Supporting Information

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SI Procedures for the Production and Characterization of iPS Cells

iPS Cell Generation and Culture. Normal fibroblasts from GFP transgenic mice [C57BL/6-Tg(UBC-GFP)30Scha/J, stock no. 004353; Jackson Laboratory] were transduced with four retroviral transcription factors, as described by Yamanaka and co-workers (1, 2). The newly generated undifferentiated mouse iPS cells were maintained on mitomycin-C–treated mouse embryo fibroblast feeder layers in ES cell medium containing knockout DMEM (KDMEM; Invitrogen), 15% (vol/vol) ES cell-qualified FBS (PAA), 2 mM L-glutamine (Invitrogen), 1×10^{-4} M nonessential amino acids (Invitrogen), 1×10^{-4} M 2-mercaptoethanol (Sigma), 1× penicillin (pen)/strep (Invitrogen), and 12.5 ng/mL LIF (Chemicon). Cultures were passaged using 0.25% trypsin (Invitrogen) at a 1:3 to 1:6 split ratio every 3–4 d. Mouse embryo fibroblast feeder cells (Millipore) were maintained in DMEM High Glucose supplemented with 10% (vol/vol) certified FBS (Invitrogen) and 1× pen/strep.

Induction of iPS Cell-Derived Pancreatic β Cells. The three-stage differentiation protocol of iPS cells into β-like cells was adapted from the differentiation of ES cells into insulin-producing cells by Wobus and co-workers (3), with modification. At stage 1, EBs were generated from iPS cells. iPS cells attached to feeder layers in 10-cm tissue culture dishes were washed one time with 1× PBS (Invitrogen) to remove residual serum. The cells were treated with 0.25% trypsin for 30 s; trypsin was then removed, and cells were disrupted by manually scraping the culture dish with a sterile cell scraper (BD Biosciences). EB medium (ES medium without LIF) was added to the cells, and the cells were further disrupted into single cells and small pieces by pipetting up and down. The cell suspension was centrifuged at $300 \times g$ for 5 min. The supernatant was removed, and the cell pellet was resuspended into 10 mL of EB medium. To deplete iPS cells of unwanted feeder cells, cells were plated onto a T75 tissue culture flask and the feeder cells were allowed to attach for 30–45 min at 37 °C in a humidified CO_2 incubator. The feeder-depleted cells were then collected and centrifuged at $300 \times g$ for 5 min. The cell pellet was resuspended in EB media at 6,000 cells per milliliter and plated as drops on 15-cm Petri dishes at 18–22 rows of drops per plate. Plates were gently flipped to invert drops and incubated at 37 °C in an incubator for 2 d. The EBs were collected and pooled by gently swirling plates with 1× PBS and transferred to a 50-mL conical tube. Pooled EBs were allowed to settle by gravity (about 10 min), resuspended in 10 mL of EB medium, transferred to 10-cm nonadherent Petri plates, and incubated for 3 more d.

At stage 2, EBs were collected and transferred to adherent culture at 10–15 EBs per 10-cm tissue culture dish to induce multilineage progenitors. For RT-PCR, immunofluorescent staining, and ELISA, 1–3 EBs were transferred into each well of 12-well plates. The EBs cultured in EB medium were differentiated for 9 d, with media changes every 3–4 d.

Finally, at stage 3, EBs were induced to differentiate into β-like cells for 7–20 d. EB medium was replaced with β-cell selective differentiation medium consisting of DMEM/F12 supplemented with 10% (vol/vol) certified FBS (Invitrogen); 20 nM progesterone (Sigma); 100 μM putrescine (Sigma); 1 $\mu g/mL^{-1}$ laminin (Sigma); 10 mM nicotinamide (Sigma); 1× ITS premix containing insulin, transferrin, and selenic acid (BD Biosciences); B27 media supplement (Invitrogen); and 1× pen/strep. After day 6 in selective medium, cells were trypsinized and replated into unused culture dishes. Again, media were changed every 3–4 d throughout stage 3.

RT-PCR. Total RNA was isolated from undifferentiated iPS cells and differentiated β-like cells using TRIzol reagent (Invitrogen) according to manufacturer's instruction. Two micrograms of total RNA was used for reverse transcription with the cDNA Archive kit (Applied Biosystems) according to manufacturer's instruction. PCR reactions were run using Platinum PCR Supermix High Fidelity (Invitrogen) according to the manufacturer's instructions. PCR sequences for endogenous/total retroviral transcripts ($n = 8$) and specific pancreatic markers ($n = 25$) are reported in Table S1.

