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Supplemental Information

Transplantation of Human Pancreatic Endoderm Cells Reverses Diabetes Post Transplantation in a Prevascularized Subcutaneous Site

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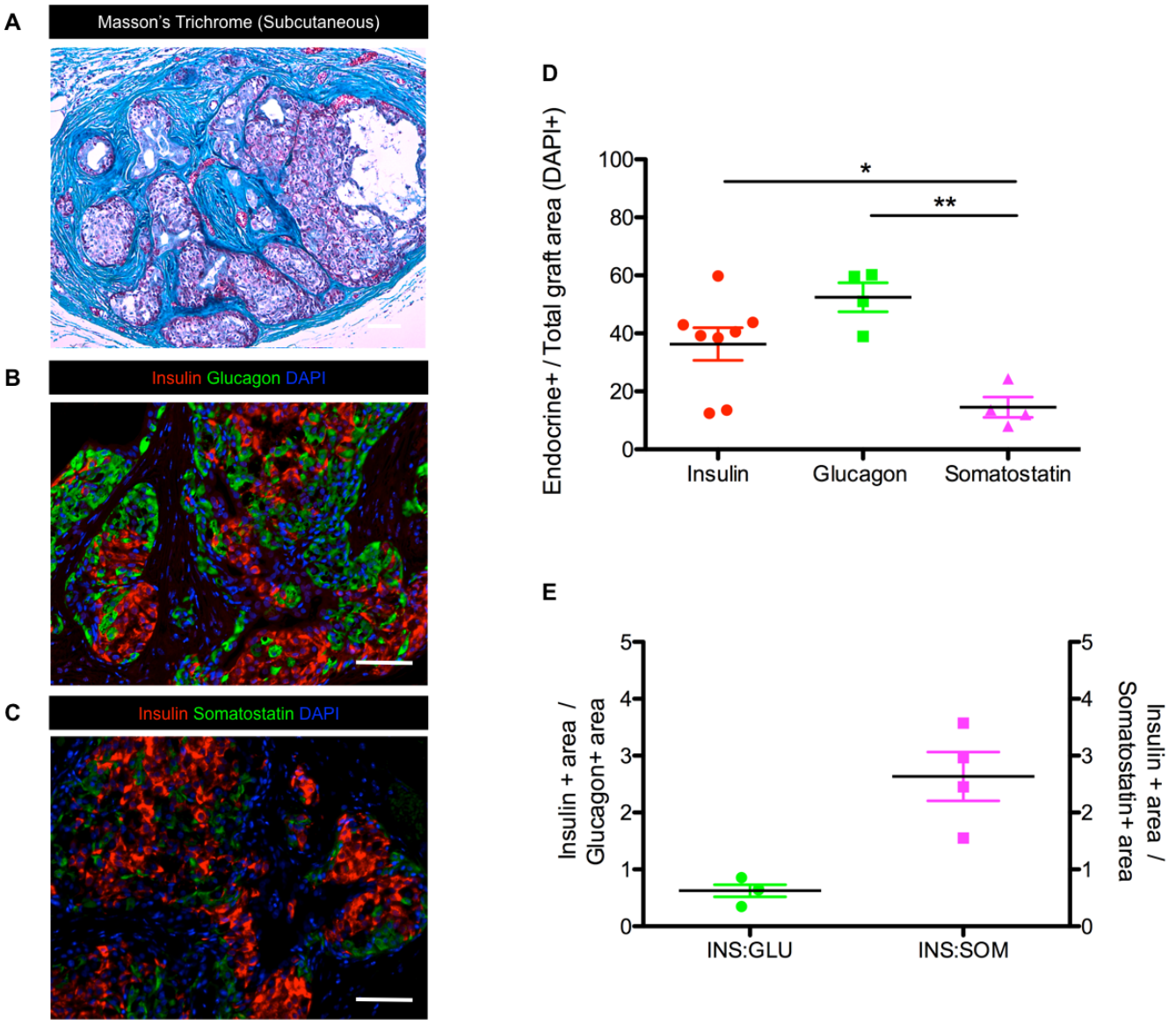

