Differentiation of bone marrow-derived mesenchymal stem cells in diabetic patients into islet-like insulin-producing cells: a new era in the treatment of diabetes

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Background The availability of donors for transplantation of human β-cells as a form of treatment for type II diabetes is limited. Introduction of differentiated human bone marrow-derived mesenchymal stem cells (MSCs) would allow transplantation of an autologous source of β-cells. This would alleviate the limitations of availability and/or allogenic rejection following pancreatic or islet transplantation.

Materials and methods Bone marrow cells were obtained from three adult type II diabetic volunteers and three nondiabetic donors. After 3 days in culture, adherent MSCs were expanded for two passages. At passage 3, differentiation was carried out in a three-staged procedure. Cells were cultured in a glucose-rich medium containing activation and growth factors including B27, betacellulin, activin A, and nicotinamide. These cells were evaluated for endocrine characteristics by flow cytometry and morphology. Differentiated cells were transplanted under the kidney capsule of diabetic nude mice and their diabetic status was tested by oral glucose tolerance tests.

Results Following differentiation, cells expressed the hormones insulin, glucagon, and somatostatin. No differences in cell characteristics were noted between diabetic and nondiabetic donors. Hormones were detectable by immunomicroscopy in some cells. Transplantation into diabetic mice resulted in secretion of human insulin, near-normalization of glucose levels, and improvements in glucose intolerance. When the MSC-bearing kidneys were removed, rapid return of diabetic state was noted. Histology of the removed kidneys identified insulin-positive cells in the graft.

Conclusion Human MSCs from diabetic and nondiabetic donors can be differentiated in vitro to form insulin-producing cells that can restore normoglycemia in diabetic mice. This source of material for autologous transplantation offers new opportunities for the treatment of diabetes.

Introduction Diabetes mellitus is a fast-growing global problem with huge social, health, and economic consequences. It is estimated that in 2010 there were globally 285 million people (~6.4% of the adult population) suffering from this disease. The main aim in the management of diabetes is to maintain blood glucose levels as near to normal as possible while avoiding hypoglycemia (Kaul et al., 2012).

A breakthrough was obtained in generating pancreatic endodermal progenitor cells from human embryonic stem cells (ESCs) and in deriving beta cells thereafter. This improved the treatment of type 1 diabetes. Stem cells hold great promise as they provide a ready source of transplantable, insulin-secreting tissues that are not limited by the supply of donor organs. ESCs have the ability to proliferate indefinitely and generate any differentiated cell in the body using an in vitro differentiation protocol, although the efficiency is relatively low. However, the application of human ESC-derived islet-like cells was largely restricted by ethical concerns. In addition, this procedure resulted in highly proliferating cells, and thereby potentially malignant, rather than mature, postmitotic cells (D’Amour et al., 2005; Jiang et al., 2007; Kroom et al., 2008).

Several fetal and adult tissues have been shown to contain stem cells that may have the potential to be engineered as insulin secretory cells. Recent demonstrations that human ESC-like-induced pluripotent stem cells can be generated from adult human somatic cells by enforced expression of defined transcription factors have raised great hope (Mali et al., 2008).

Mesenchymal stem cells (MSCs) are of advantage compared with other adult stem cells. MSCs can be obtained from a patient’s autologous tissue – for example, from bone marrow – and then expanded and differentiated into insulin-producing cells (IPCs) and transplanted into the same patient. Bone marrow-derived MSCs carry the more important implications, because they are easily accessible for an autograft and routinely collected from adults without the ethical concern inherent to fetal embryonic tissues. Some studies suggested that bone marrow-derived MSCs can differentiate into IPCs both in vitro and in vivo. MSCs are universal suppressors of immune reactivity. Cells differ-
entiated from MSCs will not cause obvious rejection (Krause et al., 2001; Tang et al., 2004; Uccelli et al., 2006; El-Badri and Ghoneim, 2013).

This work aims at studying the differentiation capacity of a diabetic patient’s bone marrow-derived MSCs, testing the feasibility of sorting the types of generating islet-like cells by hormonal expression in in-vitro studies and verifying the possibility of transplantation of IPCs into streptozotocin-induced diabetic mice with further differentiation and reduction of hyperglycemia.

Materials and methods
Experiment I
Retrieval of human bone marrow cells
The required approvals for this study were obtained from the Ethical Committee of the Faculty of Medicine, University of Mansoura. Bone marrow aspirates (BMAs) were collected from iliac crests in heparin from six consenting donors. Three donors were type II, insulin-dependent diabetic patients and three were normoglycemic and acted as controls.

Isolation, expansion, and differentiation of bone marrow-derived mesenchymal stem cells
The BMAs were diluted 1:1 with low-glucose Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St Louis, Missouri, USA) and layered on top of a density gradient (Ficoll-Paque, 1.077 g/ml; Pharmacia, Uppsala, Sweden) and centrifuged for 20 min at 600 rpm. Cells were collected from the media/Ficoll interface, washed twice in PBS, and resuspended in 10 ml of low-glucose complete DMEM supplemented with 10% fetal bovine serum, (Hyclone, Logan, Utah, USA), 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma). One milliliter of the BMA yielded about 1.5 x 10^6 nucleated cells. The collected cells were cultured in complete DMEM at a density of 5 x 10^5 cells/ml (10 ml in 25 cm^2 tissue culture flasks) and incubated at 37°C in a 5% CO_2 incubator. Aliquots were frozen in liquid nitrogen for subsequent expansion and examination.

