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Original Article

In vitro generation of functional insulin-producing cells from human bone marrow-derived stem cells, but long-term culture running risk of malignant transformation

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Abstract: Efforts involving therapeutic islet cell transplantation have been hampered by limited islet availability and immune rejection. *In vitro* transdifferentiation of human bone marrow-derived stem (hBMDS) cells into functional insulin-producing cells promises to provide a tissue source for autologous cell transplantation. In this study, we isolated hBMDS cells, developed a single-cell-derived stem cell line, and induced the cells to differentiate into islet-like clusters. These islet-like cells expressed multiple genes related to islet development and beta cell function (e.g., Pdx-1, Ngn-3, Islet-1, Neuro-D, Pax4, IAPP, and insulin) and produced insulin and C-peptide within these cells. These islet-like cells demonstrated time-dependent glucose-stimulated insulin release, and the ability to ameliorate hyperglycemia in chemically induced diabetic mice. However, these transplanted differentiated cells became tumorigenic in diabetic immunocompromised mice and their spontaneous transformation was confirmed by a marked increase in growth rate and inactivation of tumor suppressor genes (P21 and P16) by promoter hypermethylation. In conclusion, while hBMDS cells can be transdifferentiated into competent insulin-producing cells, and while such cell might be a potential source for autologous cell therapy for type 1 diabetes, caution is strongly advised in view of the neoplastic propensity of hBMDS cells, especially after a long-term culture *in vitro.*

Keywords: Bone marrow, mesenchymal stem cells, differentiation, insulin-producing cells, long-term culture, malignant transformation

Introduction

Type 1 diabetes is an insulin-dependent, autoimmune disorder characterized by the destruction of insulin-producing beta cells [1]. Current therapeutic options for individuals with type 1 diabetes include insulin replacement therapy, whole pancreas transplantation, and islet cell transplantation. With insulin replacement therapy, it is nearly impossible to achieve euglycemia consistently, resulting in aberrantly fluctuating blood glucose levels that can lead to acute and long-term complications. Pancreas transplantation often establishes an exogenous insulin-free euglycemic state, reduces long-term complications and improves neural and vascular function [2-5]. Major drawbacks of this procedure include the limited number of human pancreases available for transplantation as well as the requirement for immunosuppressive drugs following transplantation, which may cause alterations in glucose homeostasis and beta cell function [6]. Islet cell transplantation, like whole pancreas transplantation, provides the possibility for internal glycemic control and independence of exogenous insulin [7]; however, this approach is also hampered by a lack of tissue availability and immunological rejection. One theoretical alternative for islet transplantation would involve the use of a renewable source of stem cells capable of self-renewal and differentiation, as well as glucose regulated insulin production. Therefore, the development of a simple, reliable procedure to obtain autologous stem cells capable of differentiation into functional insulin producing cells (IPC) would provide a potentially unlimited source of islet cells for transplantation and alleviate the major limitations of availability and allogeneic rejection.

Recent studies have shown that bone marrow-derived stem (BMDS) cells have the ability to be induced to differentiate into a number of neuroectodermal, endothelial, mesenchymal, epithelial, and endodermal cell types [8-14]. Several recent *in vivo* studies exploring the feasibility of bone marrow-derived cells to differentiate into beta-cells in pancreas have come to different conclusions [15-18], a situation likely resulting from various systems and differentiating conditions. We and other investigators have recently demonstrated that rodent BMDS cells could be induced under high-glucose culture conditions *in vitro* to become competent insulin-producing cells capable of reducing hyperglycemia in diabetic mice [19, 20]. These findings raised the important question of whether hBMDS cells could also be induced to do the same.

To address this, we hypothesized that hBMDS cells could be induced *in vitro* to differentiate into functional pancreatic islet-like IPC. In this study, we tested this hypothesis in three steps. First, we derived an hBMDS cell line after long-term *in vitro* culture, isolated a single cell-derived cell clone, and characterized this cloned cell line. Second, we induced the cloned hBMDS cells undergoing the transdifferentiation to form IPC utilizing culture conditions containing high-glucose and beta-cell maturation factors, followed by confirmation for the presence of insulin and C-peptide production. Third, we tested the functionality of these differentiated (D)-hBMDS cells by their responsiveness to glucose challenge in terms of insulin release, in both *in vitro* and *in vivo* settings. Taken together, our results indicate that hBMDS cells can be induced *in vitro* to differentiate into competent IPC under suitable culture conditions.

Materials and methods

Bone marrow (BM)

Bone marrow was obtained from 10 healthy donors (age two to 30 years) according to guidelines from the University of Florida Institutional Review Board. Human BM mononuclear cells were obtained by Ficoll-Plaque density gradient centrifugation (Sigma Chemical, St. Louis, MO) to remove mature leukocytes and red blood cells.

Cell line culture

The rat INS-1 cell line (clone 832/13) was a generous gift from Dr. Christopher Newgard (Duke University). This cell line was derived from stable transfection of a plasmid containing the human proinsulin gene and expresses and processes both rat and human insulin in response to glucose stimulation. The cells were maintained in RPMI 1640 medium with 11.1 mM D-glucose supplemented with 10% fetal bovine serum [21].

Antibodies

Antibodies against CD45, CD34, CD117, CD38, CD64, CD14, CD13, CD33, CD11b, CD56, CD44, CD90, CD49b, CD19, CD20, CD2, CD5, CD4, CD8, CD3, CD7, HLA-DR, Class I HLA, and β2 microglobulin were from Becton Dickinson Biosciences (San Jose, CA). Rabbit anti-insulin polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for immunogold study, polyclonal guinea pig anti-insulin (DAKO Corporation, Carpinteria, CA), rabbit anti-rat-C-peptide antibody (LINCO Research, St. Charles, MO), anti-rabbit IgG and Guinea pig serum, Cy3-coupled anti-guinea pig IgG (DAKO) were utilized for immunocytochemistry.

Serum and cytokines

Culture reagents included fibroblast growth factor (FGF; Sigma, St. Louis, MO), epidermal growth factor (EGF; Peprotech, Rocky Hill, NJ), hepatocyte growth factor (HGF; Peprotech), vascular endothelial growth factor (VEGF; Peprotech), nicotinamide (10 mM; Sigma) and exendin 4 (10 nM; Sigma) and fetal calf serum (FCS; HyClone, Logan, Utah.).

Culture of hBMDS cells

The human BM mononuclear cells were plated in RPMI 1640 plus 20% FCS for 24 to 48 hours (37°C/5% CO_2). Unattached cells were removed by washing twice, with adherent cells grown in the same medium until 70 to 80% confluence before passage. Following three to four passages, hBMDS cells became morphologically homogeneous. At this stage, single cell-derived hBMDS cell lines were cloned by using a cloning cylinder (Fisher Scientific, Pittsburgh, PA), with the selected cells expanded and used for immunophenotypic characterization and for *in vitro* differentiation. The studies of the *in vitro* differentiation and characterization of the D-hBMDS cells utilized a single cell-derived clone from bone marrow of a 10-year-old donor without diabetes and hematological disorders. This cell line demonstrated the capacity for unlimited *in vitro* expansion without showing senescence.

