

Continuous inhibition of Sonic hedgehog signaling effectively leads to differentiation of human-induced pluripotent stem cells into functional insulin-producing β cells

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Research

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Abstract

Background: Human-induced pluripotent stem cell (iPSC)-derived insulin-producing cells (IPCs) can be used for islet cell transplantation in type 1 diabetic patients and as patient-specific cells for the development of novel anti-diabetic drugs. Therefore, it is necessary to develop a method for generating functional IPCs from iPSCs and simplifying the stepwise protocol.

Methods: We compared combinations of small molecules that could efficiently induce the differentiation of cells into a definitive endoderm, and preferentially into islet precursor cells. IPCs, generated using the optimal combination of small molecules, were confirmed to demonstrate insulin secretion in response to glucose stimulation. Finally, we re-constructed spheroid IPCs and verified the optimized culture and maturation conditions.

Results: It was confirmed by quantitative polymerase chain reaction that definitive endoderm-specific markers were expressed differently depending on the combination of the small molecules used. Small molecule SANT-1 induced the differentiation of cells into functional IPCs by acting as an inhibitor of Sonic hedgehog signaling. Images of 2D culture showed that IPCs were spheroid-shaped from day 5 and demonstrated sustained insulin secretion. We developed a simple differentiation method using small molecules that produced functional IPCs that responded efficiently to glucose stimulation in a relatively short time.

Conclusions: We posit that this method along with a method that refines the process of differentiation can be used for growing IPCs that can be employed in clinical trials.

Background

Human embryonic stem cell (ESC)- or human-induced pluripotent stem cell (iPSC)-derived insulin-producing cells (IPCs) can be used not only for transplantation of islet cells, which are destroyed due to autoimmunity in patients with type 1 diabetes mellitus (DM), but also for identifying novel targets for the development of anti-diabetic drugs *in vitro*. Several reports on the generation of IPCs from ESCs or iPSCs are available [1–5]. In particular, a number of studies have successfully differentiated ESC or iPSC into IPCs by mimicking pancreatic developmental and established a stepwise protocol for the same [3, 4, 6–8]. This protocol involves the addition of various differentiation-inducing factors to the culture media at each differentiation stage, because of which the expression of transcription factors that are specific to that particular pancreatic developmental stage is induced. Small molecules are also being widely used as differentiation-inducing factors, and IPCs generated using small molecules have shown robust function *in vivo* and *in vitro* [9–12].

A growing number of small molecules that can be used both *in vitro* and *in vivo* to grow stem cells [13–15], direct their differentiation [10, 11, 16–18], and reprogram somatic cells into a more naïve state [19] have been identified. These molecules also provide useful information on the signaling and differentiation mechanisms that regulate stem cell biology. Small molecules with unique biological

activities enable the establishment of new biological studies and the development of new treatments by significantly lowering the cost of production. Therefore, we leveraged the advantages of these small molecules for IPC differentiation. We screened for small molecules that induced and regulated the differentiation of stem cells into pancreatic progenitor cells. These include dorsomorphin (Dor, a selective inhibitor of bone morphogenetic protein (BMP) signaling) [9, 20], SB431542 (an inhibitor of the transforming growth factor- β (TGF- β) type I receptor/ALK5) [9, 21, 22], SANT-1 (an inhibitor of Sonic hedgehog signaling) [23–26], FR180204 (an ATP-competitive inhibitor of ERK1 and ERK2) [20, 27], and retinoic acid (RA) [28–30]. Using this screen, we developed functional IPCs that responded to glucose in response to combinatorial treatment with small molecules.

Many researchers generated ESC- or iPSC-derived IPCs that had functions similar to those of natural islet cells. However, they were reported to lack the ability of functional insulin secretion in response to glucose stimulation [5, 20, 24] because the differentiation and culture conditions generated *in vitro* did not accurately mimic the natural developmental stages of the human pancreas. Therefore, although the combination of the differentiation-inducing factors is important, the method of producing these cells in culture must also be investigated.

In this study, we developed a simple and efficient stepwise protocol for generating IPCs from iPSCs using SANT-1 and FR180204. Combined treatment with small molecules induced the efficient differentiation of iPSCs into a definitive endoderm, pancreatic progenitor cells, and finally IPCs. In addition, the functional IPCs were continuously cultured and matured to obtain a spheroid morphology similar to that of natural islet cells.

Methods

Small molecules

Several small molecular compounds were used to differentiate iPSCs into IPCs. In most cases, the small molecules inhibited the signaling pathways in cells. The differentiation process was divided into three steps to mimic the process of pancreatic development in the embryonic stage. The first step was the formation of a definitive endoderm, the second step was its differentiation into the pancreatic endoderm and pancreatic progenitor cells, and the third step was the induction of specific differentiation of the cells into IPCs. Therefore, the differentiation-inducing small molecules were processed at each step, and the results were confirmed by comparing and analyzing the combinations of small molecules (Table 1).

Cell culture

iPSCs were kindly provided by the Center for Stem Cell Research of Asan Institute (Seoul, Korea). The cells were maintained on a vitronectin-coated dish with essential 8TM basal medium (Gibco, NY, USA). For differentiation into a definitive endoderm, iPSCs were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 1% B27TM supplement (minus insulin, Invitrogen) with or without human activin A (100 ng/mL) (PeproTech, Rocky Hill, NJ), 3 μ M CHIR99021 (Sigma-Aldrich, MO, USA), and 5 μ M

LY294002 (Sigma-Aldrich) for 24 h, and then in RPMI 1640 medium containing 1% B27 with activin A (100 ng/mL) for 48 h. The iPSCs were subsequently incubated in Improved MEM Zinc Option culture medium (Invitrogen) containing 1% B27 with 1 μ M Dor (Sigma-Aldrich), 4 μ M RA, and 10 μ M SB431542 (Tocris Bioscience, Bristol, UK), with or without FGF2 (50 ng/mL, PeproTech), 3 μ M FR180204 (Sigma-Aldrich, MO, USA), and 0.25 μ M SANT-1 (Sigma-Aldrich, MO, USA) for differentiation into pancreatic progenitor cells. The cells were then cultured in differentiation-inducing medium for 6 d, and the medium was changed daily. Finally, for differentiation into IPCs, 10 μ M forskolin (Fors, Sigma-Aldrich, MO, USA), 10 μ M dexamethasone (Dexa, Sigma-Aldrich, MO, USA), and 10 μ M nicotinamide (Nico, Sigma-Aldrich, MO, USA) were added to the medium, and the cells were cultured for 10 d.

