, we will need at least 7.2 L culture to provide sufficient cells in the clinical setting to cure the diabetic patients.

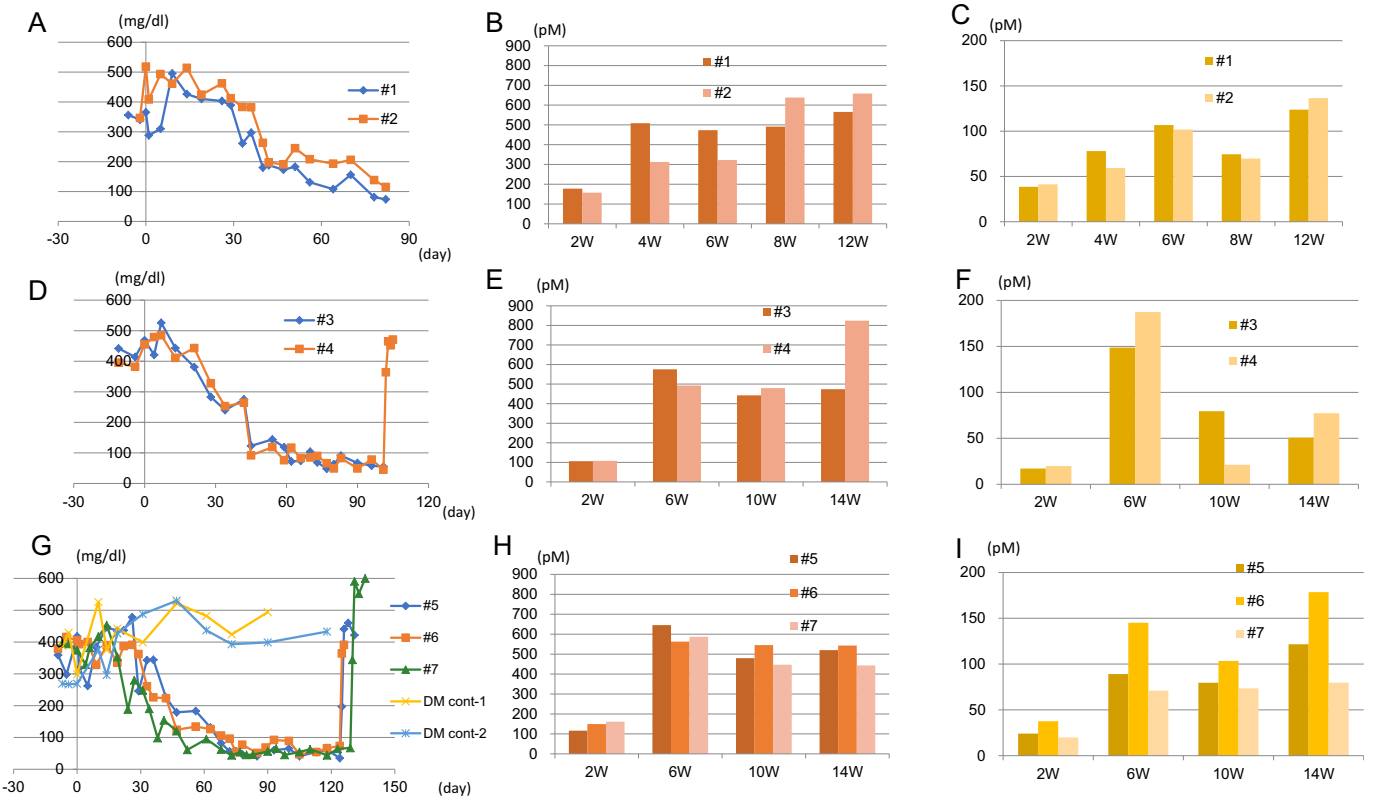

Fig. 3. Transplanted cells ameliorate hyperglycemia in diabetic mice Approximately 6×10^6 iPSC-derived cells were injected under the left kidney capsule of the STZ-induced NOD/SCID diabetic mice. Three different batches of spheroids were used for transplantation experiments. Batch A spheroids were then transplanted into mice #1 and #2, batch B spheroids; mice #3 and #4, batch C spheroids; mice #5, #6 and #7, respectively. PBS was injected in the same site of diabetic mice as a control (mouse DM-cont-1and DM-cont-2 in G). Non-fasting blood glucose levels were examined once or twice a week (A, D, G). Blood human C-peptide (B, E, H) and glucagon levels (C, F, I) were also measured every 2 weeks or 4 weeks for transplanted mice. Survival nephrectomies were performed on some mice (mouse #4, #5, #6, #7).