Teratoma Formation in Nude Mice. Evaluation of proliferation and differentiation of iPS cells was tested via teratoma formation in nude mice. iPS cells were dissociated with 0.25% trypsin for 2 min and then manually scraped using a cell scraper, collected and transferred into a 15-mL conical tube, and centrifuged at $320 \times g$ for 5 min. The cell pellet was then resuspended in EB differentiation medium. A total of 2×10^6 dissociated iPS cells in 100 μL of DMEM High Glucose was mixed with an equal volume of Geltrex (Invitrogen) and injected s.c. into the flanks of two nude mice (Taconic). Thirty days postinjection, teratomas were removed, dissected, and fixed with formalin (Fisher Scientific). Paraffin-embedded tissues were sectioned and stained with H&E.

Cellular Therapy of Diabetes Mellitus Mouse Models. Cellular transplantation was done by portal vein injection using previously established protocols (4). Under anesthesia, 200,000 FACS-sorted $GFP^+/SSEA1^-$ stage 3 day 7 iPS cell-derived β-like cells in 300 μL of basal DMEM/F12 medium were injected into the hepatic portal vein using a 32-gauge Hamilton syringe. Stage 3, day 7 cells were more efficient than Stage 3, day 13 or day 20 cells in reversing the hypoglycemic phenotype. The injected iPS cell-derived β-like cells most likely contained a mixture of precursor cells, including α, β-like, and mature β cells. Intraportal vein injection of in vitro-derived β-like cells is an efficient way to engraft the cells directly into the murine hepatic sinusoids. The engrafted cells stably expressed GFP, enabling us to recognize and distinguish the transplanted cells from other hepatic cell types. Glucose levels were obtained every 2–3 d posttransplantation from fasted mice via the tail vein using a handheld glucometer. Five microliters of blood was collected from three transplanted mice with normal glucose levels 4 wk posttransplantation ($n = 3$), and the samples were sent to an independent company (Diabetes Technologies, Inc.) to obtain the hemoglobin A1c levels

Immunofluorescent and Tissue Staining. Cell cultures were washed three times with 1× PBS and fixed for 15–30 min at room temperature (RT) in 4% (wt/vol) paraformaldehyde in PBS (EMD Chemicals). They were then washed three times in PBS and permeabilized with 0.2% Triton X (Fisher Scientific) in PBS for 15–30 min at RT. For surface markers such as SSEA-1, the permeabilization step was omitted. The cells were again washed three times with PBS and blocked for 15–30 min in 5% (wt/vol) BSA (Fisher Scientific) in PBS at RT. Primary and secondary antibodies are reported in Table S2. Primary antibodies in 1% BSA were added to the cells and incubated for 2 h at 37 °C. The cells were washed again three times with PBS, and secondary antibodies diluted in PBS were added and incubated for 1 h at 37 °C. The cells were washed again, and the nuclei were counterstained with 1 mg/mL 4′,6-diamidino-2-phenylindole DAPI (Sigma) for 10 min, followed by a final three washes in PBS.

Immunohistochemical Confirmation of Cell Engraftment. Liver tissues were prepared by two methods, and paraffin-embedded tissues

were used for immunohistochemistry, as shown in Fig. 5 *A–C*. Frozen sections were prepared for the fluorescent images, as shown in the same figure (Fig. 5 *D–F*).