Teratoma analysis

The iPSCs were harvested and dissociated into a single cell suspension using TrypLE™ Express (Gibco, NY, USA). The cells (2×10^6 cells) were subcutaneously injected dorsally into 8-week-old mice with severe combined immunodeficiency (SCID). They were maintained under non-specific pathogen-free (SFP) conditions at an experimental animal facility in Asan Institute. They were euthanized after the development of tumors $>1 \text{ cm}^3$, or following an observation period of 40 d. The tumor-containing tissue was fixed with 4% paraformaldehyde (PFA), embedded in paraffin, and serially sectioned (4 μ m sections). The tissue sections were stained with hematoxylin and eosin and subjected to histological analysis using a microscope.

Immunostaining

Cells were fixed with 4% PFA for 30 min at room temperature (RT) and then washed thrice with phosphate-buffered saline (PBS). The cells were permeabilized with 0.1% Triton X-100 for 5 min, and then washed with PBS. For blocking, cells were incubated with normal horse serum (1:30) for 30 min, and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-Oct4, 1:50 (abcam, MA, USA); mouse anti-Nanog, 1:200 (abcam, MA, USA); rabbit anti-Sox2, 1:1000 (abcam, MA, USA); mouse anti-SSEA4, 1:200 (abcam, MA, USA); mouse anti-FoxA2, 1:100 (abcam, MA, USA); goat anti-CXCR4, 1:300 (abcam, MA, USA); mouse anti-insulin, 1:200 (Santa Cruz, TX, USA); rabbit anti-glucagon, 1:200 (Santa Cruz, TX, USA); and goat anti-Pdx1, 1:10000 (abcam, MA, USA). After washing with PBS, the cells were incubated with Alexa Fluor 488- or 594-conjugated donkey or goat antibodies directed against rabbit, goat, or mouse IgG at 1:250 dilutions at RT for 1 h. Nucleotides were stained with Hoechst 33342 (Thermo Scientific, Germany).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen), and used to synthesize first strand cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). The SYBR pre-mixed system SsoAdvanced™ Universal SYBR Green Supermix (BIO-RAD, CA), and specific primers were used to determine the level of each mRNA transcript to relative to that of 18sRNA. Primer sequences used were the following: Sox17, forward 5'-CGCTTTCATGGTGTGGGCTAAGGACG-3', reverse 5'-

TAGTTGGGGTGGTCCTGCATGTGCTG-3'; FoxA2, forward 5'- ACTGGAGCAGCTACTTAGCAGAGC-3', reverse 5'-TCATGGAGTTCATGTTGGCGTAG-3'; CXCR4, forward 5'-GGTGGTCTATGTTGGCGTCT-3', reverse 5'-TGGAGTGTGACAGCTTGGAG-3'; Pdx1, forward 5'-GCATCCCAGGTCTGTCTTCT-3', reverse 5'-ATCCCCTGCCAGAAAGGTT-3'; Ngn3, forward 5'-CTTGCTGCTCAGGAAATCCC-3', reverse 5'-CTTCTGGTCGCCAAGTTCAG-3'; Nkx6.1, forward 5'-CGTTGGGGATGACAGAGAGT-3', reverse 5'-TGGGATCCAGAGGCTTATTG-3'; Sox9, forward 5'-TACGACTACACCGACCACCA-3', reverse 5'-TCAAGGTCGAGTGAGCTGTG-3'; NeuroD, forward 5'-GCCGACGGAGATTAGGAGAA-3', reverse 5'-TCTTGTCCTGACACTGGCAT-3'; insulin, forward 5'-ATCCTGGATCTCAGCTCCCT-3', reverse 5'-CTCACAGCCCTTCAGAGACA-3'; glucagon, forward 5'-ATTGCTTGGCTGGTGAAAGG-3', reverse 5'-TATAAAGTCCCTGGCGGCAA-3'; and 18sRNA, forward 5'-GAGCCTGCGGCTAATTTGA-3', reverse 5'-AACTAAGAACGGCCATGCAC-3'. PCR was performed at 45 cycles of 20 s at 98°C, 20 s at 55°C, and 20 s at 72°C. The relative level of each gene mRNA transcript to the 18sRNA level was analyzed by the $2^{-\Delta\Delta CT}$ method.

Insulin release assay

Differentiated cells were pre-incubated in Krebs-Ringer bicarbonate HEPES buffer without glucose (Krebs buffer; 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM HEPES, and 0.5% BSA) at 37°C for 2 h. Then, the cells were sequentially incubated with 2.8 mM, 20 mM, and 30 mM glucose and 30 mM KCl-Krebs buffer for 1 h. The insulin secretion of the Krebs buffer samples and differentiation-induced media was measured using an insulin ELISA kit (ALPCO, NH, USA).

Flow cytometry

Differentiated cells were dissociated to single cells using Accutase, fixed with 4% PFA, and permeabilized with Perm buffer III (BD Biosciences). The cells were incubated with normal horse serum for 10 min and then with mouse anti-insulin, rabbit anti-glucagon, and rabbit anti-Ngn3 antibody for 30 min at RT. The cells were then stained with Alexa Fluor 488-conjugated donkey antibodies directed against mouse or rabbit IgG for 30 min at RT, and flow cytometry was performed using FACS Aria II (Becton Dickinson).

Statistics and reproducibility

Statistical tests performed for specific data sets are described in the corresponding figure legends. Two-tailed unpaired *t*-tests (Student's *t*-test) were used to measure standard deviation (SD). Two-way ANOVA test for multiple comparisons was used to calculate the significance, including P values. All statistical tests were performed using GraphPad Prism Software v8.

