Indolactam V/GLP-1-mediated Differentiation of Human iPS Cells into Glucose-Responsive Insulin-Secreting Progeny

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Abstract

Nuclear reprogramming of somatic tissue enables derivation of induced pluripotent stem (iPS) cells from an autologous, non-embryonic origin. The purpose of the current study was to establish efficient protocols for lineage-specification of human iPS cells into functional glucose-responsive, insulin-producing progeny. We generated human iPS cells, which were then guided with recombinant growth factors that mimic the essential signaling for pancreatic development. Reprogrammed with four stemness factors, human fibroblasts were here converted into authentic iPS cells. Under feeder-free conditions, fate-specification was initiated with activin A and Wnt3a that triggered engagement into definitive endoderm, followed by priming with FGF10 and KAAD-cyclopamine. Addition of retinoic acid, boosted by the pancreatic endoderm inducer indolactam V (ILV), yielded pancreatic progenitors expressing PDX1, NGN3 and NEUROD1 markers. Further guidance, under IGF-1, HGF and DAPT, was enhanced by glucagon like peptide-1 (GLP-1) to generate islet-like cells that expressed pancreas-specific markers including insulin and glucagon. Derived progeny demonstrated sustained expression of PDX1, and functional responsiveness to glucose challenge secreting up to 230 pM of C-peptide. A pancreatogenic cocktail enriched with ILV/GLP-1 offers a proficient means to specify human iPS cells into glucose-responsive hormone-producing progeny, refining the development of a personalized platform for islet-like cell generation.
INTRODUCTION

Due to their high self-renewing capacity and potential to generate cells of all organ systems in the body, embryonic stem (ES) cells have been considered as a natural source for cell replacement therapy. However, their use is dependent on embryonic tissue procurement and restricted by allogeneic mismatch, preventing application in clinical practice. The alternative bioengineered approach, based on nuclear reprogramming technology, enables generation of autologous pluripotent stem cells from a non-embryonic source through ectopic expression of multiple stem cell-related genes. Derived human induced pluripotent stem (iPS) cells, which recapitulate salient features of human ES cells, have in fact been differentiated into a wide variety of cell types.

Type 1 diabetes (TID) is a chronic autoimmune disease, which destroys pancreatic-beta cells and induces an absolute insulin deficiency. Patients with T1D depend on the administration of exogenous insulin for survival; however fluctuations of blood glucose levels often lead to severe diabetes-related complications. Transplantation of whole pancreas or primary islets is a promising therapeutic option for T1D. Yet, shortage of matched islet-replacement tissues has prevented the widespread use of this therapy. Alternative therapeutic approaches include transplantation of insulin-secreting cells generated from self-renewable cell sources. In this regard, efficient differentiation of human iPS cells would offer the potential for generation of replenishable, islet-like cells, which could be used to restore euglycemia and insulin independence.

Directed differentiation of pluripotent stem cells into pancreatic lineage requires understanding of pathways controlling pancreas development. The critical steps in differentiation are the generation of cells comprising the definitive endoderm, and ultimately generation of pancreatic endoderm. Based on developmental biology insight, strategies have been recently developed to generate endoderm and pancreatic beta-like cells from ES cell sources. Activin A and low serum concentrations have been shown to differentiate mouse and human ES cells into definitive endoderm, respectively. Retinoic acid (RA) signals are required for development of Pdx1-positive cells from the endoderm in zebrafish and mouse models. RA has been reported to promote generation of Pdx1-positive cells from mouse and human stem cells in vitro. Recently, the small molecule Indolactam V (ILV), in combination with fibroblast growth factor 10 (FGF10), has also been reported to generate PDX1-positive pancreatic endoderm cells from human ES cells. Based on incremental advances in the decoding of signaling networks in pancreatic development, stepwise differentiation protocols have been established for the generation of pancreatic beta-like cells from ES cells. Although up to 12% of insulin-expressing cells have been derived from a human ES cell pool, responsiveness to glucose stimulation has remained poor.
iPS have also been most recently differentiated into C-peptide-positive cells through stimulation with a combination of activin A and sodium butyrate with additional growth factors. Moreover, iPS cells from two diabetic patients were successfully differentiated into pancreatic beta-like cells in vitro. However, iPS-derived islet-like cells have been reported to secrete low levels of C-peptide in response to glucose stimulation, and variation in differentiation propensities were observed among iPS clones. Thus, despite remarkable progress, it still remains challenging to achieve efficient differentiation of human pluripotent stem cells into functional insulin-producing cells, a prerequisite for the establishment of robust, functional platforms for bioengineered islet generation.

In this study, we generated human iPS under feeder-free conditions, which were then guided with recombinant growth factors that mimic the essential signaling for pancreatic development. Inclusion of ILV achieved efficient generation of PDX1-, NGN3- and NEUROD1-expressing pancreatic endoderm through the FOXA2- and SOX17-positive definitive endoderm. Further guided differentiation with glucagon like peptide-1 (GLP-1), known to increase beta cell mass and enhance insulin gene expression, resulted in generation of functional islet-like cells. Importantly, iPS-derived islet-like cells sustained PDX1 expression, and secreted up to 230 pM of C-peptide. Our data therefore demonstrate that inclusion of ILV and GLP-1 in pancreatogenic cocktail regimens facilitates generation of glucose-responsive islet-like cells from a human iPS cell source.