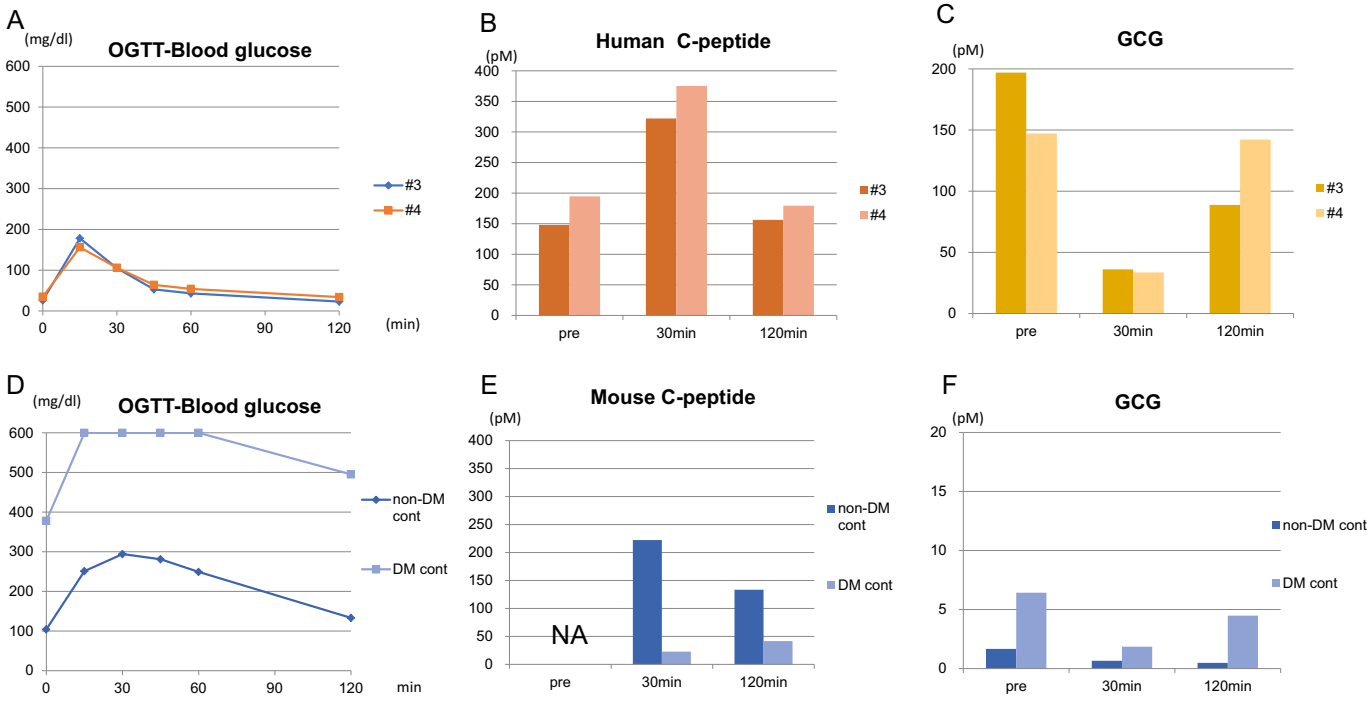

Fig. 4. Glucose tolerance test and hormone secretion A: Blood glucose levels were measured during oral glucose tolerance test (glucose load; 2.0 g/kg) at 12 weeks after transplantation (mouse #3, #4). Blood human C-peptide (B) and glucagon (C) levels were also measured before glucose load (pre), 30 min and 120 min after glucose load by the ELISA kits. D: The same OGTT procedure was performed in a DM control mouse and a non-DM control mouse. Blood mouse C-peptide (E) levels were measured 30 min and 120 min after glucose load. Glucagon (F) levels were also measured before glucose load (pre), and 30 min and 120 min after glucose load by the ELISA kits.

