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Endodermal differentiation of human pluripotent stem cells to insulin-producing cells in 3D culture

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Insulin-producing cells (IPCs) derived from human pluripotent stem cells (hPSCs) may be useful in cell therapy and drug discovery for diabetes. Here, we examined various growth factors and small molecules including those previously reported to develop a robust differentiation method for induction of mature IPCs from hPSCs. We established a protocol that induced PDX1-positive pancreatic progenitor cells at high efficiency, and further induced mature IPCs by treatment with forskolin, dexamethasone, Alk5 inhibitor II and nicotinamide in 3D culture. The cells that differentiated into INSULIN-positive and C-PEPTIDE-positive cells secreted insulin in response to glucose stimulation, indicating a functional IPC phenotype. We also found that this method was applicable to different types of hPSCs.

At present, islet transplantation is considered to be one of the most effective therapies for the treatment of patients with severe diabetes^{1–3}. However, the shortage of donor human pancreases limits such clinical therapy. Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs)^{4,5}, have replication competence and the ability to differentiate into many cell types. Furthermore, they are reported to be a potential source of β cells for cell therapy^{6,7}. hPSCs may also be used as tools in drug discovery research, such as development of new drugs using disease model cells derived from disease-specific iPSCs^{8–11}. For the realization of such therapies it is crucial to efficiently generate insulin-producing cells (IPCs) from PSCs.

Several protocols have been reported to induce IPCs from hPSCs^{11–22}, which mimic the differentiation process during pancreatic development. These methods are effective in IPCs induction. Pancreatic development is regulated by transcriptional factors, including *PDX1*, *NGN3*, *NEUROD1*, *PAX4*, *NKX2.2* and *NKX6.1*, and controlled by various morphogens^{23,24}. The pancreas is derived from definitive endoderm (DE) that develops from pluripotent cells in the early embryo. DE differentiates into gut endoderm containing pancreatic progenitors expressing PDX1 (PDX1⁺), a transcription factor that plays important roles in organogenesis of the pancreas including the endocrine and exocrine tissues. PDX1⁺ pancreatic progenitors give rise to INSULIN positive (INS⁺) cells, and other transcription factors such as *NGN3*, *NEUROD1*, *PAX4*, *NKX2.2* and *NKX6.1* are expressed during this process. Subsequently, INS⁺ cells undergo “maturation” to fully functional islet β cells that secrete insulin in response to glucose. These mature IPCs are thought to be characterized by their expression of maturation marker genes such as *MAFA*.

Although previous reports have succeeded to differentiate mature IPCs from DE cells, the efficiency of differentiation into PDX1⁺ cells is generally low and not consistent among hPSC lines. These problems have hampered the generation of sufficient numbers of mature IPCs for the treatment of patients with severe diabetes. It has been suggested that the differentiation conditions have not been optimized for induction of PDX1⁺ and INS⁺ cells. Because the generation of PDX1⁺ and INS⁺ cells is one of the most critical steps for mature IPC differentiation, we considered it to be necessary to establish an efficient differentiation method to induce PDX1⁺ and INS⁺ cells for production of mature IPCs. In addition to the difficulty to produce INS⁺ cells, previously generated INS⁺ cells have been reported to exhibit inferior functions compared with those of mature IPCs²². Therefore, it is also indispensable to devise a method to induce mature IPCs.

In this study, we focused on development of a differentiation method to induce PDX1⁺ and INS⁺ cells by examining combinations and concentrations of various growth factors and small molecules that have been



reported to promote differentiation of hPSCs into INS^+ cells. We applied a three step protocol to induce mature IPCs from hPSCs. DE cells were induced from hPSCs in Stage I, $PDX1^+$ cells were induced from DE cells in Stage II, and INS^+ cells and mature IPCs were induced from $PDX1^+$ cells in Stage III. We found that optimal induction of each differentiation step enables efficient differentiation of hPSCs into mature IPCs. $FOXA2^+/SOX17^+$ cells were approximately 91% of total cells, and $PDX1^+$ cells were approximately 76%. INS^+ cells were approximately 10% of total cells using a combination of small molecules, but these INS^+ cells did not secrete insulin in response to glucose stimulation. Using 3D culture, INS^+ cells derived from different hPSC lines were approximately 30% of the total cells and secreted insulin in response to glucose stimulation and our method can be applied to various hPSC lines.

Results

High efficiency differentiation of hESCs into DE. First, we examined the efficiency of DE cell induction from hPSCs. Activin A has been used to induce DE cells in most reports. More recently, it has been shown that the combination of activin A with Wnt3a results in more efficient induction of DE cells^{11,14,16,18,21,25,26}. In addition to these factors, we chose six growth factors and small molecules from other relevant reports, and examined the differentiation efficiency of DE cells by changing the combinations and concentrations of these factors (Table S1). Screening for maximal induction conditions was performed by analyzing the expression of DE marker genes, *CXCR4*, *FOXA2* and *SOX17*, and primitive gut tube marker genes, *HNF1B* and *HNF4A*, by quantitative PCR. The percentage of *FOXA2* and *SOX17* double-positive ($FOXA2^+/SOX17^+$) cells was evaluated by flow cytometry. KhES-3 cells, a hESC line, were treated with activin A for 4 days and then examined for their expression of *FOXA2*, *SOX17*, *CXCR4*, *HNF1B* and *HNF4A*. The expression of these genes increased by treatment with activin A (Figs. 1A and S1A). Differentiation efficiency was evaluated by flow cytometric analysis of $FOXA2^+/SOX17^+$ cells, revealing that the percentage of $FOXA2^+/SOX17^+$ cells was $37.3 \pm 8.5\%$ (Fig. 1B). We treated the cells with activin A for 4 days because any longer caused cell death. Next, we evaluated combinations of activin A and other factors, which resulted in higher levels of marker gene expression than those achieved by activin A treatment alone. Treatment with both activin A and Wnt3a resulted in higher expression levels of marker genes compared with that by activin A treatment alone, and the percentage of $FOXA2^+/SOX17^+$ cells increased to $59.7 \pm 8.6\%$ (Fig. S1B). We next elucidated the pathway of Wnt3a in differentiation of DE cells. CHIR99021, a glycogen synthase kinase 3β (GSK3- β) inhibitor, is a specific activator of the canonical Wnt pathway. Therefore, we examined the effect of CHIR99021 instead of Wnt3a. After treatment with activin A and CHIR99021, the percentage of $FOXA2^+/SOX17^+$ cells was $60.5 \pm 2.0\%$, which was similar to that achieved by treatment with activin A and Wnt3a (Figs. 1B and S1B). The results showed that Wnt acted via the canonical pathway rather than the non-canonical Wnt pathway in the induction of DE cells.

