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CLINICAL RESEARCH

Characterization of pancreatic stem cells derived from adult human pancreas ducts by fluorescence activated cell sorting

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Abstract

AIM: To isolate putative pancreatic stem cells (PSCs) from human adult tissues of pancreas duct using serum-free, conditioned medium. The characterization of surface phenotype of these PSCs was analyzed by flow cytometry. The potential for pancreatic lineage and the capability of β-cell differentiation in these PSCs were evaluated as well.

METHODS: By using serum-free medium supplemented with essential growth factors, we attempted to isolate the putative PSCs which has been reported to express *nestin* and *pdx-1*. The Matrigel™ was employed to evaluate the differential capacity of isolated cells. Dithizone staining, insulin content/secretion measurement, and immunohistochemistry staining were used to monitor the differentiation. Fluorescence activated cell sorting (FACS) was used to detect the phenotypic markers of putative PSCs.

RESULTS: A monolayer of spindle-like cells was cultivated. The putative PSCs expressed *pdx-1* and *nestin.* They were also able to differentiate into insulin-, glucagon-, and somatostatin-positive cells. The spectrum of phenotypic markers in PSCs was investigated; a similarity was revealed when using human bone marrow-derived stem cells as the comparative experiment, such as CD29, CD44, CD49, CD50, CD51, CD62E, PDGFR-α, CD73 (SH2), CD81, CD105(SH3).

CONCLUSION: In this study, we successfully isolated PSCs from adult human pancreatic duct by using serum-free medium. These PSCs not only expressed *nestin* and *pdx-1* but also exhibited markers attributable to mesenchymal stem cells. Although work is needed to elucidate the role of these cells, the application of these PSCs might be therapeutic strategies for diabetes mellitus.

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Key words: Putative pancreas stem cell; *Nestin*; *pdx-1*; Phenotypic marker

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INTRODUCTION

Diabetes mellitus (DM)[1], one of the global diseases, is the basis for insulin deficiency either due to the inability of insulin secreting β-cells (type Ⅰ) or insulin resistance (type Ⅱ) in somatic cells. In the case of type Ⅰ DM treatment, islets of Langerhan transplantation has been demonstrated to restore normoglycemia[2]. Nevertheless, prevalent application is still limited by the shortage of donor pancreas, emphasizing the importance of producing β-cells *in vitro* before their transplantation into patients. The putative pancreatic stem cells (PSCs) have been reported in endocrine, acinar, and duct cells of human[3-5] and mouse studies[6-8], and the capacity to differentiate pancreatic lineage cells has been demonstrated *in vitro*. However, the existence and the biological role of putative PSCs in β-cell neo-regeneration is still doubtful[9].

Although the existence of PSCs has been reported in mice and humans, the methodological characterization of these PSCs is still ambiguous. The identification of PSC-specific biomarkers is required not only to define the PSCs operationally, but also to provide an efficient access for further purification. *Nestin*, an intermediate filament first identified in neuroepithelial stem cells, has been maintained as a marker of multi-lineage progenitor cells[10]. In regeneration studies of pancreas, some *nestin* positive cells have been observed[5]; nestin positive cells isolated from islets[11,12], mesenchymal cells[13], pancreatic ducts[14] and vascular endothelial cells[15] have been reported. In murine embryonic stem cell (mESC) studies, *nestin* positive cells could be selected and enriched by conventional medium cultivation for further neurogenesis[16], the application in pancreas was worth contemplating[17]. Pancreas duodenum homeobox-1 (*pdx-1*) is also essential for pancreatic development, insulin production, and glucose homeostasis. In animal models of partial pancreatectomy [18], diabetic models of streptozotocin (STZ) treatment in mice[19], injury and embryology studies[20,21], the expression of *pdx-1* was detected. For the biological role of the interaction with multiple transcription factors and co-regulators, it was thought as a direct indicator of cells with pancreatic differentiation potentials[22].