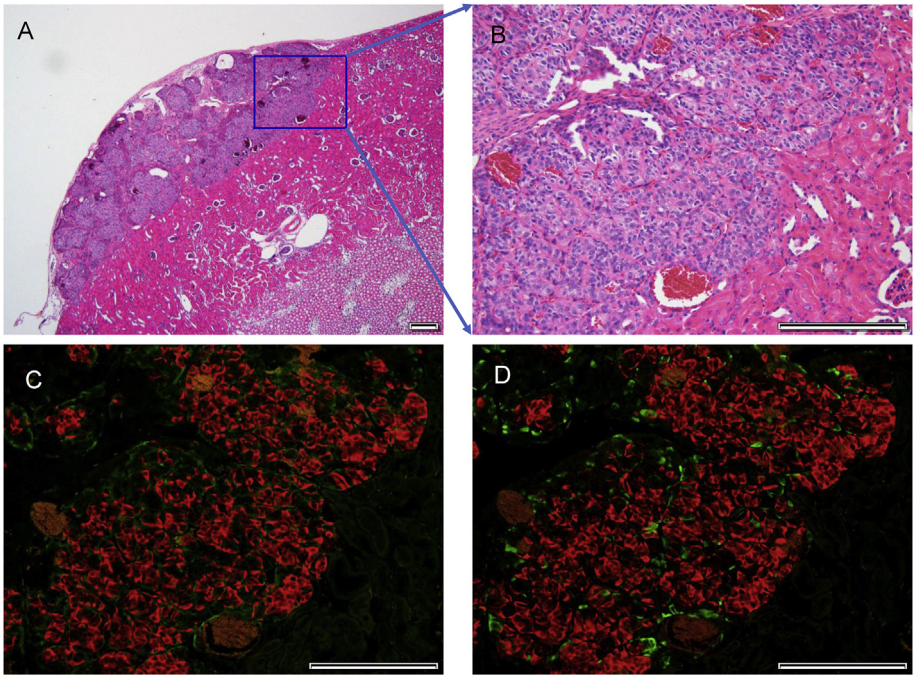

Fig. 5. Immunohistochemical analysis of grafts in the kidney A: Representative HE staining image of the kidney (mouse #3) resected 101 days after transplantation. Square area in A is enlarged in B. C, D: Double immunofluorescent staining of the same area as B. (C) red; human C-peptide, green; glucagon, (D) red; human C-peptide, green; somatostatin. All scale bars = 200 μm.

In terms of cell number, Rezania et al. reported that addition of vitamin C during stages 2–4 increased total cell numbers in their differentiation protocol for human ES cells [5]. We added vitamin C to the medium during stages 2–4, but unexpectedly, total cell numbers decreased in our case (data not shown). They also stated that their protocol did not work for the iPSC line as efficiently as with the H1 hESC line. These reports suggest that each cell line may have their own optimal conditions. Our suspension protocol achieved efficient generation of pancreatic β cells; almost all the cells were PDX1 positive (92.1 ± 4.5%) and more than 30% of the cells in spheroids were C-peptide as shown in Fig. 2A. In our previous paper [14], we obtained the "iPS -derived functional β cells" by culturing them on the dish (2D) until fifth stage and forming spheroids at the last stage. They produced insulin responding to the high glucose concentration in the culture medium *in vitro*, but 2D culture cells didn't respond to the high glucose concentration *in vitro*. In term of the final ratio of beta cells, the positive rate of PDX1 was 92.1 ± 3.4% and C-peptide 33.6 ± 4.2% in the previous 2D-3D culture, which is almost the same ratio as in this 3D culture. Although 3D cultured cells secreted a certain amount of C-peptide (more than 2000 pM) in low glucose conditions *in vitro*, we were not able to detect significant glucose-stimulated C-peptide secretion at high glucose conditions as shown in Fig. 2B. These results indicate that our spheroids are still immature in terms of glucose response *in vitro* because mature β cells are supposed to respond. Rezania et al. [5] reported that they identified R428, a selective small-molecule inhibitor of the tyrosine kinase receptor AXL, as an inducer of MAFA, one of the maturation markers of β cells. They achieved 200 times more *MAFA* expression by adding R428 at the last stage of culture, which contributed to the maturation of the β cells. We added R428 to the medium of the last stage of culture in batch C (#5,6,7), but we did not detect any upregulation of *MAFA* gene expression (data not shown). Comparison of gene expression profiles of differentiated cells before and after transplantation revealed that the gene expression of *MAFA*, *UCN3, SLC30A8, and PCSK2* was upregulated, while the expression of *NGN3* was downregulated after transplantation as shown in Supplementary Fig. 1A. Since NGN3 is known as a marker of endocrine progenitors, downregulation of this gene *in vivo* is consistent with the previous work [8]. Both MAFA and UCN3 are known as maturation markers of β cells [20] and both SLC30A8 and PCSK2 are reported to relate with the function of β cells [9]. Upregulation of these four genes support the proof of maturation of β cells *in vivo* in terms of gene expression profiles although our data are still preliminary.

