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Human Placenta-Derived Mesenchymal Stem Cells and Islet-Like Cell Clusters Generated From These Cells as a Novel Source for Stem Cell Therapy in Diabetes

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Abstract

Placental tissue holds great promise as a source of cells for regenerative medicine due to its plasticity, and easy availability. Human placenta-derived mesenchymal stem cells (hPDMSCs) have the potential to differentiate into insulin-producing cells. Upon transplantation, they can reverse experimental diabetes in mice. However, it is not known whether culture-expanded undifferentiated hPDMSCs are capable of restoring normoglycemia upon transplantation in streptozotocin (STZ)-induced diabetic mice. Hence we prepared long-term cultures of hPDMSCs from the chorionic villi of full-term human placenta. Flow cytometry analyses and immunocytochemistry study revealed bonafide mesenchymal nature of the isolated hPDMSCs. These cultures could differentiate into adipogenic, oesteogenic, chondrogenic, and neuronal lineages on exposure to lineage-specific cocktails. Furthermore, we showed that hPDMSCs can form islet-like cell clusters (ILCs) on stepwise exposure to serum-free defined media containing specific growth factors and differentiating agents. qRT-PCR showed the expression of insulin, glucagon, and somatostatin in undifferentiated hPDMSCs and in ILCs. Differentiated ILCs were found to express human insulin, glucagon, and somatostatin by immunocytochemistry. Additionally, ILCs also showed abundance of pancreatic transcription factors ngn3 and isl1. Both undifferentiated hPDMSCs and ILCs exihibited insulin secretion in response to glucose. Transplantation of hPDMSCs or ILCs derived from hPDMSCs in

STZ-induced diabetic mice led to restoration of normoglycemia. Our results demonstrate, for the first time, reversal of hyperglycemia by undifferentiated hPDMSCs and ILCs derived from hPDMSCs. These results suggest human placenta-derived MSCs as an alternative source for cell replacement therapy in diabetes.

Keywords: human placenta-derived stem cell, diabetes, mesenchymal stem cell, transplant, beta-cell, islet, differentiation, macrocapsule, immunoisolation

Introduction

Diabetes mellitus is becoming one of the main threats to human health in the 21st century [1]. The global prevalence of diabetes is shifting significantly from the developed countries to the developing countries [2]. Beta-cell replacement is an effective treatment for type 1 diabetes, but its applicability is limited by the lack of sufficient donor tissue, raising the need for alternative tissue sources. Human embryonic stem cells (hESCs) have received much attention in the last few years because of their promise as a renewable source of tissue for beta-cell differentiation [3]. However, ethical issues surrounding the use of hESCs limit its use in clinical application of cell replacement therapy. Also, tissue-resident stem cells have demonstrated self-renewal and multipotent differentiation potential, which could be of great value for the successful progress of cellular therapies for diabetes.

The placenta is a temporary organ that accompanies pregnancy connected to the fetus via the umbilical cord. Besides playing a fundamental and essential role in fetal development, nutrition, and tolerance, placenta may also represent a reserve of progenitor/stem cells. Recently, the placenta was shown to be an important hematopoietic organ, containing cells in chorionic villi that showed hematopoietic cell lineage differentiation along with presence of the hematopoietic markers CD34 and CD45 [4]. In addition to hematopoietic stem cells, the placenta has been reported to contain a population of multipotent stem cells exhibiting some of the characteristics of pluripotent ESCs including expression of stem cell markers c-kit, Thy-1, oct-4, SOX2, hTERT, SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 [5]. These cells resemble mesenchymal stem cells and can be induced to differentiate into hepatocyte, vascular endothelial, cartilage, and neural-like cells [5-16]. Differentiation of these human placenta-derived mesenchymal stem cells (hPDMSCs) into insulin positive cells [17, 18] has raised hopes for the use of these cells as an alternative source for cell therapy in diabetes.

It is known that Insulin is the only gene that is imprinted exclusively in the yolk sac placenta [19]. The yolk sac placenta and the pancreas of both human and mouse are the only tissues that produce insulin [20-22]. Rodent yolk sac is a known major source of insulin imprint [19, 20, 23], which starts by day 14.5 [24]. This suggests that yolk sac placental imprinting of insulin is an ancestral trait [25]. However, mechanisms for regulating insulin gene expression in placental tissue differ from those in pancreatic beta-cells [23].

Based on these findings, we hypothesized that hPDMSCs retaining imprinted insulin may act as surrogate beta-cells. Therefore, we supposed it is likely that transplantation of hPDMSCs under the kidney capsule of experimental diabetic mice may help to reduce hyperglycemia through insulin secretion. MSCs from bone marrow and placenta share similar characteristics, and possess immunomodulatory properties. Both have been found suitable for allogeneic stem cell therapy eliminating the need of immunosupression [26].

In the present study, we demonstrate long term maintenance of hPDMSCs in a medium supplemented with human umbilical cord blood serum (hUCBS), and confirm multilineage differentiation potential of hPDMSCs. Also, we show that culture-expanded, undifferentiated hPDMSCs, and islet-like cell clusters (ILCs) generated from hPDMSCs, are capable of restoring normoglycemia when transplanted into STZ-induced diabetic mice.

