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Supplemental Information

Targeted Derivation of Organotypic Glucose- and GLP-1-Responsive β Cells Prior to Transplantation into Diabetic Recipients

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Supplemental Figure S1. Zhu et al.

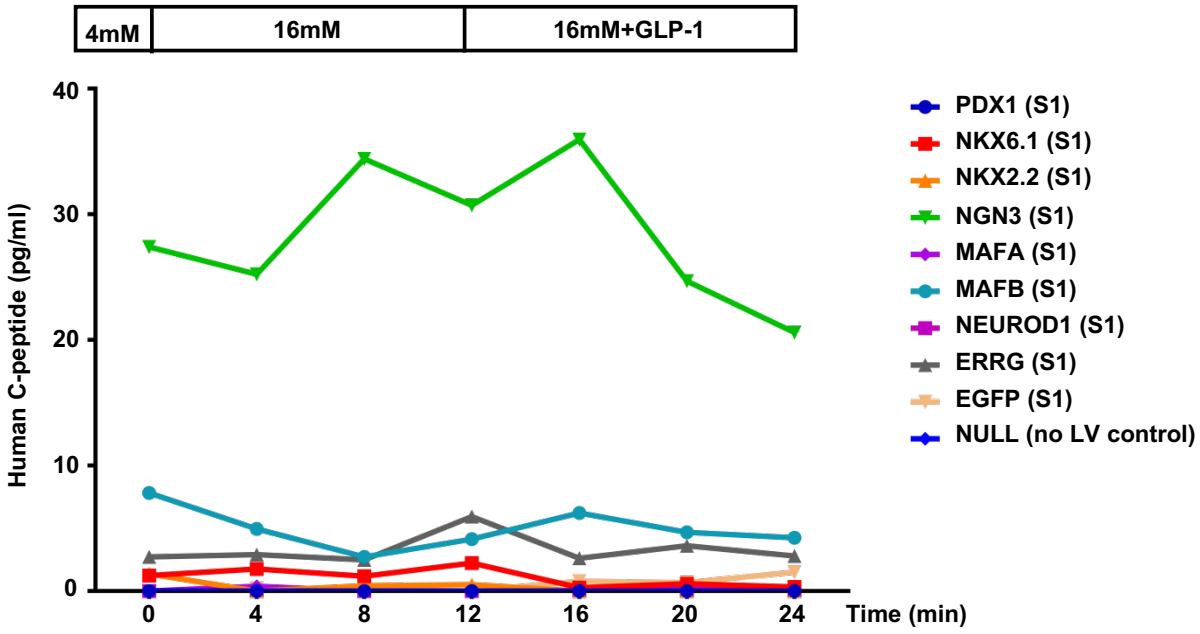


Figure S1. Secretion of human C-peptide by psBCs, Related to Fig.1e. Secretion of human C-peptide by psBCs in response to 4mM, 16mM and 16mM+GLP1 glucose medium for an indicated period of time. Different lentiviral vectors were introduced at the indicated stage.

Supplemental Figure S2. Zhu et al.

