이학석사 학위논문

유도만능줄기세포 유래 인슐린 생성세포 클러스터의 동결보존법에 대한 연구

A Study on the Cryopreservation of Induced Pluripotent Stem Cell-Derived Insulin Producing Cell Clusters

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ABSTRACT

Type 1 diabetes, resulting from autoimmune destruction of beta cells, challenges regulating blood glucose due to diminished beta cell functionality. Islet cell transplantation offers a promising solution, yet the limited islet cell supply hinders widespread application. To overcome this limitation, induced pluripotent stem cells (iPS) have been investigated as a source of islet cells. iPS cells can be differentiated into insulin-producing cells (IPCs) that are similar to native islets. However, IPCs from iPS cells need to be cryopreserved for long-term storage.

The slow freezing method using the controlled rate freezer allows for the adjustment of freezing rate and time. A freezing rate slower than –1°C/min provides sufficient equilibration time for the cryoprotective agent (CPA) to be uniformly supplied to the cell interior, preventing crystal formation and minimizing cell damage.

The investigation involves slow freezing with a CPA containing trehalose in dimethyl sulfoxide (DMSO). Initial experiments on human islets establish cryopreservation conditions, which are then applied to iPSC-derived IPCs for viability and functionality assessments. Experimental groups include conventional freezing, slow freezing with CRF, and CRF slow freezing with trehalose-added CPA.

The research findings indicate that iPS-derived IPCs, when frozen using the CRF method and subsequently thawed, maintained over 80% viability even after three days or more long-term preservation. Moreover, post-differentiation, the presence of the IPC differentiation marker, c-peptide, was confirmed to be above 17%, demonstrating the sustained differentiation capability of the clusters after thawing. Additionally, it was observed that transitioning from 2D differentiation to 3D cluster formation at the pancreatic progenitor stage during iPS differentiation resulted in enhanced functionality post-differentiation.

These results indicate that iPS-derived IPC clusters can be successfully frozen using the slow freezing method, allowing for long-term preservation. Further optimization of CRF could improve the efficiency of cryopreservation and increase the clinical potential of iPS-derived IPCs for islet transplantation.

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LIST OF ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
ATP	adenosine triphosphate
CPA	cryoprotective agent
CRF	controlled rate freezer
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
ES cell	embryonic stem cell
FBS	fetal bovine serum
GSIS	glucose-stimulated insulin secretion
IPC	insulin-producing cells
iPS cell	induced pluripotent stem cell
PBS	phosphate-buffered saline
Tre	trehalose

INTRODUCTION

Type 1 diabetes is primarily caused by the destruction of beta cells due to autoimmune disorders (1). Individuals with type 1 diabetes often struggle to regulate their blood glucose levels because a majority of their beta cells lose functionality. The defining characteristic of type 1 diabetes is the inability of beta cells to produce insulin. Insulin deficiency leads to hyperglycemia, which has adverse effects on both large and small blood vessels, increasing the risk of complications and contributing to disease prevalence and mortality in humans (2). From 1980 to 2014, the number of people with diabetes increased by 390%, reaching approximately 4 billion worldwide (3).

Type 1 diabetes can be treated through continuous insulin supply, and pancreas transplantation or pancreatic islet cell transplantation produces insulin in the body, enabling long-term blood glucose level control. Islet cell transplantation offers a higher level of safety compared to the invasive procedure of pancreas transplantation. It can improve the quality of life and reduce the risk of complications in patients with chronic diabetes who require periodic insulin administration. However, there is a limitation in the supply of islet cells compared to the quantity required for treatment. Achieving effective blood glucose level control may necessitate more than one donor for a single patient (4), and in some cases, multiple transplantations have been necessary (5).

As an alternative for addressing the issue of donor shortage, one method is xenotransplantation of pig pancreatic islet cells. Pigs are a plentiful source of islets, with the ability to isolate high-purity islets (80-95%) in quantities exceeding 255,000 (6). Porcine pancreatic cells are structurally similar to human islets, and the efficacy of porcine insulin administration is well-established. However, there is a risk of excessive immune reactions of recipients or infection from xenogenic viruses. Another approach for an alternative source is differentiating stem cells to produce beta cells (7). The advantage of stem cell-derived islets lies in obtaining a consistent quality of islets through a standardized process, allowing for mass production. Stem cells are mainly classified into embryonic stem cells (ES cells), adult stem cells, and induced pluripotent stem cells (iPS cells). The differentiation of ES cells can lead to ethical debates, and adult stem cells have limited differentiation potential and lower proliferation rates. iPS cells are genetically modified adult cells but function similarly to ES cells. Therefore, they are ethically unproblematic, and personalized therapy can be achieved by establishing a cell line from a patient's cells. When human stem cell-derived pancreatic endoderm cells were

transplanted, there was minimal immune rejection, an increase in the patient's serum C-peptide, and enhanced insulin secretion in response to glucose (8).

Stem cell-derived beta cells that are differentiated into clusters have been shown to have improved function compared to IPCs that are differentiated using conventional attachment culture methods (9) (10). Also, according to a paper by Ribeiro, Kvist et al., in terms of the cellular microenvironment within the pancreas and the multicellular microenvironment of the islet, β-cells produced through 3D differentiation rather than 2D differentiation have a higher degree of mimicking the human body in terms of in vitro phenotype. It is also more effective in function (11).

Cryopreservation is a critical technique for long-term preservation of cells for research. Classically, glycerol or DMSO (dimethyl sulfoxide) has been used to prevent ice crystal the types of cells used in research and clinical applications are becoming increasingly diverse, including genetically engineered cells, cell therapies, and multicellular spheroids. Therefore, research is needed to develop cryopreservation techniques that can maintain the survival and function of these cells (12).

Trehalose is a non-toxic disaccharide that stabilizes cell membranes and prevents damage during freezing (13). The addition of trehalose or other sugars to the cryoprotective agent (CPA) such as DMSO has been shown to increase cell recovery in several studies, including islet (14) (15), and single cell (16).

Vitrification is the process of rapidly cooling a biomaterial to achieve a glass-like state, thus avoiding the formation of ice that can cause cryoinjuries. However, vitrification involves exposing cells to high concentrations of cytotoxic CPA. To minimize cell damage, a small sample volume and a reduced amount of CPA are used in the vitrification process. To achieve a high cooling rate, cell-laden microdroplets with added CPA are directly plunged into liquid nitrogen. This method has been shown to preserve mouse embryos (17) and is also applicable to islet cryopreservation (18). Nevertheless, droplet vitrification carries the risk of cell loss and contamination due to the direct exposure of cells to liquid nitrogen.

During freezing, controlling the freezing and thawing rates is a crucial factor influencing cell survival (19). Particularly, preventing the formation of ice crystals through slow freezing and reducing the toxicity caused by CPA is essential in the cell freezing process. Therefore, the commonly employed method involves using a Controlled

rate freezer (CRF) to regulate the freezing rate and the gradual addition of DMSO to minimize cell damage (20). When frozen using CRF, islet viability is maintained at over 80% (21). Due to the difficulty in uniformly supplying cryoprotectants to clusters, the conditions for freezing cell clusters differ from those for freezing single cells. However, given that IPC clusters structurally and functionally resemble islets, it is expected that their functionality will be preserved when frozen using islet cryopreservation protocols. Additionally, using CRF provides the advantage of freezing cells in large quantities.

