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Supplemental Information

Treating Diet-Induced Diabetes and Obesity with Human Embryonic Stem Cell-Derived Pancreatic Progenitor Cells and Antidiabetic Drugs

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Supplemental Data

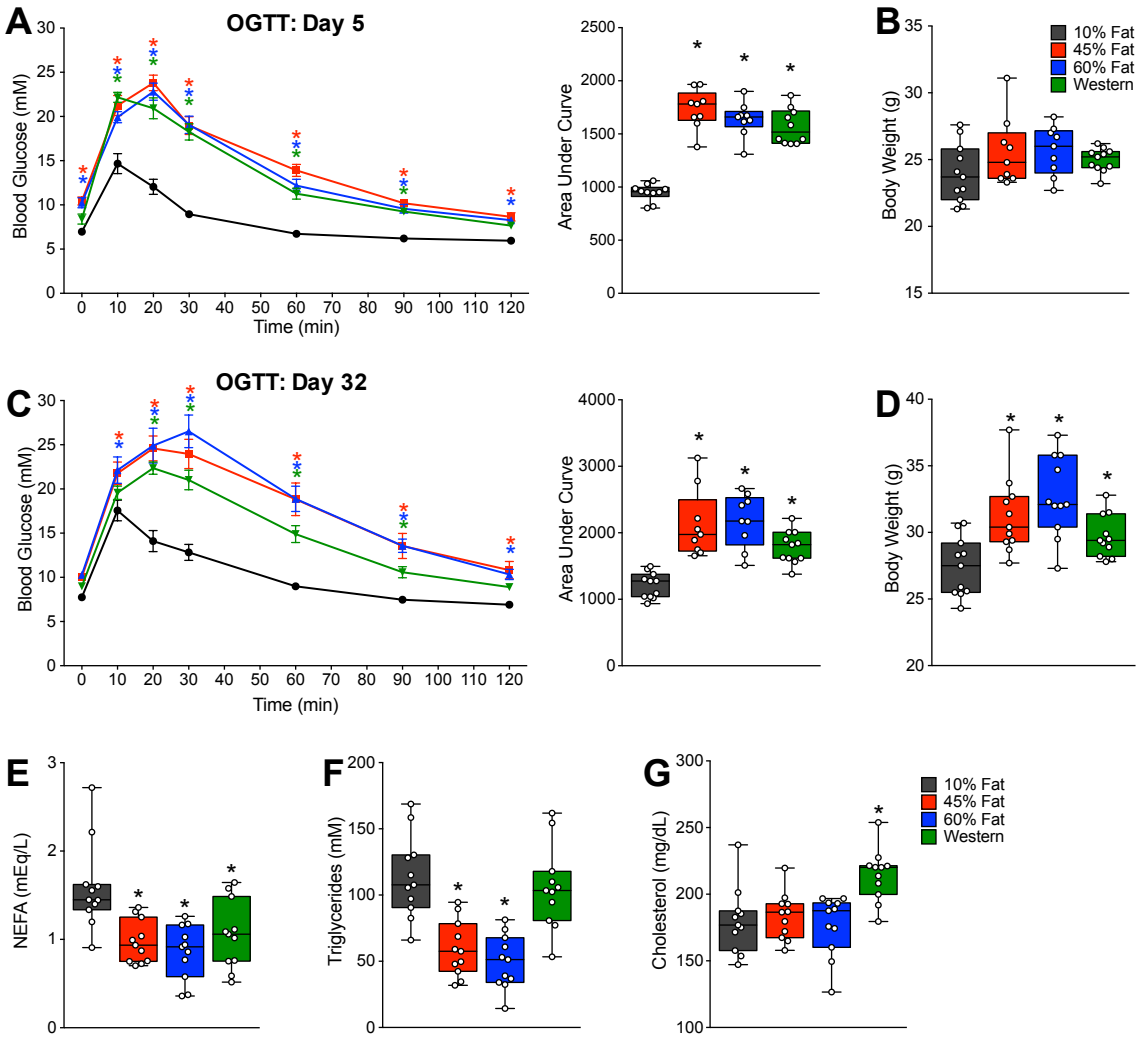

Figure S1: **SCID-beige mice rapidly develop glucose intolerance prior to differences in body weight.** Oral glucose tolerance tests (OGTT) were performed on: **A)** day 5 and **C**) day 32 after administration of the diets (10%: n = 11, 45%: n = 9, 60% n = 9, Western: n = 10-11). Area under the curve is also shown for each OGTT. Body weight was also measured at **B)** day 5 and **D)** day 32. **E-G)** Between days 47-49, plasma was collected following a 4-6 hour morning fast and levels of free fatty acids (NEFA; panel **E**), triglycerides (panel **F**) and cholesterol (panel **G**) were measured. * $p<0.05$ vs 10% controls (two-way ANOVA for multiple time point comparisons of line graphs and one-way ANOVA for bar graphs). Data are represented as mean ± SEM (line graphs) or as box and whisker plots with individual mice shown as separate data points.