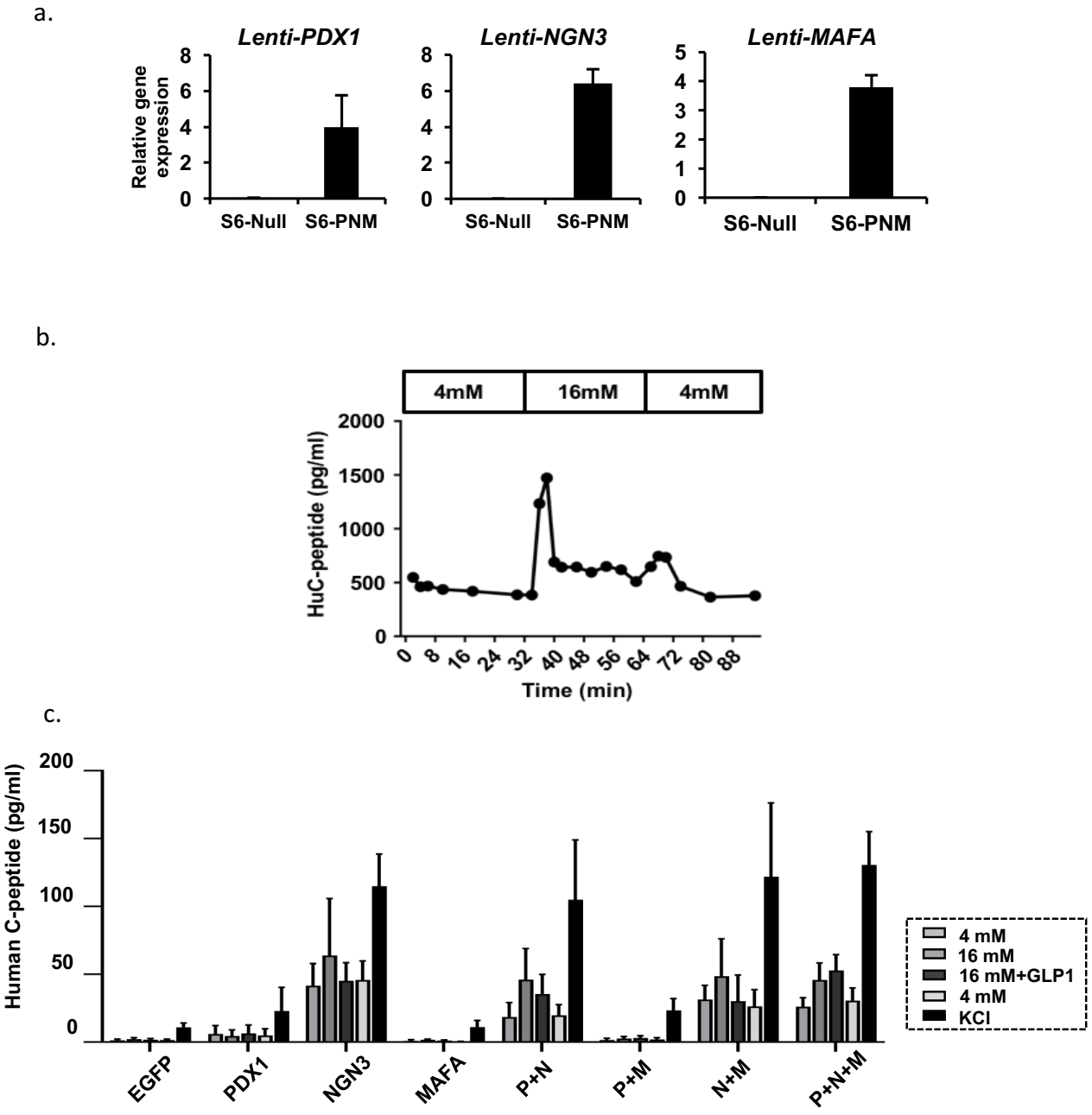
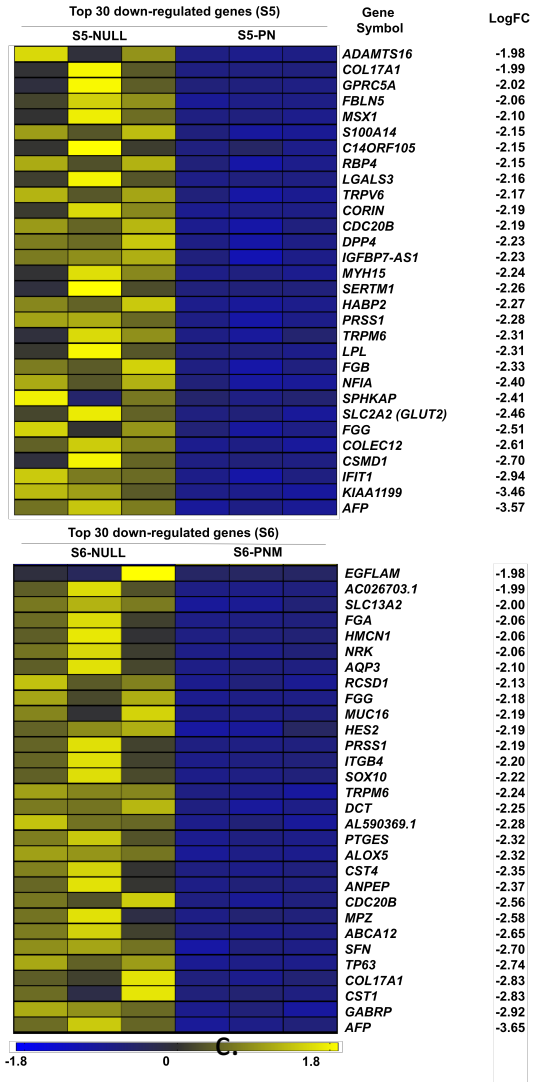


