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A unique human blood-derived cell population displays high potential for producing insulin

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Abstract

Blood can provide a valuable source for the generation of stem cells. Herein we identified a novel cell population from adult human blood, designated peripheral blood insulin-producing cells (PB-IPC). Phenotypic analysis demonstrated that PB-IPC displayed the embryonic stem (ES) cell-associated transcription factors including Oct-4 and Nanog, along with the hematopoietic markers CD9, CD45, and CD117; but lacked expression of the hematopoietic stem cell marker CD34 as well as lymphocyte and monocyte/macrophage markers. Notably, *in vitro* and *in vivo* characterization revealed that PB-IPC demonstrated characteristics of islet β cell progenitors including the expression of β cell-specific insulin gene transcription factors and prohormone convertases, production of insulin, formation of insulin granules, and the ability to reduce hyperglycemia and migrate into pancreatic islets after transplantation into the diabetic mice. These findings may open up new avenues for autologous blood stem cell-based therapies for diabetes.

Keywords: Diabetes; Insulin-producing cell; Blood; Stem cell

Stem cell-derived insulin-producing cells may provide a rational tool for treatment of both type 1 (T1D) and type 2 diabetes (T2D). Previous studies suggest that either embryonic as well as adult stem cells can serve as potential sources of β cell surrogates for therapeutic applications [1–3]. However, the immune system will recognize and attack any foreign cells utilized for the restoration of euglycemia due to the immune surveillance, even the application of allogeneic embryonic stem (ES) cells [4]. To circumvent these barriers, many novel approaches are being investigated including those directed at manipulating the host immune responses, altered nuclear transfer, and other aspects directed at modulating disease pathogenesis [5]. However, despite these efforts, no suitable solution has been achieved.

Application of autologous stem cells-derived insulin-producing cells is a potentially attractive strategy and may serve as a means to overcome many of the major issues that can complicate cell-based therapies, such as immune rejection and shortage of suitable donors. To this end, human bone marrow, peripheral blood and umbilical cord blood represent valuable sources for provision of various types of autologous stem cells [6–10]. Although stem cells derived from bone marrow usually possess higher potential of proliferation than those obtained from peripheral blood, it remains problematic to obtain bone marrow cells in comparison to that of peripheral blood. Herein, based on our previous cord blood works [8], we identified a unique cell population obtained from adult human peripheral blood, designated peripheral blood insulin-producing cells (PB-IPC). PB-IPC displayed characteristics of both embryonic and hematopoietic stem cells, along with high potential for producing insulin. The application

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of autologous PB-IPC may provide a promising approach for the treatment of diabetes.

Materials and methods

Cell culture. Human buffy coats (50–60 ml/U) were obtained from 12 healthy donors (7 male and 5 female), aged 20–62 years (averaged 45 ± 13) (Life-Source Blood Services, Glenview, IL). Human buffy coats were initially added 30–40 ml RPMI 1640 medium and mixed thoroughly with 10 ml pipette, and then used for isolation. Mononuclear cells were isolated from buffy coats using Ficoll-Hypaque ($\gamma = 1.077$, Sigma), followed by removing red blood cells using Red Blood Cell Lysis buffer (eBioscience, San Diego, CA). After three washes with PBS, the whole peripheral blood mononuclear cells (PBMC) were seeded in the 150 × 15 mm Style Petri dishes [8] (Becton Dickinson Labware, Franklin Lakes, NJ) at 1×10^6 cells/ml, 25 ml/dish in RPMI 1640 medium supplemented with 7% FBS (Invitrogen, Carlsbad, CA) without adding any other growth factors, and incubated at 37 °C, 8% CO_2 conditions [8]. It is important to note that tissue culture dishes treated with Vacuum Gas Plasma did not support the growth of PB-IPC.