Flow cytometric analysis

Approximately 3 x 10^5 undifferentiated hBMDS cells from each of the 10 donors and the cloned cell line were stained with either fluorescein isothiocyanate-conjugated (FITC) or phycoerythrin-conjugated (PE) antibodies (Becton Dickinson) against cell surface antigens [22] to obtain the phenotype of hBMDS cells. The data were analyzed with FCS express 2 software (De-Novo software, Ontario, Canada). Controls utilized FITC- and PE- conjugated isotype-matched immunoglobulins. Samples were analyzed in triplicate with data on $3x10^4$ cells acquired per run.

Testing stem cell properties

The hBMDS cells were induced *in vitro* to test the ability of differentiation into endothelial cells by culturing the cells in medium containing 10 ng/ml VEGF for 14 days. The endothelial cell phenotype was examined by detecting surface expression of various vascular antigens including CD31 and von Willebrand factor (DAKO Corporation) [22, 23].

Differentiation cultures

To induce hBMDS cells to undergo pancreatic endocrine cell differentiation, the cloned cells were cultured in basic RPMI 1640 medium with the addition of 17.5 mM glucose and 10% FCS for two to four months. To further expand and promote cellular differentiation, the cells were cultured in basic medium plus three growth factors including 1 ng/ml FGF, 10 ng/ml EGF, and 10 ng/ml HGF for an additional two months. To promote cellular maturation, the cells were cultured for five days in RPMI 1640 medium with a low glucose concentration (5.5 mM), a lower concentration of FCS (5%), plus nicotinamide (10 mM) and exendin 4 (10 nM). The low glucose and low FCS medium without growth factors was necessary for inhibiting cell proliferation and promoting cell differentiation and maturation to increase the sensitivity to glucose stimulation.

RT-PCR

Total RNA was prepared from BMDS cell cultures at various stages, including low-glucose culture, and four- and twelve-weeks of high-glucose cultures using TRIzol reagent. Transcriptional gene expression related to pancreatic organ genesis from these cultures was determined by RT-PCR according to a published protocol [24] with minor modifications. The forward and reverse primers of each PCR set were designed to be located in different exons based on sequences obtained from GenBank (Table 1). PCR products were separated by electrophoresis in 2.5% agarose gel and the sequence of each PCR product confirmed by DNA sequence analysis.

Immunocytochemistry and immunofluorescence

Cytospin slides from D-hBMDS cells were prepared, air-dried, and kept frozen at -70°C until assay for insulin and C-peptide. Immunocytochemistry was performed with polyclonal guinea pig anti-insulin (1:500) and rabbit anti-rat-C-peptide antibody (1:200) as previously described [19]. After washing three times, the cells were incubated with Cy3-coupled secondary antibodies (1:1000) for 30 min. Guinea pig or rabbit serum was used as a negative control.

Human insulin ELISA

D-hBMDS cells were cultured in the presence or absence of 10-mM nicotinamide, or exendin 4, or both for five days in RPMI 1640 containing 5% FBS, and 5.5 mM glucose after the cells were confirmed to express insulin genes by RT-PCR. The cells were switched to serum-free medium containing 0.5% BSA for 12 hrs, washed twice with PBS, then stimulated by the addition of 17.5 mM additional glucose (final concentration of 23 mM) for various times. The culture media were collected and frozen at -70°C until assay for insulin release (in triplicate). The serum-free culture medium containing 0.5% BSA was used as a control for secreted insulin measurements. Insulin release was detected by using

Table 1. Primer utilized for detection of pancreatic genes.

Gene	Forward Primer	Reverse Primer	Size	GenBank #
Actin	gtggggcgccccaggcacca	ctccttaatgtcacgcacgatttc	540bp	NM_001101
Pdx-1	cccatggatgaagtctacc	gtcctcctcctttttccac	262 bp	NM_000209
Ngn-3	aagagcgagttggcactgagc	cgtacaagctgtggtccgc	229 bp	NM_020999
NeuroD	tgtctcagttctcaggacgag	gctccagcgctgcgctgtagg	688 bp	NM_002500
Nkx2.2	accatgtcgctgaccaacacaaag	tcatcttgtagcggtggttctgga	553bp	NM_002509
Nkx6.1	agagagtcaggtcaaggtctggtt	acttgtgcttcttcaacagctgcg	215bp	NM_006168
Islet-1	cgtctgatttccctatgtgttgg	ttcttgctgaagccgatgctg	233 bp	NM_002202
Pax6	tcagcaccagtgtctaccaaccaa	atcataactccgcccattcaccga	239bp	NM_000280
IAPP	atattccagtggatacaagctt	gagagagccactgaattacttgcc	362bp	NM_000415
GK (Pancreas)	aagggaatgcttgccgactcgt	tcagcagtgccggtcatgg	572bp	NM_000162
Insulin	ctgcatcagaagaggccatcaagc	ggctttattccatctctctcggtgc	438 bp	NM_000207
Glucagon	tgttctacagcacactaccag	ctgttcctcttggtattcatc	287	NM_002054
PP	ctgctgctcctgtccacctgcgtg	ctccgagaaggccagcgtgtcctc	207	NM_002722
Somatostatin	cgtcagtttctgcagaagtccctggct	ccatagccgggtttgagttagcagatc	196	NM_001048

* All PCR performed with 35 Cycles.

human insulin ELISA kit (ALPCO Diagnostics, Windham, NH) with sensitivity of 0.15 μU/ml following the manufacturer's protocols. This assay does not detect proinsulin.

Deconvolution microscopy

Cells were stained with Cy3-conjugated secondary antibodies after incubation with antibodies specific for insulin or C-peptide. The nuclei were counter-stained with DAPI and the cells were subjected to analysis using Delta Vision/ Olympus OMT deconvolution microscopy. The images depict 3-demensional projections of 25 optical slices (0.2 micron each) through the cell, center focused on the DAPI stained chromatin in the nuclei. All images were scale-matched isotype antibody conjugates serving as negative control.

Transplantation studies

NOD-SCID mice (Jackson Laboratories) received five intraperitoneal injections of streptozotocin (STZ) (50 μg/g body weight) every day, according to our previously published procedures [19, 25] with minor modifications. The blood glucose levels were monitored using a One-Touch Profile blood glucose monitoring system (LifeScan Inc., Milpitas, CA). Within 12 days after the last injection, all mice became hyperglycemic with blood glucose levels >350mg/dl. D-hBMDS cells ($2x10^6$/mouse) were then transplanted into to the locations of both left renal subcapsular space and the distal tip of the spleen of the mice (n=6). Six control mice received sham surgery injected with the same amount of culture medium. Blood glucose levels were monitored in the afternoon between 16:00 to 18:00 hours every three days following transplantation without food deprivation. Two of the six implanted mice underwent splenectomy and nephrectomy to assess metabolic activity of the transplanted cells. The remaining mice were terminated at day 56 post-transplantation. The pancreatic tissues were harvested for analysis of residual beta cells.

