

ORIGINAL ARTICLES

In vitro cultivation of islet-like cell clusters from human umbilical cord blood-derived mesenchymal stem cells

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A major obstacle to successful islet transplantation for both type 1 and 2 diabetes is an inadequate supply of insulin-producing tissue. *In vitro* transdifferentiation of human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) into insulin-producing cells could provide an abundant source of cells for this procedure. For this study, we isolated and characterized human UCB-MSCs and induced them *in vitro* to differentiate into islet-like cell clusters using a 15-day protocol based on a combination of high-glucose, retinoic acid, nicotinamide, epidermal growth factor, and exendin-4. These clusters appeared about 9 days after pancreatic differentiation; expressed pancreatic β -cell markers, including *insulin*, *glucagon*, *Glut-2*, *PDX1*, *Pax4*, and *Ngn3*; and could synthesize and secrete functional islet proteins at the end of the inducing protocol. The insulin-positive cells accounted for (25.2–3.36)% of whole induced cells. Although insulin secretion of those insulin-producing cells did not respond to glucose challenge very well, human UCB-MSCs have the ability to differentiate into islet-like cells *in vitro* and may be a potential new source for islet transplantation. (Translational Research 2008;151:293–302)

Abbreviations: BM = bone marrow; BM-MSCs = bone marrow-derived mesenchymal stem cells; ECM = extracellular matrix; EDTA = ethylenediamine tetra-acetate; EGF = epidermal growth factor; ESC = embryonic stem cell; FBS = fetal bovine serum; H-DMEM = high glucose Dulbecco modified Eagle medium; L-DMEM = low glucose Dulbecco modified Eagle medium; MSC = mesenchymal stem cell; MNC = mononuclear cell; NSE = neuron-specific enolase; PBS = phosphate-buffered solution; RA = retinoic acid; RT-PCR = reverse transcription polymerase chain reaction; UCB = umbilical cord blood; UCB-MSC = human umbilical cord blood-derived mesenchymal stem cell.

Recently, significant advances (Edmonton protocol) in the transplantation of human primary islets of Langerhans into individuals with type 1 diabetes have largely removed the insulin dependency.^{1,2} But

based on Edmonton protocol, as many as 2 to 3 donor pancreases are required to obtain sufficient islets for a single type 1 diabetic patient becoming insulin independent. So the application of this treatment is restricted by the very limited availability of primary human islets from donors. Nowadays, a widely studied potential source for insulin-producing cells is stem/progenitor cells. Possible beta-cell stem/progenitors have been hypothesized to reside within the pancreatic ductal epithelium,^{3–6} acinar tissue, and/or pancreatic small cells.^{7,8} Moreover, it has been reported that embryonic stem cells (ESCs)^{9,10} or stem cells derived from bone marrow can be differentiated *in vitro* and *in vivo* into insulin-expressing cells.^{11,12}

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AT A GLANCE COMMENTARY**Background**

Based on the Edmonton protocol, as many as 2 to 3 donor pancreases are required to obtain sufficient islets for a single type 1 diabetic patient becoming insulin independent. So the application of islet transplantation is restricted by the very limited availability of primary human islets from donors. Recently many attempts have been made to generate new insulin-producing cells *in vitro* from non- β cells.

Translational Significance

Umbilical cord blood (UCB) is abundantly available and can be routinely harvested without risk to the donor, and infectious agents such as the cytomegalo virus are rare exceptions. This study revealed for the first time that mesenchymal stem cells derived from human cord blood were capable of differentiating into insulin-secreting cells, which may be a potential new source for islet transplantation.

As an adult stem cell, recent studies have shown that mesenchymal stem cells (MSCs) have the ability to differentiate into several neuroectodermal, endothelial, mesenchymal, and endodermal cell types,^{13–16} and the ability to differentiate into insulin-expressing cells has also been demonstrated.^{17,18} The multipotential of these cells as well as their high *ex vivo* expansive potential makes these cells an attractive therapeutic tool for diabetes. Currently, bone marrow (BM) represents the main source of MSCs for both experimental and clinical studies,¹⁹ but the source of BM is limited, and the amount of bone marrow–derived MSCs and their differentiation capacity decline with age.²⁰ In addition, obtaining a BM sample requires a painful invasive procedure. These facts have led investigators to search for good BM substitutes as MSC sources.

Umbilical cord blood (UCB) is abundantly available and is known to contain extremely immature stem cells (including MSCs), which can be routinely harvested without risk to the donor. In addition, infectious agents such as cytomegalo virus are rare exceptions.²¹ These characteristics make UCB a very promising candidate suitable for cellular therapy and regenerative medicine. Some researchers have demonstrated that the population of stem cells from UCB that expressed at embryo stage or ESC marker could be differentiated into insulin-producing cells *in vitro*.^{22,23} But until now, a key question has re-

mained: Do human UCB-derived MSCs have the plasticity to differentiate into insulin-producing cells *in vitro*. In this study, we demonstrate that UCB-derived MSCs can be *in vitro* induced into pancreatic endocrine cells. This study provides support for continuing efforts aimed at using adult stem cells as a steady and renewable source of insulin-producing cells for transplantation in patients with type 1 diabetes.

MATERIALS AND METHODS

Collection of human UCB. Human UCBs (n = 42, 36–40 weeks) were obtained from the Department of Obstetrics and Gynecology, the Second Hospital of Harbin Medicine University. UCB was collected from the umbilical cord vein with informed consent of the mother. A bag system containing 17 mL of citrate phosphate dextrose anticoagulant was used. All UCB units were processed within 3 h after deliveries. The study protocol was approved by the Ethical Committee of the Second Hospital of Harbin Medicine University.

Culture of MSCs from UCB. To isolate mononuclear cells (MNCs), each UCB unit was diluted 1:1 with phosphate-buffered saline (PBS) and carefully loaded onto Ficoll-Hypaque solution (1.077 g/mL; Sigma-Aldrich Co, St. Louis, Mo). After density gradient centrifugation at 800g for 16 min at room temperature, MNCs were removed from the interphase and washed twice with PBS and resuspended in low glucose Dulbecco modified Eagle medium (L-DMEM, 5.5 mmol/L glucose; Invitrogen Corporation, Grand Island, NY) supplemented with 30% fetal bovine serum (Invitrogen). After counting, cell suspension was seeded in uncoated T25 culture flasks (Orange Scientific, Belgium) at a concentration of 1×10^6 cells/mL. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂, and the medium was changed 8 days later. When fibroblast-like cells at the base of the flask reached confluence, they were harvested with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA; Sigma-Aldrich) and passaged at 1:3 dilution as passage 1. Then the medium was half-changed with L-DMEM containing 20% fetal bovine serum (FBS) every other day to make 30% FBS concentration decrease to 20%.