Figure S1: Immunohistochemistry of representative hESC-derived pancreatic endoderm cell (PEC) grafts transplanted into the subcutaneous (SC) space contain glucose regulatory cells (related to Figures 3,5,6 and S2). (**A**) Representative images of Masson's trichrome staining of a PEC graft cross-section in SC site >175 days post transplant, surrounded by collagen (blue), smooth muscle and erythrocytes (red). (**B,C**) Representative immunofluorescent staining of PEC engrafted in the SC site stained with insulin (red), glucagon (green) (**B**), somatostatin (green) (**C**), nuclear DAPI staining (blue) (scale bars represents 100μm). (**D**) Area of insulin (INS), glucagon (GLU) and somatostatin (SOM) immunoeactivity relative to the total graft area (DAPI+). (**E**) Areas of insulin+ relative to glucagon+ or somatostatin+ area for each graft. *P<0.05, **P<0.01; one-way ANOVA with Tukey test for multiple comparisons. All data are presented as mean ± s.e.m (n=4 animals (grafts)/2-4 sections per graft).

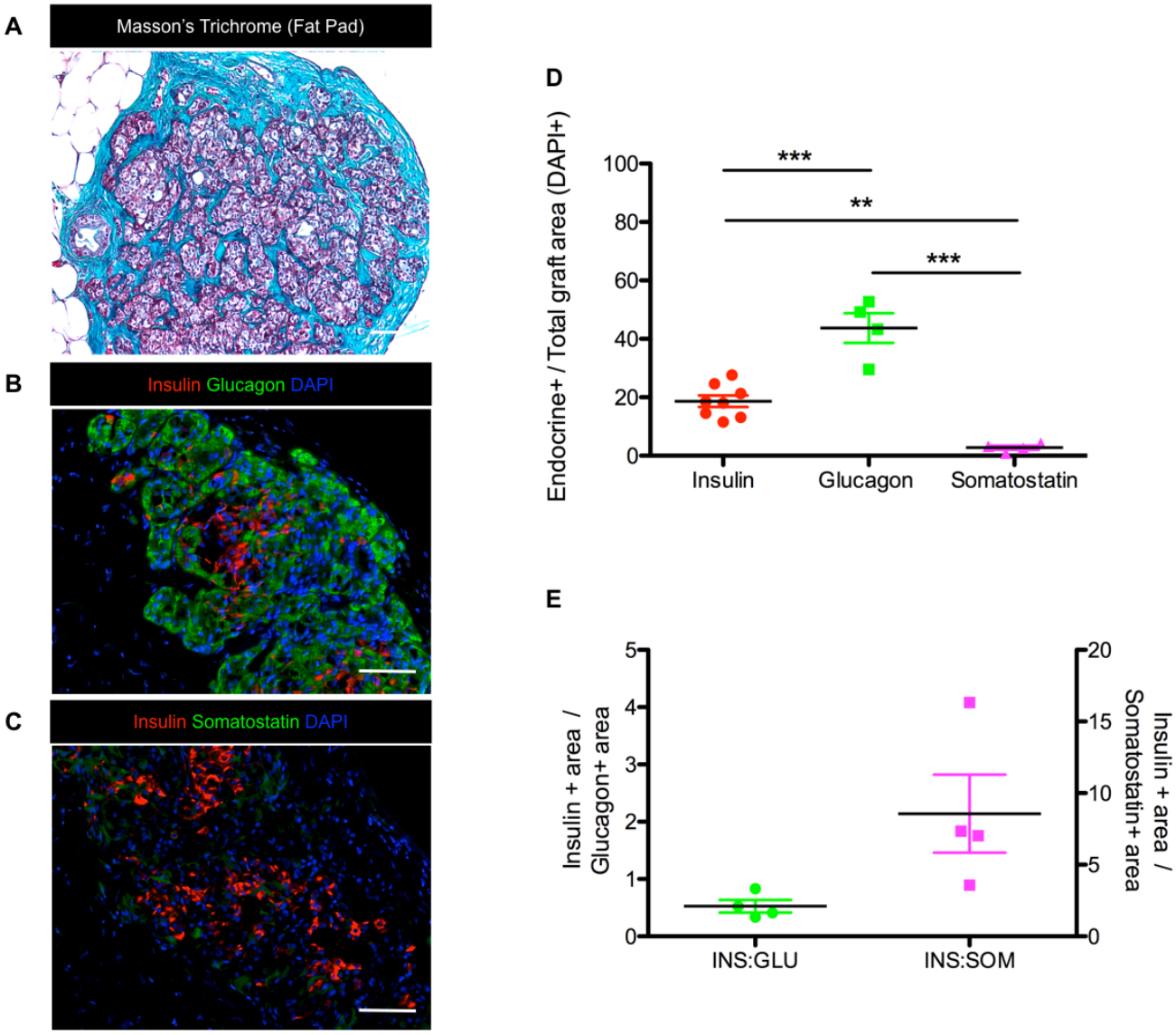

Figure S2: Immunohistochemistry of representative hESC-derived pancreatic endoderm cell (PEC) grafts transplanted into the fat pad (FP) contain glucose regulatory cells (related to Figures 3,5,6 and S1). (**A**) Representative images of Masson's trichrome staining of a PEC graft cross-section in FP site >175 days post transplant, surrounded by collagen (blue), smooth muscle and erythrocytes (red). (**B,C**) Representative immunofluorescent staining of PEC engrafted in the FP stained with insulin (red), glucagon (green) (**B**), somatostatin (green) (**C**), nuclear DAPI staining (blue) (scale bars represents 100μm). (**D**) Area of insulin (INS), glucagon (GLU) and somatostatin (SOM) immunoeactivity relative to the total graft area (DAPI+). (**E**) Areas of insulin+ relative to glucagon+ or somatostatin+ area for each graft. **P<0.01, ***P<0.001; one-way ANOVA with Tukey test for multiple comparisons. All data are presented as mean ± s.e.m (n=4 animals (grafts)/2-4 sections per graft).