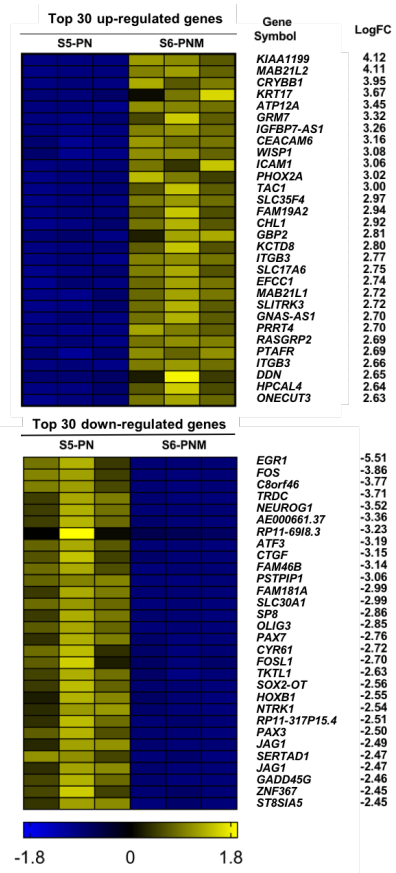
Figure S2. Glucose- and GLP-1-responsiveness of PNM-transduced psBCs, Related to Fig.2. (**a**) Levels of exogenous PDX1, NEUROG3 and MAFA gene expression in S6 psBCs were determined by qRT-PCR. (**b**) Secretion of human C-peptide by human islets in response to 4mM, 16mM and 4mM glucose medium for an indicated period of time. (**c**) Glucose- and GLP-1-responsive insulin secretion by psBCs transduced by different combinations of PNM factors. Levels of human C-peptide by psBCs in response to sequential 4mM, 16mM, 16mM+GLP-1, 4mM glucose medium and 30mM KCL incubation were analyzed by the perifusion system. Data represent the mean ± S.E.M. of the last time point of the first basal 4mM treatment, 4-minute time point for 16mM glucose treatment, 16mM glucose +GLP1 treatment, the second 4mM glucose treatment and KCL treatment.

Supplemental Figure S3. Zhu et al.

a.



b.



c.

	Gene Symbol	S6-PNM vs S5-PN (LogFC)
Beta cell & islet markers	*INS*	0.84
	CHGA	0.35
	CHGB	0.90*
	PDX1	-0.35
	NKX6.1	1.00*
	MAFB	-0.60*
	GLP1R	0.38
	GCGR	0.88*
	GCG	2.19*
	SST	0.21
GSIS-associated genes	*ADRA2A*	0.92*
	DOC2B	0.09
	ERRG	-0.36*
	GCK	0.93*
	MCT1	-0.44*
	MUNC18-1	0.70*
	MUNC18C	0.21
	PCSK1 (PC1)	1.54*
	PCSK2 (PC2)	0.47*
	PTPRN (IA-2)	0.98*
	SLC2A2 (GLUT2)	0.77
	SLC30A8 (ZnT8)	0.98*
	SLC6A5	2.25*
	SNAP25	1.25*
K^+/Ca^{2+} channel genes	*ABCC8*	0.91*
	ATP2B2	-0.18
	CACNA1D	0.80*
	CACNA2D3	1.70*
	KCNJ11	0.83*
	KCNK3	0.87*
	KCNQ1	0.51*
	MCU	-0.16*
	RYR2	-0.78
	SCGN	0.92*

d.

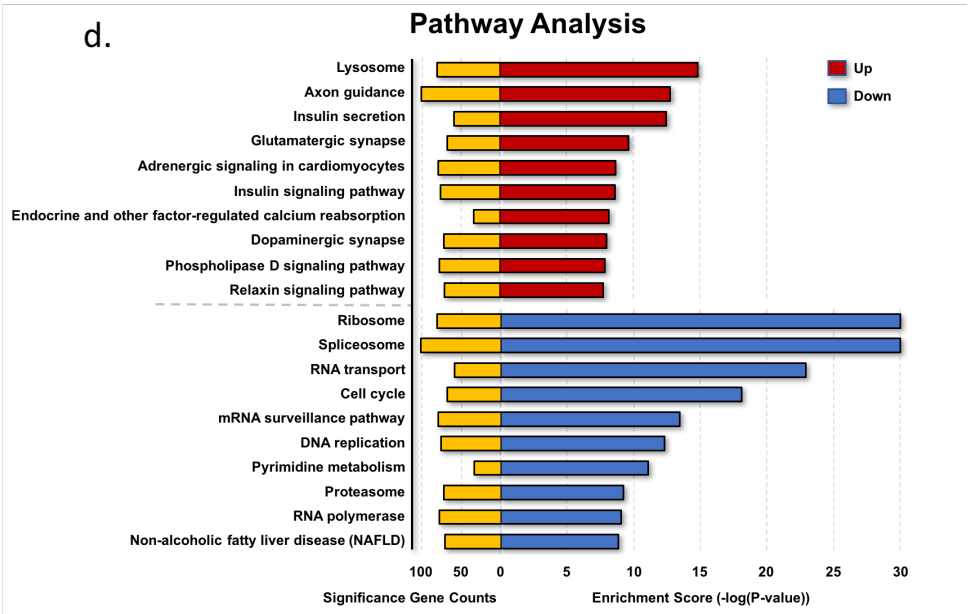


Figure S3. Characterization of PN and PNM transduced psBCs, Related to Fig.4. (**a**) Heatmaps of top 30 down-regulated genes for S5-PN and S6-PNM psBCs. LogFC stands for the $\log_2$ fold change of expression level relative to control. (**b**) Heatmaps of top 30 up-regulated and down-regulated genes for S6-PNM vs. S6-PN psBCs. LogFC stands for the $\log_2$ fold changes of expression level in S6-PNM psBCs relative to S5-PN psBCs. (**c**) Summary of differentially expressed genes between S5-PN and S6-PNM psBCs. LogFC stands for the $\log_2$ fold change of expression level of S6-PNM relative to S5-PN psBCs. **(d)** The top 10 up-regulated and down-regulated KEGG pathways of significantly differentially expressed genes between S5-PN and S6-PNM psBCs. KEGG, Kyoto Encyclopedia of Genes and Genomes. *represents statistically significant difference; Student's unpaired t-test.