Materials and methods

The work was conducted following the approval from the National Centre for Cell Science (NCCS) Institutional Ethical Committee.

Sample collection

Human placenta of segment cesarean section deliveries of full term pregnancies were collected after informed written consent of the parents. Abnormal placentas showing retrogradation and calcification, and those from proven gestational diabetes, were excluded from the study. Collected placenta tissue was stored aseptically and transported at ambient temperature to the lab in L15 collection medium containing antibiotics (penicillin 200 U/ml and streptomycin 200 µg/ml, Sigma Aldrich, St. Louis, MO, USA).

Isolation and expansion of human placenta-derived mesenchymal stem cells (hPDMSCs)

A total of 32 placentas were screened for isolation of MSCs. Isolation of hPDMSCs was carried out as per the protocol described by Miao *et al*. [12]. The chorionic plate of each placenta was exposed by stripping off the amnion. This chorionic plate was then washed with phosphate buffer saline pH 7.2 (PBS) to remove traces of cord blood. Placental tissue was cut into smaller pieces using sterile scissors. The pieces of placenta were digested with 0.25% trypsin-EDTA (Sigma, St. Louis, MO, USA) for 30 min at 37°C on magnetic stirrer (REMI, India). The digest was then centrifuged at 1000 rpm (Eppendorf 5810R, Hamburg, Germany) to separate the cells. The cells were washed three times with PBS, and finally collected by centrifugation (1000 rpm). Cell pellet was suspended in alpha-minimal essential medium (α-MEM, Invitrogen, Carlsbad, CA, USA), supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% hUCBS. Then, the cells were cultured at 37°C in a CO_2 incubator (Forma Scientific Inc., Ohio, USA) gassed with 5% CO_2 and 95% humidity.

Medium was changed every 48 h and replenished with α-MEM supplemented with 10% hUCBS. Cells were first passaged after 21-25 days, and again every 7th day. Cell growth was analyzed by direct cell count to determine the log, lag, and stationary phases. For this purpose, the cells were seeded onto 12 well tissue culture dish at a density of 5 x 10^3 cells/cm^2. Viable cells were counted every 48 h using the trypan blue dye exclusion test.

Characterization of isolated cells using immunocytochemistry and flow cytometry

The cells from early passages [4-6] were used for characterization studies. Cells were grown in monolayers on coverslips, and were fixed using 4% paraformaldehyde. The cells were then permiabilized using 50% methanol for 5 min, followed by blocking using 5% bovine serum albumin (BSA) in PBS for 1 h. The cells were then exposed to nestin, vimentin, desmin, smooth muscle actin, Ki 67, oct-4, and SSEA4 primary non-labeled mouse anti-human antibodies (1:100 dilutions, Chemicon, Temecula, CA, USA) for 12 h at 4°C, followed by respective secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C. The coverslips were mounted in mounting medium containing antifade (Vectashield, Vector Laboratory, Burlingame, CA, USA) and 4',6-diamidoino-2-phenylindole (DAPI). The slides were then viewed using confocal laser scanning microscope (Zeiss LSM510). DAPI (Invitrogen, Carlsbad, CA, USA) was used for nuclei visualization.

The cells from passages 4-6 were dislodged using 0.05% trypsin and 0.02% EDTA in PBS, and resuspended in α-MEM. Then, the cells were fixed in chilled 70% ethanol, and incubated in mouse anti-

human FITC/PE conjugated antibodies against CD29, CD33, CD34, CD44, CD45, CD73, CD90, CD105, and CD117 (1:100 dilution) for 1 h on ice (all antibodies were purchased from Becton Dickinson, San Diego, CA, USA). Finally, the cells were counted using a flow cytometry laser 488 nm, and the data were analyzed using BD Cellquest Pro software.

Induction of adipogenic, chondrogenic, osteogenic, and neuronal differentiation

Human PDMSCs at passage 3 were fed with alternate cycles of adipogenic induction medium (PT-3102B Cambrex, Walkersville, MD, USA) and adipogenic maintenance medium (PT-3102A Cambrex, Walkersville, MD, USA). Adipogenesis was induced as per the manufacturer's instruction. Adipogenesis was confirmed using Oil Red O staining.

For chondrogenic, osteogenic, and neuronal differentiation, 3 x 10^3 hUCMSCs/cm^2 were plated onto tissue culture flasks. Cells were allowed to adhere to culture surface for 24 h at 37°C. Chondrogenic, osteogenic, and neuronal lineages were induced by replacing the growth medium (α-MEM) with chondrogenic, osteogenic, and neuronal differentiation bullet kit (PT-3003, PT-3002, and CC-3229 respectively Cambrex, Walkersville, MD, USA) respectively, as per manufacturer's instructions. Chondrogenesis was confirmed using Safranin-O staining; osteogenesis by staining with Alizarin Red S, while neuronal differentiation was confirmed by immunostaining with neuron-specific markers Map2 and NeuN (Chemicon, Temecula, CA, USA).

