



Differentiation of human umbilical cord blood-derived mononuclear cells to endocrine pancreatic lineage

Vishal S. Parekh, Mugdha V. Joglekar, Anandwardhan A. Hardikar*

Stem Cells and Diabetes Section, Lab #12, National Center for Cell Science, Ganeshkhind Road, Pune, MH 411007, India

ARTICLE INFO

Article history:

Received 22 February 2009

Received in revised form

3 July 2009

Accepted 20 July 2009

Keywords:

Human umbilical cord blood

Transplantation

Differentiation

Insulin

Diabetes

ABSTRACT

Generation of insulin-producing cells remains a major limitation for cellular replacement therapy in treatment of diabetes. To understand the potential of human umbilical cord blood (hUCB)-derived mononuclear cells (MNCs) in cell replacement therapy for diabetes, we studied MNCs isolated from 270 human umbilical cord blood samples. We characterized these by immunostaining and real-time PCR and studied their ability to differentiate into insulin-producing cells. We observe that freshly isolated MNCs as well as mesenchymal-like cells grown out by in vitro culture of isolated MNCs express key pancreatic transcription factors: *pdx1*, *ngn3*, *isl1*, *brn4* and *pax6*. However, after 32-fold expansion, MNCs show decreased abundance of *pdx1* and *ngn3*, indicating that islet/pancreatic progenitors detected in freshly isolated MNCs die or are diluted out during in vitro expansion. We therefore transplanted freshly isolated MNCs in NOD/SCID (immuno-incompetent) or FVB/NJ (immuno-competent) mice to check their ability to differentiate into insulin-producing cells. We observe that after 9 weeks of transplantation, ~25% grafts exhibit human insulin-producing (16% immunopositive) cells. The number and abundance of pro-insulin transcript-containing cells increased when the animals underwent partial pancreatectomy, 15 days after transplantation. Our results indicate that such hUCB-derived MNC population contains a subset of “pancreas-committed” cells that have the potential to differentiate into insulin-producing cells in vivo. Further studies in understanding the differentiation potential of this subset of pancreas-committed hUCB-derived MNCs will provide us with an autologous source of “lineage-committed” progenitors for cell replacement therapy in diabetes.

© 2009 International Society of Differentiation. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Cell replacement therapy for diabetes requires sufficient number of insulin-producing cells to be transplanted successfully into diabetic individuals so as to achieve normoglycemia. However, the success of such islet cell grafts is limited by 2 major factors. First of all, the complex immunosuppressive regimen that these patients need to be maintained on and the number of cells or number of transplants that each patient has to undergo for successful reversal of diabetes. Though allogeneic transplantation of cadaveric human pancreatic islets (Shapiro et al., 2000) has been, till now, the only successfully used cell replacement therapy for diabetes, limited availability of cadaveric human pancreas restricts transplantation procedures to few diabetic individuals. Moreover, allogeneic transplantations impose physician to keep patients on immunosuppressive drugs, which subsequently develop major side effects (Hirshberg et al., 2003; Ojo et al.,

2003). These limitations have initiated intense research in generating alternate sources of transplantable islet-like cells. Several cell types such as embryonic stem cells (Lumelsky et al., 2001), human pancreatic ductal cells (Hardikar et al., 2003; Bonner-Weir et al., 2004, 2005) as well as adult human pancreatic islet-derived mesenchymal cells (Gershengorn et al., 2004) have been proposed to have the potential to differentiate into insulin-producing cells. However, since transplantation of such hormone-producing cell aggregates would need to be worked with immunosuppression, there is still a need to look for an alternate stem/progenitor cell source that can be differentiated into insulin-producing cells for autologous transplantation.

Several studies carried out till now have demonstrated the use of umbilical cord blood-derived mononuclear cells in autologous transplantation. Few reports propose that human umbilical cord blood (hUCB)-derived mononuclear cells (MNCs) (Erices et al., 2000; Kogler et al., 2004) possess mesenchymal stem cells-like characteristics (expression of CD44, CD90 and CD105) and have the ability to differentiate in all three germ layer cells. Differentiation potential of these MNCs into different cell types including insulin-producing cells was assessed by different groups. Though studies carried out in mice (Yoshida et al., 2005, Ende et al., 2004) demonstrate the ability of these cells as

* Corresponding author. Tel.: +91 20 2570 8150, +91 985 026 2853 (mobile); fax: +91 20 2569 2259.

E-mail address: anand@isletbiology.com (A.A. Hardikar).

URL: <http://www.isletbiology.com/> (A.A. Hardikar).

potential islet progenitors, there is a need to carry out detailed characterization of hUCB-derived MNCs so as to understand their potential to differentiate efficiently into cells of the endocrine pancreatic lineage. During the same time, it was also demonstrated (Pessina et al., 2004) that freshly isolated MNCs express important pancreas-specific transcription factor, *pdx1*. We demonstrate here that cord blood-derived mononuclear cells are similar to other known and established pancreatic precursor cells discussed till now. We describe here a process wherein hUCB-derived MNCs can be maintained as islet-progenitor cells for at least 5 passages of in vitro expansion. These 32-fold expanded populations of MNCs express pancreas-specific transcription factors. Upon transplantation of these MNCs into immunoincompetent as well as immunocompetent mice, they differentiate into insulin-producing cells. Furthermore, we also observe that more number of animals showed hormone-containing cells when transplanted animals underwent partial pancreatectomy. These studies demonstrate that human umbilical cord blood-derived MNCs differentiate following transplantation in mice and that paracrine factors from the regenerating pancreas may help in such differentiation process.

2. Materials and methods

2.1. Isolation and expansion of human umbilical cord blood-derived mononuclear cells (MNCs)

Human umbilical cord-blood samples were collected in citrate phosphate dextrose-buffer after donors' written consent as per institutional ethical committee's approval. MNCs are isolated by density gradient centrifugation using Histopaque[®]-1077 (Sigma, St. Louis, MO) as per the manufacturer's instructions. Briefly, cord blood is layered over Histopaque (1:2 v/v) and centrifuged at 400g, for 30 min, to separate the MNC layer at the interface. MNCs are then incubated in RBC lysis solution (Sigma, St. Louis, MO) and then washed once in DMEM:IMDM medium. MNCs are then seeded at a density of 3×10^3 cells/cm² in DMEM containing 10% UCBS in a humidified chamber at 37 °C and 5% CO₂. Cells were then passaged every 7 days with a split ratio of 1:2.

We optimized growth medium for MNCs using MTT assay. MNCs after isolation were seeded at 2000 cells/well in 100 μl of respective medium in quadruplet in tissue culture-treated flat-bottom 96-multiwell plates. Each plate is then taken at predetermined time points (days 1, 2, 3 and 7) and 10 μl of MTT (5 mg/ml in PBS pH 7.4, filter sterilized) was added to each well. Plates were incubated at 37 °C in 5% CO₂ for 3 h. Formazan crystals formed were dissolved in acidified iso-propanol and spectrophotometric reading was taken on spectra max 250 (Molecular devices, Sunnyvale, CA).

