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Generation of Insulin-secreting Islet-like Clusters from Human Skin Fibroblasts*[S]◆

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Increasing evidence suggests that islet cell transplantation for patients with type I diabetes holds great promise for achieving insulin independence. However, the extreme shortage of matched organ donors and the necessity for chronic immunosuppression has made it impossible for this treatment to be used for the general diabetic population. Recent success in generating insulin-secreting islet-like cells from human embryonic stem (ES) cells, in combination with the success in deriving human ES cell-like induced pluripotent stem (iPS) cells from human fibroblasts by defined factors, have raised the possibility that patient-specific insulin-secreting islet-like cells might be derived from somatic cells through cell fate reprogramming using defined factors. Here we confirm that human ES-like iPS cells can be derived from human skin cells by retroviral expression of OCT4, SOX2, c-MYC, and KLF4. Importantly, using a serum-free protocol, we successfully generated insulin-producing islet-like clusters (ILCs) from the iPS cells under feeder-free conditions. We demonstrate that, like human ES cells, skin fibroblast-derived iPS cells have the potential to be differentiated into islet-like clusters through definitive and pancreatic endoderm. The iPS-derived ILCs not only contain C-peptide-positive and glucagon-positive cells but also release C-peptide upon glucose stimulation. Thus, our study provides evidence that insulin-secreting ILCs can be generated from skin fibroblasts, raising the possibility that patient-specific iPS cells could potentially provide a treatment for diabetes in the future.

Diabetes is a life-long disease defined by hyperglycemia. Although type I diabetes is caused by autoimmune destruction of the pancreatic endocrine beta cells of the patient, type II diabetes results from insulin resistance. Although current treatment for type I diabetes is obliged to rely on insulin injection, due to the difficulties in adjusting the amounts of insulin administration to accommodate fluctuations in physiological conditions, this treatment often results in episodes of hypo- and hyperglycemia. Even under strictly controlled conditions, such treatment delays but does not always prevent development of multiple complications including microvascular disease, diabetic retinopathy, nephropathy, and neuropathy (1).

Regeneration of insulin-producing beta cell as well as islet transplantation are the most promising long term solutions for diabetes treatment (1). For example, clinical trials with islet cell transplantation from cadaver donors have resulted in promising outcomes (2–4). Insulin independence with good glycemic control was achieved and sustained in some patients for more than 2 years. Although promising, this approach faces multiple challenges. A major obstacle is the shortage of donors when compared with the large patient population. In addition, because the low yield of islet cells from cadaver tissue, it requires large number of donor cells to generate sufficient insulin-producing beta cells that are capable of producing and releasing adequate amounts of insulin in response to normal physiological signals (2–4). Furthermore, chronic immunosuppression is necessary after allograft transplantation. These factors have made it impossible for this treatment to be used for the general diabetic population.

Although the recurring autoimmune response has to be prevented to avoid destruction of the transplanted cells, derivation of patient-specific islet-like cells from adult tissues may allow both shortage of organ donors and allograft rejection to be resolved. However, generation of functional islet-like clusters (ILCs)[4] from adult progenitor cells including pancreatic progenitor cells has been tested with limited success (5–7). When compared with adult progenitor cells, human ES cells remain the most promising source for ILC generation due to their unlimited replicative capacity and differentiation potentials. Recent studies have proved that human ES cells can be differentiated into functional islet-like clusters using an *in vitro* differentiation protocol, although the efficiency is relatively low (8). In addition, transplantation of pancreatic endoderm derived from human ES cells *in vitro* can rescue mice with induced insulin deficiency (9). However, the application of human ES cell-derived ILC was largely restricted by generation of patient specific ES cells. Recent demonstrations that human ES cell-like induced pluripotent stem (iPS) cells can be generated from adult human somatic cells by enforced expression of defined transcription factors (10–14) have raised the possibility that genetically matched insulin-secreting ILCs can be derived

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[4] The abbreviations used are: ILC, islet-like clusters; ES, embryonic stem; hES, human ES; iPS, induced pluripotent stem; HESM, human embryonic stem cells medium; MEF, mouse embryonic fibroblast; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; DAPI, 4′,6-diamidino-2-phenylindole; RT-PCR, reverse transcription-PCR; H&E, hematoxylin and eosin.

from somatic cells through differentiation of patient-specific iPS cells.

Here we confirm that human ES-like iPS cells can be derived from skin cells by retroviral expression of OCT4, SOX2, c-MYC, and KLF4. Importantly, using a serum-free *in vitro* protocol, we successfully generated insulin-producing ILCs from the iPS cells. Our studies thus raise the possibility that patient-specific iPS cells could potentially provide an alternative source for drug screen, study for disease mechanisms, and cell therapy for diabetes in the future.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Retroviruses Production—The human Oct4, Sox2, Klf-4, and c-Myc cDNAs were PCR-amplified from expressed sequence tag clones and cloned into pMXs at blunted BstXI sites (15). All sequences were confirmed by DNA sequencing. To generate retroviruses expressing the four factors, pGag-pol, pVSVG, and pMXs were co-transfected into 293T cells on one 6-well plate at cell density of 8.0×10^5 cells/well using SuperFect (Qiagen) according to the manufacturer's instructions. Six hours after transfection, the medium was changed to complete Dulbecco's modified Eagle's medium medium. The viruses were harvest 48 and 72 h after transfection. The harvested viruses were filtered through 0.45-μm filter membrane (Millipore) and concentrated by spin column (Millipore).

