Human islet-derived precursor cells can cycle between epithelial clusters and mesenchymal phenotypes

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Abstract

We showed previously that undifferentiated, proliferating human islet-derived precursor cells (hIPCs) are a type of mesenchymal stem/stromal cell (MSC) that can be induced by serum deprivation to form clusters and ultimately differentiate in vitro to endocrine cells. We also demonstrated that partially differentiated hIPC clusters, when implanted under the kidney capsules of mice, continued to differentiate in vivo into hormone-producing cells. However, we noted that not all hIPC preparations yielded insulin-secreting cells in vivo and that in some animals no hormone-expressing cells were found. This suggested that the implanted cells were not always irreversibly committed to further differentiation and may even de-differentiate to a mesenchymal phenotype. In this study, we show that human cells with a mesenchymal phenotype are indeed found in the grafts of mice implanted with hIPCs in epithelial cell clusters (ECCs), which are obtained after 4-day in vitro culture of hIPCs in serum-free medium (SFM); mesenchymal cells were predominant in some grafts. We could mimic the transition of ECCs to de-differentiated mesenchymal cells in vitro by exposure to foetal bovine serum (FBS) or mouse sera, and to a significantly lesser extent to human serum. In a complementary series of experiments, we show that mouse serum and FBS are more effective stimulants of mesenchymal hIPC migration than is human serum. We found that proliferation was not needed for the transition from ECCs to de-differentiated cells because mitomycin-treated hIPCs that could not proliferate underwent a similar transition. Lastly, we show that cells exhibiting a mesenchymal phenotype can be found in grafts of adult human islets in mice. We conclude that epithelial-to-mesenchymal transition (EMT) of cells in hIPC ECCs can occur following implantation in mice. This potential for EMT of human islets or differentiated precursor cells must be considered in strategies for cell replacement therapy for diabetes.

Keywords: mesenchymal progenitor cells • pancreatic islets • cell proliferation • epithelial and mesenchymal markers

Introduction

β-Cell replacement therapy via islet transplantation is a promising treatment for type 1 diabetes; however, such an approach is limited by the shortage of donor organs. Significant efforts are currently being directed at the generation of large numbers of insulin-producing cells in vitro from embryonic or adult stem cells, adult pancreatic islets, liver cells or other sources including mesenchymal stem/stromal cells (MSCs) [1–4]. So far, these approaches have met with limited success in vivo.

We [5] and others [6–8] previously reported that mesenchymal-like cells derived from adult human islets can be expanded and induced to differentiate to insulin-expressing islet-like cells, acquiring epithelial character in vitro. The proliferating human islet-derived precursor cells (hIPCs) that we have isolated display many characteristics of MSCs including adherence to plastic tissue culture dishes, expression of mesenchymal cell-surface antigens and differentiation to various mesodermal tissues such as fat, cartilage and bone [9]. Recently, we provided evidence that hIPCs may be a specialized type of MSC that can differentiate to insulin-expressing cells in vivo [10] and that have epigenetic markings at the insulin gene similar to those found in adult β cells, which may reflect a commitment towards endocrine differentiation [11].

Vimentin is a type of intermediate filament protein normally expressed in cells of mesenchymal origin and generally absent.
ECCs can differentiate further to insulin-expressing cells in vitro. This is an important component of an epithelial phenotype. hIPC exhibits cell surface distribution [16].

To study the commitment for epithelial differentiation of hiPSCs in vitro and their potential for further differentiation in vivo, we transplanted 4-day ECCs under the kidney capsules of NOD-SCID mice. This is the protocol we used previously to show that hiPC ECCs differentiate to insulin-expressing cells after transplantation [10]. As reported previously, ECCs exhibit cell surface distribution of β-catenin, which is consistent with an epithelial phenotype, whereas mesenchymal hiPSCs exhibit nuclear and diffuse cytoplasmic localization of β-catenin [16]. We also showed that the transcript level of a β-catenin target gene Dickkopf-1 (DKK1), which is a Wnt/β-catenin antagonist, was several hundred-fold higher in proliferative hiPSCs than in islets and that DKK1 peptide was secreted from proliferative hiPSCs at high levels [16]. We predicted that the level of DKK1 mRNA would decrease towards that in islets when hiPSCs transitioned to ECCs in vitro and that this would be another indication of mesenchymal hiPSCs transitioning to a more epithelial phenotype. Figure 1 illustrates the time course of the decrease in DKK1 mRNA levels that is consistent with mesenchymal hiPSCs transitioning to a more epithelial phenotype.