***In vitro* differentiation cultures.** For differentiation into osteogenic cells, the human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) at third passage were plated at 1×10^4 cells/well in a 24-well plate. At 90% confluency, the cells were cultured for ~21–22 days in L-DMEM supplemented with 10% FBS, 10^{-7} M dexamethasone (Sigma-Aldrich), 50 μ mol/L ascorbic acid-2 phosphate (Sigma-Aldrich), and 10 mmol/L β -glycerol phosphate (Sigma-Aldrich).²⁴ Osteogenic differentiation was confirmed by Von Kossa staining.

For differentiation into adipogenic cells, the cells at third passage were plated at 1×10^4 cells/well in a 24-well plate. At 80% confluency, the cells were cultured for ~21–22 days in L-DMEM supplemented with 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 1 μ mol/L dexamethasone, 0.1 mmol/L indomethacin (Sigma-Aldrich), and 10% FBS.²⁵ Adipogenic

differentiation was evaluated by the cellular accumulation of neutral lipid vacuoles that were stained with Oil-red O.

For differentiation into neural cells, the cells at third passage were plated at 1×10^4 cells/well in a 24-well plate. At 70% confluency, the cells were cultured with L-DMEM supplemented with 1 mmol/L β -mercaptoethanol (Shanghai Shenggong, Shanghai, China) and 10% FBS for 24 h. Then the media were switched to L-DMEM supplemented with 2% dimethylsulfoxide (Shanghai Shenggong) and 200 mmol/L butylated hydroxyanisole (Sigma-Aldrich) for 5 h.²⁶ Neural differentiation was confirmed by immunofluorescence staining of neuron-specific enolase (NSE).

For pancreatic endocrine differentiation, expanded MSCs from passage 3 were allowed to become 80% to 90% confluent and were induced to differentiate into insulin-secreting cells by a 3-step protocol. In step 1, the cell monolayer was treated for 24 h with high glucose Dulbecco modified Eagle medium (H-DMEM; 25 mmol/L glucose) supplemented with 10% FBS and 10^{-6} M of retinoic acid (RA; Sigma-Aldrich); then the medium was switched to H-DMEM with only 10% FBS for 2 days. In step 2, the cells were detached with 0.25% trypsin-EDTA and seeded in extracellular matrix (ECM) gel (Sigma-Aldrich) coated 12-well plates. The medium was switched to L-DMEM, supplemented with 10% FBS, 10 mmol/L nicotinamide (Sigma-Aldrich), and 20-ng/mL epidermal growth factor (EGF; Peprotech, London, UK) for 6 days. In step 3, to mature the insulin-producing cells, the low glucose medium was supplemented with 10% FBS and 10 nmol/L exendin-4 (Sigma-Aldrich) for 6 days. Cellular differentiation was monitored by observation of 3-dimensional, islet-like cell cluster formation, the expression of genes related to pancreatic endocrine cell development and insulin production. As a control group, the cells were cultured in L-DMEM containing only 10% FBS.

Electron microscopic analysis. Preinduced cells and differentiated pancreatic endocrine cells were fixed in 5% glutaraldehyde for 2 h at 4°C, washed in PBS, transferred to 1% osmic acid for 2 h at 4°C, then washed in PBS again, dehydrated in acetic acid, and embedded. Ultra-thin sections were counterstained using uranyl acetate and lead citrate, and they were viewed by electron microscope JEM-1220 (JEOL, Tokyo, Japan).

Immunofluorescence. Preinduced and induced cells were released by 0.25% trypsin and washed 3 times by PBS. Then cytospin slides were made for insulin, C-peptide, glucagon, and NSE protein expression. The cells were fixed with 4% formaldehyde for 30 min at room temperature and were incubated overnight at 4°C with primary antibodies, including mouse anti-human NSE 1:50 (Zhongshan Goldenbridge Biotech Co., Beijing, China), mouse anti-human insulin 1:100 (Sigma-Aldrich), mouse anti-human C-peptide 1:100 (Abcam Co., Cambridge, UK), and goat anti-human glucagon 1:100 (Santa Cruz Biotechnology, Santa Cruz, Calif). Subsequently, the cells were washed with PBS 3 times and were incubated at 37°C for 1 h with a fluorescence-labeled second antibody, including FITC-labeled goat anti-mouse IgG 1:50, rhodamine-labeled goat anti-mouse IgG 1:50, and rhodamine-labeled rabbit anti-goat IgG 1:50 (Zhongshan Goldenbridge). After

being washed with PBS, cells were mounted with glycerol-PBS (9:1). The cells were visualized and photomicrographed by a laser-scanning confocal microscope TE2000-U (Nikon, Tokyo, Japan).

Western blot analysis. Preinduced and induced cells were released by 0.25% trypsin and harvested under centrifugation. Then they were lysed in buffer containing 50 mmol/L Tris-Cl (pH 8.0), 0.02% sodium azide, 1- μ g/mL aprotinin, 1% NP-40, and 100- μ g/mL phenylmethylsulfonyl fluoride. Final protein concentrations were determined using the BCA protein assay kit (Beyotime, Haimen, China) according to the manufacturer's specifications. Equal amounts of protein (100 μ g) from each cell of lysates were added to a well of 18% SDS-polyacrylamide gels and electrophoresed. The separate proteins then were transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) and blocked for 2 h in 5% fat-free dry milk, 0.1% Tween 20, 150 mmol/L NaCl, and 50 mmol/L Tris. Then blots were incubated overnight at 4°C with primary antibodies, including mouse anti-human insulin 1:50 (Sigma-Aldrich) and rabbit anti-human C-peptide 1:50 (Abcam Co.). After extensive washing, polyclonal anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (diluted at 1:500) was applied for 1 h at room temperature. Bands were visualized using an ECL kit (Amersham) according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted by using TRIzol (Invitrogen) according to the manufacturer's instructions and was quantified by ultraviolet spectroscopy. To prepare RNA for PCR analysis, 2- μ g total RNA was converted to the cDNA using SuperScript II reverse transcriptase (Invitrogen) with oligo (dT) (Promega, Madison, Wis) and random hexamer primers (Promega). PCR was performed by using Taq DNA polymerase (Invitrogen). All PCR experiments were performed using a PCR system TC-XP-G (Bioer, Hangzhou, China). Products were tested with polyacrylamide gel electrophoresis. The name and sequences of the primers, the sizes of PCR products, cycles, and annealing temperature for each pair are listed in Table I. Housekeeping gene β -actin was used as an internal control.