Generation and Culture of Human iPS Cells—A total of 8×10^5 human foreskin fibroblast HFF-1 cells on one 10-cm plate were transduced with concentrated retroviruses with Polybrene (8 μg/ml) two times sequentially. Four days after the second transduction, 5×10^4 cells were transferred onto mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder cells. On the second day, the medium was changed to HESM (Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) supplemented with 20% knock-out serum replacement and supplemented with basic fibroblast growth factor (10 ng/ml)). Colonies began to appear 9 days after the second transduction and were picked based on their morphology on days 16, 23, and 30 after transduction. iPS cell lines and the human ES cell line H9 (WiCell) were maintained as described previously (WiCell protocols) on mitomycin-treated primary MEF in HESM (Invitrogen) containing nonessential amino acids, L-glutamine, 2-mercapthoethanol, penicillin-streptomycin, and 10 ng/ml basic fibroblast growth factor (Peprotech) or on fibronectin (Sigma) using MEF-conditioned HESM. iPS cells were initially propagated manually and by enzymatic digestion with collagenase IV (Invitrogen) at later passages.

Immunostaining and TUNEL Assay—iPS cells cultured on MEFs were fixed with 4% paraformaldehyde overnight at 4 °C, permeabilized with 0.4% Triton X-100 for 15 min, and blocked in 1% goat serum in phosphate-buffered saline for 2 h. Cells were incubated with primary antibodies overnight at 4 °C. After being washed with phosphate-buffered saline, they were incubated with Alexa Fluor (Invitrogen) secondary antibodies and DAPI for 1 h. SSEA-1, SSEA-4, TRA-1–60, SOX2, and OCT4 antibodies were obtained from Millipore. Alkaline phosphatase staining was done according to the manufacturer's recommendations (Millipore). For immunostaining of differentiated cells, cell clusters were fixed in 4% paraformaldehyde at room temperature for 30 min, permeabilized in 0.25% Triton X-100, and blocked with sera/phosphate-buffered saline at room temperature for 1 h. The cells were incubated with primary antibodies overnight at 4 °C. The sources of the primary antibodies are: mouse anti-SOX17 (R&D), goat anti-Foxa2 (R&D), goat anti-PDX1 (AbCam), mouse anti-C-peptide (AbCam), and rabbit anti-glucagon (Zymed Laboratories Inc.). Fluorescein isothiocyanate- or rhodamine-labeled second antibody was used, and DAPI or Draq5 were used for nuclear staining. Images were captured under a fluorescent microscope or by confocal microscope (Carl Zeiss). The TUNEL assay of the islet-like clusters under high glucose was performed using an ApopTag kit (Chemicon), and the clusters were double-stained with anti-C-peptide antibody.

Real-time RT-PCR and Bisulfite Sequencing Analysis—Total RNA from H9 and iPS cells cultured on fibronectin or under the differentiation conditions were isolated using the RNeasy kit (Qiagen). Following DNase I treatment, reverse transcription was performed using the ImProm-II reverse transcription system (Promega). Real-time PCR reactions were performed with SYBR GreenER mix (Invitrogen) using primer sets listed in supplemental Table 1. Relative gene expression levels were normalized to actin mRNA. Bisulfite sequencing was performed as described previously with minor modifications (16). Five micrograms of sodium bisulfite-treated DNA samples was subjected to nested PCR amplification. The PCR products were subcloned using the TA cloning kit (Invitrogen). All PCR and subcloning reactions were performed in duplicate for each sample. The clones were sequenced using an M13 reverse primer.

Teratoma Formation and Analysis—Teratoma formation was completed using an Institutional Animal Care and Use Committee approved protocol. Approximately 2×10^6 iPS cells cultured on MEFs were collected manually by scrapping, resuspended in a mixture of HESM and Matrigel (ratio of 1:1), and injected subcutaneously into the hind skin flap or intramuscularly into the hind limb of 2–3-month-old immune-compromised $Rag2^{-/-}$/gamma-$C^{-/-}$ mice. Xenografted masses were observed 4–6 weeks after injection and dissected 6 weeks after injection. Samples were fixed in 4% paraformaldehyde, embedded in paraffin, and processed for hematoxylin and eosin and immunofluorescent staining. The antibodies against the following proteins were used for immunostaining: nestin (BD Biosciences), cytokeratin 19 (AbCam), α-smooth muscle actin (Sigma), and smooth muscle myosin (Biomedical Technologies).

In Vitro Differentiation of Insulin-secreting Islet-like Clusters—*In vitro* differentiation of insulin-secreting islet-like cluster cells was performed using a protocol described previously with slight modifications (8). Undifferentiated iPS and hES cells were cultured in RPMI 1640 supplemented with B27 (Invitrogen), 11.1 mM glucose, and 4 nM activin A (Peprotech) for 7 days. Sodium butyrate was added on day 1 at a final concentration of 0.1 mM (stage 1). After stage 1, the cells were dissociated with treatment of 1 μg/ml collagenase IV at 37 °C for 5 min and transferred into ultra-low attachment six-well plates (Corning). The cell aggregates were cultured in RPMI 1640 supplemented

with B27, 20 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 100 ng/ml noggin (Peprotech) for 2 weeks (stage 2). At stage 3, cell clusters were cultured in suspension in RPMI 1640 supplemented with B27, 20 ng/ml epidermal growth factor, and 100 ng/ml Noggin (Peprotech) for 1 week. Finally, the cells were incubated in RPMI 1640 medium with 0.5% bovine serum albumin, 10 mM nicotinamide, and 50 ng/ml insulin-like growth factor II for 5 days and without insulin-like growth factor II for another 2 days (stage 4).