Results

Characterization of iPSCs

We cultured the iPSCs on a vitronectin-coated culture dish without feeder cells. It was confirmed that colonies of iPSCs could be maintained stably *in vitro* for a long period of time (Fig. 1A). The specific markers of iPSCs, Oct4 (Fig. 1B), Nanog (Fig. 1C), and Sox2 (Fig. 1D), which are expressed in the nucleus, could be identified by immunostaining of the undifferentiated cells. In addition, we confirmed that SSEA4, one of the representative cell surface markers of iPSCs, was expressed (Fig. 1E). We first performed a teratoma analysis to observe the pluripotency of the iPSCs. The iPSCs that were transplanted into the mice grew into a solid mass of tissue for 40 d, and the surface of the tumor showed various types of tissue clusters (Fig. 1F). Sections of the solid masses were observed to show histological variation. As a result, representative tissues of the endoderm, ectoderm, and mesoderm were identified. Based on the identified characteristics of the iPSCs, we confirmed that the iPSCs were pluripotent and potentially capable of differentiating into IPCs.

Efficient induction of differentiation of iPSCs into a definitive endoderm using combinations of small molecules

We investigated the small molecules capable of efficiently inducing differentiation of iPSCs into a definitive endoderm (Fig. 2A). Differentiation efficiencies of treatment combinations of CHIR99021 and LY294002 small molecules were compared based on high concentration of activin A (Fig. 2B) only on day 1 of the total induction period of 3 d. On immunostaining, the group treated with CHIR99021 and LY294002 together showed higher expression of FoxA2, a typical marker of the endoderm, than the groups treated with CHIR99021 and LY294002 separately (Fig. 2C). More CXCR4-expressing cells were observed in the group treated with both compounds than in the groups treated with the compounds separately (Fig. 2D). Based on the quantitative analysis using qPCR, FoxA2 expression was found to be slightly lower in the group treated with both compounds than in the CHIR99021-treated group. However, the expression of Sox17 and CXCR4 was higher in the group treated with both compounds (Fig. 2E). Comprehensively, we concluded that treatment with CHIR99021 and LY294002 simultaneously was more effective in inducing the differentiation of iPSCs into a definitive endoderm than treatment of these compounds separately.

The induction of differentiation of iPSCs into IPCs using combinations of small molecules

Although iPSCs have differentiated into a definitive endoderm, their internal ability to differentiate into mesodermal and ectodermal cells remains. Therefore, we used small molecules that are capable of inhibiting the signaling of cell differentiation pathways, excluding the pancreatic progenitor cell pathway (Fig. 2A). In particular, we compared the effects of small molecules, which can inhibit the differentiation of cells into hepatic progenitor cells, based on the differentiation-inducing media containing small molecules such as Dor and SB431542 that inhibit differentiation of cells into ectodermal and mesodermal cells, respectively. In addition, we compared groups that were treated with growth factor FGF2, ERK signaling inhibitor FR180204, and hedgehog signaling inhibitor SANT-1 to cause differentiation of cells into mature pancreatic progenitor cells. It was confirmed that the morphologies of 6-day cultured cells were different under different differentiation-inducing conditions. In general, all

conditions induced vigorous cell growth, resulting in high cell density. However, no significant difference in the morphology and growth frequency of cells of different groups could be observed under the microscope (Fig. 3A). Therefore, the expression of characteristic transcription factors of pancreatic progenitor cells, including Pdx1, Ngn3, Nkx6.1, Sox9, and NeuroD, was analyzed under all conditions. The overall expression of transcription factors was higher than that of basic differentiation factors in group 1. However, the expression of each transcription factor in SANT- treated groups 4 and 6 was significantly increased compared to that of each transcription factor in the other groups (Fig. 3B). These results demonstrated that inhibiting hedgehog signaling of a particular differentiation pathway in the differentiation of pancreatic progenitor cells was more effective for the differentiation of cells into pancreatic progenitor cells.

After differentiation of cells into pancreatic progenitor cells, induced by different conditions in step 2, the differentiation into IPCs was induced in a similar manner using the culture medium that contained Dexa, Nico, and fors (Fig. 4A). After 8 d of treatment with differentiation factors, gene expression of representative hormones of mature pancreatic islet cells, such as insulin and glucagon, and major transcription factors, such as Pdx1 and Nkx6.1, was confirmed by qPCR. The expression of each marker in all groups showed a relatively immaterial increased mean value compared to that in group 1, but no statistical significance was observed. However, SANT-1-treated groups 4 and 6 showed a marked increase in the gene expression compared to group 1, and due to this, statistical significance of all groups, including group 1, was confirmed (Fig. 4B). Immunostaining and flow cytometry analysis were performed for group 6, which showed a relatively successful differentiation compared to other groups. Insulin and glucagon-expressing cells were found scattered between the cells. In addition, it was confirmed that differentiation occurred in groups of small numbers than in large clusters (Fig. 4C). The Pdx1-expressing cells were identified in most of the groups, but were rarely observed along with insulin-expressing cells (Fig. 4D). Based on flow cytometry analysis, about 38% of all differentiated cells expressed insulin and 23% expressed glucagon. Expression of Ngn3, the most important transcription factor of pancreatic islet cells, was observed in about 66% of all differentiated cells (Fig. 4E). These results indicated that even in the presence of a large number of Pdx1- and Ngn3-expressing islet progenitor cells, relatively few cells could directly differentiate into islet cells, suggesting that differentiation conditions could be improved to obtain higher differentiation efficiency.

Comparison of functional insulin secretion ability of differentiated IPCs

We analyzed the quantitative differentiation of IPCs based on the different combinations of differentiation-inducing factors and determined whether there was a change in the glucose-regulated quality improvement of functional IPCs. First, the concentration of the spontaneously secreted insulin in the culture medium was analyzed on day 5 of differentiation of cells into mature IPCs. It was confirmed that all groups secreted insulin, but significance among the groups could not be observed (Fig. 5A). On day 8, significantly higher insulin concentrations, approximately 15 times higher than those on day 5, were observed in all groups. In particular, SANT-1-treated groups 4 and 6 showed higher insulin concentrations than the other groups, and there was no difference in the increase in concentrations

among the other groups (Fig. 5B). fors is an adenylate cyclase activator that is involved in the vitality and growth of cells during IPC differentiation, but it also causes mature pancreatic cells to release more insulin. Therefore, we cultured the cells for another 4 d after removing fors from the differentiation culture media and then checked the concentration of insulin in the media. The group without SANT-1 showed reduced insulin concentration, more than twice less than that with fors, whereas the group treated with SANT-1 showed only a slight decrease in the insulin concentration (Fig. 3C).