Flow cytometric analysis. Immunophenotyping of human UCB-MSCs were performed using antibodies against human antigens CD19, CD14, CD29, CD34, CD44, CD45, CD90 (BD Biosciences, San Jose, Calif) and CD105 (Caltag Laboratories, Burlingame, Calif). The MSCs cells at third passage were released by 0.25% trypsin-EDTA. A total of 1×10^6 cells were resuspended in 200- μ L PBS and were incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies for 30 min at room temperature. After being washed, the cells were analyzed with a flow cytometer.

To determine the amount of insulin-positive cells, preinduced cells and differentiated pancreatic endocrine cells were released by trypsinization and were analyzed by flow cytometry. Briefly, cells were fixed with cold 1% formaldehyde, incubated with 0.01% Triton X-100, washed twice with PBS, and then stained with anti-insulin antibodies and rhodamine-labeled second antibodies. After being washed twice with PBS, cells were analyzed using a FACSCalibur (BD).

Table I. Primers used for PCR amplification

Gene	Size (bp)	Strand	Sequence 5'-3'	Annealing temperature	Cycles
Glucagon	221	F	CCC AAG ATT TTG TGC AGT GGT T	45°C	30
		R	GCG GCC AAG TTC TTC AAC AAT		
Insulin	263	F	GCA GCC TTT GTG AAC CAA CA	45°C	30
		R	GTT GCA GTA GTT CTC CAG GTG		
Ngn3	313	F	GGT AGA AAG GAT GAC GCC TC	45°C	30
		R	CCG AGT TGA GGT CGT GCA T		
Pax 4	496	F	AGG AGG ACC AGG GAC TAC CGT	45°C	30
		R	TTT AGG TGG GGT GTC ACT CAG		
PDX1	262	F	CCC ATG GAT GAA GTC TAC C	43°C	30
		R	GTC CTC CTC CTT TTT CCA C		
GLUT2	398	F	GTA CAA TGA CAG AAG ATA AG	43°C	30
		R	TGC TAC TAA CAT GGC TTT GA		
β -actin	160	F	GTC AGG TCA TCA CTA TCG GCA AT	57°C	30
		R	AGA GGT CTT TAC GGA TGT CAA CGT		

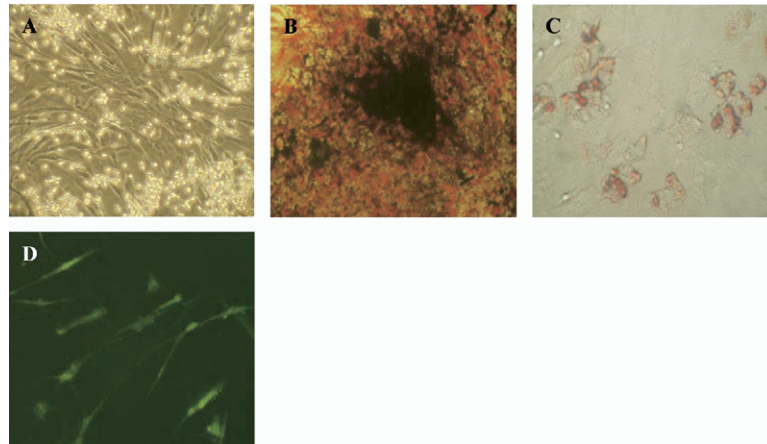


Fig 1. Osteogenic, adipogenic and neural differentiation *in vitro*. (A) After first inoculation, spindle-shaped cells colony formation could be observed at eighth day while osteoclast-like cells were rare ($\times 200$); (B) *In vitro* osteogenic induction of MSCs. Enhanced mineralization revealed by von Kossa staining ($\times 100$); (C) Adipogenic induction was verified by staining with Oil-Red O ($\times 200$); (D) Neural differentiation was verified by staining with NSE ($\times 200$). (Color version of figure is available online.)

Insulin detection assay. To test whether the insulin release of induced endocrine cells was glucose-dependent, 3 glucose concentrations (5.5 mmol/L, 16.5 mmol/L, and 28 mmol/L) were used. The differentiated cells plated in 12-well dishes were washed twice with PBS and incubated in 0.5-mL serum-free DMEM containing 5.5 mmol/L glucose for 4 h at 37°C. The media were collected and measured for basal insulin secretion, and then fresh media with 5.5 mmol/L, 16.5 mmol/L, or 28 mmol/L glucose were added. At the end of this second 4 h of incubation, the final media were collected and frozen at -70°C until they were assayed for insulin content. To estimate total cellular insulin levels, the total cell protein content was tested using the BCA Protein Assay Kit (Beyotime). Measurement of cellular and secreted insulin was performed with chemiluminescence immunoassay system ADVIA centaur (Bayer, Tarrytown, NY). Preinduced MSCs performed as above were used as a control group.

Statistics analysis. Data are presented as mean \pm SD.

Results were analyzed by independent-samples *t*-test and 1-way analysis of variance. Statistical significance was set at $P < 0.05$.