Glucose-stimulated Insulin Release Assay—Insulin secretion analysis was performed as described previously (8). Cell clusters after stage 4 were attached to a Matrigel-coated plate and were washed with glucose-free RPMI 1640 medium. The cells were first incubated with RPMI 1640 medium containing 2.8 mM glucose for 3 h, at which point the supernatant was collected. The cells were then treated with RPMI 1640 medium containing 40 mM glucose for another 3 h. The C-peptide levels in the supernatants were measured using the Mercodia ultrasensitive human C-peptide enzyme-linked immunosorbent assay kit, and the total amount of C-peptide in the supernatants was normalized according to the DNA content of islet-like clusters.

RESULTS

Generation of iPS Cells from Human Foreskin Fibroblasts—Recent studies indicate that ES-like pluripotent iPS cells can be generated through ectopic expression of defined transcription factors in differentiated mouse and human cells (10–14, 17–20). Although human iPS cells are capable of generating all three germ layers in mice, whether they can differentiate into cells of pancreatic endocrine lineages is not known. To address this question, we attempted to generate human iPS cells by retroviral expression of human *Oct4, Sox2, Klf4*, and *c-Myc* in the HFF-1 human foreskin fibroblast cells. To this end, we expressed the four transcription factors in the HFF-1 foreskin fibroblasts by retroviral transduction. Colonies were visible 9 days after the second transduction, and about 80–100 colonies were observed at day 16 from the initial 5×10^4 cells seeded in each 10-cm plate. A total of 26 colonies, picked on days 16, 23, and 30 after transduction, were propagated in hES cell culture medium on primary MEFs and passaged manually every 5–6 days. From the 26 colonies picked, we successfully established nine iPS cell lines (see below).

Characterization of Human iPS Cells—Each of the nine iPS cell clones exhibited and maintained morphology distinct from the original human fibroblasts and resembled hES cells for at least 14 passages in culture (Fig. 1*A* and supplemental Fig. S1). The various iPS clones propagated at a similar rate but varied in their percentage of spontaneous differentiation during culture. To confirm that these iPS cells exhibit hES-like properties, we examined a number of hES cell markers that include alkaline phosphatase activity, ES cell-specific transcription factors Sox2 and Oct4, and hES cell-specific extracellular antigens TRA-1–60 and SSEA-4. As a negative control, we also analyzed SSEA-1, a marker for differentiation of hES cells. Results shown in Fig. 1*B* and supplemental Fig. S1 demonstrate that the iPS clones exhibit hES-like properties, including high alkaline phosphatase (*AP*) activity, and positive staining for Sox2, Oct4, TRA-1–60, and SSEA-4. The majority of the colonies were negative for the SSEA-1, with a few positive cells observed on the periphery of some colonies. Staining intensity for all markers tested was similar to that observed with the well characterized hES cell line H9 (supplemental Fig. S1). The original HFF-1 fibroblast cell line was negative for these markers (supplemental Fig. S1).

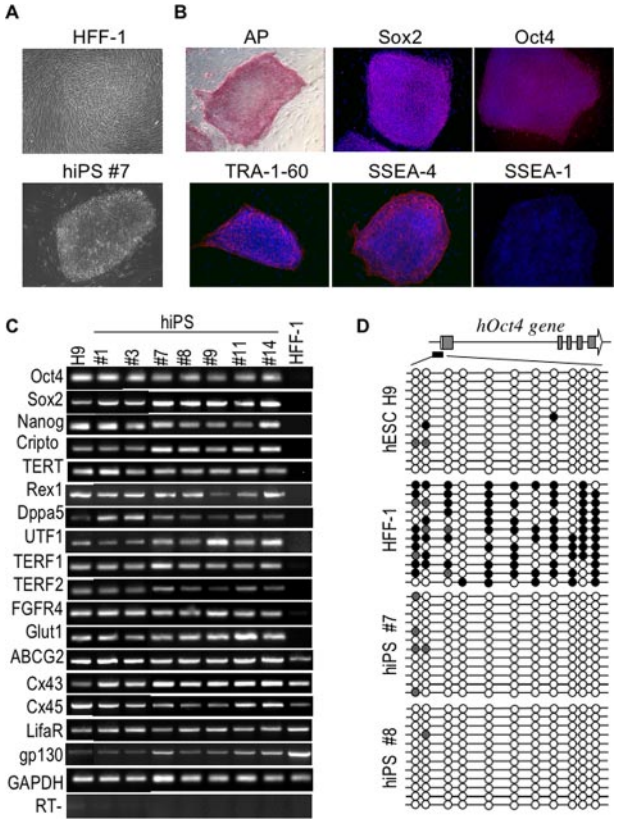

FIGURE 1. **iPS cells generated from human foreskin cells exhibit hES cell characteristics.** *A*, a phase-contrast micrograph of a representative iPS clone showing hES-cell like morphology that is distinct from the parental human foreskin fibroblast cell line HFF-1. *B*, alkaline phosphatase (*AP*) activity analysis and hES cell marker staining demonstrate ES-like features of a representative iPS clone 7. Alkaline phosphatase activity and immunostaining (*red*) of iPS cells, counterstained by DAPI (*blue*), confirmed expression of the ES cell markers throughout the colonies. Little or no staining for SSEA-1 was observed within the colonies confirming the undifferentiated state of the cells. *C*, iPS cells express genes important for hES cell maintenance. RT-PCR analysis of the gene expression profile of seven representative human iPS (*hIPS*) cell lines demonstrates a similar expression profile to that of the hES cell line H9 but different from their parental fibroblast HFF-1 cells. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *D*, the DNA methylation state of the Oct4 promoter is reprogrammed in iPS cells. The DNA methylation state of the Oct4 promoter in the hES cell line H9, the parental fibroblast cell line HFF-1, and two iPS cell lines was analyzed by bisulfite sequencing. Each *line* represents one clone. *Black*, *open*, and *gray circles* represent methylated, unmethylated, and difficult to read CG dinucleotides within the Oct4 promoter, respectively.

