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Supplemental Information

Mitigating Ischemic Injury of Stem Cell-Derived Insulin-Producing Cells after Transplant

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Supplementary Data:

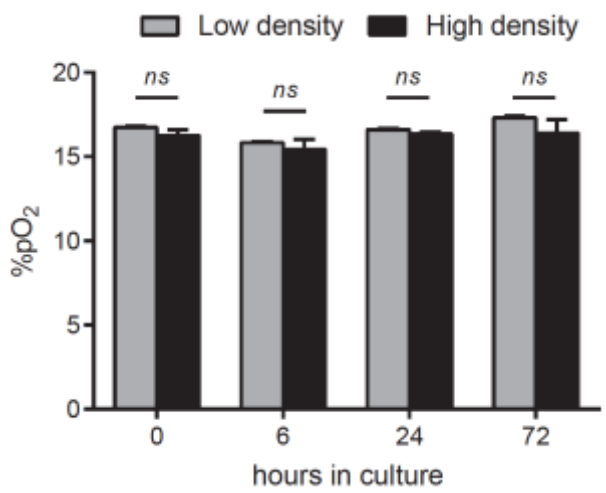

Figure S1. Oxygen tension in low density and high density cultures. Oxygen tensions were measured in low density and high density cultures over time. Data are expressed as mean ± SEM and are a compilation of results from 3 independent experiments (n= 14 per condition). Statistical significance of the difference between low density and high density group was calculated using multiple t test.

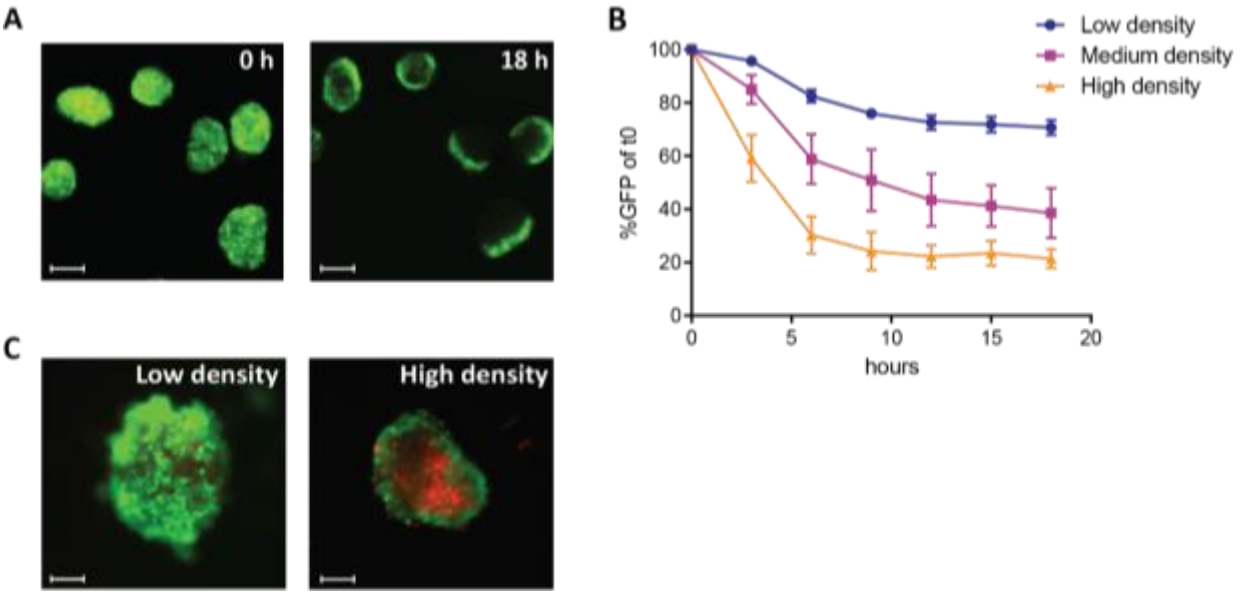

Figure S2. Nutrient-dependent cell death *in vitro*. (A) GFP-expressing mouse islets were cultured at high density (1000 islets/mL) and cell death resulted in loss of membrane integrity and GFP signal. Scale bar 100µm. (B) Kinetics of cell death of various culture densities were monitored in real time for 18 hours. Data shown as mean ± SEM are a summary of 3 independent experiments (n=3 per condition). (C) MIP.GFP islets cultured in low- and high-density cultures stained for Propidium Iodide (red) after 6 hours. Images are representative of 3 independent experiments. Scale bar 40µm.