Livers were isolated and fixed in 10% (vol/vol) neutral-buffered formalin and embedded in paraffin. Five-micrometer sections were cut, placed on precleaned slides, deparaffinized using xylene, and then hydrated using an alcohol gradient. Immunohistochemical staining using rabbit anti-mouse GFP primary antibody was performed using a RMR622G Rabbit HRP Polymer kit (Biocare Medical). Deparaffinized slides were placed in 1× Rodent Decloaker solution (Biocare Medical) and heated to 125 °C for 30 min using Biocare's Decloaking Chamber. The slides were removed and washed three times with deionized water. Rodent Block M (Biocare Medical) was applied for 30 min to reduce both nonspecific background staining and endogenous mouse IgG. The slides were washed two times with PBS, and GFP primary antibody in 1% antibody dilution buffer (1:100) was applied and incubated overnight at 4 °C in a humidified chamber. The slides were washed with PBS, and Rabbit HRP Polymer solution (Biocare Medical) was applied for 20 min. The slides were again washed in PBS, and Betazoid DAB Chromogen reagents (one drop of Betazoid DAB Chromogen added to 1 mL of Betazoid DAB Substrate Buffer, both from Biocare Medical) were applied for 5 min. The slides were then rinsed with deionized water and treated with hematoxylin for 30 s. The slides were washed with warm alkaline tap water to blue the nuclei. The slides were then dehydrated, cleared, and coverslipped. Cytoplasm appears brown, and nuclei appear blue.

Five-micrometer frozen liver sections were placed on positively charged slides and allowed to air-dry at RT for 5 min before fixing with 3% (vol/vol) formaldehyde in methanol for 15 min at RT, followed by 5 min of incubation in methanol at −20 °C. The sections were washed two times with PBS for 5 min and incubated for 10 min at RT in 3% (vol/vol) hydrogen peroxide in methanol. The sections were washed two times with PBS for 5 min and then incubated with 5% (wt/vol) BSA in PBS for 1 h at RT. The blocking buffer was removed, and 400 μL of insulin monoclonal primary antibody (1:100; Table S2) in 1% BSA as added and incubated overnight at 4 °C. The antibody solution was removed, and the sections were washed three times with PBS for 5 min each. Secondary antibody diluted 1:1,000 with PBS was then added and incubated for 30 min at RT. The secondary antibody solution was removed, and the sections were washed three times in PBS for 5 min each. Nuclei were stained with 1 mg/mL DAPI for 10 min, and sections were washed three times with PBS for 5 min each. Images were immediately captured on a fluorescent microscope.

Statistics. Statistical differences between groups ($P < 0.05$) were determined by Excel's one-tail Student *t* test. A *P* value less than 0.05 was considered statistically significant.

1. Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. *Nature* 448:313–317.
2. Takahashi K, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.
3. Schroeder IS, Rolletschek A, Blyszczuk P, Kania G, Wobus AM (2006) Differentiation of mouse embryonic stem cells to insulin-producing cells. *Nat Protoc* 1:495–507.
4. Kemp CB, Knight MJ, Scharp DW, Lacy PE, Ballinger WF (1973) Transplantation of isolated pancreatic islets into the portal vein of diabetic rats. *Nature* 244:447.

