Stem Cell Reports, Volume *12*

Supplemental Information

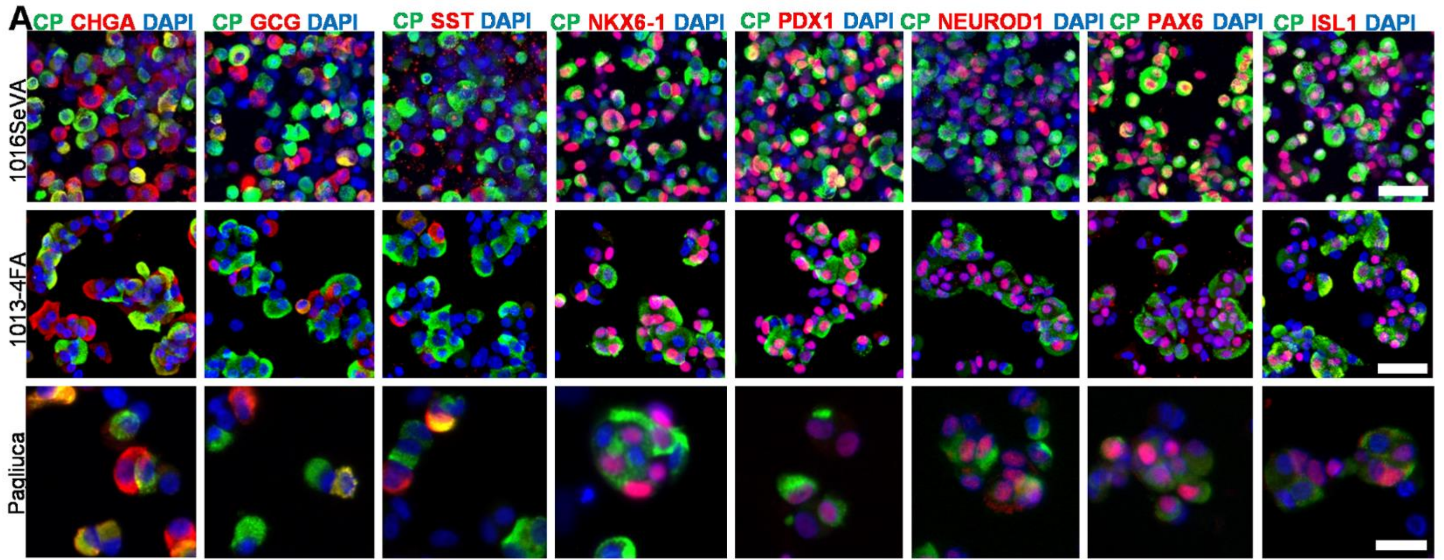
Acquisition of Dynamic Function in Human Stem Cell-Derived β Cells

Leonardo Velazco-Cruz, Jiwon Song, Kristina G. Maxwell, Madeleine M. Goedegebuure, Punn Augsornworawat, Nathaniel J. Hogrebe, and Jeffrey R. Millman

Supplemental Figures

Figure S1. Pilot data leading to our differentiation strategy and hiPSC reproduction, related to Figure 1. (**A**) Human insulin secretion of Stage 6 cells generated in CMRLS or ESFM, with or without resizing, and with or without factors (Alk5i and T3) in a static GSIS assay. The combinations investigated were (1) CMRLS, no resize, no factors (*n*=3), (2) CMRLS, yes resize, no factors (*n*=6), (3) ESFM, no resize, no factors (*n*=3), (4) ESFM, yes resize, no factors (*n*=3), (5) ESFM, yes resize, yes factors (*n*=3). HUES8 cell line used. (**B**) Flow cytometric dot plots of Stage 6 cells generated in CMRLS or ESFM, with or without resizing, and with or

without factors (Alk5i and T3) immunostained for C-peptide and NKX6-1. HUES8 cell line used. (**C**) Human insulin secretion in a static GSIS assay of three hiPSC lines (*n*=3 each). *P < 0.05, **P < 0.01, and ***P < 0.0001 by one-sided paired *t*-test. (**D**) Dynamic human insulin secretion of Stage 6 cells generated with two hiPSC lines in a perfusion GSIS assay. Cells are perfused with low glucose (2 mM) except where high glucose (20 mM) is indicated (*n*=3 for 1013-4FA and *n*=4 for 1016SeVA).



B
1016SeVA
Gating Control
22 38 16 25
C-Peptide
NKX6-1
99.9
31 58 8 3
CHGA
NKX6-1
99.8
34 25 39 3
C-Peptide
GCG
99.9
49 9 41 1
C-Peptide
SST
99.8

C
1013-4FA
Pagliuca
Gating Control
23 37 16 25
21 23 32 24
C-Peptide
NKX6-1
99.7
30 57 9 4
23 35 30 12
CHGA
NKX6-1
99.8
33 26 39 3
33 16 48 3
C-Peptide
GCG
99.7
52 6 42 1
46 2 52 0
C-Peptide
SST
99.8

Figure S2. Additional immunostaining data for Stage 6 cells, related to Figure 2. (**A**) Immunostaining of Stage 6 clusters single-cell dispersed, plated overnight, and stained for the indicated markers. Stage 6 cells were generated from two hiPSC lines with the protocol from this paper and the HUES8 cell line with the Pagliuca protocol. Scale bar=50 μm for 1016SeVA and 1013-4FA and 25 μm for Pagliuca protocol. (**B-C**) Flow cytometric dot plots of Stage 6 cells generated from two hiPSC lines with the protocol from this paper and the HUES8 cell line with the Pagliuca protocol stained with the indicated markers.