In this study, the aim is to investigate whether the function of insulin-producing cell clusters is maintained even after thawing when cryopreserved using a slow freezing technique with a CPA incorporating trehalose in DMSO. The details of the cryopreservation experimental conditions are outlined in Fig. 1a. Initially, experiments were conducted with human islets to verify the cryopreservation conditions, and subsequently, viability and functionality in IPCs were examined using the same approach. The experimental groups were divided into three categories for result comparison: conventional cell freezing condition, slow freezing condition using CRF, and slow freezing condition using CRF with the addition of trehalose to the CPA to aid in cell preservationThis study is expected to optimize the conditions for long-term preservation of iPS-derived pancreatic islet cells without damaging their function and increase the possibility of clinical application of IPC.

MATERIALS AND METHODS

1. Isolation of human pancreatic islet

   The use of human material was approved by the Institution Review Board of Asan Medical Center, Korea (IRB number: 2022-0251) and informed consent was obtained from the participants. Isolation of human pancreatic islets follows the islet isolation protocol developed by Ricordi et al. (22). After isolation, the pancreas tissue is transported to the laboratory in an icebox. In the lab, the pancreas tissue is weighed, and an enzyme (collagenase HA, Vitacyte) is injected to inflate the pancreatic tissue. Subsequently, the pancreas is trimmed to remove fat tissues, blood vessels, and other unwanted components. The remaining pancreas is then cut into pieces and placed into the Ricordi chamber, which contains stainless beads and the remaining enzyme. Within an automated pump-driven circuit, the tissue is physically agitated while media is perfused, allowing digestion to take place. Samples of the perfused solution are periodically collected, and the presence of an appropriate number of isolated islets is confirmed through staining with dithizone. Once the desired islet quantity is achieved, digestion is halted by reducing the temperature of the culture medium to 4 degrees Celsius and adding a buffer to collect the isolated cells. The collected cells are then combined into a 250 mL conical tube, undergo three rounds of washing, and are left to stabilize in a 4°C Custodiol solution for a minimum of 30 minutes. Subsequently, purification is achieved through continuous density gradient separation using a COBE 2991 cell processor and OptiPrep solution (Sigma-Aldrich). The purity of the isolated islets is determined via Dithizone staining. Before their use in experiments, the isolated pancreatic islet cells are cultured in CMRL1066 medium (Gibco), supplemented with 10% Fetal Bovine Serum (FBS, Gibco), and 1x Antibiotic-Antimycotic (AA, Gibco). The culture is maintained at 37°C with 5% CO_2, and the culture medium is changed daily.

2. iPS cell culture and pancreatic differentiation

   For the experiments conducted in this study, ethical approval was obtained from the Institutional Review Board (IRB: 2020-1373) at Asan Medical Center. Following the manufacturer's guidelines, somatic cells underwent transduction using the CytoTune-iPS Reprogramming Kit (Life Technologies). Selected colonies were cultivated on vitronectin (Life Technologies) coated culture plates in StemMACS

medium (Miltenyi Biotec) under conditions of 37°C and 5% CO2.

Human iPS cells are cultured on 100mm cell culture dishes coated with Vitronectin (Gibco) diluted at a ratio of 1:100 in StemMACS culture medium. When the cells reach over 70% confluence, they are passaged using sodium citrate solution. For 2D culture, dissociated cells are plated on 100mm cell culture dishes coated with BME (Cultrex® Reduced Growth Factor BME, Type 2 PathClear® BME, KOMED) diluted at a ratio of 1:100 in StemMACS™. Once the cells reach over 90% confluence, they are cultured in TeSR™-E8™ media (STEMCELL Technologies) for 24 hours before initiating differentiation.

For 3D culture, the cells are dissociated using TryPLE (Gibco) and then inoculated into a 100mm petri dish at a total volume of 12 mL containing 2 x 10^7 cells. Y-27632 is added at a concentration of 10 μM. Suspension culture is conducted at 50 rpm, 37°C, and 5% CO_2 in stage 5 medium. After 24-36 hours of seeding, clusters are formed, and differentiation is initiated following a 24-hour culture in TeSR-E8 media.

The reaggregation process follows these steps: After completing stage 4 differentiation, 2D cells are washed with PBS and dissociated into single cells using TryPLE treatment. Subsequently, they are inoculated into a 100mm petri dish at a total volume of 12 mL, containing 2 x 10^7 cells, with the addition of Y-27632 at a concentration of 10 μM. Suspension culture is conducted at 50 rpm, 37°C, and 5% CO_2 in stage 5 medium. The differentiation protocol is based on Maxwell et al.'s work (23), and the composition and duration of the medium used in the differentiation are as follows.

Stage 1: Definitive endoderm (4 days)

day1: MCDB131 (Gibco) media with 0.1 % BSA (Proliant Biologicals), 1.6 g/L glucose (Sigma-Aldrich), 1.174 g/L sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich), 1x Glutamax (Gibco), 100 μg/mL ActivinA(Peprotech), 3 μM CHIR99021 (LC Laboratories), 10 μM Y-27632 (LC Laboratories), 1x AA. day2-4: MCDB131 media with 0.1 % BSA + 10 mM glucose, 1x Glutamax, 100 μg/mL ActivinA, 1x AA

Stage 2: Primitive gut tube (2 days)

MCDB131 media with 0.1% BSA, 0.8g/L glucose, 1.174g/L $NaHCO_3$, 1x Glutamax, 44mg/L vitamin C (Sigma-Aldrich), 50 ng/mL KGF (Peprotech), 1x AA

Stage 3: Pancreatic Progenitor 1 (2 days)

MCDB131 media with 2% BSA, 0.44 g/L glucose, 1.754g/L $NaHCO_3$, 44mg/L vitamin C, 1xGlutamax, 0.5 x Insulin-Transferrin-Selenium-Ethanolamine (ITS-X, Gibco), 50 ng/mL KGF, 0.2 μM TPPB (TOCRIS), 0.25 μM SANT1 (Sigma-Aldrich), 2 μM RA (Sigma-Aldrich), 0.2 μM LDN (Stemgent), 1x AA

Stage 4: Pancreatic Progenitor 2 (4 days)

MCDB131 media with 2% BSA, 0.44 g/L glucose, 1.754g/L $NaHCO_3$, 44mg/L vitamin C, 1x Glutamax, 0.5 x ITS-X, 50 ng/mL KGF, 0.2 μM TPPB, 0.25 μM SANT1, 0.1 μM RA, 0.2 μM LDN, 1x AA

Stage 5: Pancreatic Endocrine (7 days)

MCDB131 media with 2% BSA, 3.6 g/L glucose, 1.754g/L $NaHCO_3$, 44mg/L vitamin C, 1x Glutamax, 0.5 x ITS-X, 1 μM XXI (MilliporeSigma), 10 μM Alk5i II (Enzo Life Sciences), 1 μM L-3,3′,5-Triiodothyronine (T3; MilliporeSigma), 0.25 μM SANT1, 0.1 μM RA, 10 μM Forskolin (LC Laboratories), 10 mg/L Heparin, 1x AA