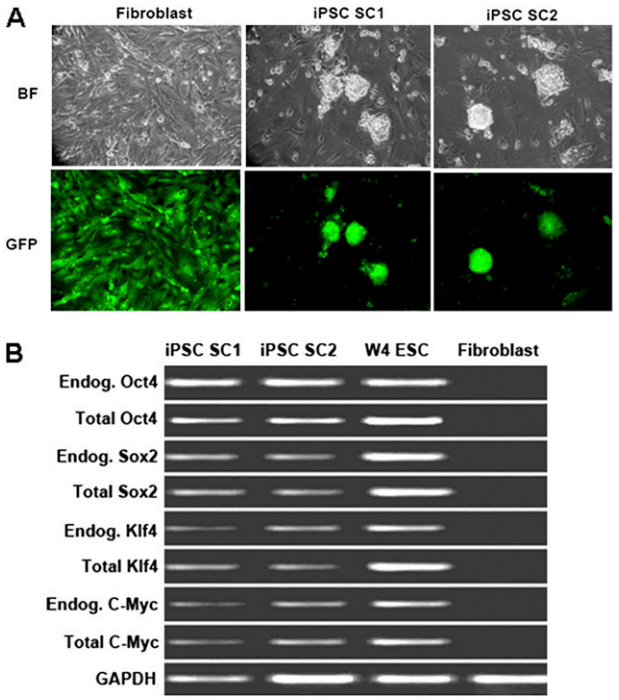

Fig. S1. Evaluation of iPS cell clones. (*A*) Morphologies of parental skin fibroblasts expressing GFP and iPS cells (two subclones) after somatic cell reprogramming. At 20 d postinduction, low-magnification phase contrast images show two subclones, iPSC SC1 and iPSC SC2, that are distinct from parental skin fibroblasts. GFP was still highly expressed after reprogramming. BF, bright field image. (*B*) Quantitative RT-PCR analysis of Oct4, Sox2, Klf4, and c-Myc in two iPS cell lines. The expression patterns of two iPS cell subclones, iPSC SC1 and iPSC SC2, demonstrate ES cell-like characteristics. W4 ES cells (mouse ES cells) were used as the positive control, and normal fibroblasts were used as the negative control. A housekeeping gene, GAPDH, was used as an endogenous control. We tested primers that only amplify endogenous genes of Oct4, Sox2, Klf4, and cMyc, labeled "Endog." on the figure. We also tested whether retroviral transcripts were silenced in newly established iPS cell clones by measuring both viral and cell expression together (1). Clones with silenced retroviral transcripts have indistinguishable "Endog" and "Total" gene expressions levels.

1. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.

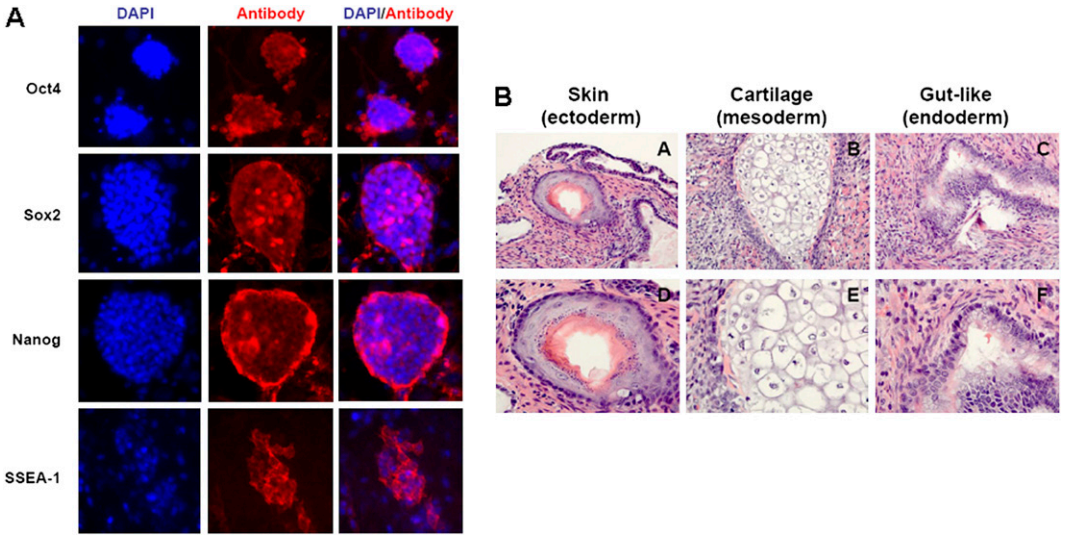

Fig. S2. Evaluation of pluripotency. (*A*) Immunofluorescent staining of iPS cells. iPS cells were characterized by immunofluorescent staining using well-established pluripotency markers: Oct3/4, Sox2, Nanog, and SSEA-1. The clones were counterstained with DAPI (blue), confirming the expression of ES cell markers throughout the iPS cell clones. (*B*) Histological analysis of teratoma. Low-magnification (*A–C*) and high-magnification (*D–F*) images confirm the presence of cell lineages belonging to the three germ layers, ectoderm (skin), mesoderm (cartilage), and endoderm (gut-like), within the tumor sections.

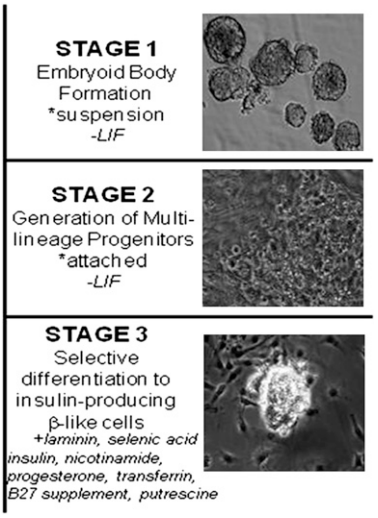

Fig. S3. Staging for the selective differentiation of iPS cells into insulin-producing β-like cells. Schematic representation of the three-stage differentiation protocol for deriving insulin-secreting β-like cells from ES cell-like iPS cells. In stage 1, iPS cells differentiate into embryo-like spheroids. In stage 2, multilineage progenitors are generated, which migrate from the EBs. In stage 3, selective differentiation of iPS cells into insulin-secreting β-like cells occurs.