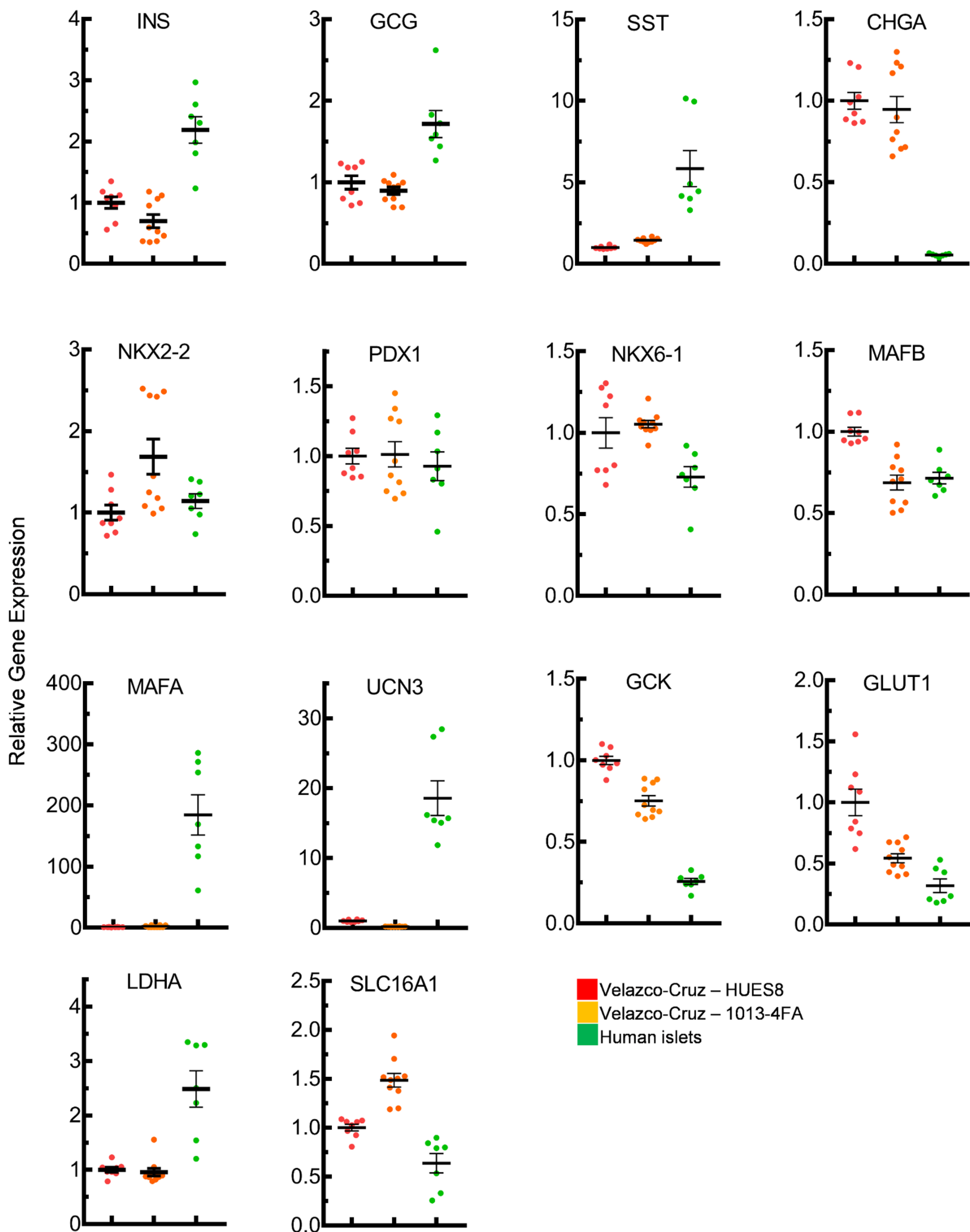


Figure S3. Additional gene expression data for Stage 6 cells, related to Figure 2. Gene expression data for Stage 6 cells generated with our differentiation protocol from the HUES8 (n=8) and 1013-4FA (n=10) lines and human islets (n=7) measured with real-time PCR. The HUES8 and human islet plotted here is the same as from Figure 2.