Stage 6: SC-β cells

MCDB131 media with 2% BSA, 0.46 g/L glucose, 44mg/L, 1x Glutamax, 1x AA, 1x MEM nonessential amino acids (Corning), 0.1x Trace Elements A (Sigma-Aldrich), 0.1x Trace Elements B (Sigma-Aldrich), 10.46 mg/L Heparin, 300 μg/L $ZnSO_4 \cdot 7H_2O$ (MilliporeSigma), 1x AA

3. Cryopreservation

A cryopreservation experiment is conducted based on the protocol of Rajotte et al. (24). A cryoprotectant solution was prepared based on CMRL1066 and 10% FBS for the islet and MCDB131 media with 1.754g/L sodium bicarbonate, 1x glutamax, 2% BSA, and 0.44g/L glucose solution for the IPC cluster. Collect islets or IPC clusters, dissolve in 250 μL of basal solution, and transfer to a cryovial. Afterward, 2M and 3M DMSO solutions were added to gradually increase the concentration of DMSO solution (2M DMSO solution 125 μL RT 5 min, 2M DMSO solution 125 μL RT 25 min, 3M DMSO solution 500 μL 4℃ 15 min). For conventional conditions, after incubation, the cryovial was placed in a Mr. Frosty™ Freezing Container (Thermo Scientific™) and placed at -80℃ to cool at a rate of -1℃/min. In the case of trehalose conditions, 200mM trehalose (D-(+)-Trehalose dihydrate, Sigma Aldrich) was added to the media 4 hours before cryopreservation for pre-incubation, and a solution containing 200mM trehalose was also used in the cryoprotectant solution. Freezing is slow freezing according to the programmed

freezer conditions of CRF (Grant): 4 to 7.4℃ rate of -2℃/min, 7.4℃ hold for 15 min, 7.4℃ to -40℃ rate of -0.3℃/min, -40℃ to -150℃ rate of -25℃/min (Table 1). After cryopreservation is completed, the cryovial is stored in a liquid nitrogen tank.

4. Cell thawing

The thawing process follows the previously described protocol (24) using the slow dilution method. After removing the cryovial from the liquid nitrogen, it is placed in a 37°C water bath for rapid thawing. Following this, centrifugation is performed at 1,000rpm for 2 minutes at 4°C to remove the CPA. The cryoprotectant solution is then replaced with a 0.75M sucrose solution, based on a basal solution. Gradual dilution is carried out with the addition of the cryoprotectant solution over time. The dilution proceeds as follows: 1mL for 5 minutes, 1mL for 5 minutes, 2mL for 5 minutes, and 4mL for 5 minutes. After dilution, supernatant removal through centrifugation is performed, and culture media is added. The entire process is conducted at 37°C in a 5% CO_2 environment during incubation.

5. Insulin ELISA (Enzyme-Linked Immunosorbent Assay)

Insulin secretion was measured using an Ultrasensitive Insulin ELISA kit, product number 80-INSHUU-E10 (ALPCO). This assay was performed according to the manufacturer's manuals.

6. Glucose-stimulated insulin secretion assays

After collecting the islets, they are washed with Kreb's Ringer bicarbonate buffer (Kreb's buffer; containing 128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1 mM Na_2HPO4, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES, and 0.1% BSA). Following the wash, the islets are incubated in Kreb's buffer containing 2mM glucose at 37℃ under shaking conditions for 1 hour. The solution is then removed, and another round of incubation is carried out with Kreb's buffer containing 2 mM glucose at 37℃ under shaking conditions for another 1 hour. After this incubation, the solution is collected, and the insulin content is measured. The islets are then subjected to another incubation with Kreb's buffer containing 20mM glucose at 37℃ under shaking conditions for 1 hour. Finally, the solution is collected, and the amount of insulin secreted is measured using a human ultrasensitive insulin ELISA kit (Alpco).

7. Flow cytometry

The 3D cell clusters are dissociated into single cells using TryPLETM and then washed with PBS. BD Horizon™ fixable viability stain 450 (BD Biosciences) is used for cell staining. The cells are incubated with this stain. After staining, the cells are washed twice with PBS. For intracellular staining, the cells are fixed for 30 minutes using BD Cytofix (BD Biosciences). The cells are washed twice with BD Perm/Wash buffer (BD Biosciences) solution. The primary antibody (rat anti-C-peptide, DSHB) is diluted 1:300 and used to incubate the cells for 40 minutes at room temperature in a dark environment. After incubation, the cells are washed twice with PBS. The secondary antibody (Goat anti-Rat IgG, Invitrogen) is diluted 1:300 and used to incubate the cells for 40 minutes at 4°C in a dark environment.

To perform nuclear permeabilization staining, the cells are fixed for 45 minutes at room temperature in the dark using 1x Fix Concentrate (BioLegend). After fixation, the cells are washed twice with 1x Perm Buffer (BioLegend). The primary antibody (goat anti-PDX, R&D Systems) is diluted 1:300 and used to incubate the cells for 40 minutes at room temperature in the dark. After incubation, the cells are washed twice with PBS. The secondary antibody (Donkey anti-goat IgG, Invitrogen) is diluted 1:500 and used to incubate the cells for 40 minutes at 4°C in the dark. The stained cells are washed twice with PBS and suspended in PBS for measurement using the Canto™ II flow cytometer (BD Biosciences). The acquired data is analyzed using FlowJo.

8. Viability assessment

After handpicking 50 IPC clusters, they were washed with PBS. Subsequently, TryPLE™ was used to dissociate the clusters into single cells, followed by dilution in media. The resulting cell solution was mixed with an equal volume of 0.4% trypan blue (Gibco) to determine viability based on the ratio of live cells to dead cells. Cell counting was performed using the LUNA-II™ Automated Cell Counter (Logos Biosystems).

Acridine orange (AO)/ propidium iodide (PI) staining method is as follows: Pick islets with a diameter of 100μm or more. Add 1 mL of a solution containing AO (0.67M) and PI (75M) in PBS to the cells and incubate for 10 minutes in the dark at room temperature (RT). Afterward, observe with the EVOS imaging system. The quantification of fluorescence images was analyzed using ImageJ. The calculation method for cell viability is as follows: % of cell viability = [AO stained area / (AO stained area + PI stained area)] x 100%"

9. ATP (Adenosine triphosphate) assay

The ATP(Adenosine triphosphate) assay was performed using CellTiter-Glo (Promega). Cluster dissociation into single cells was carried out using the same method as for the viability assay. Cells and medium were mixed and added to a 96-well opaque plate at 50μL per well. An ATP standard solution was prepared using Adenosine 5′-triphosphate disodium salt hydrate (Sigma-Aldrich) and added at 50μL per well. CellTiter-Glo Reagent was added to the wells at 50μL per well. The mixture was shaken for 2 minutes on an orbital shaker. After incubation at room temperature for 10 minutes, luminescence was measured using the VICTOR X instrument (PerkinElmer).

10. C-peptide ELISA

C-peptide levels were measured using the Human C-peptide ELISA kit from Alpha Diagnostic. The total amount of C-peptide was normalized to the cell number.