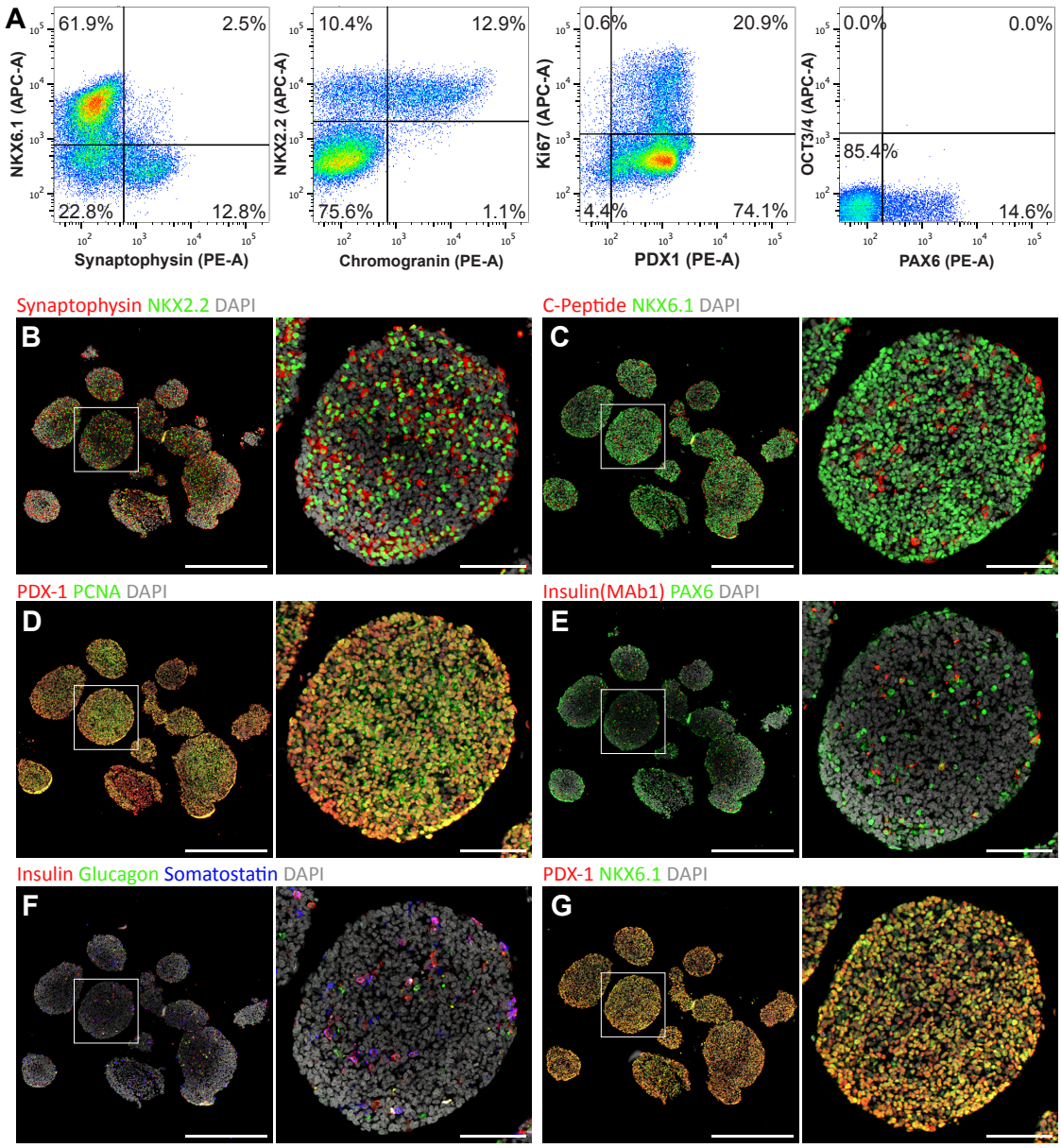

Figure S2: Profile of hESC-derived pancreatic progenitor cells prior to transplant. **A)** Flow cytometry was used to measure key markers of stage 4, day 4 pancreatic progenitor cells following our 14-day differentiation protocol. Co-expression of the following proteins were assessed: synaptophysin and NKX6.1, chromogranin and NKX2.2, PDX1 and Ki67, PAX6 and OCT3/4. **B-G)** Immunofluorescent staining of pancreatic progenitor cell clusters prior to transplant for: **B)** synaptophysin and NKX2.2, **C)** C-peptide and NKX2.2, **D)** PDX-1 and PCNA, **E)** insulin (MAb1 antibody) and PAX6, **F)** insulin (GP antibody), glucagon (Ms antibody) and somatostatin (Rb antibody), and **G)** PDX-1 and NKX6.1. DAPI nuclear staining was used for all conditions. Scale bars on low magnification images represent 500 μm and high magnification insets represent 100 μm.