The 4-day ECCs that were transplanted did not express insulin or other endocrine hormones prior to transplantation [10]. Cells positive for human C-peptide were detected by immunostaining in some grafts harvested 30–90 days after implantation but not in others, demonstrating that ECCs may not always mature into endocrine cells in vivo. We hypothesized that these graft failures represent a transition to a mesenchymal phenotype of cells in some ECCs after implantation. Indeed, using antibodies specific for human proteins, immunostaining of 4 of 12 ECC grafts from two hiPSC preparations recovered at various times between 1 week and 5 weeks demonstrated a mixed population of cells expressing either the endocrine markers, C-peptide and glucagon, or the mesenchymal markers, vimentin and SMA (Fig. 2A–D). Moreover, cells within 8 of 12 grafts after 1 to 5 weeks stained positively for vimentin and SMA but not for insulin or glucagon (Fig. 2E–H), supporting the likelihood of partial or full transition to cells with a mesenchymal phenotype in these grafts. These data suggest that cells within hiPSC ECC grafts may continue to differentiate into endocrine cells or may revert to a mesenchymal phenotype. Due to the extended time between implantation and immunohistochemical analysis, we cannot exclude the possibility that a very few mesenchymal cells originally present in the ECCs continue to proliferate in the graft and their number represents proliferation in vivo rather than EMT of hiPSCs in ECCs. In view of our in vitro data (see below), we think this is unlikely.

To establish a model in which to study this phenomenon in more detail, we determined whether similar transitions of cells within ECCs could occur in vitro. When ECCs formed during incubation in serum-free medium (SFM) for 6 days (6-day ECCs), a time at which insulin mRNA expression is observed, were transferred to serum-containing medium (SCM) with 10% foetal bovine serum (FBS) for 36 hrs, mesenchymal-like cells migrated out from the ECCs to form a proliferating, adherent monolayer of
spindle-shaped cells similar to undifferentiated, proliferating hIPCs (Fig. 3A). In addition to differences in morphology, mesenchymal cells in mammals differ from epithelial cells by exhibiting higher levels of expression of genes of the intermediate filament network such as vimentin and fibroblast-specific protein-1 (S100A4) (an early fibroblast lineage marker) [17]. In contrast, epithelial cells express higher levels of tight junction proteins such as claudins 3 and 4. We performed quantitative real time PCR and observed that during the transition from undifferentiated mesenchymal hIPCs to ECCs, mRNAs for claudins 3 and 4 as well as those specific for endocrine cells including insulin, glucagon and somatostatin increased whereas mRNAs for vimentin, S100A4 and two other mesenchymal marker genes, snail homologues 1 and 2, decreased. Upon addition of FBS, these cells exhibited changes in gene expression consistent with de-differentiation to a mesenchymal character with transcript levels for epithelial/endocrine genes decreasing and those for the mesenchymal phenotype increasing (Fig. 3B). These data show that cells in ECCs can transition in vitro to a de-differentiated mesenchymal phenotype.

BrdU incorporation demonstrates that hIPCs migrating out from ECCs in medium containing 10% FBS begin to proliferate. The level of BrdU-positive cells incubated 1 day or 4 days in medium containing 10% FBS was 20.4 ± 1.1% and 35 ± 3.3%, respectively (Fig 4A). Consistent with the increase in the percentage of BrdU-positive cells, cell cycle analysis showed that ECCs maintained in FBS for 6 days exhibited an increase in the percentage of cells in S-phase from 1.5% ± 0.3% to 12.6% ± 1.7% (Fig 4B). By comparison, 7.0% ± 2.3% of proliferating, undifferentiated hIPCs were in S-phase (n = 4).

