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Supplemental Information

β Cell Replacement after Gene Editing of a Neonatal Diabetes-Causing Mutation at the Insulin Locus

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Figure S1. Related to Figure 1.

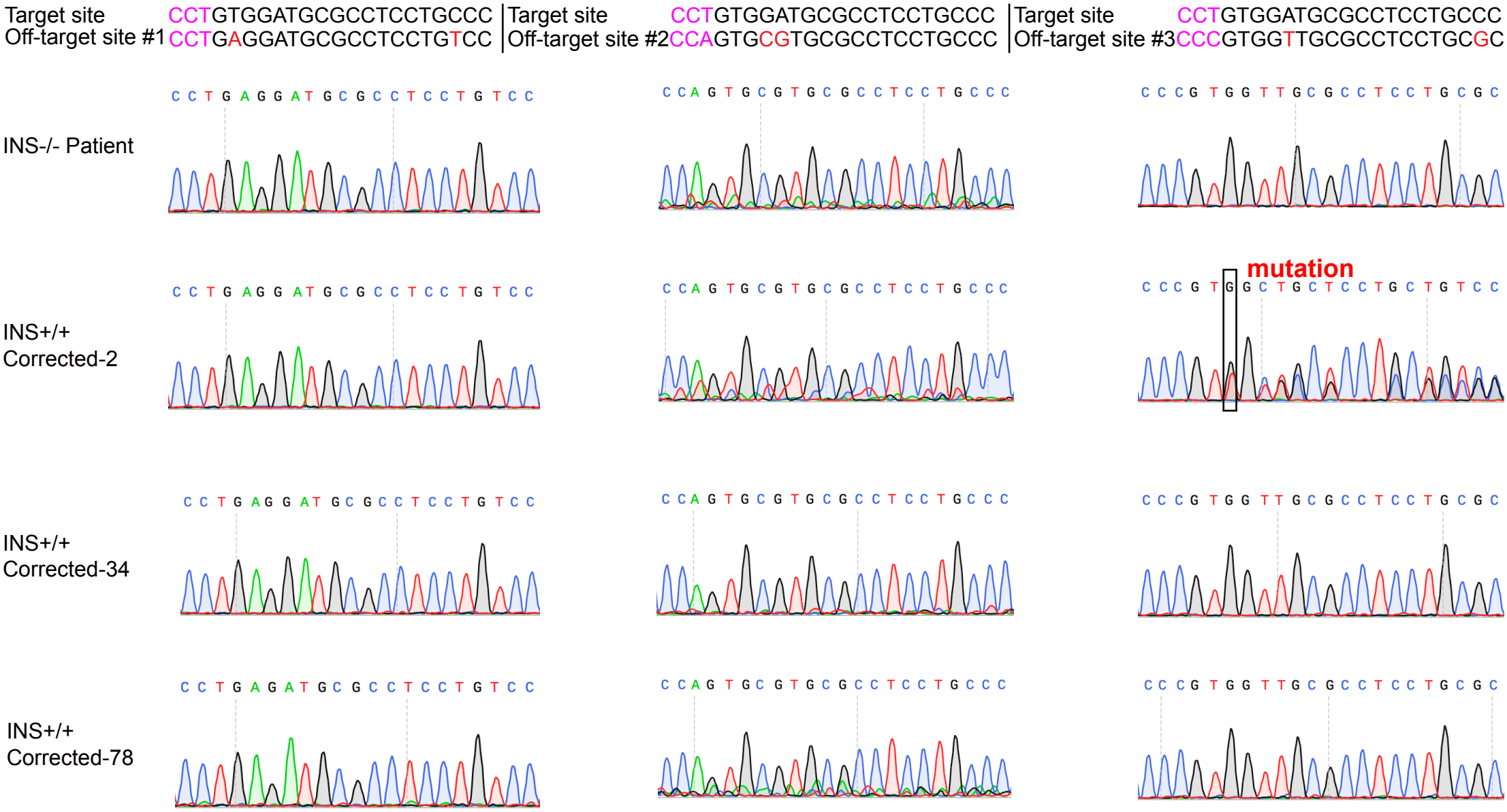
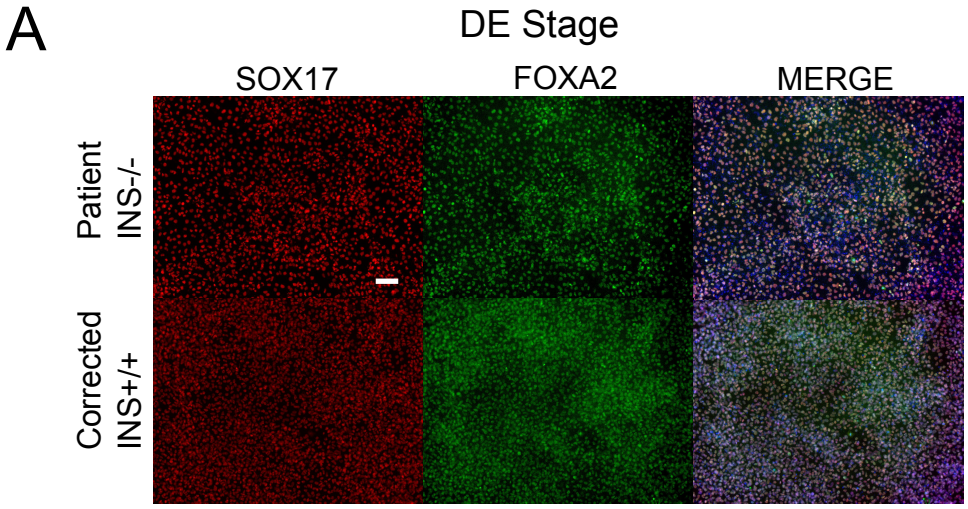
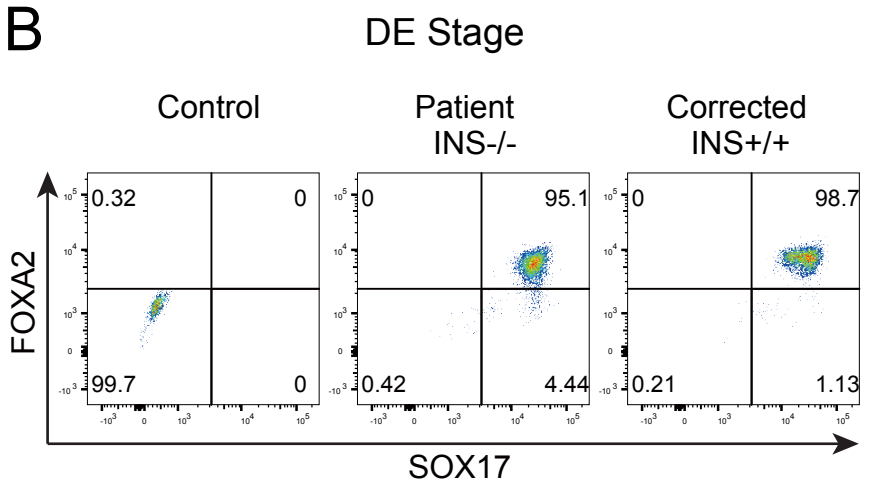