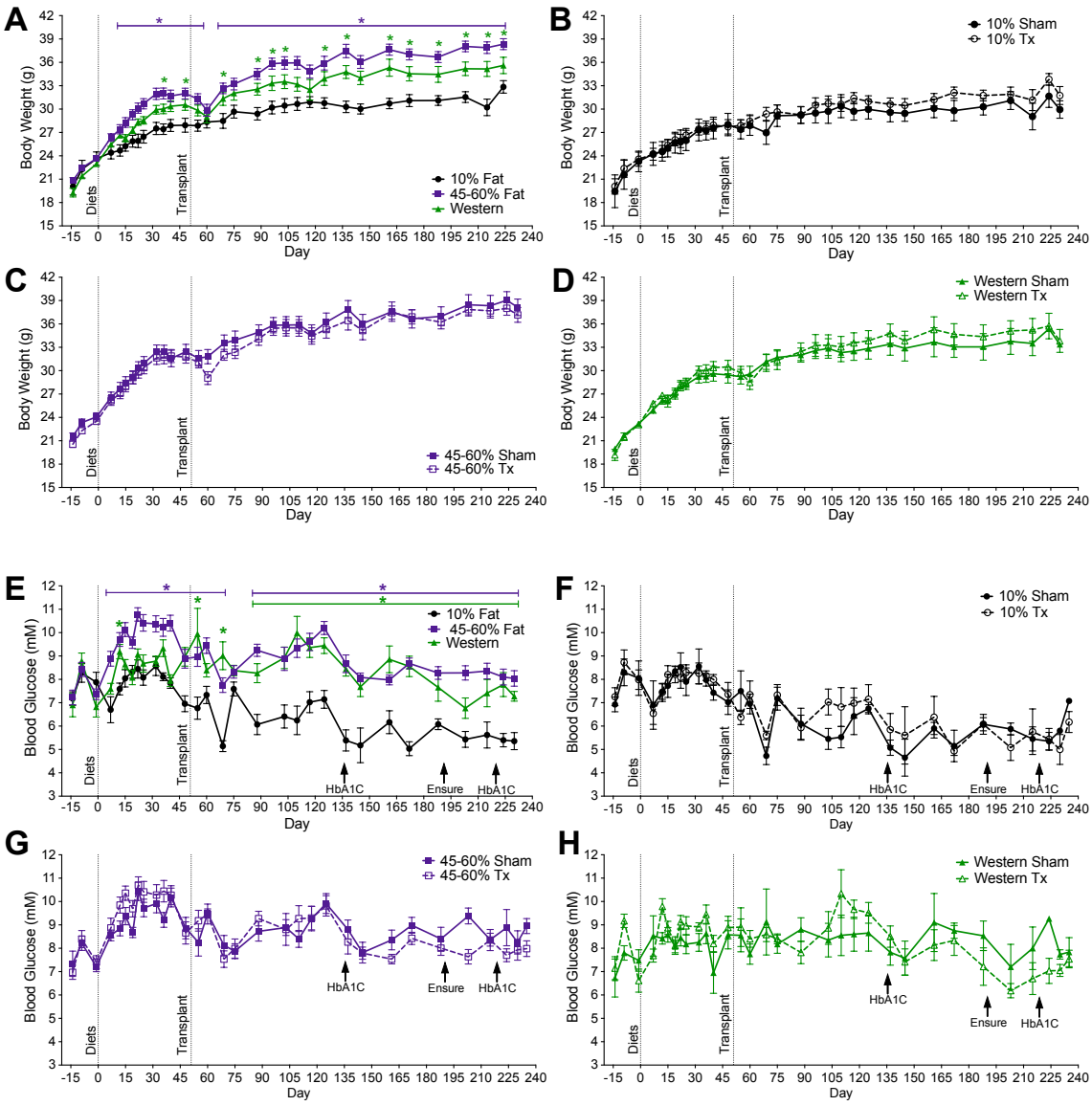

Figure S3: Cell transplants had no effect on fasting glucose or body weight levels. Fasting body weight and blood glucose levels were assessed throughout the study duration (before and after progenitor cell transplants on day 51) in mice on 10% fat (black; Tx: n = 7, Sham: n = 4), 45% or 60% fat (purple; Tx: n = 14, Sham: n = 4), and western (green; Tx: n = 7, Sham: n = 4) diets. **A)** Body weight and **E)** fasting blood glucose levels in all mice, regardless of cell transplant (* $p<0.05$, two-way ANOVA vs 10% control). **B-D)** Fasting body weight and **F-H)** blood glucose levels are shown separately for sham mice (solid lines, closed symbols) and transplant recipients (Tx; dashed lines, open symbols) from each diet group. Arrows in panels **E-H** indicate the time of sampling for HbA1C and glucose levels during the Ensure challenge.