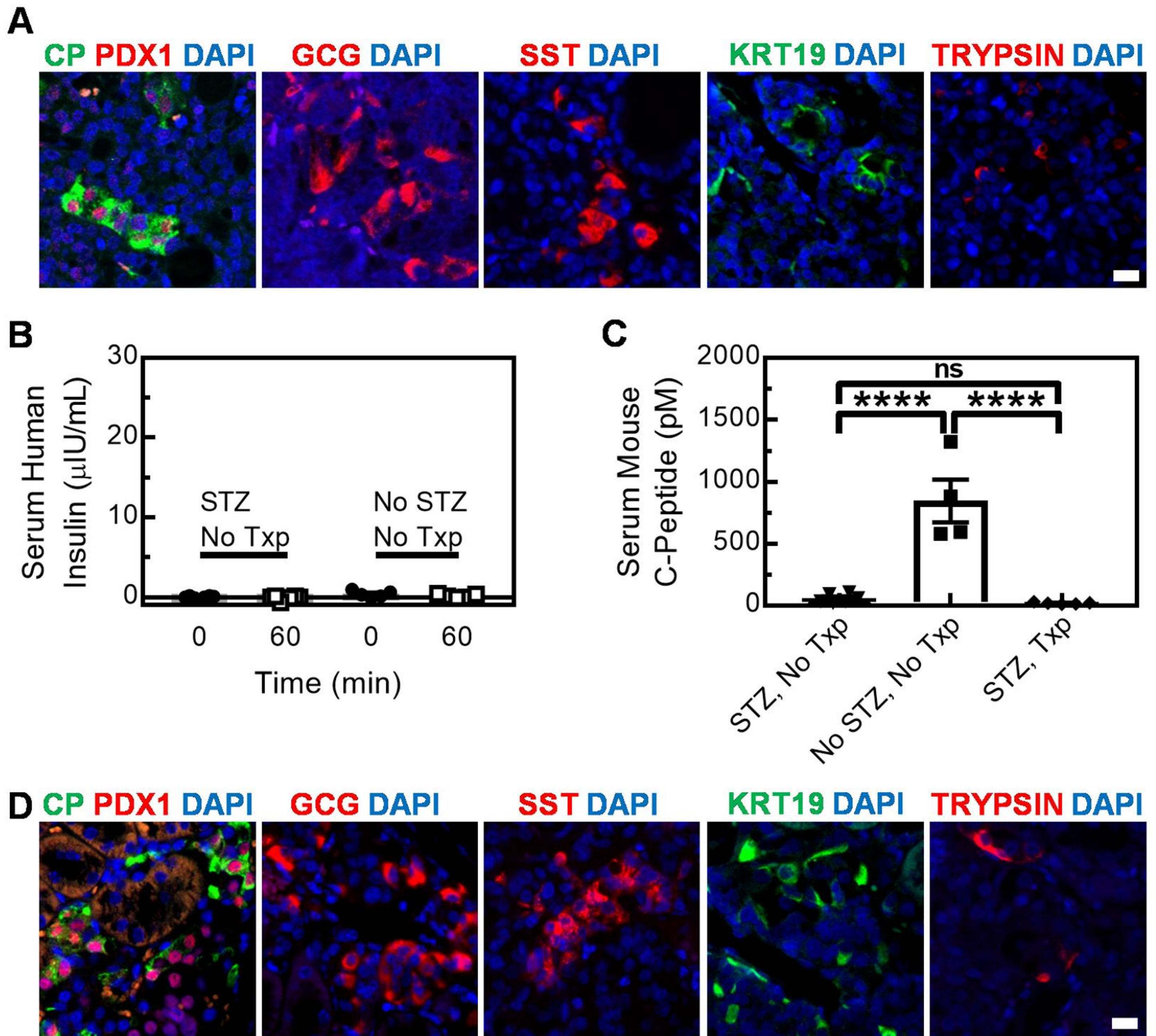


Figure S4. Additional immunostaining, serum human insulin measurements, and mouse C-peptide measurements, related to Figure 3. (**A**) Immunostaining of sectioned paraffin-embedded explanted kidneys of non-STZ-treated mice 6 months after transplantation for C-peptide (CP; β cell marker), PDX1 (β cell marker), glucagon (GCG; α cell marker), somatostatin (SST; δ cell marker), KRT19 (ductal marker), and trypsin (acinar marker). Scale bar=25 µm. (**B**) Serum human insulin of STZ, No Txp mice (*n*=6) and No Stz, No Txp (*n*=5) fasted overnight 0 and 60 min after an injection of 2 g/kg glucose. (**B**) Serum mouse C-peptide of STZ, No Txp (*n*=6), No STZ, No Txp (*n*=4), and STZ, TXP (*n*=5). ****P < 0.0001 and ns by one-way ANOVA Tukey multiple comparison test. (**C**) Immunostaining of sectioned paraffin-embedded explanted kidneys of STZ-treated mice 11 wk after transplantation for the indicated markers. Scale bar=25 µm. HUES8 cell line used.

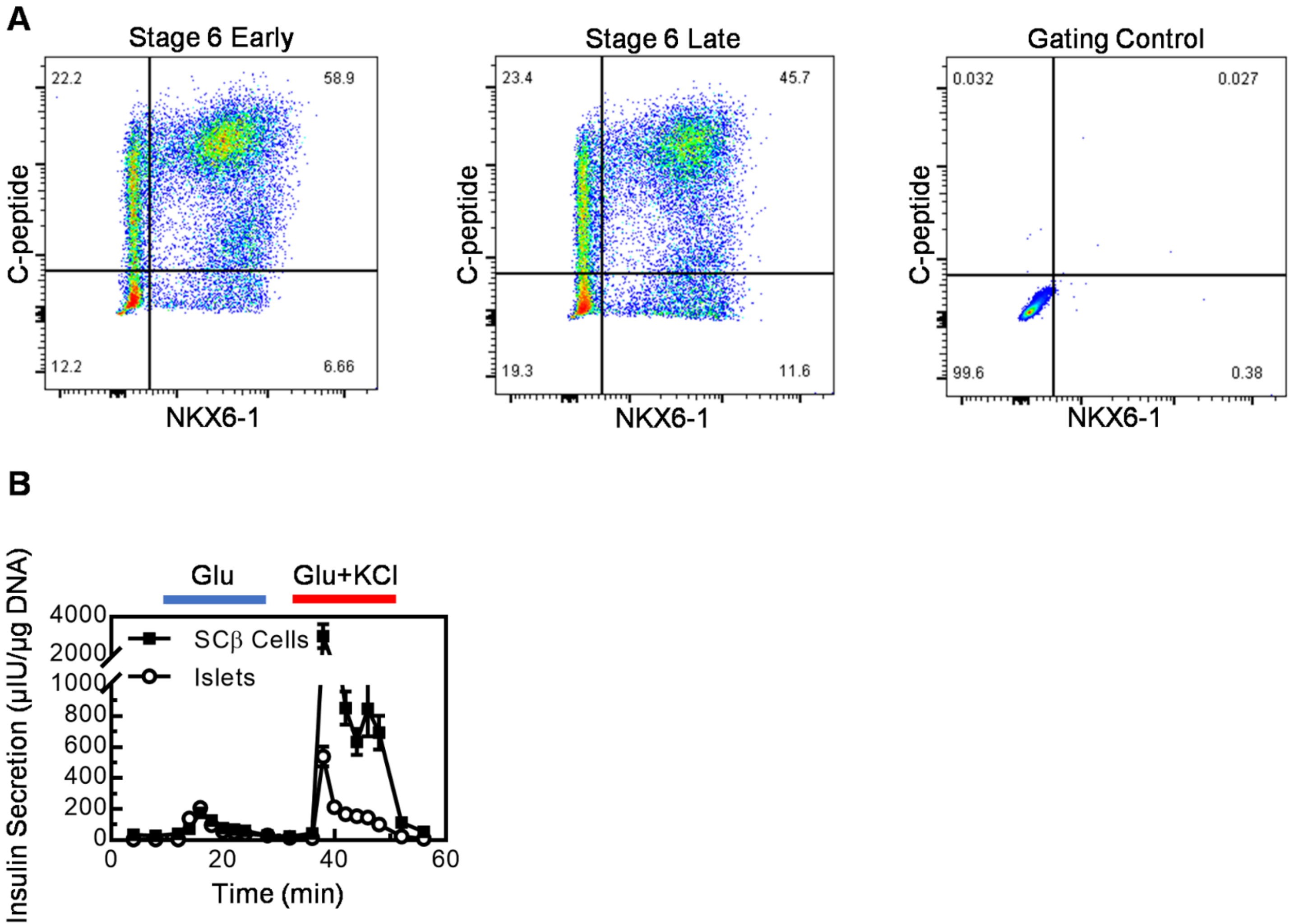


Figure S5. Temporal flow cytometry during Stage 6 and KCl challenge of human islets, related to Figure 4. (**A**) Flow cytometric dot plots of Stage 6 cells at early (9 d) and late (26 d) time points stained for C-peptide and NKX6-1. HUES8 cell line used. (**B**) Dynamic human insulin secretion of human islets in a perfusion GSIS assay perfused with low glucose (2 mM) except where high (20 mM) glucose is indicated (Glu), then perfused with a second challenge of high glucose with KCl where indicated (Glu+Factor) (*n*=4).