2.2. Immunostaining and confocal microscopy

Kidney or TheraCyte grafts were sectioned (20 μm) after embedding them in either cryomatrix or paraffin and taken for immunostaining. Freshly isolated MNCs and graft sections were immunostained using specific primary antibodies: guinea pig insulin (Linco Research Inc. St Charles, MO), mouse glucagon (Sigma, St. Louis, MO), mouse smooth muscle actin (Sigma, St. Louis, MO), rabbit Ki67 (Dako, Carpinteria, CA), and mouse vimentin (Chemicon, Temecula, CA) and rabbit von Willebrand Factor (Chemicon, Billerica, MA) were used at 1:100 dilution. Rabbit anti-human GLP1R, as well as C-terminal and N-terminal-specific PDX1 antibodies (raised against peptide sequence kindly provided by Doris Stoffers), was used at 1:100 dilution. Alexa

Fluor-488, Alexa Fluor-546 or Alexa Fluor-633 secondary antibodies (Molecular Probes, Eugene, OR) were used at 1:100 dilution. Hoechst 33342 was used to visualize nuclei. MNCs are cytospun, fixed with freshly prepared 4% paraformaldehyde, permeabilized using 50% chilled methanol (v/v in water) and blocked with 4% NDS (v/v in PBS) for 1 h at room temperature. Cells are then incubated in primary antibody (1:100 dilution) prepared in blocking buffer, and kept in a humidified chamber at 4 °C overnight. The next morning, cells were washed with PBS and incubated in secondary antibodies for 1 h at 37 °C. After washing with PBS, cells were mounted in vectahield mountant (Vectoc Laboratories, Burlingame, CA) and observed using a confocal microscope (Zeiss LSM 510, Germany). Detector gains, laser power and pinhole settings were kept identical for all scans and the detectors were confirmed to be set below saturation.

2.3. Hematoxylin and eosin staining

Therocyte graft sections were incubated at 60 °C for 10 min, deparaffinised in xylene and then stained for hematoxylin (Sigma-Aldrich, St. Louis, USA). Sections were then dehydrated using gradients of ethanol and finally with 100% ethanol to be stained with Eosin Y (Shandon, Cheshire, England). Slides were then rehydrated in decreasing grades of ethanol and mounted in glycerol for visualization through an inverted microscope (Nikon TE-2000, Japan).

2.4. Flowcytometry analysis

Cells were trypsinized at different passages after washing with chilled PBS (Ca/Mg-free). Blocking was carried out using 4% NDS (v/v in Ca/Mg-free PBS) for 30 min on wet ice. Cells were then incubated with fluorescence-tagged primary antibody at 4 °C for 30 min in dark. Data were acquired on FACS vantage (Becton Dickinson, Franklin Lakes, NJ). R-phycoerythrin conjugated mouse anti-human CD34, anti-mouse CD44, anti-mouse CD45, anti-human CD90 (all from BD Pharmingen, TM) antibodies were used at 1:100 dilution. Rabbit PDX1 C-terminus primary antibody and secondary Alexa fluor 546 antibody (Molecular Probes, Eugene, OR) were used (1:100 dilution in 4%NDS) to sort PDX 1-positive subpopulation from freshly isolated MNCs. Freshly isolated MNCs were washed with chilled PBS (Ca/Mg-free), permeabilized with 50% chilled methanol, and blocked using 4% NDS at 4 °C for 30 min. Cells were incubated in primary antibody and secondary antibody at 4 °C for 30 min, and washed before sorting on FACS Aria (Becton Dickinson, Franklin Lakes, NJ). Following sorting, PDX1⁺ and PDX1⁻ cell populations were taken in Trizol (Invitrogen, Carlsbad, CA) for transcript analysis using qRT-PCR.

2.5. RNA isolation and real-time PCR

RNA isolation and cDNA synthesis were carried out as described elsewhere (Joglekar et al., 2007b). Briefly, cell samples from different passages were frozen in Trizol (Invitrogen, Carlsbad, CA). RNA was isolated as per manufacturers' instruction, quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and taken for reverse transcription and quantitative real-time PCR (qRT-PCR). cDNA was synthesized using a 'high capacity cDNA archive kit' (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out in 5 μl total volume in 96-well plates using cDNA prepared from 50 to 100 ng of total RNA on a 7500 FAST real-time PCR cycler (Applied Biosystems, Foster City, CA). Assay-on-demand primers and probe mixes were used as per the manufacturers' recommendations (Applied Biosystems, Foster City, CA). Single-cell PCR was carried

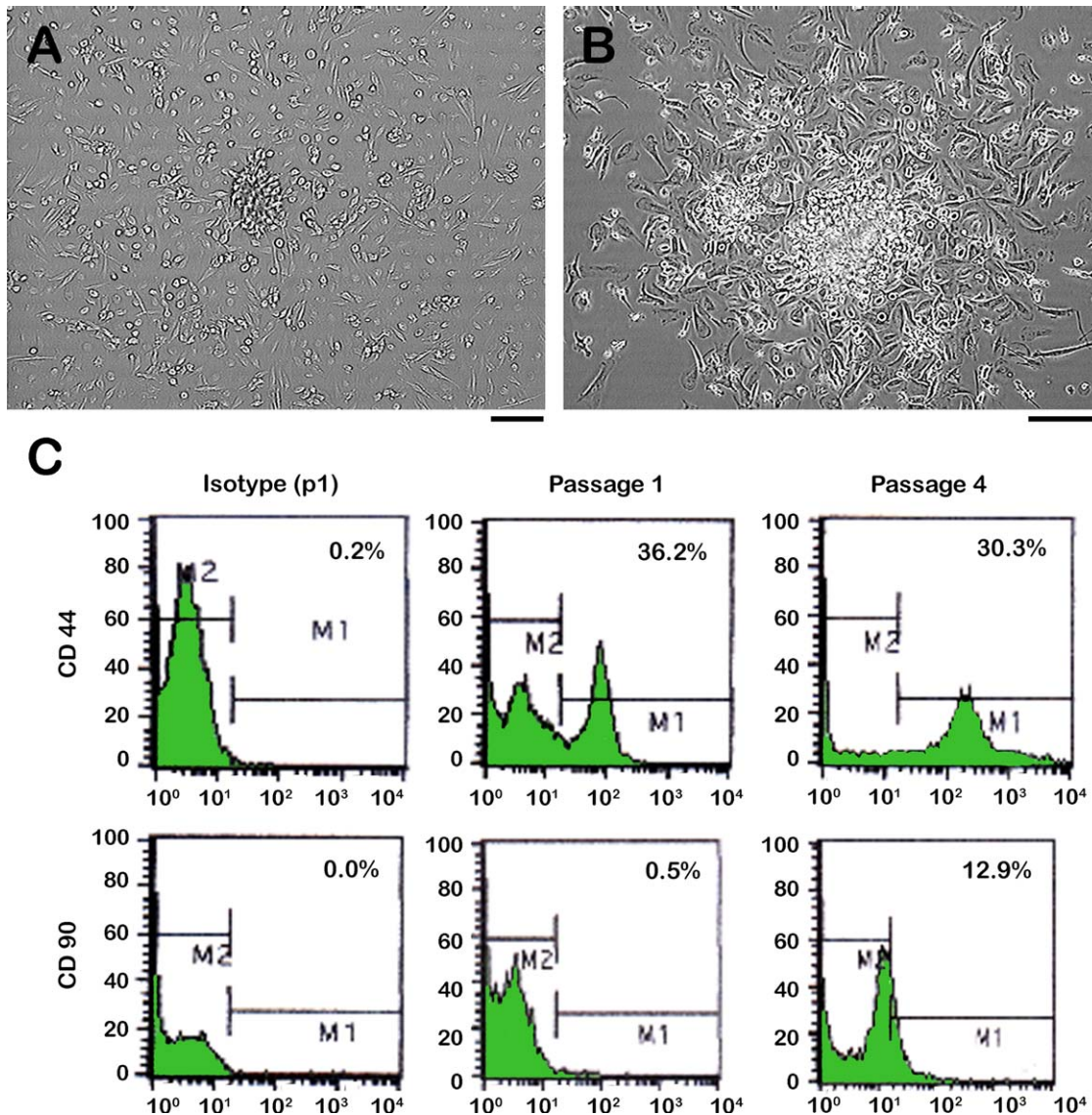


Fig. 1. In vitro-expanded human umbilical cord blood (hUCB)-derived mononuclear cells (MNCs). Human umbilical cord blood-derived mononuclear cells were isolated from freshly collected umbilical cord blood and the mononuclear cells (MNCs) or cell clusters obtained after isolation were seeded into tissue culture-treated plates. Cells adhered to culture plates by day 4 (A) and migrated out to form confluent monolayers of cells by day 10 (B). A subset of these cells was seen to express CD44 as well as CD90 (C) during passaging. Bar = 25 μ m.