Supplemental Figure S4. Zhu et al.

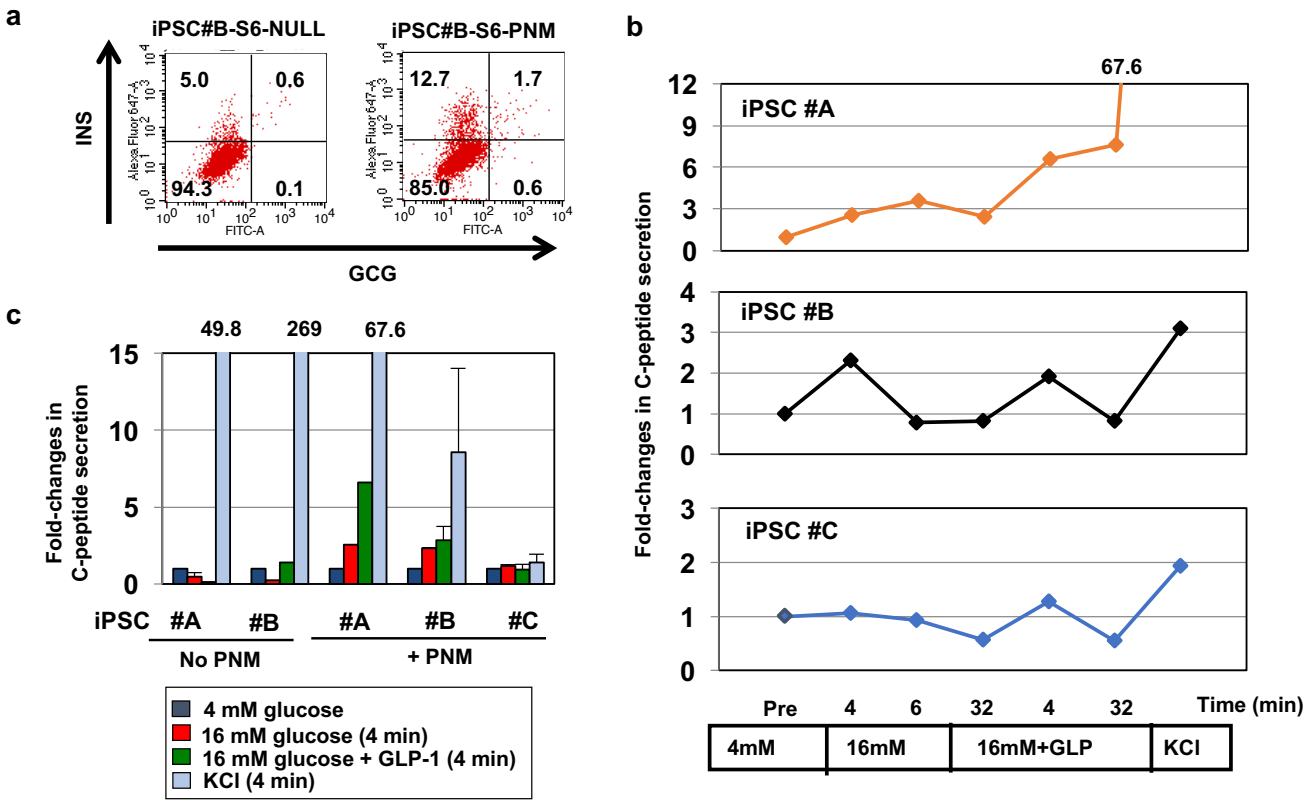
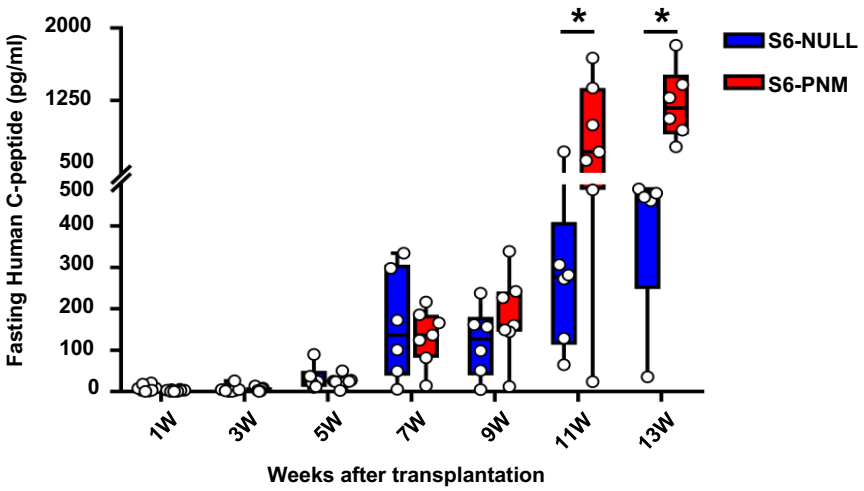