To further support the idea that epithelial-like hIPCs within ECCs transition to mesenchymal-like cells upon exposure to FBS, we monitored the intracellular localization of β-catenin because we had shown that β-catenin translocated from the nucleus to the cell periphery when hIPCs transition from a proliferating monolayer to ECCs [16]. We confirmed our previous observation and found that 81% of 248 cells within ECCs exhibited peripheral β-catenin; there was only 1 of 248 cells that had nuclear staining for β-catenin and 18% of the cells displayed neither nuclear nor surface β-catenin. When ECCs were
transferred to medium containing 10% FBS, most of the cells migrating out from the ECCs after 72 hrs exhibited nuclear β-catenin staining (Fig. 4C) consistent with the idea that cells with epithelial-like features within clusters de-differentiated to a proliferating mesenchymal phenotype.

Undifferentiated, proliferating hiPCs express cell surface markers CD73, CD90 and CD105 that are characteristic of MSCs [10]. To determine whether the levels of expression of these cell-surface antigens changed with changes in cell morphology, we analysed these CD markers by flow cytometry in undifferentiated, proliferating hiPCs, in ECCs formed after 4-day incubation in SFM, and in ECCs switched back to media containing 10% FBS for 3, 7 or 11 days. Cells at all three stages express the three CD markers. However, expression of CD90 and CD105 decreased in 4-day ECCs in comparison with hiPCs and increased upon return to medium containing 10% FBS. In contrast, expression of CD73 did not change significantly, which may reflect slower protein turnover at the cell surface for this marker (Fig. 5). The reversible reduction in...
cell surface expression of CD90 and CD105 supports the conclusion that hiPCs may transition between epithelial and mesenchymal characteristics in vitro and suggests that this ability may be at least partially responsible for the high proportion of vimentin-expressing cells in grafts.

To determine whether FBS was unique among sera in causing hiPCs to transition from cells in ECCs to mesenchymal cells, 4-day ECCs in SFM were transferred to medium containing 10% FBS, 10% adult mouse serum or 10% adult human serum. As illustrated in Fig. 6A, mesenchymal-like cells migrated out from ECCs faster in adult mouse serum than in FBS whereas adult human serum was least active in causing this transition. Time-lapse video recordings demonstrated that cells in mouse serum adopt a mesenchymal-like morphology within 18 hrs (Movie S1). The same transition takes 36 hrs in FBS (data not shown) and is delayed up to seven days for ECCs cultured in human serum (Movie S2).

The transition from cells in ECCs to a monolayer of mesenchymal cells involves a complex series of events, one of which is migration of the cells out from the cluster. We, therefore, tested...
whether different sera stimulated migration of undifferentiated hIPCs. As shown in Fig. 6B, mouse serum was more effective as a stimulant for hIPCs to migrate than FBS (P < 0.01) and both of these sera were more effective than human serum.

Although the data presented are most consistent with a transition of the majority of hIPCs within ECCs to a mesenchymal-like phenotype upon exposure to FBS, it was possible that the epithelial-like cells died and a minor population of mesenchymal hIPCs within ECCs proliferated rapidly. To test this possibility, we blocked the proliferation of hIPCs by treating them with mitomycin C, a DNA crosslinker and anti-proliferative agent. Figure 7 shows that mitomycin C-treated hIPCs: (i) are indistinguishable from untreated hIPCs when cultured in FBS (A); (ii) form clusters like untreated hIPCs when incubated in SFM (B) and (iii) migrate out from ECCs and transition to a mesenchymal phenotype like hIPCs when ECCs are re-exposed to FBS (C). As expected, the number of cells in mitomycin C-treated ECC cultures did not increase when exposed to FBS whereas those in cultures of untreated hIPCs showed a progressive increase (Fig. 7D). The absence of proliferation during transition back to a mesenchymal phenotype of mitomycin C-treated hIPCs was confirmed by absence of BrdU incorporation in these cultures (data not shown). These findings demonstrate that hIPCs can transition from a mesenchymal to an epithelial phenotype and cycle back to a mesenchymal phenotype in the absence of their proliferation.

Since mouse serum appeared to be an effective stimulus for the transition of cells within ECCs to a mesenchymal phenotype, we tested whether mesenchymal cells may arise in grafts of adult human islets placed under the kidney capsule of NOD-SCID mice. There were very few vimentin-positive cells in freshly isolated adult human islets (data not shown). In two experiments, we found numerous vimentin-positive cells in islet grafts (Fig. 8). Some transplanted human islets contained cells expressing endocrine hormones scattered in an extensive network of cells expressing vimentin filaments, which is not found in adult human islets in situ [18, 19].