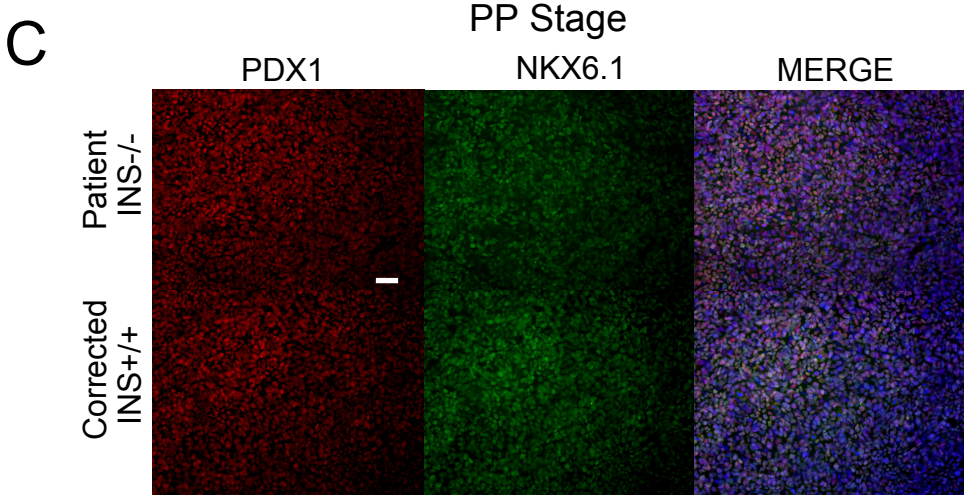
Figure S1. Off-target effect analysis at three top sites using PCR and Sanger sequencing, Related to Figure 1.