11. Statistical analysis

All data measurements were presented as mean ± standard deviation. Statistical comparisons between groups were performed using one-way ANOVA followed by Tukey's test, and results were considered statistically significant when $P < 0.05$. Statistical analysis was conducted using Prism software (GraphPad, version 8.0.2), and graphs were generated using Prism (GraphPad).

RESULTS

1. Morphological and functional evaluation of islet after cryopreservation

When observing the islets isolated from humans after cryopreservation and thawing, it was noticed that in all groups, after one hour of thawing, the clusters had a rough and fragmented appearance, but by the third day, their morphology had significantly recovered. In comparison to the conventional group, the Tre group exhibited a firmer and smoother morphology (Fig. 2a). Viability assessed through AO/PI fluorescence intensity in the Conventional, CRF, and Tre groups were 75.2%, 79.6%, and 86.3%, respectively. The conventional group had a higher proportion of dead cells, while in the CRF-frozen group, dead cells were mainly observed within the cells, resulting in an overall higher proportion of live cells (Fig. 2b). Evaluation of insulin responsiveness to glucose in each group revealed that only the Fresh and CRF groups exhibited a significant difference. The GSIS index values were 4.22 ± 0.77 for the fresh group, 1.41 ± 0.5 for the conventional group, 2.42 ± 0.31 for the CRF group, and 3.08 ± 0.2 for the CRF with 0.2M Tre group.

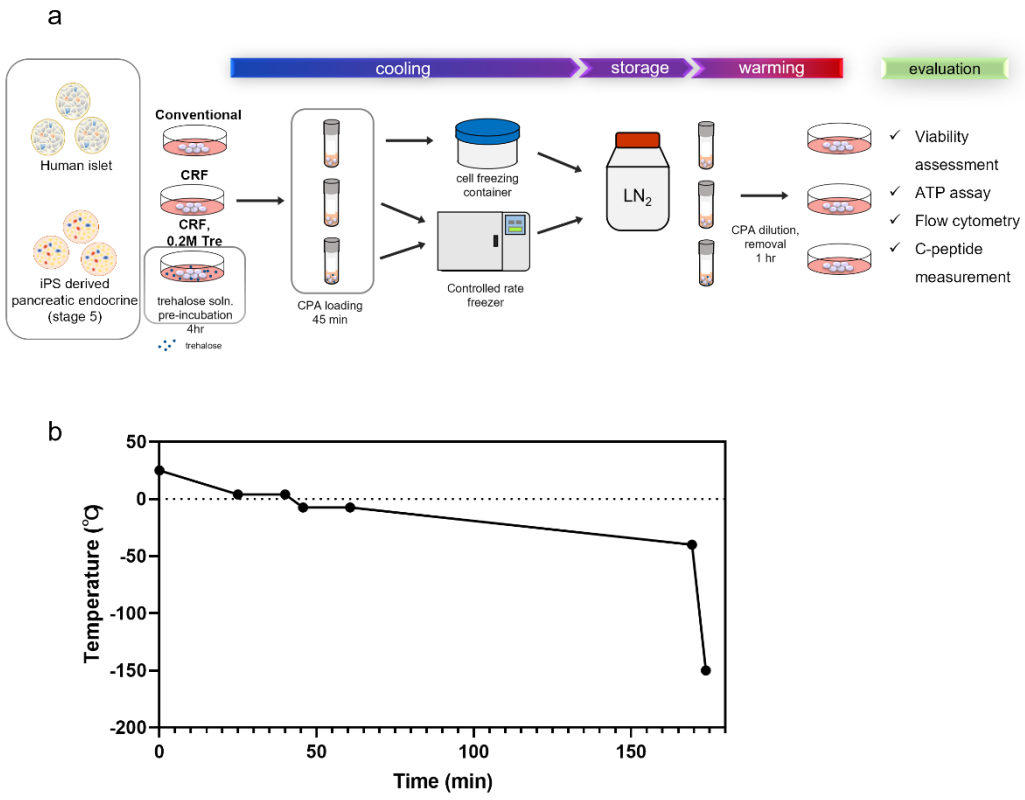

Figure 1. Schematic overview

(a) Cryopreservation workflow and (b) slow cooling process over time and temperature.

Step	Start temp. (℃)	End temp. (℃)	Rate (℃/min)	Duration time (min)
1	25	4	-	25
2	4	4	-	15
3	4	-7.4	-2	5.7
4	-7.4	-7.4	-	15
5	-7.4	-40	-0.3	108.7
6	-40	-150	-25	4.4