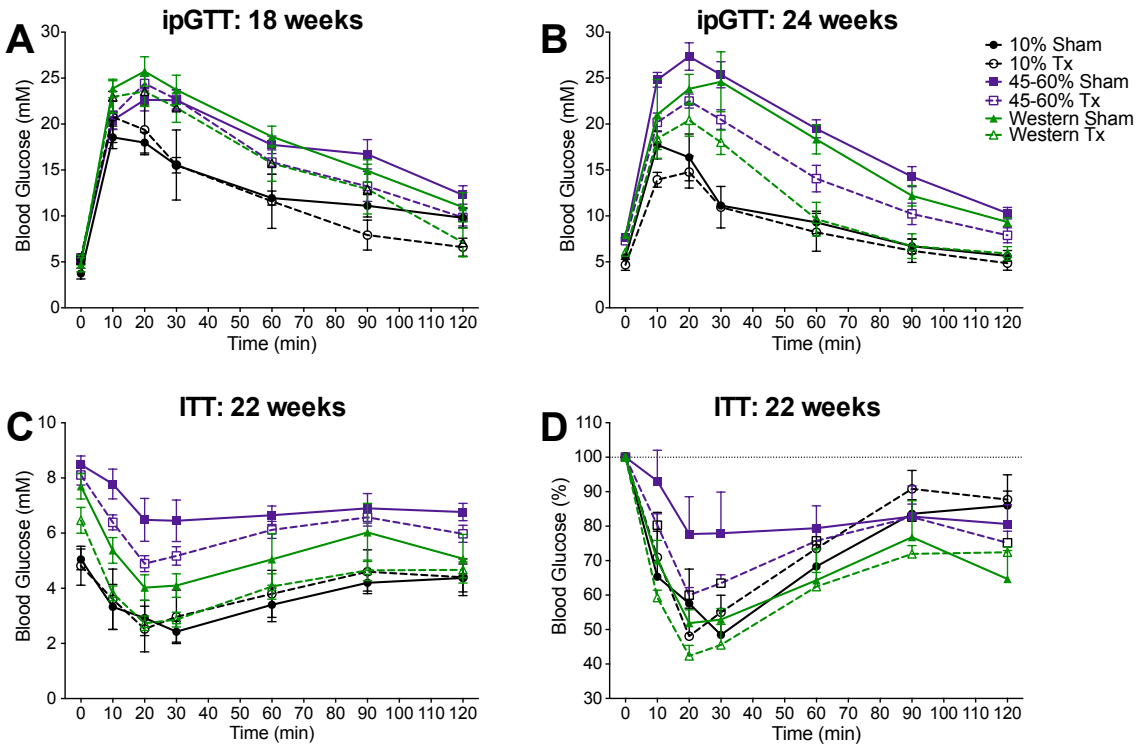

Figure S4: A-B) Intraperitoneal glucose tolerance tests (ipGTTs) were performd at 18 (panel **A**) and 24 (panel **B**) weeks post-transplant in sham mice (solid lines, closed symbols) and transplant recipients (Tx, dashed lines, open symbols) on 10% fat (grey; 18 weeks: Tx/Sham, n = 4; 24 weeks: Tx, n = 5 and Sham, n = 4), 45% or 60% fat (purple; 18 weeks: Tx, n = 12 and Sham, n = 7; 24 weeks: Tx, n = 13 and Sham, n = 6) and western (green; 18 weeks: Tx, n = 5 and Sham, n = 4; 24 weeks: Tx, n = 6 and Sham, n = 3) diets. **C-D)** An insulin tolerance test (ITT) was performed at 22 weeks post-transplant in sham and transplant recipients from each diet group. Data are presented as both raw values (mM, panel **C**) and percentage of basal glucose levels (%, panel **D**). All data are represented as mean ± SEM.

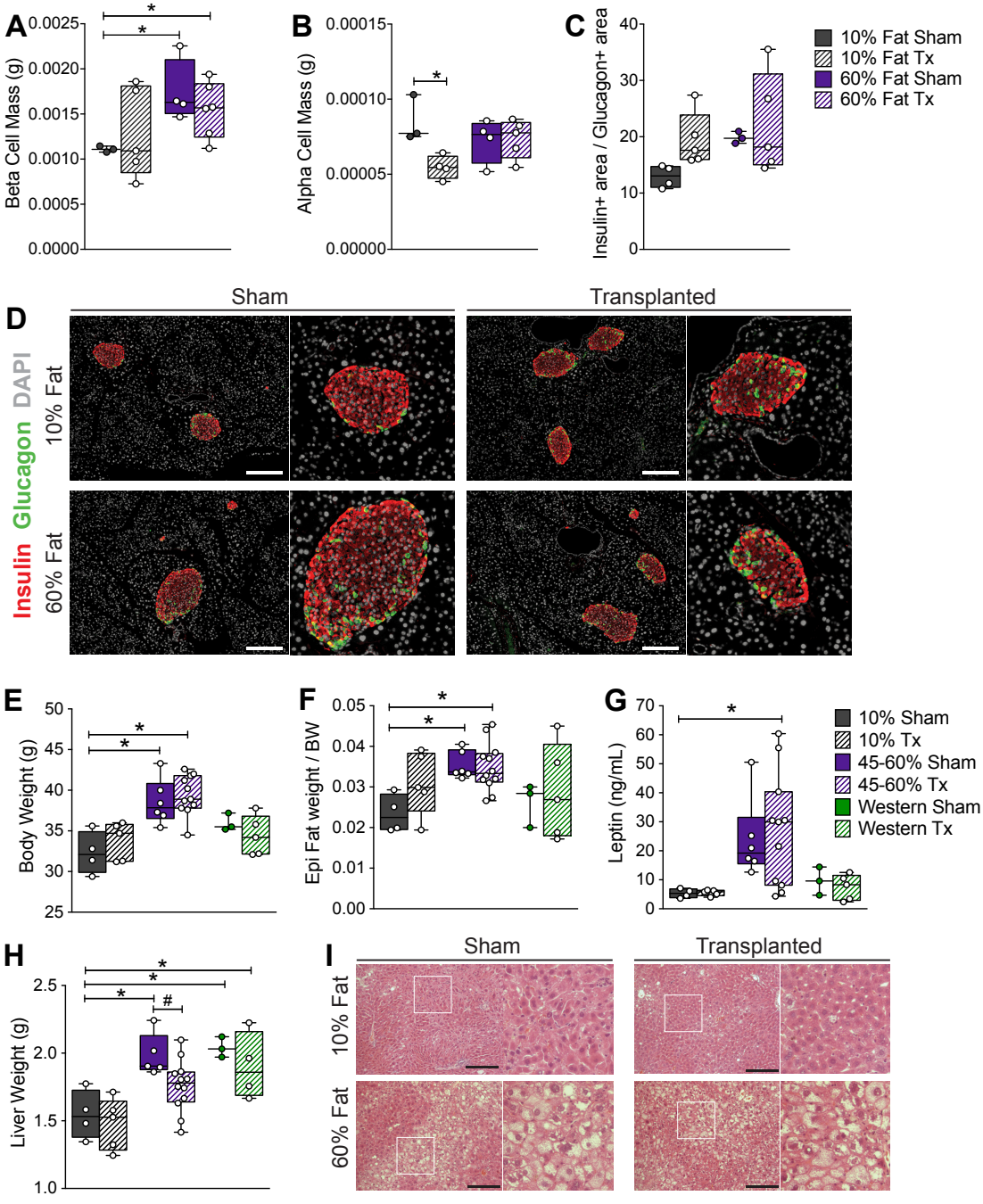

Figure S5: Transplantation of hESC-derived progenitor cells did not affect endogenous beta cell mass or the obesity phenotype in HFD-fed mice. Tissues were harvested at 29 weeks after transplantation of hESC-derived cells (Tx, striped bars) or sham surgery (Sham, solid bars) and 36 weeks after administration of diets (10% fat, grey bars; 45% or 60% fat, purple bars; western, green bars). **A)** Beta cell mass, **B)** alpha cell mass, and **C)** the ratio of insulin to glucagon immunostaining in pancreas sections from mice on 10% or 60% fat diets. **D**) Representative images of pancreas tissue immunostained for insulin (red; rabbit antibody), glucagon (green; mouse antibody) and nuclei (DAPI, white). Magnified insets of islets are shown to the right of each image;

scale bars = 200 μm. **E)** Body weight, **F)** epididymal fat weight relative to body weight, **G)** plasma leptin levels, and **H)** liver weight. **I)** Representative H&E images of liver sections from sham and transplanted mice on 10% or 60% fat diets illustrate lipidosis as a result of HFD exposure. Scale bars = 300 μm. Magnified insets are shown to the right of each image. * $p<0.05$, one-way ANOVA vs 10% sham controls; # $p<0.05$, two-tailed t-test, sham vs tx. Data points from individual mice are shown on box and whisker plots.