out using protocol described elsewhere (Joglekar et al., 2008). Comparative transcript analysis between different pancreatic progenitor/stem cells was carried out using TaqMan Low Density Arrays (TLDA; Applied Biosystems, Foster City, CA). TLDA were customized for assessment of 46 different pancreas-specific genes transcripts. Here, 2 μ g of cDNA per sample was loaded in one lane (48 wells) of each TLDA card as per the manufacturers' specifications. Real-time PCR was carried out using 7900 HT FAST real-time PCR cyclers TLDA block (Applied Biosystems, Foster City, CA). Cycle threshold values for gene transcripts obtained using real-time PCR were normalized with 18S rRNA. Normalized data sets gene expression profiles were taken as input data for bi-directional clustering. Bi-directional clustering is one of the most widely used algorithms to recognize patterns in data sets with similar expression profiles (Joglekar et al., 2007b). Since functional modules of genes are generally regulated together; such modules can be identified from the similarity of expression patterns in such an analysis. Two-way clustering is performed in MatLabTM, using the Bioinformatics Tool-box (MatLabTM v 7.0, R 14), which basically groups the samples with similar matching gene profiles

together across the X-axis. Genes within these grouped samples that show similar expression patterns are grouped together along the Y-axis. Bi-directional clustering thus offers an important tool to assess closely related samples as well as similar gene expression pattern within these sample groups.

2.6. Surgical procedures

Immuno-competent (FVB/NJ) or immuno-incompetent (NOD/SCID) mice were obtained after approval from animal ethics committee of National Center for Cell Science (NCCS), Pune, India, and housed as per the guidelines at experimental animal facility, NCCS. MNC grafts were prepared just prior to transplantation by mixing 1000 islet equivalent MNC-clusters (1 IEq \approx 3000 cells) and mouse blood so as to form a clot. NOD/SCID mice were sedated using isoflurane and shaved on the lower left back side of the pelvic region. Vertical incision of not more than 1 cm was made on peritoneal skin to pull out the left kidney. A pocket was made in the superior renal capsule opposite to the renal pelvis and graft

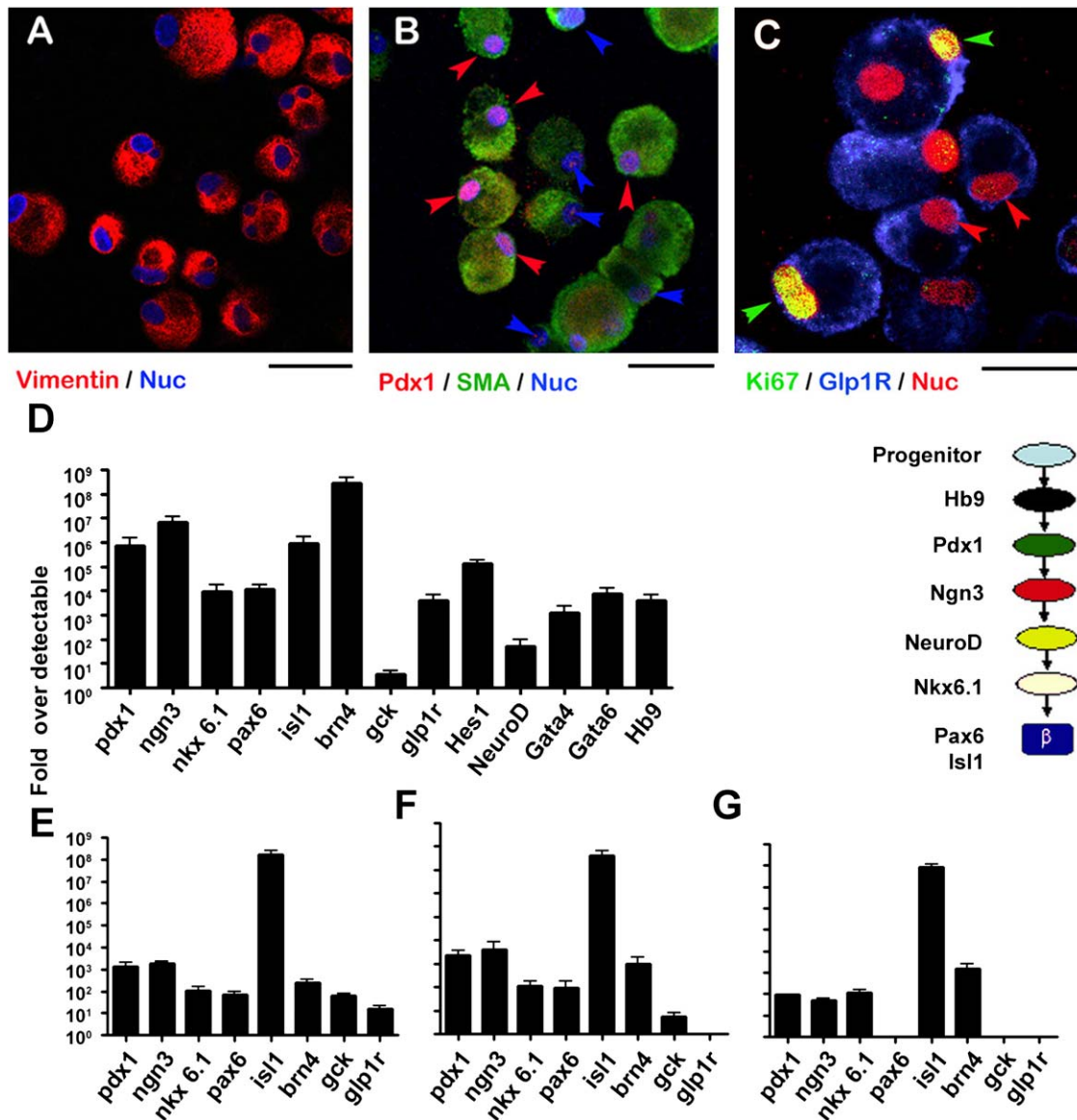


Fig. 2. hUCB-derived MNCs express transcription factors essential for endocrine pancreas development. Freshly isolated hUCB-derived MNCs show immunopositivity to PDX1 (B) in some nuclei (red arrowheads in B) but not in all (blue arrowheads in (B)). Some of the MNCs also show immunopositivity to GLP1R (C) and a subset of these cells also seem to be proliferating (green arrowheads in (C)). Majority of these cells are immunopositive for mesenchymal markers such as vimentin (A) and smooth muscle actin (B). We then measured the relative abundance of different endocrine pancreas-specific transcription factors (outlined in the schematic)/receptors/enzyme by qRT-PCR. These analyses suggest that MNCs contain majority of pancreatic markers (D) and continue to express some of them at passage 3 (E), passage 4 (F) or passage 5 (G). Data represent fold over detectable for 7 different hUCB preparations. Ct values above or equal to 39 were considered to be undetectable. At least 600 cells from 2 different preparations were counted for immunostaining analysis. Bar = 25 μ m. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