In the present study, we attempted to isolate putative PSCs from adult human pancreatic duct tissue rather than as in previous studies which used the animal model[13] or the human fetus[5]. Furthermore, to seek the potential biomarkers on these PSCs, the spectrum of phenotypic markers of human BMSCs was utilized and analyzed. These efforts attempt to investigate the properties of putative PSCs and demonstrate that β-cells could be induced by autogenous pancreatic tissue and possibly apply to diabetes therapy.

MATERIALS AND METHODS

Putative pancreatic stem cells (PSC) isolation

This research follows the tenets and regulations of the Declaration of Helsinki and has been reviewed by the Institutional Review Committee at Taipei Veterans General Hospital. Human pancreatic duct tissues at close proximity to the duct, originating from 4 identical donors, were dissected and digested by collagenase P (Roche Molecular Biochemicals, Mannheim, Germany) with HEPES-buffered saline for 7 h at 37℃. The digested tissue was washed two times with a HBS solution, pipetted up and down several times using a 10 mL syringe with a 22G needle, and placed into 10 cm Petri dishes with 10 mL of CMRL 1066 (5.6 mmol/L glucose, Gibco™, USA) media plus 10 mL/L Fetal bovine serum (FBS, Biological Industries, Israel). After two days incubation a sphere-like floating structure was observed. This suspended cell mass was collected by centrifugation, re-suspended using new serum-free ITSFn medium (composed: 1:1 of DMEM/F12, 0.6 g/L glucose, 25 μg/mL insulin, 100 μg/mL transferrin, 20 nmol/L progesterone, 60 μmol/L putrescine, 30 nmol/L selenium chloride, 2 mmol/L glutamine, 3 mmol/L sodium bicarbonate, 5 mmol/L HEPES buffer, 2 μg/mL heparin, 20 ng/mL human epidermal growth factor (EGF), 20 ng/mL human basic fibroblastic growth factor (b-FGF) and 20 ng/mL human hepatocyte growth factors, all growth factors were purchased from PerproTech, Israel) and placed into a new dish. The procedure was repeated twice to get rid of non-spherical masses and suspended cells, then the suspended cell mass was transferred to a 6 cm Falcon non-treated cultivation dish for plating, and cultivated using 10mL modified serum-free ITSFn medium. The medium was changed twice and sub-cultured once at a ratio of 1:5 in a week. The proliferation ability of putative PSC cells in passage 5, 10, 15, 20, 25 was examined by doubling time calculation.

Human bone marrow mesenchymal stem cell (BMMSC) isolation

Bone marrow aspirates were taken from the posterior iliac crest of normal adult donors (5 mL each; n = 4) and the isolation procedure followed our previous protocol[23]. Briefly, the bone marrow was washed twice with equal volume of PBS and centrifuged at 300 g for 10 min at room temperature. All washed cells were re-suspended in PBS to 10 mL and nucleated cells were isolated with a Percoll density gradient (diluted with equal volume of 1.073 g/mL Percoll solution, then centrifuged at 900 g for 30 min). The mononuclear cells (MNCs) were then suspended in plates. Expansion medium consisted of Dulbecco's modified Eagle's medium with 1 g/L glucose (DMEM-LG, Gibco) and 10% fetal bovine serum (FBS; Gibco) supplemented with 10 ng/mL bFGF, 10 ng/mL EGF, 10 ng/mL PDGF-BB (R&D), 100 Units/mL penicillin, and 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (Gibco). All of the nucleated cells were plated in 20 mL medium in a 75 cm^2 culture dish and incubated at 37℃ with 5 ml/L CO_2. After 24-48 h, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). These adherent cells were then cultured through 5 passages and used in flow cytometry studies.