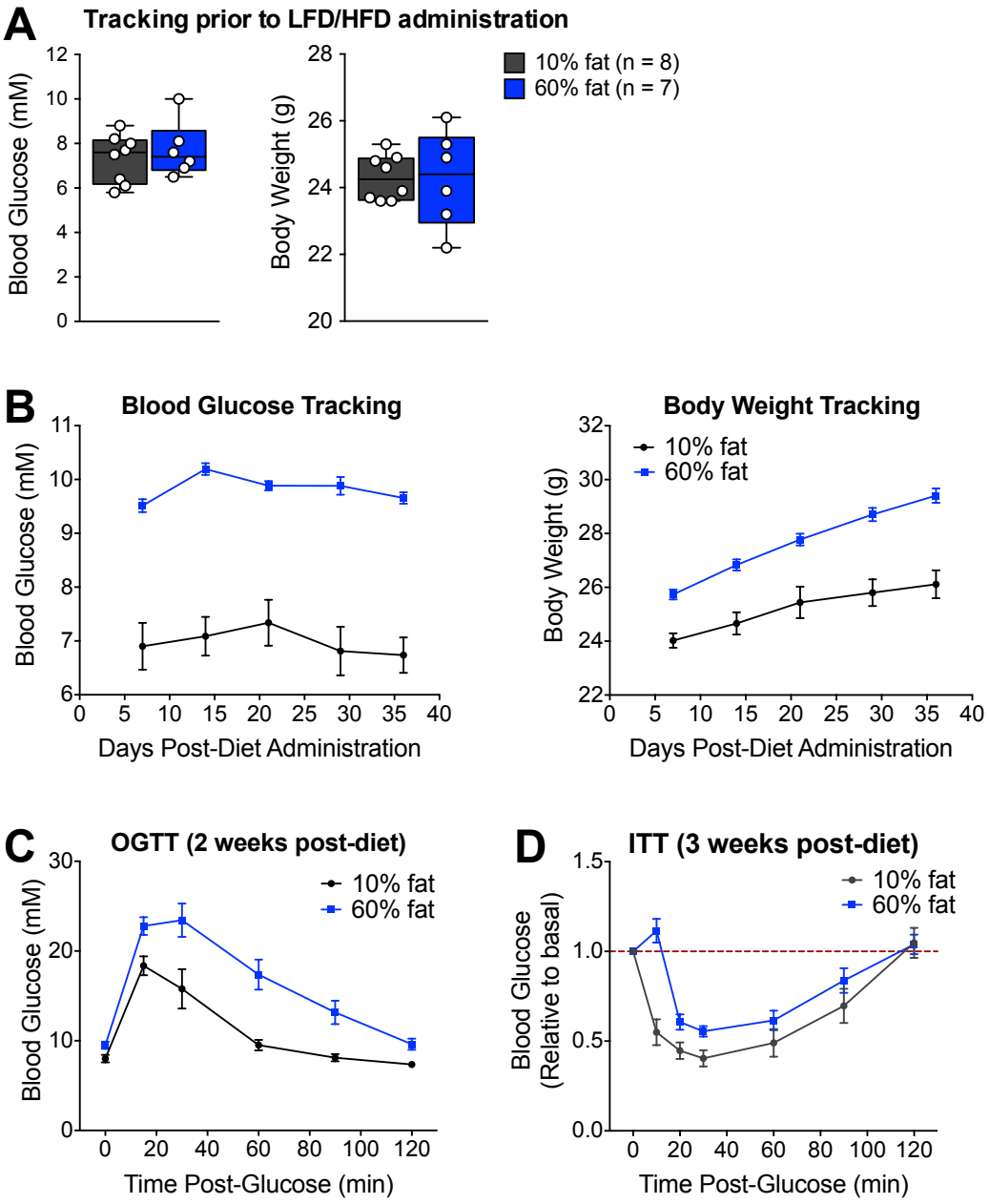

Figure S6: Confirmation of the high fat diet-induced phenotype in cohort 2. A) Fasting blood glucose and body weight levels in a subset of mice from cohort 2 during their acclimatization period on normal chow. Data points from individual mice are presented on box and whisker plots. **B)** Fasting blood glucose and body weight tracking following administration of either low fat diet (LFD, 10% fat, n = 8 mice) or high fat diet (HFD, 60% fat, n = 82 mice). **C)** At 2 weeks following administration of 10% and 60% fat diets, an oral glucose tolerance test (OGTT) was performed (n = 7 mice per group). **D)** An insulin tolerance test (ITT) was performed after 3 weeks on the 10% (n = 8 mice) or 60% fat (n = 10 mice) diets. Blood glucose levels were normalized to the basal levels at time 0 for each animal.