was carefully placed and massaged to restrict this inside the pocket. The peritoneal cavity was then suture closed using 4.0 absorbable suture (Davis-Geck, Manati, PR) and then the skin was closed using auto wound clippers (Becton Dickinson, Franklin Lakes, NJ). Soframycin[®] ointment (Aventis Pharmaceuticals, India) was applied to the suture-closed area and animals were given analgesics as per the recommendations of the facility veterinarian. Partial pancreatectomy (Px) involves surgical removal of 50% pancreas. Mice undergoing Px become transiently diabetic and exhibit >200 mg/dL fasting blood glucose concentrations during initial 8–12 days. Px was carried out on mice as discussed elsewhere (Hardikar et al., 1999) on day 15 after transplantation. Unless otherwise mentioned, all animals were euthanized at the end of 7 weeks after Px. Prior to euthanasia, 2 g/kg body weight glucose was injected intra-peritoneally and blood samples were

collected at 30 min (prior to euthanasia). Sera isolated were used for further analysis using a human insulin ELISA kit (Merckodia, Sweden). Grafts appear integrated within the host tissue and appeared viable as determined by Hoechst 33342 and PI staining (not shown). Graft-bearing kidneys were isolated and UCB grafts carefully removed and taken for RNA isolation or immunostaining and confocal microscopy.

FVB/NJ mice were taken to test the differentiation potential of hUCB-MNCs in immuno-competent mice. Here, we performed partial pancreatectomy (Hardikar et al., 1999) 24 h prior to transplantation of MNCs. In this case, MNCs were placed inside TheraCyt[™] devices (TheraCyt, Laguna Hills, CA). This device is made up of a proprietary biocompatible membrane that allows for vascularization of the device and immuno-isolation of encapsulated cells within the device. Devices were then placed

subcutaneously in FVB/NJ mice, which vascularize on the outermost membrane. Skin was carefully sutured using 3.0 catgut suture (Davis-Geck, Manati, PR) and Soframycin ointment was applied. The graft was retrieved after 9 weeks and cells inside the device were scraped and collected for gene expression analysis. For eliminating any contamination of mouse tissue in the collected graft sample (under kidney capsule or within Theracytes), human- and mouse-specific *gapdh* were measured as controls and gene expression (Ct values) was normalized to correct for any contaminating mouse tissue.

3. Results

3.1. MNCs contain proliferative mesenchymal-like cells

Based on the previous efforts to grow hUCB-derived MNCs, we began by checking several different media conditions that support optimal cellular metabolism (better proliferation). Cells seeded with DMEM, HF12K or DMEM:IMDM (1:1) media consistently exhibited better metabolic activity (Supplementary Fig. 1) and were therefore selected for optimization of cell expansion. Since cellular morphology was seen to be better in DME media rather than HF12K or DMEM:IMDM, we decided to continue expansion/growing of these cells using DME media. Under these conditions, freshly isolated MNCs appear as either single cells or several cell aggregates in suspension. By 2–5 days after isolation, majority of these cell aggregates adhere to the culture dishes and mesenchymal-like cells are seen to grow out from them (Fig. 1A). Cells in cluster migrate out to form monolayer by day 10 (Fig. 1B). Such confluent cultures at this time largely comprise mesenchymal-like cells. However, though these conditions supported growth and proliferation of hUCB-MNCs in the initial passages, we failed to continue expansion of these cells beyond passage 5 (32-fold expansion).

We then characterized mesenchymal cell population obtained from MNCs through passage 1 to passage 4 for CD44 and CD90, two of the known cell surface markers present on the mesenchymal cells, using flow cytometry. We found that 36% of MNCs expressed CD44 at passage 1 while 30% of cells continue to be immunopositive at passage 4, suggesting CD44-positive fraction of MNCs was fairly stable during expansion. However, 1% CD90-positive fraction of MNCs at passage 1 increased to 13% of the total population by passage 4 (Fig. 1C). Though we did not see any immunopositivity for CD34 cells in these MNCs, we did see a significant amount of CD45-producing cells at passage 4 (Supplementary Fig. 2).

3.2. Mesenchymal-like cells express pancreas-specific genes during expansion

For further characterization of cells obtained from hUCB, we immunostained freshly isolated MNCs for mesenchymal marker vimentin (Fig. 2A) as well as an important pancreatic transcription factor PDX1 (pancreas and duodenal homeobox gene 1). Interestingly, PDX1 protein was detected in 67% of freshly isolated hUCB-derived mononuclear cells (Fig. 2B). Furthermore, Ki67, a nuclear antigen expressed by proliferating cells, was seen to be present in 9.8% of freshly isolated MNCs (Fig. 2C). Though the presence of *pdx1* gene transcript has been demonstrated earlier (Pessina et al., 2004), we confirm that PDX1 protein translocates to the nucleus in hUCB-derived MNCs and is possibly involved in regulation of downstream transcription factors (outlined in Fig. 2) as is known during endocrine pancreas development (Habener et al., 2005; Watada et al., 2000). We

also observed that hUCB-derived MNCs show immunopositivity to GLP1R (Fig. 2C). GLP1R has been known to be involved in glucose-stimulated insulin secretion as well as endocrine pancreas development/regeneration (Bonner-Weir and Weir, 2005; Xu et al., 1999).

To better assess the level of these pancreatic gene transcripts during in vitro expansion, we quantitated gene transcript levels using TaqMan[®]-based quantitative real-time PCR (qRT-PCR). Though presence of pancreatic transcription factors has been documented earlier in freshly isolated hUCB cells (Pessina et al., 2004), we observed that these gene transcripts were not only detected but also retained in expanded MNCs that we maintain in vitro (Fig. 2D–G). In freshly isolated MNCs *pdx1* transcripts were found to be expressed a million-fold above the detectable level. Though these levels decreased by almost 1000-fold during in vitro expansion, possibly due to death or dilution of these cell types, hUCB-derived MNCs were seen to contain *pdx1* transcripts even after 32-fold expansion. Surprisingly, we observe that *isl1* transcript abundance was increased by 1000-fold during expansion of MNCs in initial 5 passages. However, single-cell PCR (Fig. 3A) carried out on these in vitro-expanded cell populations suggests that such increase in the level of *isl1* is mainly due to the