RESULTS

Isolation and characterization of human UCB-MSCs. In this study, we successfully isolated 16 MSCs from 42 term UCB units (16/42, 38.09%). The onset of fibroblast-like cell colony formation could be observed approximately during 6 to 12 days after first seeding, and attachment of osteoclast-like cells to culture flasks was rare (Fig 1, A). Osteogenic differentiation and adipogenic differentiation could be induced in those cells defined by von Kossa and Oil-red O staining (Fig 1, B and C). Neural-like cells could be induced and defined by expression of NSE (Fig 1, D). Flow cytometric

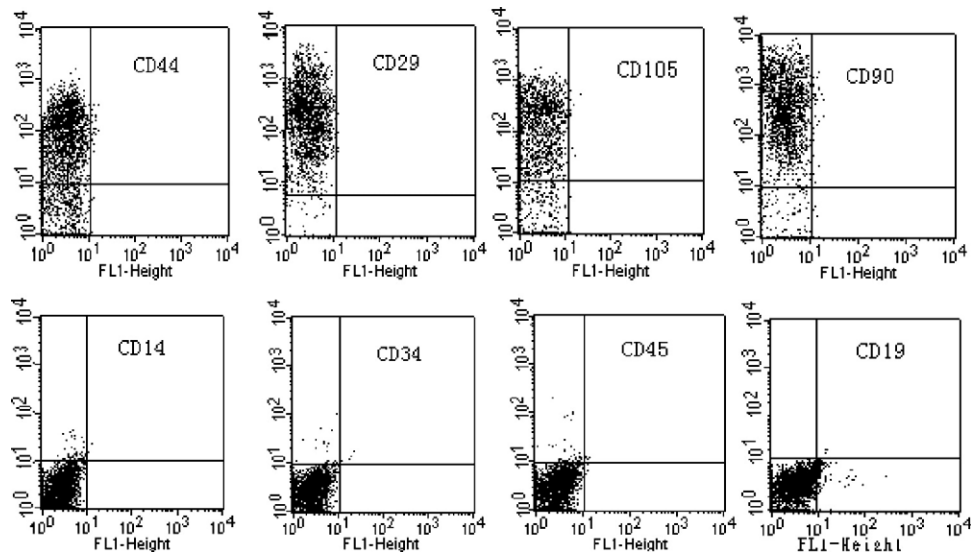


Fig 2. Immunophenotyping of MSCs from human UCB. Cells at passage 3 were examined by flow cytometry. UCB-MSCs were strongly positive for MSC-specific markers such as CD29, CD44, CD105 and CD90, while negative for CD14, CD19, CD34 and CD45.

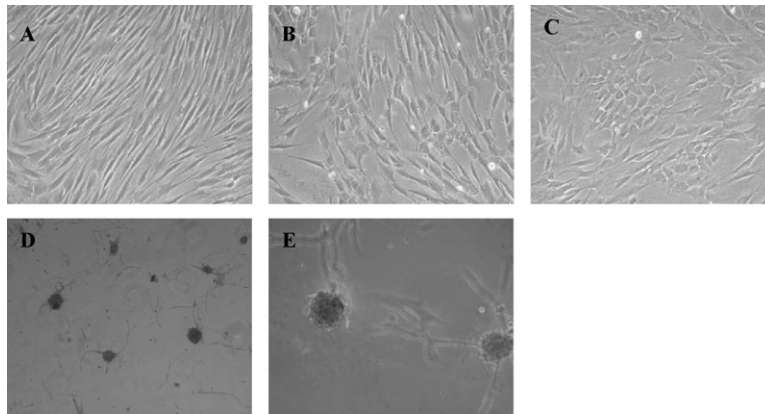


Fig 3. Morphological changes in endocrine differentiation. (A) The cells were spindle-shaped and fibroblast-like before induced; (B, C) In step 2, the induced cells turned shorter and changed into round epithelial-like ($\times 200$); (D, E) Islet-like cell clusters appeared at end of step 2, and more islet-like clusters were formed in step 3 (D $\times 100$, E $\times 400$).

analysis showed that MSC-specific markers CD29, CD44, CD90, and CD105 were detected, but that hematopoietic antigens CD34, CD45, lymphomonocyte antigen CD14, and endothelial antigen CD19 could not be detected (Fig 2).

***In vitro* differentiation of UCB-MSCs into insulin-producing cells.** UCB-MSCs were induced into islet-like cells by a 3-step, 15-day protocol. At step 1, changes in cell morphology could not be observed. During continued culture, the rate of cell proliferation became slower, and these spindle-like cells became short and changed into round and epithelial-like at the end of step 2, about 9

days after differentiation. Meanwhile, some new islet-like clusters started to appear, ranging from 100 to 150 μm in diameter. At step 3, more islet-like clusters were formed, and there were 3 ± 1.4 clusters per cm^2 (Fig 3). Ultrastructural analysis showed that induced cells contained small secretory granules, which is a characteristic of pancreatic endocrine cells (Fig 4).

Moreover, we found that islet-like clusters did not form when UCB-MSCs were induced on dishes without ECM gel in step 2, and the control MSCs cultured on dishes with ECM gel coated could not produce clusters either.

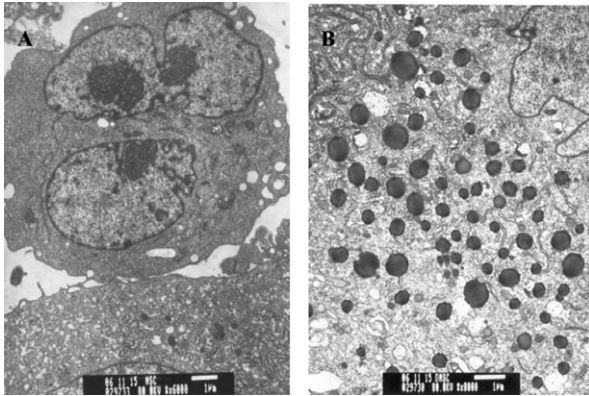


Fig 4. Electron microscopic analysis of islet-like cells. (A) Few secretory granule was found in pre-induced human UCB-MSCs ($\times 6000$); (B) But within the cytoplasm of induced cells, small secretory granules were often found ($\times 8000$).

RT-PCR analysis of gene expression of insulin-producing cells. To determine whether UCB-MSCs had differentiated into pancreatic endocrine cells, gene expression profiles for pancreatic β -cell differentiation markers and hormones were assessed by RT-PCR. As illustrated in Fig 5, endocrine cell differentiation-related genes were not expressed in preinduced cells. After differentiation, many characteristic pancreatic endocrine cell marker genes were expressed at the end of our protocol, such as PDX-1, Pax4, Ngn3, insulin, glucagon, and Glut-2. When induced by the 3-step protocol without the ECM gel coated, the differentiated cells only weakly expressed PDX-1, Pax4, and Ngn3, and small cluster formation could not be observed.

