Supporting Online Material

Epithelial-to-Mesenchymal Transition Generates Proliferative Human Islet Precursor Cells

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Materials and Methods

Cell culture. Human pancreatic islet isolations were performed at the Clinical Center of the National Institutes of Health according to the procedure of Ricordi et al. (1). Preparations are composed of 60% to 90% mature islets with residual duct and exocrine tissue. Islet preparations from twelve donors, ages 3 to 58 years, were used. Samples were enriched for islets and depleted of debris and single cells by retention in a 40 µm filter. Approximately 2,000 islet equivalents were seeded onto 100 mm tissue culture-treated dishes in 10 ml CMRL-1066 medium containing 5.5 mM glucose (Gibco) supplemented with 2mM L-glutamine and 10% fetal bovine serum (serum-containing medium, SCM). The majority of islets attached to the dish within 1 to 2 days. Cultures were re-fed on day 2 and thereafter as needed to replenish nutrients and remove debris. They were sub-cultured (1:2) when nearly confluent or when the outgrowth extended beyond one diameter from the original islet (usually once or twice between days 3-14). During the first several days in culture, adherent cells migrated out from the islets until there were virtually no islets remaining and a monolayer of cells comprised mostly of “fibroblast-like” cells with a few cobblestone-appearing
cells was established. We define "passage 0" as 14 days after the islets are received and placed in tissue culture-treated dishes. Beginning at passage 0, cells were harvested with trypsin and sub-cultured every 3-4 days for at least 3 months. During this period, angular "fibroblast-like" cells dominate the culture. At each passage, a portion of the cells was cryopreserved in 90% FBS / 10% DMSO for subsequent study. To measure the cell doubling rate, one million hIPCs were seeded at each passage in a 10-cm dish in SCM and harvested for counting and re-seeding every three days.

To generate islet-like cell aggregates (ICAs), hIPCs at various passages were monodispersed with trypsin on "day 0" and 0.2 to 1x10^6 cells were added to each well of 6-well tissue culture-treated plates in serum-free CMRL-1066 medium supplemented with 1% BSA, insulin (10 µg/ml), transferrin (5.5 µg/ml) and sodium selenite (6.7 ng/ml) (SFM). The medium was replaced every other day, using unit gravity to retain ICAs that had become detached from the culture dish and separate ICAs from single or dead cells and debris that were in suspension.

PANC-1 cells were cultured and induced to form ICAs as described previously (2).

**Real time quantitative RT-PCR.** Total RNA was purified using Trizol (Invitrogen). For RNA preparation from single cells, 10 µg of glycogen was added to 150 µl of Trizol. First strand cDNA was prepared using a High Capacity cDNA Archive Kit (Applied Biosystems). PCR was performed in 25 µl reactions in 96-well plates using cDNA prepared from 100 ng of total RNA and Universal PCR Master Mix (Applied Biosystems). Primers and probes were Assay-on-Demand (Applied Biosystems) or for cytokeratin-19 (Ck-19) were custom designed with 5' to 3' sequences: forward CCCGCGACTACAGCCACTAC, reverse TGTTGGCACCAAGAATCTTG, probe CACGACCATCCAGGACCTGCGG. We have confirmed linear amplification of proinsulin and glucagon transcripts by qRT-PCR on samples containing varying proportions of total RNA from human islets and HeLa cells. Thus, for each 2-fold reduction in the fraction of human islet RNA in a 100 ng sample of combined total RNA, the fluorescence cycle threshold (Ct) value for proinsulin or
proglucagon mRNA increased by one cycle. This linear relationship continued until the Ct values were 38, and we therefore consider Ct values greater than 38 to be undetectable. For quantitative expression of fold-changes by qRT-PCR when the initial transcript levels were undetectable, the initial Ct value was assigned to be 38, which would lead to a possible underestimation of the actual fold-change. qRT-PCR results generally were normalized to GAPDH or \( \beta \)-actin levels to correct for differences in RNA input. For Table 1, 18S RNA was used for normalization.

For single cell measurements, cells were monodispersed with trypsin, washed 3 times with buffer and \( 10^4 \) cells were seeded in 15 ml SCM into 10-cm tissue culture-treated dishes. After 5 to 10 min, individual cells were handpicked under microscopic visualization from cells that had settled to the surface of the dish and become loosely adherent. Thus, proinsulin mRNA was measured in single cells that were selected to be part of the proliferating hIPC cultures. Similar results were obtained when single cells were acquired by dilution after monodispersion. Proinsulin transcript was detected in single cells with fluorescence Ct values of 23 or more and comparable aliquots of medium not containing a cell gave Ct values of 38 or higher, presumably from leakage of mRNA from damaged cells. Therefore, single cells were scored positive for proinsulin transcript if Ct values were between 23 and 37. This \( 2^{37-23} = 2^{14} \sim 16,000 \)-fold range for detectable insulin expression in single human islet-derived cells is consistent with the estimate for proinsulin II mRNA of \( 10^5 \) copies per mouse beta cell (3).