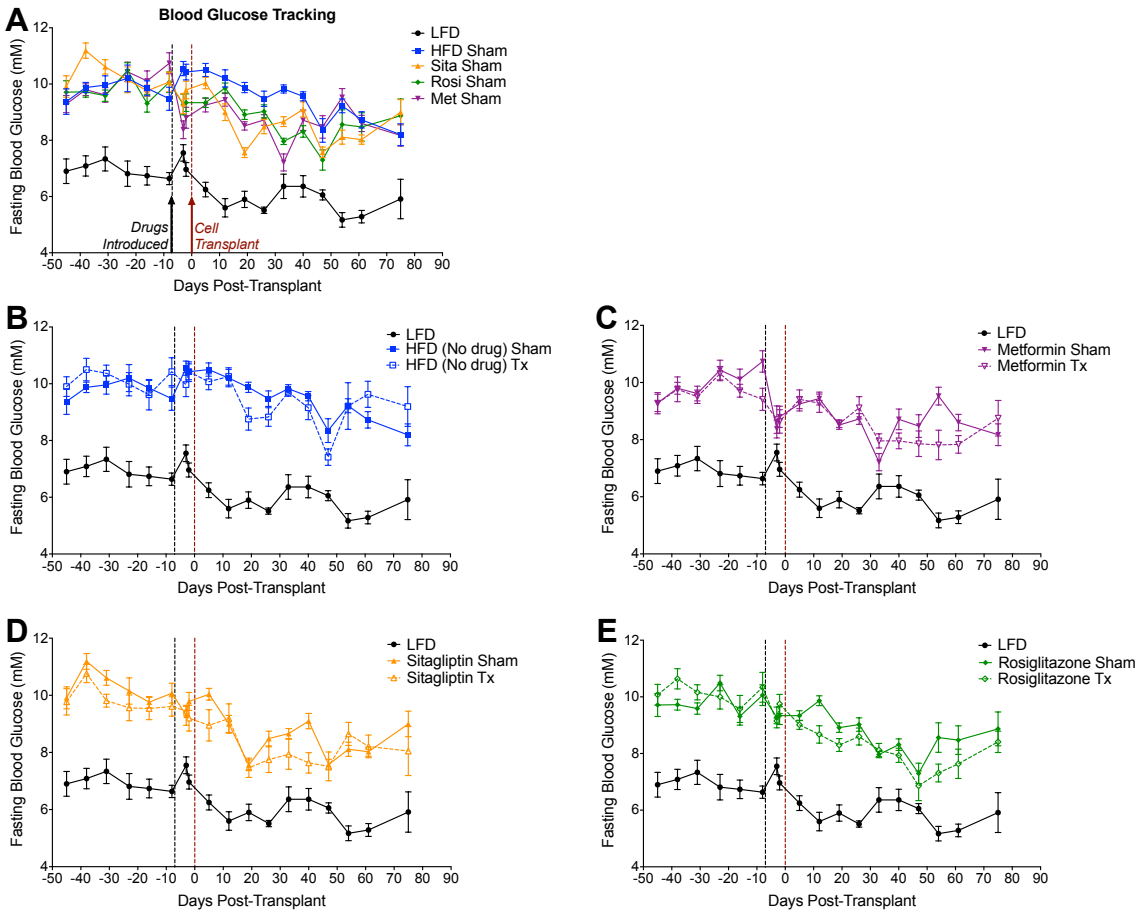

Figure S7: Fasting blood glucose levels were not affected by treatment with the progenitor cell therapy alone or in combination with antidiabetic drugs. A-E) Fasting blood glucose levels were assessed in mice fed 10% fat diet without drug (black/grey; all panels; n = 8 mice), 60% fat diet with no drug (blue; panels **A,B**; n = 7-8 mice), 60% fat diet plus metformin (purple; panels **A,C**; n = 7-8 mice), 60% fat diet plus sitagliptin (orange; panels **A,D**; n = 8 mice per group) and 60% fat diet plus rosiglitazone (green; panel **A,E**; n = 8 mice per group). Blood glucose tracking for sham mice from all treatment groups is shown together in panel **A**. Blood glucose for sham mice (solid lines, closed symbols) and transplant recipients (Tx; dashed lines, open symbols) from each treatment group are shown separately with the LFD control as a reference (panels **B-E**). Data are represented as mean ± SEM.

Table S1: Summary of H&E-stained tissues evaluated by an independent pathologist using light microscopy.

Tissues Evaluated by Light Microscopy		
Adipose Tissue, Perirenal	Ileum	Skeletal Muscle
Cecum	Jejunum	Spleen
Colon	Kidney	Stomach, Glandular
Duodenum	Liver	Stomach, Nonglandular
Heart	Lung	Testis

Table S2: Summary of treatment groups for *in vivo* transplant (Tx) studies

Cohort #	Diet	Drug	Tx/Sham	Sample Size
1	10% fat	None	Tx	7
1	10% fat	None	Sham	4
1	45% fat	None	Tx	7
1	45% fat	None	Sham	4
1	60% fat	None	Tx	7
1	60% fat	None	Sham	4
1	Western	None	Tx	7
1	Western	None	Sham	4
2	10% fat	None	Sham	8
2	60% fat	None	Tx	8
2	60% fat	None	Sham	8
2	60% fat	Rosiglitazone	Tx	8
2	60% fat	Rosiglitazone	Sham	8
2	60% fat	Sitagliptin	Tx	8
2	60% fat	Sitagliptin	Sham	8
2	60% fat	Metformin	Tx	8
2	60% fat	Metformin	Sham	8

Table S3: List of qPCR primers.