Immunofluorescence and western blot analysis of functional islet proteins. To investigate the expression of pancreatic hormones, immunofluorescence analyses was performed for insulin, C-peptide, and glucagon in MSCs-derived clusters (Fig 6). The result showed that the cells were positive staining for insulin and C-peptide. Because the anti-C-peptide antibody recognizes both C-peptide and proinsulin, the positive staining of C-peptide indicated that the differentiated cells were likely to synthesize and process insulin. Furthermore, we found some cells were positive staining for glucagon. But do insulin-positive cells also coexpress glucagon? Or the cell expressing insulin is different from the cell expressing glucagon. We performed double immunofluorescent staining of insulin and glucagons and found that the insulin-positive cells were negative staining for glucagons. However, considering the insulin content and responsibility to glucose stimulation, we suspected the induced cells were most likely to represent immature pancreatic endocrine cells that coexpressed insulin and glucagon. So the result needs additional testing.

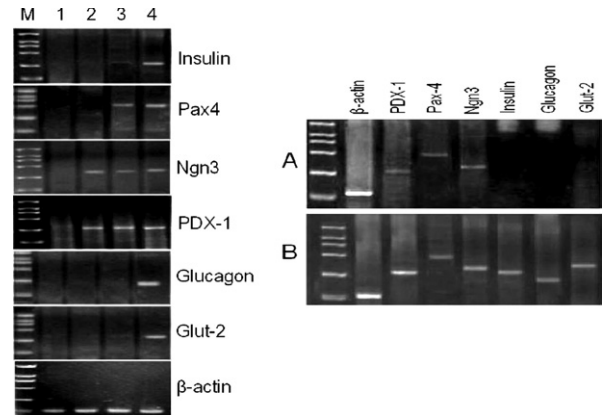


Fig 5. Gene expressions during human UCB-MSCs differentiation. M: DNA marker; 1: Pre-induced MSCs; 2: Cells at end of step 1; 3: Cells at end of step 2; 4: Cells at end of step 3; A: Without ECM gel; B: ECM gel coated.

Furthermore, Western blot analysis showed that only proinsulin was detectable in the cell lysate, but that insulin and C-peptide were not. The reason may be the detection of C-peptide and insulin in Western blot is relatively difficult, because each of them is only about 5 kDa big. The other probable reason is that the cells maybe immature and store insulin in its proinsulin form (Fig 7).

Flow cytometric analysis of insulin-positive cells. It was found that $(1.11 \pm 0.15)\%$ of preinduced cells were insulin-positive, whereas $(25.2 \pm 3.36)\%$ of differentiated cell were insulin-positive ($n = 6$, $t = 17.50$, $P < 0.01$) (Fig 8, A).

Insulin content and release in response to glucose stimulation. Insulin secretion analysis indicated that preinduced cells showed no significant release of insulin in the presence or absence of glucose challenge (Fig 8, B). After differentiation, these islet-like cells produced much more insulin and secreted it into extracellular medium. However, those differentiated cells were not responsive to glucose challenge very well. No significant increased insulin secretion was observed in higher glucose concentration. Because β cells of the pancreatic islets of Langerhans act as glucose sensors, adjusting insulin output to the glucose level, the relative defect in the insulin response to glucose indicates those differentiated cells are most likely incompetent immature islet-like cells.

As an additional assessment, we evaluated the content of total cellular insulin in both preinduced (0.14 ± 0.02 -ng/mg protein) and induced cells (12.28 ± 4.59 -ng/mg protein) and found that induced cells showed a significant increase ($n = 7$, $t = 6.99$, $P < 0.01$). The proportion of insulin release to insulin content in differentiated cells was approximately 9.2%.

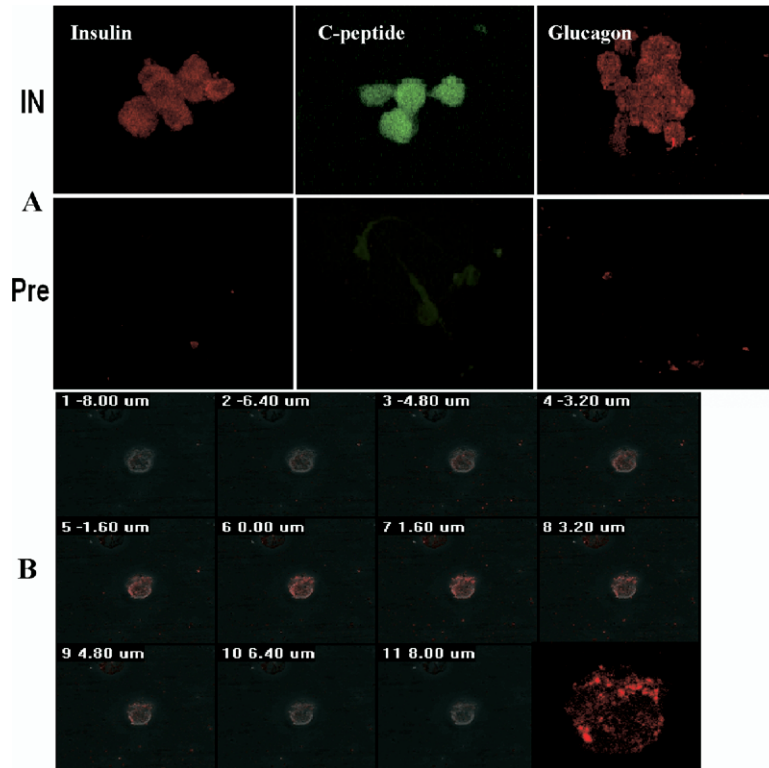


Fig 6. Insulin, C-peptide and glucagon production by induced human UCB-MSCs. (IN: induced cells; Pre: pre-induced cells) (A) Pre-induced human UCB-MSCs were negative for islet hormones staining, but induced UCB-MSCs showed the staining of insulin, C-peptide and glucagon. (B) Series of optical sections by a laser-scanning confocal microscope through the insulin positive cells were acquired at 1.6 μm intervals in the axial dimension. Small light insulin secretory granules were in cytoplasm ($\times 400$). (Color version of figure is available online.)