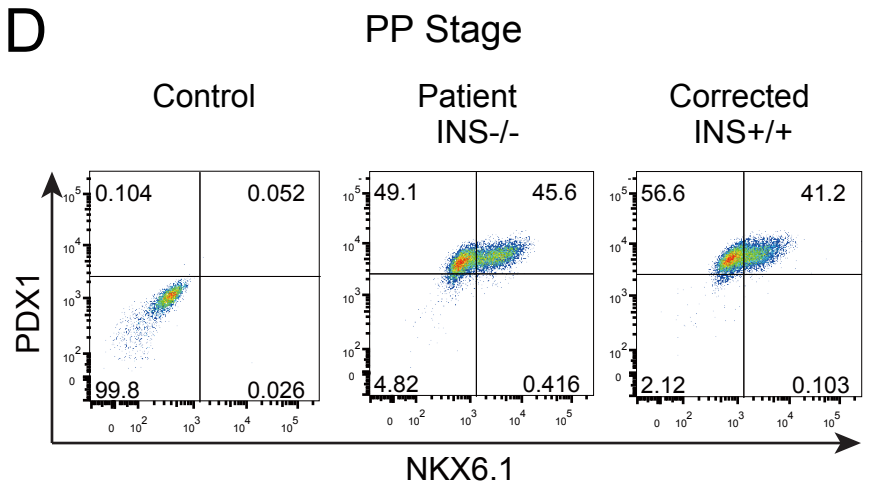
The top three off-target sites where chosen based on results (http://crispr.mit.edu/). Mismatches are highlighted in red. The site with an off-target effect is in a noncoding region 1.7kb upstream of the *PAX2* locus, a gene that is not expressed at detectable levels in stem cell derived endocrine cells.

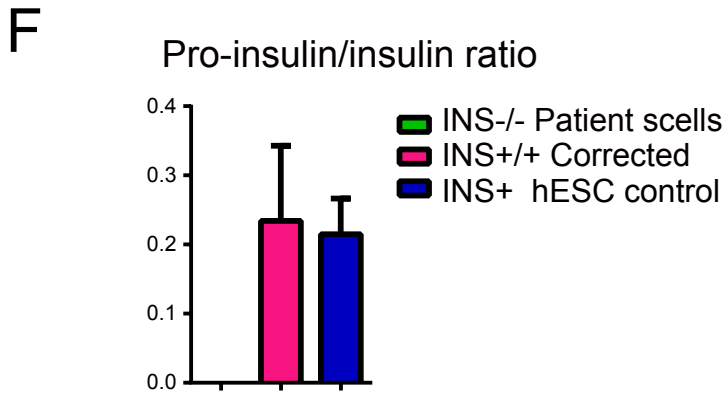
Figure S2. Related to Figure 2.











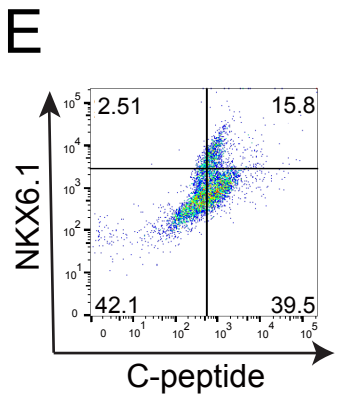


Figure S2. DE and PP differentiation results for mutant and corrected patient iPSCs, Related to Figure 2.

(A) Immuno-staining for DE marker genes, *SOX17* and *FOXA2*. Scale bar is 100 μM. (B) Flow cytometry results for DE marker genes, *SOX17* and *FOXA2.* (C) Immuno-staining for PP marker genes, *NKX6.1* and *PDX1*. Scale bar is 50 μM. (D) Flow cytometry results for PP marker genes, *NKX6.1* and *PDX1*. (E) Flow cytometry for Nkx6.1 and insulin. (F) Pro-insulin to insulin ratio results for *INS* mutant, corrected and WT β-like cells. n = 3 independent experiments.