Quantitative real-time PCR. Expression of different mRNAs was analyzed by quantitative real-time PCR. Total RNA was extracted using a Qiagen kit (Valencia, CA). First-strand cDNA was synthesized from 1 mg total RNA using random hexamer primers according to the manufacturer's instructions (Fermentas, Hanover, MD). Real-time PCR was performed on each sample in triplicate using the Mx3000p Quantitative PCR System (Stratagene, La Jolla, CA), under the following conditions: 95 °C for 15 min, 40 cycles of (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s), using the validated gene-specific RT^2 PCR Primer sets for each gene (SuperArray, Frederick, MD). Relative expression level of each transcription factor was, respectively, corrected for that of the housekeeping gene β-actin as an internal control.

In situ hybridization. To examine expression of insulin mRNA, we performed in situ hybridization as previously described [11]. In brief, cells were incubated with the probes in hybridization buffer (DakoCytomation, Carpinteria, CA) at dilution 1:100 (1 μg/ml), 37 °C for 16–18 h. The signals were detected using the Tyramide Signal Amplification kit (DakoCytomation).

To confirm human PB-IPC migrated into mouse pancreatic islets, we initially performed immunostaining using human C-peptide antibody (1:2000, Linco Research, St. Charles, MO) and then performed fluorescence in situ hybridization (FISH) using the human CEP® X/Y DNA Probe Kit (Abbott Molecular Inc., Des Plaines, IL), following the manufacturer's protocols.

Transplantation of PB-IPC into diabetic NOD-scid mice. Diabetes in NOD-scid male mice, aged 8–10 weeks, was induced with a single dose of streptozotocin (STZ) (Sigma, 180 mg/kg of body weight, i.p.), freshly dissolved in citrate buffer (pH 4.5) [8]. PB-IPC were transplanted at 5 million cells/mouse (i.p.). PBMC or physiological saline with equal volume served as controls. Blood glucose levels were monitored using an AccuChek glucose detector (Roche Diagnostics, Indianapolis, IN). To measure human C-peptide, blood samples were collected from the tail vein. Blood human C-peptide level was detected by using an ultrasensitive human C-peptide enzyme-linked immunosorbent assay (ELISA) kit (Alpco Diagnostics, Windham, NH). This assay does not detect mouse C-peptide [8]. All above procedures were performed according to a protocol approved by the Animal Care Committee (ACC).

Statistics. Statistical analyses of data were performed by the two-tailed Student's *t* test to determine statistical significance. Values are given as means ± SD (standard deviation).

Results and discussion

Identification of the insulin-producing cells from adult human peripheral blood

Our recent works has characterized a novel type of stem cells from human umbilical cord blood [8], displaying embryonic stem (ES) cell markers [8,9,13], and differentiation into insulin-producing cells [8,9]. Based on these studies, we hypothesized that adult human peripheral blood may contain similar cells capable of producing insulin. To test this hypothesis, we initially took peripheral blood mononuclear cells (PBMC) from healthy donors and examined the insulin gene transcription factors [14] such as the basic leucine zipper MafA, Pdx-1 (pancreatic duodenal homeobox factor 1), Nkx6.1 (Nk homeobox gene), NeuroD1 (neurogenic differentiation 1), HNF6 (hepatocyte nuclear factor 6, also known as Onecut1), and Nkx2.2, the five kinds of pancreatic islet-produced endocrine hormones and the insulin release-related K(ATP) channel proteins (Table 1). Quantitative real-time PCR revealed that there are cells among PBMC, expressing insulin mRNA and its transcription factors including MafA and Nkx6.1; results also showed expressions of somatostatin (a pancreatic islet δ cell product) and ghrelin (a pancreatic islet ε cell product); however, other hormones (such as the α cell product glucagon and the PP cell product pancreatic polypeptide) and transcription factors (Pdx-1, NeuroD1, and Nkx2.2) were undetectable in comparison with positive control human islets (Table 1).