For higher efficiency of differentiation into DE cells, we screened combinations of other factors. Treatment with the combination of activin A, CHIR99021 and wortmannin, a phosphoinositide 3-kinase (PI3K) inhibitor, for 2 days and then activin A and CHIR99021 for a further 2 days increased the expression of *FOXA2*, *SOX17*, *HNF1B* and *HNF4A* (Figs. 1A and S1A). The percentage of $FOXA2^+/SOX17^+$ cells was $91.6 \pm 0.3\%$ of total cells (Fig. 1B). Wortmannin treatment for 4 days resulted in extensive cell death. Expression of *FOXA2* and *SOX17* proteins was examined by immunocytochemistry, revealing colocalization of these proteins (Fig. 1C). Treatment with other factors did not affect the expression of *FOXA2*, *SOX17*, *HNF1B* or *HNF4A* as shown by quantitative PCR analysis (data not shown). These results showed that the combination of activin A,

CHIR99021 and wortmannin synergistically induced DE cells from hPSCs.

Differentiation of DE cells into pancreatic progenitor cells. Because we established a highly efficient differentiation method that induced up to 90% of the total cell population into DE cells, we next tried to improve the differentiation efficiency of $PDX1^+$ (pancreatic progenitor) cells from DE cells. It has been reported that treatment with Noggin, an inhibitor of bone morphogenetic protein (BMP) signalling, retinoic acid and fibroblast growth factor (FGF)7 or FGF10 induces $PDX1^+$ cells from DE cells^{16,22,25,27,28}. To evaluate these factors in differentiation of $PDX1^+$ cells (Table S1), the expression of pancreatic progenitor markers *PDX1* and *HNF6* were analysed by quantitative PCR and the percentage of $PDX1^+$ cells was examined by an immunochemical assay using an anti-*PDX1* antibody. Treatment with Noggin or dorsomorphin, an inhibitor of BMP type I receptors ALK2, 3 and 6, increased the expression of *HNF6* and *PDX1* to similar levels (Fig. 2A), and the percentage of $PDX1^+$ cells was also comparable ($33.7 \pm 10.3\%$ and $33.7 \pm 11.1\%$, respectively) (Fig. 2B). These results showed that both BMP signalling inhibitors acted with similar efficiencies, and the combination of these factors increased $PDX1^+$ cells to $39.2 \pm 6.2\%$ (Fig. 2B). Next, we used retinoic acid for differentiation of $PDX1^+$ cells, and found that the percentage of $PDX1^+$ cells was $18.4 \pm 6.5\%$ (Fig. 2B). Because it was reported that activation of the ERK pathway antagonizes the effects of retinoic acid²⁹, we examined the combination of FR180204, an inhibitor of ERK1/2, and retinoic acid. As a result, the percentage of $PDX1^+$ cells was increased to $58.2 \pm 6.2\%$ (Fig. 2B). Next, we evaluated the combination of Noggin, dorsomorphin, retinoic acid and FR180204, which increased the expression of *HNF6* and *PDX1* (Fig. 2A), and $PDX1^+$ cells were $67.3 \pm 4.8\%$ of total cells in the immunochemical assay (Fig. 2B). Moreover, we found that the combination of FGF10, retinoic acid, Noggin, dorsomorphin and FR180204 increased the expression of *HNF6*, *PDX1* and endocrine progenitor markers, including *NKX6.1*, *NKX2.2* and *NGN3* (Figs. 2A and S2A). In addition, the percentage of $PDX1^+$ cells was increased to $76.5 \pm 6.7\%$ (Fig. 2B). FGF10 treatment was performed for 3 days because the expression of *PDX1* and the percentage of $PDX1^+$ cells were not different at 3 and 6 days (Fig. S2B). The expression of *PDX1* was not significantly increased by treatment with other factors (data not shown). These results suggested that the combination of these five factors acted synergistically to efficiently induce $PDX1^+$ cells from DE cells.

Next, we applied our differentiation method developed using KhES-3 cells to other hPSC lines, including H9, iPS (IMR90)-4 and iPS 253G1. All four cell lines showed differentiation into $PDX1^+$ cells at an efficiency of 70–80%, suggesting our method is applicable to a wide variety of PSC lines (Fig. 2C).

Induction of pancreatic progenitor cells into INS^+ cells. $PDX1^+$ cells could be differentiated at a high efficiency of more than 70%. Next, we developed a differentiation method to induce INS^+ cells. It has been reported that the factors used to induce $PDX1^+$ cells have no effect on the expression of *INS*²². Therefore, we hypothesized that other signals participated in the differentiation of $PDX1^+$ cells into INS^+ cells^{19,30}. To generate INS^+ cells, we screened growth factors and small molecules that have been reported to regulate differentiation^{11,14–16,18–22} (Table S1). Although single treatments with dexamethasone, glucocorticoid, nicotinamide, or Alk5 inhibitor II did not increase the expression of *INS* or *GLUCAGON* (*GCG*), treatment with forskolin alone or forskolin, Alk5 inhibitor II and nicotinamide increased the expression of those genes (Fig. 3A). Moreover, the combination of forskolin, dexamethasone, Alk5 inhibitor II and nicotinamide obviously increased the expression of *INS*, *GCG* and pancreatic endocrine marker genes, including *NGN3*, *NKX2.2*, *PAX4* and *NEUROD1*, but had no effect on the expression of *NKX6.1* and the maturation marker gene, *MAFA*. (Figs. 3A and S3A).