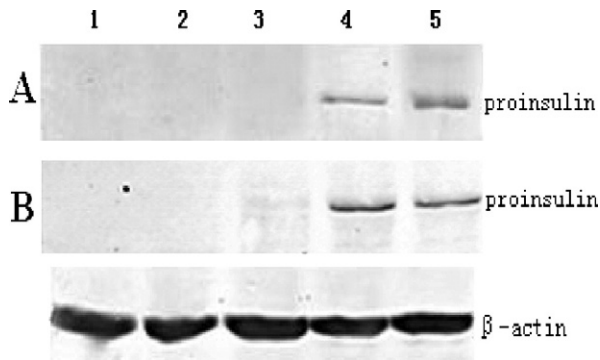


Fig 7. Western blot analysis of insulin and C-peptide in differentiated human UCB-MSCs. A: Western blot analysis of insulin; B: Western blot analysis of C-peptide. 1: Pre-induced MSCs; 2: Cells at end of step 1 (3 days after differentiation); 3: Cells at end of step 2 (9 days after differentiation); 4: Cells at 12 days after differentiation; 5: Cells at end of step 3 (15 days after differentiation).

DISCUSSION

Nowadays many attempts have been made to generate new insulin-producing cells *in vitro* from non- β cells.^{3-12,17,18,27} As an excellent candidate for cell-

based therapy, MSCs are multipotent, and it has been demonstrated that mouse bone marrow-derived mesenchymal stem cells (BM-MSCs) can differentiate into insulin-expressing cells. The use of MSCs to treat diabetes seems promising. In the study reported here, we obtained islet-like clusters from human UCB-MSCs using a 3-step protocol. These induced cells express pancreatic β -cell markers, including *insulin*, *glucagon*, *Glut-2*, *PDX1*, *Pax4*, and *Ngn3*, and they can synthesize and secrete functional islet proteins, such as insulin, glucagon, and C-peptide. It indicates the potential for deriving functional β cells from human UCB-MSCs and warrants additional investigation.

Recently, several different laboratories have successfully isolated MSCs from UCB,²⁸⁻³⁰ but some researchers do not think UCB is a rich source of MSCs for the low frequency of MSCs in term UCB.³¹ In this study, we have obtained fibroblast-like cells that showed some characteristic features of MSCs from term UCB with an efficiency of greater than 38%, using L-DMEM containing 30% FBS. Those cells showed plastic-adherent capability. The immunophenotype of these cells, which seemed similar to that of MSCs

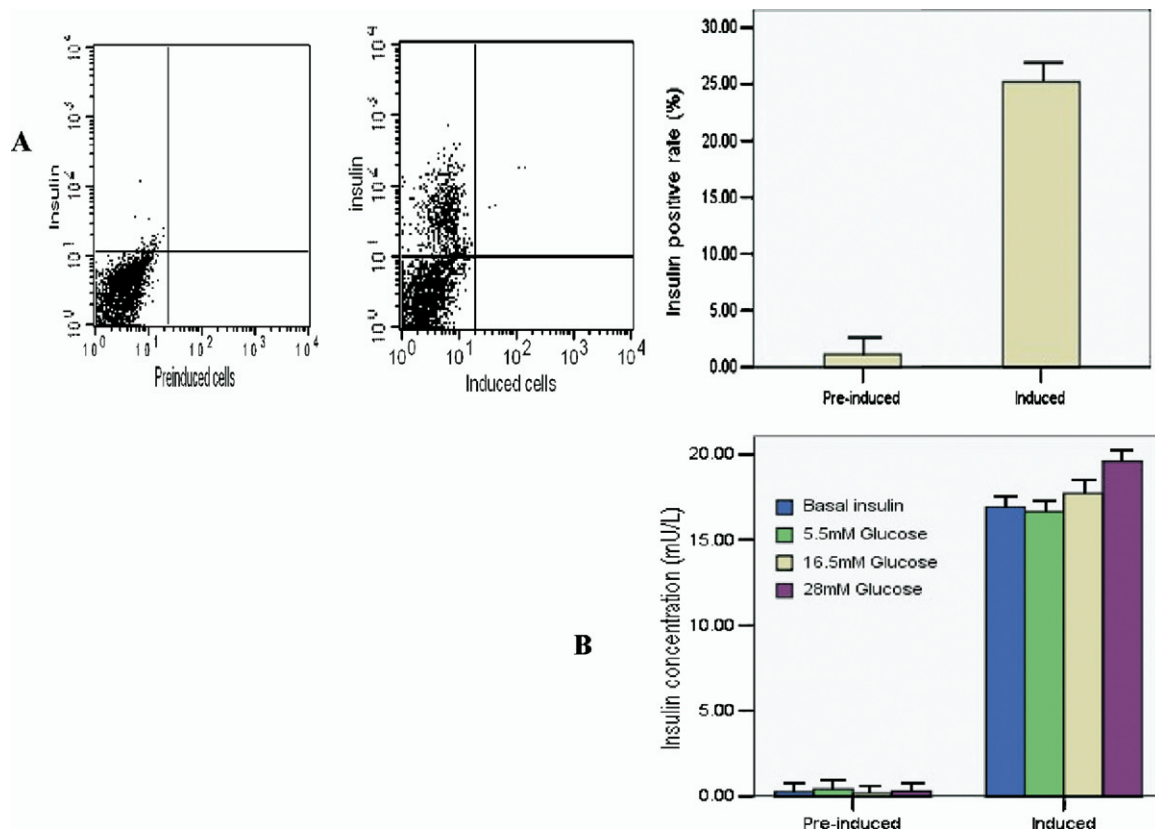


Fig 8. Frequency of insulin positive cells and insulin secretion of pre- and induced human UCB-MSCs. (A) Insulin positive rate of pre-induced cells was $(1.11 \pm 0.15)\%$, while that of induced cells was as high as $(25.20 \pm 3.36)\%$ ($n = 6$, $t = 17.50$, $P < 0.01$); (B) Basal insulin secretion of pre-induced MSCs was low to 0.25 ± 0.28 mU/L, but that of induced cells was dramatically increased to 16.91 ± 3.70 mU/L ($n = 6$, $t = 10.98$, $P < 0.01$). Insulin secretion of induced cells in response to glucose showed that although insulin concentration increased with glucose challenge (5.5 mM: 16.65 ± 3.29 mU/L; 16.5 mM: 17.71 ± 2.54 mU/L; 28 mM: 19.57 ± 3.66 mU/L), the difference was not statistically significant ($n = 6$, $F = 1.05$, $P = 0.38$). (Color version of figure is available online.)

reported by Kern et al,²⁷ is negative for CD45, CD34, CD19, and CD14, but positive for CD29, CD44, CD90, and CD105. In addition, these MSC-like cells have the capability to differentiate into osteoblasts, adipocytes, and neural-like cells. These findings are consistent with the characterization of MSCs that had been reported.^{32,33} Therefore, we believe that the cells we have obtained most likely represent mesenchymal stem cells derived from human UCB.