Bisulfite modification and methylation-specific PCR (MSP) of the p16 and p21 gene

Sample DNA (1 μg) was modified with sodium bisulfite, converting all unmethylated but not methylated cytosine to uracil followed by amplification with primers specific for methylated *versus* unmethylated DNA. Methylation of 5-CpG islands in the promoters of tumor suppressor p16 and p21 genes was analyzed by MSP as described by Harmann JG et al [26] using a DNA methylation Kit (ZYMO Research, CA). Methylated primer for p16 (150 bp) is: forward, 5-TTATTAGAGGGTGGGGCGGATCGC-3, and reverse, 5-GACCCCGAACCGCGACCGTAA-3; Unmethylated primer for p16 (161 bp) is: forward, 5-TTATTAGAGGGTGGGGTGGATTGT, and reverse, 5-CAACCCCAAACCACAACCATAA-3; Methylated for p21 is (133bp): 5-TACGCGAGGTTTCGGGA TCG-3 and 5-AAAAACGACCCGCGCTCG-3; Unmethylated for p21 is (142bp): 5-TATGTGAGGTT

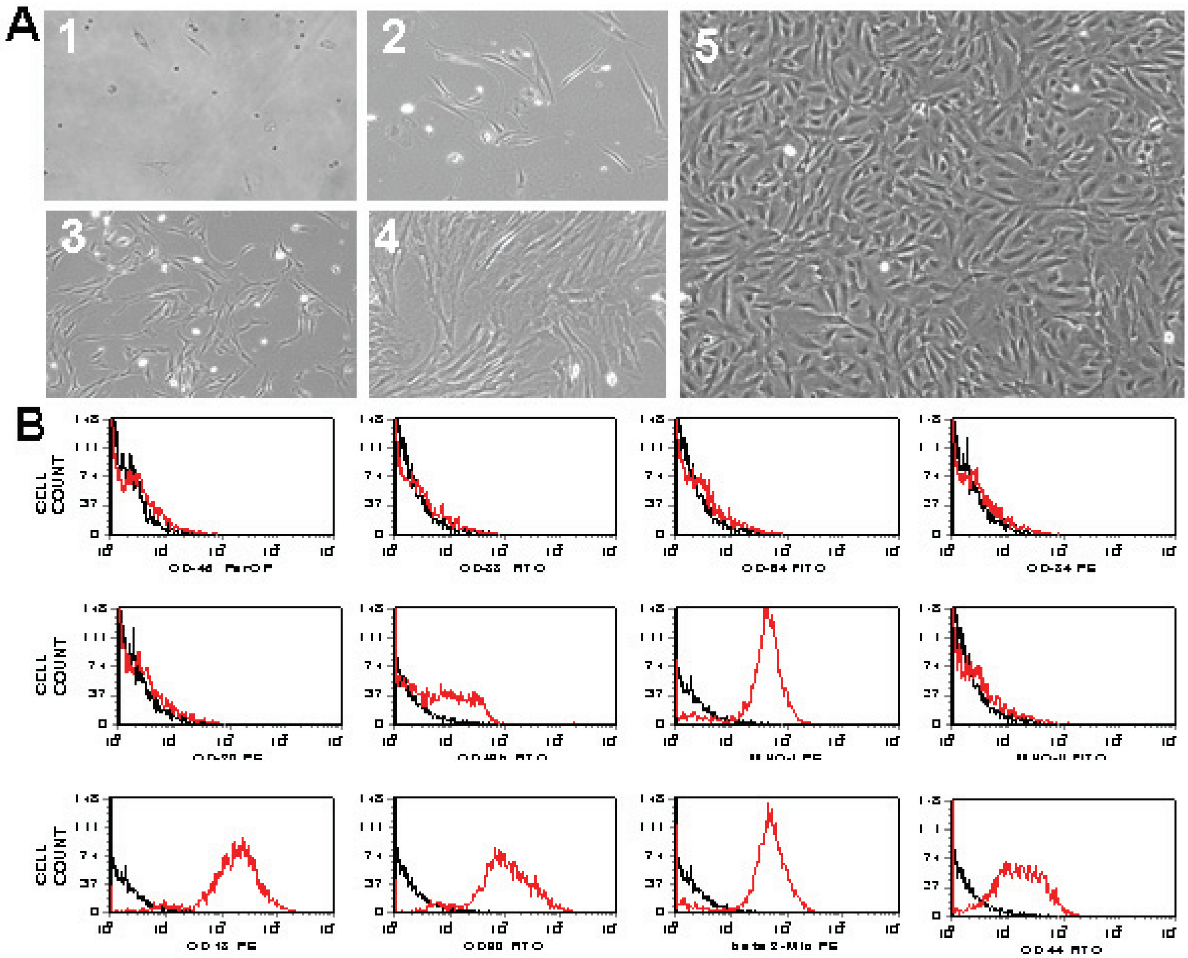

Figure 1. 1A. Isolation and derivation of clonal hBMDS cells. Human bone marrow-derived mononuclear cells (1x10^6 cells/ml) were plated and cultured for 2 days to obtain the adherent hBMDS cells (**Figure 1A-1** and **-2**). These cells were given fresh medium for continuing cell growth until the cells reached 50-70% of confluence (**Figure 1A-3** and **-4**). A single-derived clonal cell line was generated and has a homogeneous appearance (**Figure 1A-5**). 1B. Immunophenotypic characterization of hBMDS cells. Human BMDS cells from 10 donors were collected by trypsinization, washed in PBS, resuspended in RPMI1640 medium containing 10%FCS and incubated at 37°C for 60 min to reduce non-specific binding to antibodies. The cells were stained with 24 FITC-, PE-, or PerCP-labeled antibodies and isotype-matched control antibodies and then analyzed by FACS for expression of surface antigens. The figure shows one of the ten representative immunoprofiles of selected antibodies against CD45, CD38, CD64, CD34, CD20, CD49b, MHC -I, MHC-II, CD13, CD90, β2-microglubulin, and CD44.

TTGGGATTGG-3 and 5-AAAAACAACCCACACTCA ACC-3. DNA-PCR amplification conditions were an initial denaturation at 95°C for 10 min followed by 35 cycles of 45 seconds each at 95°C, 60°C, and 72°C, and a final extension at 72°C for 7 min. PCR products were analyzed on 3% agarose gels.

Results

Isolation of hBMDS cells

Human BM cells from healthy donors were used to obtain hBMDS cells. Adherent hBMDS cells were derived from cultures of unsorted BM mononuclear cells. The unattached cells were removed after 48-hours of culture, and rare adherent cells (**Figure 1A-1**) were cultured for an additional two weeks until the spindle-shaped adherent cells reached 70-80% confluence (**Figure 1A- 2, 3, 4**). The cells were then released from the surface with trypsin-EDTA, replated, and expanded at a 1:3 dilution under the same culture conditions for several passages. A single cell derived cell clone (**Figure 1A-5**) was obtained by trypsinization of a single cell derived cell cluster with a cloning cylinder. The single cell derived cells as well as the mixed hBMDS cells were then immunophenotypically characterized.