Figure S4. Effectiveness of PNM transduction in multiple iPSC lines, Related to Result 'PNM transduction in other iPSC lines'. We used five iPSC lines which were established by Sendai viral reprogramming and validated for their pluripotency and capacity to differentiate into insulin-positive cells (Kudva et al., Stem Cells Translational Medicine 2012). iPSC#A to #E were differentiated with or without PNM transduction and tested for their glucose and GLP-1 responsiveness by the perifusion system. (**a**) Same as Fig. 3b with iPSC#B. (**b**) Representative perifusion data of glucose- and GLP-1-responsiveness by iPSC clones #A, #B and #C. Experiments were performed the same way as Fig. 1e. (**c**) Summary of fold changes in C-peptide secretion, measured by perifusion. #C, #D and #E showed no notable glucose- and GLP-1-responsiveness (data not shown for #D and #E).

Supplemental Figure S5. Zhu et al.

a



b

Figure S5. Characterization of psBCs *in vivo*, Related to Fig.5. (**a**) Fasting human C-peptide levels in mice that received S6-NULL and S6-PNM psBCs transplantation. C-peptide levels from individual mice are shown on box and whisker plots. Student's unpaired *t*-test. (**b**) IPGTT 7 weeks after transplantations in control mice (n=4), endoC cells transplanted mice (n=3), S6-Null transplanted mice (n=6), S6-PNM transplanted mice (n=7). Letter 'a' represents statistically significant difference between control and S6-PNM, letter 'b' represents difference between control and S6-NULL, letter 'c' represents difference between S6-NULL and S6-PNM, letter 'd' represents difference between control and beta cells, letter 'e' represents difference between S6-NULL and beta cells, letter 'f' represents difference between S6-PNM and beta cells; One-way ANOVA with Tukey test for multiple comparisons.

Table S1. Primer sequence used in this study, Related to Fig.4c.

Gene Name	FWD (5' to 3')	REV (5' to 3')
ABCC8	CGT CTT AGC TGT GCT TCT GT	CTT GGT CTG TAT TGC TCC TCT C
CACNA1D	GGG AGC AGG AGT ATT TCA GTA G	GAT GTT TCT GCC TGG GTA TCT
CACNA2D3	GAT GGC CTC CAA CTG GTA AA	CAT GTT TCA GGT GTG CTT CTT C
ESRRG	TCT CTA CCC TTC TGC TCC TAT C	GCA TCG AGT TGA GCA TGT ATT C
GAPDH	GCG CCC AAT ACG ACC AA	CTC TCT GCT CCT CCT GTT C
GCK	GAT GCA CTC AGA GAT GTA GTC G	TGA AGG TGG GAG AAG GTG AG
GLP1R	CTC CTT CTC TGC TCT GGT TAT C	CAG GTT CAG GTG GAT GTA GTT
INS	CTT CAC GAG CCC AGC CA	ATC AGA AGA GGC CAT CAA GC
KCNJ11	ATG AGG ACC ACA GCC TAC T	GGA ATC TGG AGA GAT GCT GAA C
PCSK1	CAG ACA GCA TCT ACA CCA TCT C	GTG TAA TCT CCG CTG CTG TAA
PCSK2	GAC CTG GCC TCC AAC TAT AAT G	TGT GTA CCG AGG GTA AGG ATA G
SLC30A8	GCA TGC CCT TGG AGA TCT ATT	GCA GAT TGG GTC GGC TAT TT
SLC6A5	GAA AGT CTG CTG GGC ATT TG	CAC CAT GGA CCA GTT AGG ATA G

METHODS

Cells. 293T cells were cultured in DMEM medium supplemented with 10% fetal calf serum and antibiotics. Commercially available iPSC lines IISH2i-BM9 (WiCell, Madison, Wisconsin), and in-house iPSC lines #A, #B, #C, #D and #E were tested for beta cell propensity. Undifferentiated iPSCs at passage 10-30 was cultured on Matrigel (Corning, Corning, NY, #354277) - coated plates in mTeSR1 medium (StemCell Technologies, Vancouver, Canada, #05850). Cultures were fed every day with mTeSR1 medium.