RESULTS

Reprogramming of Human Fibroblasts with Stemness Factors
HCF and BJ fibroblasts were infected with lentiviral vectors encoding OCT4, SOX2, KLF4 and c-MYC, and transduced cells re-seeded on mitomycin C-inactivated SNL feeder cells or replated on matrigel-coated plates to ensure feeder-free culture. On SNL feeder cells, reprogrammed colonies, characterized by distinct morphology of sharp-edged, flat, tightly-packed structures were visible 2 weeks after viral vector transduction (Fig. 1A). Under feeder-free conditions, similar colonies were observed as early as day 6 after viral vector infection (Fig. 1B) with clusters of 30–50 cells expressing alkaline phosphatase (Supplementary Fig. S1). The number of expandable colonies formed on feeders or on non-feeders plates were 5 to 20 clones per $10^5$ transduced cells. Identified colonies were picked at 3 to 6 weeks to allow sufficient growth after viral transduction.

Expression of Pluripotency Markers in Derived iPS Clones
Over 3–9 months or 30–90 passages, putative iPS clones cultured under feeder-free and serum-free conditions exhibited a distinctive morphology similar to that of human ES cells over long-term culture (Fig. 1C). Tested clones expressed high levels of alkaline phosphatase (Fig. 1D). Immunocytochemistry revealed expression of SSEA-4, TRA-1-60, TRA-1-81, OCT4, SOX2, KLF4 and NANOG in multiple clones (Fig. 2A and Fig. 2B). These clones were negative for SSEA-1 expression. RT-PCR of total cellular RNA further demonstrated induction of endogenous pluripotency-associated genes, including OCT4, SOX2, GDF3, telomerase (TERT), KLF4, c-MYC and NANOG (Fig. 2C). No notable difference was observed between clones isolated from BJ and HCF fibroblasts, or with
clones isolated with SNL feeder cells. Morphology and expression of stem cell genes indicate establishment of human iPSC clones from fibroblasts, and maintenance in an undifferentiated state under feeder-free conditions.

**Pluripotency Validated through Three Germ Layer Differentiation**

Human iPSC clones were assayed, through embryoid body (EB) formation, for the ability to spontaneously differentiate *in vitro* into cells of the three embryonic germ layers. All iPSC clones assayed formed EBs (Fig. 3A). After variable times in suspension, EBs were transferred to adherent conditions and further cultured. Immunostaining for lineage-specific markers confirmed that human iPSC cells differentiated into ectoderm (beta-III tubulin, Fig. 3A), endoderm (FOXA2, Fig. 3A) and mesoderm (CD31, Fig. 3A) lineages. Moreover, *in vivo* human iPSC cells formed teratomas after injection into nude mice. These subcutaneous tumors enlarged up to 1 cm in diameter within 3 months post-injection (Fig. 3B). Histology revealed diverse cell types, including glandular epithelium (ectoderm, Fig. 3C), adipose (endoderm, Fig. 3C) and muscular (mesoderm, Fig. 3C) tissues. Thus, human iPSC cells generated from BJ and HCF fibroblasts exhibit hallmark properties of pluripotent stem cells.

**Differentiation of Human iPSC cells into Pancreatic Endoderm**

Normal differentiation of a pluripotent precursor into lineage-specified pancreatic endodermal tissue encompasses multiple steps. Here, verified iPSC cells were treated first with activin A and Wnt3a for generation of definitive endoderm cells, and then with FGF10 and CYC for derivation of gut tube endoderm (Fig. 4A). Derived cells were further treated with FGF10, RA and CYC in the absence or presence of ILV for generation of pancreatic endoderm, followed by culture in HGF, IGF and DAPT in the absence or presence of GLP-1 for generation of pancreatic hormone-expressing cells (Fig. 4A). In this way, human iPSC clones were induced to form definitive endoderm by treatment with activin A and Wnt3a initially for 1 day followed by culture in activin A and 2% FBS for 2 additional days. Immunostaining of treated cells revealed efficient SOX17 and FOXA2 induction, markers of definitive endoderm (Fig. 4B). Similar results were observed with clones generated from human cardiac fibroblasts or foreskin (Supplementary Fig. S2). Flow cytometry demonstrated that 92%, 72% and 84% cells were positive for SOX17 in three distinct clones, respectively (Fig. 4C; Supplementary Fig. S2). Next, we evaluated the efficiency of definitive endoderm transformation into pancreatic endoderm. Our initial attempts to generate pancreatic endoderm by stimulating definitive endoderm cells with FGF10 and CYC for two days, followed by FGF10, RA, and CYC stimulation resulted in cells with low levels of PDX1 expression (data not shown). We therefore included ILV, which was recently demonstrated to efficiently induce pancreatic endoderm from definitive endoderm. In our protocol, treatment of iPSC-derived definitive endoderm cells with FGF10, RA, and CYC in the presence of ILV resulted in cells expressing PDX1, NEUROD1 and NGN3, markers of pancreatic endoderm (Fig. 4D). Similar results were observed for iPSC cells derived from human cardiac fibroblasts or foreskin (Supplementary Fig. S3). These results indicate successful induction of pancreatic endoderm from iPSC-derived definitive endoderm.
**Induction of Stage-Specific Pancreatic Genes through Guided Differentiation**