Characterization of the hBMDS cells

The hBMDS cells were enumerated at each passage utilizing a hemocytometer. After three to four passages, the cells were labeled with FITC-, PE-, or Per-CP-coupled antibodies against CD45, CD34, CD117, CD38, CD64, CD14, CD13, CD33, CD11b, CD56, CD44, CD90, CD49b, CD19, CD20, CD2, CD5, CD4, CD8, CD3, CD7, HLA-DR, Class I HLA, and β2 microglobulin. Isotype-matched immunoglobulin served as control antibodies. Cells were analyzed using a FACS and prototypic results are presented in **Figure 1B**. The phenotype of cultured mixed and cloned hBMDS cells were identical; both were negative for leukocyte common antigen CD45, hematopoietic stem cell markers (CD34, CD38, and CD117), monocytic markers (CD64 and CD14), myeloid lineage markers (CD33, CD11b), a nature killer cell marker (CD56), T-cell markers (CD2, CD5, CD3, CD5, CD4, CD8, and CD7), and B-lymphocyte markers (CD19, and CD20). These cells also do not express class II HLA-DR. However the cells weakly expressed CD49b and CD44, and strongly expressed CD90, CD13, beta-2-microglobulin and class I HLA (**Figure 1B**). Thus, the morphology and phenotype were similar between early and late passages. The hBMDS cell phenotype was similar to that of human BM mesenchymal stem cells. These cells have the capacity of cell renewal and differentiation into endothelial-like cells after 14 days of incubation with VEGF (data not shown), demonstrating stem cell like properties. The cells were stored in liquid nitrogen and, when re-started, the morphology and immunophenotype remained unchanged.

In vitro differentiation of hBMDS cells

To induce cell differentiation, the cloned hBMDS cells (**Figure 2A**) were switched into RPMI 1640 medium containing 10% FCS, high glucose (23mM), and with or without various growth factors as described in the Methods section. After two to four months of *in vitro* induction, the cells began to form three-dimensional cluster (**Figure 2B**) similar to that shown in our previous study [24]. To promote maturation of the precursors of BM-derived pancreatic endocrine cells, the cells were switched to the medium containing 5% FCS with 10mM nicotinamide, exendin 4, and low concentration of glucose (5.5 mM) after the expansion of the differentiated cells. This step facilitated cluster formations (**Figure 2C**) both in number and mass with increased the sensitivity of glucose responsiveness. The cell differentiation was monitored by RT-PCR for islet-related gene expression and glucose-responsive insulin secretion (see below).

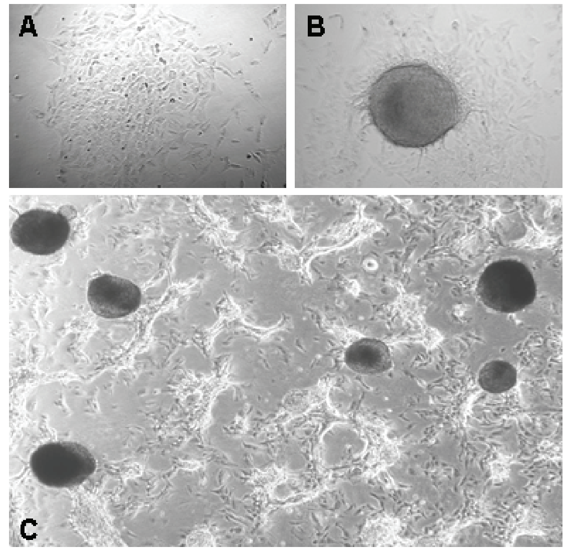

Figure 2. In vitro differentiation of hBMDS cells into islet-like clusters. There are many single cell-derived islet-like clusters at various development stages morphologically similar to the islets generated from both pancreatic stem cells and hepatic stem cells as shown in previous studies after two-four months of induction of cell differentiation. Panel A represents a single-cell derived cell line and panel B shows a single-cell-derived three-dimensional cell cluster. Panel C shows numerous cell clusters and non-clustered cells after induction of further differentiation with beta-cell promoting factors.

Gene expression of hBMDS cells and D-hBMDS cells

To determine if the islet-like clusters appearing in the hBMDS cell cultures had differentiated to endocrine-hormone expressing cells, the gene expression of endocrine cell differentiation markers and hormones was measured using RT-PCR at various stages of *in-vitro* high-glucose differentiation. Undifferentiated hBMDS cells, cultured under low glucose conditions, expressed no detectable levels of any of the tested pancreatic transcription factors (Figure 3, lane 1). In contrast, the 4-week high-glucose D-hBMDS cells expressed *Pdx1, Isl-1*, and *Ngn 3* genes (Figure 3, lane 2). The *Ngn3* gene exhibited transient expression at four-weeks of high-glucose culture but was undetectable at 12-weeks of high-glucose culture, which is consistent with its expressing pattern during developing pancreas [27]. In contrast, *NeuroD, Pax4, IAPP*, and *insulin* genes were activated at 12-weeks of high-glucose differentiation, along with persistently increasing expression of *Pdx1* and *Isl-1* genes (Figure 3, lane 3). No detectable gene expression of *Nkx6.1, Pax6,* and *GK*, as well as other pancreatic hormones including glucagon, pancreatic polypeptide, and somatostatin were observed, which is consistent with pancreatic precursor-like cells at the tested stages. Total RNA from human islets expressed all of the expected islet-related genes (Figure 3, lane 4). The negative control showed no DNA contamination (Figure 3, lane 5). These results indicated that the hBMDS cells, under *in-vitro* high-glucose induction, transdifferentiated into cells exhibiting a genotypic expression profile similar to pancreatic beta-like cells.

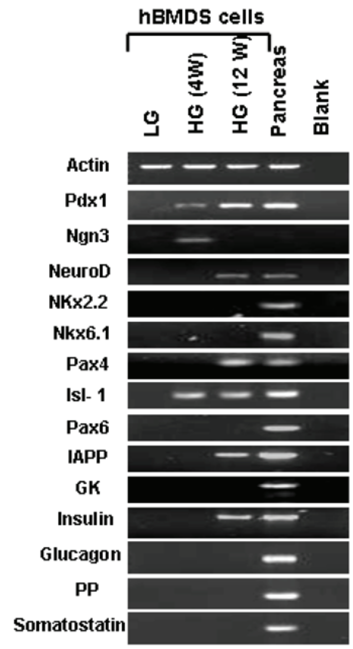

Figure 3. Gene expression in cultures of undifferentiated, and various stages of differentiated hBMDS cells. Total RNA was isolated from cultures of undifferentiated (Low-glucose (LG), Lane 1), four (HG (4W), Lane 2, and twelve-weeks(HG (12W), Lane 3) of high-glucose differentiated hBMDS cells and RT-PCR was performed to detect gene expression. All primers were designed cross intron(s) and details regarding primers are presented in Table 1. RNA isolated from human islets served as positive controls (Pancreas, Lane 4) for beta cells related gene expression. No template, water controls were included in all PCR assays (Blank, lane 5).