Figure S3. Related to Figure 4.

INS-/- graft tissue

INS+/+ graft tissue

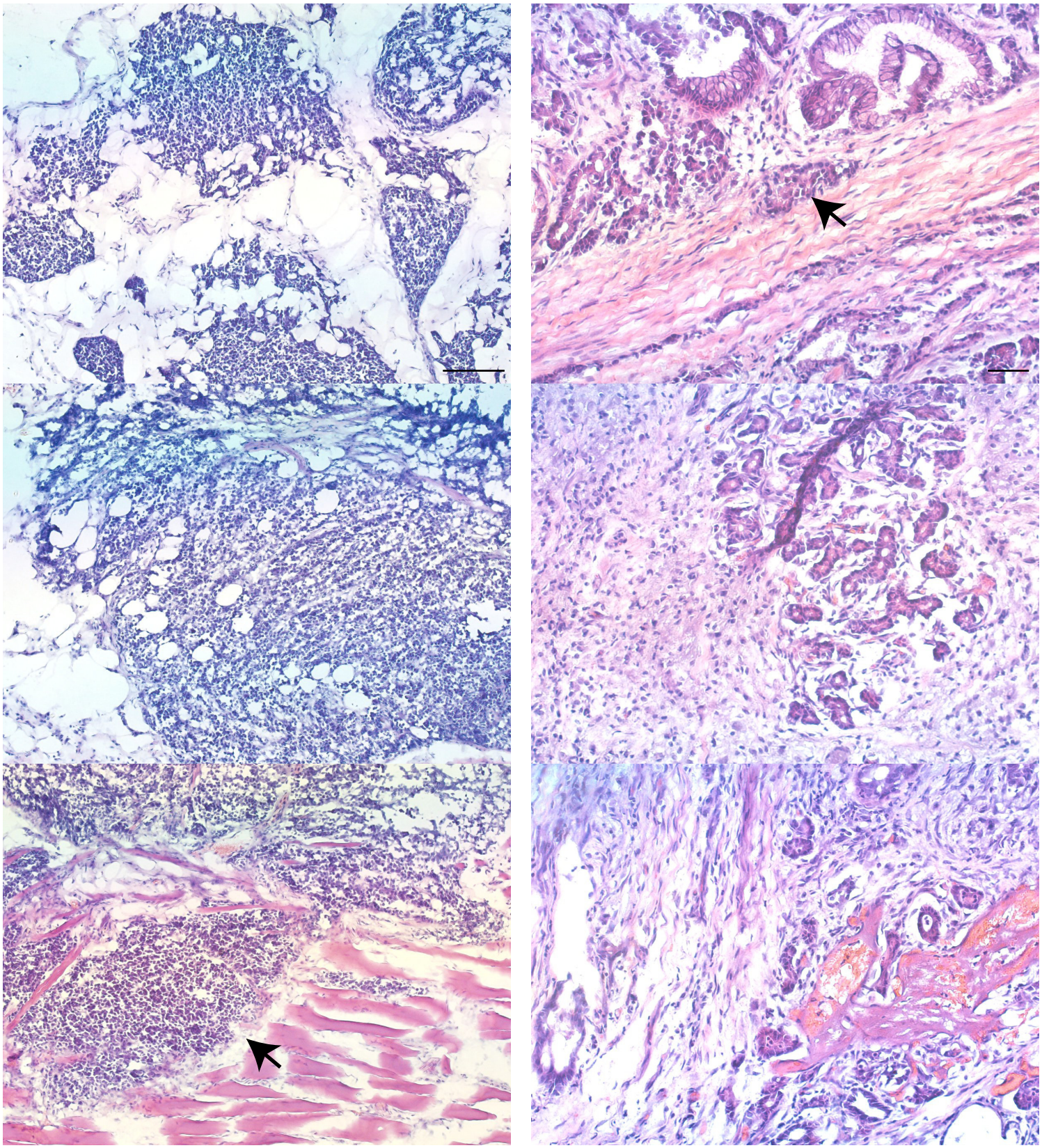


Figure S3. H&E staining of grafts, Related to Figure 4. Arrow points to a cluster of endocrine cells. Growth of non-pancreatic cells is also seen. Scale bar for INS+/+ is 50 μm and for INS-/- is 5 mm.