We also determined whether the differentiated IPCs could secrete insulin by glucose stimulation. Group 1 without SANT showed increased concentration of insulin in the medium when KCL was added during differentiation, but no significant secretion based on glucose concentration was observed (Fig. 5D). However, the group with SANT showed an increase in insulin secretion in response to a high concentration of glucose (Fig. 5E, F). Overall, high levels of natural insulin and the ability to control insulin secretion based on glucose concentration were observed only in the group with SANT-1 that inhibited hedgehog signaling mechanisms. These results suggested that inhibiting hedgehog signaling played a major role in the differentiation of iPSCs into mature pancreatic islet cells.

IPC culture to manufacture cells similar to islet cells

The IPCs, differentiated using small molecules, were cultured in a 2D culture that was adhered to the culture dish. Thus, we attempted to cultivate spheroid clusters of IPCs similar to natural islet cells. After 10 d of step 3 of differentiation, the cells were separated, and a high density of cells was cultured. As a result, small aggregations of cells were observed on day 1, and smaller spheroid clusters of constant size were formed on day 5 (Fig. 6A). The spheroid IPCs were well-coordinated and coherent clusters of insulin- and glucagon-expressing cells (Fig. 4B). To confirm the *in vitro* conditions that allowed long-term culture of differentiated cells before *in vivo* transplantation, it was confirmed that the culture conditions could maintain the spheroid morphology and basic insulin secretion ability of the IPCs.

The spheroid IPCs were cultured for a considerable period under any culture condition. It was confirmed that the insulin secretion ability of IPCs gradually decreased when the cells were cultured with differentiation-induction medium without FBS.

When fors was removed from the differentiation-induction medium, the basal c-peptide secretion was low but decreased significantly on day 11. Similarly, when the CMRL 1066 medium was used for islet cell culture, the c-peptide secretion was lower than that when fors was added, but remained stable for 7 d (Fig. 4C). We suggest that culturing spheroid IPCs in CMRL 1066 medium for 7 d represents the best culture condition because it is difficult to stably perform *in vitro* culture of normal islet cells for a long time.

Discussion

In this study, we demonstrated the differentiation of iPSCs into IPCs by a simple method using small molecules. As most small molecules are target inhibitors of biological pathways, we used small

molecules to inhibit the differentiation pathways except that of IPCs. In other words, a combination of small molecules was developed to ensure the natural differentiation of iPSCs in only one direction towards IPCs.

To induce the differentiation of iPSCs into a definitive endoderm, the GSK3 β -specific inhibitor CHIR99021 [20] and the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 were used in combination with activin A. As a result, the expression of FoxA2 was slightly higher in the group treated with the combination of CHIR99021 and activin A. However, based on the results of the expression of other specific markers, we found that addition of LY294002 would have a better effect. Differentiation of cells into pancreatic progenitor cells was induced by the treatment of the definite endoderm with FGF family members, RA, and Dor, as reported in previous studies. Therefore, we investigated whether IPC differentiation was better after treating with FR180204, an ATP-competitive inhibitor of ERK1 and ERK2 [20, 27], and SANT-1, a Sonic hedgehog pathway antagonist [23, 24], to inhibit differentiation into hepatocyte precursor cells instead [23-26]. In conclusion, the present study demonstrated that treatment with SANT-1 and FR180204 resulted in increased induction of differentiation into IPCs, increased insulin levels in the culture medium, and in particular, production of functional IPCs that were responsive to glucose.

In this study, IPCs were made more similar to natural islet cells by culturing them to obtain spheroid morphology because 2D IPCs were less capable of secreting insulin in response to glucose stimulation. Although we used combinations of small molecules to induce differentiation into 2D functional IPCs that respond to glucose, spheroid IPCs were developed by further maturing and growing the cells because it is easy to transplant them and they survive well even after transplantation.

We suggest that inducing the differentiation of iPSCs into IPCs using this method was suitable for use during human transplantation. The iPSCs were maintained and differentiated on xeno-free matrix vitronectin that supported the growth and differentiation of human iPSCs under serum-free feeder-free conditions. In addition, small molecules, which are more stable and safer than protein growth factors and are economical for mass production, were used to induce differentiation. Therefore, we will develop a method to ensure that the matured IPCs are functionally more similar to natural pancreatic islet cells and are suitable for use in clinical trials in further studies.

Declarations

Ethical approval and consent to participate

Human iPSCs were obtained from Han Choe (the Center for Stem Cell Research of Asan Institute) and ethics approval received from the Institutional Review Board (IRB) of Asan Medical Centre (AMC), Seoul, South Korea. The study was performed according to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the medical faculty of Asan Medical Center, Seoul, Korea.

Consent for publication

Not applicable

Availability of data and materials

The majority of the data generated or analyzed during this study are included in this article. Unpublished data are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SL, JHJ, JYO, EHS, and SCK conceptualized and designed the study, performed data analysis and interpretation, and drafted and approved the article. EJ and IKS conducted the study and approved the article.

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Abbreviations

iPSCs, human-induced pluripotent stem cells; IPCs, insulin-producing cells; ESCs, human embryonic stem cells; DM, diabetes mellitus; SCID, severe combined immunodeficiency; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; GSIS, glucose-stimulated insulin secretion; Fors, forskolin; Dexam, dexamethasone; RA, retinoic acid; Dor, dorsomorphin; Nico, nicotinamide; PFA, paraformaldehyde

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Table

Due to technological limitations, Table 1 is only available as a download in the supplementary files section.