Synthesis and process of insulin by D-hBMDS cells

To determine if the D-hBMDS cells actually synthesize and process insulin after the continued cell differentiation and maturation, the cells were evaluated for insulin and C-peptide expression by immunofluorescence (Figure 4). There was a marked increase in the percentage of the IPC that represent approximately 20% of examined cells, with strong cytoplasmic staining for insulin (Figure 4A left-middle) and C-peptide (Figure 4A left bottom). INS-1 cells were used as a positive control for insulin and C-peptide immunostaining. These results indicate that the D-hBMDS cells can be further induced *in vitro* to differentiate into more mature IPC. To examine the distribution of insulin granules, we used deconvolution microscopy to visualize the insulin granule and C-peptide distribution in the *in-vitro*-D-hBMDS cells and compare with INS-1 insulinoma cells. Figure 4B shows the distribution of intracellular insulin and C-peptide. Interestingly, the insulin granules in the differentiated cells were arranged in a polarized fashion, with most of the granules being situated within one side of the cell similar to the location in INS-1 cells. This pattern is consistent with insulin being released in a physiologic response to glucose stimulation.

Insulin release in response to glucose stimulation

To determine whether the differentiated cells are responsive to a glucose challenge, the time-course of insulin release from the D-hBMDSC cells with various culture conditions was measured. In order to increase the sensitivity of the cells to a high-glucose challenge, the cells were switched to low serum, low-glucose medium plus either exendin 4, nicotinamide, or both for five days. The cells then were switched to serum -free low-glucose medium containing 0.5% BSA overnight, then stimulated by the addition of 23 mM glucose for various times up to eight hours.

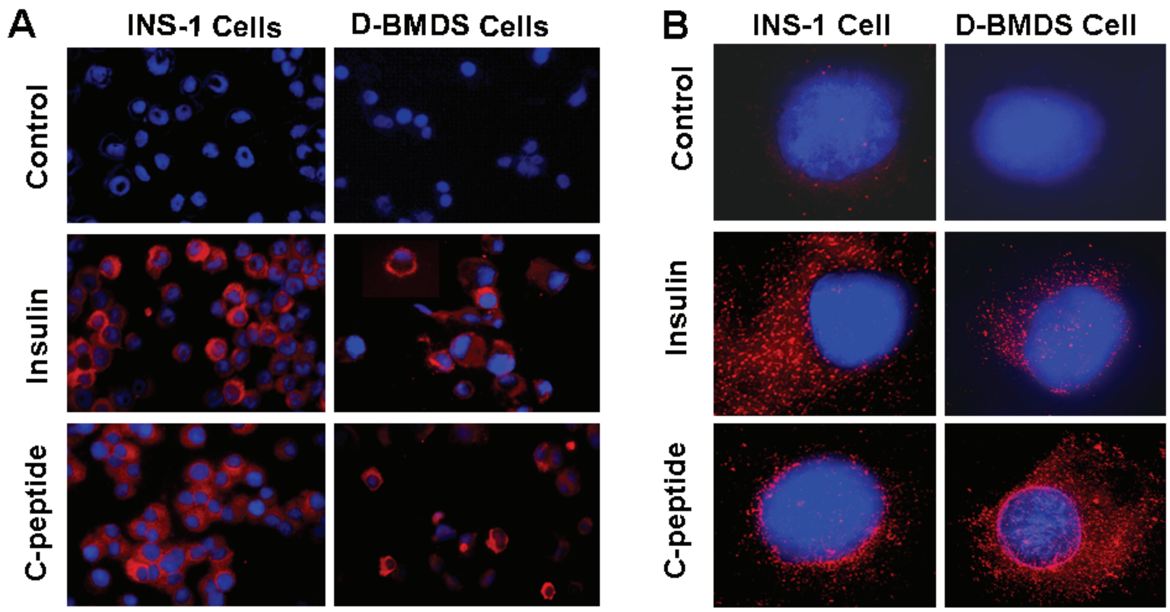

Figure 4. Immunofluorescence analysis of insulin and C-peptide. 4A. Differentiated hBMDS cells on cytospin slides were stained with anti-insulin (middle panel) and c-peptide (lower panel) antibodies and visualized by immunofluorescence analysis. Rat insulinoma cell line, INS-1 cells, was used as a positive control for insulin and c-peptide as indicated. There is strong cytoplasmic insulin and C-peptide staining in both INS-1 and differentiated hBMDS cells. DAPI was used to stain cell nuclei (blue). Original magnification is 40x. Figure 4B illustrates the distribution of insulin granules (red color) visualized by deconvolution microscopy in a representative single cell after immunostaining with anti-insulin and C-peptide antibodies. Nucleus was stained with DAPI (blue color).

As demonstrated in **Figure 5A**, the peak of insulin release occurred at two hours after the glucose challenge in the culture condition of pretreatment with exendin 4. Interestingly, insulin release in cells pretreated with both nicotinamide and exendin 4 occurred much earlier and peaked within a few minutes in response to a glucose challenge and returned to a lower level at one hr (**Figure 5B**). Moreover, the intensity of insulin release after pretreatment with both nicotinamide and exendin 4 was much stronger than after exendin 4 treatment alone. Surprisingly, there was no detectable insulin release when the cells were treated with nicotinamide alone. These results demonstrated that the D-hBMDS cells are functional IPC capable of releasing insulin in response to a glucose challenge.

Amelioration of hyperglycemia in NOD-SCID mice

To determine if the D-hBMDS cells were able to reduce blood glucose levels in STZ-induced hyperglycemic NOD-*SCID* mice, animals were transplanted with or without the D-hBMDS cells. Approximately one week following transplantation, glucose levels in D-hBMDS cell-transplanted mice were reduced by almost one-half ($p<0.01$, **Figure 6**). However, blood glucose levels were not completely normalized (i.e., euglycemia). These data suggest that even though the numbers of implanted cells were sufficient to maintain blood glucose reduction over the entire monitoring period, it may not have been enough for a complete reversal of hyperglycemia. By comparison, control mice did not exhibit any decrease in blood glucose levels. When two mice from the transplant group underwent surgery to remove implants, both were observed to have a rapid increase in blood glucose levels (> 350mg/dl after 24hr post surgery), suggesting that the transplanted cells were responsible for the reduction in blood glucose levels. Post-mortem examination of the pancreas of all mice demonstrated few scattered residual insulin-positive cells, yet no significant differences were observed between pancreata of control and implanted mice (data not shown). This observation suggests that the STZ treatment of mice used in this study eliminates a majority of pancreatic beta cells and the residual beta cells