Kern et al²⁹ indicated that in contrast to BM- and adipose tissue-MSCs, human UCB-MSCs did not show adipogenic differentiation capacity, and the difference may be related to tissue source. However, in this study, we successfully differentiated human UCB-MSCs into adipocytes, which is consistent with what Lee et al²⁵ have reported. The conflicting data may be caused by UCB-MSCs' less sensitivity toward the adipogenic differentiation and loss of adipogenic potential in Kern

et al's postconfluence inducing system because of progressive aging. Some studies have reported changes in MSCs morphology and phenotype with repeated passages *in vitro* and have also emphasized the loss of adipogenic potential when they approached the end of their proliferative life span.^{14,34} Actually, although MSCs are multipotent, MSCs isolated from different tissues might exhibit diverse differentiation capacities. For example, MSCs isolated from different fetal tissues during the second trimester showed diverse adipogenic and osteogenic differentiation potentials.³⁵ The adipogenic capacity was markedly lower, but the osteogenic capacity was higher, in the MSCs derived from fetal spleen than from bone marrow, liver, or lung.³⁵ Chang et al³⁶ also reported a higher adipogenic potential in BM-MSCs than UCB-MSCs. Therefore, although several studies have demonstrated that mouse BM-MSCs can be transdifferentiated into insulin-secreting cells *in vitro*, there is still the key question of whether human

UCB-MSCs can differentiate into insulin-secreting cells *in vitro*.

During embryonic development, a cascade of transcription factors is activated to initiate the development of pancreas. A key player in this system is the transcription factor *PDX-1*, which is commonly expressed in all pancreatic progenitor cells.³⁷ RA is an important signaling molecule in the development of the early embryonic pancreas, which can induce *PDX-1*+ endoderm formation.^{38,39} Moreover, a high-glucose culture represents a critical factor for adult stem cell transdifferentiation into insulin-producing cells.^{17,40} In this study, the induced cells cultured with high-glucose and RA for 3 days expressed *PDX-1* and another pancreatic endocrine cell marker-*Ngn3*. The combination of high glucose and RA specifically activates pancreatic endocrine cell differentiation from UCB-MSCs. To promote the maturation of induced cells, some maturation factors, such as nicotinamide, EGF, and exendin-4, were used in subsequent culture. Nicotinamide is a poly (ADP-ribose) synthetase inhibitor that can induce islets forming from the pancreatic progenitor cells, transdifferentiation, and maturation of the liver stem cells into insulin-producing cells.^{41,42} EGF can increase the number of undifferentiated endocrine precursor cells. Upon removal of EGF, many β cells are differentiated.⁴³ In this study, morphologic changes of cells were observed after nicotinamide and EGF were added in the second step. RT-PCR analysis showed that in addition to expression of *Pdx1* and *Ngn3*, induced cells also expressed *Pax4* at the end of step 2. The data show that nicotinamide and EGF accelerate the differentiation of pancreatic precursor cells into endocrine cells. Exendin-4 is a potent GLP-1 agonist that has been shown to stimulate both β -cell replication and neogenesis from ductal progenitor cells. With the withdrawal of nicotinamide and EGF, exendin-4 was added in the induction system in step 3. The cells matured quickly in this stage with more islet-like clusters formed and mRNA expression of *insulin*, *glucagon*, and *Glut-2*. The functional islet proteins could also be detected. However, the insulin content of insulin-secreting cells is low, and glucose-induced insulin secretion and its proportion (secretion expressed as percent of content) are somewhat lower than those in native islets. In addition, the differentiated cells also expressed early pancreatic genes, such as *Pdx1*, *Ngn3*, and *Pax4*, which suggests that the *in vitro*-generated insulin-secreting cells are immature, and some unknown inducing factors are necessary for the last differentiation.

Previous study has demonstrated that microenvironments are important in differentiation of stem cells.⁴⁴ ECM has been shown to play a crucial role in cell differentiation through rearrangement of the cytoskeletal net-

work.⁴⁵ Matrigel, a mixture of ECM, is essential for pancreatic progenitor cell migration, the 3-dimensional cystic structures formation, and protrusion of the islet bud.⁶ If ESCs are cultured on the Matrigel, they can form insulin-positive, islet-like clusters.²⁷ In our study, we found that if UCB-MSCs were induced without ECM gel coated, clusters formation and functional islet proteins secretion could not be observed in 15 days. Moreover, the morphology of differentiated cells seemed better on ECM than without it. Although some studies obtained insulin-secreting cells from mouse MSCs without ECM gel coated, the induction strategies took about 2–4 months¹⁷ or cluster formations were not observed.¹⁸ Therefore, matrix and growth factors in ECM are very critical for formation of 3-dimensional structures and pancreatic endocrine cell maturation.

In conclusion, this study shows that human UCB-MSCs are capable of differentiating into insulin-secreting cells *in vitro* by a 3-step protocol. However, these cells are immature in terms of insulin production and glucose responsiveness. It has been reported that mouse BM-MSCs could transdifferentiate into insulin-producing cells that were responsive to glucose stimulation under culture conditions containing high concentrations of glucose within 2–4 months.¹⁷ But in our previous study, we found human UCB-MSCs are more susceptible to high-glucose-induced apoptosis. The cells were aging early within 10 days of culture in a high-glucose condition. This result indicated that some biological characters might be different between mouse BM-MSCs and human UCB-MSCs, or more multifactorial influences are involved in transdifferentiation of human UCB-MSCs into competent insulin-producing cells. Obviously, more research is needed to make possible the use of human UCB-MSCs in diabetes therapeutic approaches.