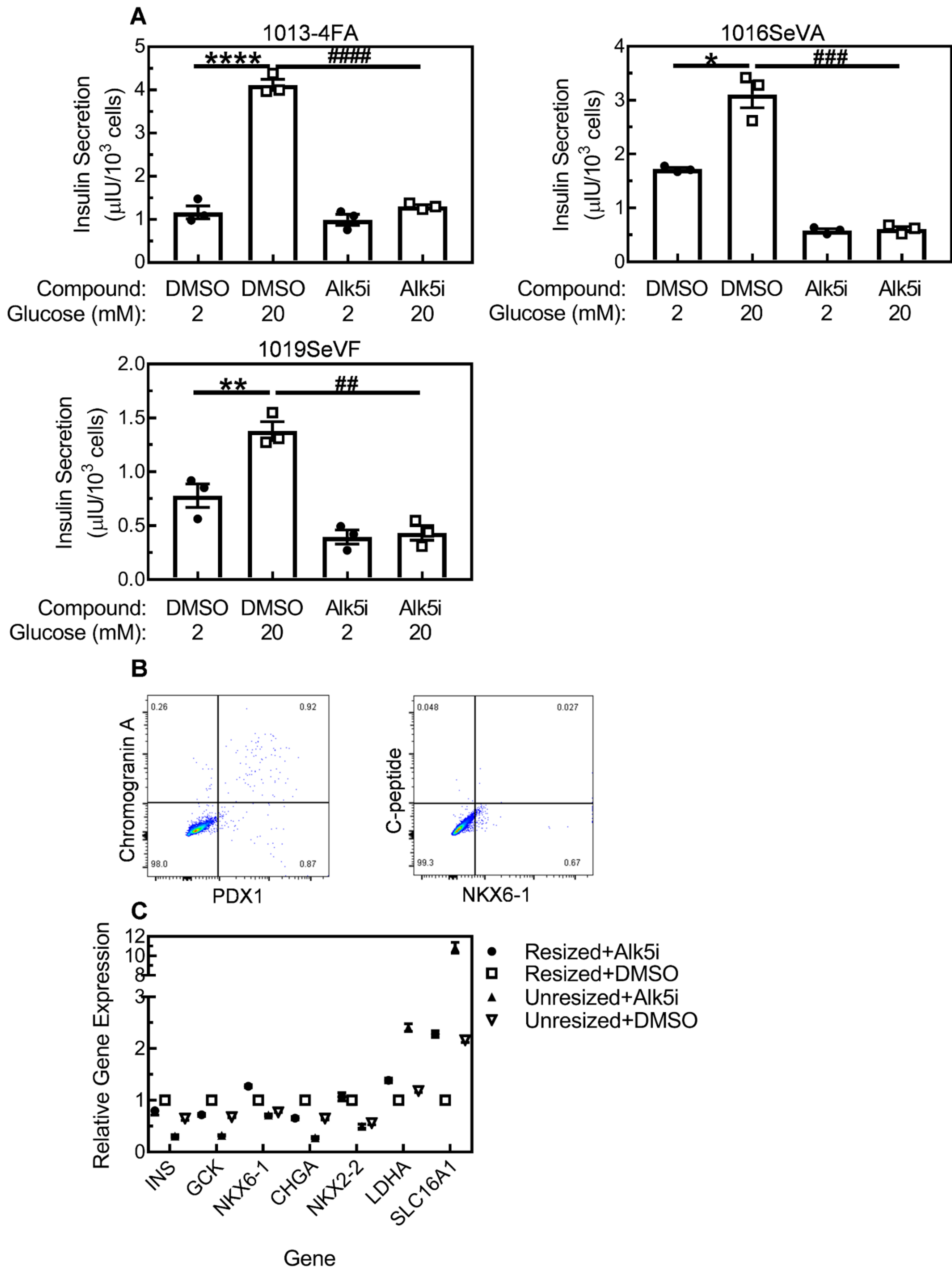


Figure S6. Stage 6 cells generated from hiPSC undergo GSIS that is inhibited by Alk5i, flow cytometry controls, and gene expression data, related to Figure 5. (**A**) Human insulin secretion of Stage 6 cells generated from three hiPSC lines (1013-4FA, *n*=4;

1016SeVA, *n*=3; 1019SeVF, *n*=3) in static GSIS assay treated with DMSO or Alk5i. *P < 0.05, **P < 0.01 , ****P < 0.0001 by two-way paired *t*-test; ##P < 0.01, ###P < 0.001, ####P < 0.0001 by two-way unpaired *t*-test. The control data here is the same data in Figure S1. (**B**) Flow cytometry controls for Figure 5. The C-peptide/NKX6-1 control is the same as shown in Figure 2. (**C**) Real-time PCR analysis of Stage 6 cells with or without resizing treated with Alk5i or DMSO (*n*=3). Data generated with the 1013-4FA cell line.