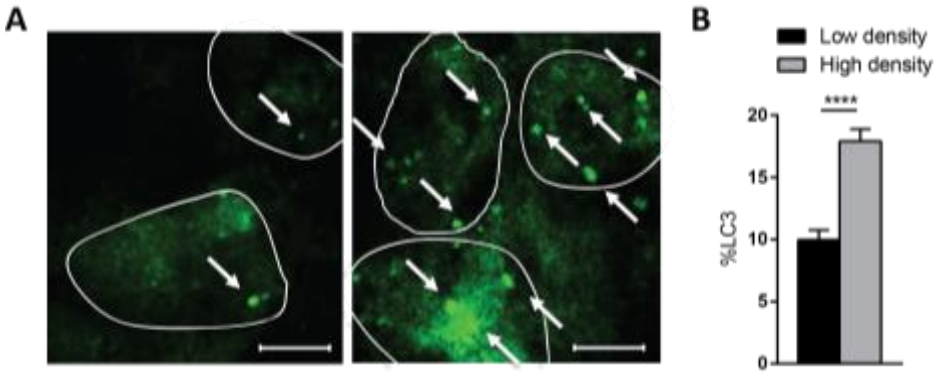

Figure S3. Nutrient-dependent cell death in vitro. (A) GFP-labeled LC3 autophagosome formations (arrows) in cells (outlined) in low-density (left), and high-density conditions (right) for 3 hours. Magnification 200x. Pictures shown are representative of 3 independent experiments (n=3 per group). Scale bar 5µm. (B) Islets are dissociated to single cells and percentage of LC3+ cells are quantified through flow cytometry (****p= 0.0007). Data shown as mean ± SEM are a summary of 3 independent experiments. Statistical significance of the difference between low-density and high-density group was calculated using paired t test.

Table S1. Primers used for RTqPCR analysis of human SCIPC.

Gene symbols	Forward primers	Reverse primers
SLC2A1 (GLUT1)	5'-GTCCCTCTCAGTGGCCATCTTT	5'-GGCCAAAGCGGTTAACGAAA
SLC2A2 (GLUT2)	5'-TGGAATTGACAGGACTCCCAAC	5-TCCAGTGGAACACCCAAAACA
HK1	5'-AATTCTGCGGGTGCAAGTGA	5'-ACTGCCGTGCACGATGTTCT
HK2	5'-CAAAGTGACAGTGGGTGTGG	5'-GCCAGGTCCTTCACTGTCTC
GCK	5'-GGAGCGTGAAGACCAAACAC	5'-GTAGTCGAAGAGCATCTCAGC
PDK1	5'-AGGGTTACGGGACAGATGCAG	5'-TCAGCCTCGTGGTTGGTGTT
LDHA	5'-GCACCCAGTTTCCACCATGA	5'-TCTTCAAACGGGCCTCTTCC
VEGFA	5'-GCTACTGCCATCCAATCGAG	5'-ACACAGGATGGCTTGAAGATG
PDX1	5'-TGGAGCTGGCTGTCATGTTG	5'-GCGCTTCTTGTCCTCCTCCT
MCT4 (SLC16A3)	5'-TCATCACTGGCTTCTCCTACGC	5'-AGCCGATCCCAAACTCCTGT
IRS1	5'-CCACTCGGAAAACTTCTTCTTCAT	5'-AGAGTCATCCACCTGCATCCA
GADPH	5'-TTGCCATCAATGACCCCTTCA	5'-CGCCCCACTTGATTTTGGA