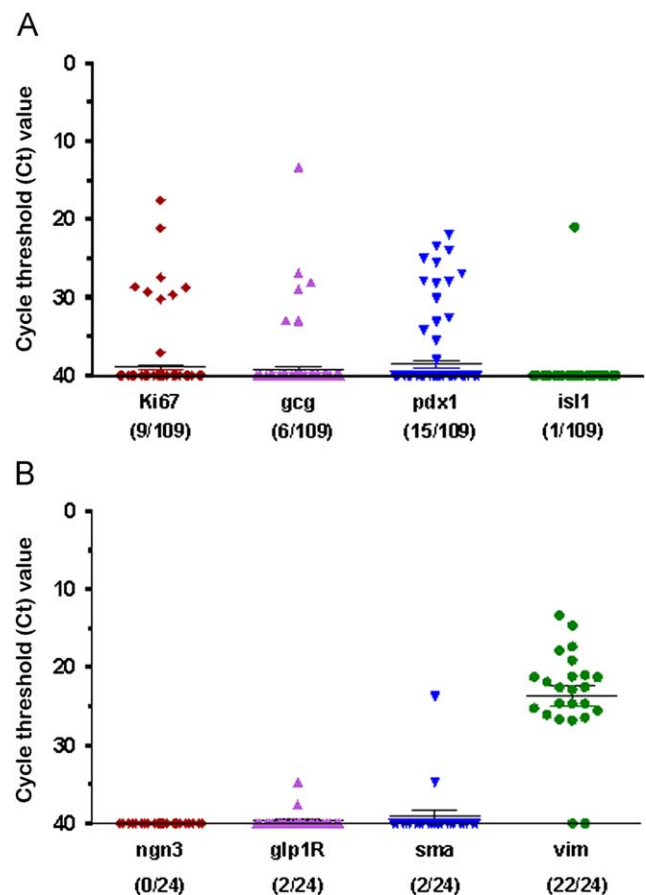


Fig. 3. Single-cell PCR of hUCB-derived MNCs. To understand whether the expression of pancreatic transcription factors was in all or few cells, we picked up at least 25 single cells from 4 different hUCB preparations. We find that pancreas specific/proliferation markers were expressed only in a relatively small subset of hUCB-derived MNCs (A). Since we can assess 12 different gene transcripts by qRT-PCR, we also assessed expression of *ngn3*, *glp1r*, *sma* and vimentin in cells that contained *pdx1*, *gcg*, *isl1* or *ki67* gene transcripts (represented in panel A). We found that these cells also contain mesenchymal transcripts such as vimentin but not the pro-endocrine marker *ngn3* (B). These analyses suggest that though pancreas-specific transcription factor (*pdx1*) is expressed in hUCB-derived MNCs, they do not show expression of the downstream endocrine marker *ngn3*. Numbers in parenthesis indicate the total number of cells assessed.

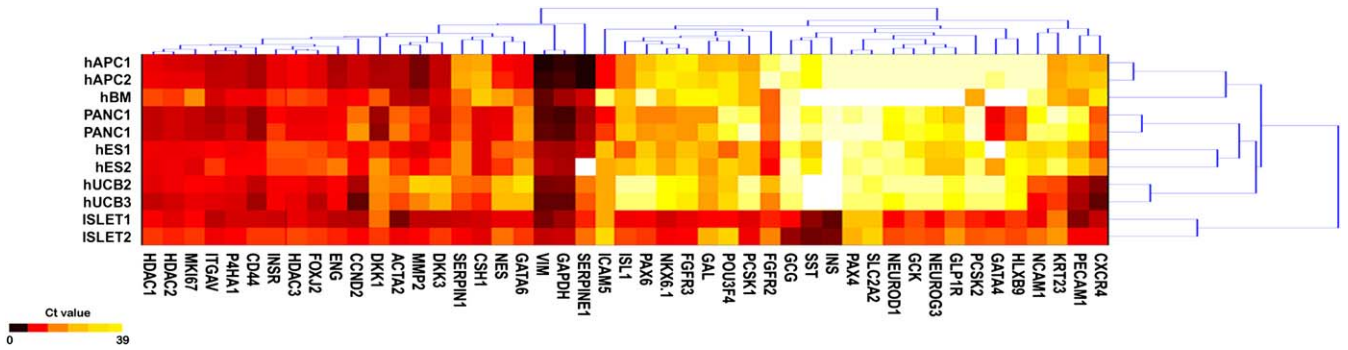


Fig. 4. Comparative gene expression profile of pancreatic progenitor cells. Since hUCB-derived MNCs express pancreas-specific transcription factors, we decided to compare the overall gene expression of hUCB-derived MNCs with other pancreatic progenitor cells as well as adult human islets. We isolated RNA and generated cDNA from human islet-derived adherent pancreatic cells (hAPCs), human bone marrow derived cells (hBM), human pancreatic duct cells (PANC1), human ES cells (hES), human umbilical cord blood-derived cells (hUCB) and adult pancreatic islets (islet). We looked at 2 different biological replicates or a pooled sample of 3 biological replicates for hBM due to the scarcity of available cells. TaqMan-based real-time PCR was carried using TLDA cards from Applied Biosystems. Data obtained were normalized to 18 s rRNA and imported into MatLab™. Bidirectional cluster analysis was carried out as discussed in methods. These data indicate that gene expression profile of human islets is much different from that of any other progenitor/stem cells and that these cell types cluster as a separate group. However, hUCB-derived MNCs cluster closest to islets suggesting that they represent a progenitor cell type that has more similarities to endocrine pancreatic cells than other progenitor/stem cells compared herein. The scale indicates a heatmap wherein black color represents high expression (Ct value close to 5) and white color represents low expression (Ct value = 39).

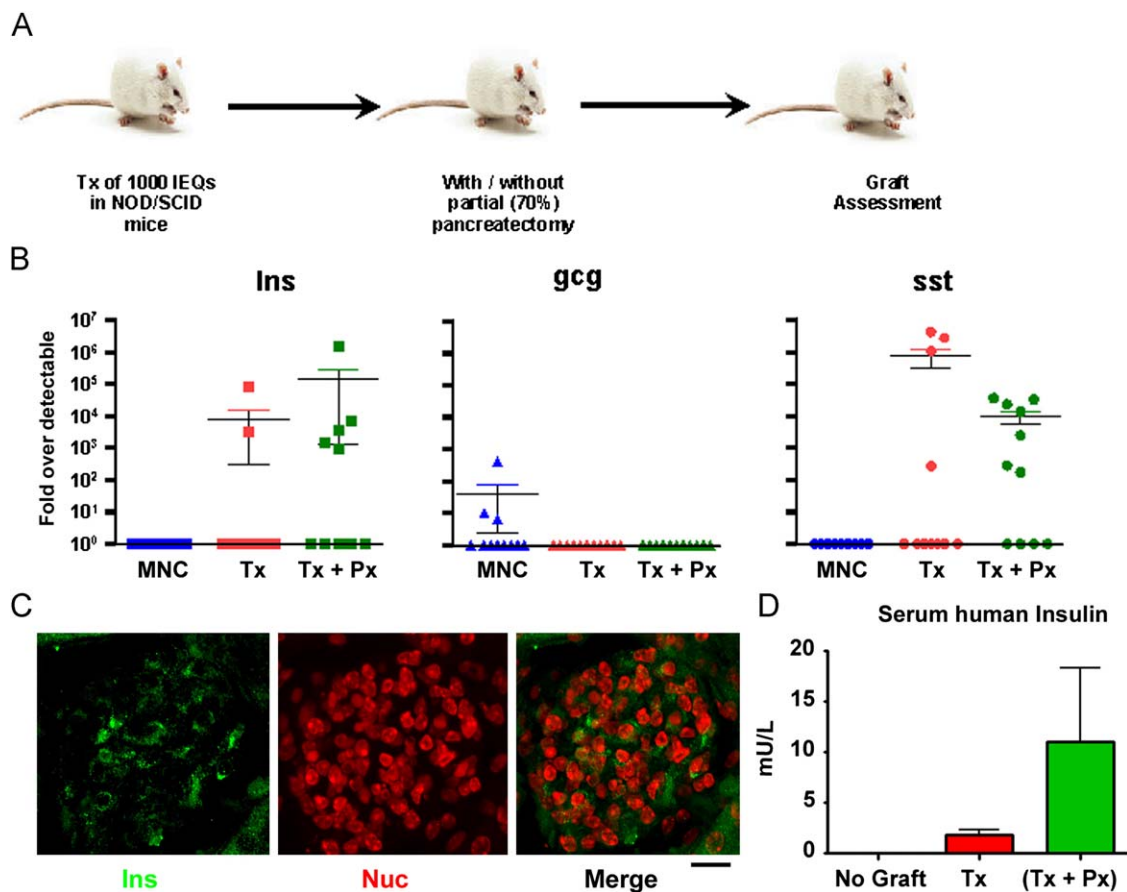


Fig. 5. hUCB-derived MNCs mature in vivo. A plan of the surgical procedure is outlined in (A). Briefly, hUCB-derived MNCs (19 different preparations) were transplanted under the kidney capsule of NOD/SCID mice ($n = 19$) on day 0. Of these, 11 mice underwent pancreatectomy as discussed in methods while the remaining 8 mice underwent a sham surgery wherein the pancreas was just handled but not removed. Data were normalized using primer-probe set specific for human (but not mouse) gapdh. The expression of islet (pro-)hormones seen in the grafts is plotted as fold above detectable. A Ct value of 39 is considered to be undetectable. We observe that human insulin and/or somatostatin was detected in almost all of the transplanted grafts at 9 weeks after transplantation in Tx+Px group (B). MNC grafts containing insulin (green) immunopositive cells are seen in the kidney sections of NOD/SCID mice (C). Sera collected from transplanted mice were assessed for insulin using a human insulin ELISA kit. We found that mice undergoing pancreatectomy have 3-fold more human insulin as compared to those that received UCB grafts but were not pancreatectomized. Bar = 25 μ m. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