Isolation of the insulin-producing cells from human peripheral blood by attachment and further in vitro characterization

Above results demonstrate that there are insulin-producing cells in peripheral blood, designated as peripheral blood insulin-producing cells (PB-IPC). Next, we try to isolate them from peripheral blood by adhering PBMC to the plastic Petri dishes (not vacuum gas-treated tissue culture dishes) as previously reported [8]. Similar to the culture

Table 1
Expression of pancreatic gene markers in the PBMC

Specific genes	PBMC Level ($\Delta C_T \pm$ SD)	Human islets Level ($\Delta C_T \pm$ SD)
Transcription factors		
MafA	+ (14.70 ± 0.38)	+++ (4.56 ± 0.35)
Nkx6.1	+ (19.79 ± 1.02)	+++ (4.27 ± 0.32)
Pdx-1	—	+++ (5.26 ± 0.26)
Onecut1	+ (20.54 ± 1.88)	+++ (8.78 ± 0.12)
NeuroD1	—	+++ (6.11 ± 0.22)
Nkx2.2	—	+++ (4.59 ± 0.16)
Pancreatic hormones		
Insulin	+ (20.14 ± 1.76)	+++++ (−4.67 ± 0.27)
Glucagon	—	++++ (−0.89 ± 0.20)
Pancreatic polypeptide	—	+++ (3.00 ± 0.34)
Somatostatin	+ (20.58 ± 0.60)	++++ (−2.89 ± 0.16)
Ghrelin	+ (12.68 ± 0.16)	+++ (4.87 ± 0.28)
K(ATP) channel proteins		
Sur-1	+ (23.22 ± 0.80)	+++ (5.10 ± 0.41)
Kir6.2	+ (16.59 ± 0.28)	+++ (6.33 ± 0.34)

Notes: Data represent their expression level (ΔC_T mean ± SD of three experiments) from non-detectable (—) to the high (+++++) after amplification for 40 cycles.

of human umbilical cord blood-derived stem cells [8], we observed that attached cells could proliferate and significantly increased in cell number after planting the PBMC in Petri dishes (Fig. 1A). To evaluate their potential of insulin production, several approaches were undertaken including examination for the insulin gene transcription factors, the insulin synthesis-related converting enzymes, insulin expression at protein and mRNA levels, and insulin granules by ultrastructure analysis.