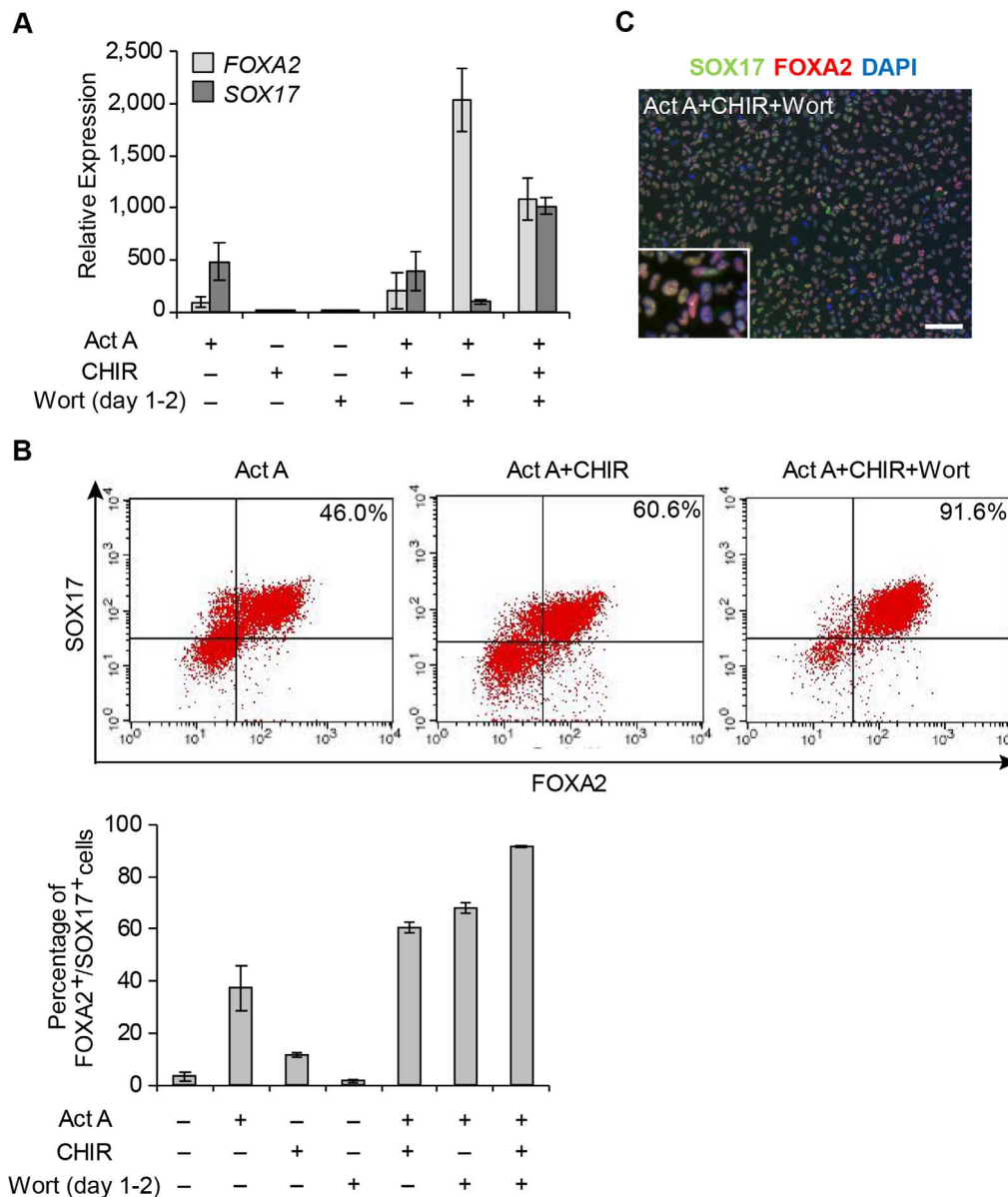


Figure 1 | Differentiation of hPSCs into DE cells. (A) Quantitative RT-PCR analysis of definitive endoderm marker genes *FOXA2* and *SOX17* at day 4. hESCs were treated with each factor for 4 days as shown in Fig. 4A. Expression levels were normalized to *TBP* expression. mRNA expression was relative to that in untreated cells at day 4. Error bars indicate SD (n = 3). (B) Upper: flow cytometric analysis of cells treated with or without three factors. Percentage in the upper right quadrant indicates the percentage of FOXA2⁺/SOX17⁺ cells. Lower: percentage of FOXA2⁺/SOX17⁺ cells among differentiated cells treated with each factor. Error bars indicate SD (n = 3). Control, no factors added; Act A, 100 ng/ml activin A; CHIR, 3 μM CHIR99021; wort, 100 nM wortmannin. Scale bar, 100 μm. (C) Expression of FOXA2 and SOX17 proteins in cells treated with three factors.

This result suggested that dexamethasone was necessary for differentiation of INS⁺ cells. Expression of *INS* was not significantly increased by treatment with other factors (data not shown). The combination of these four factors induced expression of *INS*, *C-PEPTIDE*, *GCG* and *SOMATOSTATIN* (*SST*) proteins. In addition, other endocrine cell markers, *INS* and *C-PEPTIDE* proteins, were colocalized in the immunochemical assay (Figs. 3B and C). The percentage of INS⁺ (*C-peptide*⁺) cells was $9.2 \pm 4.1\%$ in differentiated cell populations (Fig. 3C). Some INS⁺ cells expressed *GCG* protein, but not *SST* protein (Fig. 3C). These results suggested that the combination of Forskolin, dexamethasone, Alk5 inhibitor II and nicotinamide induced INS⁺ cells from PDX1⁺ cells.

Ability to secrete insulin by pharmacological stimulation. To examine whether the INS⁺ cells induced using these four factors

secreted insulin in response to glucose stimulation, we measured the C-peptide level in the culture medium after glucose stimulation. There was no significant difference in C-peptide levels by treatment with D-glucose at low (2.5 mM) or high (25 mM) concentrations (395.2 ± 95.0 pM C-PEPTIDE/μg DNA and 424.5 ± 54.3 pM C-PEPTIDE/μg DNA, respectively) (Fig. 3D). On the other hand, treatment with potassium chloride (KCl), a depolarizing agent, and 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, resulted in a higher C-peptide level than that obtained by D-glucose treatment at low and high concentrations (C-peptide levels in KCl and IBMX were 1498.8 ± 293.0 pM/μg DNA and 1690.3 ± 397.2 pM/μg DNA, respectively) (Fig. 3D). These results indicated that the INS⁺ cells induced using these four factors could not secrete insulin in response to glucose stimulation, but these cells had the capacity to secrete insulin in