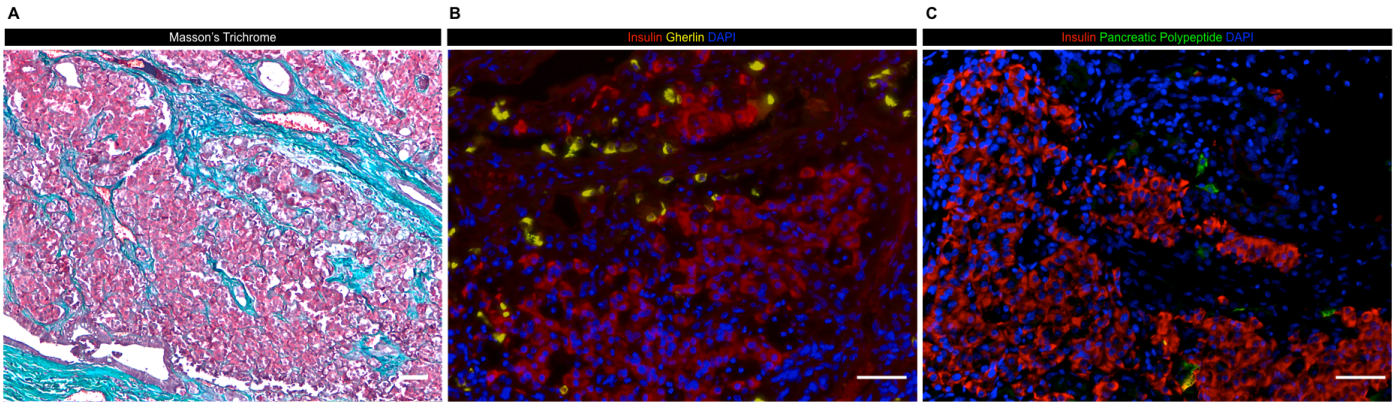

Figure S3. Long term hESC-derived pancreatic endoderm cell (PEC) grafts transplanted into the DL space contain additional glucose regulatory cells (related to Figure 3 and 5). (**A**) Representative images of Masson's trichrome staining of a PEC graft cross-section in DL site >175 days post transplant, surrounded by collagen (blue), smooth muscle and erythrocytes (red). (**B,C**) Representative immunofluorescent staining for insulin (red), ghrelin (yellow) (**B**), pancreatic polypeptide (green) (**C**) and nuclear DAPI staining (blue). Scale bars represents 100μm.

Supplemental Experimental Procedures

ESC cell culture differentiation and processing

Briefly, hESC were expanded in adherent culture in DMEM/F12 (Life Technologies, cat#10565) containing GlutaMAX (Life Technologies, cat#10565), supplemented with 10% v/v of Xeno-free KnockOut Serum Replacement (Life Technologies, cat#12618-001), 1% v/v non-essential amino acids (Life Technologies, cat#11140-050), 1% v/v penicillin/streptomycin (Life Technologies, cat#15070-063), 10 ng/mL heregulin-1b (Peprotech, cat#100-03) and 10 ng/mL activin A (R&D Systems, cat#338-AC). Cells were plated at 50,000 or 33,000 cells/cm^2 for three and four day growth cycles, respectively. Suspension aggregates of hESC were formed in roller bottles in StemPro medium. The next day, differentiation was initiated in suspension in roller bottles and the differentiation media and timing were as described previously (Schulz et al., 2012). At differentiation day 12, PE cells was harvested and cryopreserved as described (Agulnick et al., 2015). For the studies described herein, PE cells were thawed from cryopreservation and cultured in 6-well plates in Stage 4 media as previously described (Schulz et al., 2012).

Glucose stimulated insulin secretion

Glucose solutions were perfused through the system at 100 µL/min in 16-min intervals with an initial concentration of 2.8 mM followed by an interval of 28 mM, concluding with a final 6-min interval of 2.8 mM. Subsequent to glucose infusion, KCL 30 mM was administered a 6-min interval to depolarize the assayed cells. Insulin concentrations (mU/L) in the effluents supernatants were measured by ELISA (Mercodia, Uppsala, Sweden).

Measurements of $[Ca^{2+}]_i$

The cell suspension was plated onto petri dishes or coverslips and cultured in RPMI medium (Life Technologies) containing 7.5mM glucose for >24h before the experiments. Dispersed human islets and PE cells were incubated in culture medium containing 1 µM Fura-2AM (Life Technologies) for 15mins. The cover slips were then mounted onto an inverted microscope and perifused with Krebs-Ringer buffer [KRB composed of (in mM) 140 NaCl, 3.6 KCl, 0.5 $MgSO_4$, 1.5 $CaCl_2$, 10 HEPES, 0.5 NaH_2PO_4, 5 $NaHCO_3$, 3 glucose (unless otherwise indicated), and pH adjusted to 7.4 with NaOH]. Fluorescence was excited at 340 and 380 nm (intensity ratio 20/8) using an Oligochrome light source (Till Photonics) and a 20x objective (Zeiss Fluar). Emission was monitored at 510 nm, and images were captured at 2 Hz using an ICCD camera and Life Acquisition software (Till Photonics).