Table 1. CRF time and temperature settings

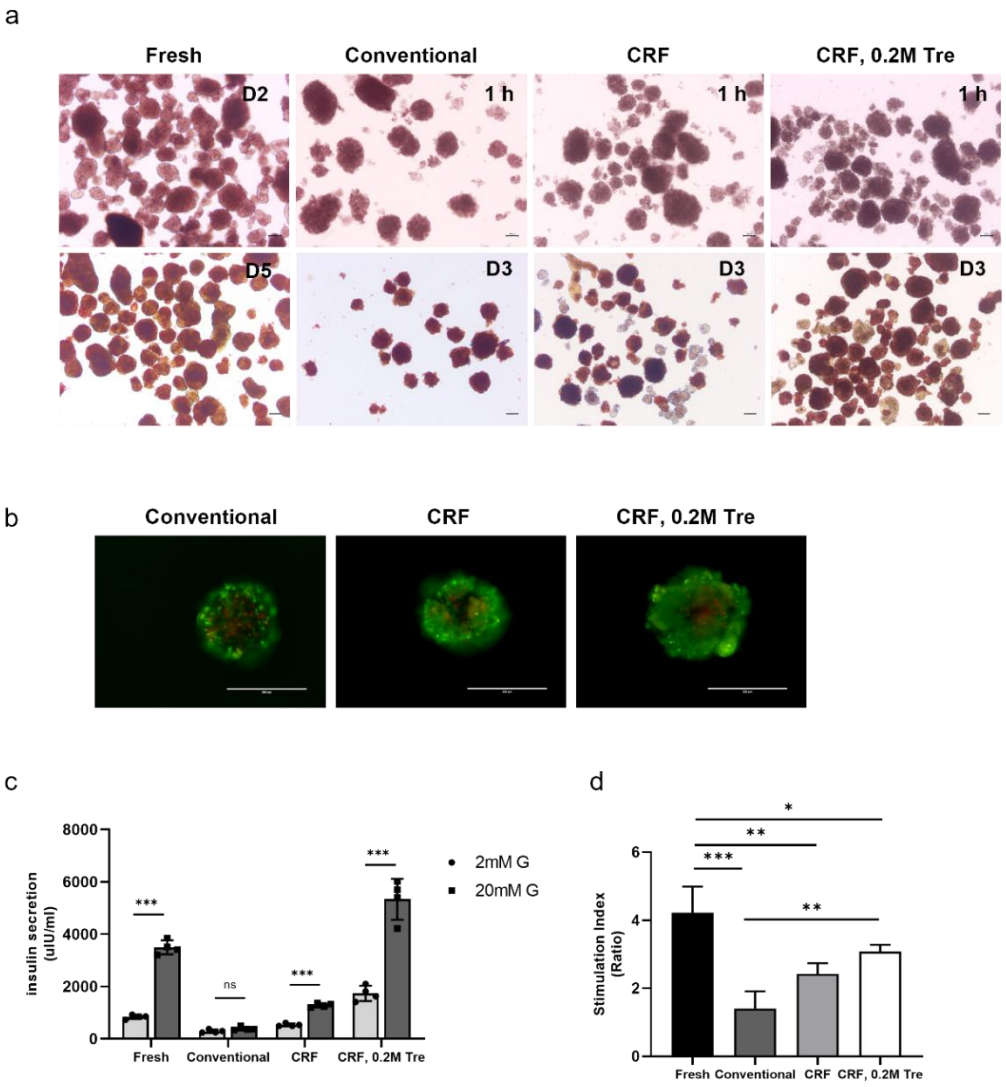

Figure 2. Evaluation of human islet after cryopreservation

(a) Brightfield images of Fresh and cryopreserved islets. Fresh cells were observed on day 2 (D2) and day 5 (D5). Cryopreserved cells were observed for 1 hour (1h) and 3 days (D3) after thawing. The islet was assessed using DTZ staining (islet; red). Scale bar, 100 μm. (b) Cells were stained for viability using Live/Dead staining on day 3 (live; green, dead; red). Scale bar, 200 μm. (c) Glucose responsiveness in a GSIS during 2 mM and 20 mM glucose concentrations 24 hours after cell thawing and (d) Stimulation index. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (n=4).

2. Evaluation of differentiation and differentiation capacity with insulin-producing cell (IPC)

We assessed the functionality of β cells by culturing iPS cells in a 3D culture format. The diameter of 3D iPS cells was approximately 200-300 μm just before differentiation. In stages 2 and 3, the cells lost their round shape and exhibited an irregular morphology. However, after stage 4, they reverted to a round shape (Fig. 3b). Flow cytometric analysis of cells on Stage 6, day 4 of differentiation revealed that the PDX-1 content in the cells was 64.7%, and C-peptide was present at 44.2% (Fig. 3c). This confirmed the differentiation of iPS cells into β cells in the differentiation protocol.

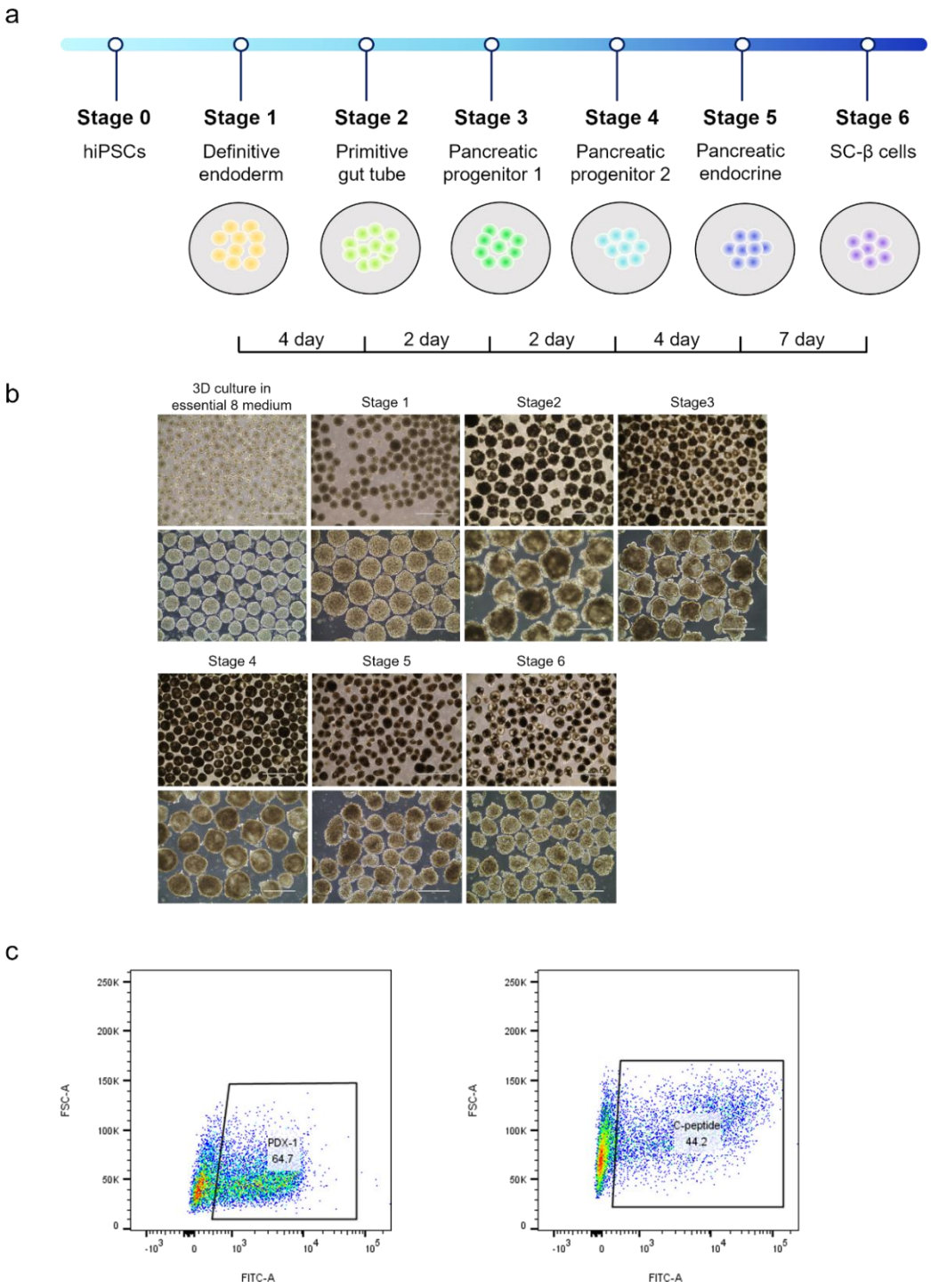

Figure 3. Human iPS differentiation into insulin-producing cells in suspension culture

(a) Differentiation scheme (b) Phase images of insulin-producing cells at different differentiation stages. scale bar 1000 μm, 400 μm. (c) Flow cytometry at stage 6 day 4.

3. Morphology, viability, and function assessment of 3D cultured IPC clusters after cryopreservation

We cryopreserved the IPC clusters formed in 3D culture at the end of stage 5 and thawed them at stage 6 for subsequent culture (Fig. 4a). Upon observation 24 hours after thawing, in the conventional group, many cells were found to be broken. However, when cryopreservation was performed using CRF, the clusters' shape was better maintained (Fig. 4b). The results of ATP measurements at 1 hour and 24 hours post-thawing were presented as a ratio compared to the ATP measurement results of the fresh group. After 1 hour of thawing, ATP measurements showed 20.30 ± 2.48% for the Conventional group, 12.57 ± 1.25% for the CRF group, and 24.34 ± 4.55% for the Tre group. Notably, the Tre group exhibited significantly higher values compared to CRF. However, the overall viability remained below 30%, indicating reduced viability compared to fresh samples. After 24 hours of thawing, the ATP measurement results also remained below 30% for all groups (Fig. 4c). After 1 hour of thawing, viability measured by trypan blue staining was 25.8 ± 4.24%, 41.55 ± 2.19%, and 48.6 ± 10.04% for the Conventional, CRF, and Tre groups, respectively. After 24 hours, viability decreased to 7.75 ± 2.90%, 7.50 ± 0.99%, and 16.6 ± 5.37% for the Conventional, CRF, and Tre groups, respectively. Viability decreased after 24 hours of thawing in all groups (Fig. 4d). After thawing, iPS cells were differentiated until day 3 of stage 6, and the quantity of the differentiation marker, C-peptide, was investigated through flow cytometry analysis. The results showed percentages of 10.8%, 7.69%, 3.42%, and 13.7% for the fresh, conventional, CRF, and Tre groups, respectively, with the Tre group exhibiting the highest levels (Fig. 4e).