high expression of these gene transcripts in a fewer cells rather than moderate increase in all/many cells. We find that pdx1 is expressed in ~14% of these cells while ~8% cells expressed ki67. Interestingly,

isl1 transcript is seen at high levels in a fraction of cells. Single cells expressing either ki67, pdx1, pro-glucagon or isl1 transcripts also contain vimentin transcript in majority of the cells (Fig. 3B).

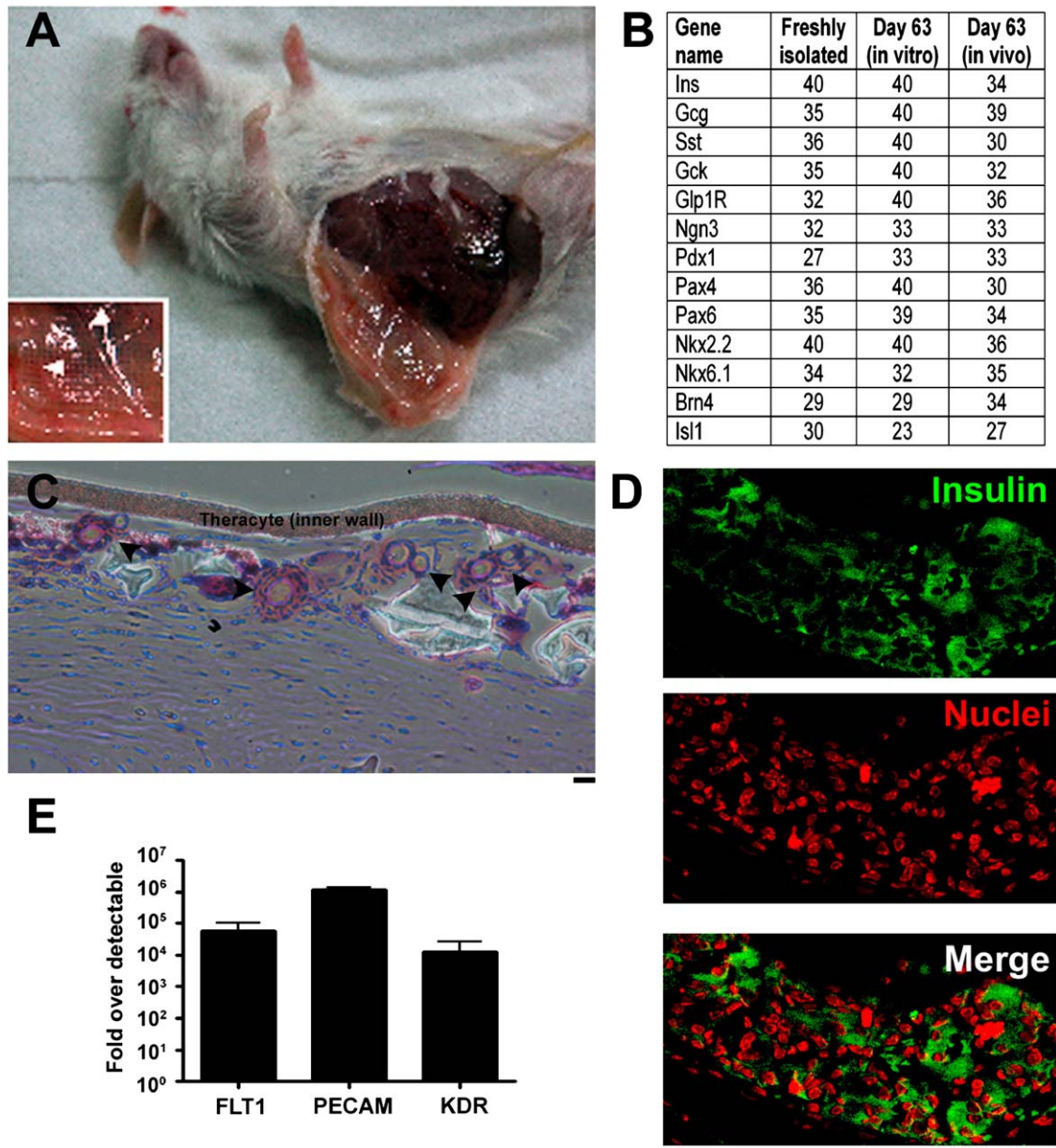


Fig. 6. hUCB-derived MNCs differentiate *in vivo* in immuno-competent mice. Freshly isolated hUCB-derived MNCs were subcutaneously transplanted using Theracyte™ devices in FVB/NJ mice. At 9 weeks after transplantation, Theracyte™ devices show good vascularization (panel A; arrowheads in inset) as well as hematoxylin and eosin staining (arrowheads in panel C). Table (B) represents the mean of Ct values ($n = 3$) obtained by qRT-PCR. A Ct value of 39 is considered to be undetectable. The respective values for these cells after 9 weeks of transplantation are given in the 3rd column. Insulin immunopositive cells (D) as well as endothelial gene transcripts (E) were detected in the retrieved grafts. Bar = 25 μ m

3.3. MNCs are potential pancreatic precursor cells

Since hUCB-derived MNCs show expression of pancreas-specific transcription factors, we decided to compare gene expression of several pancreas-specific genes in these MNCs with those expressed in other known islet-progenitor cells. From our initial microarray analysis of human islets and islet-derived progenitor cells, we have arrived at a list of 46 important genes that were seen to be highly regulated during differentiation to endocrine pancreatic lineage. We looked at these gene transcripts in hUCB-derived MNCs, other known human pancreatic stem/progenitor cells and human pancreatic islets using custom-printed TaqMan® low density arrays (TLDA). Normalized

data were taken into MatLab™ software and bi-directional clustering was carried out on the heatmap generated to understand hierarchical clustering of progenitor cell types as per the gene expression patterns assessed using TLDA (Fig. 4). We found that known and established pancreatic progenitor cells, human bone marrow-derived mesenchymal cells as well as hUCB-derived MNCs have similar gene expression patterns. Interestingly, though all of the pancreatic progenitor cells have distinctly different gene expression profile from adult human islets (which cluster separately as indicated by blue connecting lines), freshly isolated MNCs cluster close to adult human islets as compared to other pancreatic progenitor/stem cells (Fig. 4).