**Antibodies, immunostaining and in situ hybridization.** Rabbit polyclonal antibody to human C-peptide (Linco Research Inc, MO) and mouse monoclonal anti-vimentin (Immunotech, France) antibodies were used at 1:100 dilution. Mouse monoclonal anti-cytokeratins-7 and -19 antibodies (Dako) and anti-smooth muscle actin (Sigma) were used at 1:200 dilution. Rabbit polyclonal antibody to human nestin was a generous gift from Dr. Eugene Major (NINDS, NIH) and was used at 1:200 dilution. Antibody against BrdU (with nuclease) was from Amersham Biosciences.
Antibody against C-peptide was used in this study to avoid detection of the insulin supplement in SFM during immunostaining procedures (4). Alexa-Fluor 488 and 633 F(ab’)2 secondary antibodies (Molecular Probes, OR) were used at 1:200 dilution. Propidium iodide was used to visualize nuclei. For immunostaining of islet or ICA outgrowths, cells were washed with DPBS. In some cases, cell monolayers or clusters were dispersed with 0.05% Trypsin/EDTA (Cellgro) and cytospun for 5 min at 700 rpm onto glass slides. Cells were then fixed in 4% fresh paraformaldehyde, permeabilized with chilled 50% methanol, blocked with 4% donkey serum and then incubated with antisera. Primary antibodies were incubated overnight at 4°C, washed with PBS and then incubated with the secondary antibodies at 37°C for 1h. Slides were then washed extensively in PBS and mounted in Mowiol. Double in situ hybridization and antibody labeling were performed as described (5, 6). Briefly, cells were fixed in fresh paraformaldehyde and first processed for hybridization by standard procedures but without proteinase treatment. Proinsulin Greenstar™ oligonucleotide anti-sense probe (Gene Detect, FL) was hybridized overnight at 37°C. Controls for hybridization included a sense probe (negative control) as well as poly(dT) (positive control) and each hybridization experiment included a positive sample (fresh human islets) and a negative sample (passage 9 hIPCs). Following hybridization, slides were washed with PBS and then processed for immunostaining as described above. Confocal images were captured with a Zeiss LSM 510 Meta laser scanning inverted microscope using a 100X/1.3 oil objective with optical slices less than 0.7 μm. Magnification, laser and detector gains were identical across samples and images are displayed in pseudocolor. Results presented are representative fields confirmed from at least 3 different experiments using cells derived from at least 2 islet preparations.
Figure S1. PANC-1 cells undergo reversible epithelial-to-mesenchymal-to-epithelial transition. (A) PANC-1 cells were induced to cluster into ICAs by incubation in SFM for 6 days (2). At time zero, SFM was replaced with SCM. By 12 h, some cells have migrated out of the ICA and a monolayer of epithelial cells has formed by 48 h. (B) PANC-1 cells migrating to form ICAs after 3 h in SFM express vimentin peptide in filamentous arrays while cells in SCM (0 h) do not. Boxed cells are shown enlarged to illustrate vimentin filaments in migrating cells. Vimentin staining (green) is superimposed on a differential interference contrast (DIC) image. Scale bars = 20 µm.
Figure S2.
Figure S2. hIPCs are derived from adult human islets by epithelial-to-mesenchymal transition in vitro. (A) Within 48 h, adherent cells migrate out from adult human islets. (B) Cells migrating out from in vitro cultures of fresh human islets at day 4 express intermediate filament proteins vimentin, nestin and smooth muscle actin (upper panels) and continue to express these characteristic mesenchymal proteins after the transition into proliferative hIPCs shown here at passage 16 (lower panels). Scale bars = 20 μm.
Figure S3. Cell size is a characteristic of different phenotypes within a single hIPC population. (A) hIPCs were sorted by flow cytometry using light scatter properties into populations that were enriched for small (left) or large (right) cells. Scale bars = 20 µm. (B) After sorting, the small cell population was immediately analyzed for viable cell size distribution using a Vi-Cell counter (Beckman-Coulter) and remaining small cells were placed in culture and analyzed on day 3 for cell size distribution. These findings are not consistent with two cell populations. If large cells were a distinct population they would have to proliferate faster than small cells and within 3 days increase from 5% to 30% of the population. The doubling time of the entire population is 60 hr (fig. S4B) so that in 3 days the number of cells increased by more than 2-fold. For the large cells to increase in
number by at least 12-fold (2 for population increase times 6 for increase in %) they would have to exhibit a doubling time of less than 20 hours. If large cells doubled at that rate they would rapidly make up the majority of cells and the doubling time of the entire population would be less than 20 hours.
Figure S4. **C-peptide-expressing cells proliferate.** Islet cultures on day 7 were labeled with BrdU for 40 hr and then harvested with trypsin, cytospun and co-stained with antibodies to BrdU (green) and to C-peptide (red). Nuclei were labeled with Hoechst 33342 (blue). Panels A and B show cells that stain for BrdU but not C-peptide (green arrowheads), C-peptide but not BrdU (red arrowheads) and BrdU and C-peptide (cyan arrowheads). Of 413 cells counted from these and additional fields, 33 (8%) stained for both BrdU and C-peptide, 153 (37%) were positive for BrdU but not C-peptide, and 125 (30%) were positive for C-peptide but not BrdU. Scale bars = 10 µm.
Figure S5. Characterization of hIPCs. (A) After 14 days in culture, adherent hIPCs were harvested and re-seeded in SCM (left). The re-seeded cells exhibited a fibroblast-like morphology and proliferated to confluence after 3 days (center). When harvested and re-seeded in SFM, hIPCs formed ICAs shown here after 9 days (right). Scale bars = 20 µm. (B) A composite growth curve from 7 hIPC preparations. For a representative hIPC preparation, proinsulin mRNA measured by qRT-PCR from 100 ng total RNA decreases until undetectable at passage 10 and beyond.
Figure S6.  Induction of proinsulin transcript in ICAs from different islet preparations. 14-day ICAs were induced from 6 different hIPC preparations at passages between 10 and 18. Proinsulin mRNA was measured by qRT-PCR and was not detectable in uninduced hIPCs from each preparation.
SOM References


2. A. A. Hardikar, B. Marcus-Samuels, E. Geras-Raaka, B. M. Raaka, M. C. Gershengorn, 