To determine the expression of endocrine-specific transcription factors and pancreas-specific genes throughout differentiation, we analyzed the gene expression pattern at each stage of differentiation. RT-PCR detected high levels of *FOXA2* expression after 3 days of differentiation, confirming induction of definitive endoderm cells (Fig. 5A). The expression of the endocrine progenitor-specific gene, *NGN3*, was observed from day 3 of differentiation, and the expression persisted throughout the differentiation process (Fig. 5A). Expression of the islet specific gene, *ISL-1*, was also found from day 3, with expression levels increasing at later time points (Fig. 5A). Moreover, *PDX1* and *NEUROD1* transcripts, which were found only after treatment with FGF10, RA, CYC and ILV, further confirmed the generation of iPS-derived pancreatic endoderm cells upon differentiation (Fig. 5A). To evaluate whether human iPS-derived pancreatic endoderm cells are capable of generating functional pancreatic islet-like cells, we proceeded with an additional step of differentiation. We initially treated the iPS-derived pancreatic endoderm with HGF, IGF, Exendin-4 and DAPT; however, we failed to detect C-peptide secretion from resulting cells (data not shown). We therefore included GLP-1, which is known to increase beta cell mass, in order to optimize the yield of glucose-responsive, islet-like cell progeny. Following inclusion of GLP-1, RT-PCR revealed positive gene expression of pancreatic hormones, including *insulin*, *glucagon* (*GCG*), and *somatostatin* (*SST*), and islet cell-specific marker genes *PDX1*, *NKX6.1*, *ISL1* and *NEUROD1* and glucose transporter 2 (*GLUT2*) (Fig. 5A). Conversely, to determine whether pluripotency genes were silenced during differentiation, we performed RT-PCR analysis for *c-MYC*, *GDF3*, *hTERT*, *NANOG*, *SOX2* and *KLF4*. We found that *c-MYC*, *GDF3*, *hTERT*, and *NANOG* gene expression levels gradually decreased during differentiation, while these gene transcripts were absent in the human pancreas (Fig. 5B). *SOX2* and *KLF4* gene expression remained throughout iPS differentiation, in line with expression of these two genes in the human pancreas (Fig. 5B). The targeted down-regulation of pluripotency genes along with sequential expression of pancreas-specific genes collectively indicated that human iPS cells are capable of undergoing guided differentiation *in vitro* into islet-like cells, with the observed combined expression of *GLUT-2*, *NKX6.1* and *NEUROD1* further suggesting derivation of tissue with properties of functional beta cells.

**Differentiation of iPS Cells into Insulin-Secreting Islet-like Progeny**

During treatment with RA, FGF10, CYC and ILV, iPS-derived pancreatic endoderm cells started to form spheroid-like cell clusters, which reached maximum size and number following further maturation with HGF, IGF, DAPT and GLP-1 (Fig. 5C). The three dimensional morphology resembled pancreatic islet-like clusters, and selected clones yielded clusters (Fig. 5C) strongly positive for C-peptide expression (Fig. 5D). Importantly, even iPS-derived islet-like cells that did not organized into typical clusters also expressed insulin, C-peptide and glucagon (Fig. 5E). The presence of insulin/C-peptide co-expressing cells (Fig. 6A.i) confirmed the potential for de novo insulin synthesis and excluded the possible artifact of insulin uptake from the media. Also, we did not observe insulin-glucagon double positive cells, indicating that the expression pattern of iPS-derived hormone-expressing islet-like cells is consistent with normal pancreatic beta-cell development. Although few insulin and somatostatin double-positive cells were found (Fig. 6A.ii),
characteristic of immature islet cells, our results indicate successful differentiation of iPS cells into hormone-expressing islet-like cells. Indeed, similar to pancreatic beta cells which co-express insulin and PDX1, the majority of the insulin-expressing cells showed nuclear-localized PDX1 signals (Fig. 6A.iii). When we quantified by flow cytometry the insulin-positive population, 1.3%, 0.7% and 0.8% of distinct clones-derived islet-like cells were insulin-positive (Fig. 6B; Supplementary Fig. S4).

**Functional Response of iPS-derived Islet-like Clusters**

Finally, we analyzed C-peptide secretion from iPS-derived islet-like clusters in response to glucose challenge, the critical physiological function of pancreatic beta cells. To determine whether islet-like cells are capable of C-peptide secretion in response to glucose induction, cells were exposed to increasing concentrations of glucose and secreted C-peptide was measured by ELISA. At extracellular glucose levels of 2.5 mM, that mimics a fasting condition, there was only marginal detection of the C-peptide signal (Fig. 6C). Raising glucose levels to 10 mM induced marked secretion of C-peptide by iPS-derived islet-like cells (Fig. 6C). Further raising glucose levels to the supraphysiological 27.7 mM range, triggered an additional bolus of secreted C-peptide, reaching cumulatively the range of 72.0–236.1 pM (HCF#1, three independent experiments, Fig. 6C) or 12.1–50.9 pM (BJ#1). iPS-derived islet-like cells differentiated without ILV or GLP-1 failed to secrete C-peptide in response to glucose challenge (Fig. 6D). Although clonal variation in responsiveness was observed, iPS-derived islet-like clusters were typically capable to secrete C-peptide in response to glucose stimulation.