3.4. MNCs differentiate into islet-hormone-expressing cells following transplantation

Based on our assessment of fresh as well as in vitro-expanded MNCs, we believe that this population contains a distinct subset of islet-progenitor cells. We therefore assessed the potential of hUCB-derived MNCs to differentiate into insulin-producing cells. We transplanted 1000 islet equivalent (IEq) of freshly isolated MNC-clusters under the kidney capsule of immuno-incompetent (NOD/SCID) mice. Transplanted mice were randomly segregated into two groups: one group of mice underwent partial pancreatectomy after 2 weeks of transplantation while the other group underwent a sham surgery (without partial pancreatectomy) as outlined in Fig. 5A. MNC grafts were then retrieved from animals in both the groups at 9 weeks after transplantation and examined for islet (pro-)hormone transcripts using qRT-PCR. We find that 6 of the 8 animals transplanted (without pancreatectomy) showed presence of either insulin or somatostatin in their grafts at 9 weeks of Tx as compared to those seen in the transplanted day 0 MNCs. However, animals that were transplanted and had undergone pancreatectomy ($n = 11$) showed presence of human insulin or somatostatin in all the grafts. Freshly isolated MNCs did not express human pro-insulin and/or somatostatin transcripts. However, pro-glucagon was seen to be expressed on day 0 MNCs, but not in the 9-week transplanted grafts (Fig. 5B). Immunostaining of kidney sections bearing these grafts demonstrates that the transplanted MNCs are immunopositive to insulin (Fig. 5C). These grafts also contain vWF-producing cells (Supplementary Fig. 3B). We performed detailed transcript analysis of the transplanted hUCB-MNC grafts using quantitative real-time PCR for several pancreas-specific transcription factors (Supplementary Fig. 3A,D) as well as endothelial cell markers (Supplementary Fig. 3C). We observe that all the grafts are vascularized after transplantation. 'Tx-only' animals contain more of pancreatic progenitor markers such as pdx1 and ptf1a, while pancreatectomized animals that were transplanted with hUCB-derived MNCs show better differentiation to endocrine pancreatic lineage as evidenced by the presence of endocrine hormone transcripts in all of the grafts (Fig. 5B). Serum insulin measurements using ELISA show that insulin is present in both the groups; however, 3-fold more insulin concentrations were detected in circulation of animals that underwent pancreatectomy after transplantation (Fig. 5D). We then studied the differentiation potential of PDX1⁺ subpopulation of hUCB-MNCs. We found that this subset of PDX1⁺ cells obtained after sorting for PDX1 is enriched for several pancreas-specific transcription factors (Supplementary Fig. 4). It therefore appears that this population is committed to pancreatic lineage and has the potential to differentiate into insulin-producing cells in vivo.

We also assessed the potential of these hUCB-derived MNCs to differentiate/mature into islet-hormone-producing cells following transplantation into immuno-competent mice. Here, we used TheracyteTM devices, a commercially available macrocapsule made of biocompatible membrane that allows transfer of nutrients and gases but prevents immune components of the host to access graft. One thousand IEq cell clusters in alginate gels were placed inside each TheracyteTM device and transplanted subcutaneously into FVB/NJ mice. Animals underwent partial pancreatectomy as discussed in the methods section of this manuscript. Grafts were retrieved at 9 weeks after transplant and analyzed for the presence of islet-hormone transcripts. We found that TheracyteTM devices were well vascularized (Fig. 6A,C) and also express endothelial cell-specific flt1, pecam and kdr gene transcripts (Fig. 6E). Cells within these devices express pro-insulin transcripts (Fig. 6B) and are also immunopositive to insulin (Fig. 6D). These studies demonstrate that hUCB-derived MNCs

have the potential to differentiate into insulin-containing cells following transplantation into immuno-incompetent or immuno-competent mice.

4. Discussion

In this study, we have explored the expansion potential of human umbilical cord blood (hUCB)-derived mononuclear cells (MNCs) and their potential to differentiate into insulin-expressing cells. The presence of pancreas-specific transcription factors in freshly isolated hUCB-derived MNCs is indeed intriguing. Our assessment of multiple pancreatic progenitor cells using gene panels on a TLDA platform help us to understand the similarity that these umbilical cord blood-derived cells share with other tissue-specific progenitor cells (Gershengorn et al., 2004), or human pancreatic ductal cells (Hardikar et al., 2003), embryonic stem cells and human bone marrow-derived stem cells (Ianus et al., 2003; Tang et al., 2004) described earlier.

Several reports suggest that hUCB-derived MNCs can differentiate into insulin-producing cells after injecting MNCs either in the peritoneal cavity (Zhao et al., 2006) of streptozotocin-diabetic mice or through the retro-orbital plexus (Kogler et al., 2004) of NOD mice. The end point for these studies was determined by either improved survival after transplantation or improved glycemic control. In each of these cases cell fusion-dependent and -independent mechanisms were proposed to be possible mechanisms by which these cells acquire differentiation into insulin-producing cells. In our study, we transplanted hUCB-derived MNCs either under the kidney capsule of NOD/SCID mice or contained within TheracyteTM capsules in immuno-competent mice. These transplanted mice were not made diabetic using any beta-cell toxins (such as STZ), but rendered transiently diabetic using a surgical procedure that we have discussed earlier (Hardikar, 2004). Our studies indicate that similar to human fetal pancreatic islets demonstrated earlier (Tuch et al., 1989), human UCB-derived MNCs also have the potential to differentiate in vivo. Amongst the transplanted groups, we found more number of grafts expressing human pro-insulin and/or somatostatin and higher serum insulin in animals that underwent pancreatectomy, 2 weeks after the transplant (Fig. 5D). These data indicate that factors secreted by the regenerating pancreas may be involved in differentiation of pancreatic progenitor cells. Indeed, the potential of regenerating pancreas extracts/secretions to induce differentiation and regeneration of the endocrine pancreas is not new. Studies carried out earlier have been suggestive of the potential of such factors in regenerating pancreas (Hardikar and Bhonde, 1999; Lipsett et al., 2007; Rosenberg, 1998). What appears more interesting is the expression of islet-specific transcription factors in umbilical cord blood-derived mononuclear cells. It seems that such progenitors may be mobilized during embryonic development and be therefore detected even at the time of birth in the fetal portion of the umbilical cord blood. It therefore seems important to store such cells that demonstrate characteristics of pancreas-specific islet progenitors. Though we have not been successful in achieving better expansion of these cells under xenoprotein-free environment, our studies clearly indicate the presence of pancreas-specific transcription factors in hUCB-derived mononuclear cells and their differentiation/maturation following transplantation in mice. However, our studies indicate that hUCB-derived MNCs, under the influence of paracrine factors secreted by the regenerating pancreas, differentiate better into insulin-producing cells in vivo. A comparative analysis of 46 different gene transcripts in fresh hUCB-MNCs, transplanted (Tx) and transplanted+pancreatectomized (Tx+Px) grafts suggests that transplanted cells show distinctly different expression patterns

(Supplementary Fig. 3A). Though we do see the presence of pro-glucagon transcripts in day 0 MNCs, we could not detect glucagon protein in these cells. The pro-glucagon gene transcript detected in hUCB-derived MNCs therefore may not be translated into glucagon hormone as has been known in certain other cell types (Drucker et al., 1994). Though islet (pro-)hormone gene transcripts were seen at significant abundance, circulating human insulin concentrations at 30 min after glucose stimulation were lower (Fig. 5D). These data suggest that though hUCB-derived MNCs differentiate to insulin-producing cells in vivo, these cells may still be immature in terms of glucose sensing and/or secretory machinery required for glucose-stimulated insulin release. Further studies presently being carried in our laboratory are focused on understanding maturation and secretion of insulin by hUCB-MNCs using incretin (Hardikar et al., 2002) and/or micro-RNAs (Joglekar et al., 2008, 2007a) to obtain mature insulin-secreting cells. Though recent studies have demonstrated that umbilical cord blood-derived mononuclear cells can be safely used for autologous transplantation in type 1 diabetes subjects (Haller et al., 2008), these studies are largely limited by inefficient differentiation of these MNCs to hormone-producing cells. In summary, our studies demonstrate that islet-specific gene transcript as well as protein-containing cells exist in human umbilical cord blood and have the potential to differentiate into insulin-producing cells. Further studies directed to identification of paracrine/autocrine factors involved in differentiation of hUCB-derived MNCs to endocrine pancreatic lineage will help in assessing these autologous islet progenitors for cell replacement therapy in diabetes.