RT-PCR detects the expression of nestin and pdx-1 in putative PSC

Trizol™ (Invitrogen, USA) reagent and GeneStrips™ (RNAture, USA) kits were employed for mRNA purification from putative PSC (passage 5). The Advantage RT-for-PCR Kit (Clontech; BD Biosciences, San Jose, CA) was used to synthesize the first strand of cDNA and 12 μL of extracted mRNA solution was utilized as the template. The experimental procedure followed the manufacturer's instructions. The sequence of primers used to detect human *nestin*, *pdx-1, insulin, glucagon* and *somatostatin* expression were as followed: *nestin*, forward: 5'-AGAGGGGAATTCCTGGAG-3', reverse: 5'-CTGAGGACCAGGACTCTCTA-3'; *pdx-1* forward: 5'-CCTTTCCCATGGATGAAGTC-3', reverse: 5'-TGTCCTCCTCCTTTTTCCAC-3'; *insulin* forward: 5'-CACACCTGGTGGAAGCTCTCT-3', reverse: 5'-GTAGAGGGAGCAGATGCTGGTA-3'; *glucagon* forward: 5'-ATCTGGACTCCAGGCGTGCC-3', reverse: 5'-AGCAATGAATTCCTTGGCAG-3'; *somatostatin* forward: 5'- TTCATCATCTACACGGC-3', reverse: 5'-GAGAG-

TAGAAGCAACCTACC-3'. Amplification was carried out with the program of 94℃ for 30 s to denature, 55℃ for 30 s for primer annealing and 72℃ for 30 s to elongate the PCR product for 30 cycles. The reaction was done on a total volume of 25 μL containing 0.5 μmol/L of each primer, 200 μmol/L dNTP, 2 units of Taq enzyme, and 5 μL of synthesized cDNA in reaction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.4, 1.5 mmol/L $MgCl_2$ and 100 μg/mL bovine serum albumin).

Pancreatic differentiation by basement-membrane-rich gel (Matrigel™)

The Matrigel™; a commercial preparation of murine basement membrane (BD biosciences, USA), was employed to induce pancreatic differentiation. Briefly, 1×10^4 cells of passage 10 and 20 were suspended with 1 mL medium and were placed on the top of the 6 cm plate coated with Matrigel™ (50 μL per cm^2) and were allowed to gel overnight before additional medium was added. Cell samples were taken for the following Dithizone and immunochemistry staining at different time points per week until the end of experimentation (4 wk).

Dithizone staining

Dithizone (DTZ, also named Diphenylthiocarbozone, Sigma, USA), which stains insulin-containing cells bright red, was used to quickly assess the presence of insulin-producing cells. The staining protocol followed was from the study by Shiroi, *et al* 2002[24].

Immunofluorescent staining

The sphere aggregated by PSC differentiated in Matrigel™ was dug and embedded by O.C.T. (Sakura Finetechnical Co., USA) for frozen section. Sections were fixed by ice-cold acetone (50 mL/L) for 2 min at 4℃, and blocked with 5 g/L skim milk at room temperature for 2 h. The sections were then incubated in rabbit anti-glucagon (1: 500, Abcam, ab11195), rabbit anti-glucagon (1:500, Abcam, ab 930), and mouse anti-insulin (1:100, BioGenex, MU029-UC) antibodies in 5 g/L skim milk at 37℃ for 2 h, washed twice by PBS, then followed with secondary antibody incubation (goat anti-mouse IgG with FITC conjugated for insulin detection, Jackson115-095-075, 1:500; goat anti-rabbit IgG with FITC conjugated for glucagon detection, Chemicon AP132F, 1:500; and goat anti- rabbit IgG- TRITC, Chemicon AP132R, 1:500). Specimens were washed in PBS three times after incubating with the secondary antibody and coverslips were applied using Fluoromount-G. The slide plating with HepG2 cells (ATCC) was used as a negative control (data not shown).

Measurement of insulin content/secretion

The differentiated cells from Matrigel™ were washed three times with PBS, and placed in 12-well dishes (Falcon, USA) with RPMI culture medium supplemented with 10 mL/L FBS and adjusted the glucose concentration up to 16 mmol/L (the RPMI contained 5 mmol/L glucose origin) then cultivated 48 h. The supernatant was collected and centrifuged to examine the insulin secretion, and kept at -80℃ before use. The attached cells were treated with cold acid-ethanol (0.1 N hydrochloric acid in absolute ethanol) and kept at 4℃ overnight to examine insulin content by ELISA (Mercodia, Sweden). The clear supernatants were used to investigate the intracellular insulin content and the values obtained were normalized relative to the total protein content (protein assay reagent, Bio-Rad, USA). The RIN-m5F insulinoma cell line (CRL-11605, ATCC) and undifferentiated PSCs were used as controls.