Lentiviral Vectors. Lentiviral vector genome plasmid, pSIN-CSGW-PKG-puro, which supports EGFP expression and puromycin selection, was kindly provided by Dr. Paul Lehner (Cambridge Institute for Medical Research). Codon-optimized ORF sequences for beta-cell factors, including PDX1, NEUROG3, NKX2.2, NKX6.1, NEUROD1, MAFA, MAFB and ESRRG, were designed and synthesized (GenScript, Piscataway, NJ), and cloned into the place of the EGFP gene in pSIN-CSGW-PKG-puro with the unique *Bam*HI and *Xho*I sites. Resulting vector plasmids were designated as pLenti-PDX1, pLenti-NEUROG3, pLenti-NKX2.2, pLenti-NKX6.1, pLenti-NEUROD1, pLenti-MAFA, pLenti-MAFB and pLenti-ESRRG, respectively. The internal spleen focus-forming virus (SFFV) promoter drives the expression of beta-cell factors. Lentiviral vectors were produced by plasmid transfection in 293T cells as described previously (Tonne and Campbell et al.,2011), concentrated by ultracentrifugation and re-suspended in phosphate buffered saline (PBS). Lentiviral titers were determined by puromycin selection.

Guided differentiation and stepwise lentiviral vector transduction. The basic differentiation strategy without viral vector transduction was a modified version of the previously published protocols. In pilot studies, we tested several reported differentiation protocols (Pagliuca and Millman et al.,2014,Rezania and Bruin et al.,2014) as well as the protocol we have been using, a modified Viacyte/Novocell protocol with additional Indolactam V for enhanced PDX1 expression (Thatava and Kudva et al.,2013,El Khatib and Ohmine et al.,2016). We also tested key ingredients targeting related pathways, including Wnt 3a vs GSK3b inhibitor, Activin A vs. GDF8, FGF10 vs. FGF7, Cyc vs. SANT1 (Shh inhibitors), and Indolactam V vs. TPB (PKC activators). Our final basic protocol was based on these pilot studies, which is similar to the Rezania's protocol(Rezania and Bruin et al.,2014), but with the use of Activin A and FCS instead of GDF8 and bovine serum albumin, and without heparin and the liquid-air interphase culture steps.
Guided differentiation was initiated 48 hours following seeding, with a 60-80% starting confluency with some spaces between iPSCs colonies. The basal medium was prepared by supplementing MCDB131 medium (Thermo Fisher Scientific, Waltham, MA, #10372019) with 1x Glutamax (Thermo Fisher Scientific, #35050061), 50 U/ml Penicillin, 50 µg/ml Streptomycin, 0.02% D-Glucose solution (45%, Sigma-Aldrich, St. Louis, MO, #G8769) and 2% Sodium Bicarbonate Solution (7.5%, Sigma-Aldrich, #S8761). Step 1 (S1, 3 days); Day 1 iPSCs were first rinsed with PBS without Mg2+ and Ca2+ and then cultured in the basal medium further supplemented with 0.5% FBS (Thermo, #A3160602), 100 ng/ml Activin A (R&D Systems, Minneapolis, MN, #338-AC-050) and 3 µM of CHIR-99021 (SelleckChem, Houston, TX, #S2924). Cells were cultured in 2 ml media each for a well of a 6-well-plate, 1 ml for a 12-well-plate. At day 2, culture supernatants were removed and cells were incubated with the basal medium with 0.5% FBS and 100 ng/ml Activin A. Cells were further infected with the Lenti-PDX1 vector once at an approximate MOI of 30 on day 2. For the screening of single lentiviral vectors for individual factors, each lentiviral vector was delivered at this time point. At day 3, culture supernatants were replaced by the basal medium with 0.5% FBS and 100 ng/ml Activin A. Step 2 (S2, 2 days); Cells were rinsed with PBS, then cultured with the basal medium 0.5% FBS, 0.25 mM ascorbic acid (Sigma-Aldrich, # A4544) and 50 ng/ml of FGF7 (R&D Systems, #251-KG-050). Culture supernatants were replaced with the same, fresh medium at day 2. Step 3 (S3, 2 days); The culture supernatants were replaced to the basal medium, further supplemented with 14 µl/ml of 7.5% sodium bicarbonate solution, 2% FBS, 0.25 mM ascorbic acid, 50 ng/ml FGF7, 0.25 µM SANT-1 (Sigma-Aldrich, # S4572), 1 µM retinoic acid (Sigma-Aldrich, #R2625), 100 nM LDN193189 (Stemgent, Lexington, MA, #04-0019), 1:200 ITS-X (Thermo Fisher Scientific, #51500056), and 100 nM alpha amyloid protein modulator (EMD Millipore, Billerica, MA, #565740). Medium was changed every day. Step 4 (S4, 3 days); The culture supernatants of S3 cells were replaced with the basal medium further supplemented with 14 µl/ml of 7.5% sodium bicarbonate solution, 2% FBS, 0.25 mM ascorbic acid, 2 ng/ml of FGF7, 0.25 µM SANT-1, 0.1 µM retinoic acid, 200 nM LDN193189, 1:200 ITS-X, and 100 nM TPB. Cells were also infected with the Lenti-NEUROG3 vector at an approximate MOI of 30 once on day 7 (end of Step 3) or day 8 (beginning of Step 4). Medium changed every day with the fresh media. Step 5 (S5, 3 days); Cells were cultured in the basal medium further supplemented with 14 µl/ml of 7.5% sodium bicarbonate solution, 4 µl/ml of 45% glucose solution, 2% FBS, 0.25 µM SANT-1, 10 µM ALK5 inhibitor II (Enzo Life Sciences, Farmingdale, New York, #ALX-270-445), 0.05 µM retinoic acid, 1 µM thyroid hormone (Sigma-Aldrich, #T6397), 100 nM LDN193189, 1:200 ITS-X, 10 µM zinc sulfate (Sigma-Aldrich, #Z0251) and 10 µg/ml heparin (Sigma-Aldrich, #H3149). Medium was replaced every day. Step 6 (S6, 7 days); Culture media were replaced with the basal medium further supplemented with 14 µl/ml of 7.5% sodium bicarbonate solution, 4 µl/ml of 45% glucose solution, 2% FBS, 1 µM thyroid hormone, 10 µM ALK5 inhibitor II, 10 µM zinc sulfate, 100nM LDN193189, 1:200 ITS-X, 10 µg/ml heparin, and 100 nM gamma secretase inhibitor XX (EMD Millipore, Billerica, MA, # 565789) for 7 days. Cells were also infected with the Lenti-MAFA vector at an approximate MOI of 30 once, on day 14, 15, or 16. Fresh medium was fed every day.