**CONCLUSIONS**

Islet cell transplantation is increasingly considered as a new generation therapy for insulin-dependent diabetes. It is however limited by the availability of donor islets. Differentiation of human iPS cells into islet-like progeny would offer an alternative approach, ensuring an unlimited supply for islet-like cells. Here, under feeder-free conditions, human iPS clones were successfully guided by a pancreatogenic cocktail - enriched with ILV/GLP-1 - into islet-like cells which expressed pancreas-specific transcription factors and islet-specific hormones. Importantly, derived islet-like cells secreted C-peptide in response to glucose stimulation. The present study therefore establishes an iPS-based feeder-free protocol for derivation of functional hormone-producing progeny, expanding the armamentarium for islet-like cell generation.

Human stem cell clones are typically maintained on non-human feeder cells impeding translation of human cell therapy protocols. Indeed, non-human feeder cells express undefined factors, which potentially compromise stem cell differentiation. Moreover, feeder cells have been reported to constitutively produce infectious endogenous retroviruses, often preventing immediate clinical application. To circumvent this limitation, we here generated feeder-free conditions using Matrigel-coated plates and an optimized serum-free medium, supplementing HEScGRO with 25% of mTeSR1. This previously untested approach achieved feeder-free/serum-free conditions, nurturing iPS cell expansion and maintaining the undifferentiated state long-term. In this way, we were able to sustain multiple iPS clones

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in feeder/serum-free milieu for >1 year without losing salient structural and genetic traits of pluripotency.

It is established that the initial step to achieve successful generation of islet-like cells from a pluripotent cell source requires differentiation into definitive endoderm. Stimulation of mouse or human ES cells with activin A, Nodal and/or Wnts leads to endoderm induction, and was here implemented with human iPS cells. Specifically, we generated definitive endoderm cells by stimulating feeder-free human iPS cells with activin A and Wnt3a in the presence of low serum. Induction of definitive endoderm was particularly efficient, such that 70–90% of derived cells expressed the definitive endoderm marker SOX17. Similar results were observed across distinct iPS clones, derived from foreskin or cardiac fibroblasts, demonstrating that the applied approach offers a robust means for definitive endoderm induction.

Further lineage specification requires conversion of definitive endoderm into pancreatic endoderm. A recent study has identified a small molecule ILV, a protein kinase C activator, which strongly directs human ES cell-derived definitive endoderm into pancreatic endoderm. Pancreatic endoderm induction by ILV alone has been shown to generate approximately 53% PDX1-positive cells, in contrast to 25% PDX1-positive cells achievable with standard inductors, FGF10, RA and CYC. Here, using human iPS-derived definitive endoderm, we demonstrated robust induction of pancreatic endoderm markers, PDX1, NGN3 and NEUROD1, only after inclusion of ILV to the FGF10, RA and CYC cocktail, underscoring the feasibility of generating pancreatic endoderm cells from human iPS cells through ILV-mediated guidance. It has been proposed that ILV may share an RA-dependent signaling pathway, allowing ultimately to streamline the process of efficient pancreatic endoderm derivation.

Human ES cells after guided differentiation to pancreatic hormone-expressing cells generate up to 12% of insulin and synaptophysin expressing cells. However, these cells show minimal glucose-responsive C-peptide secretion and do not maintain expression of key beta cell markers, NKX6.1 and PDX1. Foreskin-derived iPS cells or iPS cells from patients with type 1 diabetes (T1D) were also recently differentiated into islet-like clusters in vitro, yet generation of glucose-responsive insulin-producing cells from human iPS cells remains inefficient. GLP-1 was shown to convert intestinal epithelial cells into functional insulin-producing cells, and could promote differentiation of mouse bone marrow or human embryonic stem cells into insulin-producing cells. Inclusion of GLP-1 in our differentiation cocktail enhanced the generation of insulin-producing cells from iPS-derived pancreatic endoderm cells. Derived islet-like cells expressed transcripts of PDX1, NKX6.1, NEUROD1, ISL-1 and GLUT2, recognized beta cell-specific markers. Co-expression of insulin and PDX1, a characteristic of pancreatic beta cells, was evident in iPS-derived insulin-producing cells by immunofluorescence. In contrast to previous reports which demonstrated limited PDX1 expression in ES cell-derived insulin-producing cells, we found prevalent insulin/PDX1-double positive cells after differentiation. Moreover, these islet-like cells secreted C-peptide in response to glucose stimulation. These observations indicate that the ILV/GLP-1-containing pancreatogenic cocktail offers proficient means to specify human iPS cells into functional beta-like cells.
Replacement of Exendin-4 (120 nM) with GLP-1 (55 nM) in the final differentiation step notably improved the differentiation of iPS-derived pancreatic endoderm into glucose-responsive, insulin-producing cells. Exendin-4, a 39 amino acid peptide secreted by the salivary gland of a venomous lizard, is a GLP-1 analog which binds and activates the GLP-1 receptor (GLP-1R). Intriguingly, Exendin-4 and GLP-1 bind GLP-1R with similar affinity but in different modes; Exendin-4 relies largely on the N-domain for interaction, while GLP-1 utilized the core domain for GLP-1R binding. We speculate that iPS-derived endoderm cells express GLP-1R with differential post-translational modification, affecting the binding of GLP-1R with Exendin-4, but not GLP-1. Alternatively, binding of GLP-1, but not Exendin-4, to GLP-1R on iPS-derived endoderm cells may facilitate activation of a critical intracellular signaling pathway necessary for successful endoderm differentiation into glucose-responsive islet-like cells.