Figures

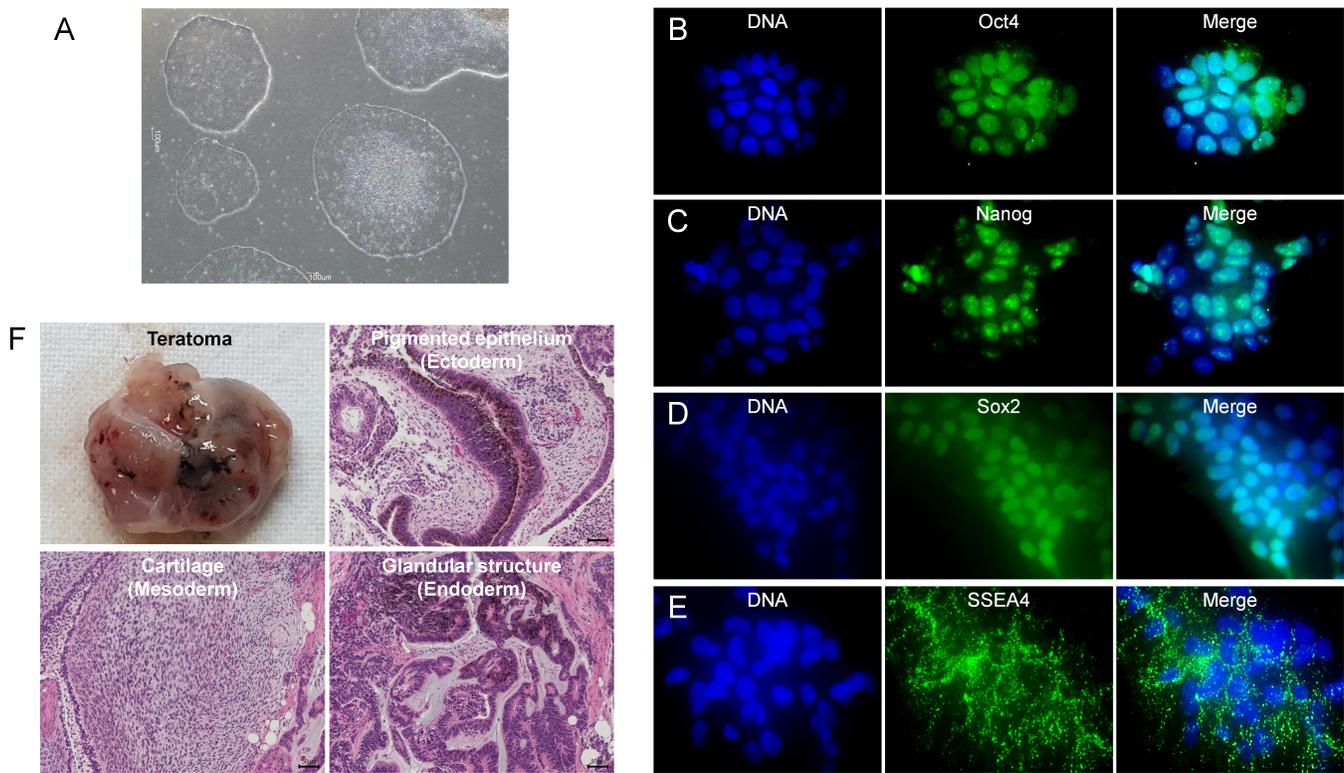
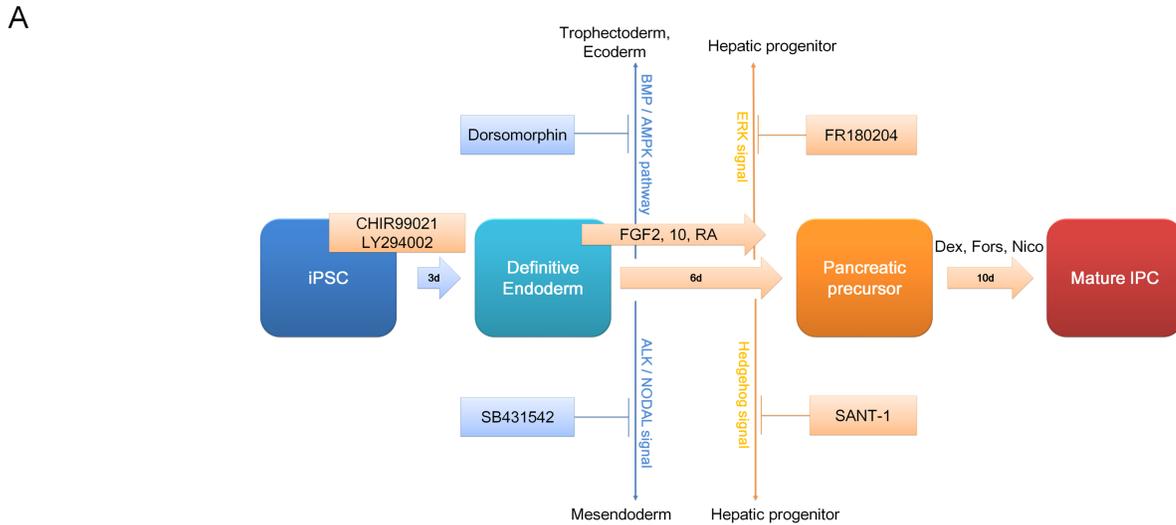


Figure 1

Characterization of human-induced pluripotent stem cells (iPSCs). (A) Undifferentiated iPSC colonies have a unique appearance. Fully reprogrammed iPSC colonies are round, compact cell aggregates with sharp, distinct borders. (B) The expression of Oct4 is detected in undifferentiated iPSCs by immunostaining. (C) Nanog-expressing cells are detected in undifferentiated iPSCs. (D) Sox2-expressing cells are observed in undifferentiated iPSCs by immunostaining. (E) The expression of SSEA4 is detected in undifferentiated iPSCs by immunostaining. (F) Teratomas are derived from immunodeficient mice injected with iPSCs. iPSCs exhibits cells from tissues representing all three embryonic germ layers, including pigmented epithelium (ectoderm), cartilage (mesoderm), and glandular structure (endoderm).