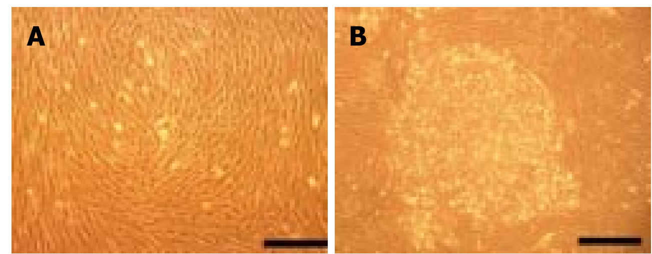

Figure 1 Putative PSC isolated from adult pancreas. **A**: Morphology of cultivated putative PSCs; **B**: Cells aggregate when saturated. bar = 100 μm.

Identification of cell phenotypic markers by FACS

Putative PSC of passage 10 and 20 was used for phenotypic marker identification by FACS. 1×10^5 cells were resuspended in 100 μL PBS and incubated with primary antibodies at 4℃ for 1 h with 1:100 dilutions. After washing twice with PBS, labeled cells were resuspended in 100 μL PBS with 1 μL goat anti-mouse IgG conjugated with FITC (Chemicon, AP124F) at 4℃ for 1 h, then examined by flow cytometry (BD, USA). The information of antibodies used in investigation was listed in Table 1.

RESULTS

Putative stem cells with nestin expression isolated from adult human pancreas

Putative PSCs with the property of sphere-like cell mass formation were cultivated by a series protocol of isolation and the divergent adhesion to bacteria Petri dishes. Cells with spindle-like shape were observed after plating and served with DMEM/F12 ITSFn serum free medium. The morphological homogeneity of putative PSC was demonstrated (Figure 1A). While the density of culture cells increased, the sphere-like cell aggregation was shown (Figure 1B). The expression of *nestin* and *pdx-1* were detected by RT-PCR, with sustained expression of both genes detected through 20 passages completing this study (RT-PCR examined, Figure 2). The protein level of nestin and pdx-1 was also examined by immunofluorescent staining (IF) revealing low protein intensity (data not shown). The experimental consistency was illustrated in the repeated examination of tissues from four individual donors.

The pancreatic differentiation potential of putative PSC

The differential competence of putative PSC was inspected by the growth in Matrigel™. The aggregation ability of the isolated PSCs (passage 10) was observed at d 3 after cell seeding (Figure 3A). Color formation was shown when stained with Dithizone at 4-wk after cell seeding, suggest-

Table 1 Comparative analysis of phenotype between human putative PSC and human BMMSC

Cell	Putative PSC								Human BMMSC	Information of antibodies
	Passage 10				Passage 20					
Antigen / Donor	1	2	3	4	1	2	3	4		
CD29	+	+	+	+	+	+	+	+	+	abcam, ab8238
CD44	+	+	+	+	+	+	+	+	+	abcam, ab6337
CD51	+	+	+	+	+	+	+	+	+	Ancell Corporation, USA
CD81	+	+	+	+	+	+	+	+	+	BD Pharmingen, clone JS81
SH2 (CD105)	+	+	+	+	+	+	+	+	+	ATCC, USA
SH3 (CD73)	+	+	+	+	+	+	+	+	+	ATCC, USA
CD14	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab760
CD38	-	-	-	-	-	-	-	-	+/-	abcam, ab1173
CD49b	+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD49d	+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD50	+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD54	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab1048
CD58	+	+	+	+	+	+	+	+	+/-	abcam, ab1420
CD61	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab7162
CD62E	+	+	+	+	+	+	+	+	+/-	abcam, ab6630
CD90	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab225
CD109	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	BD Pharmingen, 556039
EGFR	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab30
PDGFR-alpha	+	+	+	+	+	+	+	+	+/-	R & D systems, USA
CD7	-	-	-	-	-	-	-	-	-	abcam, ab1249
CD34	-	-	-	-	-	-	-	-	-	abcam, ab8147
CD45	-	-	-	-	-	-	-	-	-	abcam, ab6329
CD62P	+	-	+	-	+	-	+	+	-	abcam, ab6632
CD62L	-	-	-	-	-	-	-	-	-	abcam, ab222
CD120a	-	-	-	-	-	-	-	-	-	Serotec, UK
AC133	-	-	-	-	-	-	-	-	-	Miltenyi Biotec., Germany