To assess the gene expression pattern of the iPS clones, we isolated RNA from iPS cells that were cultured under feeder-free conditions for at least two passages. All of the selected clones maintained the morphological characteristics observed for hES cell lines when cultured on fibronectin or similar matrixes (data not shown). RT-PCR analysis confirmed expression of the genes essential for hES cell maintenance, including Nanog, Crypto, telomerase (*TERT*), *Rex1*, *Dppa5*, *UTF1*, *TERF1*, *TERF2*, fibroblast growth factor receptor 4 (*FGFR4*), and *Glut1* glucose transporter. None of these genes were expressed in the original HFF-1 fibroblasts (Fig. 1*C*). Expression

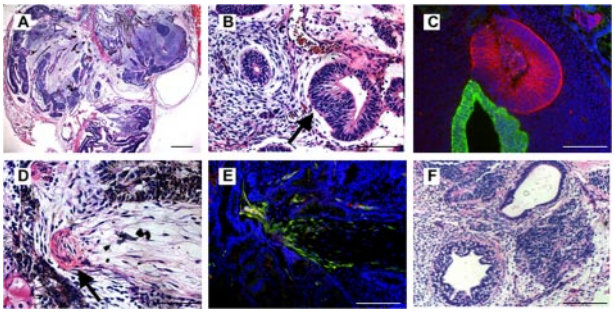

FIGURE 2. **iPS cells generated from human foreskin cells have the potential to differentiate into the three germ layers *in vivo*.** *A*, low magnification of H&E staining of a section from a representative human iPS cell-derived teratoma. Cells of different lineages were observed within the tumor section. *B* and *C*, cells of ectoderm lineage, such as the neuroprogenitor rosettes and epidermal cells suggested by H&E staining (*B*, arrow), were confirmed by nestin (*red*) and cytokeratin 19 (*green*) staining (*C*). *D* and *E*, cells of mesoderm lineage, such as the fat tissue and smooth muscle cells seen in H&E staining (*D*, *arrow*), were confirmed by α-smooth muscle actin (*red*) and myosin heavy chain (*green*) staining (*E*). *F*, H&E staining reveals gut-like and airway epithelial structure, representative of endoderm lineage.

of other genes such as *ABCG2*, *Cx43*, *Cx45*, *LifaR*, and *gp130*, which play more general roles in cell maintenance, was maintained in both iPS cells and the parental fibroblasts (Fig. 1*C*). RT-PCR analysis using primers that only amplify endogenous Sox2 and Oct4 genes confirmed activation of these loci (supplemental Fig. S2). Previous studies have established a tight connection between the efficiency of somatic cell reprogramming and the DNA methylation status of the Oct4 promoter (21). We therefore analyzed the DNA methylation status of the Oct4 promoter by bisulfite sequencing. Results shown in Fig. 1*D* demonstrate that, similar to the H9 hES cells, the promoter of Oct4 is hypomethylated in the iPS cells, which is in stark contrast to the heavy degree of methylation observed in the original HFF-1 fibroblasts, further supporting successful reprogramming.

To confirm the multipotency of the human iPS cells, we injected five lines of iPS cells subcutaneously or intramuscularly into Rag2$^{-/-}$/gamma-C$^{-/-}$ mice. Teratomas formed 4–6 weeks after injection. H&E and immunostaining of tumor sections from teratomas dissected 6 weeks after injection demonstrated the presence of cell types of all three germ layers (Fig. 2*A*), including neural and non-neural ectoderm (Fig. 2, *B* and *C*), pigmented epithelium (Fig. 2*D*), smooth muscle and fat tissue (mesoderm) (Fig. 2, *D* and *E*), and airway and gut-like epithelial tissues (endoderm, Fig. 2*F*). Collectively, the above results demonstrate that the cell lines that we generated exhibit all the features of blastocyst-derived human ES cells and are therefore qualified to be human iPS cells.

Differentiation of iPS Cells into Insulin-secreting Islet Clusters—Having confirmed the multipotency of the iPS cells, we asked whether they are capable of differentiating into the pancreatic endocrine lineage as successful generation of insulin-secreting cells has great potential in the development of regenerative therapy for diabetes. To address this possibility, we adapted a recently developed serum-free differentiation procedure from which hES cells have been successfully differentiated into insulin-secreting islet-like clusters (8). Four randomly chosen iPS cell lines, numbers 1, 3, 7, and 8, and the hES H9 cell line were subjected to the four-stage differentiation procedure outlined in Fig. 3*A*. Although all four iPS cell lines express human ES cell markers (Fig. 1, *B* and *C*, and supplemental Fig. S1), only iPS lines 3 and 7 survived the differentiation procedures, indicating that iPS lines 1 and 8 may not be truly multipotent. As reported previously, activin A and sodium butyrate treatment leads to considerable morphological change of hES cells (Fig. 3*B*, *panels a* and *d*). Similar morphological changes were also observed for the iPS cells 3 and 7 (Fig. 3*B*, *panels b* and *e* and *panels c* and *f*). After stage 2, those cells, from either hES or iPS cells, formed cellular aggregates under low attachment conditions (Fig. 3*B*, *panels g–i*). By stage 4, the aggregates became fused to each other, and some small bud-like clusters appeared (Fig. 3*B*, *panels j–l*, *arrows*). The results suggest that iPS clones 3 and 7 undergo similar morphological changes to those of hES cells in response to the pancreatic cell lineage differentiation procedure.