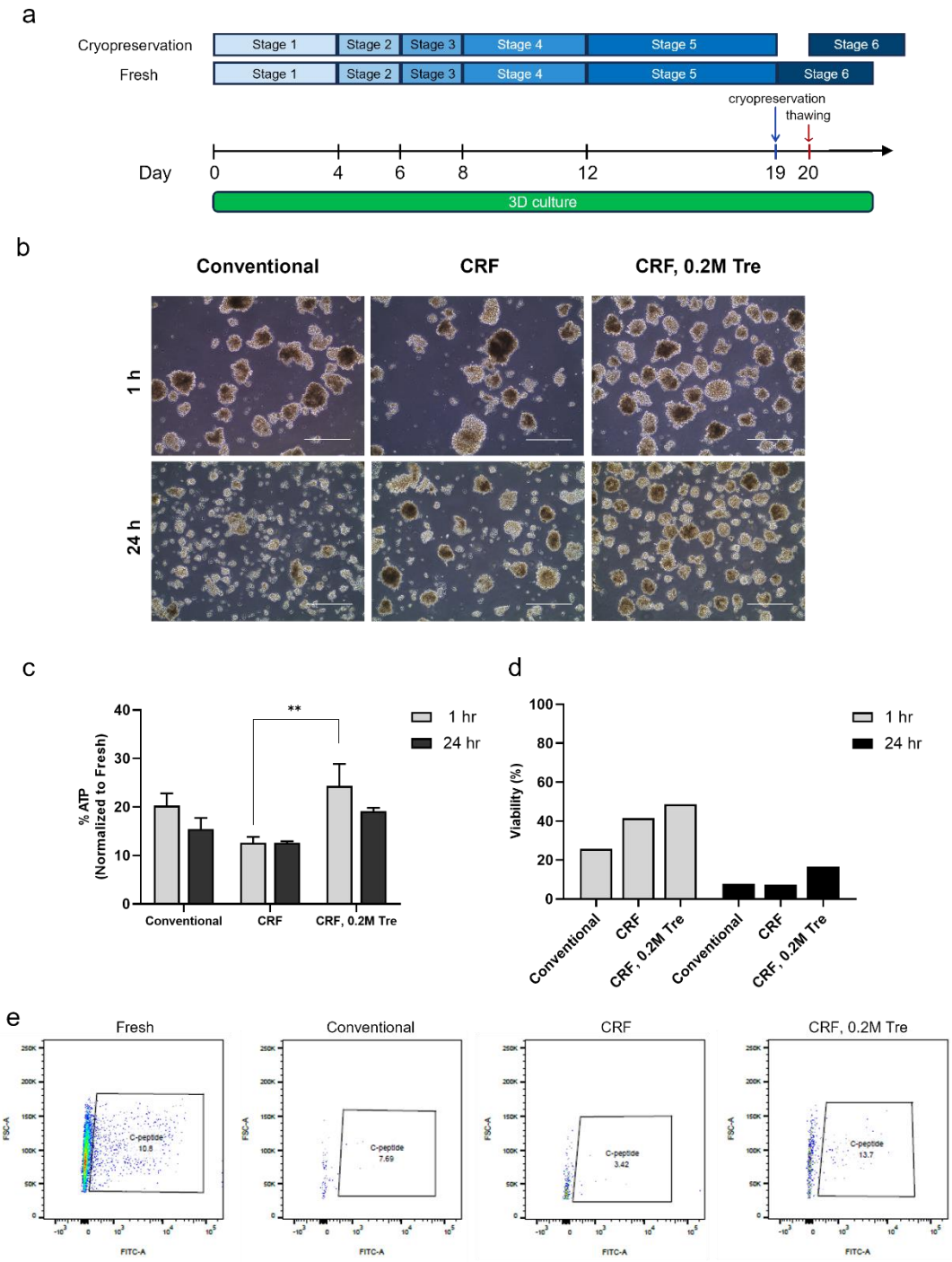

Figure 4. Viability and differentiation assessment of cryopreserved IPC clusters

(a) Differentiation scheme of Cryopreserved and Fresh Cells. (b) The morphology after thawing 1 hour (1 h) and 24 hours (24 h). Scale bar, 400 μm. (c) The ATP measurement normalized to fresh (n=4). (d) The assessment of viability after thawing 1 h and 24 h. (e) Flow cytometry analysis(c-peptide). *p < 0.05, **p < 0.01, ***p < 0.001.

4. Morphology, viability, and function assessment of IPC cluster formed through 2D reaggregation

After differentiating the cells through stage 4 in 2D culture, they were detached and separated into single cells, and reaggregation was performed at stage 5. Well-formed clusters started to appear from stage 5 day 2, and cryopreservation was done on day 3 (Fig. 5a). Subsequently, clusters were thawed, observed for morphology, and evaluated for viability and functionality. One hour after thawing, the cells appeared to be in a fragmented state, but when observed 48 hours later, all groups showed well-aggregated clusters (Fig. 5b). After 24 hours of thawing, ATP measurements showed 62.86%, 40.06%, and 54.71% for the Conventional, CRF, and Tre groups, respectively, with the CRF group displaying the lowest value. Viability analysis using the Trypan blue staining method 24 hours post-thawing resulted in 65.4%, 16.8%, and 67.3% for the Conventional, CRF, and Tre groups, respectively, with CRF showing the lowest viability. When viability was measured again after 3 days, it was found to be 79.1%, 81.7%, and 80.9% for the Conventional, CRF, and Tre groups, respectively. After 24 hours of thawing, viability, which was lower in the CRF group, recovered after 3 days (Fig. 5d).

After thawing, iPS cells were differentiated until day 6 of stage 5, and the functionality of the clusters was evaluated. ELISA was performed to measure the C-peptide secretion, and the results showed that in the fresh group, C-peptide secretion was the highest at 12.91 ng/mL (Fig. 6a). However, when analyzed by normalizing to cell count, CRF had the highest value at 21.5 ng/mL (Fig. 6b). Additionally, the analysis of C-peptide through flow cytometry revealed percentages of 13.0%, 15.5%, 30.8%, and 17.4% for the fresh, conventional, CRF, and Tre groups, respectively, with CRF exhibiting the highest levels (Fig. 6c).