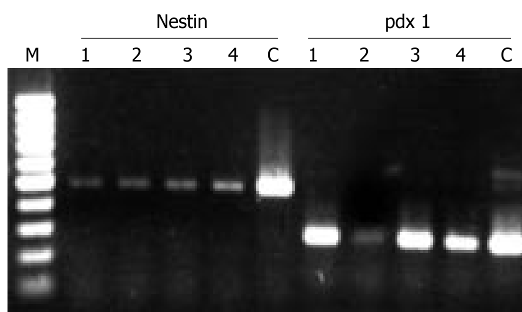

Figure 2 Nestin and pdx-1 expressed in putative PSCs after 5 passages of cultivation. cDNA oriented from 4 individual donors (lane No. 1 to 4), 100 bp marker (M) and the plasmid cloned human nestin and pdx-1 gene with positive control (C) were shown.

ing that cells went through β-cell differentiation in Matrigel™ (Figure 3B).

The expression of *insulin*, *glucagon* and *somatostatin* via RNA level was detectable in the putative PSCs (passage 10) growth in Matrigel™, comparatively; expression was barely illustrated in the group of cells without Matrigel™ (data not shown). Furthermore, the spheroid body of aggregated PSCs showed positive immune reactivity in IF staining (Figures 3 C-E). A similar result was observed in the examination of PSCs after 20 passages, suggesting the differentiation capacity of cells of pancreatic lineage could be preserved.

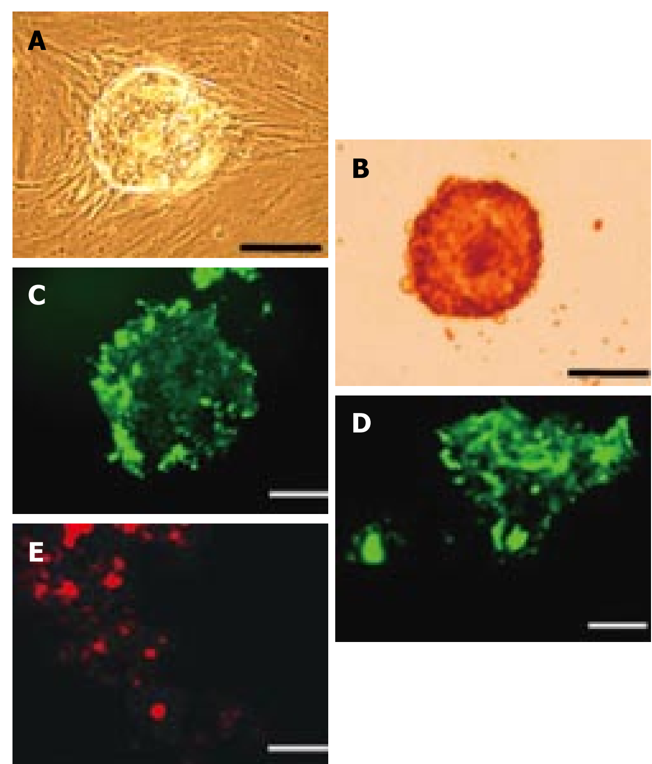

Figure 3 The differentiation of putative PSCs (4 wk). **A**: Growth in Matrigel™; **B**: Dithizone stain; **C-E**: Immunohistochemistry staining by anti-insulin (**C**), glucagon (**D**) and somatostatin (**E**) immunoglobulins. bar = 100 μm.