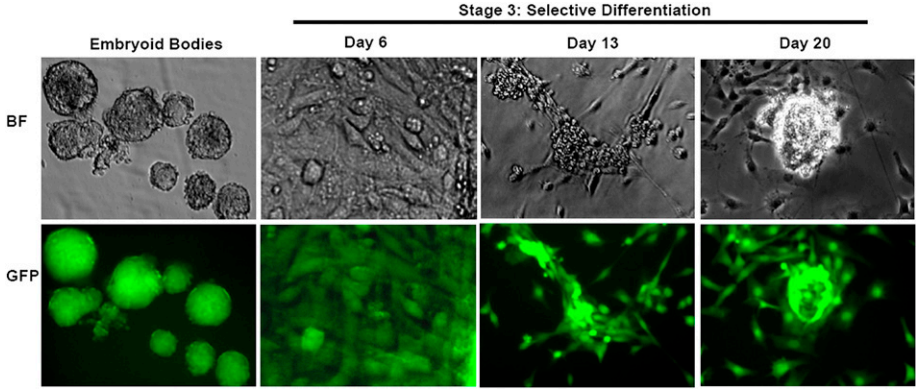

Fig. S4. Morphologies of differentiating iPS cells into β-like cells. Images of iPS cells at three different time points during stage 3 differentiation into β-like cells in vitro. GFP continues to be expressed throughout all stages. BF, bright field image.

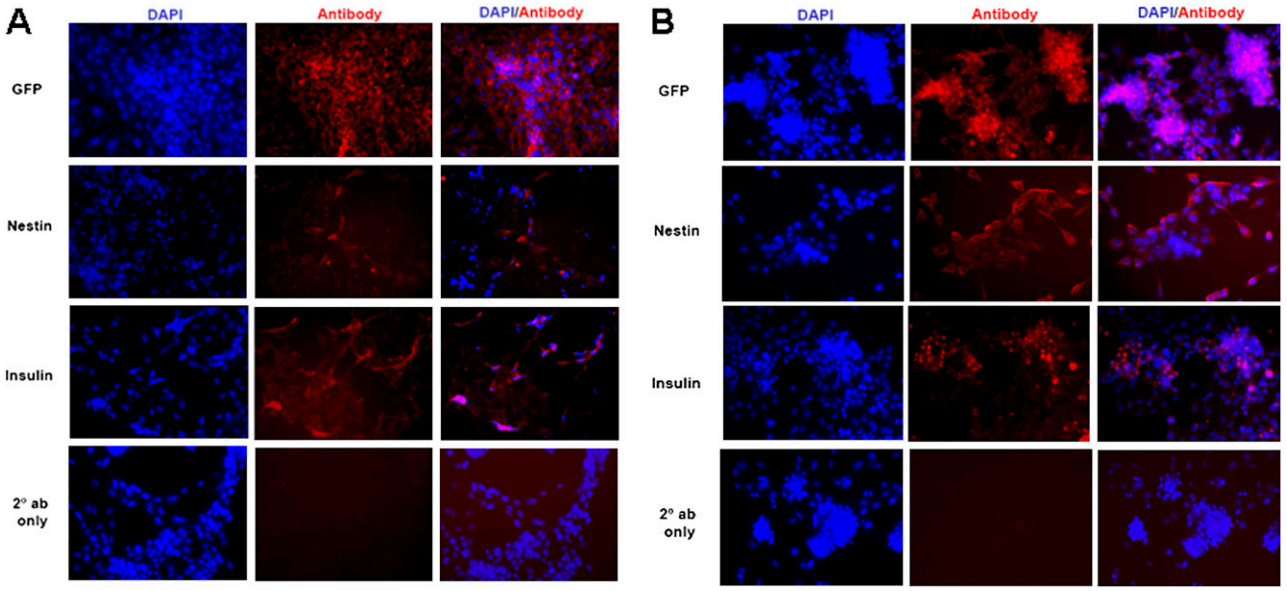

Fig. S5. Immunofluorescent staining of stage 3 cultures. (*A*) Stage 3 on day 7: In vitro-derived β-like cells are stained with antibodies to GFP, nestin, and insulin at day 7 of stage 3. Cells are counterstained with DAPI. (*B*) Stage 3 on day 13: In vitro-derived β-like cells are stained with antibodies to GFP, nestin, and insulin at day 13 of stage 3. Cells are counterstained with DAPI. Both nestin and insulin continue to stain strongly.