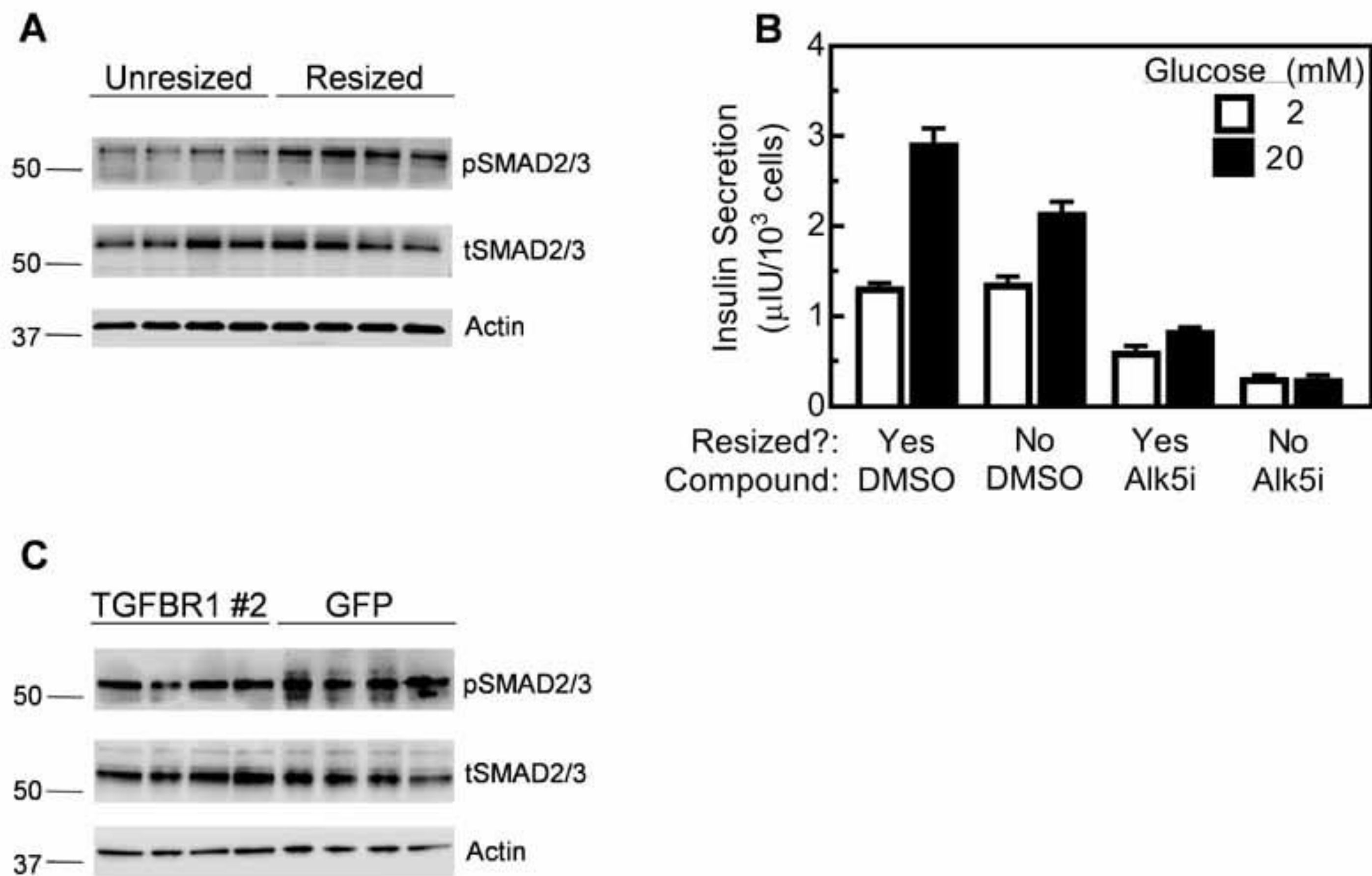


Figure S7. Resized and unresized Stage 6 clusters have SMAD2/3 phosphorylation and reduced GSIS with Alk5i treatment and TGFBR1 #2 western blot, related to Figure 6. (**A**) Western blot of Stage 6 cells with and without resizing stained for phosphorylated SMAD 2/3 (pSMAD2/3), total SMAD 2/3 (tSMAD2/3), and Actin. Data shown is from 1013-4FA. (**B**) Human insulin secretion of Stage 6 cells in static GSIS assay resized or unresized with treatment of DMSO or Alk5i. Data shown is from 1013-4FA. (**C**) Western blot of Stage 6 cells transduced with lentiviruses containing GFP or TGFBR1 #2 shRNA. Data shown is from HUES8.

Supplemental Experimental Procedures

Culture of Undifferentiated Cells. The HUES8 hESC line, 1013-4FA (a non-diabetic hiPSC line referenced as ND-1 in Millman et al., 2016), 1016SeVA (a non-diabetic hiPSC line referenced as ND-2 in Millman et al. 2016), and 1019SeVF (a type 1-diabetic hiPSC line referenced as T1D-1 in Millman et al. 2016) have been previously published (Millman et al., 2016; Pagliuca et al., 2014) and were generously provided by Dr. Douglas Melton (Harvard University). Undifferentiated cells were cultured using mTeSR1 (StemCell Technologies; 05850) in 30-mL spinner flasks (REPROCELL; ABBWVS03A) on a rotator stir plate (Chemglass) spinning at 60 RPM in a humidified 5% CO_2 37 °C incubator. Cells were passaged every 3-4 days by single cell dispersion using Accutase (StemCell Technologies; 07920), viable cells counted with Vi-Cell XR (Beckman Coulter), and seeded at 6 x 10^5 cells/mL in mTeSR1+ 10 µM Y27632 (Abcam; ab120129).