Measurement of insulin content in differentiated cells

The intracellular insulin content in the differentiating putative PSCs (the samples collected from individual donors were pooled together for measurement) were measured at

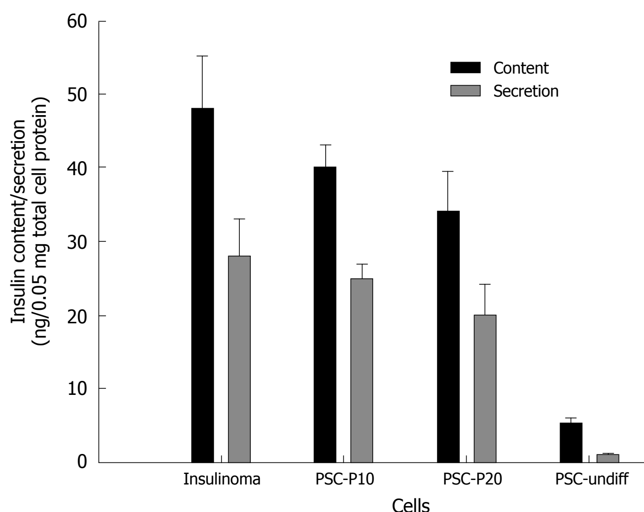

Figure 4 Measurement of Insulin content and secretion in differentiated putative PSCs.

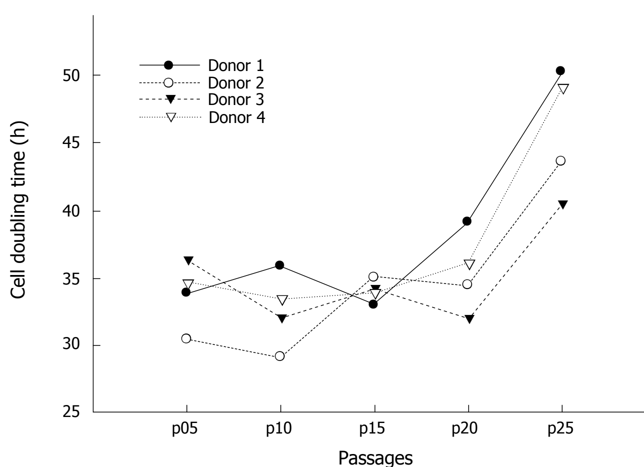

Figure 5 Cell doubling time of putative PSC.

4-wk after cell seeding on Matrigel™ (Figure 4). Compared to the undifferentiated putative PSCs, differentiated PSCs collected at passage 10 and 20 demonstrated the obvious increase in intracellular insulin content/secretion when normalized to the total protein content ($P > 0.01$).

Comparative phenotypic markers spectrum between putative PSC and BMMSC was detected by flow cytometry

To further investigate the potential phenotypic markers in PSCs, the biomarkers that are commonly examined in bone marrow- derived cells were used as the candidates for selection, and the human BMSC was employed as a comparative study (Table 1). The similar patterns of CD29, CD44, CD51, CD81, SH2 and SH3 were illustrated from both cells. Comparatively, differential intensity was demonstrated on the markers CD38, CD49b, Cd49d, CD50, CD58, CD62E and CDGFR-alpha; the stronger intensity was shown in putative PSCs. The markers of CD62P were revealed only in some PSC but not in BMMSC. No significant difference was shown between the putative PSC batches collected from 4 individual donors, moreover, the consistency was demonstrated in the studies of putative PSCs of passages 10 and 20.

Cell viability was monitored in long-period cultivation

A simplified cell proliferation method was used to monitor cell viability. The doubling time of 5, 10, 15, 20, 25 passages was illustrated in Figure 5. The cells proliferated rapidly at the beginning (32 h, approximately), however, a remarkable decrease was observed after 20 passages, and failure to proliferate occurred at approximately 30 passages.