Preparing a prevascularized subcutaneous transplant site

Briefly, four weeks prior to PE cell transplant, 2 cm segments of a 5-French nylon radiopaque angiographic catheter (Torcon NB® Advantage Beacon® tip Cook Medical, Indiana, USA) were implanted subcutaneously into the lower left quadrant of 20-25 gram male B6.129S7-Rag1^{tm1Mom}(B6/Rag$^{-/-}$) immunodeficient mice (Jackson Laboratory, Canada).

Fat Pad Cell Transplantation

PE cells were transferred onto approximately 7mm diameter × 1.5 mm thick absorbable gelatin sponge (Gelfoam®, Pfizer, Michigan USA) discs soaked in culture media. The resultant PE-constructs were placed in an incubator set at 37°C and 5% CO2 for 20 min and transferred to culture dishes with medium until implantation. The epididymal fat pad was externalized through a midline incision and a single PE-gelfoam construct was wrapped in the fat pad and secured by tissue adhesive (VetBond, 3M, London, ON) prior to returning back into the peritoneal cavity.

Evaluation of graft function

At 8, 12, 20, 24 weeks post-transplant serum from recipient mice were assayed for human c-peptide at basal and post-stimulation. Prior to testing mice were fasted overnight and blood was collected from the tail vein, for basal analysis, prior to received an intraperitoneal glucose bolus (3 g/kg). Blood was collected again from the tail vein 60 min post-glucose injections to determine stimulated human C-peptide levels. C-peptide results are expressed as ng/ml. Serum human C-peptide concentrations were measured by ELISA (Mercodia, Uppsala, Sweden).

In addition, glucose tolerance tests were conducted 24 weeks post-transplant, as a means to further assess metabolic capacity in response to a glucose bolus. Animals were fasted overnight prior to receiving an intraperitoneal glucose bolus (3 g/kg). Blood glucose levels were monitored at 0, 15, 30, 60, 90 and 120 minutes post injection, allowing for area under the curve (AUC-blood glucose) to be calculated and analyzed between transplant groups.

Histological assessment

Briefly, following deparaffinization and antigen heat retrieval, the graft sections were washed with phosphate buffered saline supplemented (PBS) with 1% goat serum, followed by blocking with 20% goat serum in PBS for 30 minutes. The sections were treated with a primary antibody of guinea pig anti-pig insulin (Dako A0564) diluted 1:100, rabbit somatostatin (Abcam ab30788)

diluted 1:400, rabbit anti-glucagon (Abcam ab43837) diluted 1:200, rabbit anti-Ghrelin (BioVision 5991) diluted 1:100 and rabbit anti-pancreatic polypeptide (Abcam 14985) diluted 1:100 for 24 hours at 4ºC. All primary antibodies were diluted in PBS with 1% goat serum. Samples were rinsed with PBS with 1% goat serum followed by secondary antibody treatment consisting of goat anti-guinea pig (Alexa 568) diluted 1:500 (PBS with 1% goat serum), and goat anti-rabbit (Vector Fl-1000) diluted 1:500 (PBS with 1% goat serum) for 1 hour at room temperature. Samples were rinsed with PBS and counter stained with DAPI in anti-fade mounting medium (ProLong®, LifeTechnologies).

Supplemental References

Agulnick, A.D., Ambruzs, D.M., Moorman, M.A., Bhoumik, A., Cesario, R.M., Payne, J.K., Kelly, J.R., Haakmeester, C., Srijemac, R., Wilson, A.Z*., et al.* (2015). Insulin-Producing Endocrine Cells Differentiated In Vitro From Human Embryonic Stem Cells Function in Macroencapsulation Devices In Vivo. Stem Cells Transl Med *4*, 1214-1222.

Schulz, T.C., Young, H.Y., Agulnick, A.D., Babin, M.J., Baetge, E.E., Bang, A.G., Bhoumik, A., Cepa, I., Cesario, R.M., Haakmeester, C*., et al.* (2012). A scalable system for production of functional pancreatic progenitors from human embryonic stem cells. PLoS One *7*, e37004.