Of note, human iPS clones demonstrated here no notable differences in morphology, pluripotency marker expression, and/or ability to differentiate into cells of the three germ layers. Although iPS clones were efficiently differentiated into definitive endoderm, they exhibited varying propensity to generate glucose-responsive insulin-producing cells. Moreover, we noted that high levels of C-peptide remained in glucose-stimulated cells (data not shown), suggesting an immature insulin secreting system in iPS-derived islet-like cells. In fact, it was notable that while certain clones displayed vigorous response to glucose with marked secretion of insulin, other clones failed to significantly secrete C-peptide even though features of insulin-expressing cells were documented. This diversity in response cannot be ascribed to variations in the genetic or tissue background of iPS clones, as variations were found among iPS clones derived from the same tissue origin. Clonal differences in differentiation propensities have been reported among pluripotent stem cells, which could be due to varying degrees of reprogramming, different copy numbers of integrated pluripotency genes, or different levels of pluripotency gene silencing/reactivation in individual clones. Further analysis using multiple iPS clones and improved islet differentiation efficiency will be necessary to establish the mechanisms underlying unique functional property of derived lineage-specified cell progeny in vitro and in vivo.

Retroviral or lentiviral vector integration has risks associated with insertional mutagenesis. Use of oncogenic c-MYC during reprogramming is also problematic for clinical application of the resulting iPS cells, as sustained c-MYC expression can increase the risk of tumor formation in iPS-derived cells in vivo. Indeed, use of c-Myc has been associated with increased tumorigenicity in chimeric mice established with iPS cells. Additionally, reactivation or sustained expression of pluripotency genes in iPS-derived islet-like cells could result in spontaneous reprogramming of iPS progeny, leading to the potential risk of teratoma formation upon transplantation. Indeed, residual expression of pluripotency-associated genes, such as c-MYC, NANOG, hTERT and GDF3, was evident in differentiating iPS cells (Fig. 5B), which is likely due to sustained vector transgene expression. Determining the levels of vector-derived and endogenous pluripotency gene expression in the different clones before and after differentiation will be necessary to understand the influence of integrating vectors on the diversity in differentiation propensities among iPS clones. In order to increase the safety of iPS-based cell therapy, it will be critical...
to generate iPS cells without integrating vectors and continuous c-MYC expression. Derivation of iPS cells with transient expression from non-integrating vectors\textsuperscript{43–45} will solve both problems, as the resulting iPS cells carry no genomic modifications. To further minimize the risk of teratoma formation, it will be necessary to develop further strategies, such as sorting out fully differentiated cell populations by flow cytometry, eliminating residual pluripotent cells by a suicide gene vector or encapsulating the iPS-derived cells before transplantation."

In summary, we demonstrate that under feeder-free conditions an ILV/GLP-1-enriched pancreatogenic cocktail allows derivation of glucose-responsive, insulin-producing cells from human iPS cells, providing a platform for islet-like cell generation. Autologous iPS derivation and iPS differentiation into insulin-producing cells would allow modeling of patient-specific disease pathogenesis, and ultimately lead to personalized approaches for T1D cell therapy with iPS-derived islet-like cells.

**MATERIALS AND METHODS**

**Plasmid Construction and Lentiviral Vector Production**

Stemness factor-expressing lentiviral pSIN-CSGWdINotI-derived transfer vectors were generated as described\textsuperscript{46}. In brief, the packaging plasmid pEX-QV was engineered with H87Q mutation in the HIV-1 capsid region for increased transduction efficiency of purified infectious supernatants\textsuperscript{47}. HIV vectors were produced by transient transfection of 293T cells and titrated by immunostaining\textsuperscript{47}. Vectors expressed pluripotency factors from a spleen focus-forming virus (SFFV) promoter\textsuperscript{47}.

**Generation and Culture of Human iPS Cells on SNL Feeder Cells**

Human neonatal foreskin fibroblasts (BJ1) (ATCC\#CRL-2522) and primary human cardiac fibroblasts (HCF) (ScienCell \#6300) were seeded one day before infection in wells of 6 well plates with DMEM containing 10% FBS, Penicillin (100U/ml) and Streptomycin (100µg/ml) (Pen/Strep) (complete DMEM). Fibroblasts were infected with lentiviral vectors expressing \textit{OCT4}, \textit{SOX2}, \textit{KLF4} and \textit{c-MYC} at a multiplicity of infection \textasciitilde 5 each. After 12 h of viral infection, cells were fed with fresh complete DMEM. Vector-transduced cells were replated 4 days after infection at \textasciitilde 5 \times 10^4 cells per 100 mm dish on mitomycin-C treated SNL feeder cells in complete DMEM. Next day, the medium was replaced with the serum-free HEScGRO medium (Millipore \#SCM020) supplemented with basic fibroblast growth factor (bFGF, 20 ng/ml; Peprotech). Cells were fed with fresh HEScGRO medium every two days. Putative iPS colonies, which began to appear 3–4 weeks after vector transduction, were picked based on size and human embryonic stem cell-like colony morphology, and expanded through dissociation with the cell dissociation buffer (Invitrogen \#13151014). BJ1-derived iPS clones, BJ#SA and BJ#SD, were generated on SNL feeder cells. Established iPS clones were maintained in feeder-free condition.