Conflict of interest

None.

Acknowledgements

The authors thank Dr. Ramanmurthy and Dr. Bankar, Animal facility, National Center for Cell Science, for assistance with post-operative animal care, and Dr. Ramesh Bhonde, Dr. (Mrs) Medha Patankar, Dr. Vaishali Biniwale and Dr. Parag Biniwale for support. This work was supported by the Intramural Research Program of National Center for Cell Science and a microRNA consortium project grant (BT/PR7975/MED/14/1211/2006) to AAH from the Department of Biotechnology (DBT), Government of India. V.S.P. is supported through this fellowship from the DBT, while M.V.J. is supported by a fellowship from Council of Scientific and Industrial Research (CSIR), Government of India.

Author contributions: VSP carried all isolations and majority of experimentation presented in this manuscript, M.V.J. carried out analysis of transplanted grafts and helped in writing and preparing this manuscript. All authors read, discussed and agreed on the submitted version of this manuscript.

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiomech.2008.10.038.

References

Bonner-Weir, S., Weir, G.C., 2005. New sources of pancreatic beta-cells. *Nat. Biotechnol.* 23 (7), 857.

- Bonner-Weir, S., Toschi, E., Inada, A., et al., 2004. The pancreatic ductal epithelium serves as a potential pool of progenitor cells. *Pediatr. Diabetes* 5 (Suppl. 2), 16.
- Drucker, D.J., Jin, T., Asa, S.L., Young, T.A., Brubaker, P.L., 1994. Activation of proglucagon gene transcription by protein kinase-A in a novel mouse enteroendocrine cell line. *Mol. Endocrinol.* 8 (12), 1646.
- Ende, N., Chen, R., Reddi, A.S., 2004. Transplantation of human umbilical cord blood cells improves glycemia and glomerular hypertrophy in type 2 diabetic mice. *Biochem. Biophys. Res. Commun.* 321 (1), 168.
- Erices, A., Conget, P., Minguell, J.J., 2000. Mesenchymal progenitor cells in human umbilical cord blood. *Br. J. Haematol.* 109 (1), 235.
- Gershengorn, M.C., Hardikar, A.A., Wei, C., Geras-Raaka, E., Marcus-Samuels, B., Raaka, B.M., 2004. Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. *Science* 306 (5705), 2261.
- Habener, J.F., Kemp, D.M., Thomas, M.K., 2005. Minireview: transcriptional regulation in pancreatic development. *Endocrinology* 146 (3), 1025.
- Haller, M.J., Viener, H.L., Wasserfall, C., Brusko, T., Atkinson, M.A., Schatz, D.A., 2008. Autologous umbilical cord blood infusion for type 1 diabetes. *Exp. Hematol.* 1.
- Hardikar, A.A., 2004. Generating new pancreas from old. *Trends Endocrinol. Metab.* 15 (5), 198.
- Hardikar, A.A., Bhonde, R.R., 1999. Modulating experimental diabetes by treatment with cytosolic extract from the regenerating pancreas. *Diabetes Res. Clin. Pract.* 46 (3), 203.
- Hardikar, A.A., Karandikar, M.S., Bhonde, R.R., 1999. Effect of partial pancreatectomy on diabetic status in BALB/c mice. *J. Endocrinol.* 162 (2), 189.
- Hardikar, A.A., Wang, X.Y., Williams, L.J., et al., 2002. Functional maturation of fetal porcine beta-cells by glucagon-like peptide 1 and cholecystokinin. *Endocrinology* 143 (9), 3505.
- Hardikar, A.A., Marcus-Samuels, B., Geras-Raaka, E., Raaka, B.M., Gershengorn, M.C., 2003. Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. *Proc. Natl. Acad. Sci. USA* 100 (12), 7117.
- Hirshberg, B., Rother, K.I., Digon III., B.J., et al., 2003. Benefits and risks of solitary islet transplantation for type 1 diabetes using steroid-sparing immunosuppression: the National Institutes of Health experience. *Diabetes Care* 26 (12), 3288.
- Ianus, A., Holz, G.G., Theise, N.D., Hussain, M.A., 2003. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J. Clin. Invest.* 111 (6), 843.
- Joglekar, M.V., Parekh, V.S., Mehta, S., Bhonde, R.R., Hardikar, A.A., 2007b. MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3. *Dev. Biol.* 311 (2), 603.
- Joglekar, M.V., Parekh, V.S., Hardikar, A.A., 2007a. New pancreas from old: microregulators of pancreas regeneration. *Trends Endocrinol. Metab.* 18 (10), 393.
- Joglekar, M.V., Joglekar, V.M., Hardikar, A.A., 2008. Expression of islet-specific microRNAs during human pancreatic development. *Gene Expr. Patterns.*
- Kogler, G., Sensken, S., Airey, J.A., et al., 2004. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J. Exp. Med.* 200 (2), 123.
- Lipsett, M., Hanley, S., Castellarin, M., et al., 2007. The role of islet neogenesis-associated protein (INGAP) in islet neogenesis. *Cell. Biochem. Biophys.* 48 (2–3), 127.
- Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R., McKay, R., 2001. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292 (5520), 1389.
- Ojo, A.O., Held, P.J., Port, F.K., et al., 2003. Chronic renal failure after transplantation of a nonrenal organ. *N. Engl. J. Med.* 349 (10), 931.
- Pessina, A., Eletti, B., Croera, C., Savalli, N., Diodovich, C., Gribaldo, L., 2004. Pancreas developing markers expressed on human mononucleated umbilical cord blood cells. *Biochem. Biophys. Res. Commun.* 323 (1), 315.
- Rosenberg, L., 1998. Induction of islet cell neogenesis in the adult pancreas: the partial duct obstruction model. *Microsc. Res. Tech.* 43 (4), 337.
- Shapiro, A.M., Lakey, J.R., Ryan, E.A., et al., 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N. Engl. J. Med.* 343 (4), 230.
- Tang, D.Q., Cao, L.Z., Burkhardt, B.R., et al., 2004. In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow. *Diabetes* 53 (7), 1721.
- Tuch, B.E., Osgerby, K.J., Turtle, J.R., 1989. Reversal of diabetes in hyperglycaemic nude mice by human fetal pancreas. *Transplant Proc.* 21 (1 Pt 3), 2665.
- Watada, H., Mirmira, R.G., Leung, J., German, M.S., 2000. Transcriptional and translational regulation of beta-cell differentiation factor Nkx6.1. *J. Biol. Chem.* 275 (44), 34224.
- Xu, G., Stoffers, D.A., Habener, J.F., Bonner-Weir, S., 1999. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* 48 (12), 2270.
- Yoshida, S., Ishikawa, F., Kawano, N., et al., 2005. Human cord blood-derived cells generate insulin-producing cells in vivo. *Stem Cells* 23 (9), 1409.
- Zhao, Y., Wang, H., Mazzone, T., 2006. Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics. *Exp. Cell Res.* 312 (13), 2454.