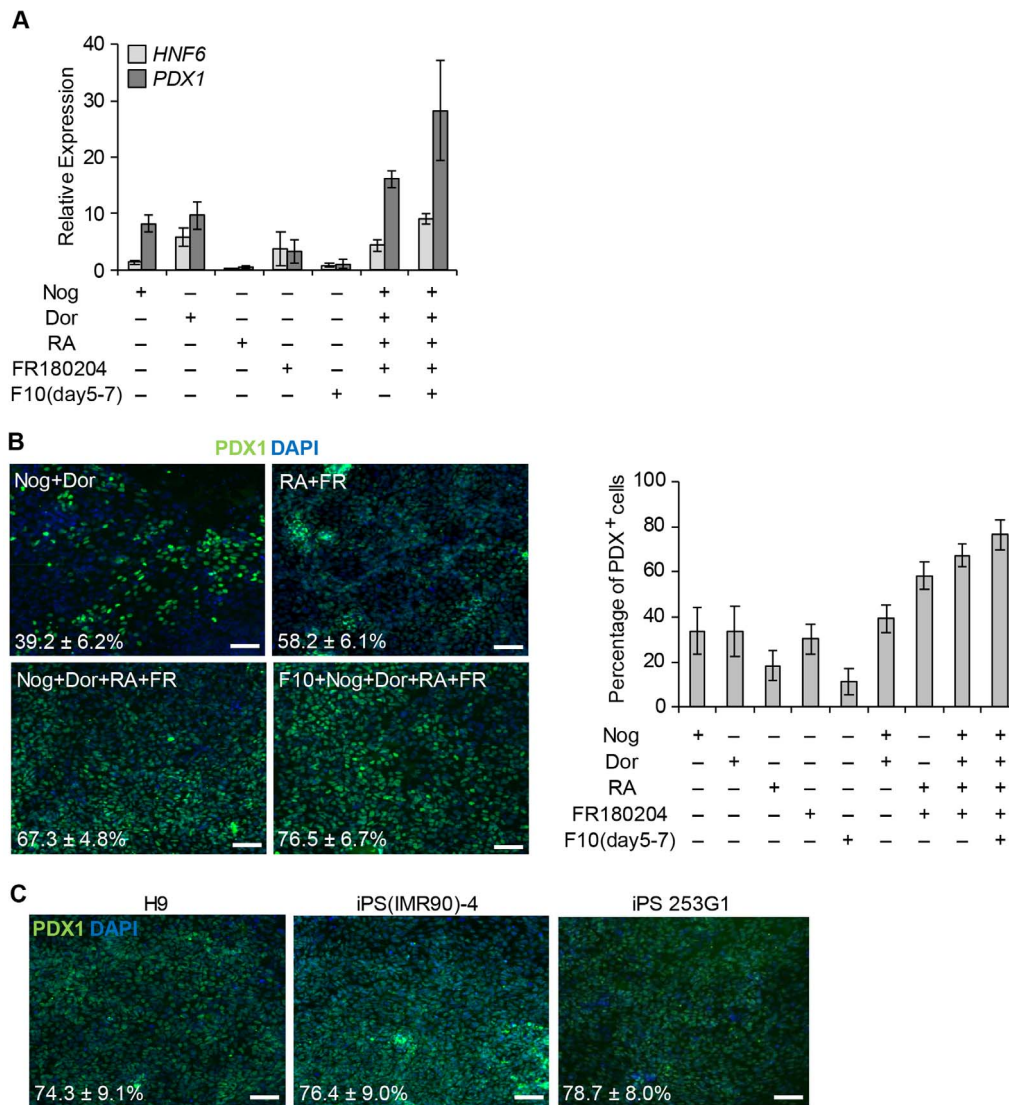


Figure 2 | Differentiation of DE cells into pancreatic progenitor cells. (A) Quantitative PCR analysis of the expression of *HNF6* and *PDX1* at day 10. Expression levels were normalized to *TBP* expression. mRNA expression was relative to that in control cells at day 10. Control cells were treated with three factors for 4 days as shown in Fig. 4A, and then without factors for 6 days. Error bars indicate SD (n = 3). (B) Left: cells were treated with each factor for 6 days and then stained with an anti-PDX1 antibody at day 10. Right: percentage of PDX1⁺ cells among differentiated cells treated with each factor. Mean ± SD (n = 5–10). (C) Each cell line, H9, iPS (IMR90)-4 and iPS 253G1, was treated with five factors for 6 days as shown in Fig. 4A, and stained then with an anti-PDX1 antibody at day 10. Mean ± SD (n = 5–10). F10, 50 ng/ml FGF10; Nog, 50 ng/ml Noggin; Dor, 1 μM dorsomorphin; RA, 2 μM retinoic acid; FR, 3 μM FR180204. Scale bar, 100 μm.

response to IBMX and KCl treatment. Taken together, the data suggested that the combination of these four factors induced premature IPCs in adhesion (2D) culture.

3D culture induces mature IPCs. We developed a differentiation method to induce INS⁺ cells from hPSCs in 2D culture, but these INS⁺ cells did not secrete insulin. In 2D culture, there is the advantage that all cells are exposed to factors added to the culture medium and immunochemical assays can be performed easily. Furthermore, it has been reported that INS⁺ cells induced from PDX1⁺ cells in 2D culture are able to secrete insulin^{14,19}. However, the INS⁺ cells induced using our method were premature IPCs. To acquire mature IPCs that biosynthesize and secrete insulin in response to glucose stimulation, we improved the differentiation method to induce mature IPCs from hPSCs (Fig. 4A). It has been reported that 3D culture is important for the acquisition of mature IPCs^{15,20,31}. Cells in 2D culture were transferred to 3D culture and treated with the combination of forskolin, dexamethasone, Alk5

inhibitor II and nicotinamide. As a result, treatment with the four factors in 3D culture increased the expression of *INS* and *GCG* compared with that in 3D culture without factors and 2D culture with the four factors (Fig. 4B). The cells expressed INSULIN and C-PEPTIDE at day 22 (Fig. 4C), but did not NKX6.1 (data not shown). The percentage of INS⁺ cells was 31.4 ± 2.7% in differentiated cell populations (Fig. 4C). *NKX6.1* expression was remarkably increased at 16 day of differentiation (Fig. S4A), and *MAFA* expression was increased at 22 day (Fig. 4B). In 3D culture, KhES-3-derived INS⁺ cells secreted insulin in response to glucose stimulation, and C-peptide levels in response to D-glucose at low and high concentrations were 845.4 ± 351.1 pM/μg DNA and 1857.3 ± 331.4 pM/μg DNA, respectively (Fig. 4D). We also found that the percentages of INS⁺ cells induced from all four cell lines, including H9, iPS(IMR90)-4 and iPS 253G1, were similar in 3D culture (Fig. S4C). In addition, these INS⁺ cells secreted insulin in response to glucose stimulation. For H9-, iPS(IMR90)-4- and iPS 253G1-derived cells, C-peptide levels in D-glucose at low and high concentrations