**Feeder-Free iPS Generation and Culture**

For feeder-free iPS generation and maintenance on Matrigel (BD Biosciences \#354277)-coated plates, we compared various commercially available stem cell media or their
combinations. Optimal results were obtained when iPS cells were maintained in a feeder-free medium, which contained HEScGRO with 25% of mTeSR1 medium (Stemcell Technologies #05850) and 20 ng/ml of bFGF (iPS medium). In order to generate feeder-free iPS clones from BJ and HCF fibroblasts, cells were transduced with pluripotency factor-expressing lentiviral vectors, 4 days after infection; cells were re-plated at a density of 5 × 10^5 cells on a Matrigel-coated 100 mm dish. Medium was replaced with fresh iPS medium every two days. Putative iPS colonies were observed 1–2 weeks after vector transduction. iPS clones were picked based on morphology and size. iPS clones were expanded with cell dissociation buffer and passaged at a 1:2–1:8 split ratio every 3–7 days depending on cell density. BJ#1, HCF#1 and HCF#6 iPS clones were generated and maintained under feeder-free conditions.

**Immunostaining and Alkaline Phosphatase Staining**

For immunostaining, iPS cells were fixed for 20 min at room temperature (RT) in 4% paraformaldehyde (PFA) in PBS, washed in PBS and blocked for 30 min with 5% FBS in PBST (PBS with 0.1% Tween-20 (Sigma). Cells were stained with primary antibodies overnight at 4°C, rinsed by PBS, and incubated with secondary antibodies 1 h at RT48. Cells at different stages of differentiation were fixed and stained with primary and secondary antibodies. Primary and secondary antibodies used for characterization of iPS and derived cells were: SSEA-1, SSEA-4, TRA-1–60, TRA-1-81 (Millipore #SCR001), OCT4 (Cell Signaling Technology #2750), SOX2 (Cell Signaling Technology #2748), KLF4 (Abcam #ab26648), NANOG (Abcam #ab21624), mouse anti-SOX17 (R&D Systems #MAB1924), rabbit anti-HNF3 beta/FOXA2 (Millipore #07-633), rabbit anti-PDX1 (Santa Cruz Biotechnology #sc-25403), rabbit anti-NGN3 (Millipore #AB5684), rabbit anti-NEUROD1 (Abcam #16508), mouse anti-insulin (Sigma #I2018), rabbit anti-C-peptide (Cell Signaling Technology #4593), rabbit anti-Insulin (Cell Signaling Technology #4590), mouse anti-proinsulin C-peptide (Millipore #CBL94), mouse anti-glucagon (Abcam #ab10988), MafA (Santa Cruz Biotechnology #sc-66958), and rabbit anti-somatostatin (Dako #A0566). Texas Red-conjugated donkey-anti-rabbit IgG (Jackson Laboratories #711-075-152), Texas Red conjugated donkey-anti-mouse IgG (Jackson Laboratories #715-075-151), FITC conjugated donkey-anti-rabbit IgG (Jackson Laboratories #711-095-152), and FITC conjugated donkey-anti-mouse IgG (Jackson Laboratories #715-095-151) were used as secondary antibodies. DAPI was used for counterstaining. Stained cells were analyzed using confocal laser-scanning microscopy (Zeiss, LSM 510 confocal scanning laser system). Alkaline phosphatase staining was performed with an Alkaline Phosphatase Detection Kit (Millipore) as described48.

**Spontaneous Differentiation**

For spontaneous differentiation, iPS clones were dissociated using collagenase IV and plated on low adhesion plates in basal HEScGRO medium (SCM 021) without bFGF. Embryoid bodies (EBs) were cultured as suspension for 7–14 days and were adherent in knockout DMEM with 20% FBS for an additional 7–14 days. For immunofluorescence analysis, cells were fixed and stained48. Primary antibodies were: FOXA2 for endoderm, beta III tubulin (Abcam #41489) for ectoderm and CD31 (Santa Cruz Biotechnology #5C1506) for mesoderm, while Texas Red-conjugated donkey-anti-rabbit IgG (Jackson Laboratories #711-075-152) was used for counterstaining.
### Teratoma Formation and Analysis

Teratoma formation assay was performed using an Institutional Animal Care and Use Committee approved protocol. iPS cells were injected subcutaneously into the flank skin of 2–3 months old athymic nude mice at 500,000 cells/50 µl medium. Tumor growth was observed 4–6 weeks after injection. Tumors were processed by rapid freezing, cut as cryosections, and stained with hematoxylin and eosin dyes.