Discussion

A variety of cell types within the endocrine, exocrine or ductal compartments of murine and human pancreases have been described as potential pancreas-specific precursor cells and different strategies for their isolation and expansion have been used [20–24]. A recent study, using an in vitro cell lineage approach, demonstrated that human β-cells can be induced to transition to cells with a mesenchymal phenotype and proliferate in vitro [25]. Another report, using adenoviral vectors to specifically label different endocrine cell types in human islets, shows de-differentiation of endocrine cells to a ductal progenitor-like cell [26]. These studies support the idea of human islet plasticity and de-differentiation in vitro.

Our laboratory [10] and others [24, 27, 28] reported that expanded precursor cells derived from adult human islets could be transplanted and secrete human insulin in mouse recipients. In our experimental paradigm, undifferentiated hIPCs are expanded in vitro in the presence of FBS, induced to transition to cells within ECCs in vitro by serum deprivation and grafted into recipient mice to differentiate further into glucose-responsive, insulin-secreting cells in vivo. However, as shown in the present study, hIPC grafts exhibit variable proportions of cells staining for pancreatic hormones and of cells that do not express hormones but express mesenchymal marker proteins (Fig. 2). Moreover, related results were seen in mouse grafts of adult human islets, where we observed a significantly higher number of vimentin-positive cells than that found in islets in situ (Fig. 8).

In hIPC ECCs formed in vitro, a majority of cells express non-filamentous vimentin but a few may be mesenchymal cells that express filamentous vimentin. It is possible that in hIPC ECC grafts within mice, some cells of a minor mesenchymal population survive and proliferate, while the major fraction of cells do not proliferate or die. Similarly, in grafts of freshly isolated adult human islets, a minor population of vimentin-expressing cells may have
Fig. 6 Different effects of foetal bovine, adult mouse and adult human sera on transition of ECCs to a mesenchymal state and on migration of mesenchymal hIPCs. (A) hIPC 4-day ECCs were incubated in fresh medium containing 10% FBS, adult mouse serum or adult human serum and photographed after four additional days. (B) Mesenchymal hIPCs in monolayer cultures were incubated overnight in SFM. Cells were monodispersed with trypsin and seeded into the upper chamber of Transwell® dishes (30,000 cells/500 µl) in SFM alone or in medium containing 10% of different sera as indicated. Medium (750 µl) containing 10% of different sera was added to the lower chamber. Cells migrating in 4 hrs through pores to the undersurface of the Transwell® were photographed and counted. (** P < 0.01).

Fig. 7 Mitomycin C-treated hIPCs that do not proliferate undergo mesenchymal-to-epithelial-to-mesenchymal transitions. (A) Monolayer cultures of mitomycin C-treated hIPCs incubated with medium containing 10% FBS. (B) Mitomycin C-treated hIPCs were harvested with trypsin and incubated in SFM for 4 days. (C) ECCs (4 days) comprised of mitomycin C-treated hIPCs were incubated in fresh medium containing 10% FBS. The photomicrograph was taken after 3 days. (D) ECCs were formed with mitomycin C-treated or untreated hIPCs by incubation in medium without serum and re-exposed to SCM for 3 days. Cell counts were made daily of untreated (—) and mitomycin-C treated (→) cells. (* P < 0.05 and **P < 0.001 versus day 0, n = 3).
proliferated whereas hormone-expressing cells did not expand. Indeed, Ko et al. [29] reported expression of vimentin in proliferating human duct cells, which these authors considered a marker of pancreatic precursor cells, whereas epithelial cells in the pancreases of normal adult human, adult rat or neonatal pig did not express vimentin protein. Similarly, Buerger and colleagues [30] suggested that cells exhibiting vimentin protein expression in invasive breast cancer may be proliferating breast progenitor cells with a bilinear (glandular and myoepithelial) differentiation potential.