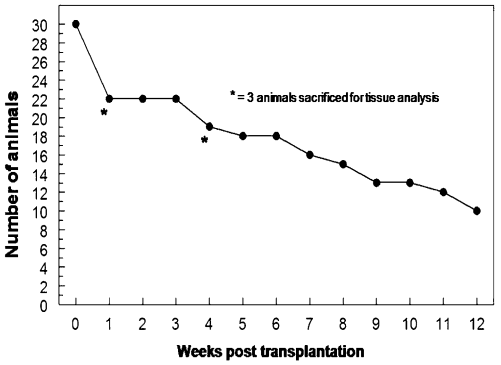

Fig. S6. Weekly number of animals after surgery. The number of treated type 2 animals at each week of the study is shown. Injections were staggered, but the data are shown for the total time each animal remained in the study. The earliest transplants had higher rates of postsurgical losses, but the success rate increased as the technique was refined. The majority of posttransplant losses, as described in main text, resulted from postsurgical hematomas. These losses dropped off with the introduction of the 32-gauge needle. *Three animals killed at this point for histology.

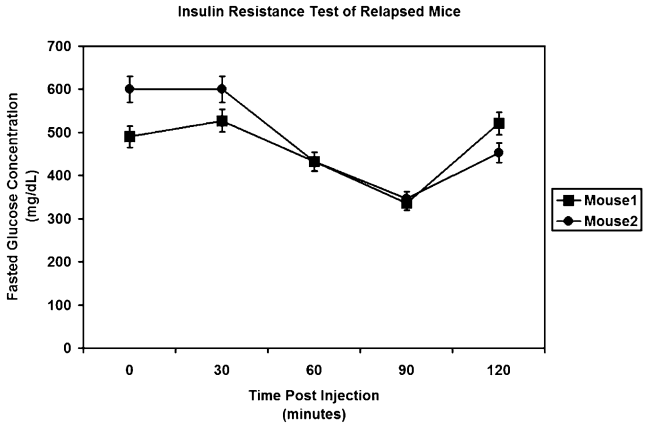

Fig. S7. Insulin resistance test of relapsed mice. Two of the 30 transplanted mice relapsed into a diabetic hyperglycemic phenotype 3–4 wk postengraftment. They were injected with 0.75 u/kg human insulin (Novo Nordisk). Glucose levels obtained at 30, 60, 90, and 120 min postinjection demonstrated that both mice had become resistant to insulin.

Table S1. PCR primer sequences for endogenous/total retroviral transcripts and specific pancreatic markers