### In Vitro Differentiation of Human iPS Cells to Insulin-Secreting Islet-like Clusters

At the first step of differentiation, human iPS clones were treated with 25 ng/ml Wnt3a (R&D systems) and 100 ng/ml activin A (Peprotech) in advanced RPMI (A-RPMI, Invitrogen) with Pen/Strep for 1 day, followed by treatment with 100 ng/ml activin A in A-RPMI supplemented with 0.2% FBS (Invitrogen) for two days. At step two, cells were cultured in A-RPMI medium containing 50 ng/ml FGF10 (R&D systems), 0.25 µM KAADCyclopamine (CYC), and 2% FBS for 2 days. Cells were then treated with 50 ng/ml FGF10, 0.25 µM CYC, and 2 µM all-trans Retinoic Acid (RA) (Sigma) in DMEM (Invitrogen) supplemented with Pen/Strep, 1x B27 supplement (Invitrogen) for 4 days at step three. Cells were then cultured in the presence of 50 ng/ml FGF10, 300 nM ILV (Axxora) and 55 nM GLP-1 (Sigma) in DMEM with 1x B27 for 4 days at step four. In step five, differentiation medium included 10 µM DAPT (Sigma) and 55 nM GLP-1 in DMEM with 1x B27 and culture lasted 6 days. Finally, in step six, cells were cultured in the presence of 50 ng/mL hepatocyte growth factor (HGF) (R&D systems), 50 ng/ml Insulin-like growth factor 1 (IGF-1) (R&D systems) and 55 nM GLP-1 in CMRL-1066 medium (Invitrogen) with 1× B27 for 6 days. All experiments were repeated more than three times.

### C-peptide Content and Glucose Stimulated Secretion Assays

C-peptide release assay was performed by incubating derived islet-like clusters in Krebs-Ringer solution with bicarbonate and HEPES (KRHB; 129 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 5 mM NaHCO₃, 10 mM HEPES, 0.1% (wt/vol) BSA). Initial incubation was performed in KRHB buffer containing 2.5 mM D-glucose for 1 h at 37°C, followed by incubation in glucose stimulation conditions containing 10 mM D-glucose and 27.7 mM D-glucose for 1 h at 37°C. C-peptide or proinsulin levels were determined using an ultrasensitive C-peptide/proinsulin ELISA kit (Alpco Diagnostics).

### Flow Cytometry

Single-cell suspensions of differentiating human iPS cells were obtained by dissociating cells with TrypLE (Invitrogen # 12605) at 37°C. Intracellular antibody staining was performed using BD Cytofix/Cytoperm and BD Perm/Wash buffer. The following antibodies were used: mouse-anti-SOX17 (R&D Systems #MAB1924), guinea pig-anti-insulin (Dako Cytomation #A0564), and goat-anti-mouse Alexa Fluor 488 (Invitrogen #A11029), donkey-anti-guinea pig-Cy5 (Jackson ImmunoResearch Laboratories).
Flow cytometry data were acquired on a Becton Dickinson FACS Calibur and analyzed using Flowjo software.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of Human iPS Clones from BJ and HCF Fibroblasts

(A) Lentiviral vector-mediated delivery of OCT3/4, SOX2, KLF4 and c-MYC resulted in iPS-like colony formation. (i) SNL feeder cells, (ii) uninfected HCF fibroblasts, (iii) HCF-derived iPS-like colony at two weeks post-infection, (iv) iPS-like cells with high magnification. iPS cells exhibited morphology similar to human ES cells, characterized by large nuclei and scant cytoplasm, (v) uninfected BJ fibroblasts, (vi) BJ fibroblasts-derived iPS-like colony at two weeks after infection, (vii) image of a BJ-derived clone expanded on feeder cells, (viii) high magnification image of BJ-derived clone. (B) Feeder-free generation
of human iPS cells allowed visualization of the early reprogramming events. (i) Uninfected BJ fibroblasts, (ii) an early stage iPS-like colony in vector-transduced BJ cells one week after infection, (iii) high magnification image of BJ fibroblast-derived iPS-like colony. (C) Morphology of iPS clones cultured under feeder-free conditions. BJ#SA was established on SNL feeder cells, while HCF#1 and BJ#1 were derived feeder-free. (D) HCF#1, BJ#SA and BJ#1 cultured under feeder-free conditions expressed high levels of alkaline phosphatase (AP).
Figure 2. Expression of Pluripotency-Associated Genes in Putative iPS Clones

(A) (B) HCF- and BJ-derived iPS clones were analyzed for expression of pluripotency markers by immunostaining. HCF#1 and BJ#SA cells were positive for pluripotency markers SSEA4, TRA-1-60, TRA-1-81, OCT4, SOX2, KLF4 and NANOG, while no notable staining was observed for SSEA1. Cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Control (m) and Control (r); control cells treated with FITC-conjugated secondary antibodies against mouse IgG and rabbit IgG. Scale bars indicate 20 µm.