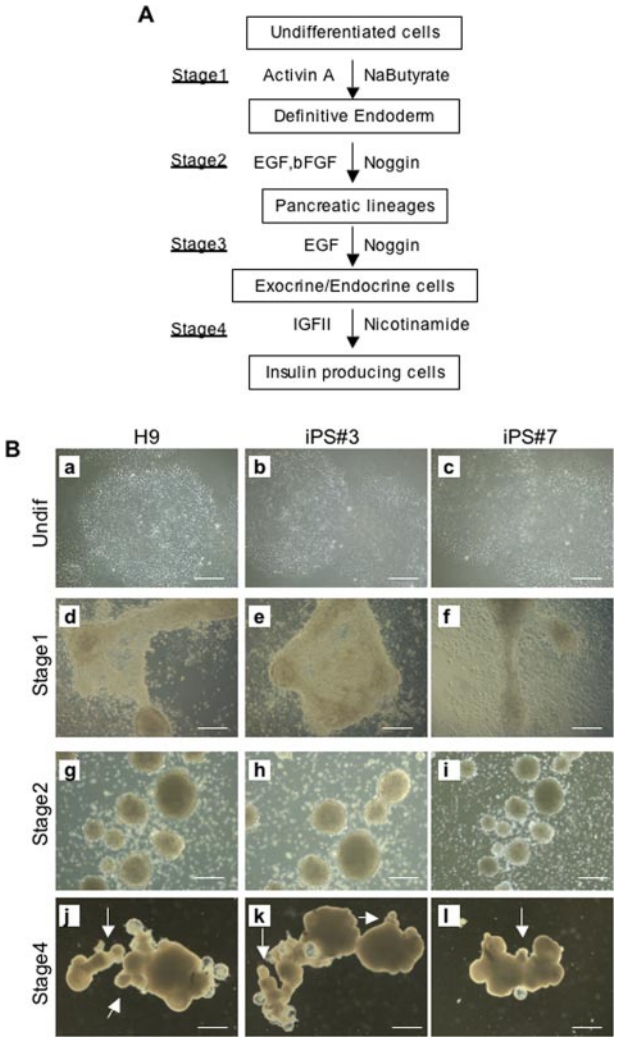

FIGURE 3. **iPS cells and hES cells exhibit similar morphological changes during islet-like cluster differentiation.** *A*, schematic representation of the procedure for generating islet-like clusters from hES and iPS cells. *bFGF*, basic fibroblast growth factor; *EGF*, epidermal growth factor; *IGF-II*, insulin-like growth factor II; *NaButyrate*, sodium butyrate. *B*, morphological changes of hES H9 cells and representative iPS cells during the differentiation procedure. *Arrows* (in *panels j–l*) indicate the budding structure in the colonies. *Bar*, 500 μm.

To determine whether these morphological changes reflect successful differentiation of both hES and iPS cells into pancre-