Some mice showed quick drops in blood glucose level soon after transplantation (Fig. 3A, G). One possible reason is that dying β cells may release insulin all at once due to damage at the very early stage of transplantation. The fact that blood glucose levels increased and returned their previous levels again indicates that this accidental insulin surge ends. We have to bear in mind that the non-fasting glucose levels tend to fluctuate more than fasting glucose levels and the effect of surgical procedure cannot be overlooked. In fact, the blood glucose levels of mouse DM-cont-1and DM-cont-2 fluctuated especially during first 2 weeks post-operation. The reason for the fluctuation of blood glucose level in these mice can be explained in part by the feeding behavior after the surgical procedure. Surprisingly, our transplantation experiments reveal that non-fasting blood glucose levels were maintained below 100 mg/dl 10 weeks after transplantation in most of the cases (Fig. 3. A, D, G). A blood glucose level below 100 mg/dl is normal in humans; however, this is much lower than is usual in mice; the normal murine blood level is 120–150 mg/dl. Schulz TC et al. reported that blood glucose was maintained at or below 100 mg/dl by human ES-derived β cells in mice in which STZ was administered 4–5 months after human cell engraftment [21]. Recently Rodriguez-Diaz et al. also reported that the pancreatic islet imposes its glycemic set point on the organism, making it the bona fide glucostat in the body, and that glucagon input from the alpha cell to the insulin-secreting beta cell is necessary to fine-tune the distinctive human set point [22]. Therefore, it is worth emphasizing that the immature β cells

induced by our protocol not only mature and function *in vivo* but also set the human glycemic set point.

Moreover, histological analysis revealed that the surviving cells were closely surrounded by capillaries, creating a suitable environment for the transplanted cells to live long within the kidney capsule space (Fig. 5A and B). Judging from the amount of plasma human C-peptide in the mice, it took at least 4 weeks for the transplanted cells to mature and ameliorate hyperglycemia as the plasma human C-peptide level increased and enough insulin was secreted (Fig. 3B, E, H). In sharp contrast, it took 16–20 weeks for pancreatic progenitor cells to maturate in *vivo* [12]. In addition, the OGTT results, in which blood glucose peaked at 15min after glucose administration, support the evidence of maturation of the transplanted cells (Fig. 4A). In particular, the data in which the plasma human C-peptide level was elevated while the plasma glucagon level was decreased 30 min after administration of glucose suggest that the transplanted cells behave physiologically like pancreatic islets (Fig. 4B and C). Our data strongly indicate that, even if the β cells are immature before transplantation, they mature and fulfill their function *in vivo*.

We wanted to observe how long the blood glucose levels were maintained by the transplanted cells. However, the 6–8 month limitation of the life span of NOD/SCID mice made this difficult. Therefore, we confirmed the rapid elevation of blood glucose level after a survival nephrectomy from the mice (Fig. 3D, G). We were not able to detect human C-peptide in mice plasma after the nephrectomy, proving that the human C-peptide ELISA kit could distinguish the difference in species between human and rodents. In contrast, we did detect glucagon in mouse plasma after nephrectomy, because the current glucagon ELISA kit could not distinguish the two types. The blood level of glucagon was 10–38pM after nephrectomy, much lower than before nephrectomy. These results suggest that the transplanted cells are the major source of insulin and glucagon and that they control blood glucose levels at the human threshold in mice.

We are currently examining the encapsulation of these cells to avoid attack by the murine immune cells after transplantation. We need to scale up the culture system for future clinical use, because we need yields of more than 10^{10} cells to be competitive with islet transplantation.

5. Conclusions

Induced islet-like cells from human iPS cells produced by suspension culture ameliorate hyperglycemia in immunocompromised diabetic mice.