Supplemental Table 1. Primer, gRNA and ssODN sequences used in this study, Related to Figure 1 and 3.

Primer sequences used in this study:

gene	Primers
PTV3.6 (for PCR)	CATCAAGCAGGTCTGTTCCA
	CGGGTCTTGGGTGTGTAGAA
span PTV3.6 (for sequence)	GTCAGGTGGGCTCAGGATT
Insulin (for qPCR)	TTCTACACACCCAAGACCCG
	CAATGCCACGCTTCTGC
TBP (for qPCR)	AACAACAGCCTGCCACCTTA
	GCCATAAGGCATCATTGGAC
XBP1 and sXBP1 (for gel imaging)	GAAGCCAAGGGGAATGAAGT
	GGGAAGGGCATTTGAAGAAC
Spliced-XBP1 (for qPCR)	CTGAGTCCGCAGCAGGTG
	TGCCCAACAGGATATCAGACT
Off-target #1	CAAGTGACCCTCCTGAAACG
	AGCCAGTTTCCAGAATGACC
Off-target #2	TCTCTGTGAAGCCTGTGTGG
	CCAGAAAGACGTCCACTTCC
Off-target #3	GGATTCGGGCTGCAAGAG

gRNA and ssODN sequences used in this study:

gRNA Sequence(5' →3')	**ssDNA Sequence**	**Location**
CCTGTGGATGCGCCTCCTGC CC	tgagcccaggggccccaaggcagggcacctggccttcagcct gcctcagccctgcctgtctcccagATCACTGTCCTTC TGCCATGGCtCTGTGGATGCGCCTCCTGC CCCTGCTGGCGCTGCTGGCCCTCTGGGG ACCTGACCCAGCCGCAGCCTTTGTGAA CCAACACCTGTGCGGCTCACACCTGGT GGAAGCTCT	*INS* exon2

Red highlights indicates PAM sequence and Yellow highlights indicates *INS* mutation site.

Supplemental Table 2. Primary and Secondary Antibody dilution used in this study, Related to Figure 1 and 2.

Primary Antibody	**Dilution**
Anti-Rabbit FOXA2	1:400
Anti-Goat SOX17	1:100
Anti-Mouse NKX6.1	1:300
Anti-Goat PDX1	1:100
Anti-Mouse CHGA	1:100
Anti-Rabbit MAFA	1:100
Anti-Rat C-peptide	1:100
Anti-Guinea Pig Glucagon	1:200
Anti-Mouse OCT4	1:200
Anti-Rabbit SOX2	1:200
Secondary Antibody	**Dilution**
Donkey Anti-Rat Alexa Fluor® 488	1:500
Goat Anti-Rat Alexa Fluor® 555	1:500
Donkey Anti-Rabbit Alexa Fluor® 555	1:500
Donkey Anti-Rabbit Alexa Fluor®488	1:500
Donkey Anti-Guinea Pig Alexa Fluor®647	1:500
Donkey Anti-Goat Alexa Fluor® 555	1:500
Donkey Anti-Goat Alexa Fluor® 488	1:500
Donkey Anti-Mouse Alexa Fluor® 488	1:500
Donkey Anti-Mouse Alexa Fluor® 555	1:500
Goat anti-Rabbit IgG (H+L), HRP	1:500

Supplemental Table 3. Stem cell differentiation protocol, Related to Figure 2.

Stage	Day	Basal Media	Supplement
S0(1d)	d0	seeded 1×10^6 hiPSCs on matrigel (1×100) coated plates with 5μM Y-27632 in mTeSR1 for 24 hours	
S1(1d)	d1	M1 start differentiation until cells are 100% confluent and monolayer	Supplement A 1×100 Supplement B 1×100
S2(3d)	d2-4 change medium every 36 hours	M1 at the end of d4, check DE stage markers	Supplement B 1×100
S3(2d)	d5-6 no changing	M2	FGF7 50ng/ml
S4(3d)	d7-9 change medium every 36 hours	M3	KAAD 0.25μM RA 2μM LDN 250nM
S5(3d)	d10-12 change medium every 36 hours	M3 at the end of d12, check PP stage markers	EGF 50ng/ml
S6(2d)	d13-14 no need to change medium and don't move plates	M3 use Tryple-LE gently dissociate cells into single cells, aggregate 4×10^4 cells per cluster into low attachment 96 well plates	KAAD 0.25μM T3 1μM Alk5i 10μM Zinc-Sulfate 10μM Heparin 10μg/ml Y-27632 5μM
S7(7d)	d15-21 change medium every 2 days	M3	LDN 100nM T3 1μM Alk5i 10μM Zinc Sulfate 10μM DBZ 10μg/ml Heparin 10μg/ml
S8(7d)	d22-28 change medium every 2 days	M3 transfer every 48 clusters into 1 well of low attachment 6 well plates	T3 1μM Alk5i 10μM Zinc-Sulfate 10μM Heparin 10μg/ml N-Cys 1mM Trolox 10μM R428 2μM