Gene	Forward primer sequences	Reverse primer sequences
Endogenous Oct3/4	TCT TTC CAC CAG GCC CCC GGC TC	TGC GGG CGG ACA TGG GGA GAT CC
Total Oct3/4	CTG AGG GCC AGG CAG GAG CAC GAG	CTG TAG GGA GGG CTT CGG GCA CTT
Endogenous Sox2	TAG AGC TAG ACT CCG GGC GAT GA	TTG CCT TAA ACA AGA CCA CGA AA
Total Sox2	GGT TAC CTC TTC CTC CCA CTC CAG	TCA CAT GTG CGA CAG GGG CAG
Endogenous Klf4	GCG AAC TCA CAC AGG CGA GAA ACC	TCG CTT CCT CTT CCT CCG ACA CA
Total Klf4	CAC CAT GGA CCC GGG CGT GGC TGC CAG AAA	TTA GGC TGT TCT TTT CCG GGG CCA CGA
Endogenous c-Myc	TGA CCT AAC TCG AGG AGG AGC TGG AAT C	AAG TTT GAG GCA GTT AAA ATT ATG GCT GAA GC
Total c-Myc	CAG AGG AGG AAC GAG CTG AAG CGC	TTA TGC ACC AGA GTT TCG AAG CTG TTC G
GADPH	GAT GCC CCC ATG TTT GTG AT	TTG CTG ACA ATC TTG AGT GAG TTG T
Sox 17	CCA TAG CAG AGC TCG GGG TC	GTG CGG AGA CAT CAG CGG AG
HNF3β (Foxa2)	ACT GGA GCA GCT ACT ACG	CCC ACA TAG GAT GAC ATG
Cytokeratin 19	CTG CAG ATG ACT TCA GAA CC	GGC CAT GAT CTC ATA CTG AC
Isl-1	GTT TGT ACG GGA TCA AAT GC	ATG CTG CGT TTC TTG TCC TT
Nestin	CTA CCA GGA GCG CGT GGC	TCC ACA GCC AGC TGG AAC TT
Ngn3 (MATH4B)	TGG CGC CTC ATC CCT TGG ATG	AGT CAC CCA CTT CTG CTT CG
Pax4	ACC AGA GCT TGC ACT GGA CT	CCC ATT TCA GCT TCT CTT GC
Pax6	TCA CAG CGG AGT GAA TCA G	CCC AAG CAA AGA TGG AAG
Pdx1 (IPF-1)	CTT TCC CGT GGA TGA AAT CC	GTC AAG TTC AAC ATC ACT GCC
Insulin 1	TAG TGA CCA GCT ATA ATC AGA GAC	CGC CAA GGT CTG AAG GTC
Glucagon	CAT TCA CAG GGC ACA TTC ACC	CCA GCC CAA GCA ATG AAT TCC
Amylase	CAG GCA ATC CTG CAG GAA CAA	CAC TTG CGG ATA ACT GTG CCA
Glut-2	TTC GGC TAT GAC ATC GGT GTG	AGC TGA GGC CAG CAA TCT GAC
IAPP	TGA TAT TGC TGC CTC GGA CC	GGA GGA CTG GAC CAA GGT TG
PP	ACT AGC TCA GCA CAC AGG AT	AGA CAA GAG AGG CTG CAA GT
Somatostatin	TCG CTG CTG CCT GAG GAC CT	GCC AAG AAG TAC TTG GCC AGT TC
β5-tubulin	TCA CTG TGC CTG AAC TTA CC	GGA ACA TAG CCG TAA ACT GC

IAPP, islet amyloid polypeptide.

Table S2. List of primary and secondary antibodies

Antigen	Primary Ab* and dilutions	Secondary Ab* and dilutions	Blocking buffer
GFP	Rabbit polyclonal, 1:100 (Cell Signaling)	Donkey anti-rabbit, 1:1,000 PE (BD Pharmingen)	5% BSA (Fisher Scientific)
Oct 3/4	Rabbit polyclonal, 1:100 (Santa Crux Biotechnology)	Donkey anti-rabbit, 1:1,000 PE (BD Pharmingen)	5% BSA (Fisher Scientific)
Sox2	Rabbit polyclonal, 1:100 (Santa Cruz Biotechnology)	Donkey anti-rabbit, 1:1,000 PE (BD Pharmingen)	5% BSA(Fisher Scientific)
Nanog	Rabbit polyclonal, 1:100 (Santa Cruz Biotechnology)	Donkey anti-rabbit, 1:1,000 PE (BD Pharmingen)	5% BSA (Fisher Scientific)
SSEA1	Mouse polyclonal, 1:100 (Santa Cruz Biotechnology)	Donkey anti-mouse, 1:1,000 PE (BD Pharmingen)	5% BSA (Fisher Scientific)
Nestin	Mouse monoclonal, 1:100 (Millipore)	Donkey anti-mouse, 1:1,000 PE (BD Pharmingen)	5% BSA (Fisher Scientific)
Insulin	Mouse monoclonal, 1:100 (Sigma)	Goat anti-mouse, 1:1,000 PE (BD Pharmingen)	5% BSA (Fisher Scientific)
Islet	Rabbit polyclonal, 1:100 (Abcam)	Goat anti-rabbit, 1:1,000 PE (BD Pharmingen)	5% BSA (Fisher Scientific)

PE, phycoerythrin.

*Primary and secondary antibodies were diluted to 1% blocking buffer before use in immunofluorescent staining.