Cell Line Differentiation. To initiate differentiation, undifferentiated cells were single-cell dispersed using Accutase and seeded at 6 x 10^5 cells/mL in mTeSR1+ 10 µM Y27632 in a 30-ml spinner flask. Cells were then cultured for 72 hr in mTeSR1 and then cultured in the following differentiation media. <u>Stage 1</u> (3 days): S1 media + 100 ng/ml Activin A (R&D Systems; 338-AC) + 3 µM Chir99021 (Stemgent; 04-0004-10) for 1 day. S1 media + 100 ng/ml Activin A for 2 days. <u>Stage 2</u> (3 days): S2 media + 50 ng/ml KGF (Peprotech; AF-100-19). <u>Stage 3</u> (1 day): S3 media + 50 ng/ml KGF + 200 nM LDN193189 (Reprocell; 040074) + 500 nM PdBU (MilliporeSigma; 524390) + 2 µM Retinoic Acid (MilliporeSigma; R2625) + 0.25 µM Sant1 (MilliporeSigma; S4572) + 10 µM Y27632. <u>Stage 4</u> (5 days): S4 media + 5 ng/mL Activin A + 50 ng/mL KGF + 0.1 µM Retinoic Acid + 0.25 µM SANT1 + 10 µM Y27632. <u>Stage 5</u> (7 days): S5 media + 10 µM ALK5i II (Enzo Life Sciences; ALX-270-445-M005) + 20 ng/mL Betacellulin (R&D Systems; 261-CE-050) + 0.1 µM Retinoic Acid + 0.25 µM SANT1 + 1 µM T3 (Biosciences; 64245) + 1 µM XXI (MilliporeSigma; 595790). <u>Stage 6</u> (7-35 days): ESFM.
Differentiation media formulations used were the following. <u>S1 media</u>: 500mL MCDB 131 (Cellgro; 15-100-CV) supplemented with 0.22 g glucose (MilliporeSigma; G7528), 1.23 g sodium bicarbonate (MilliporeSigma; S3817), 10 g bovine serum albumin (BSA) (Proliant; 68700), 10 µL ITS-X (Invitrogen; 51500056), 5 mL GlutaMAX (Invitrogen; 35050079), 22 mg vitamin C (MilliporeSigma; A4544), and 5 mL penicillin/streptomycin (P/S) solution (Cellgro; 30-002-CI). <u>S2 media</u>: 500mL MCDB 131 supplemented with 0.22 g glucose, 0.615 g sodium bicarbonate, 10 g BSA, 10 µL ITS-X, 5 mL GlutaMAX, 22 mg vitamin C, and 5 mL P/S. <u>S3 media</u>: 500mL MCDB 131 supplemented with 0.22 g glucose, 0.615 g sodium bicarbonate, 10 g BSA, 2.5 mL ITS-X, 5 mL GlutaMAX, 22 mg vitamin C, and 5 mL P/S. <u>S5 media</u>: 500mL MCDB 131 supplemented with 1.8 g glucose, 0.877 g sodium bicarbonate, 10 g BSA, 2.5 mL ITS-X, 5 mL GlutaMAX, 22 mg vitamin C, 5 mL P/S, and 5 mg heparin (MilliporeSigma; A4544). <u>ESFM</u>: 500mL MCDB 131 supplemented with 0.23 g glucose, 10.5 g BSA, 5.2 mL GlutaMAX, 5.2 mL P/S, 5 mg heparin, 5.2 mL MEM nonessential amino acids (Corning; 20-025-CI), 84 µg $ZnSO_4$ (MilliporeSigma; 10883), 523 µL Trace Elements A (Corning; 25-021-CI), and 523 µL Trace Elements B (Corning; 25-022-CI). Cells were sometimes cultured with 0.01% DMSO. Cells were resized the first day of Stage 6 by incubating in Gentle Cell Dissociation Reagent (StemCell Technologies; 07174) for 8 min, washed with ESFM, passed through a 100 µm nylon cell strainer (Corning; 431752), and cultured in ESFM in 6-well plates on an Orbi-Shaker (Benchmark) set at 100 RPM. Assessment assays were performed between 10-16 days of stage 6 unless otherwise stated. Human islets were acquired from Prodo Labs for comparison. A subset of Stage 6 experiments were performed without cluster resizing, with Alk5i and T3, with Alk5i, and/or CMRL 1066 Supplemented (CMRLS) (Mediatech; 99-603-CV) + 10% fetal bovine serum (FBS) (HyClone; 16777) + 1% P/S rather than ESFM, as indicated. To perform the Pagliuca protocol, the protocol outlined in Pagliuca, Millman, Gürtler et al. 2014 was followed in 30-mL spinner flasks.

Light Microscopy. Light Microscopy images were taken of unstained or stained with 2.5 µg/mL DTZ (MilliporeSigma; 194832) cell clusters using an inverted light microscope (Leica DMi1).

Immunostaining. To immunostain *in vitro* cell clusters or *ex vivo* transplanted grafts within mouse kidneys, samples were fixed with 4% paraformaldehyde (Electron Microscopy Science; 15714) overnight at 4 °C. After fixation, cell clusters were embedded in Histogel (Thermo Scientific; hg-4000-012). Embedded cell clusters and grafts were placed in 70% ethanol and submitted for paraffin-embedding and sectioning to the Division of Comparative Medicine (DCM) Research Animal Diagnostic Laboratory Core at Washington University. Paraffin was removed using Histoclear (Thermo Scientific; C78-2-G), samples rehydrated, and antigens retrieved with 0.05 M EDTA (Ambion; AM9261) in a pressure cooker (Proteogenix; 2100 Retriever). Samples were blocked and permeabilized for 30-min with staining buffer (5% donkey serum (Jackson Immunoresearch; 017-000-121) and 0.1% Triton-X 100 (Acros Organics; 327371000) in PBS), stained overnight with primary antibodies at 4 °C, stained for 2 hr with secondary antibodies at 4 °C, and treated with mounting solution DAPI Fluoromount-G (SouthernBiotech; 0100-20). To immunostain plated cells, clusters were single-cell dispersed using TryplE Express (Fisher, 12604039), plated down onto Matrigel (Fisher, 356230)-coated plates, cultured in ESFM for 16 hr, and fixed for 30 min with 4% paraformaldehyde at RT. Fixed cells were blocked and permeabilized with staining buffer for 45 min at RT, stained overnight with primary antibodies at 4 °C, stained for 2 hr with secondary antibodies at RT, and stained with DAPI for 5 min. Imaging was performed on a Nikon A1Rsi confocal microscope or Leica DMI4000 fluorescence microscope.

Primary antibody solutions were made in staining buffer with the following antibodies at 1:300 dilution unless otherwise noted: rat-anti-C-peptide (DSHB; GN-ID4-S), 1:100 mouse-anti-nkx6.1 (DSHB, F55A12-S), mouse-anti-glucagon (ABCAM; ab82270), goat-anti-pdx1 (R&D Systems; AF2419), rabbit-anti-somatostatin (ABCAM; ab64053), mouse-anti-pax6 (BDBiosciences;561462), rabbit-anti-chromogranin a (ABCAM; ab15160), goat-anti-neurod1 (R&D Systems; AF2746), mouse-anti -Islet1 (DSHB, 40.2d6-s), 1:100

mouse-anti-cytokeratin 19 (Dako; MO888), undiluted rabbit-anti-glucagon (Cell Marque; 259A-18), 1:100 sheep-anti-trypsin (R&D Systems; AF3586). Secondary antibody solutions were made in staining buffer with the following antibodies at 1:300 dilution: anti-rat-alexa fluor 488 (Invitrogen; a21208), anti-mouse-alexa fluor 594 (Invitrogen; a21203), anti-rabbit-alexa fluor 594 (Invitrogen; a21207), anti-goat-alexa fluor 594 (Invitrogen; a11058).