Supplemental Table 4. Basal medium, chemical and other components for stem cell differentiation protocol, Related to Figure 2.

Basal medium for stem cell differentiation protocol:

Basal Medium name	Component
M1	Stemdiff™ Definitive Endoderm Basal Medium
M2	RPMI-1640 Penstrep 1×100 B-27 Serum free Supplement (50×)
M3	DMEM with high glucose, GlutamaxtTM Supplement, pyruvate Penstrep 1×100 B-27 Serum free Supplement (50×)

Chemicals and other components used in this study:

Component	Vendor	Cat. No.
DMEM with high glucose	Thermo fisher Scientific	10569-044
Knockout DMEM	Life technology	10829-018
mTeSR1	Stem cell technologies	05850
Y-27632	Selleckchem	S1049
Definitive Endoderm kit	Stem cell technologies	05110
RPMI-1640	Life technology	61870-127
B-27 Serum free Supplement	Life technology	17504044
Penstrep 1×100	Life technology	15140163
Matrigel	Fisher Scientific	354277
FGF7	R&D Systems	251-KG-050
KAAD (KAAD-Cyclopamine)	Stemgent	04-0028
RA (All-Trans Retinoic Acid)	Stemgent	04-0021
LDN (LDN-193189)	Stemgent	04-0074-10
EGF	R&D Systems	236-EG-01M
Alk5i	Stemgent	04-0015
Zinc-sulfate (Zinc sulfate heptahydrate)	Sigma-Aldrich	Z0251-100G
Heparin	Sigma-Aldrich	H13149-10KU
T3 (3,3',5-Triiodo-L-thyronine)	Sigma-Aldrich	T6397-100MG
DBZ (γ-Secretase Inhibitor XX)	EMD Millipore	565789
N-Cys (N-Acetyl-L-cysteine)	Sigma-Aldrich	A9165-5G
Trolox	EMD Millipore	648471-500MG
R428	ApexBio	A8329

Supplemental Experimental Procedures

Generation of induced pluripotent stem cells and mutation correction by CRISPR/Cas9 system

Skin fibroblast cells were cultured in fibroblast culture media. iPSCs were generated from fibroblasts by manufacturers' instruction (SCR550, EMD Millipore) and a previously published protocol (Yoshioka et al., 2013). In brief, fibroblasts of 90% confluence were split one day before initiation of reprogramming onto four wells of a 12-well plate. Multiple wells with different splitting ratios were used to ensure at least one well was 80% confluent at the start of reprogramming. One day after splitting, cells were transfected for 1 day with VEE-OKS-iG mRNA expressing OCT4, KLF4, SOX2 and GLIS1, and B18R RNA by RiboJuice (TM) mRNA transfection kit (TR-1013, EMD Millipore). From day 2 to day 4, cells were treated with 0.05 μg/mL puromycin (A1113803, Gibco) to select for transfected cells and then allowed to recover without puromycin to 80% confluence from day 5 to day 8. On day 9, transfected fibroblasts were re-plated from one well of a 12-well plate to one well of a CF-1 IRR mouse embryonic fibroblasts (MEF) (A34181, Gibco) coated 6-well plate using MEF conditioned human embryonic stem cell (ESC)/induced pluripotent cell (iPSC) media with addition of 10 ng/ml bFGF (1791281B, Invitrogen), 1 μl/ml Human iPSC Reprogramming Boost Supplement II (SCM094, EMD Millipore) and 200 ng/mL B18R protein until the colonies began to form on day 16. From day 17 onward, cells were cultured with MEF conditioned human ES media with 10 ng/mL bFGF and 1 μl/mL Human iPSC Reprogramming Boost Supplement II until day 23 when individual colonies were picked and expanded. Patient iPSCs were karyotyped by G-band analysis by Cell Line Genetics.