On the other hand, it is possible that hIPCs within ECCs de-differentiate in vivo to a mesenchymal phenotype and thereby lose their commitment to endocrine differentiation. A number of studies in development and cancer biology have shown that epithelial cells can transition in response to environmental signals to migratory mesenchymal phenotypes [13–15, 31, 32]. For example, it has been suggested that EMT occurs in kidney and lung allografts and plays a role in progressive deterioration of the grafts [17, 33–35]. Weir and co-workers reported expression of vimentin mRNA as a marker of stromal fibroblasts in islet grafts from normal and diabetic recipients and suggested that these cells may be part of the process of angiogenesis [36]. If this were the case, the stromal cells could have been formed by EMT. Abraham and colleagues [21] found vimentin-positive cells concentrated along the graft-kidney border after transplantation of human nestin-positive islet-derived stem/progenitor cells under the kidney capsule of immunocompetent C57BL/6 mice also.

To provide support for the hypothesis that vimentin-expressing cells in hIPC grafts are derived by EMT, we developed an in vitro experimental paradigm that attempts to mimic the situation observed in hIPC ECC grafts. The majority of cells within ECCs formed in vitro exhibit decreased mesenchymal characteristics, that is, epithelial morphology, cell surface β-catenin [16] and no migration, no filamentous vimentin and decreased expression of DKK1, CD90 and CD105 mRNAs. Within 36 hrs of addition of FBS, hIPCs migrate out of ECCs, exhibit mesenchymal morphology and up-regulate expression of mesenchymal mRNAs for vimentin, S100A4, and transcription factors snail homologues 1 and 2, while transcripts for endocrine hormones and epithelial markers, claudins 3 and 4, decrease. Although the proportion of proliferating cells also increased, the rapid kinetics of the changes in morphology, migration and gene expression strongly suggested that these changes represented FBS-induced EMT rather than selective expansion of a few residual mesenchymal cells. We confirmed that this was the case by showing that mitomycin C-treated hIPCs that could not proliferate underwent the same mesenchymal-to-epithelial-to-mesenchymal transitions as untreated hIPCs. Complementary findings were reported by Rukstalis et al. [40] who showed that several endocrine cell lines could reversibly de-differentiate to mesenchymal-like cells and that this was mediated, at least in part, by the transcription factors snail 1 and 2, and in studies using mouse islets in which it was shown by lineage tracing that β cells could undergo de-differentiation and generate short-term cultures of mesenchymal cells [41, 42].

Our data also show that adult mouse serum was a more effective stimulus than FBS for EMT of ECCs, while adult human serum was much less effective. The observation that mouse and FBS are more effective than human serum as inducers of EMT raises some concerns regarding choice of serum for culture of MSCs as suggested recently [43, 44]. Mouse serum was the best chemotactic stimulus of undifferentiated hIPCs also. The results of these experiments are consistent with the idea that similar signalling pathways control transition to a mesenchymal phenotype and migration of mesenchymal hIPCs. These data further support the hypothesis that vimentin-expressing cells in hIPC grafts in mice represent cells that transitioned (de-differentiated) to a mesenchymal phenotype. The finding that adult human serum is less effective
than adult mouse serum in inducing hIPC EMT and migration suggests that the use of mouse models, which are the most commonly used animal models for these transplantation experiments, may overestimate EMT as a potential problem in transplantation of islet precursor cells into human recipients.

Lastly, based on their cell-surface marker expression and their capacity to differentiate to fat, bone, cartilage and insulin-producing cells [10] and their exhibition of epigenetic marks at their insulin gene similar to those found in β cells [11], we suggested that undifferentiated hiPCs are a specialized type of MSC that retains ‘memory’ to differentiate to insulin-expressing cells. Of note, we have presented preliminary evidence that mesenchymal cells with characteristics of hiPCs are present in adult human islets [10]. Thus, hiPCs may represent pancreatic adult stem cells but this has not been proven. However, the studies reported herein offer a cautionary note for the use of hiPCs for replacement therapy in type 1 diabetes. Despite the potential of undifferentiated hiPCs to differentiate into fully functional hormone-secreting cells, differentiated hiPCs, and perhaps all types of differentiated mesenchymal precursor/stem cells, are capable of de-differentiating to a mesenchymal phenotype that is indistinguishable from undifferentiated hiPCs. Indeed, Song et al. [45] recently showed that human bone marrow-derived MSCs after differentiation to osteocytes, chondrocytes and adipocytes could de-differentiate in vitro to cells that appear indistinguishable from undifferentiated MSCs. Thus, we conclude that cells within hiPC ECCs have the capacity to de-differentiate to a mesenchymal phenotype, a process that might compete with their capacity to differentiate into mature insulin-expressing cells and diminish their potential in cell therapy.