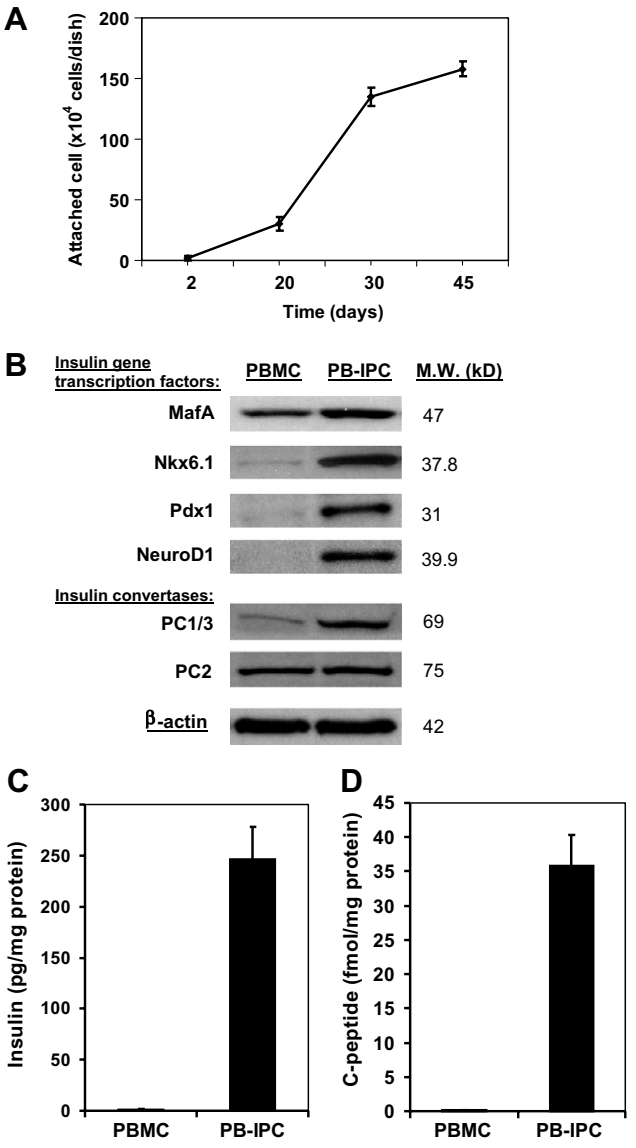


Fig. 1. *In vitro* characterization of PB-IPC for insulin production. (A) Quantitation of attached cells per dish over 45 days. Freshly isolated PBMC were planted in the 150×15 mm style Petri dishes at 1×10^6 cells/ml, 25 ml/dish in RPMI 1640 medium supplemented with 7% FBS. (B) Western blot for insulin gene transcription factors including MafA, Nkx6.1, Pdx-1, NeuroD1, along with prohormone convertases PC1/3 and PC2. PBMC served as negative control; β-actin served as internal control. (C) Quantification of insulin content per total cell protein by ultrasensitive human insulin ELISA kit (Alpco Diagnostics). (D) Quantification of C-peptide content per total cell protein by ultrasensitive human C-peptide ELISA kit (Alpco Diagnostics).

First, we examined the insulin gene transcription factors [14] by Western blot. We found that the attached cell population strongly expressed these transcription factors and had significantly increased their levels in comparison with PBMC (Fig. 1B); specifically for the β cell specific transcription factor MafA expression, immunostaining showed that $93 \pm 2\%$ of attached cell population clearly displayed nuclear staining for MafA (Fig. S1). We observed that PBMC also expressed MafA and Nkx6.1 at weak levels, but failed to show other transcription factors (Fig. 1B), and consistent to the real-time PCR results (Table 1). Second, Western blot showed that both attached cell population and PBMC expressed the prohormone convertases: PC1/3 and PC2 (Fig. 1B), which are usually present in islet β cells and other cellular tissues involved in insulin and/or other neuropeptide synthesis [15]. These data imply that these attached cell population display a high potential to produce insulin and PB-IPC may be isolated from adult peripheral blood by adhering to Petri dishes.

To detect their insulin production, levels of total insulin and its by-product C-peptide in PB-IPC were evaluated by the enzyme-linked immunosorbent assay (ELISA); results showed insulin level was around 246.07 ± 32 pg/mg cell protein (Fig. 1C), and 35.76 ± 4.5 fmol/mg cell protein for C-peptide level (Fig. 1D); while PBMC showed background levels for insulin protein and C-peptide. To exclude the possibility that insulin was uptaken from culture medium [16], insulin production in PB-IPC was further confirmed by in situ hybridization with a human insulin oligonucleotide probe for insulin mRNA (Fig. S2). Moreover, conventional transmission electron microscopy showed that there were numbers of granules in the cytoplasm of PB-IPC, with size ranged from 200 to 300 nm in diameter; some of granules exhibited a "halo" structure, which is the unique characteristic of insulin granules of human islet β cells (Fig. S3, top panels); the immunogold-labeling transmission electron microscopy [12] further confirmed they were insulin granules (Fig. S3, bottom panels).

In addition to insulin production, we also found PB-IPC expressed other β cell-related markers including glucose transporter 2 (Glut-2), insulin-releasing-associated K^+_{ATP} channel protein suphonylurea receptor-1 (Sur-1), calcium channel subunit α 1C, and glucokinase regulator protein (GCKR); whereas the α cell product glucagon was undetectable (Fig. S4). Taken together, these data indicate that PB-IPC possess human islet β cell characteristics and can produce insulin.

PB-IPC display embryonic markers and a unique hematopoietic cell phenotype

To distinguish PB-IPC from other blood cells, we performed flow analysis by using monoclonal antibodies to the specific markers that characterize the different cell lineages [7,8]. Results showed that PB-IPC highly expressed hematopoietic cell phenotype including tetraspanin CD9, leukocyte common antigen CD45, and stem cell factor receptor CD117 (Fig. 2A); however PB-IPC