qRT-PCR. For quantitative reverse transcription polymerase chain reaction (qRT-PCR), total RNA from differentiated iPSCs at indicated time points was isolated using Trizol according to the manufacturer instructions. cDNAs were then synthesized by reverse transcription from 200 ng of total RNA using SuperScript III Reverse Transcriptase, dNTP solutions, RNaseOUT and Random Hexamer. Hotstart Taq DNA polymerase and primer pairs for human *INS, GCK, GLP1R, ESRRG, SLC6A5, SLC30A8, ABCC8, KCNJ11, CACNA1D, CACNA2D3, PCSK1* and *PCSK2* were used. Sequence of the primers used for qRT–PCR was listed in Table S1. The PCR conditions were 95°C for 10 minutes enzyme activation, 95°C for 15 seconds denaturation, 60°C for 60 seconds annealing and extension, and overall 40 cycles were performed. The transcript levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RNA sequencing. For RNA sequencing, a total 200 ng RNA from differentiated iPSCs at indicated time points was isolated using RNeasy Mini Kit. Library preparation (TruSeq mRNA v2 (TMRNA)) and next-generation sequencing and analysis (standard secondary analysis pipeline, MAPRSeq) were performed in Mayo Clinic Sequencing and Bioinformatics Cores. Heatmap was depicted using Graphpad Prism.

Immunofluorescence staining. Lentiviral vector-infected 293T cells were fixed with 4% paraformaldehyde (PFA) for 20 min. After fixation cells were washed once with PBS and were then permeabilized with 0.3% Triton X-100 in PBS for 10 min. Cells were then washed with PBS twice and were blocked with 5% FBS in PBS for 1 hours. Cells were incubated overnight with rabbit anti-human PDX1 (1:200, Abcam, Cambridge, MA, #AB47267), rabbit anti-human NEUROG3 (1:50, DSHB, Iowa City, IA, #F25A1B3), rabbit anti-human MAFA (1:200, Abcam, #AB47267), goat anti-human NKX6.1 (1:100, R&D Systems, #AF5857), rabbit anti-human NEUROD1(1:200, Sigma, #N3663), mouse anti-human NKX2.2 (1:50, DSHB, #74.5A5), anti-human ESRRG (1:100, Abcam, #AB131593), mouse anti-human MAFB (1:25, R&D Systems, #MAB3810) overnight at 4°C, followed by incubation with secondary antibodies for 1 hour at room temperature.
For the characterization of psBCs, undifferentiated iPSCs were seeded on chamber slides and went through the differentiation protocol described above. Differentiated cells were fixed at the indicated time points and were permeabilized and blocked as described above. Cells were incubated overnight with guinea pig anti-human insulin (1:400, Dako, Santa Clara, CA, # A056401), goat anti-human NKX6.1 (1:100), rabbit anti-human NEUROD1(1:200), mouse anti-human NKX2.2 (1:50), mouse anti-human glucagon (1:300, Abcam, #ab10988-100), rabbit anti-human somatostatin (1:100, Santa Cruz, Dallas, Texas, #sc-20999) or mouse anti-human C-peptide (1:400, Thermo Fisher Scientific, #MA1-19159) overnight at 4°, followed by incubation with secondary antibodies for 1 hour at room temperature. Nuclei were counterstained by DAPI (blue)
Mouse Kidneys with the grafts were harvested and frozen in OCT Compound. 7 μm pancreatic cryosections were immediately fixed, permeabilized and then blocked as described above .Slides were then incubated with guinea pig anti-human insulin (1:400), goat anti-human NKX6.1 (1:100), rabbit anti-human NEUROD1 (1:200), mouse anti-human NKX2.2 (1:50), mouse anti-human glucagon (1:300), rabbit anti-human somatostatin (1:100) overnight at 4°, followed by secondary antibody incubation for 1 hour at room temperature. Nuclei were counterstained by DAPI (blue). Images were taken using a Zeiss LSM 780 confocal laser scanning microscope and analyzed with Zeiss imaging software. Fluorescence intensity were analyzed by Image J software.