Gene Name	Assay ID
ABCC8	Hs00165861_m1
CHGB	Hs01084631_m1
G6PC2	Hs01549773_m1
GAPDH	Hs99999905_m1
GCG	Hs00174967_m1
GCGR	Hs01026191_g1
IAPP	Hs00169095_m1
INS	Hs00355773_m1
ISL1	Hs00158126_m1
MAFA	Hs01651425_s1
NKX6.1	Hs00232355_m1
PAX6	Hs00240871_m1
PCSK1	Hs00175619_m1
PCSK2	Hs01037347_m1
SLC30A8	Hs00545183_m1
SST	Hs00356144_m1
UCN3	Hs00846499_s1

Table S4: Antibody information for immunofluorescent staining.

Antigen	Species	Source	Dilution
CK19	Mouse	Dako; Denmark	1:100
C-Peptide	Guinea Pig	Abcam; Cambridge, MA	1:100
F4/80	Rat	AbD Serotec; Kidlington, UK	1:100
FGF21	Rabbit	Abcam; Cambridge, MA	1:50
Glucagon (Ms)	Mouse	Sigma-Aldrich; St Louis, MO	1:1000
Glucagon (Rb)	Rabbit	Cell Signaling; Danvers, MA	1:500
Insulin (GP)	Guinea Pig	Sigma-Aldrich; St Louis, MO	1:1000
Insulin (Rb)	Rabbit	Cell Signaling; Danvers, MA	1:100
Insulin (MAb1)	Mouse	Millipore; Billerica, MA	1:200
MAFA	Rabbit	Custom Antibody, Lifespan Biosciences; Seattle, WA	1:1000
NKX6.1	Rabbit	Custom Antibody, Lifespan Biosciences; Seattle, WA	1:1000
NKX2.2	Mouse	Developmental Studies Hybridoma Bank; University of Iowa, Iowa City, IA	1:100
PAX6	Rabbit	Covance; Princeton, NJ	1:250
PCNA	Mouse	BD Biosciences; Mississauga, ON	1:100
PDX1	Guinea Pig	Abcam; Cambridge, MA	1:1000
Somatostatin (Ms)	Mouse	Sigma-Aldrich; St Louis, MO	1:100
Somatostatin (Rb)	Rabbit	Sigma-Aldrich; St Louis, MO	1:500
Synaptophysin	Rabbit	Novus Biologicals; Littleton, CO	1:50
Trypsin	Sheep	R&D Systems; Minneapolis, MN	1:100

Table S5: Antibody information for FACS.

Type	Antibody	Source	Dilution
Unconjugated primary antibodies	Mouse anti-NKX6.1	Developmental Studies Hybridoma Bank University of Iowa (Cat #F55A12)	1:400
	Rabbit anti-Synaptophysin	Abcam (Cat #ab52636)	1:800
	Rabbit anti-Chromogranin A	DAKO (Cat# IS502)	1:10
	Mouse anti-NKX2.2	Developmental Studies Hybridoma Bank University of Iowa (Cat# 74.5A5)	1:100
Conjugated primary antibodies	Alexa Fluor 647 mouse anti-human Ki67	BD cat# 561126	1:10
	PE mouse anti-PDX1	BD cat# 562161	1:40
	PE mouse anti-human Pax6	BD cat# 561552	1:20
	Alexa Fluor 647 mouse anti-Oct3/4	BD cat# 560329	1:20
Secondary antibodies	Goat Anti Mouse IgG AF647	Invitrogen (Cat# A21235)	1:4000
	PE-Goat anti-Rabbit Fab2 IgG (H+L)	Invitrogen (Cat #A10542)	1:800
Isotype control antibodies	Purified Rabbit IgG, k Isotype	BD cat# 550875	1:1000
	Purified Mouse IgG, k Isotype	BD cat# 557273	1:50
	PE Mouse IgG1,k, Isotype Control	BD cat # 555749	1:40
	Alexa Fluor 647 IgG1, Isotype control	BD cat# 557732	1:40

Supplemental Experimental Procedures

Flow cytometry

Differentiated cells were released into a single-cell suspension, fixed, permeabilized, and stained for various intracellular markers, as described previously (Rezania et al., 2012). Dead cells were excluded during FACS analysis and gating was determined using isotype antibodies. Refer to Table S5 for antibody details.

Transplantation of hESC-Derived Pancreatic Progenitor Cells

All mice were anaesthetized with inhalable isoflurane and transplant recipients received ~$5x10^6$ hESC-derived pancreatic progenitor cells subcutaneously (s.c.) within a 20 µl Theracyte™ macroencapsulation device (TheraCyte Inc., Laguna Hills, CA) on the right flank, as previously described (Bruin et al., 2013). Sham mice received the same surgical procedure, but no macroencapsulation device was implanted.

Metabolic Assessments

Glucose tolerance tests (GTTs) were performed following a 6-hour morning fast and administration of glucose by oral gavage or intraperitoneal (i.p.) injection (2 g glucose/kg BW, 30% solution; Vétoquinol, Lavaltrie, QC). Glucose-stimulated human C-peptide secretion from engrafted cells was assessed following an overnight fast and an i.p. injection of glucose (2 g/kg). Insulin tolerance tests (ITTs) were performed following a 4-hour morning fast and administration of human synthetic insulin (0.7 IU/kg body weight; Novolin ge Toronto, Novo Nordisk, Mississagua, Canada). For monthly mixed-

meal challenges, mice received an oral gavage of Ensure® (8 uL/g body weight; Abbott Laboratories, Abbott Park, Illinois, USA) following an overnight fast (~16 hours). For arginine tolerance tests (ArgTT), mice received an i.p. injection of arginine (2 g/kg, 40% solution; Sigma-Aldrich) following a 4-hour morning fast.