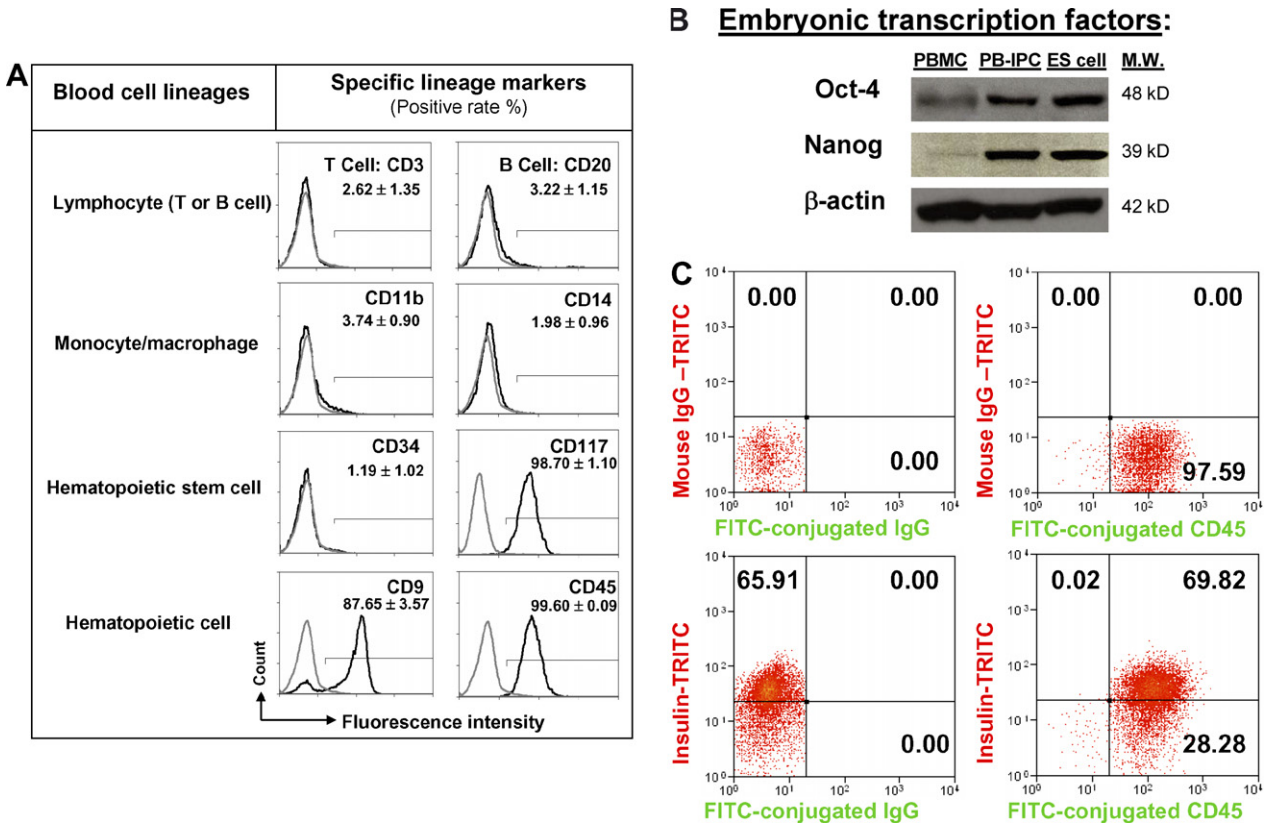


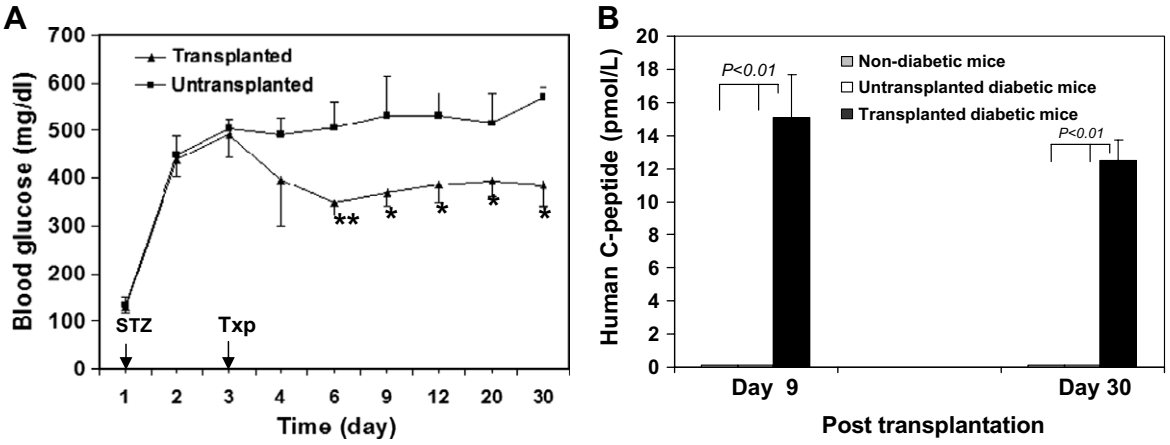
Fig. 2. Phenotypic characterization of PB-IPC. (A) Flow analysis of different blood cell lineage markers on PB-IPC. Isotype-matched IgG served as negative control (grey histogram) for different specific antibodies (black histogram). Data represent results from four experiments with the similar results. (B) Western blot for embryonic transcription factors Oct-4 and Nanog. Human embryonic stem (ES) cell lysate served as positive control; freshly isolated peripheral blood mononuclear cells (PBMC) served as additional control; β-actin served as internal control. (C) Flow analysis on PB-IPC for insulin and leukocyte common antigen CD45. Isotype-matched IgG served as negative control for insulin (red) and CD45 (green). Data represent results from six experiments with the similar results.

were considered to be negative for hematopoietic stem cell marker CD34, and lymphocyte markers CD3 (T cells) and CD20 (B cells) (Fig. 2A); specifically, the uniform expression of CD45 at high level confirms that PB-IPC belong to hematopoietic ($CD45^+$) cells, not mesenchymal ($CD45^-$) cells circulating in peripheral blood.

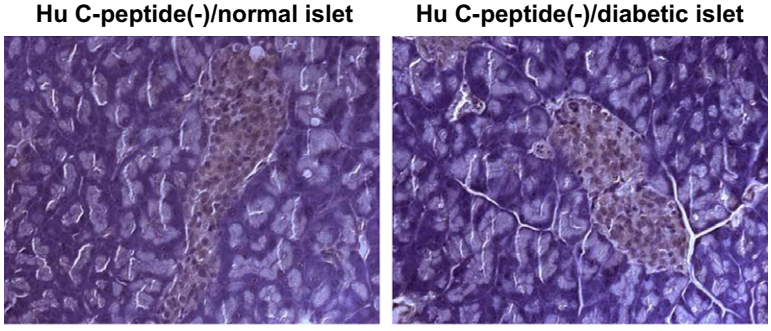
Attached cells in blood are usually regarded as "macrophages". However, PB-IPC failed to express human monocyte/macrophage specific antigens [7] CD14 and CD11b/Mac-1 (Fig. 2A). To further discriminate PB-IPC from monocytes/macrophages, we examined PB-IPC for other monocyte/macrophage-related phenotypes [7] such as human leukocyte antigens (HLA): HLA-DR, HLA-DQ, and HLA-ABC, together with costimulating molecules: CD40, CD80, and CD86 (Fig. S5). Compared with freshly isolated blood monocytes, PB-IPC were negative for HLA-DR, CD40, and CD80 (Fig. S5); less than 10% of cells were positive for CD86 and HLA-DQ; but strongly expressed HLA-ABC (usually expressed on all nucleated adult cells) (Fig. S5). Thus, these results further confirm that PB-IPC are different from monocytes/macrophages. The potential reason for absence of monocytes/macrophages in PB-IPC cultures may be associated with the culture vessel—Petri dishes we used, which are different from the regularly used tissue culture dishes (treated with vacuum gas plasma by manufacturers). The surface of Petri dishes is hydrophobic with a positive charge; however the regularly used tissue culture dishes become hydrophilic with a negative charge, which