Flow cytometry. iPSC-derived psBCs were dispersed into single-cell suspension by incubation in Trypsin at 37°C for 10 minutes, quenched with 3-4 volumes of FCS-containing culture media and cells were spun down for 5 min at 800g. Cells were transferred to a 1.7ml microcentrifuge tube, fixed in 4%PFA, permeabilized with 0.3% Triton-X and then blocked with 5% FBS for 1 hours. Cells were incubated overnight with guinea pig anti-human insulin (1:400), mouse anti-human glucagon (1:300), mouse anti-human C-peptide (1:400) at 4°C. After washing three times with 5% FBS, cells were stained with secondary antibodies. Cell were then washed and filtered through a 35μm mesh Falcon tube, and analyzed using the LSR-II flow cytometer (BD Biosciences). Analysis of the results was performed using FlowJo software.

Electron microscopy. iPSC-derived psBCs were fixed at room temperature. Cell samples were processed and analyzed by transmission Electron Microscopy at Mayo Clinic Microscopy and Cell Analysis Core.

***In vitro* GSIS assay.** We used an islet perifusion system (Biorep technologies, Miami Lakes, FL). Approximately 1 x 106 psBCs differentiated in wells of 48-well plates, or cadaver human islets (n=200 hand-picked islets) were first incubated in 4mM glucose Krebs Ringer Bicarbonate buffer supplemented with 0.2% BSA for 30 minutes at 37°C. Cells were then gently scraped off from the wells, transferred into a sterile 1.5ml eppendorf tube, and centrifuged at 37°C for 5 minutes. Cell pellets were transferred into the perifusion chamber and were washed for 40 minutes in the perifusion system with 4mM glucose buffer, which were preheated to 37°C and oxygenized with 95% O2 and 5% CO2. After washing, cells were exposed to 4mM glucose perifusate for 32min, followed by 32 minutes of 16mM glucose buffer, 32 minutes of 16mM glucose buffer supplemented with 100nM GLP-1 (Peprotech, Rocky Hill, NJ, #130-08-1MG), 32 minutes of 4mM glucose buffer and finally 8 minutes of 16mM glucose buffer supplemented with 30mM KCL. Effluent was collected in 2-minute intervals and assayed for human C-peptide by ELISA (Alpco, Salem, NH, #80-CPTHU-CH01) for subsequent determination of basal and GSIS.

Mice transplantation studies. All animal experiments were performed in accordance with Mayo Clinic International Animal Care and Use Committee (IACUC) regulations. Immunodeficient Fox Chase SCID-Beige mice, aged 8-10 weeks, were purchased from Charles River Laboratory. To induce diabetes, mice received 50 mg/kg body weight streptozotocin (STZ, Sigma, #S0130) intraperitoneally over the course of five consecutive days. Mice with non-fasting blood glucose levels over 250 mg/dl after STZ administration were used and randomized into four groups in the following experiments. PsBCs clusters (approximately 50 million cells per mouse), and human beta-cell line (EndoC-βH2 cells, approximately 5 million cells per mouse) were gently scraped off and transferred into a 15ml conical vial. After spinning for 5 minutes at 800g, cells were loaded into a catheter for cell delivery into the kidney capsules (El Khatib and Sakuma et al.,2015). 1 day, 4 day and 1, 2, 3, 5, 7, 9, 11, 13 weeks after the surgery, fasting (16 hours) blood glucose was tested using glucose monitor and strips. To measure glucose-responsive C-peptide secretion, fasting blood and 30 minutes blood after an intraperitoneal injection of D-(+)-glucose at 2g/kg body weight was collected through retro orbital bleeding every two weeks. Serum was separated out using Microvettes (Sarstedt, Nümbrecht, Germany, #20.1278.100) and stored at 80°C until ELISA analysis. At indicated time points, kidneys containing the grafts were dissected from the mice, embedded and frozen in OCT compound. Immunostaining was performed as described above. No statistical method was used for sample size estimation. Investigators were not blinded to the group allocations. Mice transplantation study was performed in duplicate.

Intraperitoneal glucose tolerance test (IPGTT). To measure glucose handling capacity *in vivo*, mice were fasted (16 hours) and blood glucose was tested at 0min, 30min, 60min, 90min, 120min after IP injection of D-(+)-glucose at 2g/kg body weight.

Sample size and statistical analysis. All data represent the means ±S.E.M. of three to nine samples, as indicated in the figure legends. Group comparisons were analyzed by unpaired or paired *t* tests, one sample *t* test and one-way ANOVA with Tukey test through IBM SPSS Statistics 22. *P<0.05, **P<0.01, ***P<0.001. Bar graphs, heatmaps, curves, box and whisker plots were generated with GraphPad Prism7 and Excel 2010.