SLC2A1 (GLUT1): solute carrier family 2 (facilitated glucose transporter), member 1 (glucose transporter 1);
SLC2A2 (GLUT2): solute carrier family 2 (facilitated glucose transporter), member 2 (glucose transporter 2);
HK1, hexokinase 1;
HK2, hexokinase 2;
GCK, glucokinase (HK4);
PDK1, pyruvate dehydrogenase kinase 1;
LDHA, lactate dehydrogenase A;
VEGFA, vascular endothelial growth factor A;
PDX1, pancreatic and duodenal homeobox 1;
MCT4 (SLC16A3), monocarboxylic acid transporter 4 (solute carrier family 16, member 3);
IRS1, insulin receptor substrate 1;
GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Supplemental Experimental Procedures

Mice

C57BL/6 (B6), B6.MIP-Luc, B6.MIP-GFP, B6.LC3-GFP, and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were housed and bred at the University of California, San Francisco Animal Barrier Facility. All animal procedures were performed under approved protocols and in accordance with ethical guidelines by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco.

Islet isolation

Mouse islets were isolated as previously (Szot et al., 2007). Purified islets were rested in RPMI medium overnight and healthy islets were handpicked for experiments. Human research islets provided by the UCSF DRC Islet Production Core following current standard protocols on research consented pancreas donors (Szot et al., 2009). All human studies were approved by local ethics committees. Pancreata were procured from multiorgan cadaveric donors using cold perfusion with University of Wisconsin solution (ViaSpan, DuPont Pharmaceuticals Ltd.).

Derivation of constitutive luciferase-expressing MEL1 INS$^{GFP/w}$ cell line

DNA encoding a firefly luciferase gene was targeted to the AAVS1 locus of the MEL1$^{INS\text{-}GFP/wt}$ cells using a published TALEN pair and a targeting vector containing homology sequences and a Puromycin selection (Addgene plasmid #58409, #59025, and #59026), which was modified by inserting a firefly luciferase gene via a T2A cleavage site after the Puromycin resistanc gene. Human ES cells were targeted by electroporation of 5ug of each TALEN vector with 20ug of the Luciferase containing targeting vector using a Biorad GenePulser (Hercules, CA) using an exponential decay and 250V and 500uF settings. Cells were plated on DR4 resistant MEFs containing plates and selected after 24-48 hours with 0.5ug/ml Puromycin for 3-4 days. Successfully targeted cells were identified using bioluminescent followed by PCR of genomic DNA to verify site directed integration of the targeting vector. Bioluminescence imaging demonstrates a cell dose-dependent increase of bioluminescence signal (not shown).

SCIPC differentiation

Human embryonic stem cell line containing a GFP gene knocked into the endogenous insulin locus, MEL1$^{INS\text{-}GFP/wt}$ (Micallef et al., 2012), was maintained as described (Russ et al., 2015). Suspension-based differentiations were carried out as described previously (Russ et al., 2015) with modification of the last differentiation stages. Cultures were incubated in DMEM (Gibco) containing 25 mM glucose, 1:100 B27 (StemCell Technologies), 50 ng/ml EGF (RnD Systems), and 50 ng/ml KGF (RnD Systems) for days 9 and 10. Thereafter, clusters were incubated in DMEM (Gibco) containing 25mM Glucose supplemented with 1:100 B27 (StemCell Technologies), 1:100 non-essential amino acids (Gibco), 1mM N-Acetyl-L-Cysteine (Sigma), 10µg/ml Heparin Sodium Sulfate (Sigma), 10µM Zinc Sulfate heptahydrate (Sigma), 10µM ALK inhibitor 2 (Axxora), 2µM T3 (3,3′,5-Triiodo-L-thyronine sodium salt) (Sigma), 500nM LDN-193189 (Stemgent), 1µM Gamma-Secretase inhibitor (XXi) (Millipore), 2µM Bay K 8644 (Tocris), and 0.5mM 2-Phospho-L-ascorbic acid (Sigma). Medium was changed daily by aspirating approximately 5ml followed by addition of 5ml fresh media.