Static GSIS. Assays were performed by collecting ~20-30 stage 6 clusters or cadaveric human islets, washed twice with KRB buffer (128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$ 1.2 mM $MgSO_4$, 1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 10 mM HEPES (Gibco; 15630-080), and 0.1% BSA), resuspended in 2 mM glucose KRB, and placed into transwells (Corning; 431752) in 24-well plates. Clusters were incubated at 2 mM glucose KRB for a 1 hr equilibration. The transwell was then drained and transferred into a new 2 mM glucose KRB well, discarding the old KRB solution. Clusters were again incubated for 1 hr at low glucose and then the transwell is drained and transferred into a new 2, 5.6, 11.1, or 20 mM glucose KRB well, retaining the old 2 mM glucose KRB. Clusters were then incubated for 1 hr at high glucose and then the transwell was drained and the old glucose KRB was retained. The retained KRB was run with the Human Insulin Elisa (ALPCO; 80-INSHU-E10.1) to quantify insulin secretion. The cells were single-cell dispersed by TrypLE treatment, counted on a Vi-Cell XR, and viable cell counts used to normalize insulin secretion.

Dynamic Glucose-Stimulated Insulin Secretion. A perifusion system was assembled as has been previously reported (Bentsi-Barnes et al., 2011). Our system used a high precision 8-channel dispenser pump (ISMATEC; ISM931C) in conjunction with 0.015" inlet and outlet two-stop tubing (ISMATEC; 070602-04i-ND) connected to 275-µl cell chamber (BioRep; Peri-Chamber) and dispensing nozzle (BioRep; PERI-NOZZLE) using 0.04" connection tubbing (BioRep; Peri-TUB-040). Solutions, tubing, and cells were maintained at 37 °C in a water bath. Stage 6 clusters and cadaveric human islets were washed with KRB twice and resuspended in 2 mM glucose KRB. Cells were then loaded onto a Biorep perifusion chamber sandwiched between two layers of Bio-Gel P-4 polyacrylamide beads (Bio-Rad; 150-4124). Cells were perfused with 2 mM glucose KRB for 90 min prior to sample collection for equilibration. For single high glucose challenges, sample collection was started with cells exposed to 2 mM glucose KRB for 12 min, followed by 24 min of 20 mM glucose KRB, and back to 2 mM glucose KRB for an additional 12 min. For multiple secretagogue challenges, sample collection was started with cells exposed to 2 mM glucose KRB for 6 min, followed by 12 min of 20 mM glucose KRB, 6 min 2 mM glucose KRB, 12 min of 20 mM glucose KRB plus treatment, and finally 6 min of 2 mM glucose KRB. Treatments with multiple secretagogues were as follows: 20 mM glucose only, 10 nM Extendin-4 (MilliporeSigma; E7144), 100 µM IBMX (MilliporeSigma; I5879), 300 µM Tolbutamide (MilliporeSigma; T0891), 20 mM L-Arginine (MilliporeSigma; A5006), and 30 mM KCL (Thermo Fisher; BP366500). Effluent was collected at a 100 µl/min flow rate with 2-4 min collection points. After sample collection, clusters were collected and lysed in 10 mM Tris (MilliporeSigma; T6066), 1 mM EDTA, and 0.2% Triton-X 100 solution and DNA was quantified using Quant-iT Picogreen dsDNA assay kit (Invitrogen; P7589). Insulin secretion was quantified using the Human Insulin Elisa kit.

Flow Cytometry. Clusters were single-cell dispersed with TrypLE, fixed with 4% paraformaldehyde for 30 min at 4 °C, blocked and permeabilized with staining buffer for 30 min at 4 °C, incubated with primary antibodies in staining buffer overnight at 4 °C, incubated with secondary antibodies in staining buffer for 2 hr at 4 °C, resuspended in staining buffer, and analyzed on an LSRII (BD Biosciences) or X-20 (BD Biosciences). Dot plots and percentages were generated using FlowJo. All antibodies were used at 1:300 dilution except where noted. The antibodies used were: rat-anti-C-peptide (DSHB; GN-ID4-S), mouse-anti -nkx6.1 (1:100; DSHB, F55A12-S), mouse-anti -glucagon (ABCAM; ab82270), rabbit-anti-somatostatin (ABCAM; ab64053), rabbit-anti-chromogranin A (1:1000; ABCAM; ab15160), goat-anti-pdx1(R&D Systems; AF2419), anti-rat-alexa fluor 488 (Invitrogen; a21208), anti-mouse-alexa fluor 647 (Invitrogen; a31571), anti-rabbit-alexa fluor 647 (Invitrogen; a31573), anti-goat-alexa fluor 647 (Invitrogen; a21447), anti-rabbit-alexa fluor 488 (Invitrogen; a21206).

Real-Time PCR. RNA was extracted using the RNeasy Mini Kit (Qiagen; 74016) with DNase treatment (Qiagen; 79254), and cDNA was synthesized using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems; 4368814). Real-time PCR reactions were performed in PowerUp SYBR Green Master Mix (Applied Biosystems; A25741) on a StepOnePlus (Applied Biosystems) and analyzed using ΔΔCt methodology. TBP was used as a normalization gene. Primer sequences used were (gene, forward primer, reverse primer): INS, CAATGCCACGCTTCTGC, TTCTACACACCCAAGACCCG; PDX1,CGTCCGCTTGTTCTCCTC, CCTTTCCCATGGATGAAGTC; GCG, AGCTGCCTTGTACCAGCATT, TGCTCTCTCTTCACCTGCTCT; SST, TGGGTTCAGACAGCAGCTC, CCCAGACTCCGTCAGTTTCT; TBP, GCCATAAGGCATCATTGGAC, AACAACAGCCTGCCACCTTA; NKX6-1, CCGAGTCCTGCTTCTTCTTG, ATTCGTTGGGGATGACAGAG; CHGA, TGACCTCAACGATGCATTTC, CTGTCCTGGCTCTTCTGCTC; NEUROD1, ATGCCCGGAACTTTTTCTTT, CATAGAGAACGTGGCAGCAA; NGN3, CTTCGTCTTCCGAGGCTCT, CTATTCTTTTGCGCCGGTAG; NKX2-2, GGAGCTTGAGTCCTGAGGG, TCTACGACAGCAGCGACAAC; TGFBR1, CGACGGCGTTACAGTGTTTCT, CCCATCTGTCACACAAGTAAA; GUSB, CGTCCCACCTAGAATCTGCT, TTGCTCACAAAGGTCACAGG; UCN3, GGAGGGAAGTCCACTCTCG, TGTAGAACTTGTGGGGGAGG; MAFA, GAGAGCGAGAAGTGCCAACT, TTCTCCTTGTACAGGTCCCG; GCK, ATGCTGGACGACAGAGCC, CCTTCTTCAGGTCCTCCTCC; MAFB, CATAGAGAACGTGGCAGCAA, ATGCCCGGAACTTTTTCTTT; LDHA, GGCCTGTGCCATCAGTATCT, GGAGATCCATCATCTCTCCC; GLUT1, ATGGAGCCCAGCAGCAA, GGCATTGATGACTCCAGTGTT; SLC16A1, CACTTAAAATGCCACCAGCA, AGAGAAGCCGATGGAAATGA