Fig. 3. Transplantation of PB-IPC into the streptozotocin (STZ)-induced diabetic NOD-scid mice. (A) Blood glucose examination post transplantation (Txp), 5 million cells/mouse, i.p., $n = 5$/group. PBMC served as control for PB-IPC. (B) Examination of human C-peptide level by ultrasensitive human C-peptide ELISA kit in mouse sera. (C) Immunostaining of human C-peptide in pancreatic tissues of non-diabetic mice (left) and PB-IPC-untransplanted diabetic mice (right). (D) Immunostaining of human C-peptide in pancreatic tissues of PB-IPC-transplanted diabetic mice. In PB-IPC-transplanted mice, the same immunostained pancreatic section showed a human C-peptide-negative islet (left panel), a single human C-peptide-positive cell located among exocrine tissues (middle panel), and human C-peptide-positive cells located inside of mouse pancreatic islets (right panel). Scale bar, 56 μm. (E) Fluorescence in situ hybridization (FISH) for human chromosome X/Y after immunostaining for human C-peptide. A human C-peptide-positive cell (brown, in green circle) is also positive for human chromosome x/x (red, pointed by green dashed arrow, female karyotype). Asterisk (*) represents for $p < 0.05$ and (**) represent for $p < 0.01$. Data represent mean (±SD).

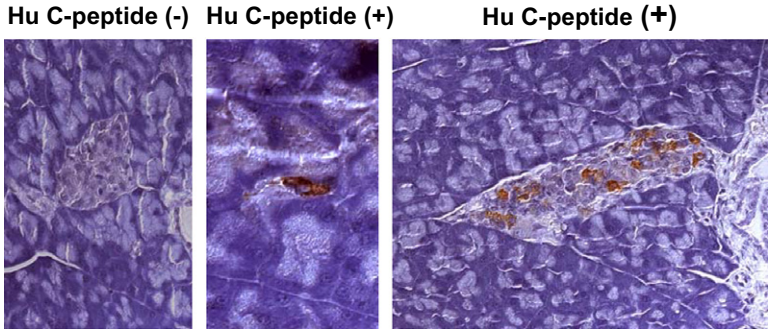
monocytes/macrophages are used to adhering. To test this possibility, we planted the same sets of PBMC in the regular tissue culture dishes instead of Petri dishes; we realized that the majority of attached cells in tissue culture dishes were



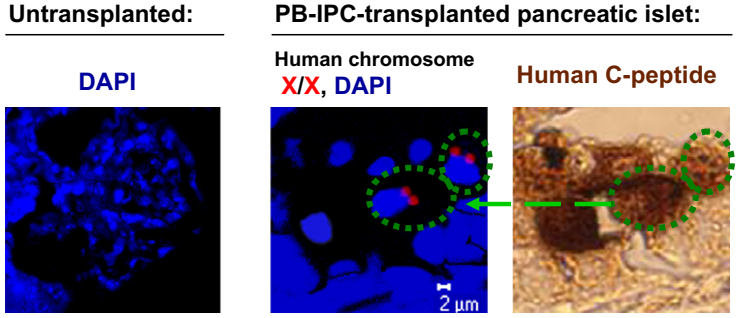
C Untransplanted normal and diabetic mice:



D PB-IPC-transplanted diabetic mice:



E In situ hybridization with human x/y probes:



monocytes/macrophages; however PB-IPC could not be generated and expanded in these tissue culture dishes (data not shown). Thus, different type of surface of culture vessels can make selections for PB-IPC and monocytes/macrophages.

The above hematopoietic phenotypes of PB-IPC are consistent with umbilical cord blood-derived stem cells (CB-SC), displaying ES cell markers [8]. To fully characterize PB-IPC, we evaluated the expression of embryonic stem (ES) cell-related transcription factors Oct-4 and Nanog (associated with the self renewal of embryonic stem cells [17]), in comparison with human embryonic stem cells (Fig. 2B). Notably, Western blot showed the strong expression of Oct-4 and Nanog in PB-IPC (Fig. 2B).

Next, using the leukocyte common antigen CD45 as an indicator for PB-IPC, we performed flow analysis and found that $70 \pm 6\%$ of PB-IPC were double-positive for insulin and CD45 (Fig. 2C). This percentage of insulin-positive cells is higher than that observed after the differentiation of human embryonic stem (ES) cells (with average 7.3%) [3].