Islet metabolic measurement

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were quantified as previously described (Wikstrom et al., 2012). XF24 plates that are specifically designed to assess islets and similar clusters were used. 20 islets or SCIPC clusters were deposited at the bottom of individual wells and restrained by a mesh to prevent clusters dispersion. Cells were incubated for 1 hour at 37°C after which respiration was measured. Samples were analyzed using Seahorse XF24 Extracellular Flux Analyzer (Seahorse, North Billerica, MA).

Bioluminescence Imaging

Luciferase-expressing islet or SCIPC graft mass was monitored using bioluminescence imaging as described previously (Faleo et al., 2016).

Islet live imaging

MIP-GFP islets were used to visualize islet survival *in vitro* in real time. Isolated islets were plated into a 96-well plate at various concentration and placed on a stage of a microscope that was encased in an environmentally controlled chamber to maintain 37°C and 5% CO_2 throughout the experiment. Green fluorescent signal and bright field images were captured every 3 hours for an 18-hour follow-up period. The pictures were then analyzed using NIS Elements

software (Nikon) to quantify GFP signals.

Oxygen tension measurements

Oxygen tensions of islet cultures were measured using the FOSPOR-PI600-2M Oxygen Sensor probe (Ocean Optics, Dunedin, FL). The probe was immersed deep into the media of each well. For each sample, 14 measurements were performed at each time point and the mean ± SEM was calculated.

Flow cytometry

To measure cell viability after *in vitro* culture, SCIPC clusters or whole islets were stained with 25µg/mL Propidium Iodide (PI, Sigma) for 15 minutes. The clusters were then washed in PBS 3 times before dissociation into single cells using Accumax (Innovative cell technologies, San Diego, CA). The cells were washed before analysis on an Accuri flow cytometer (BD Bioscience, San Jose, CA). To quantify cells expressing GFP after transplant, grafts were harvested from the renal capsule at day 7 and the dissociated cells were stained with PI and anti-HLA-ABC-PE-Cy5 (W6/32, A18548, Invitrogen Antibodies, Carlsbad, CA) before analysis on Accuri. Flow cytometric analyses of expression of β cell maturation markers by SCIPC were performed as previous described. Data were analyzed with FlowJo software (FLOWJO LLC, Ashland, Oregon).

GSIS assay

GSIS was measured as described previously for islets (Kloster-Jensen et al., 2016). Insulin concentrations in supernatant were measured using enzyme-linked immunosorbent assays (ELISA) for mouse insulin (Mercodia, Uppsala, Sweden) and human C-peptide (ALPCO, Salem, NH). Stimulation index is calculated using the following formula: $SI=[Insulin]_{high\ glucose}/[Insulin]_{low\ glucose}$.

RTqPCR Analysis

Total RNA was isolated from SCIPC using the RNeasy MicroKit (Qiagen, Hilden, Germany) first-strand cDNA was prepared using iScript cDNA Kit (Bio-Rad, Hercules, CA), and quantitative RT-PCR was performed on a CFX Connect Real-Time System (Bio-Rad) with FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland). Relative changes in gene to *GADPH* ratio between hypoxic and normoxic conditions were computed using the $2^{-\Delta\Delta Ct}$ method. See Table 1 in supplementary methods for primer sequences.

Immunohistochemistry

Tissue-bearing SCIPC grafts were fixed in paraformaldehyde (Sigma), incubated overnight in 30% sucrose (Sigma), before being embedded in tissue-Tek® OCT (Sakura Finetek, Torrance,CA). A polyclonal guinea pig anti-insulin antibody (1:100, A0564, Dako, Glostrup, Denmark) was applied overnight and Alexa Fluor® 568 goat anti-guinea pig IgG (1:200, A11075, Invitrogen) was incubated for 1 hour. Images were taken using Leica SP5 upright confocal microscope (Leica Microsystem, Buffalo Grove, IL) and processed with Imaris software (Bitplane, Concord, MA).

Statistical Analysis

Statistical analyses were performed with the aid of GraphPad software (GraphPad Prism, La Jolla, CA).

Supplemental References

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