Differentiation to islet-like clusters (ILCs)

Differentiation was carried out in three stages, as indicated in Table Table1.1. To induce differentiation, undifferentiated hPDMSCs from T75 culture flasks (BD Falcon, Franklin Lakes, NJ, USA) were trypsinized and reseeded at cell density 1 x 10^6 cells/ cm^2 in SFM-i and plated on ultralow attachment tissue culture plates (Corning, Fisher Scientific International, Hampton, NH, USA). SFM-i contained α-MEM with 17.5 mM glucose, 1% BSA Cohn fraction V, fatty acid free (Sigma-Aldrich, St. Louis, MO, USA), 1x insulin-transferrin-selenium (ITS) (5 mg/l insulin, 5 mg/l transferrin, 5 mg/l selenium, Sigma Aldrich, St Louis, MO, USA). The cells were cultured in this medium for 2 days. On the third day, the medium was changed to SFM-ii, which contained α-MEM with 17.5 mM glucose, 1% BSA, ITS, and 0.3 mM taurine. The cell aggregates were cultured in this medium for another 4 days, and shifted to SFM-iii on the seventh day. SFM-iii contained α-MEM with 17.5 mM glucose, 1.5% BSA, ITS, 3 mM taurine, 100 nM glucagon-like peptide (GLP)-1 (amide fragment 7-36;), and 1 mM nicotinamide. The cell aggregates were fed with

fresh SFM-iii every 2 days for further 4 days. All chemicals were purchased from Sigma Aldrich, St. Louis, MO, USA, unless indicated otherwise.

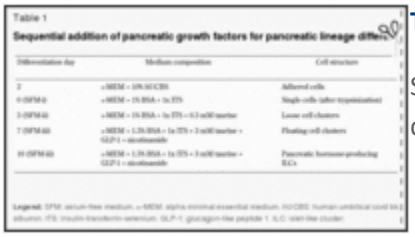

Table 1

Sequential addition of pancreatic growth factors for pancreatic lineage differentiation

This three step protocol, developed by our group, is well proven for differentiation of ILCs from mesenchymal stem cells derived from human umbilical cord and amnion [27, 28].

Characterization of ILCs using diphenylthiocarbazone (DTZ) staining

Specificity of ILCs was determined by diphenylthiocarbazone (DTZ) staining. ILCs were incubated with 10 µl DTZ stain for 1 h at 37°C, and viewed under inverted phase contrast microscope (Olympus IX 70, Tokyo, Japan).

Assessment of hPDMSCs and ILCs for the presence of pancreatic hormones and transcription factors

ILCs and hPDMSCs pellets were homogenized and frozen in Trizol (Invitrogen, Carlsbad, CA, USA). RNA was isolated according to the manufacturers' instruction, measured on ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and subjected to reverse transcription using a high capacity cDNA archival kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using TaqMan based assay-on-demand (AoD) from Applied Biosystems. Target genes were detected with FAM-MGB probes, while VIC-MGB 18S were used in duplex/single-plex on a 7500 FAST real time PCR system (Applied Biosystems, Foster City, CA, USA). Real time PCR was performed in 5 µl total volume in 96-well plates, using cDNA prepared from 100 ng equivalent total RNA. All qRT-PCR data was normalized to 18S rRNA, and carried out in duplex/single-plex reaction to correct for any differences in RNA input in each well.

All values are represented as cycle threshold (Ct) values. Ct values are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). Samples with a Ct value of 39 and above have no target gene expression. In an ideal PCR reaction, the number of target molecules doubles in each PCR cycle. Therefore, a difference

of 1 in the Ct value corresponds to a concentration difference of 2. For comparative purposes, fold difference over the detectable Ct value (i.e. < 39) was calculated for each target gene, and was compared with ILCs and undifferentiated hPDMSCs.

Insulin release and C-peptide assay

100 ILCs were handpicked and incubated with 200 µl Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mM/l HEPES (now called KRBH) and 5.5 mM glucose at 37°C for 1 h. Supernatant was collected and stored at -80°C. Then, ILCs were transferred to KRBH supplemented with 16.5 mM glucose. Similarly, undifferentiated hPDMSCs were exposed to basal and stimulated glucose solution in KRBH. After incubating them for 1 h at 37°C, the supernatant was collected and stored at -80°C. Secreted insulin was quantified using insulin ELISA kit (Mercodia, AB, Sweden). For stage-specific C-peptide assay during ILC development, conditioned medium was collected at each time point from all experimental groups. The medium was quantified using ultra-sensitive C-peptide human and mouse ELISA kit (Mercodia, AB, Sweden).

Induction of diabetes in Balb/C mice

Balb/C male mice aged 6 to 8 weeks were obtained from an inbred colony maintained at the experimental animal facility of the National Centre for Cell Science (NCCS), Pune, India. Animals were housed under controlled conditions of light (12 h of light and 12 h of darkness), temperature (24°C) and humidity (50%). Mice were maintained on normal chow and water. Experimental protocols were approved by the NCCS Animal Ethical Committee.

After 6-8 h of fasting, mice were injected i.p. with freshly prepared STZ (Sigma Aldrich, St. Louis, MO, USA) at a dose of 200 mg/kg body weight in chilled citrate buffer (pH 4.5). Blood from the tail veins of 6-8 h fasted mice, were assessed for blood glucose (BG) concentrations (Accutrend Sensor Comfort blood glucose meter, Roche Diagnostic, Germany) at 24 h intervals to confirm the diabetic status of the animals. Body weights were recorded at the same time. Mice showing BG more than 300 mg/dl were consistently considered diabetic.