PB-IPC reduced hyperglycemia and migrated into pancreatic islet after transplantation into the diabetic mouse model

To better characterize this cell population, we next examined whether PB-IPC could secret insulin in response to high glucose. Therefore, we transplanted these cells into the streptozotocin (STZ)-induced diabetic NOD-scid mice (5 million cells/mouse, i.p., $n = 5$). Compared with control mice, PB-IPC transplantation could reduce blood glucose level approximately 20–30% (Fig. 3A). To demonstrate these glucose responses were associated with transplanted PB-IPC, we utilized an assay that is specific for human C-peptide as an indicator (*no cross-reaction with mouse C-peptide*) to evaluate human insulin secretion [8,18]. Data showed that human C-peptide level was significantly increased after transplantation (Fig. 3B) ($p < 0.01$); however, human C-peptide was undetectable in mouse sera of the control diabetic mice and non-diabetic normal mice (Fig. 3B). Based on these serum human C-peptide levels following transplantation of PB-IPC into diabetic mice, we estimate that 5 million PB-IPC equals to approximately 5% of serum human C-peptide levels observed after transplantation with 2000 human islets [18].

One month later following transplantation, immunohistochemistry showed human C-peptide-positive cells were present in pancreatic tissue of PB-IPC-transplanted mice, such as in exocrine tissue (Fig. 3D, middle panel) and in residual mouse pancreatic islets (Fig. 3D, right panel). Thirty out of 46 pancreatic islets from 5 PB-IPC-transplanted diabetic mice showed human C-peptide-positive cells; the number of positive cells ranged from 2 to 42 positive cells/islet. However, human C-peptide-positive cells were not observed in pancreatic islets ($n = 35$) of the normal mice or PB-IPC-untransplanted diabetic mice (Fig. 3C). Double immunostaining showed human C-peptide-positive cells were in proximity to glucagon-positive α cells within mouse pancreatic islets (Fig. S7). To confirm these human C-peptide-positive cells were derived from PB-IPC, we performed fluorescence in situ hybridization (FISH) using human X/Y chromosome probes and demonstrated that these human C-peptide-positive cells displayed human chromosome X/X karyotyping (Fig. 3E). These results indicate that PB-IPC can migrate into pancreatic islets after transplantation into the peritoneal cavity.

In addition, we performed immunohistochemistry on other tissues of PB-IPC-transplanted diabetic mice, e.g., liver, kidney, and adipose tissue of peritoneal cavity. Analysis of serial tissue sections demonstrated that these tissues failed to display human C-peptide staining (data not shown). It suggests homing of PB-IPC to pancreatic islets is not a random process and may be controlled by certain mechanism. To date, the chemokine stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, play an essential role in mediating hematopoietic stem cell homing [19]. Mechanistic studies showed the strong expression of SDF-1 in pancreatic islets of diabetic NOD-scid mice (Fig. S8), which is consistent with previous reports [19]. Notably, flow analysis revealed that PB-IPC strongly expressed SDF-1 receptor CXCR4 (Fig. S9). SDF-1 is a highly conserved chemokine since there is only one amino acid difference between human and mouse SDF-1 [19]. Therefore, chemokine SDF-1/its receptor CXCR4 may be one of potential mechanism for PB-IPC homing to islets.

In conclusion, the major goal for treatment of type 1 and type 2 diabetes is to obtain β cell surrogates and overcome the shortage of insulin-producing cells. Our current studies demonstrated that insulin-producing cells exist in peripheral blood. Using attachment, these cells can be isolated and expanded in a large scale. *In vitro* characterization and *in vivo* transplantation into the diabetic mice have demonstrated the authentic insulin production at different levels; especially homing of PB-IPC into islets suggests they act as islet β cell progenitors. Application of PB-IPC is easy to access, culture, expand, differentiate, and safe, without any ethical issues and immune rejection. Further optimizing with the β cell inducers like glucagon-like peptide-1 (GLP-1) may improve their therapeutic potential. These findings may lead to develop new approaches for autologous transplantation of these blood insulin-producing cells to treat diabetes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.035.

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