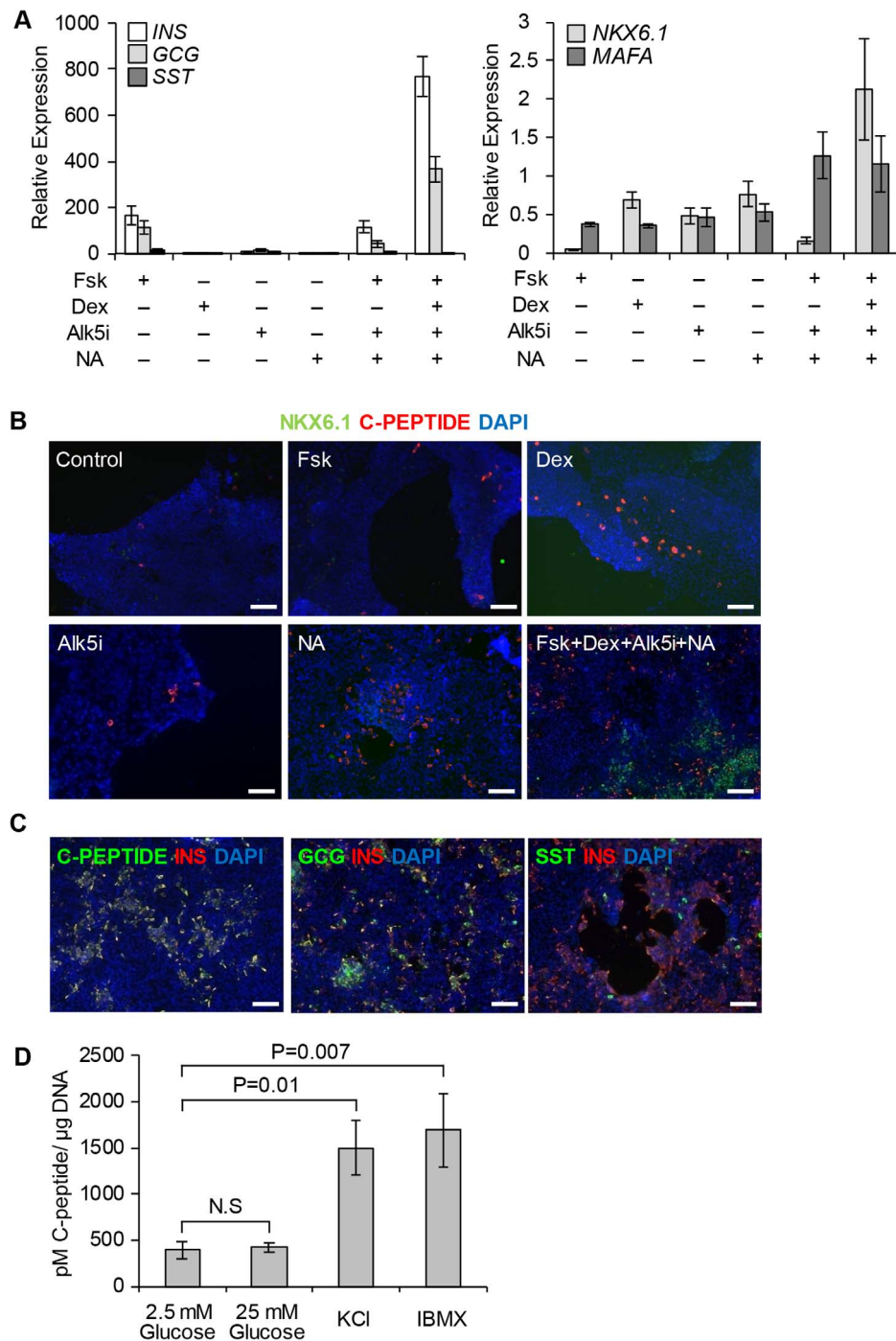


Figure 3 | Differentiation of pancreatic progenitor cells into INS positive cells. (A) Quantitative PCR analysis of the expression of *NKX6.1*, *MAFA*, *INS*, *GCG* and *SST* in cells treated with each factor at day 22. Expression levels were normalized to *TBP* expression. mRNA expression was relative to that in control cells at day 22. Control cells were treated with each factor for 10 days as shown in Fig. 4A, and then without factors for 12 days in 2D culture. Error bars indicate SD (n = 3). (B) Expression of *NKX6.1* and C-PEPTIDE proteins in cells treated with each factor at day 22. (C) Cells were treated with four factors at day 22 and then stained with anti-C-PEPTIDE, -INS, -GCG and -SST antibodies. *INS*⁺ (C-PEP⁺) cells were $9.2 \pm 4.1\%$ of total cells. *INS*⁺/*GCG*⁺ cells were $5.2 \pm 4.0\%$ of total cells. Mean $\pm$ SD (n = 3). (D) Secreted C-peptide in response to low (2.5 mM) or high (25 mM) concentrations of D-glucose, 30 mM potassium chloride (KCl) or 0.5 μ M IBMX was measured with a C-peptide ELISA kit at day 22. Error bars indicate SD (n = 3). ns; not significant. Fsk, 10 μ M forskolin; Dex, 10 μ M dexamethasone; Alk5i, 5 μ M Alk5 inhibitor II; NA, 10 mM nicotinamide. Scale bar, 100 μ m.

were 764.0 ± 27.6 pM/ μ g DNA and 1306.2 ± 255.7 pM/ μ g DNA, 592.6 ± 151.9 pM/ μ g DNA and 1056.1 ± 296.3 pM/ μ g DNA, and 734.8 ± 276.2 and 1471.2 ± 202.8 pM/ μ g DNA, respectively (Fig. 4D). These results suggest that our method efficiently induces mature IPCs, and it is a robust method that can be applied to various hPSC lines.