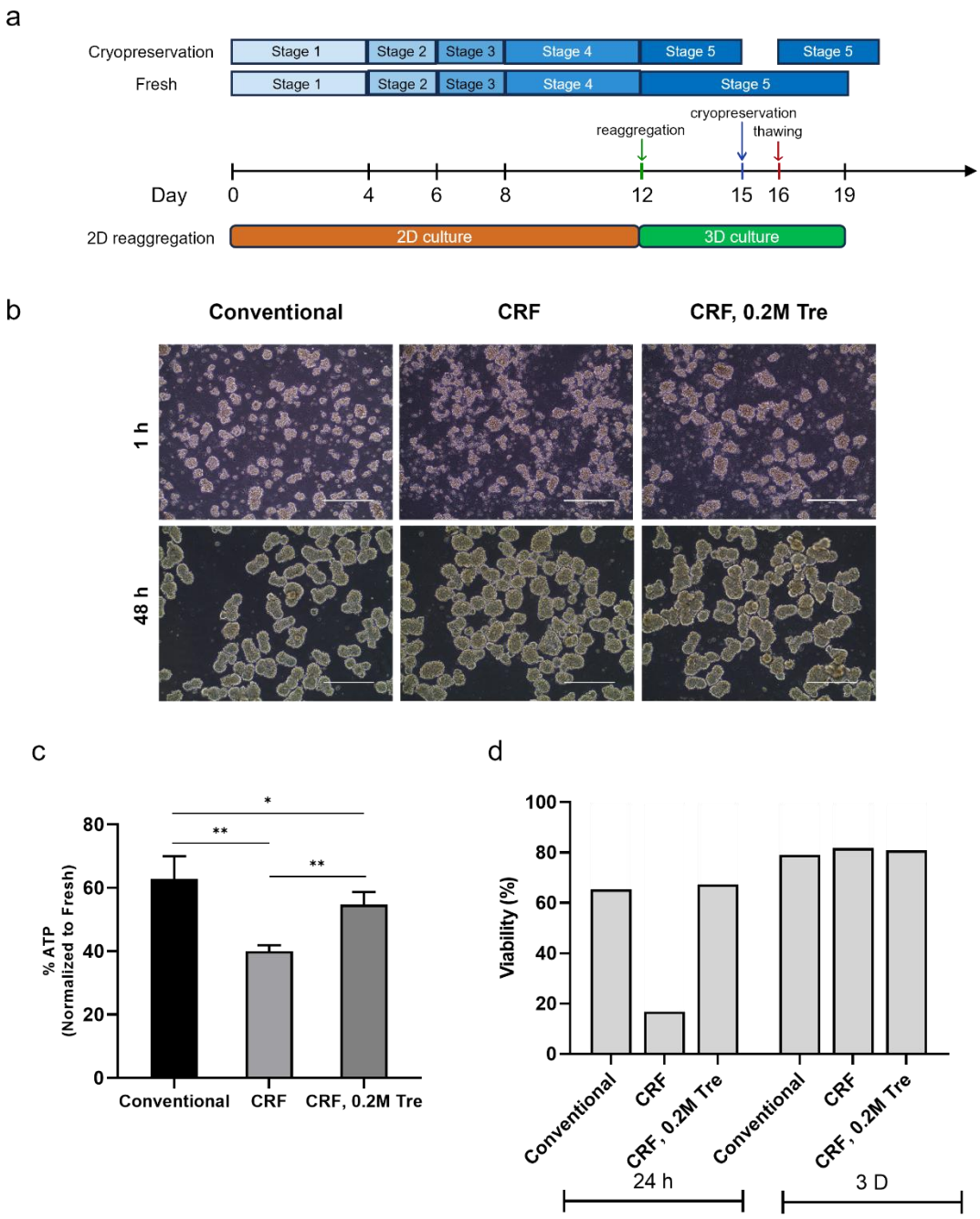

Figure 5. Viability and differentiation assessment of cryopreserved IPC clusters formed through 2D reaggregation

(a) Differentiation scheme of cryopreserved and fresh cells. (b) The morphology after thawing 1 hour (1 h) and 48 hours (48 h) Scale bar, 400 μm. (c) The ATP measurement normalized to fresh after thawing 24 hours (n=4). (d) The assessment of viability after thawing 24 hours (24 h) and 3 days (3 D). *p < 0.05, **p < 0.01, ***p < 0.001.

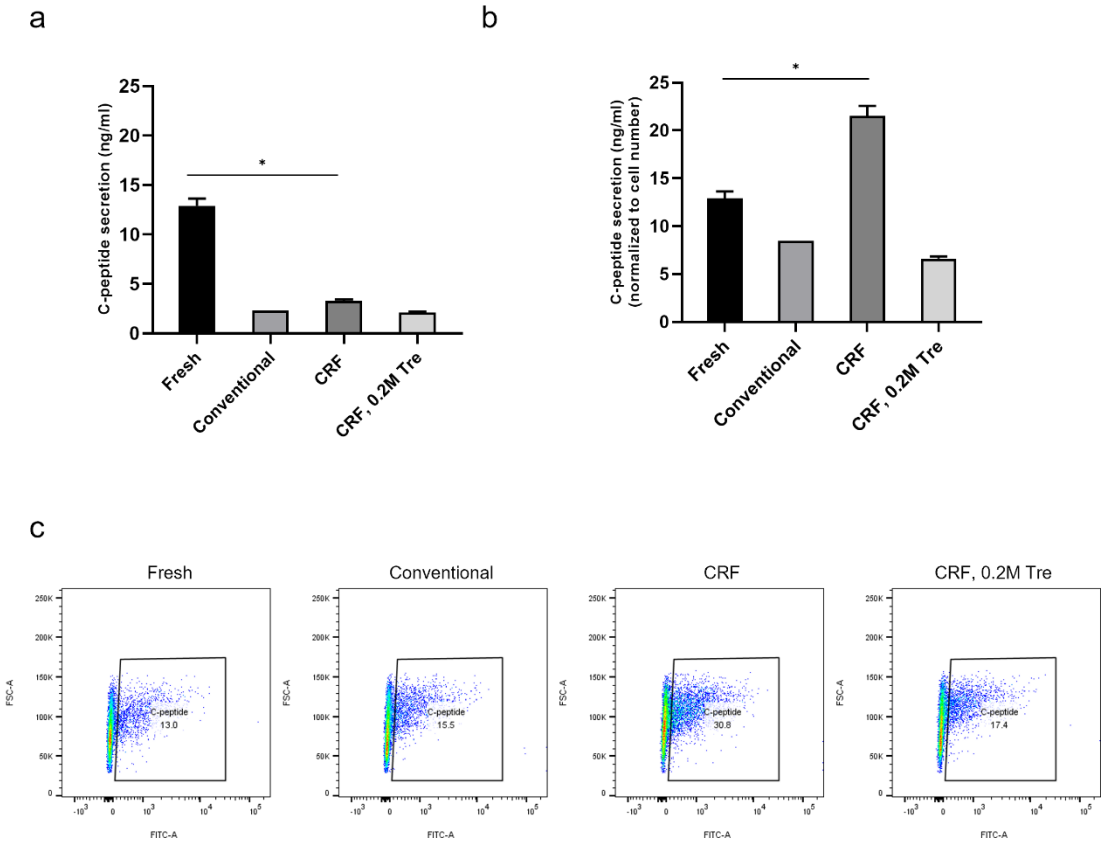

Figure. 6 Functional evaluation of IPC clusters formed via 2D reaggregation after cryopreservation

(a) C-peptide secretion at stage 5 day 6 and (b) normalized result to the total cell number (n=2). (c) Quality evaluation from flow cytometry analysis. *p < 0.05, **p < 0.01, ***p < 0.001.