Transplantation of hPDMSCs in diabetic mice under kidney capsule

The obviously diabetic mice (5 mice/group) were anesthetized (ketamine, 150 mg/kg), and xylazine, 10 mg/kg, i.p.), shaved, and cleaned. A small incision was made on the left flank of the mouse and the kidney was exposed. The kidney was kept moist with normal saline swab. Using a sterile surgical blade, a

small scratch on the right flank of the kidney was made, creating a nick in the kidney capsule, not too large or too deep. Blood was drawn from tail vein, allowed to clot, and the clot was carefully aspirated off the bottom of a 1.5 ml microcentrifuge tube using a p200 pipette and a straight, thin-wall pipette tip. A blood clot containing the hPDMSCs (1.5×10^5) was slowly delivered under the kidney capsule (animals form this group then called hPDMSCs Tx). The kidney was placed back into the cavity and the peritoneum and skin were sutured using absorbable 6-0 catgut sutures (Stericat Gutstrings, Delhi) and autoclipper (Becton Dickinson, Bedford, USA).

All animals (control and experimental) received an i.p. injection of gentamycin (3 mg/kg body weight), ampicillin and cloxacillin (20 mg/kg body weight), and diclofenac sodium (0.5 mg/kg body weight), for 3 days (starting from the day of operation). Also, they received the topical ointment (Soframycin, Aventis Pharma. Ltd., Pune, India), and were placed in a cage on a heating pad. Animals were administered analgesics (buprenorphine 0.05 mg/kg every 12 h for 3 days). The second group consisted of diabetic control/sham transplanted (sham Tx) animals. They received blood clot only. Non-transplanted diabetic animals were considered as positive control, while non-diabetic untreated animals were considered as untreated control.

Transplantation of ILCs in diabetic mice in biocompatible macrocapsules

For ILC transplantation (ILC Tx) we used another protocol employing an immune isolation device. Animals in this experimental group received approximately 1000 islet equivalent clusters i.p. packed in biocompatible macrocapsules made up of polyurethane-polyvinylpyrrolidone semi-inter-penetrating network (PU-PVP semi-IPN). The macrocapsule transplantation protocol was followed as described earlier by our group [27, 28].

A total of five groups, with five animals in each group, was created to study the *in vivo* functionality of hPDMSCs and differentiated ILCs. A biocompatible and immunoisolatory PU-PVP semi-IPN macrocapsule was used to encapsulate the differentiated ILCs to avoid immune rejection. All encapsulation procedures were carried out under strictly aseptic conditions. These capsules were washed thoroughly for 60 days with sterile distilled water followed by PBS (pH 7.2) and DMEM medium washes of 24 h each. For transplantation, around 1000 islet equivalent clusters were suspended in sodium alginate solution (1-2% w/v alginic acid, Sigma Chemical Co., St. Louis, MO, USA; in 0-85% saline) at a ratio of 1000 islets/ml alginate solution. The solution containing ILCs was then transferred into biocompatible capsules, and the open ends of the capsules were sealed using heat. The capsules were then transplanted into the

peritoneal cavity of STZ-induced diabetic mice. Animals were fasted overnight and anesthetized by i.p. administration of thiopentalsodium at a dose of 40 mg/kg body weight. About 2 mm long incisions were made in the abdomen, and macrocapsules with or without islets were introduced.

Blood glucose and body weights were monitored for all groups every 48 h (while values for every 7th day have been represented). After 30 days of transplantation, animals from the ILC Tx group and the hPDMSCs Tx group were subjected to intra-peritoneal glucose tolerance test (IPGTT). After 8 h of fasting, mice were injected with 2 g/kg body weight of glucose i.p. Glucose disposal was analyzed by measuring blood glucose at 0, 15, 30, 45, 60, and 120 min post injection using Accutrend Sensor Comfort blood glucose meter.

Estimation of serum insulin

For serum preparation, blood was collected from all mice groups by retro-orbital bleeding and incubated at 37°C for 30 min. The blood was later centrifuged at high speed; clear serum was collected and stored at -80°C. Human and mouse insulin concentrations were measured (both Mercodia, ultrasensitive human and mouse insulin ELISA kits, Sweden) for all the abovementioned time points according to the manufacturer's instruction. Secreted insulin was quantified using insulin ELISA kits (Mercodia, AB, Sweden).

At the end of the study period (after 30 days), ILC-transplanted PU-PVP semi-IPN macrocapsules and undifferentiated hPDMSCs transplanted kidneys were surgically removed. Kidney grafts were examined by H&E staining and the viability of ILCs packed inside the capsules were checked by trypan blue staining.

Statistical analysis

Values are expressed as mean ± SEM or median and interquartile range, from at least three different experiments. Experimental groups were compared using Anova or 't' test. Prism 5 (Graphpad Software, San Diego) was used for analysis.

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Results

Isolation and expansion of hPDMSCs

The results reported here represent data obtained from the study of 28 (out of 32) successfully isolated human placentas. The isolated cells from these placentas at passage 0 represented a mixed population of epithelial and fibroblast-like cells (Figure (Figure1A).1A). Cell colonies from placental tissue began to appear after 7-10 days of cells isolation. After passaging with trypsin, the epithelioid population rapidly disappeared from culture and were no longer apparent by the second passage (Figure (Figure1B).1B). The 100% confluency was reached after 21 to 25 days of culture. In contrast, the fibroblastoid population of cells continued to proliferate, even after 25 passages. After subjecting these cells to different media, optimum growth was obtained in the α-MEM supplemented with 10% hUCBS (Data not shown).