Discussion

We established a differentiation method that induced PDX1⁺ cells from hPSCs at high efficiency, suggesting that inhibition of transforming growth factor (TGF)- β -induced AP-1 activation has an important role in PDX1⁺ cell induction. In addition, we showed that 3D culture plays an important role in the induction of functional

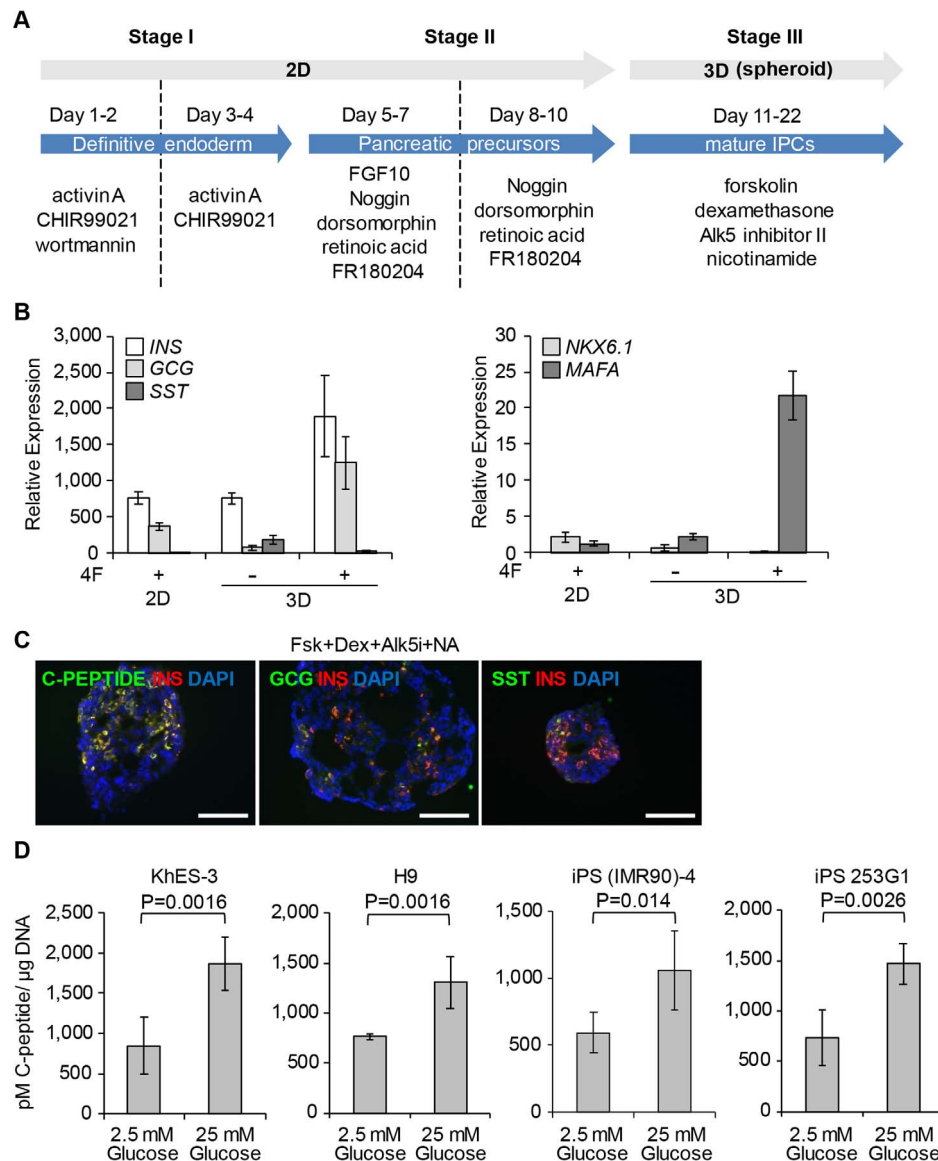


Figure 4 | Differentiation into mature IPCs in 3D culture. (A) Scheme of differentiation into IPCs in 3D culture. (B) Quantitative PCR analysis of the expression of *NKX6.1*, *MAFA*, *INS*, *GCG* and *SST* in cells treated with each factor at day 22. Expression levels were normalized to *TBP* expression. mRNA expression was relative to that in control cells at day 22. Control cells were treated with each factor for 10 days as shown in Fig. 4A, and then without factors for 12 days in 2D culture. Error bars indicate SD (n = 3). (C) Immunostaining of C-PEPTIDE, GCG, SST and INS proteins in cells treated with each factor in 3D culture at day 22. INS^+ cells were $31.4 \pm 2.7\%$ of total cells. INS^+/GCG^+ cells were $6.8 \pm 2.4\%$ of total cells. Mean $\pm$ SD (n = 5). (D) C-peptide secreted in response to low (2.5 mM) and high (25 mM) concentrations of D-glucose was measured with a C-peptide ELISA kit at day 22 (3D culture) in each hPSC line. Error bars indicate SD (n = 5). Fsk, 10 μ M forskolin; Dex, 10 μ M dexamethasone; Alk5i, 5 μ M Alk5 inhibitor II; NA, 10 mM nicotinamide. Scale bar, 100 μ m.

INS^+ cells with insulin secretion. Approximately 30% of total cells were mature IPCs that secreted insulin in response to glucose stimulation. Furthermore, this method allows robust differentiation that can be applied to various hPSC lines. The mature IPCs induced by this method may promote the development of new therapeutic drugs and cell therapies for diabetes.

It has been reported that activin A and Wnt3a can be used to differentiate DE cells from hPSCs^{11,14,16,18,21,22,25,26}. Wnt is known to participate in at least three pathways including β -catenin, PCP and Ca^{2+} signalling. We found that DE cells were induced from hPSCs by treatment with CHIR99021 instead of Wnt3a. This result suggests that inhibition of GSK3- β is important in multiple pathways. Furthermore, we found that the combination of activin A, CHIR99021 and wortmannin remarkably increased the expression of *FOXA2*, *SOX17*, *HNF1B* and *HNF4A* (Figs. 1A and S1A). Our

findings suggest that activation of nodal/activin signalling is important for differentiation into DE cells, and activation of β -catenin signalling and inhibition of PI3Ks promote induction of DE cells.

For differentiation of DE cells into $PDX1^+$ cells, we found that FR180204 induced $PDX1^+$ cells effectively. FR180204 is known to selectively inhibit the kinase activity of ERK1/2 as well as TGF- β -induced AP-1 activation³¹. Furthermore, inhibition of ERK activity has been suggested to restore the activity of retinoic acid²⁹. The combination of FGF10, Noggin, dorsomorphin, retinoic acid and FE180204 induced $PDX1^+$ cells at high efficiency (Fig. 2B). To evaluate the effect of ERK inhibition or inhibition of TGF- β -induced AP-1 activation, we investigated the combination of FGF10, Noggin, dorsomorphin, retinoic acid and PD0325901, a MEK inhibitor, or SR11302, an AP-1 inhibitor. Using the combination of the four factors and PD0325901 or SR11302, the percentage of $PDX1^+$ cells was



48.4 ± 11.1% and 73.9 ± 9.0%, respectively (Fig. S2C). Inhibition of BMP signalling is known to mediate the activation of ERK1/2. Therefore, the inhibition of BMP signalling suggested the importance of several pathways, including Smad, and that the ERK pathway was not important because the differentiation efficiency of PDX1⁺ cells increased using the combination of the four factors and FR180204 or SR11302. This result suggests that FR180204 is involved in differentiation to PDX1⁺ cells by inhibition of TGF-β-induced AP-1 activation rather than inhibition of ERK. These findings indicate that inhibition of BMP signalling, inhibition of TGF-β-induced AP-1 activation and the addition of retinoic acid are important for differentiation of DE cells into PDX1⁺ cells.