Blood glucose levels were measured using a handheld glucometer (Lifescan; Burnaby, Canada). Mouse hormone and lipid profiles were assessed in plasma using the following kits: leptin (Mouse Leptin ELISA, Crystal Chem Inc., Downers Grove, IL), insulin (Ultrasensitive Mouse Insulin ELISA, Alpco Diagnostics, Salem, NH), C-peptide (Mouse C-peptide ELISA, Alpco Diagnostics), triglycerides (Serum Triglyceride kit, Sigma-Aldrich), free fatty acids (NEFA-HR(2) kit, Wako Chemical, Richmond, VA) and cholesterol (Cholesterol E kit, Wako Chemical). Hormone secretion from engrafted hESC-derived cells was assessed by measuring plasma human C-peptide (C-peptide ELISA, 80-CPTHU-E01.1; Alpco Diagnostics) and human insulin and glucagon levels (K15160C-2; Meso Scale Discovery, Gaithersburg, MD). Hemoglobin A1c (HbA1c) levels were measured with a Siemens DCA 200 Vantage Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY) from whole blood collected from the saphenous vein with EDTA as an anticoagulant.

Quantitative RT-PCR

Theracyte devices were cut in half at the time of tissue harvest and stored in RNA*later* Stabilization Solution (Life Technologies, Carlsbad, CA) at -80°C until use. Excess

mouse tissue was first removed from the outside of the device before placing the device in 2 mL PBS. The edge of the device was cut off, the outer membranes peeled back, and the device isolated and placed into 400 µl Qiagen Buffer RLT Plus (Qiagen Inc., Valencia, CA) containing 0.1% (v/v) β-mercaptoethanol. The PBS was collected and centrifuged at 2000xg for 4 min to collect any cells that spilled out of the device. The cell pellet was resuspended in the same RLT Plus buffer used for lysing the corresponding device. RNA was isolated using Qiagen RNeasy Plus Mini kit (Qiagen Inc) and eluted in 16 µl nuclease-free water. RNA concentration was measured using the NanoDrop8000 (Thermo Scientific).

Human islets were obtained from four organ donors (23-48 years of age; two males and two females) as a positive control for qPCR analysis (Prodo Labs; Irvine, CA). Islet purity ranged from 85-95% and viability from 90-95%. All human islet preparations showed a 2 to 4-fold increase in human insulin secretion after incubation with high glucose concentration (data not shown) using a static glucose-stimulated insulin secretion assay, as previously described (Rezania et al., 2014).

Due to a low amount of human cells/tissue in the device, and the high probability that some of the RNA would be from the surrounding mouse tissue, the amount of human RNA was measured using a standard curve. First, all RNA was converted into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific/Life Technologies) with the following program: 25°C for 10 minutes, 37°C for 2 hours, 4°C hold. Pre-amplification was performed using a primer pool specific for the genes run

(Table S3) and TaqMan PreAmp 2x Master Mix (Thermo Fisher Scientific/Life Technologies) with the following cycling conditions: 95^{0}C 10 min, 8 cycles of 95^{0}C 15s and 60^{0}C 4 min, 99^{0}C 10 min, and 4^{0}C hold.

To determine the amount of human cDNA, real-time PCR was performed on the Pre-amplified cDNA using primers specific to human *GAPDH* and mouse *Gapdh* and run against a standard curve made from known amounts of cDNA from a human cell line. Sixteen ng of calculated human cDNA was run on a custom TaqMan Low Density Array (Thermo Fisher Scientific/Life Technologies; Table S3) using the Quant Studio 12K Flex Real Time PCR instrument (Thermo Fisher Scientific/Life Technologies). Data were analyzed using Expression Suite software (v1.0.3, Thermo Fisher Scientific/Life Technologies) and normalized to undifferentiated H1 cells using the $\Delta\Delta$Ct method.

Immunofluorescent staining and image quantification

To measure endogenous pancreatic beta and alpha cell area three pancreas sections per animal, separated by at least 200 μm, were immunostained for insulin and glucagon. Whole slide fluorescence scanning was performed using the ImageXpressMicro™ Imaging System, and images were stitched together and analyzed using MetaXpress Software (Molecular Devices Corporation, Sunnyvale, CA). The beta cell or alpha cell fraction was calculated as the insulin-positive or glucagon-positive area / total pancreas area and the average of three sections per animal was then multiplied by the pancreas weight. To quantify the endocrine composition within devices, the number of DAPI-positive nuclei were counted using the Multi Wavelength Cell Scoring module in

MetaXpress and the number of cells that were immunoreactive for insulin, glucagon or both hormones was counted manually by an investigator who was blinded to the treatment groups.

Statistical Analysis

Two-way repeated measure ANOVAs were performed with a Fisher's LSD post-hoc test to compare HFD mice with LFD controls at different time points. One-way repeated measures ANOVA were performed with Dunnett post-hoc to compare values at different time points to baseline levels (time 0) within each treatment group. One-way ANOVAs were performed with a Dunnett post-hoc test for multiple comparisons to 10% fat controls or a Student-Neuman-Keuls test to compare between multiple groups. qPCR data was assessed by one-way ANOVA with a Fisher's LSD post-hoc test to compare grafts from various treatment groups with either human islets or HFD-fed mice without drug treatment. Unpaired t-tests were used to compare the effect of transplantation within a single treatment group (i.e. sham vs tx) and paired t-tests were used when comparing samples pre- and post-administration of a insulin or glucagon secretogogue (glucose, oral meal, arginine). Area under the curve was calculated with $y = 0$ as the baseline. For ITTs, area above the curve was calculated using the fasting blood glucose level for each animal as the baseline.

Supplemental References

Rezania, A., Bruin, J.E., Arora, P., Rubin, A., Batushansky, I., Asadi, A., O'Dwyer, S., Quiskamp, N., Mojibian, M., Albrecht, T., et al. (2014). Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nat Biotechnol *32*(11), 1121-1133.