Transplantation Studies. All animal work was performed in accordance to Washington University International Animal Care and Use Committee regulations. Mice were randomly assigned to transplantation or no transplantation groups, mouse number was chosen to be sufficient to allow for statistical significance based on prior studies (Millman et al., 2016; Pagliuca et al., 2014; Song and Millman, 2016). All procedures were performed by unblinded individuals. Two mouse cohorts were used in this study. The first consisted of non-STZ treated SCID/Beige male mice 50-56 days of age purchased from Charles River. The second consisted of STZ-treated and control-treated NOD/SCID male mice 6 weeks of age purchased from Jackson Laboratories. Mice were anaesthetized with isoflurane and injected with ~$5x10^6$ Stage 6 cells or saline (no transplant control) under the kidney capsule, similar to as previously reported (Millman et al., 2016; Pagliuca et al., 2014). Mice were monitored up to 6 months after transplantation by performing glucose-tolerance tests and *in vivo* GSIS. Mice were fasted 16 hr and then injected with 2 g/kg of glucose. Blood was collected via tail bleed. Blood glucose levels were measured with a handheld glucometer (Contour Blood Glucose Monitoring System Model 9545C; Bayer). Human insulin was determined by collecting blood and separating serum in microvettes (Sarstedt; 16.443.100) and quantifying using the Human Ultrasensitive Insulin ELISA (ALPCO Diagnostics; 80-ENSHUU-E01.1). Serum mouse C-peptide concentration was determined by collecting blood from fed mice, separating serum in microvettes, and quantifying using a Mouse C-peptide ELISA (ALPCO Diagnostics; 80-CPTMS-E01).

Insulin and Proinsulin Content. Stage 6 clusters were washed thoroughly with PBS, immersed in a solution of 1.5% HCl and 70% ethanol, kept at -20 °C for 24 hr, retrieved and vortexed vigorously, returned and kept at -20 °C for an additional 24 hr, retrieved and vortexed vigorously, and centrifuged at 2100 RCF for 15 min. The supernatant was collected and neutralized with an equal volume of 1 M TRIS (pH 7.5). Human insulin and pro-insulin content were quantified using Human Insulin Elisa and Proinsulin Elisa (Mercodia; 10-1118-01) respectively. Samples were normalized to viable cell counts made using the Vi-Cell XR.

Western Blot. Protein was extracted from cell clusters after washing with PBS by placing in western blot lysis buffer consisting 50 mM HEPES, 140 mM NaCl (MilliporeSigma; 7647-14-5), 1 mM EDTA (MilliporeSigna; 1233508), 1% Triton X-100, 0.1% Na-deoxycholate (MilliporeSigma: D6750), 0.1% SDS (ThermoScientific; 24730020), 1mM Na_3VO_4 (MilliporeSigma; 450243), 10 mM NaF (MilliporeSigma; S7920), and 1% Protease Inhibitor Cocktail (MilliporeSigma; p8340), incubating on a shaker for 15 min at 4 °C, and centrifuging at 10000 RCF for 10 min at 4 °C. Protein amount was quantified with the Pierce BCA Protein Assay (Thermo Scientific; 23228). Protein (30 µg) was loaded onto a 4-20% gradient polyacrylamide gel (Invitrogen; SP04200BOX), resolved by electrophoresis, and transferred onto a 0.45 µm nitrocellulose membrane (BioRad; 1620115). The nitrocellulose membrane was blocked with Blotting Grade Blocker (BioRad; 170-6404) and incubated with rabbit-anti-phospho-SMAD2/3 1:1000 (Cell Signaling Technologies; 8828) and rabbit-anti-Actin 1:1000 (Santa Cruz Biotechnology; SC1616) antibodies in blocker overnight at 4 °C. Membrane was washed and stained with rabbit secondary antibody 1:2500 (Jackson Immuno Research Laboratories; 211-032-171) in blocker for 2 hr at 4 °C and developed using SuperSignal West Femto (Thermo Scientific; 34096). Images were taken on an Odyssey FC (Li-COR). After imaging, the nitrocellulose membrane was stripped using Restore Western Blot Stripping Buffer (Thermo Scientific; 21059), incubated withrabbit-anti-SMAD2/3 (Cell Signaling Technologies; 8685) antibody overnight at 4 °C, washed and stained with rabbit secondary antibody 1:2500 in blocker for 2 hr at RT, developed using SuperSignal West Femto, and imaged using the Odyssey FC.

Lentivirus. pLKO.1 TRC plasmids containing shRNA sequences were received from the RNAi Core at the Washington University containing the following sequences: shRNA GFP, GCGCGATCACATGGTCCTGCT; shRNA TGFBR1 #1, GATCATGATTACTGTCGATAA; shRNA TGFBR1 #2, GCAGGATTCTTTAGGCTTTAT. Lentivirus particles were generated and tittered by the Hope Center Viral Vectors Core at Washington University using pMD-Lgp/RRE and pCMV-G, and RSV-REV packaging plasmids to contain shRNA. Stage 6 Day 1 cells were single cell dispersed using TrypLE, and 3 million cells were seeded in 4 mL ESFM lentivirus particles at MOI 3-5 on the shaker. Transduced cells were washed with fresh ESFM 16 hr post transduction. RNA extraction and static GSIS was performed on stage 6 day 13.