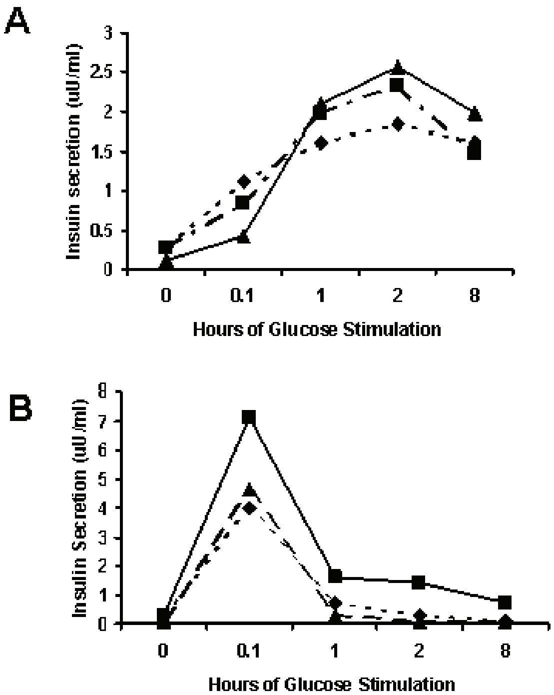

Figure 5. Time course of insulin release in response to glucose stimulation in the differentiated hBMDS cells. Cells were cultured in low glucose (5.5mM) and low FCS (5%) for one week before addition of 10 nM exendin 4, 10 mM nicotinamide, or both for five days in the same medium. The cells were washed twice with PBS and switched to a serum-free cell culture medium containing 0.5% BSA for 12 hrs. The cells then were stimulated with 23 mM glucose. Samples of cell culture medium were collected for insulin release assay by ELISA at indicated times. Nicotinamide alone did not increase insulin release (data not shown). Pretreatment with exendin 4 for five days caused slow increase in insulin release with a peak at 2 hr (upper panel). Pretreatment with both nicotinamide and exendin 4 resulted in a rapid release of insulin with a peak at 0.1 hour in response to a glucose challenge (lower panel). Figure 5 shows three parallel experiments.

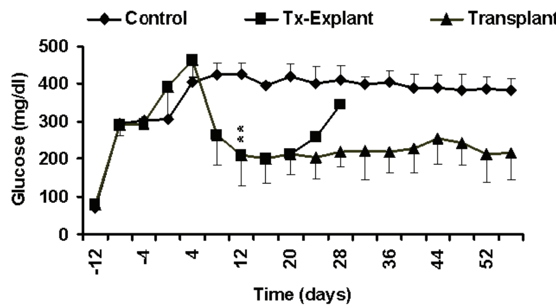

Figure 6. Reduction of blood glucose levels after implantation of the differentiated hBMDS cells. Blood glucose levels in sham-operated diabetic NOD-SCID mice (¨) (n=6), Differentiated hBMDS cell-implanted mice (5) (n=6) and the implanted mice that become hyperglycemic after removal of implants (<) (n=2). Blood glucose levels were evaluated between 16:00 and 18:00 hours under nonfasting conditions at two to four day intervals. Arrow indicates the implantation day (shown as day 0). Arrowhead indicates the day of removal implants (shown as day 20). Values are means ± SE. ** P< 0.01.

were insufficient for maintaining normal glucose levels.

At 40 to 45 days post-transplantation, we noted that the remaining mice receiving D-hBMDS cells developed an enlarged tumor mass around the left renal region, palpable to touch. Furthermore, the blood glucose levels of these mice gradually rose to levels above 300 mg/dl. These animals were sacrificed between 45 to 56 days post-transplantation and the histology of the tumor examined. These studies showed the tumor cells had malignant cell morphology with an infiltrating border adjacent to normal renal tissue and the frequent presence of atypical mitosis. These findings suggested that the transplanted D-hBMDS cells likely represented a mixture of differentiated and undifferentiated cells, capable of either reducing hyperglycemia or formation of a tumor mass, respectively.

Hypermethylation of p16 and p21 promoter region in transformed hBMDS cells

The *in vivo* data from these animal studies prompted us to further study the nature of our hBMDS cell line. Since aberrations in the DNA methylation patterns are recognized as a hallmark of the cancer cell, and silencing of tumor suppressor genes such as p16 and p21 has established promoter hypermethylation serve as a common mechanism for tumor suppressor inactivation in human cancer [28, 29]. Using sensitive methylation-specific PCR (MSP), we examined the methylation status of the tumor suppressor gene p16 and p21 using sodium bisulfite-treatment of DNA. Specifically, the CpG island methylation in p16 and p21 were examined by MSP in early passage hBMDS cells as well as the clonal line from hBMDS cells. A human multiple myeloma-derived cell line (H929) and normal human DNA is served as controls. Hypermethylation of p16 and p21 promoter

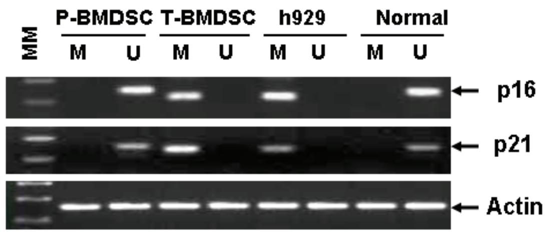

Figure 7. Hypermethylation of *p16 and p21* tumor suppressor genes in cloned hBMDS cells. Viewed from left to right, four samples are as following sequence: primary human BMDS cells (P-BMDSC), cloned hBMDS cells (T-BMDSC), human tumor cell line (H929), and normal human lymphocytes (normal). In the *p16*, and *p21* promoter regions, the DNA PCR products showed hypermethylated (M) in T-BMDSC, whereas the PCR products from P-BMDSC were unmethylated (U). Human tumor cell line H929 serves as a positive control for hypermethylated DNA (M), normal lymphocyte DNA as a negative control for unmethylated DNA (U). Expression of house-keeping actin gene serves as sample DNA quality control.

regions was detected in the transformed hBMDS cells as well as H929 tumor cell line, as demonstrated by the presence of the amplified 150 bp and 133 bands, respectively, using the methylation primers (Figure 7). For comparison, 161 bp and 142 bp bands for p16 and p21 were detected by unmethylated primers in normal human DNA and early passages of non-clonal HBMDS cells that served as normal as well as internal controls for the quality of bisulfite-treatment of DNA. These studies provided molecular evidence that the cloned hBMDS cells were spontaneously transformed into neoplastic cells, and acquired features of malignant cells during a long-term *in vitro* derivation and selection.

Discussion

Recent studies have demonstrated the feasibility of generating IPC from progenitor cells of various sources including pancreas [30, 31], liver [24], intestinal epithelium [32], and pluripotent embryonic stem cells of mouse [33, 34] and human [35] origin. However, even with the conceptual advance offered by these findings, some obstacles, such as immune rejection and autoimmunity against newly formed beta cells from pancreatic stem cells, remain. Furthermore, it may prove very difficult to obtain enough autologous adult stem cells from these organs.