(C) HCF- and BJ-derived iPS-like clones were analyzed for pluripotency-associated gene expression by RT-PCR. Total cellular RNA from parental BJ and HCF fibroblasts and no template (water) samples were included as controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcript was amplified as an internal RNA control.
Figure 3. Spontaneous Differentiation of HCF- and BJ Fibroblast-derived iPS Cells into Cells of Three Embryonic Germ Layers

(A) In vitro differentiation of HCF#1, BJ#1 and BJ#SA clones in suspension culture as embryoid bodies (EB) was followed by monolayer culture for spontaneous differentiation. HCF#1, BJ#1 and BJ#SA clones generated EBs with varying sizes. Cells of ectoderm, endoderm and mesoderm lineages were confirmed by beta III tubulin (green), FOXA2 (red) and CD31 (PECAM-1) (green), respectively. Cells were counterstained with DAPI. Scale bars on right 50 µm and left 20 µm (B) Teratoma formation. iPS cells 500,000 were injected subcutaneously into athymic nude mice. Tumor growth was detected only from sites injected with iPS cells. After 3 months tumors were harvested. Scale bar indicates 2 mm. (C) H&E staining of teratoma sections demonstrated multiple lineages within the complex architecture of the tumor, including ectoderm (glandular tissue), endoderm (adipose tissue) and mesoderm (muscular tissue) tissues.
Figure 4. Differentiation of Human iPS Cells into Pancreatic Endoderm Cells

(A) Schematic representation of the stepwise differentiation protocol for generation of islet-like clusters from human iPS cells. DE, definitive endoderm; GTE, gut tube endoderm; PP, pancreatic progenitor; EN, endocrine hormone expressing cells. CYC, KAAD-cyclopamine; RA, all-trans retinoic acid; ILV, indolactam V; HGF, hepatocyte growth factor; IGF, insulin like growth factor; GLP-1, glucagon-like peptide-1. (B) Induction of definitive endoderm cells. iPS cells were treated with activin A and Wnt3a for one day, followed by activin A with 2% FBS for two days. iPS-derived cells were immunostained with antibodies against...
SOX17 (green) and FOXA2 (red). Cells were counterstained by DAPI. Bars indicate 20 µm.

(C) Flow cytometric analyses of iPS-derived definitive endoderm cells. iPS-derived definitive endoderm cells were dissociated and stained with anti-SOX17 antibody. Staining with the secondary antibody alone was used as a control. (D) iPS-derived definitive endoderm cells were treated with FGF10, CYC, RA and ILV for induction of pancreatic endoderm and immunofluorescence analysis was performed to detect, PDX1 (red), NEUROD1 (red) and NGN3 (red).
Figure 5. Successful Differentiation of Human iPS Cells into Pancreatic Hormone-expressing Cells

(A) Induction of stage-specific pancreatic genes through guided differentiation. RT-PCR analysis was performed to determine the expression of key pancreatic genes at different stages of differentiation. Undifferentiated human iPS cells (d0), definitive endoderm cells after treatment with activin A and Wnt3a (d3), foregut endoderm cells induced with FGF10 and CYC (d9), pancreatic endoderm cells were generated after exposure with FGF10, RA, CYC and ILV (d18) and islet-like clusters in presence of HGF, IGF, and GLP-1. Human pancreas RNA was used as a positive control. No template (water) was included as negative control.

(B) Down-regulation of pluripotency-associated genes upon differentiation. RT-PCR analysis was performed to analyze the expression of pluripotency genes (c-MYC, GDF3, hTERT and NANOG) after stepwise differentiation. The same RNA samples as Fig. 5A were used.

(C) Formation of islet-like clusters in HCF#1-derived cells upon differentiation. iPS-derived pancreatic endoderm were differentiated into islet-like cells with HGF, IGF, DAPT and GLP-1. Prominent islet-like cluster formation was observed in HCF#1-derived cells.

(D) Islet-like clusters expressed high levels of human C-peptide. (E) Detection of pancreatic hormones insulin, C-peptide and glucagon in iPS-derived islet-like cells. Immunofluorescence analysis identified iPS-derived islet-like cells which expressed insulin (green), C-peptide (red) and glucagon (red).
Figure 6. Sustained PDX1 Expression and Glucose-Responsive C-Peptide Secretion by iPS-derived Islet-like Cells

(A) iPS-derived islet-like cells demonstrated beta cell characteristics. (i) Double-staining of iPS-derived islet-like cells revealed co-localization of insulin (green) and C-peptide (red), indicating de novo insulin synthesis. (ii) Some cells were double-positive for insulin (green) and somatostatin (red). (iii) Sustained PDX1 expression (red) in the iPS-derived insulin-producing cells after differentiation. Cells were counterstained with DAPI. (B) Flow cytometric analysis of iPS-derived islet-like cells for insulin expression.
like clusters were dissociated with TrypLE, and analyzed for insulin expression by an anti-human insulin antibody. Insulin staining was observed in HCF#1-derived islet-like clusters. (C) Glucose-responsive C-peptide secretion by the iPS-derived islet-like clusters. The islet-like clusters were sequentially exposed to low (2.5 mM), intermediate (10 mM) and high concentrations (27.7 mM) of glucose. Supernatants of HCF#1-derived islet-like cells were collected and analyzed for C-peptide secretion by ELISA. Error bars indicate standard deviation. (D) Glucose-responsive C-peptide secretion by HCF#1-derived islet-like clusters generated with pancreatogenic cocktails including GLP-1 and ILV (left), GLP-1 without ILV (middle) or ILV without GLP-1 (right). The islet-like clusters were sequentially exposed to low (2.5 mM), intermediate (10 mM) and high concentrations (27.7 mM) of glucose. Cumulative C-peptide secretion upon glucose stimulation with 10 mM and 27.7 mM were shown. Error bars indicate standard deviation.