In addition to *INS*, *NGN3* and *PAX4* can be used to monitor the differentiation of INS⁺ cells from PDX1⁺ cells, because *NGN3* is an essential transcription factor in the development of pancreatic endocrine tissue^{32–34}, and *PAX4*, a downstream gene of *NGN3*, plays a critical role in the development of β cells³⁵. We found that the combination of forskolin, dexamethasone, Alk5 inhibitor II and nicotinamide achieved the highest expression of *NGN3*, *PAX4* and *INS* as reported previously (Figs. 3A and S3A). Under this condition, the percentage of INS⁺ cells was increased to about 10% (Fig. 3C). It has been suggested that an increase of the cAMP level and inhibition of TGF-β signalling play an important role in differentiation of PDX1⁺ cells into INS⁺ cells. In our study, although INS⁺ cells were generated at a certain efficiency, we did not observe insulin secretion by the induced INS⁺ cells in response to glucose stimulation, which is consistent with a previous reported²². It has been shown that mature IPCs can be induced from NKX6.1-expressing cells²⁰. Furthermore, *MAFA* has important roles in secretion of insulin in response to glucose stimulation in mice³⁶. In contrast to *INS* expression, we found no increase in the expression of these genes. Co-expression of *INS* and *GCG* or *SST* is suggested to be a marker of premature IPCs, because such co-expression is found at the embryonic stage of pancreatic development in rats and humans^{37,38}. Collectively, our results indicated that treatment with the four factors induced premature IPCs cells in 2D culture.

To achieve further maturation of INS⁺ cells, we employed a 3D culture system in the presence of the four factors. 3D culture is thought to mimic the in vivo microenvironment^{39,40}. Moreover, aggregated mouse insulinoma-derived MIN6 cells, which display characteristics of pancreatic β cells, secrete insulin in response to glucose stimulation⁴¹. Indeed, the combination of 3D culture and the four factors increased the expression of *NKX6.1*, *MAFA* and *INS* (Figs. 4B and S4A). The percentage of INS⁺ cells was increased to about 30%, but only a small proportion of INS⁺ cells co-expressed *GCG* protein (Fig. 4C). Importantly, the INS⁺ cells induced by 3D culture secreted insulin in response to glucose stimulation (Fig. 4D). Compared with conventional 2D culture, these results indicate that 3D culture has an advantage for maturation of INS⁺ cells. Furthermore, it has been reported that the differentiation efficiency into INS⁺ cells varies among hPSC lines. However, our differentiation method induced INS⁺ cells with glucose-dependent INSULIN secretion at a similar efficiency among different hPSCs (Figs. 2C and S4D).

In conclusion, we established a differentiation method that allowed highly efficient differentiation of hPSCs to mature IPCs via induction of DE cells and then PDX1⁺ cells. In particular, we found that treatment with FR180204 was effective for differentiation into PDX1⁺ cells. We also efficiently induced mature IPCs that secreted insulin in response to glucose stimulation by treatment with forskolin, dexamethasone, Alk5 inhibitor II and nicotinamide in 3D culture. Moreover, our differentiation method is robust and optimization for individual cell lines is unnecessary.

To date, the shortage of donor human pancreases limits application to clinical therapy for islet transplantation. In addition, the lack of suitable models for diabetes makes it difficult to develop new anti-

diabetic drugs. Our method might have advantages for clinical application, because it does not use foetal bovine serum (FBS) or mouse embryonic fibroblasts (MEFs). Moreover, our method might be useful for development of new drugs using disease model cells derived from disease-specific iPSCs, because it is a robust method that induces mature IPCs at a similar efficiency from various hPSC lines. Furthermore, the data obtained in this study might be useful for understanding the mechanism of insulin secretion in human pancreatic cells.

Methods

hPSC culture. The hESC line KhES-3 and hiPSC line 253G1 were maintained on mitomycin-C (Wako)-treated MEF feeder cells as described previously⁴². The hESC line WA09 (H9) and hiPSC line iPS (IMR90)-4 were purchased from the National Stem Cell Bank (NSCB). These cells were maintained in accordance with the NSCB protocols. All cells were passaged every 5 days at ratios of 1:3 to 1:7.

For pancreatic differentiation, hPSCs were passaged on Matrigel (Becton Dickinson)-coated dishes at a density of 4.4×10^4 cells/cm² to remove MEF feeders using CTX. hPSCs were cultured for 4–5 days to near confluency. During induction, the above-mentioned cells were not dissociated and treated with each factor. For DE induction, hPSCs were washed with PBS(+) (Sigma) and then cultured in RPMI 1640 containing 1 × Glutamax I and 1% B27 without insulin (All purchased from Life Technologies) supplemented with 100 ng/ml activin A (eBioscience), 3 μM CHIR99021 (Stemgent) and 100 nM wortmannin (Sigma) for 2 days, and then 100 ng/ml activin A and 3 μM CHIR99021 for a further 2 days. Subsequently, the cells were washed with PBS(+), the supplements were changed to 50 ng/ml FGF10 (R&D systems), 50 ng/ml Noggin (R&D systems), 1 μM dorsomorphin (Stemgent), 2 μM retinoic acid (Sigma) and 3 μM FR1890204 (Calbiochem), and the cells were cultured for 3 days. Then, the cells were treated with 50 ng/ml Noggin, 1 μM dorsomorphin, 2 μM retinoic acid and 3 μM FR1890204 in RPMI 1640 containing 1 × Glutamax I and 1% B27 without insulin for 3 days. The cells were rinsed with PBS(+) and then cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (Sigma), 1% B27 without insulin, 10 μM forskolin (Sigma), 10 μM dexamethasone (Sigma), 5 μM Alk5 inhibitor II (Calbiochem) and 10 mM nicotinamide (Sigma) for 12 days. At day 22, the cells were cultured in low glucose DMEM (Sigma) containing B27 without insulin and supplemented with 10 μM forskolin, 10 μM dexamethasone, 5 μM Alk5 inhibitor II and 10 mM nicotinamide.