Figure 1

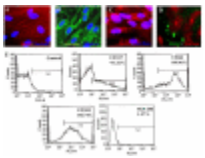
A: hPDMSCs exhibiting mixed population of epithelial cells and fibroblast-like cells at initial passages (0 to 2). At later passages, they exhibited fibroblast-like cell appearance only (**B**). Growth analysis of these cells showed a mean doubling time of (more ...)

The combined data of the cell count showed that the lag phase of these hPDMSCs lasts for approximately 72 h, leading to 120 h of log phase (Figure (Figure1C).1C). It was observed that hPDMSCs had a doubling time of 21.84 h.

Immunocytochemistry and flow cytometry

The confocal microscopy study showed that hPDMSCs were positive for mesenchymal markers such as Stro-1 (Figure (Figure2A),2A), vimentin (Figure (Figure2B),2B), nestin (Figure (Figure2C),2C), and embryonic oct-4 (Figure (Figure2D).2D). Flow cytometry analysis of the hPDMSCs showed that they were strongly positive for CD44, CD105, and CD117 (Figure (Figure2E),2E), and negative for CD10, CD34, CD45, and CD166.

Figure 2



Physical characterization of hPDMSCs

Multi-lineage differentiation studies

Human PDMSCs were differentiated into an adipogenic cell type after approximately 21 days of incubation in adipogenic induction medium. The adipocytic phenotype in induced hUCMSCs was

signaled by the change in cell morphology from spindle-shaped through round to oval shaped cells, and by the appearance of numerous large, rounded intracytoplasmic lipid droplets. These lipid droplets were stained positive by Oil Red O (Figure (Figure33A).

Figure 3

Multilineage differentiation potential of hPDMSCs

Subsequently, hPDMSCs differentiated into a chondrogenic cell lineage after 3 weeks of incubation in chondrogenic medium. The chondrogenic phenotype in induced hPDMSCs was signaled by the changes in cell morphology, from spindle-shaped to larger and rounded cell aggregates, and by the accumulation of sulfated proteoglycans which were present in cartilage. These proteoglycans in the matrix stained positive with Safranin-O (Figure (Figure33B).

Upon exposure to osteogenic differentiation medium for three weeks, hPDMSCs showed changes in cell morphology. They changed from fibroblast-like to cuboidal-shaped, as they differentiated and mineralized. Calcium phosphate mineralization, which stained positive by alizarin red S stain, indicated direct evidence of calcium deposits as an amorphous accumulation between cells (Figure (Figure3C)3C) after the third week of osteogenic induction.

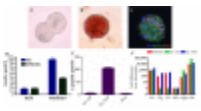
When exposed to a neuronal differentiation cocktail medium for 21 days, hPDMSCs changed their morphology to neuronal cell. These cells stained positive for the neuronal markers Map2 and NeuN (Figure (Figure33D).

Differentiation to islets-like clusters and their functional study

The induction of hPDMSCs with serum-free medium containing a cocktail of ITS, nicotinamide, and taurine has microscopically shown progressive cell clustering from day 2 onwards. This led to typical ILC formation at the end of day 10 (Figure (Figure4A).4A). hPDMSCs that proliferated as an adherent monolayer aggregated into spherical cell clusters when the medium was changed from serum-containing medium to day 1 SFM (SFM-i). Cellular aggregation occurred as a gradual process, and complete clusters were formed by 24-36 h of incubation in SFM-i. The cells formed tight clusters that resembled pancreatic islets. Henceforth, these clusters were termed ILCs. ILCs gradually matured to pancreatic hormone-expressing cells when exposed to SFM-ii followed by SFM-iii supplemented with beta-cell maturation factors like GLP-1 and nicotinamide (SFM-iii). After 10 days of induction, these

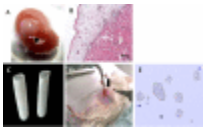
mature ILCs stained positive for islet-specific DTZ stain (Figure (Figure4B).4B). DTZ is known to selectively stain pancreatic beta-cells because of its high zinc content; non-islet tissue remained unstained. After 30 days, viable ILCs were found by trypan blue dye exclusion method (Figure (Figure5E).5E). These clusters were also found positive for insulin and glucagon (Figure (Figure4C)4C) by immunocytochemistry. We obtained about 600-650 ILCs from a dish containing 3 x 10^5 cells. Hence, the efficiency and fraction of hPDMSCs undergoing differentiation ranged between 65-70%, depending on the yield of ILCs. This represents 20-25% beta-cells per islet.