B

	1	2	3
Activin A	+	+	+
LY294002			+
CHIR99021		+	+

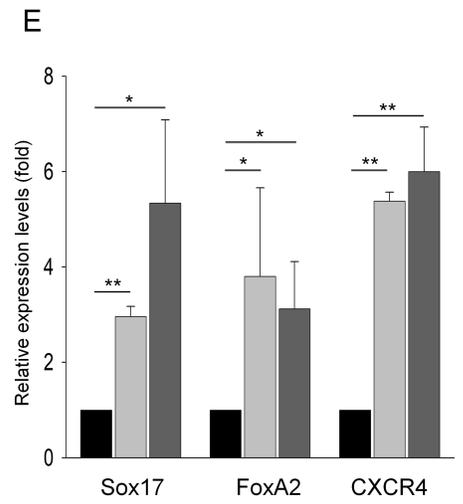
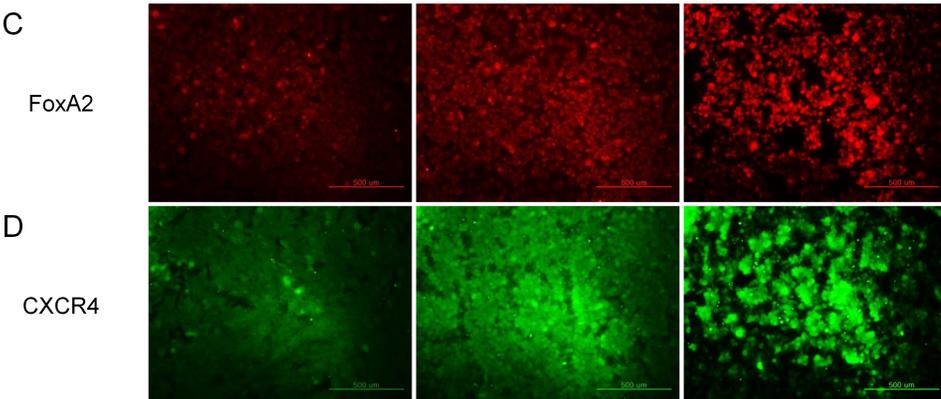


Figure 2

Comparison of small molecule combinations used to induce differentiation of cells into a definitive endoderm. (A) Scheme depicting the mechanism of action of small molecules involved in IPC generation. (B) Groups treated with activin A, LY294002, or CHIR99021 to induce differentiation of cells into a definitive endoderm. (C) The FoxA2-expressing cells are identified in groups treated with the combination of activin A, LY294002, and CHIR99021 by immunostaining and observed most frequently. (D) CXCR4 is highly expressed in groups treated with the combination of activin A and CHIR99021 and activin A, LY294002, and CHIR99021. (E) Based on qPCR analysis, the mRNA expression of Sox17 and CXCR4 is highest in groups treated with activin A, LY294002, and CHIR99021, while the mRNA expression of FoxA2 is relatively lower in this group than that in groups treated with activin A and CHIR99021. Each bar represents the mean \pm standard deviation. *P < 0.05, **P < 0.005.

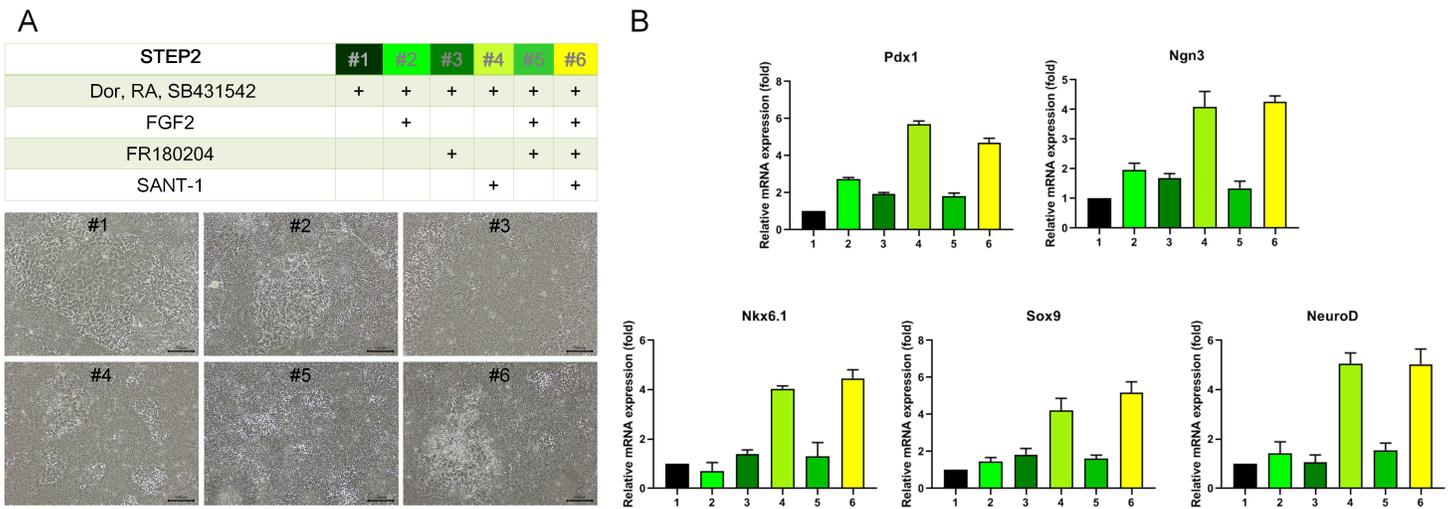


Figure 3

Combinations of small molecules used for differentiating cells into pancreatic progenitor cells. (A) Observation of cell morphology based on the combinations of small molecules used in the three stages of differentiation to induce differentiation into islet precursor cells. Cells of homogeneous morphology are observed in groups 4 and 6, where SANT-1 is added to the basic differentiation culture medium during step 3 of differentiation. (B) The expression of major transcription factors in pancreatic progenitor cells under all conditions is compared using qPCR. The expression of major transcription factors (Pdx1, Ngn3, Nkx6.1, Sox9, and NeuroD) in other groups is slightly higher than that in group 1, which is treated with only the basic differentiation factor. The group with SANT shows a higher expression pattern.