REFERENCES

1. Ryan EA, Lakey JRT, Rajotte RV, et al. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton Protocol. *Diabetes* 2001;50:710–9.
2. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with Type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regime. *N Engl J Med* 2000;343:230–8.
3. Zulewski H, Abraham EJ, Gerlach MJ, et al. Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate *ex vivo* into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 2001;50:521–3.
4. Bonner-Weir S, Taneja M, Weir GC, et al. *In vitro* cultivation of human islets from expanded ductal tissue. *Proc Natl Acad Sci USA* 2000;97:7999–04.
5. Petropavlovskaja M, Rosenberg L. Identification and characterization of small cells in the adult pancreas: potential progenitor cells? *Cell Tissue Res* 2002;310:51–8.
6. Gao R, Ustinov J, Pulkkinen MA, Lundin K, Korsgren O, Otonkoski T. Characterization of endocrine progenitor cells and critical factors for their differentiation in human adult pancreatic cell culture. *Diabetes* 2003;52:2007–15.

7. Rooman I, Lardon J, Bouwens L. Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. *Diabetes* 2002;51:686–90.
8. Wang RN, Kloppel G, Bouwens L. Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats. *Diabetologia* 1995;38:1405–11.
9. 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;292:1389–94.
10. Leon-Quinto T, Jones J, Skoudy A, Burcin M, Soria B. In vitro directed differentiation of mouse embryonic stem cells into insulin-producing cells. *Diabetologia* 2004;47:1442–51.
11. Jahr H, Bretzel B. Insulin-positive cells *in vitro* generated from rat bone marrow stromal cells. *Transplant Proc* 2003;35:2140–1.
12. 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;111:843–50.
13. Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci USA* 1999;96:10711–6.
14. Oswald J, Boxberger S, Jørgensen B, et al. Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* 2004;22:377–84.
15. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–7.
16. Kang XQ, Zang WJ, Bao LJ, et al. Fibroblast growth factor-4 and hepatocyte growth factor induce differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocytes. *World J Gastroenterol* 2005;11:7461–5.
17. Tang DQ, Cao LZ, Burkhardt BR, et al. In vivo and in vitro characterization of insulin-producing cells obtained from murine bone marrow. *Diabetes* 2004;53:1721–32.
18. Chen LB, Jiang XB, Yang L. Differentiation of rat marrow mesenchymal stem cells into pancreatic islet beta-cells. *World J Gastroenterol* 2004;10:3016–20.
19. Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfection of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol* 2000;18:307–16.
20. Mueller SM, Glowacki J. Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J Cell Biochem* 2001;82:583–90.
21. Rubinstein P, Rosenfield RE, Adamson JW, Stevens CE. Stored placental blood for unrelated bone marrow reconstitution. *Blood* 1993;8:679–90.
22. Denner L, Bodenbun Y, Zhao JG, et al. Directed engineering of umbilical cord blood stem cells to produce C-peptide and insulin. *Cell Prolif* 2007;40:367–80.
23. Sun B, Roh KH, Lee SR, Lee YS, Kang KS. Induction of human umbilical cord blood-derived stem cells with embryonic stem cell phenotypes into insulin producing islet-like structure. *Biochem Biophys Res Commun* 2007;354:919–23.
24. Okamoto T, Aoyama T, Nakayama T, et al. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun* 2002;295:354–61.
25. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004;103:1669–75.
26. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000;61:364–70.
27. Shi Y, Hou L, Tang F, et al. Inducing embryonic stem cells to differentiate into pancreatic β cells by a novel three-step approach with activin A and All-*Trans* retinoic acid. *Stem Cells* 2005;23:656–62.
28. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000;109:235–42.
29. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;24:1294–301.
30. Lu FZ, Fujino M, Kitazawa Y, et al. Characterization and gene transfer in mesenchymal stem cells derived from human umbilical-cord blood. *J Lab Clin Med* 2005;146:271–8.
31. Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal stem cells but umbilical cord and mobilized adult blood are not. *Br J Haematol* 2003;121:368–74.
32. Jackson L, Jones DR, Scotting P, Sottile V. Adult mesenchymal stem cells: Differentiation potential and therapeutic applications. *J Postgrad Med* 2004;53:121–7.
33. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8:315–7.
34. Park JS, Kim HY, Kim HW, et al. Increased caveolin-1, a cause for the declined adipogenic potential of senescent human mesenchymal stem cells. *Mech Ageing Dev* 2005;126:551–9.
35. In 't Anker PS, Noort WA, Scherjon SA, et al. Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica* 2003;88:845–52.
36. Chang YJ, Shih DT, Tseng CP, Hsieh TB, Lee DC, Hwang SM. Disparate mesenchyme-lineage tendencies in mesenchymal stem cells from human bone marrow and umbilical cord blood. *Stem Cells* 2006;24:679–85.
37. Edlund H. Pancreatic organogenesis—developmental mechanisms and implications for therapy. *Nat Rev Genet* 2002;3:524–32.
38. Maden M. Role and distribution of retinoic acid during CNS development. *Int Rev Cytol* 2001;209:1–77.
39. Micallef SJ, Janes ME, Knezevic K, Davis RP, Elefanty AG, Stanley EG. Retinoic acid induces pdx1-positive endoderm in differentiating mouse embryonic stem cells. *Diabetes* 2005;54:301–5.
40. Kojima H, Fujimiya M, Matsumura K, Nakahara T, Hara M, Chan L. Extrapancratic insulin-producing cells in multiple organs in diabetes. *Proc Natl Acad Sci USA* 2004;101:2458–63.
41. Yang L, Li S, Hatch H, et al. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. *Proc Natl Acad Sci USA* 2002;99:8078–83.
42. 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;6:278–82.
43. Cras-Meneur C, Elghazi L, Czernichow P, Scharfmann R. Epidermal growth factor increases undifferentiated pancreatic embryonic cells *in vitro*: a balance between proliferation and differentiation. *Diabetes* 2001;50:1571–9.
44. Watt FM, Hogan B. Out of Eden: stem cells and their niches. *Science* 2000;287:1427–30.
45. Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 2002;99:4391–6.