Figure 4



Differentiation of hPDMSCs to pancreatic lineage

Figure 5



In vivo transplantation study

These newly generated ILCs showed insulin secretion upon glucose stimulation. Basal insulin secretion of approximately 15 pmol/l was observed; stimulation insulin secretion was around 100 pmol/l (Figure (Figure4D).4D). Undifferentiated hPDMSCs showed 18 pmol/l insulin secretion at basal glucose level (5.5 mM glucose), and 48 pmol/l for stimulated glucose levels (16.5 mM glucose) (Figure (Figure4D).4D). Also, C-peptide secretions from these ILCs were around 108.54 pmol/l (Figure (Figure4E),4E), and was found negligible for undifferentiated hPDMSCs. The absence of C-peptide release in day 0 supernatant is due to the fact that day 0 PDMSCs represents the undifferentiated state. Amylase secretion in day 10 culture supernatant was negligible for differentiated ILCs and for hPDMSCs culture supernatant.

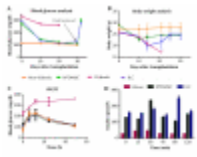
Undifferentiated placental MSCs along with day 4 and day 10 differentiated ILCs were analyzed for the pancreatic hormones and beta-cell development transcription factors by Taqman-based real-time PCR. An abundance of proinsulin, glucagons, and somatostatin in differentiated and undifferentiated ILCs was observed. Day 4 ILCs (i.e. intermediate differentiated cells) did not show the abundance of glucagon and somatostatin, but exhibited an abundance of Ngn3 and Isl1 transcripts. Also, undifferentiated hPDMSCs showed abundance of Ngn3 and Isl1 transcripts, along with ILCs (Figure (Figure4F).4F). This expression indicated their propensity towards islet differentiation. hPDMSCs differentiated and acquired islet-like

architecture only at the end of the differentiation process. Insulin secretions from hPDMSCs and ILCs were similar to those obtained after transplanting hPDMSCs/ILCs. This feature is important because ILCs generated from hPDMSCs serve the same purpose as hPDMSCs. It suggests that hPDMSCs are an alternative equivalent to ILCs.

Transplanted hPDMSCs/ILCs restored normoglycemia in STZ-induced diabetic mice

Mice transplanted with hPDMSCs and ILCs did not show graft rejection. Diabetic mice without transplantation showed hyperglycemia throughout the study period and died after 20 days of STZ-induced diabetes. Non-transplanted, non-diabetic mice showed normal blood glucose values. Mice transplanted with ILCs or undifferentiated hPDMSCs showed reductions in blood glucose levels, and reversal of experimental diabetes after 15 days. This status was maintained at blood glucose values of less than 140 mg/dl. Diabetic control mice did not restore normoglycemia and died after day 15 of diabetes induction (Figure (Figure6A),6A), with constant loss in body weight. Whereas, increased body weight was observed in ILC Tx, hPDMSC Tx, Sham Tx, and the negative control group (Figure (Figure66B).

Figure 6



Post-transplantation study

Transplanted hPDMSCs/ILCs were responsive to glucose challenge in vivo

The function of implanted ILCs/hPDMSCs was further evaluated by a standard 2-hour IPGTT. The IPGTT of ILC/hPDMSC-transplanted and non-diabetic control group mice (n = 5 for all groups) resulted in a typical bell-shaped curve. This means that glucose concentrations were elevated at 15 min, followed by a return to near normal by 90 min, after glucose infusion for plotted time vs. blood glucose (Figure (Figure6C).6C). In contrast, blood glucose concentrations in the diabetes group mice remained elevated throughout the entire study period. Although blood glucose concentrations in ILC/hPDMSC-transplanted mice were elevated initially, as compared to non-diabetic control mice, they displayed a similar glucose tolerance test profile by 90 min of glucose injection. Diabetic animals continued to display elevated glucose concentrations even beyond 120 min. Since all diabetic control mice showed significant signs of morbidity and weight loss (20% of their initial body weight), they had to be euthanized as per the institutional guidelines for care of laboratory animals.

Simultaneous analysis of human and mouse insulin was carried out to understand whether normal circulating glucose was maintained as a result of insulin (human) released from hPDMSCs and ILCs derived from hPDMSCs. We observed detectable levels of human insulin in the blood of hPDMSC- and ILC-transplanted mice. This increased following glucose challenge (Figure (Figure6\D).6\D). Significant increases in human basal insulin were seen at 30 min in hPDMSC- and at 60 min in ILC-transplanted mice.

To confirm whether normal glucose concentrations observed in transplanted mice were caused by hPDMSCs and ILCs derived from hPDMSCs, we surgically removed the grafted capsules and hPDMSC-grafted kidneys after 8 weeks of STZ injection (12 weeks post-transplant). In the graft-removed animals, hyperglycemia was observed within three days of removal (Figure (Figure6A),6A), and almost all mice (~90%) died within 2 weeks of graft removal.

Histopathology of transplanted kidneys revealed that hPDMSCs were present in the kidney capsule of mice. There were no signs of neovascularization (Figure (Figure5B).5B). The viability of ILCs in the transplanted capsules was checked by trypan blue stain. Most of the ILCs in the capsules were found viable even 30 days after transplantation (Figure (Figure55E).

Discussion

The main focus of our study was to evaluate the potential of culture-expanded undifferentiated hPDMSCs as a novel source of insulin-producing cells. Obtaining adequate numbers of human stem cells has been problematic for several reasons. First, the isolation of normally occurring populations of stem cells in adult tissues has been technically difficult, expensive, and very limited in quantity. Second, procurement of these cells from embryos or fetal tissue, including abortions, has raised ethical and moral concerns. Alternative sources that do not violate the sanctity of independent life are essential for further progress in the clinical use of stem cells. Hence, the search continues for an ethically conducive, easily accessible, and controllable source of stem cells. Placental tissue draws great interest as a source of cells for regenerative medicine because of the phenotypic plasticity of many of the cell types isolated from this tissue.