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Appendix A. Supplementary data

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References

[1] Kieffer TJ, Woltjen K, Osafune K, Yabe D, Inagaki N. Beta-cell replacement strategies for diabetes. J Diabetes Investig 2017;9:457–63.
[2] Melton DA. Applied developmental biology: making human pancreatic beta cells for diabetics. Curr Top Dev Biol 2016;117:65–73.
[3] Pellegrini S, Cantarelli E, Sordi V, Nano R, Piemonti L. The state of the art of islet transplantation and cell therapy in type 1 diabetes. Acta Diabetol 2016;53:683–91.
[4] D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006;24:1392–401.
[5] Vegas AJ, Veiseh O, Gurtler M, Millman JR, Pagliuca FW, Bader AR, et al. Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. Nat Med 2016;22:306–11.
[6] Toyoda T, Mae S, Tanaka H, Kondo Y, Funato M, Hosokawa Y, et al. Cell aggregation optimizes the differentiation of human ESCs and iPSCs into pancreatic bud-like progenitor cells. Stem Cell Res 2015;14:185–97.
[7] Shahjalal HM, Shiraki N, Sakano D, Kikawa K, Ogaki S, Baba H, et al. Generation of insulin-producing beta-like cells from human iPS cells in a defined and completely xeno-free culture system. J Mol Cell Biol 2014;6:394–408.
[8] Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al. Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol 2014;32:1121–33.
[9] Pagliuca FW, Millman JR, Gurtler M, Segel M, Van Dervort A, Ryu JH, et al. Generation of functional human pancreatic beta cells in vitro. Cell 2014;159:428–39.
[10] Mihara Y, Matsuura K, Sakamoto Y, Okano T, Kokudo N, Shimizu T. Production of pancreatic progenitor cells from human induced pluripotent stem cells using a three-dimensional suspension bioreactor system. J Tissue Eng Regen Med 2017;11:3193–201.
[11] Hosoya M, Kunisada Y, Kurisaki A, Asashima M. Induction of differentiation of undifferentiated cells into pancreatic beta cells in vertebrates. Int J Dev Biol 2012;56:313–23.
[12] Chen S, Borowiak M, Fox JL, Maehr R, Osafune K, Davidow L, et al. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat Chem Biol 2009;5:258–65.
[13] Kondo Y, Toyoda T, Ito R, Funato M, Hosokawa Y, Matsui S, et al. Identification of a small molecule that facilitates the differentiation of human iPSCs/ESCs and mouse embryonic pancreatic explants into pancreatic endocrine cells. Diabetologia 2017;60:1454–66.
[14] Yabe SG, Fukuda S, Takeda F, Nashiro K, Shimoda M, Okochi H. Efficient generation of functional pancreatic beta-cells from human induced pluripotent stem cells. J Diabetes 2017;9:168–79.
[15] Takeuchi H, Nakatsuji N, Suemori H. Endodermal differentiation of human pluripotent stem cells to insulin-producing cells in 3D culture. Sci Rep 2014;4:4488.
[16] Rungarunlert S, Techakumphu M, Pirity MK, Dinnyes A. Embryoid body formation from embryonic and induced pluripotent stem cells: benefits of bioreactors. World J Stem Cells 2009;1:11–21.
[17] Olmer R, Lange A, Selzer S, Kasper C, Haverich A, Martin U, et al. Suspension culture of human pluripotent stem cells in controlled, stirred bioreactors. Tissue Eng C Methods. 2012;18:772–84.
[18] Matsuura K, Wada M, Shimizu T, Haraguchi Y, Sato F, Sugiyama K, et al. Creation of human cardiac cell sheets using pluripotent stem cells. Biochem Biophys Res Commun 2012;425:321–7.
[19] Yabe SG, Iwasaki N, Yasuda K, Hamazaki TS, Konno M, Fukuda S, et al. Establishment of maturity-onset diabetes of the young-induced pluripotent stem cells from a Japanese patient. J Diabetes Investig 2015;6:543–7.
[20] Blum B, Hrvatin S, Schuetz C, Bonal C, Rezania A, Melton DA. Functional beta-cell maturation is marked by an increased glucose threshold and by expression of urocortin 3. Nat Biotechnol 2012;30:261–4.
[21] Schulz TC, Young HY, Agulnick AD, Babin MJ, Baetge EE, Bang AG, et al. A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS One 2012;7, e37004.
[22] Rodriguez-Diaz R, Molano RD, Weitz JR, Abdulreda MH, Berman DM, Leibiger B, et al. Paracrine Interactions within the Pancreatic Islet Determine the Glycemic Set Point. Cell Metabol 2018;27. 549-558.e544.