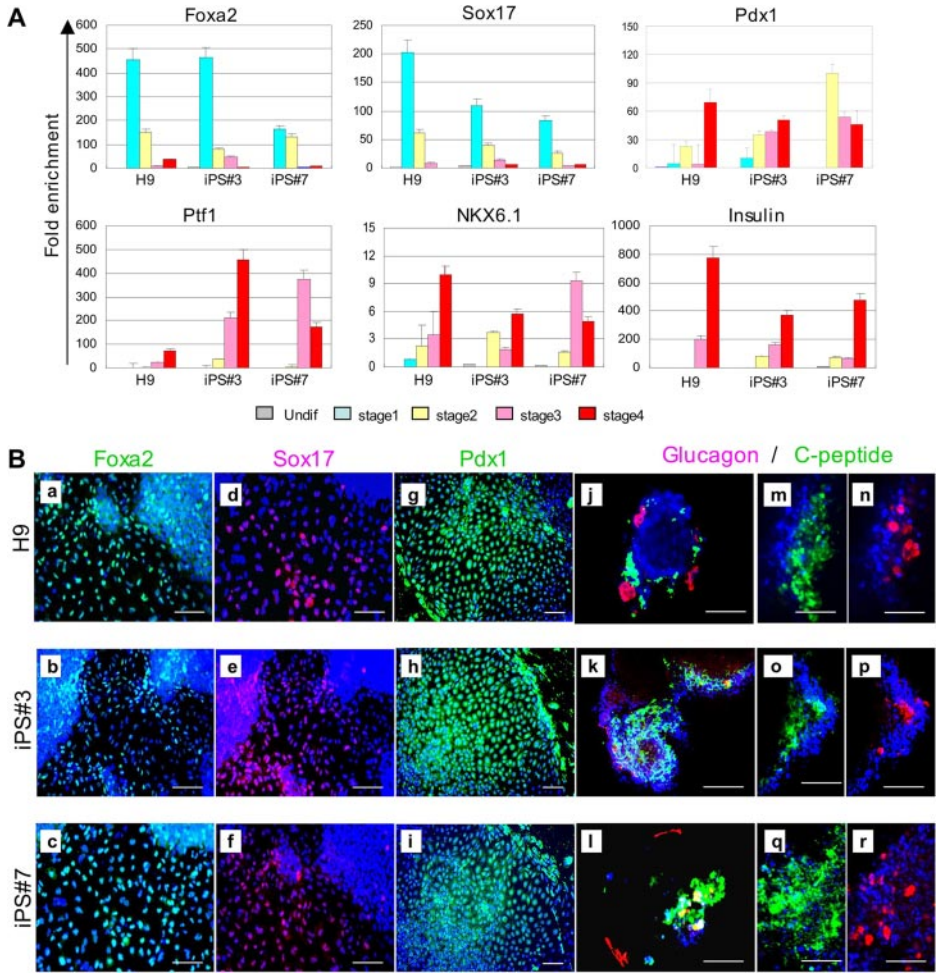

FIGURE 4. **iPS cells can be differentiated into insulin-producing islet-like clusters.** *A*, quantitative RT-PCR analysis of the expression of the various cell lineage markers at the different stages of differentiation. Relative levels of gene expression were normalized to the actin mRNA level. The value of undifferentiated (*Undif*) human ES cells is arbitrarily set as 1. *B*, immunostaining of pancreatic lineage marker proteins in hES (panels *a*, *d*, *g*, *j*, *m*, and *n*) and iPS (*panels b*, *c*, *e*, *f*, *h*, *i*, *k*, *l*, *o*, *p*, *q*, and *r*)-derived cells. *panels a–f*, stained after stage 1; *panels g–i*, stained after stage 2; *panels j–l*, C-peptide and glucagon staining after stage 4. *Panels m–r*, high magnification of C-peptide and glucagon staining. *Bar* = 500 μm (*panels a–l*); 10 μm (*panels m–r*).

atic cell lineages, we analyzed the gene expression pattern of pancreatic lineages at each stage of the differentiation process by quantitative RT-PCR. As shown in Fig. 4*A*, the definitive endoderm-specific genes, such as *Foxa2* and *Sox17*, are expressed at high levels after completion of stage 1. After treatment with basic fibroblast growth factor, epidermal growth factor, and noggin, expression of Pdx1, markers of pancreatic lineage cells, is readily detectable. At stage 3, NKX6.1, Ptf1, and insulin mRNAs are detectable, and insulin mRNA reaches the highest level at stage 4. Importantly, both iPS line 3 and iPS line 7 in general exhibited similar gene expression profiles as that of the human H9 ES cells, indicating that iPS cells and hES cells have similar differentiation potential for endoderm pancreatic lineage. To further confirm expression of key genes during the differentiation process, we analyzed protein expression of Foxa2 and Sox17 in stage 1, Pdx1 in stage 2, and C-peptide and glucagon in stage 4 by immunostaining. Results shown in Fig. 4*B* demonstrate that activin A and sodium butyrate induced the expression of Foxa2 and Sox17 in the nuclei of iPS cells as well as hES cells (Fig. 4*B*, *panels a–f*). In addition, Pdx1 protein was clearly detected in cells derived from both hES and iPS cells after stage 2 (Fig. 4*B*, *panels g–i*). Finally, both C-peptide and glucagon-positive cells were detected in the cells of stage 4 (Fig. 4*B*, *panels j–r*). Consistent with a previous report (8), the expression of pancreatic hormones was predominantly localized in the small budding clusters. Therefore, we conclude that, similar to hES cells, the iPS cells derived from foreskin fibroblasts can be differentiated into islet-like endocrine cells.

Next we performed a functional test for the differentiated islet-like clusters. To this end, we measured insulin secretion upon glucose stimulation using C-peptide enzyme-linked immunosorbent assay. At low glucose concentration (2.8 mM), levels of insulin secretion, as measured by the level of C-peptide, are comparable among islet-like cells derived from hES and iPS cells (Fig. 5*A*). At high glucose concentration (40 mM), hES cell-derived islet-like cells responded to glucose stimulation (Fig. 5*A*) (8). Importantly, islet-like cells derived from iPS clone 3 also responded to high glucose stimulation by insulin secretion, although the response from iPS clone 7 is marginal (Fig. 5*A*). Previous studies indicate that long time exposure to high concentration glucose can cause apoptosis of human islet cells (22). To exclude the possibility that the increased levels of C-peptide in the supernatant under high glucose concentration were caused by increased cell death, we examined apoptosis of insulin-positive cells by TUNEL assay. Results shown in Fig. 5*B* demonstrate that most C-peptide-positive cells were TUNEL-negative, thus ruling out the possibility that the increased release of C-peptide after high glucose treatment is related to apoptosis. Therefore, we conclude that iPS cells, like hES cells, have the potential to differentiate into functional islet-like clusters *in vitro*, although clonal variation exists. We note that although the two iPS lines used in our differentiation protocol exhibited no noticeable difference from the H9 hES cells in terms of marker gene expression and teratomas formation, the islet-like clusters generated from them exhibited quantitative differences in glucose response. It is possible that variation in transgene copy number and/or their degree of silencing (supplemental Fig. S2) might contribute to the observed differences. However, more extensive studies are needed to determine the basis of the variation. We note that similar variations in the differentiation potential of different hES cell lines have been reported (23, 24).