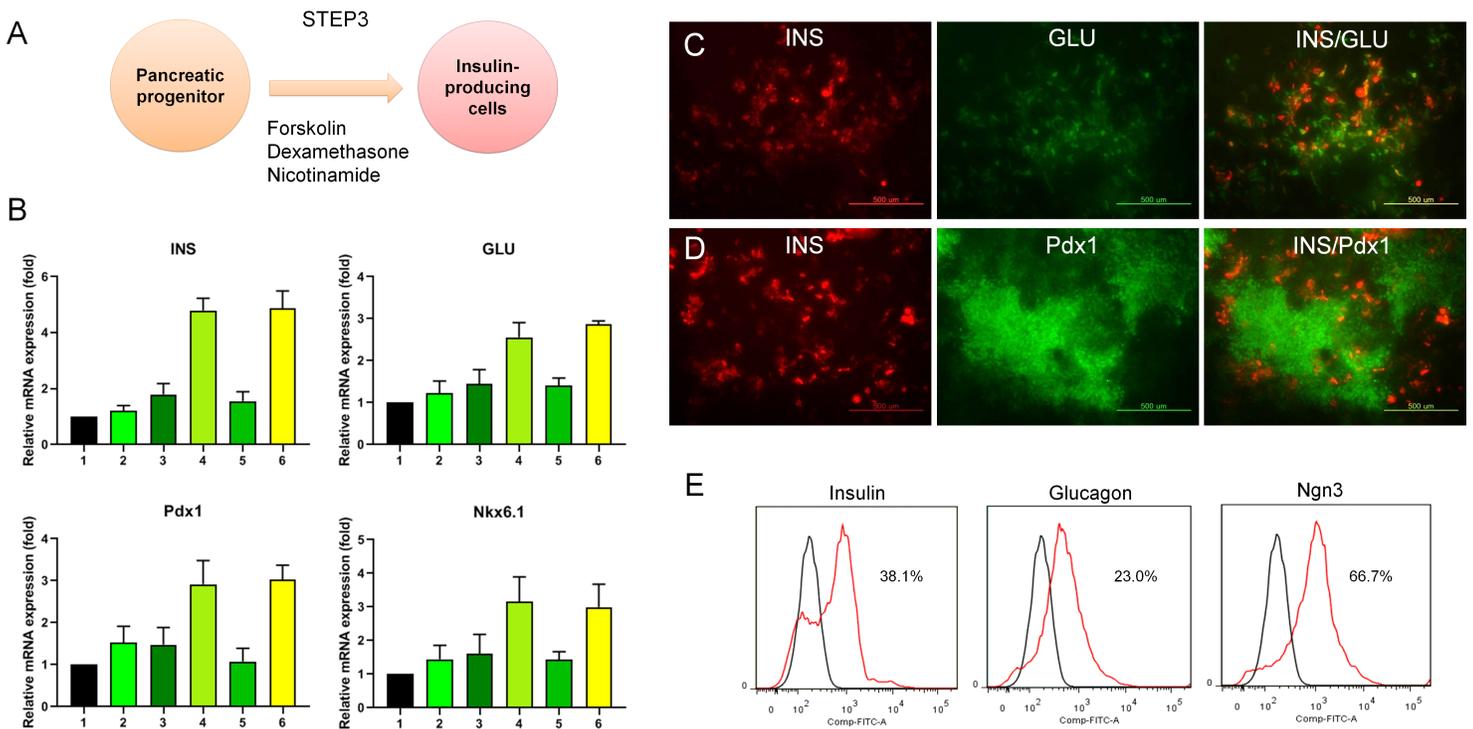


Figure 4

Differentiation of pancreatic progenitor cells into IPCs. (A) The final stage of differentiation includes treatment with forskolin, dexamethasone, and nicotinamide to induce differentiation into IPCs. (B) Insulin and glucagon levels in differentiation-induced cells are observed using qPCR after 10 d, and the SANT-1-treated group shows higher expression than other groups. The expression of Pdx1 and Nkx6.1 follows a similar pattern. (C) Insulin- or glucagon-expressing cells are identified by immunostaining on day 10 of step 3 of differentiation. (D) The expression of insulin or Pdx1 is detected on day 10 of step 3 of differentiation. (E) The distribution of insulin-, glucagon-, and Ngn3-expressing cells in the differentiated cells is analyzed by FACS.