Patient iPSCs with normal karyotypes were used for generating *INS* mutation corrected cells by Crispr/Cas9 system. gRNAs against *INS* gene locus close to the mutation were designed with the online tool (http://crispr.mit.edu/). gRNAs of 19 nucleotides (19 nt) long were cloned into vectors by PCR and Gibson assembly method. Human iPSCs were transfected with Cas9-GFP, guide RNA vectors and 198 nucleotide long single strand DNA (IDT, Integrated DNA Technologies) for mutation replacement by using human embryonic stem cell Nucleofector Kit (VVPH-5012, Lonza) and hESC medium supplemented with ROCK inhibitor (Y26732, Selleckchem) onto feeder cells. Fourty-eight hours post transfection, GFP positive cells were sorted by flow cytometry sorting machine (BD Influx Cell Sorter) and re-plated onto a 10-cm dish by using hESC medium with ROCK inhibitor. One day after sorting, change medium to normal hESC without Y26732 and after that change the same medium for every 2 days. After 2 weeks, individual clones were picked up and analyzed by Sanger sequencing for mutation correction events. PCR primers were listed in Supplemental Table 1.

Fibroblast culture medium consisted of DMEM-high glucose (10569-044, Gibco), 10% HyClone Fetal Bovine Serum (U.S.) (SH30088.03HI, GE Healthcare), and Pen Strep (15140-122, Gbico).
MEF conditioned human ESC/iPSC media were made with MEF conditioned media by adding 10 ng/mL bFGF, which was used only for generating iPSC.
Human ESC culture medium were made with KnockOut DMEM (10829018,Gibco), KnockOut Serum Replacement (10828028, Gibco), GlutaMAX Supplement (35050061, Gibco), MEM NEAA (11140050, Gibco), Pen Strep (15140-122, Gibco), 2-Mercaptoethanol (21985023, Thermo Fisher Scientific) supplemented with 0.01 μg/ml bFGF (1791281B, Invitrogen). Human

ESC culture medium was used for routinely culturing and passaging human pluripotent stem cells.

Flow cytometry analysis

Cells were dissociated to single cells and suspended in FACS buffer (PBS with 5 % FBS). First LIVE⁄DEAD stain diluted 1000 times in FACS buffer (Molecular Probes L34955, 1:1,000) was used to stain dissociated cells for 15 minutes in order to differentiating between live and dead cells during flow cytometry analysis. Then cells were fixed and permeabilized with Foxp3 staining buffer set (eBioscience) for 30 minutes. After fixed cells were washed twice with FACS buffer, they were incubated with primary antibodies for 30 to 60 minutes at 4℃. At last, cells were washed twice and stained with secondary antibodies for 30 to 60 minutes at 4℃. After finish staining, cell were analyzed by flow cytometer and gated along with isotype control samples only for live cells. All staining was performed at 4 degrees.

Primary antibodies used in the study are as follows: OCT4 (sc-8628, Santa Cruz biotechnology), SOX2(09-0024, Stemgent), anti-FOXA2 (3143S, Cell Signaling Technology), anti-SOX17 (AF1924, R&D Systems), anti-NKX6.1 (F55A10, Developmental Studies Hybridoma Bank), anti-PDX1 (AF2419, R&D Systems), anti-CHGA (MAB5268, Millipore), anti-MAFA (ab26405, Abcam), anti-C-peptide (GN-ID4, Developmental Studies Hybridoma Bank), anti-Glucagon (M182, Takara).
Second antibodies used in the study were listed as follows: Donkey Anti-Rat Alexa Fluor® 488 (712-545-153, Jackson Immuno Research Laboratories), Goat Anti-Rat Alexa Fluor® 555 (A-21434, Life Technologies), Donkey Anti-Rabbit Alexa Fluor® 555 (A-31572, Life Technologies), Donkey Anti-Rabbit Alexa Fluor® 488 (A-21206, Life Technologies), Donkey Anti-Guinea Pig Alexa Fluor® 647 (706-605-148, Jackson Immuno Research Laboratories), Donkey Anti-Goat Alexa Fluor® 555 (A-21432, Life Technologies), Donkey Anti-Goat Alexa Fluor® 488 (A-11055, Life Technologies), Donkey Anti-Mouse Alexa Fluor® 488 (A-21202, Life Technologies), Donkey Anti-Mouse Alexa Fluor® 555 (A-31570, Life Technologies). Information on Antibody dilution is provided in Supplemental table 2.