To overcome this latter limitation, we explored the possibility of using hBMDS cells as sources for transdifferentiation into IPC under specific *in -vitro* culture conditions. Bone marrow has been known for years to represent a safe and abundant source for large quantities of adult stem cells. In the present study, we isolated, cloned, and characterized the immunophenotype of hBMDS cells. We also generated IPC from hBMDS cells using *in vitro* differentiation procedures, with RT-PCR, and immunofluorescence confirming the presence of insulin synthesis and process in the cells. Determination of insulin release, in response to increased glucose concentration in cells cultured with different maturation factors, demonstrated that insulin was released in a time dependent fashion. Moreover, the release of insulin occurred earlier in more mature cells than less mature cells. We believe that this pattern mimics the physiological release of insulin by native pancreatic beta cells. Our study provides direct evidence that human bone marrow contains pluripotent cells capable of being reprogrammed *in vitro* to become IPC. These cells demonstrate the ability to respond to the glucose challenge to release insulin and reduce blood glucose levels in diabetic mice. This finding has also been confirmed by several other studies, demonstrating that BM -derived stem cells can be induced to transdifferentiate into insulin-releasing cells using *in vitro* system [19, 20].

A study by Ianus et al [15] also provides *in vivo* evidence of adult mouse BM harboring cells that can transdifferentiate into glucose-competent pancreatic endocrine cells. The results of that study suggested that the *in vivo* generation of IPC is likely due to transdifferentiation of BMDS cells into beta cells rather than of cell fusion. Kojima, et al surprisingly observed that proinsulin- and insulin-positive cells could be detected in the multiple organs including liver, adipose tissue, spleen, bone marrow, and thymus in hyperglycemic mice or rats and further bone marrow transplantation experiments showed that most of the extrapancreatic proinsulin-producing cells originated from the bone marrow [16]. In contrast, two recent studies [17, 18] showed that there is little evidence of transdifferentiation of BM-derived cells into pancreatic beta cells in the chemically induced diabetic mice. A current unresolved issue in terms of the plasticity of BMDS cells in *in-vivo* animal studies is transdifferentiation versus cell fusion as an underlying mechanism for adult stem cell plasticity. In our current study as well as other *in vitro* studies [19, 20], since homogeneous hBMDS cells were used to induce *in vitro* differentiation into IPC, cell fusion is clearly not the explanation for the presence of competent IPC although a few binucleated cells were observed in the culture dishes.

To confirm that we have generated IPC from hBMDS cells which may have features of pancreatic islet beta-like cells, we determined the following islet cell characteristics: 1) expression of otherwise silent islet cell differentiation transcription factors including *Isl-1, Ngn 3, Pdx-1, NeuroD, Pax4, IAPP,* and *insulin* by RT-PCR; 2) presence of C-peptide (by-product of *de novo* insulin synthesis) and insulin detected by immunofluorescence; 3) insulin and C-peptide release as determined by deconvolution microscopy; 4) glucose stimulated time-dependent insulin release; and 5) reduction of hyperglycemia in diabetic mice. Rajapogal *et al.* reported that false-positive insulin-producing human embryonic stem cells did not stain with an antibody for C-peptide, and did not contain transcripts for insulin mRNA. In contrast, our transdifferentiated IPC possessed these islet cell traits including the presence of C-peptide and insulin. The identified islet-like cells contained transcripts for the insulin gene along with *Isl-1, Pdx-1, NeuroD, Pax4,* and *IAPP* and they also contained insulin and C-peptide. In addition, the islet-like cells exhibited a time-dependent rapid insulin release (**Figure 5**) that closely matched the response of native islet cells to a glucose challenge. Finally, in our study, insulin release did not involve absorbed insulin, since the culture medium did not contain insulin. Thus, our data clearly demonstrate the ability of hBMDS cells to differentiate into IPC that have many characteristics of pancreatic beta-cells.

There are two key steps in our cell culture conditions that appear to be critical for inducing differentiation of hBMDS cells into insulin-producing islet-like cells. First, the hBMDS cells initially need to be cultured in medium containing a high-glucose concentration (23mM) for various durations of time until certain genes such as *islet-1, Ngn3, Pdx1, NeuroD*, and *insulin* become detectable. Second, the transdifferentiated hBMDS cells require subsequent culture with maturation factors, such as exendin-4 and nicotinamide, in a medium containing low FCS and low glucose, in order to promote cell maturation and to restore the sensitivity to a glucose challenge. Exendin-4 is a potent glucagon-like peptide-1 (GLP-1) agonist that has previously been shown to stimulate both beta cell replication and neogenesis from ductal progenitor cells [36]. GLP-1 stimulates insulin secretion and augments beta cell mass via activation of beta cell proliferation and islet neogenesis [37]. Nicotinamide is a potent activator of beta cell regeneration [38] and it promotes liver stem cell *in vitro* transdifferentiation and maturation into insulin-producing cells [24]. Our protocol of transdifferentiation employed two different cellular factors in addition to high-glucose conditions to convert hBMDS cells into IPC. We demonstrated that exendin-4 treatment alone of the hBMDS cells was not capable of producing a rapid glucose-stimulated response, whereas exendin-4 plus nicotinamide treatment of hBMDS cells resulted in an islet-like glucose-stimulated instantaneous insulin secretion (**Figure 5**). This suggests that multiple pathways of islet-cell differentiation must be induced for complete maturation and development of islet-like cells from hBMDS cells. For example, exendin-4 treatment of hBMDS cells may result in upregulation of islet cell differentiation factors. However, exendin-4 may be necessary but not sufficient for complete islet-like cellular differentiation. Whereas, in the presence of both exendin-4 and nicotinamide, this may induce upregulation of required co-factors of islet cell differentiation that allow for sufficient transdifferentiation of islet-like cells.

Since there are multifactorial influences in the transdifferentiation of hBMDS cells into competent IPC, many questions are left unanswered and unresolved issues do remain. For example, how much BM is required to generate a sufficient amount of islet-like cells in a cell based therapy for type 1 diabetes? What is the expansion capacity of the D-hBMDS cells? Does the patient's age affect the quality of the hBMDS cells? Is there genetic predisposition affecting the process of *in vitro* transdifferentiation? In this study, for the purpose of reproducibility of our results, we used the clonal hBMDS cell line derived from a 10-year-old patient without type 1 diabetes and with no genetic manipulation in order to explore the feasibility of generating IPC *in vitro*. Although development of clonal expansible hBMDS cell line is a useful tool for study the mechanism and conditions for cell transdifferentiation, we also noticed from our data that one of the disadvantages for a long-term *in vitro* manipulation is the potential for spontaneous cell transformation. This adverse effect has also been observed recently by other groups during in vitro long-term culture of both mouse and human mesenchymal stem cell [39-41]. Therefore, it raises concern in terms of clinical applications that BMDS cells should not be manipulated in vitro for long time in order to prevent from possible cell spontaneous malignant transformation. Therefore, it is still a remaining and arduous task to find the decisive steps (e.g., addition of exogenous factors) and shortest time for in-vitro for the transdifferentiation without potential for neoplastic transformation.