DISCUSSION

Islets, being non-proliferative cells, face challenges in long-term culture, necessitating cryopreservation for clinical applications. Consequently, extensive research has been conducted on islet cryopreservation, and widely recognized protocols are available. Studies have shown that even after cryopreservation for over 17 years and subsequent thawing, islets can effectively regulate blood glucose levels in diabetic mice, suggesting the feasibility of long-term preservation for more than a decade (25).

Cryopreservation involves several steps that can potentially damage cells, and there are various variables to consider. Further improvements may be possible by optimizing other parameters such as the thaw rate, cell density in the vials, freezing volume, and cryovial type. Additionally, it's important to note that the iPS cell-derived IPCs used in this study may have differences from islets, particularly in their differentiation state

CPA conditions play a crucial role in cryopreservation. Setting the concentration of substances that protect cells during the freezing process, while minimizing toxicity to the cells, is important. CPAs can be classified into permeating and non-permeating agents. Permeating CPAs penetrate the cell and prevent the formation of intracellular ice crystals during the freezing process through the dehydration process (26). Non-permeating agents enhance recovery during the freezing and thawing processes. Saccharide solutions prevent the formation of ice crystals due to their viscosity and help maintain moisture within the cell by regulating osmotic pressure. The combination of both types of agents is expected to enhance the protective effects. In this study, DMSO was used as the permeating CPA, and trehalose was used as the non-permeating CPA.

To confirm the cryopreservation conditions for IPCs, we first evaluated the functionality of human islets after freezing and thawing. The results showed that following the fresh islets, the trehalose group preserved the best functionality. Subsequently, we conducted cryopreservation experiments with iPS cells differentiated into IPCs. The IPCs were frozen at Stage 5 when markers like CHGA and NKX6.1, which are associated with β cell function, were expressed, indicating their proximity to islet-like status.

Upon observing the morphology of IPC clusters cultured in 3D after cryopreservation and thawing, it appeared that immediately after thawing, there were many broken clusters, indicating potential damage. However, after 24 hours, the clusters seemed to have recovered with a solid surface. However, when assessing viability, it was observed that overall viability

decreased with an extended culture period. This suggests that cells may not recover from the damage inflicted during freezing and thawing, possibly due to the inability of cells to replicate (27).

To enhance cell viability, we initially differentiated iPS cells from a 2D state to pancreatic progenitors and formed 3D IPC clusters through aggregation before further differentiation into pancreatic endocrine cells. Cryopreservation and thawing of the IPC clusters generated through this method resulted in increased viability even after long-term culture, leading to an overall improvement in C-peptide secretion capabilities. While the overall cell count significantly decreases, reaggregation may appear inefficient. However, some studies have highlighted the significance of this cell loss, as it eliminates irrelevant cell types and enhances the final cell population's enrichment for endocrine cells (28). Therefore, in this experiment, it is likely that the CRF group retained only well-functioning cells after removing those that did not perform properly, leading to improved overall functionality.

CONCLUSION

In conclusion, this research elucidates the efficacy of CRF-mediated slow freezing for the cryopreservation of IPC derived from iPS. The study reveals that these clusters, when frozen using CRF, exhibit remarkable resilience with over 80% viability maintained even after more than three days of long-term preservation. The sustained expression of the IPC differentiation marker, c-peptide, exceeding 17% post-thawing, underscores the preservation of functional integrity. Moreover, the transition to 3D cluster formation during differentiation at the pancreatic progenitor stage proves beneficial, enhancing post-thaw functionality. Further experiments are needed to determine the optimal stage of IPC differentiation for cryopreservation, one that maximizes both survival and functionality.

These findings signify the potential clinical applicability of CRF-based cryopreservation in ensuring the viability and function of iPS-derived IPC clusters. The optimized conditions presented here contribute to the advancement of long-term preservation strategies for such clusters, addressing critical challenges in diabetes treatment and fostering progress in the field of regenerative medicine. This research paves the way for further exploration and development of cryopreservation protocols to meet the demands of diverse applications in both research and clinical settings.

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국문 요약

제1형 당뇨는 인슐린 의존성 당뇨병으로, 일반적으로 인슐린 투여나 췌도세포 이식을 통해 혈당을 조절하고 합병증을 예방한다. 췌도세포 이식은 한 번의 수술로 장기적인 혈당 조절이 가능하므로, 주기적인 인슐린 투여가 필요한 만성 당뇨 환자들의 삶의 질을 향상시키고 합병증 발생 위험을 줄일 수 있다. 그러나, 췌도세포는 공급이 한정적이라는 한계가 있다. 이러한 한계를 극복하기 위한 대안으로 줄기세포를 분화하여 췌도세포의 공급원으로 사용되는 방법이 많이 연구되고 있다. 특히 유도만능 줄기세포(induced pluripotent stem cell, iPS)는 당뇨병 치료에 효과적인 인슐린 생성 세포(insulin producing cell, IPC)로 분화될 수 있다. 그러나 이 분화 과정에는 최소 3주가 소요되며, 추가적인 품질평가 과정을 고려한다면 분화된 iPS 유래 인슐린 IPC를 장기보관 하기 위한 동결보존법이 필요하다. 자동세포 동결 장비(controlled rate freezing, CRF)를 사용한 완만 동결 방법은 동결 속도와 시간을 조절할 수 있게 해준다. -1°C/min 이하의 동결 속도는 세포 동결 시 동결보호제가 세포 내부에 균일하게 공급될 수 있는 평형시간을 주어 결정 형성을 방지하고 세포 손상을 최소화한다. CRF 장비를 이용하면 세포의 군집인 클러스터 형태인 IPC 클러스터를 효율적인 동결보존이 가능할 것으로 예상하였다. 본 실험을 진행하기 전에, 췌도세포를 사용한 사전 실험을 통해 동결보존 조건을 먼저 검증하고 이를 iPSC에서 유래된 IPC 클러스터의 생존률 및 기능 평가에 적용한다. 연구 결과에 따르면 iPS에서 유래된 IPC 클러스터가 CRF 방법을 사용하여 동결되고 이후 해동될 때 3일 이상의 장기 보존 후에도 80% 이상의 생존률을 유지하는 것으로 나타났다. 또한 분화 후 IPC 분화 마커인 c-펩타이드가 17% 이상으로 확인되어 해동 후에도 클러스터의 분화능이 유지됨을 확인하였다. 추가적으

로 췌장 전구체 단계에서 2D 분화에서 3D로 클러스터를 형성하며 분화하면 분화 후 기능이 향상되었다는 것을 관찰하였다. 이러한 결과는 iPS에서 유래된 IPC 클러스터를 CRF를 이용한 완만동결방법으로 동결할 수 있으며, IPC 클러스터의 임상 가능성을 높일 수 있을 것으로 기대한다.