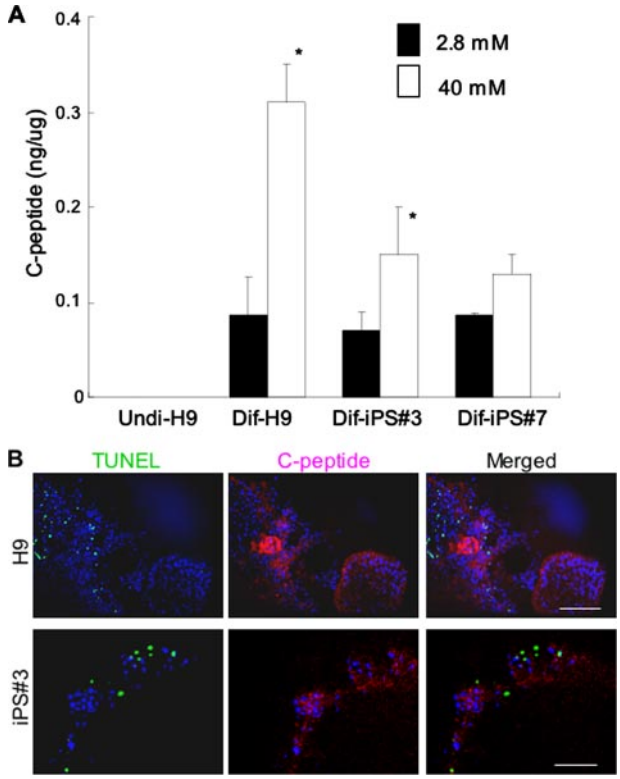

FIGURE 5. **The iPS-derived islet-like cluster secretes insulin upon glucose stimulation.** *A*, iPS-derived islet-like cluster responses to glucose-stimulated insulin secretion. The islet-like clusters were sequentially treated with low (2.8 mM, *black bar*) and high (40 mM, *white bar*) concentrations of glucose, and the supernatants were collected and analyzed for C-peptide concentration by enzyme-linked immunosorbent assay. The amount of C-peptide was normalized to DNA content. Data were collected in triplicate. *undi-H9* and *dif-H9* indicate undifferentiated H9 and H9-derived islet-like clusters, respectively. *, $p < 0.05$. *B*, TUNEL analysis of islet-like clusters derived from H9 (*upper*) and iPS (*lower*) cells under high concentration of glucose. *Green*, TUNEL-positive cells; *red*, C-peptide-expressing cells.

DISCUSSION

Recent success in generating ES cell-like pluripotent iPS cells from somatic cells using defined factors has provided a complementary method to somatic nuclear transfer for nuclear reprogramming (10–13, 17–20). This technical breakthrough has significant implications for overcoming the ethical issues associated with hES cell derivation from human embryos. In addition, it also makes the generation of patient-specific multipotent stem cells possible. Despite the fact that several safety issues, such as oncogenic and viral integration, which has been associated with genome instability, must be overcome before iPS technology can be used in patients, the implication of this technology in replacement therapy is far reaching.

Among all the diseases that can be potentially treated by cell therapy, type I diabetes is one of the most attractive candidates as pancreatic transplantation or islet infusion has shown great promise in achieving independence from insulin injection (2–4). For this reason, we tested the differentiation potential of human iPS cells into insulin-producing ILCs. We demonstrated that the iPS cells derived from human skin cells can be differentiated into pancreatic ILCs. Our results provided evidence that functional endocrine cells can be generated *in vitro* from human somatic cells. Given that islet cell transplantation therapy has been proven to be beneficial to patients with type I diabetes (2, 3), generation of patient-specific iPS cells and subsequent differentiation of these iPS cells into insulin-secreting cells may prove to be a potential cell source for transplantation therapy. However, before this approach can become practical, several obstacles must be overcome. First, although the iPS cells and human ES cells have comparable efficiency of differentiation into insulin-secreting islet clusters, the general efficiency is extremely low. In addition, the levels of C-peptide secreted by *in vitro* differentiated islet-like cells from both iPS cells and human ES cells are much lower when compared with that of adult human β-cells. The low efficiency of these two aspects is caused by the limitation of current *in vitro* differential protocol but not the intrinsic properties of the iPS cells. For this reason, it is essential to establish efficient differentiation procedures that significantly increase the formation of insulin-secreting cells and their response to glucose. One approach that has shown great promise is *in vivo* maturation of endocrine cells through transplantation of *in vitro* differentiated pancreatic endoderm cells (9). Second, since type I diabetes is an autoimmune disease, the iPS-derived insulin-producing cells can be attacked by the autoreactive T cells. Although the immunological problem is serious, the self-renewal capacity of iPS cells might partially address the continuous retreatment of the disease by the availability of autologous iPS-derived cells without sensitization by donor tissues, which is one of the major problems in repeated infusion of islets derived from multiple donors (25). Third, the obvious obstacles for iPS cell-based therapy that are associated with the reprogramming process, such as the introduction of the oncogenes and possible mutations due to the use of integrating viral vectors, must also be overcome. Given the amazing progress that has been made in the past 2 years, we anticipate that it will not be long before these obstacles are resolved.

It is worth noting that although individual iPS cell clones showed no significant difference in cell morphology, expression of pluripotent marker genes, or DNA demethylation at the Oct4 promoter, we observed clonal variability in their potential to differentiate into the pancreatic lineage cells. Of the four iPS cell clones that we tested in the same differentiation procedure, two iPS clone failed to maintain islet-like cell aggregates (data not shown). The clonal variability in terms of differentiation potential indicates that not all human iPS clones are fully pluripotent although they passed the standard tests. This raises the question regarding the criteria for "true" human iPS cells. Because the differentiation protocol used in this study has clearly defined developmental stages, we will be able to define the specific blockage of the differentiation process for some of the "incompletely" reprogrammed clones. This information, in combination with other factors, such as epigenetic modifications and microRNA profiling, might help us to establish the criteria for true human iPS cells with full differentiation potential.