The placenta is involved in maintaining fetal tolerance. It contains cells that display immunomodulatory properties. This feature could prove useful for future cell therapy-based clinical applications. Placental tissue is readily available and easily procured without invasive procedures. Its use does not elicit ethical

debate. Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic progenitor cells that are being explored as a promising new source. Although their immunomodulatory properties are not yet completely understood, their low immunogenic potential together with their effects on immune response make them a promising therapeutic tool for severe refractory autoimmune diseases [29].

In the present study, we have successfully isolated hPDMSCs from human chorionic villi of full term placenta. The chorionic villi of human term placenta are a rich source of mesenchymal stem cells [30]. These hPDMSCs showed typical fibroblast-like appearance, which accords with the observations by Yen *et al*. and Miao *et al*. [11, 12]. Our lab has reported earlier that hUCBS supports attachment, propagation, and differentiation of human bone marrow-derived mesenchymal cells [31], which was further confirmed by Shetty *et al*. [32]. Hence, hPDMSCs supplemented by hUCBS instead of the fetal calf serum (FCS) showed better proliferation. We deliberately avoided the use of FCS in order to grow the cells in medium free of xenoproteins, and to make them suitable for human transplantation studies. hPDMSCs have been cultured for more than 18 population doublings without any change in morphology, MSC characteristics, and differentiation potential. These isolated hPDMSCs exhibited mesenchymal proteins stro1, vimentin, nestin, and surface markers CD44, CD105, CD117, and were negative for CD45, CD34, CD90. These observations confirmed their identity as MSCs. Also, the hPDMSCs weakly expressed the embryonic cell marker oct-4 [13, 17], indicating their closeness with embryonic stem cells. Presence of embryonic characteristics may be responsible for beta-cell function in hPDMSCs [17].

The isolated hPDMSCs differentiated into various lineages like adipocyte, chondrocyte, osteocyte, and neurons after exposure to lineage-specific differentiation cocktails of differentiating agents, as reported earlier by several groups [5, 8, 10, 11, 13]. Our results documented for the first time that there are transcripts of insulin, glucagon, somatostatin, Ngn3, and Isl1 in undifferentiated hPDMSCs (Figure (Figure4).4). This is a striking feature of our study. Such transcripts were not detected in undifferentiated MSCs derived from human umbilical cord and amnion [27, 28]. It is well known that only placenta is highly vascularized among the fetal membranes. Whereas, amnion is avascular and umbilical cord Wharton's jelly acts as a matrix to support two arteries and a vein. This anatomical variation makes placenta an endocrine organ compared to other fetal membranes.

The presence of insulin in undifferentiated hPDMSCs could be explained by the insulin gene imprinting reported in yolk sac placenta of rat and human [19, 22, 23]. The closely related peptide insulin and the insulin-like growth factors (IGF)-I and II have been well characterized in a variety of tissues, including human placenta [33]. The placenta expresses high amounts of insulin receptors relative to other tissues

in the body [34], facilitating the transport of nutrients to the developing fetus across the placenta. The presence of proinsulin gene in undifferentiated hPDMSCs could be due to the involvement of insulin-like growth factor 2 (IGF2) in embryonic and placental development [35].

Transplanting undifferentiated hPDMSCs under the kidney capsules of STZ-induced diabetic mice, and transplanting biocompatible macrocapsules packed with ILCs in diabetic mice, both resulted in a reduction of hyperglycemia and restoration of normoglycemia, 15 days post transplantation. This was accompanied by increased body weight, indicating signs of diabetes reversal.

In a recent study, Chang *et al*. have demonstrated the differentiation potential of hPDMSCs into insulin-producing cells [17]. The total time needed for differentiation into insulin-producing spheroid bodies was 4 weeks, in presence of serum-free medium containing ITS. In our modified pancreatic lineage differentiation protocol, we have added nicotinamide and GLP1 along with ITS sequentially into serum-free differentiation medium, as detailed in Table 1, and reported previously by us [27, 28]. This procedure resulted in the differentiation of hPDMSCs into islet-like clusters within merely 10 days. Thus, this procedure reduces the time needed for islet differentiation.

The identity of these differentiated ILCs was then confirmed as true islets by DTZ-staining and positive immune-staining for all islet hormones (Figure (Figure4B4B and and4C).4C). The islets obtained were found to be responsive to glucose challenge, as evidenced by a threefold increase in insulin secretion over basal stimulation (Figure (Figure4D).4D). This indicated that the generated islets were able to synthesize, store, and release insulin in response to glucose.