3D culture. Differentiated cells cultured in the combination of FGF10, Noggin, dorsomorphin, retinoic acid and FR180204 in adhesion culture for 10 days were transferred and incubated in 3D culture for 12 days. To aggregate the cells, differentiated cells in 2D culture were dissociated with 200 U/ml collagenase type IV (Life Technologies) and then passed through a 100-μm cell strainer. The dissociated cells were centrifuged at 330 g for 5 min at 4°C three times. The cells were then cultured in 6-well plates with an Ultra-Low Attachment Surface (Corning) for 3 days in DMEM with 1% B27 without insulin, 10 μM forskolin, 10 μM dexamethasone, 5 μM Alk5 inhibitor II, 10 mM nicotinamide and 10 μM Y27632 (Wako). Then, Y27632 was omitted from the medium that was changed every 3 days for 8 days. At day 22, aggregated cells were cultured in low glucose DMEM (Sigma) containing B27 without insulin and supplemented with 10 μM forskolin, 10 μM dexamethasone, 5 μM Alk5 inhibitor II and 10 mM nicotinamide for 1 day.

Quantitative real-time PCR. Total RNA was isolated from cells in 2D culture with Trizol (Life Technologies) and treated with recombinant DNase I (Takara Bio). In 3D culture, total RNA was isolated from cells using a PureLink RNA Mini Kit (Life Technologies). Reverse transcription was performed with SuperScript III (Life Technologies) using random hexamers according to the manufacturer's instructions. Primers for RT-PCR are shown in Table S2. Quantitative PCR was carried out using a 7500 Real-Time PCR system (Life Technologies). The reaction mixture consisted of Power SYBR Green PCR Master Mix (Life Technologies), 0.5 nM of each primer and cDNA. The expression levels of each gene were normalized to those of the housekeeping gene *TATA box binding protein (TBP)*. All quantitative PCR primers were obtained from Sigma Genosys.

Flow cytometric analysis. Differentiated hPSCs were dissociated by treatment with 4.8 mM EDTA (Sigma) in PBS(-) (Sigma) for 2–5 min and then 0.05% trypsin/EDTA (Life Technologies) in DMEM for 1 min. After washing with 2% FBS (Cell Culture Bioscience) in DMEM and then 2% FBS in PBS(-), the cells were fixed with 2% paraformaldehyde (Nacalai Tesque) for 15 min on ice. After washing with 2% FBS in PBS(-), the cells were treated with 90% methanol (Wako) in PBS(-) for 15 min on ice. After rinsing with 2% FBS in PBS(-), the cells were incubated for 30 min at 4°C with a rabbit anti-FOXA2 antibody (1:50, CST) diluted in 0.1% bovine serum albumin (BSA; Sigma)/PBS(-). Then, the cells were washed with 2% FBS in PBS(-) and incubated for 30 min at 4°C with Alexa Fluor 488 donkey anti-rabbit IgG (1:1000, Life Technologies) and an APC-conjugated goat anti-SOX17 antibody (1:50, R&D systems) diluted in 0.1% BSA/PBS(-). The cells were then rinsed twice with 2% FBS in PBS(-). Fluorescence intensities were analysed on a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest software.



Immunochemical assay. Cells were rinsed with DMEM or RPMI 1640 three times and then fixed in 4% paraformaldehyde for 15 min at room temperature. The cells were then blocked with 5% donkey serum (Millipore) and 0.3% Triton X-100 (Nacalai Tesque) in PBS(-) for 1 h at room temperature. Aggregated cells were rinsed DMEM twice and fixed in 4% paraformaldehyde for 25–30 min at room temperature and then rinsed with 0.1% Triton X-100. Cryosections (5 μ m) were obtained using OCT compound (Sakura) and a Leica CM1850 (Leica). The sections were incubated with 5% goat (Life Technologies) and 5% donkey serum and 0.1% Triton X-100 in PBS(-) for 1 h at room temperature. The cells were then incubated overnight at 4°C with the following primary antibodies: rabbit anti-FOXA2 (1 : 400; CST), mouse anti-SOX17 (1 : 6000; Origene), rabbit anti-PDX1 (1 : 100; CST), mouse anti-NKX6.1 (1 : 300; DSHB), mouse anti-C-PEPTIDE (1 : 300; Sanbio), guinea pig anti-INSULIN (1 : 2000), rabbit anti-GLUCAGON (1 : 200; Sigma) and rabbit anti-SOMATOSTATIN (1 : 1000; Dako). After washing with PBS(-) three times, the cells were incubated for 1 h at room temperature with Alexa Fluor 488- or 594-conjugated donkey or goat anti-rabbit, -mouse or -guinea pig IgG (1 : 1000; Life Technologies). Nuclei were counterstained with DAPI (Sigma). To quantify the percentage of PDX1⁺ cells, 16 images/well were acquired using an ArrayScan VTI HCS Reader (Thermo) and analyzed with Cellomics BioApplications (Thermo). The percentage of PDX1⁺ cells was normalized to the percentage of cells stained with the secondary antibody only. The number of INS⁺/C-PEPTIDE⁺ cells relative to the total number of nuclei stained with DAPI is indicated as the percentage of C-PEPTIDE⁺ cells ($n = 3$, total aggregated cells = 6).

C-peptide enzyme-link immunosorbent assay (ELISA). After pre-incubation with Krebs-Ringer solution containing bicarbonate, HEPES and D-glucose (KRBH; 129 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM HEPES, 2.5 mM D-Glucose (all purchased from Wako) and 0.1% (w/v) BSA) at 37°C for 2 h, the samples were incubated with KRBH containing 2.5 mM or 25 mM D-glucose or 30 mM KCl (Wako) or 0.5 μ M IBMX (Sigma) at 37°C for 1 h. The C-peptide level in culture supernatants was measured with a human C-PEPTIDE ELISA kit (Mercodia) according to manufacturer's instructions. Cells were lysed in Spheroid Lysis Buffer (Scivax), and dsDNA content was measured by a PicoGreen dsDNA Quantitation Kit (Life Technologies) and Wallac 1420 multilabel/Luminescence counter (PerkinElmer).

Statistical analysis. Data are presented as the means $\pm$ standard deviation (SD) of three or five independent experiments. Statistical significance was evaluated by the Student's t-test.

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Author contributions

H.T. and H.S. designed research. H.T. performed research. H.T., N.N. and H.S. analyzed data. H.T., H.S. wrote the paper.

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