In our experience, the differentiated cells are different from beta cell-derived cell lines such as β-TC and INS-1 cells in terms of gene expression profile, cell maturity, and capacity of processing and release of insulin in response to glucose stimulation. Hence, one can also question whether these cells can really be pushed to the level of maturity like true beta cells by changing the *in vitro* culture conditions. Another relevant clinical question concerning the issue of autoimmunity is whether the immune response to beta cell antigens will result in recognition and destruction of the newly generated insulin-producing cells derived from hBMDS cells. While further research is obviously required to settle these important issues, the results presented here lend a sense of optimism to the notion that transdifferentiation of stem cells to insulin-producing cells may represent a viable therapeutic option for type 1 diabetes as well as caution for further clinical applications.

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Reference

[1] Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001; 358: 221-229.
[2] Jahansouz C, Kumer SC, Ellenbogen M, Brayman KL. Evolution of β-Cell Replacement Therapy in Diabetes Mellitus: Pancreas Transplantation. Diabetes Technol Ther 2011; 13: 395-418.
[3] Boggi U, Vistoli F, Amorese G, Giannarelli R, Coppelli A, Mariotti R, Rondinini L, Barsotti M, Piaggesi A, Tedeschi A, Signori S, De Lio N, Occhipinti M, Mangione E, Cantarovich D, Del Prato S, Mosca F, Marchetti P. Results of pancreas transplantation alone with special attention to native kidney function and proteinuria in type 1 diabetes patients. Rev Diabet Stud 2011; 8: 259-267.
[4] Otsuki K, Yoshikawa K, Kenmochi T, Maruyama M, Akutsu N, Iwashita C, Ito T, Asano T. Evaluation of segmental pancreatic function using 11C-methionine positron emission tomography for safe living donor operation of pancreas transplantation. Transplant Proc 2011; 43: 3273-3276.
[5] Gruessner AC, Sutherland DE. Pancreas transplant outcomes for United States (US) cases as reported to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) Clin Transpl. 2008; 45-56.
[6] Han DJ, Sutherland DE. Pancreas transplantation. Gut Liver 2010; 4: 450-465.
[7] Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343: 230-238.
[8] Ma K, Fox L, Shi G, Shen J, Liu Q, Pappas JD, Cheng J, Qu T. Generation of neural stem cell-like cells from bone marrow-derived human mesenchymal stem cells. Neurol Res 2011; 33: 1083-1093.
[9] Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-967.
[10] Wan SY, Zhang TF, Ding Y. Galectin-3 enhances proliferation and angiogenesis of endothelial cells differentiated from bone marrow mesenchymal stem cells. Transplant Proc 2011; 43: 3933-3938.
[11] Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998; 279: 1528-1530.
[12] Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 1999; 401: 390-394.
[13] Wei Y, Chen YH, Li LY, Lang J, Yeh SP, Shi B,

Yang CC, Yang JY, Lin CY, Lai CC, Hung MC. CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. Nat Cell Biol 2011; 13: 87-94.
[14] Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, Lenvik T, Johnson S, Hu WS, Verfaillie CM. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002; 109: 1291-1302.
[15] Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 2003; 111: 843-850.
[16] Kojima H, Fujimiya M, Matsumura K, Nakahara T, Hara M, Chan L. Extrapancreatic insulin-producing cells in multiple organs in diabetes. Proc Natl Acad Sci USA 2004; 101: 2458-2463.
[17] Choi JB, Uchino H, Azuma K, Iwashita N, Tanaka Y, Mochizuki H, Migita M, Shimada T, Kawamori R, Watada H. Little evidence of transdifferentiation of bone marrow-derived cells into pancreatic beta cells. Diabetologia 2003; 46: 1366-1374.
[18] Lechner A, Yang YG, Blacken RA, Wang L, Nolan AL, Habener JF. No evidence for significant transdifferentiation of bone marrow into pancreatic beta-cells in vivo. Diabetes 2004; 53: 616-623.
[19] Tang DQ, Cao LZ, Burkhardt BR, Xia CQ, Litherland SA, Atkinson MA, Yang LJ. In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow. Diabetes 2004; 53: 1721-1732.
[20] Oh SH, Muzzonigro TM, Bae SH, LaPlante JM, Hatch HM, Petersen BE. Adult bone marrow-derived cells trans-differentiating into insulin-producing cells for the treatment of type I diabetes. Lab Invest 2004; 84: 607-617.
[21] Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes 2000; 49: 424-430.
[22] Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418: 41-49.
[23] Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 2001; 98: 2615-2625.
[24] Yang LJ, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci USA 2002; 99: 8078-8083.
[25] Cao LZ, Tang DQ, Horb ME, Li SW, Yang LJ. High glucose is necessary for complete maturation of pdx-1-vp16-expressing hepatic cells into functional insulin-producing cells. Diabetes 2004; 53: 3168-3178.
[26] Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996; 93: 9821-9826.
[27] Wilson ME, Scheel D, German MS. Gene expression cascades in pancreatic development. Mech Dev 2003; 120(1):65-80.
[28] Nuovo GJ, Plaia TW, Belinsky SA, Baylin SB, Herman JG. In situ detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis. Proc Natl Acad Sci USA 1999; 96: 12754-12759.
[29] Paz MF, Fraga MF, Avila S, Guo M, Pollan M, Herman JG, Esteller M. A systematic profile of DNA methylation in human cancer cell lines. Cancer Res 2003; 63: 1114-1121.
[30] Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil JJ. In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci USA 2000; 97: 7999-8004.
[31] Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG. Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nat Med 2000; 6: 278-282.
[32] Suzuki A, Nakauchi H, Taniguchi H. Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells. Proc Natl Acad Sci USA 2003; 100: 5034-5039.
[33] Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes 2000; 49: 157-162.
[34] Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 2001; 292: 1389-1394.
[35] Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M. Insulin production by human embryonic stem cells. Diabetes 2001; 50: 1691-1697.
[36] Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 1999; 48: 2270-2276.
[37] Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. J Biol Chem 2003; 278: 471-478.
[38] Akiyama T, Takasawa S, Nata K, Kobayashi S,

Abe M, Shervani NJ, Ikeda T, Nakagawa K, Unno M, Matsuno S, Okamoto H. Activation of Reg gene, a gene for insulin-producing beta - cell regeneration: poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly(ADP-ribosylation. Proc Natl Acad Sci USA 2001; 98: 48-53.

[39] Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis Mortari A, McElmurry RT, Bell S, Xia L, Zhou N, Riddle M, Schroeder TM, Westendorf JJ, McIvor RS, Hogendoorn PC, Szuhai K, Oseth L, Hirsch B, Yant SR, Kay MA, Peister A, Prockop DJ, Fibbe WE, Blazar BR. Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 2007; 25: 371-379.

[40] Rubio D, Garcia-Castro J, Martin MC, de la FR, Cigudosa JC, Lloyd AC, Bernad A. Spontaneous human adult stem cell transformation. Cancer Res 2005; 65: 3035-3039.

[41] Ren Z, Wang J, Zhu W, Guan Y, Zou C, Chen Z, Zhang YA. Spontaneous transformation of adult mesenchymal stem cells from cynomolgus macaques in vitro. Exp Cell Res 2011; 317: 2950-2957.