DISCUSSION

The existence of putative PSC in pancreatic duct tissue of adult humans was illustrated in the present study. By using serum free medium with essential growth factors, cells with sphere-like mass aggregation were isolated. The putative PSC expressed both *nestin* and *pdx-1*, and these cells were able to differentiate into pancreatic lineage cells that express *insulin*, *glucagon* and *somatostatin* by Matrigel™. The potential biomarkers were evaluated by FACS, and the antibodies that identify common biomarkers of human bone marrow were used. As a comparative study of human BMSCs, the biomarkers of CD29, CD44, CD51, CD81, SH2 and SH3 (mesenchymal stem cell markers) were all detected on the surface of both cells and higher intensity in PSCs was illustrated in the markers of CD38, CD49b, Cd49d, CD50, CD58, CD62E and CDGFR-alpha. We also found that the expression of CD34, AC 133 (hematopoietic stem cell markers) and CD45 (endothelial marker) were not detected in either PSCs or BMSCs. The consistency of phenotypic patterns was demonstrated between individual (original cells from 4 identical donors) and the passages (to 20 passages).

Bone marrow (BM)-derived stem cells can be aspirated directly from donors. They can be cultured for *ex vivo* expansion. Previous studies demonstrated the pluripotency of these stem cells. They were able to differentiate into ectodermal[25,26], endodermal[26,27], mesodermal[27], hepatic[28], cardiac muscle[29] and skeletal muscle[30] progenitor cells. By using negative selection, a cell subpopulation isolated from bone marrow, muscle and brain cells have been shown to be able to differentiate into all three germ layers[31]. This type of mesenchymal stem cells, termed multipotent adult progenitor cells (MAPCs), have the remarkable potential to differentiate not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endodermal features at the single cell level. Recently, Zhang, *et al*[32] demonstrated that the *nestin*-positive progenitor cells isolated from human fetal pancreas, and these cells also have the phenotype markers identical to mesenchymal stem cells (MSCs). In agreement with these results, our results supported these findings and further isolation of *nestin*- and *pdx-1* -positive adult human pancreatic stem cells, which co-expressed the identical MSC markers. These interesting findings also provide evidence to support the interpretation of the study by Ianus *et al*[33], that bone marrow derived cells have the capacity to be competently pancreatic islet beta cells. Moreover, the differential capacities and properties of MSCs from different organs are

worth additional attention and may be investigated in a subsequent study.

Nestin is a well-discussed marker of the pancreatic stem cells in embryology, *in vitro* cultivation and pancreatectomy-regeneration in animal models[1,11,15,32]. However, a contradictory study provided evidence that *nestin*-lineage cells contribute to the microvasculature of pancreas but not endocrine cells of the islet[1]. The elegant experiment performed by Suzuki *et al*[3], had clearly demonstrated that the *nestin*-positive cells were isolated from the aggregated insulin-producing precursor and endothelium cells of pancreas by FACS assay[3]. This result provided a reasonable explanation that the *nestin* antigen is expressed in a much broader precursor population of pancreas, including differentiated cells. The *pdx-1*, compared to the *nestin*, is more specific for pancreatic differentiation[18-20]. In the present study, the strategy that both the *nestin*- and *pdx-1*- positive cells enriched by serum-free medium was employed followed the previous study of neuronal lineage stem cells isolated from murine embryonic stem cells[9,16]. Our data supported the claim that the *nestin*- and *pdx-1* expressed stem cells can be isolated from adult pancreatic ducts and possess the differentiation potential of the pancreatic lineage. Moreover, we found that the sustained expression of *nestin* and *pdx-1* in PSCs further exhibited and correlated to the stem cell characteristics of the insulin-producing ability, stable passage and long-term maintenance in vitro. Thus, *nestin* and *pdx-1* could not only be the markers of pancreatic stem cells but also play an important role in the self-renewal of beta progenitor cells.

In summary, we use the serum-free method and successfully isolate pancreatic stem cells from adult human pancreatic duct. These PSCs not only expressed the *nestin* and *pdx-1* but also exhibited the markers of mesenchymal stem cells. PSCs and the usage of serum free medium may avoid the potential immune problem of xenogenic protein contamination. Furthermore, this approach should overcome the ethical and immunologic concerns associated with the use of fetal tissues and embryonic stem cells. Although more work is needed to elucidate the role of these PSCs, the application of these PSCs can further be extended and used as an alternative source for therapeutic strategies of diabetes mellitus.

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