Experimental procedures

Chemicals

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Cell culture

hiPCs were maintained in 150-mm tissue culture dishes (Falcon, BD, Franklin Lakes, NJ, USA) in serum-containing medium (SCM). SCM (‘growth medium’) was CMRL-1066 (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS, 2 mM L-glutamine (GIBCO). Cells were passaged every 3–4 days at 80–90% confluence. Briefly, SCM was removed and 10 ml trypsin-EDTA, 1X (Cellgro, Mediatech INC, Herndon, VA, USA) was added per dish. Trypsin was inactivated by the addition of an equal volume of growth medium and cells were collected by centrifugation (160 × g, 5 min.). Cells were resuspended in fresh SCM and counted using a VI-Cell XR analyser (Beckman Coulter, Inc., Fullerton, VA, USA) and seeded in new dishes at 1.5 × 10^4 cells/cm^2.

For formation of ECCs, cells were trypsinized and washed twice with differentiation medium. Differentiation medium was CMRL-1066 supplemented with 2 mM L-glutamine, 1% (w/v) fatty acid free bovine serum albumin, fraction V and 1× insulin-transferrin-selenium-A (100×, GIBCO). Clusters were harvested by gentle pipetting 4 or 6 days after serum withdrawal and were filtered through a 70-μm cell strainer (BD Biosciences, Bedford, MA, USA) to remove small cell aggregates or individual cells for further processing. For EMT, 10% FBS (Biosources, Rockville, MD, USA) and HyClone, Logan, UT, USA), 10% mouse serum (US Biologicals, Swampscott, MA, USA and Atlanta Biologicals, Lawrenceville, GA, USA) or 10% human serum (NIH Blood Bank) was added to cell clusters.

hiPCs were incubated with 10 μg/ml mitomycin C for 4 hrs at 37°C. Cells were trypsinized and frozen. After thawing, mitomycin C-treated cells were maintained in SCM and incubated in SFM to form ECCs and then re-exposed to SCM in a fashion identical to untreated hiPCs.

Quantitative real-time PCR analysis

Total RNA was purified using RNeasy kit (Qiagen, Valencia, CA, USA). First strand cDNA was prepared using a high Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed in an MX3000P instrument (Stratgene, La Jolla, CA, USA) using TaqMan probes and Universal PCR Master Mix (both from Applied Biosystems). Expression levels were normalized to expression of human GAPDH.

BrdU incorporation and cell proliferation

Monolayer hiPCs were seeded in two-chamber Lab-Tek slides in growth medium. BrdU, (Amersham Biosciences, Piscataway, NJ, USA) was added to the medium for 24 hrs prior to fixation and staining as described in the section ‘Antibodies and immunostaining’. A monoclonal anti-BrdU antibody (Amersham Biosciences) was used and signals were visualized using secondary antibody and DAPI. For BrdU immunolabelling of ECCs, 6-day cells were transferred to the designated medium in presence of BrdU for 24 or 96 hrs and paraformaldehyde-fixed for immunostaining.

Migration assay

Migration assays were performed in 24-well Transwell® dishes (BD Falcon) with 8-μm-pore filters. Overnight serum starved cells (30,000 cells/500 μl) were added to the upper chamber, and 750-μl migration medium with chemotactic factors was added to the lower chamber. After 4 hrs incubation of the Transwells at 37°C, 5% CO2, the medium was removed from upper chamber and the upper surface of the filters was carefully wiped with a cotton-swab.
ECCs, which typically ranged from 50 to 200
were transplanted into each mouse. ECCs were maintained on ice
equipped with xenon and halogen lamps and an Orca-ER
coordinated every 15 min. for up to 3 days using
Cell cycle analysis
For flow cytometric analysis of cell cycle, hiPCs were harvested with trypsin, collected by centrifugation at 200 × g for 5 min. and re-suspended in PBS. Cells were again collected by centrifugation, monodispersed in 0.5 ml PBS, fixed in 4.5 ml cold (−20°C) 70% ethanol, and stored at −20°C overnight. Before analysis, the samples were centrifuged and the pellets were re-suspended in 0.5 ml PBS containing propidium iodide (50 μg/ml) and deoxyribonucle-ase-free ribonuclease A (50 μg/ml) for 1 hr at room temperature. A total of 10,000 gated cells (to exclude debris) were analysed by flow cytometry in a FACS Calibur cytomter. The percentages of
Statistical analyses
Statistical significance was determined by using Student’s t-test.