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REFERENCES

1. Efrat, S. (2008) *Adv. Drug Delivery Rev.* **60,** 114–123
2. Lakey, J. R., Mirbolooki, M., and Shapiro, A. M. (2006) *Methods Mol. Biol.* **333,** 47–104
3. Shapiro, A. M., Ricordi, C., Hering, B. J., Auchincloss, H., Lindblad, R., Robertson, R. P., Secchi, A., Brendel, M. D., Berney, T., Brennan, D. C., Cagliero, E., Alejandro, R., Ryan, E. A., DiMercurio, B., Morel, P., Polonsky, K. S., Reems, J. A., Bretzel, R. G., Bertuzzi, F., Froud, T., Kandaswamy, R., Sutherland, D. E., Eisenbarth, G., Segal, M., Preiksaitis, J., Korbutt, G. S., Barton, F. B., Viviano, L., Seyfert-Margolis, V., Bluestone, J., and Lakey, J. R. (2006) *N. Engl. J. Med.* **355,** 1318–1330
4. Gangemi, A., Salehi, P., Hatipoglu, B., Martellotto, J., Barbaro, B., Kuechle, J. B., Qi, M., Wang, Y., Pallan, P., Owens, C., Bui, J., West, D., Kaplan, B., Benedetti, E., and Oberholzer, J. (2008) *Am. J. Transplant.* **8,** 1250–1261
5. Sapir, T., Shternhall, K., Meivar-Levy, I., Blumenfeld, T., Cohen, H., Skutelsky, E., Eventov-Friedman, S., Barshack, I., Goldberg, I., Pri-Chen, S., Ben-Dor, L., Polak-Charcon, S., Karasik, A., Shimon, I., Mor, E., and Ferber, S. (2005) *Proc. Natl. Acad. Sci. U. S. A* **102,** 7964–7969
6. Suzuki, A., Nakauchi, H., and Taniguchi, H. (2003) *Proc. Natl. Acad. Sci. U. S. A* **100,** 5034–5039
7. Zalzman, M., Anker-Kitai, L., and Efrat, S. (2005) *Diabetes* **54,** 2568–2575
8. Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G., and Majumdar, A. S. (2007) *Stem Cells* (*Durham*) **25,** 1940–1953
9. Kroon, E., Martinson, L. A., Kadoya, K., Bang, A. G., Kelly, O. G., Eliazer, S., Young, H., Richardson, M., Smart, N. G., Cunningham, J., Agulnick, A. D., D'Amour, K. A., Carpenter, M. K., and Baetge, E. E. (2008) *Nat. Biotechnol.* **26,** 443–452
10. Lowry, W. E., Richter, L., Yachechko, R., Pyle, A. D., Tchieu, J., Sridharan, R., Clark, A. T., and Plath, K. (2008) *Proc. Natl. Acad. Sci. U. S. A* **105,** 2883–2888
11. Park, I. H., Zhao, R., West, J. A., Yabuuchi, A., Huo, H., Ince, T. A., Lerou, P. H., Lensch, M. W., and Daley, G. Q. (2008) *Nature* **451,** 141–146
12. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) *Cell* **131,** 861–872
13. Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II, and Thomson, J. A. (2007) *Science* **318,** 1917–1920
14. Mali, P., Ye, Z., Hommond, H. H., Yu, X., Lin, J., Chen, G., Zou, J., and Cheng, L. (2008) *Stem Cells* (*Durham*) **26,** 1998–2005
15. Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T., and Kumagai, H. (2003) *Exp. Hematol.* **31,** 1007–1014
16. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) *Nucleic Acids Res.* **22,** 2990–2997
17. Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K., and Hochedlinger, K. (2007) *Cell Stem Cell* **1,** 55–70
18. Okita, K., Ichisaka, T., and Yamanaka, S. (2007) *Nature* **448,** 313–317
19. Takahashi, K., and Yamanaka, S. (2006) *Cell* **126,** 663–676
20. Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E., and Jaenisch, R. (2007) *Nature* **448,** 318–324
21. Simonsson, S., and Gurdon, J. (2004) *Nat. Cell Biol.* **6,** 984–990
22. Federici, M., Hribal, M., Perego, L., Ranalli, M., Caradonna, Z., Perego, C., Usellini, L., Nano, R., Bonini, P., Bertuzzi, F., Marlier, L. N., Davalli, A. M., Carandente, O., Pontiroli, A. E., Melino, G., Marchetti, P., Lauro, R., Sesti, G., and Folli, F. (2001) *Diabetes* **50,** 1290–1301
23. Osafune, K., Caron, L., Borowiak, M., Martinez, R. J., Fitz-Gerald, C. S., Sato, Y., Cowan, C. A., Chien, K. R., and Melton, D. A. (2008) *Nat. Biotechnol.* **26,** 313–315
24. Wu, H., Xu, J., Pang, Z. P., Ge, W., Kim, K. J., Blanchi, B., Chen, C., Sudhof, T. C., and Sun, Y. E. (2007) *Proc. Natl. Acad. Sci. U. S. A* **104,** 13821–13826
25. Campbell, P. M., Senior, P. A., Salam, A., Labranche, K., Bigam, D. L., Kneteman, N. M., Imes, S., Halpin, A., Ryan, E. A., and Shapiro, A. M. (2007) *Am. J. Transplant.* **7,** 2311–2317