In the present study, we have used ILCs generated from human tissue. We transplanted these ILCs into mice (xenotransplantation) using biocompatible PU-PVP-semi IPN macrocapsules. There was no graft failure during the study period. Proper glucose sensing and insulin release by the transplanted islets was evidenced by normal IPGTT. This indicated adequate graft functionality upon transplant. Upon retrieval, transplanted islets were in good condition with well-defined morphology retained inside the macrocapsule. In the study by Chang *et al*., transplantation of spheroid bodies derived from placental MSCs into STZ-induced diabetic mice led to restoration of normoglycemia, while animals transplanted with untransformed, undifferentiated hPDMSCs remained diabetic [17]. This result conflicts with our observations, where both undifferentiated hPDMSCs and differentiated ILCs showed reduction in blood glucose levels with progressive increase in body weight (Figure (Figure6A6A and and6B).6B). For the transplantation of ILCs, we used an immunoisolation device (Figure (Figure5C)5C) transplanted into the

peritoneum of diabetic mice. This novel way of transplanting islets packed into a biocompatible capsule was reported recently by our group [28].

As shown in the present study, reversal of STZ-induced diabetes after transplantation of hPDMSCS, or ILCs, is evidenced by a decrease in hyperglycemia and an increase in body weight. The restoration of normoglycemia observed in these mice could be attributed to insulin secretion by the transplanted hPDMSCs or ILCs, since graft removal led to recurrence of hyperglycemia. Moreover, estimated serum insulin of transplanted mice clearly showed an increase in the level of human insulin in mouse serum. The level of mouse insulin observed in these mice was negligible, suggesting that endogenous pancreas regeneration did not occur. The maintenance of glucose homeostasis in these mice affirms our hypothesis that transplanted hPDMSCS or ILCs are capable of insulin production in response to glucose. It also supports our hypothesis that hPDMSCs are capable of insulin secretion due to insulin gene imprinting.

As expected, we did not observe regeneration of mouse pancreas. This was due to the transplantation of undifferentiated hPDMSCs under the kidney capsule to act as surrogate beta-cells, and to satisfy the insulin requirement. However, MSCs have already been co-transplanted systemically with islets through the tail vein, and features of regeneration, neovascularization, and immunomodulation have been observed [36, 37]. Therefore, it would be very interesting to explore whether systemic administration of hPDMSCs could reverse experimental diabetes through endogenous pancreatic beta-cell regeneration. Previously, we have reported that multiple injections of bone marrow could reverse experimental diabetes in mice by inducing pancreatic regeneration [38]. Additional studies involving systemic injections of hPDMSCs via the tail vein in diabetic mice could clarify the role and mechanism of action caused by hPDMSCs.

Transplantation of undifferentiated hPDMSCs, or newly formed ILCs, into STZ-induced diabetic mice were safe. Undifferentiated hPDMSCs did not show signs of immune rejection, as evidenced by graft maintenance. This was further affirmed by the lack of HLA-DR markers on their cell surface (Figure (Figure2E).2E). Also, the cells did not cause teratoma formation upon xenotransplantation [39].

Our data confirms earlier findings of insulin gene expression and release by rat yolk sac [23]. In the study by Giddings and Carnaghi, insulin mRNA was present in extra-placental membranes before pancreatic differentiation [23]. Their study also showed that membrane fragments, maintained in culture, produced approximately 10 ng of radio-immunoassayable insulin/mg membrane protein/day. Over a 4-

day period, approximately 50 times more insulin accumulated in medium than that present in membranes at the time of isolation. In the light of this finding, our study indicates that human placental mesenchymal stem cells are a promising source for insulin-producing cells.

In our study, reversal of experimental diabetes through ILC or hPDMSC transplant was also confirmed by IPGTT performed on normoglycemic mice. Simultaneous analysis of human and mouse insulin was carried out to understand whether normoglycemia was restored as a result of human insulin released from grafted ILCs. It showed that human insulin was detectable, and increased following glucose challenge in ILC transplanted mice (Figure (Figure6D).6D). Increased fasting and glucose-stimulated serum levels of human insulin at 30 min, indicated that the implanted ILCs synthesized, stored, and secreted insulin in response to both fasting-induced hypoglycemia and glucose challenge. To find out whether normal glucose concentrations observed in transplanted mice were due to ILC graft, we surgically removed the grafted capsules after 8 wk of STZ injection (12 wk post-transplant). Mice reverted to hyperglycemia within three days of graft removal (Figure (Figure6A),6A), and almost all mice (~90%) died 2 weeks later.

Earlier studies with undifferentiated placenta-derived MSCs from umbilical cord and amniotic membrane did not show production and secretion of insulin. As transplants, they were unable to reverse STZ-induced diabetes [27, 28]. Presently, human bone marrow-derived mesenchymal stem cells are applied in human clinical trials, and their use is advocated for allogeneic stem cell therapy. However, our study shows that hPDMSCs offer a worthy alternative.

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Conclusion

The present study shows that placenta-derived mesenchymal stem cells can be easily isolated and expanded in medium supplemented with hUCBS, without alterations in morphological and functional characteristics. Due to the easy accessibility, lack of ethical concerns, and abundant availability, hPDMSCs may well be an attractive, alternative source of progenitor/stem cells for basic or translational research.

Our data confirms that hPDMSCs are able to differentiate into islets that can secrete insulin in response to glucose *in vitro* and *in vivo*. The transplantation of culture-expanded, undifferentiated hPDMSCs in experimental diabetic mice reversed hyperglycemia. Thus, these cells offer another non-pancreatic,

readily available, noninvasive, and inexhaustible source of allogeneic stem cells for cell replacement therapy in diabetes.

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