Transplantation

ECCs generated from mesenchymal hiPCs that had been expanded by 10 or more doublings were used for transplantation. This means that the cells used for implantation have prolif-erated to generate at least 1000-fold (215) more cells than were initially present in the islets used to establish the hiPc culture. For each mouse to be transplanted, 1000–1500 4-day
were transplanted into each mouse. ECCs were maintained on ice
equipped with xenon and halogen lamps and an Orca-ER
coordinated every 15 min. for up to 3 days using
Cell cycle analysis
For flow cytometric analysis of cell cycle, hiPCs were harvested with trypsin, collected by centrifugation at 200 × g for 5 min. and re-suspended in PBS. Cells were again collected by centrifugation, monodispersed in 0.5 ml PBS, fixed in 4.5 ml cold (−20°C) 70% ethanol, and stored at −20°C overnight. Before analysis, the samples were centrifuged and the pellets were re-suspended in 0.5 ml PBS containing propidium iodide (50 μg/ml) and deoxyribonucle-ase-free ribonuclease A (50 μg/ml) for 1 hr at room temperature. A total of 10,000 gated cells (to exclude debris) were analysed by flow cytometry in a FACS Calibur cytomter. The percentages of
Statistical analyses
Statistical significance was determined by using Student’s t-test.

Antibodies and immunostaining
Rabbit anti-human C-peptide (Linco, St. Charles, MO, USA), mouse anti-human glucagon (Sigma-Aldrich), mouse anti-human α-SMA (Sigma), mouse anti-human β-catenin (Zymed, Carlsbad, CA, USA) and mouse anti-human vimentin (Immunotech, Marseilles, France) antibodies were used at 1:100 dilution in blocking buffer (4% donkey serum in DPBS). Alexa-Fluor 488 and 546 F(ab‘)2 secondary antibodies (Molecular Probes, Carlsbad, CA, USA) were used at 1:200 dilutions. Graft-containing kidneys from killed mice

Statistical analyses
Statistical significance was determined by using Student’s t-test.

Inserts were transferred to a second 24-well plate containing 4% paraformaldehyde in Dulbecco’s phosphate-buffered saline (DPBS) and stored over night at 4°C. Inserts were then washed three times
were transferred to a second 24-well plate containing 4%

Time-lapse microscopy
ECCs formed after 6 days in SFM were transferred and placed in 6-well plates in CMRL-1066 containing 10% FBS or adult human

Phase-contrast microscopy
Cells were viewed using an inverted Axiovert microscope (Carl Zeiss). Image acquisition was performed with a digital camera.

For islet transplantation, 500 human islets were transplanted as described above.

Antibodies and immunostaining
Rabbit anti-human C-peptide (Linco, St. Charles, MO, USA), mouse anti-human glucagon (Sigma-Aldrich), mouse anti-human α-SMA (Sigma), mouse anti-human β-catenin (Zymed, Carlsbad, CA, USA) and mouse anti-human vimentin (Immunotech, Marseilles, France) antibodies were used at 1:100 dilution in blocking buffer (4% donkey serum in DPBS). Alexa-Fluor 488 and 546 F(ab‘)2 secondary antibodies (Molecular Probes, Carlsbad, CA, USA) were used at 1:200 dilutions. Graft-containing kidneys from killed mice

Statistical analyses
Statistical significance was determined by using Student’s t-test.
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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Movie S1. ECCs formed during 4 days in SFM were exposed to SCM (10% adult human serum) and observed by time-lapse photography every 5 min. for the next 46 hrs. Cells in the ECCs migrated out of clusters to form flattened cell monolayers that appear similar to the original undifferentiated hIPCS in SCM. Movie S2. ECCs formed during 4 days in SFM were exposed to SCM (10% adult human serum) and observed by time-lapse photography every 5 min. for the next 46 hrs. ECCs remained as clusters and an adoption of a mesenchymal-like character was delayed.

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