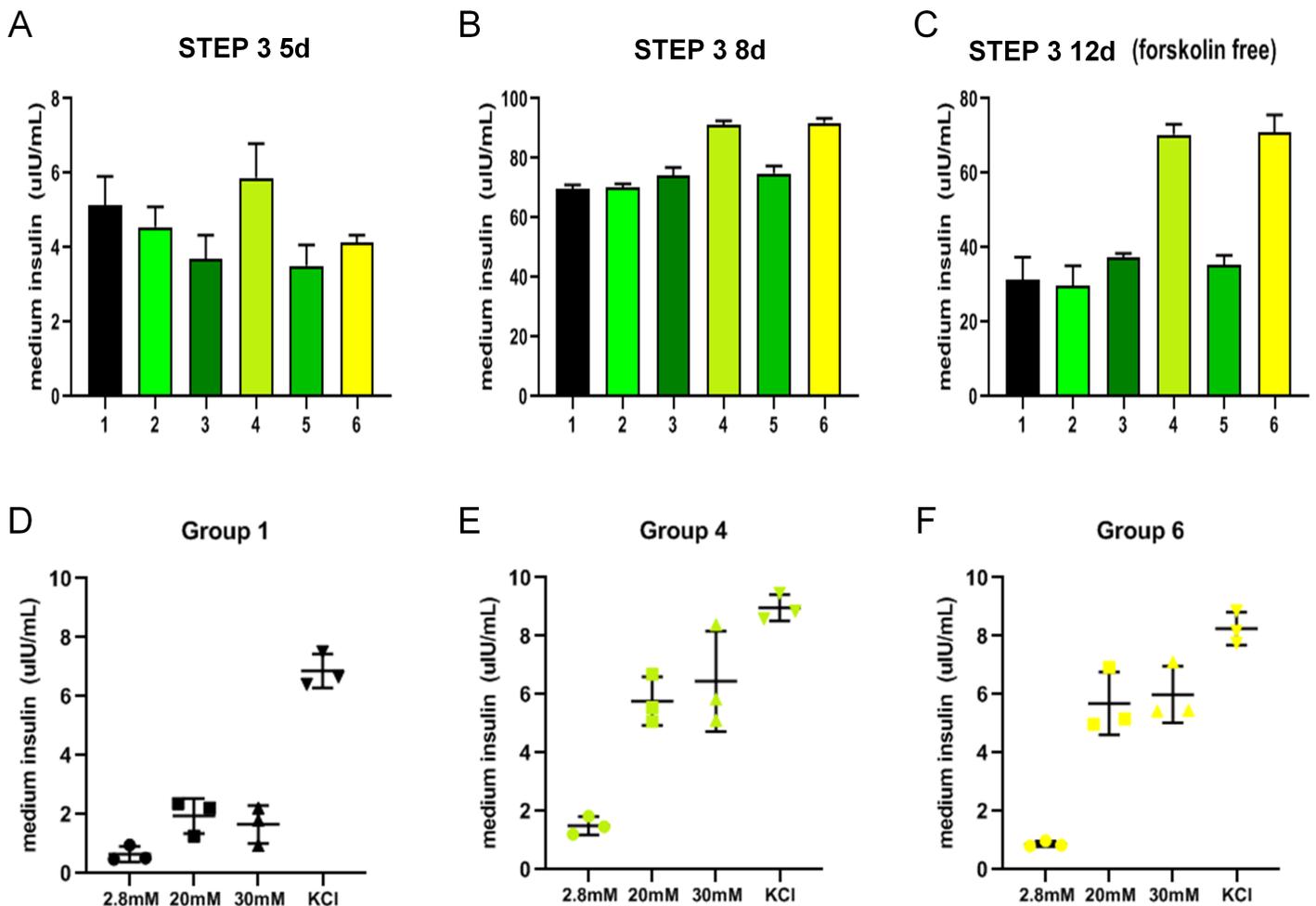


Figure 5

Functional capabilities of differentiated IPCs. (A) Insulin is detected on day 5 of step 3 of differentiation in the culture media of all groups. SANT-1-treated group 4 shows higher insulin levels than the other groups. (B) On day 8 of step 3, the secretion of insulin significantly increases in all groups, especially in groups 4 and 6, where the amount of insulin exceeds the detected measurement range. (C) In general, the concentration of insulin in the culture medium without forskolin is low, but groups 4 and 6 show high insulin secretion. (D) Based on the glucose-stimulated insulin secretion, the expression of insulin is not significantly affected by the glucose concentration in the group treated with only the basic differentiation factor in step 2 of differentiation. (E) In the SANT-treated group 4, insulin secretion increases in response

to 20 mM and 30 mM glucose. (F) In group 6, as in group 4, differentiated cells respond to high concentrations of glucose and show increased insulin secretion.

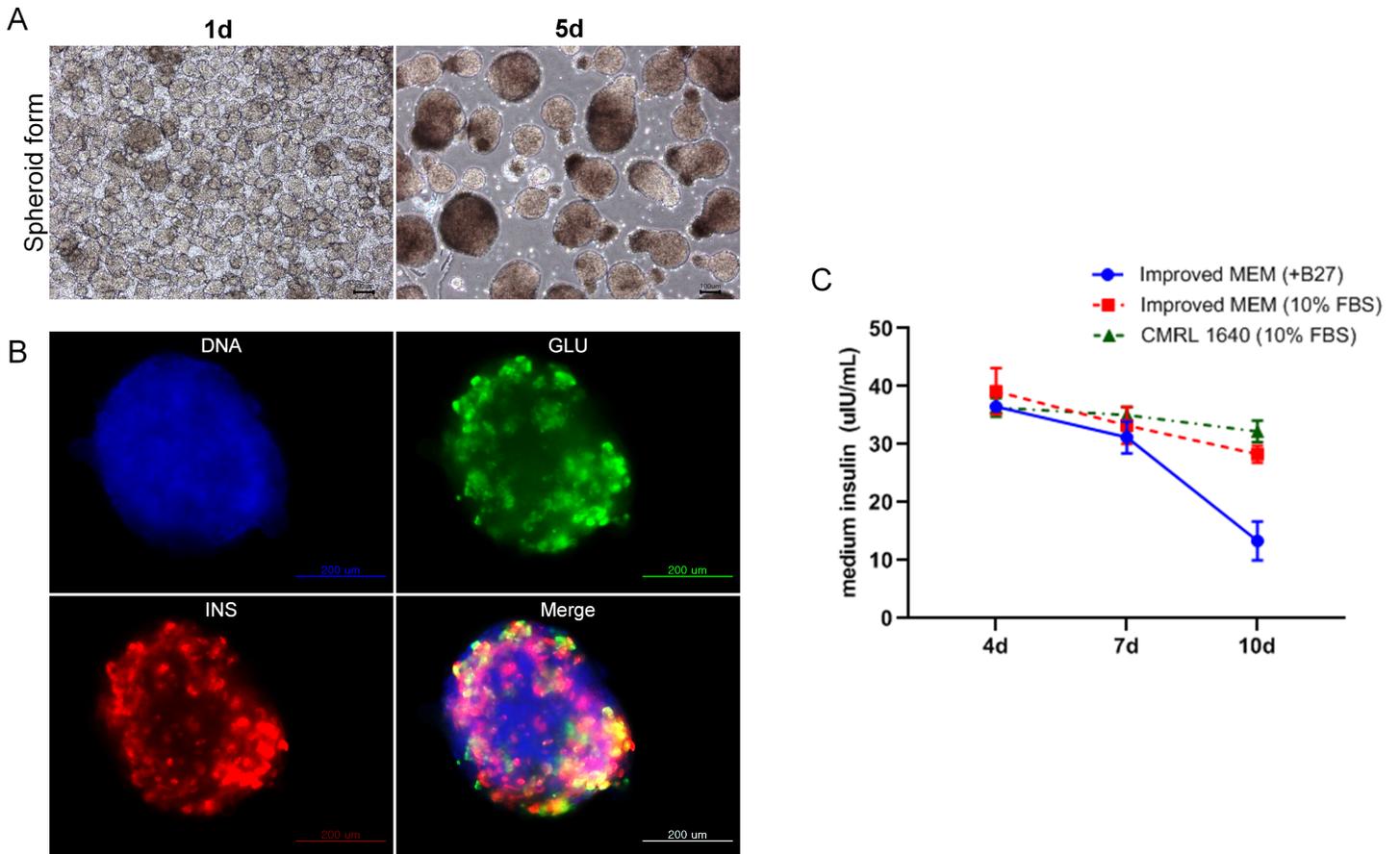


Figure 6

Spheroid formation and sustained maintenance of IPCs. (A) Many small clusters of IPCs are observed after day 1 of IPC dissociation, and a large number of spheroid IPCs are generated after 5 d. (B) Many insulin- or glucagon-expressing cells are detected in the generated spheroid IPCs. (C) Differentiated IPCs show decreased levels of insulin in the culture medium with improved MEM (1x B27) that was used for differentiation after continuous culture for 10 d. However, in the culture medium containing FBS 10%, cells stably secrete insulin.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.tif](#)