Insulin content and secretion analysis method

For insulin secretion assay, we treated live clusters with KRB medium for two hours. Clusters were first stimulated in 3.3 mM glucose and stimulated with 30.5 mM KCl for one hour respectively. After one hour of stimulation, supernatant was collected in order to measure insulin secretion by Mercodia Insulin ELISA kit (10-1113-01, Mercodia).
For insulin content, dissociated clusters were spun down and a total of 10^5 cells were sonicated in 50 µl water and mixed with acid ethanol (0.18 M HCl in 96% ethanol (v/v)) in a 1:3 proportion of sonicated product and acid ethanol. To extract insulin, the mixed sample was incubated at 4℃ for 12 hours. After that, acid ethanol extracts was stored at -70℃ or used for measuring insulin content with Mercodia Insulin ELISA kit (10-1113-01, Mercodia) .

KRB medium consisted of 128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2mM $MgCl_2$, 1mM Na_2HPO_4, 1.2 mM KH_2PO_4, 5 mM $NAHCO_3$, 10 mM HEPES supplement with 0.1% BSA in deionized water, adjusted with NaOH to pH 7.4 and was sterilized by 0.22 µm filter (09-761-106, Fisher Scientific).

RNA analysis by RT-qPCR

RNA was extracted following the total RNA Purification Micro Kit (35300, Norgen Biotek) protocol. cDNA was generated from RNA by iScript Reverse Transcription Supermix (170-8841, Bio-Rad). RT-qPCR reaction system was setup by mixing cDNA and SsoFast EvaGreen Supermix (172-5202, Bio-Rad) and ran on the ABI thermal cycler (C1000 Touch Thermal Cycler). RT-qPCR primers were listed in Supplemental Table 1.

Transplantation and graft analysis methods

To transplant differentiated cells, around 200 clusters at day 27 of differentiation stage were suspended in 80 μl matrigel and transplanted into leg muscle of NSG mice (stock number 005557, The Jackson Laboratory). After three weeks post-transplantation, blood was collected from mouse tails, followed by subsequent collection every 2 weeks to analyze human C-peptide levels using a Mercodia Ultrasensitive C-peptide ELISA kit (10-1141-01, Mercodia). Daytime random measurements were performed. When human C-peptide reached up to 400 pmol/l or after19 weeks, mice were treated with STZ for amount of 150 mg/kg per mice (S0130-1G, Sigma Aldrich) to ablate mouse β cells in order to test whether transplanted human endocrine cells could help mice maintain normoglycemia. Mouse C-peptide was measured 7 days after STZ treatment with a Mouse C-Peptide ELISA Kit (90050, Crystal Chem) to make sure mouse C-peptide was eliminated before starting intraperitoneal glucose tolerance test (IPGTT). Mice had continuous access to food (standard diet).

IPGTT was performed 12 days after successful STZ treatment. For the IPGTT, mice were first fasted for 16 hours to keep them in a low blood glucose status. After fasting, an initial blood draw was done prior to each glucose challenge. For the glucose challenge, mice were injected intraperitoneally with 20% glucose of 10 times volume (μl) of body weight (g). Blood were drawn at 15, 30, 60 and 120 min after glucose injection. Glucose in the blood was measured by glucose meter (FreeStyle).

To monitor glucose dynamic homeostasis difference during day time and night, we performed 24 hour glucose measurement in 2 hour interval for STZ treated mice transplanted with corrected endocrine cells and WT mice by glucose meter (FreeStyle). Mice had continuous access to standard diet.

Supplemental References

Yoshioka, N., Gros, E., Li, H.R., Kumar, S., Deacon, D.C., Maron, C., Muotri, A.R., Chi, N.C., Fu, X.D., Yu, B.D., et al. (2013). Efficient generation of human iPSCs by a synthetic self-replicative RNA. Cell stem cell 13, 246-254.