After 3 days, the nonadherent cells were discarded by changing the medium. The remaining adherent MSCs were cultured to 80% confluence before passaging with trypsin. Cells were resuspended with complete DMEM and replated at a ratio of 1:2 and cultured for a further 8 days. This step was repeated for a second passage. The cells then had the appearance of fibroblast-like cells.

Differentiation of mesenchymal stem cells to form endocrine cells
At passage 3, MSCs were cultured at a density of 1 x 10^5 cells/ml in serum-free, glucose-rich DMEM (25 mmol/l) containing 0.5 mmol/l β-mercaptoethanol (Sigma) and incubated for 2 days. The medium was replaced with serum-free, glucose-rich medium containing 1% nonessential amino acids (Sigma), 20 mg/ml basic fibroblast growth factor (Sigma), 20 ng/ml epidermal growth factor (Sigma), 2% B27 supplement (Gibco BRL; Life Technologies, UK), and 2 mmol/l l-glutamine (Sigma) and cultured for a further 8 days. Finally, the cells were cultured for an additional 8 days in serum-free, glucose-rich DMEM containing 10 ng/ml β-cellulin (Sigma), 10 ng/ml activin A (Sigma), 2% B27 supplement, and 10 mmol/l nicotinamide (Sigma) as per the protocol followed by Tayaramma et al. (2006). The samples from each donor were examined in duplicate for the in-vitro and in-vivo components of this study.

Evaluation of cells
Characterization of isolated mesenchymal stem cells by flow cytometry
For flow cytometric analysis, the bone marrow-derived MSCs at passage 3 were trypsinized, centrifuged at 300 g for 8 min, and resuspended in PBS at a concentration of 1 x 10^6 cells/ml. Aliquots of 100 μl were labeled (30 min) with antibodies against CD14, CD45 [fluorescein isothiocyanate (FITC)], CD73, CD34 phycoerythrin (Becton–Dickinson, USA), and CD105 phycoerythrin, CD90 and class II HLA (FITC) (Becton–Dickinson), washed with 1 ml of stain buffer (BD-Pharmlingen), and resuspended in 500 μl of stain buffer. The labeled cells were analyzed using an argon ion laser with a wavelength of 488 nm (FACS Calibur; Becton–Dickinson). A total of 10,000 events were obtained and analyzed with the Cell Quest software program (Becton–Dickinson). Control staining with appropriate isotype-matched monoclonal antibodies was included.

Multilineage differentiation potential
Human bone marrow (HBM)–MSCs were induced to differentiate into adipocytes, chondrocytes, and osteocytes using a differentiation protocol as described previously by Pittenger et al. (1999). Oil-Red-O was used to determine the phenotype of adipocytes, Alcian blue for chondrocytes, and alizarin red for osteocytes.

Immunolabelling
Cell preparations and engrafted IPCs were immunolabeled for insulin (rabbit monoclonal; Cell Signaling Technology, Danvers, Massachusetts, USA), glucagon (rabbit polyclonal anti-glucagon; Cell Signaling Technology), rabbit anti-human somatostatin (DakOcytometry, Glostrup, Denmark), or human c-peptide (rabbit polyclonal; Cell Signaling Technology). Specimens were fixed in 4% paraformaldehyde and examined in situ on chamber slides (Nunc). The secondary antibodies used were swine anti-rabbit immunoglobulin labeled with FITC (Dako-Cytomation), anti-mouse TRITC for immunofluorescence, or the biotin avidin complex and DAB for immunohistochemistry.

The frequency of cell division was detected by labeling for Ki-67 (Cell Marque, USA).

Preliminary confocal images were captured after labeling for insulin (in house guinea-pig antibody to human B-chain) and glucagon (mouse monoclonal antibody; Sigma).

For nanogold immunostaining, the IPCs were fixed in 2% glutaraldehyde and 0.5% glutaraldehyde (both EM grade from Ted Pella) in PBS overnight at 4°C. After washing for 3 x 15 min with PBS containing 0.05% Tween 20 (BioRad
Laboratories, Hercules, California, USA), they were blocked for 6 h in casein blocking buffer (BioRad Laboratories) with 0.05% Tween 20 at room temperature. Subsequently, the samples were incubated overnight with mouse monoclonal c-peptide (1H8) antibody (ab8297, Abcam) at 1: 50 dilution in blocking buffer at 4°C. The next day, the samples were washed for several hours with PBS-0.05% Tween 20 and incubated overnight at 4°C with anti-mouse antibody conjugated with 1.4 nm gold (Nanoprobes, Yaphank, New York, USA) diluted at 1:50 with blocking buffer. The next day, the samples were washed for 3 x 15 min with PBS-0.05% Tween 20, postfixed for 20 min in 1% glutaraldehyde in PBS-0.05% Tween 20, washed for 3 x 15 min in water, silver enhanced, and then photographed as a whole mount embedded in Epon. Thin (70–100 nm) sections were examined and photographed with a JEOL 1200 transmission electron microscope (Kloc et al., 2007). Undifferentiated MSCs as well as human islet cells were similarly labeled to serve as negative and positive controls, respectively.

**In-vivo transplantation studies in mice**

The ability of differentiated cells to establish normoglycemia in diabetic nude mice (Swiss Nu/Nu; Charles River Laboratories, Paris, France) was examined by their implantation in the renal subcapsular space. The animals were divided into four groups: normal mice (n = 10); diabetic mice treated with culture media only (n = 10); diabetic mice treated with undifferentiated MSCs (n = 10); and diabetic mice treated with differentiated cells generated from MSCs of the donor (1) (n = 20). Induction of diabetes was done with a single dose (220 mg/kg of body weight) of streptozotocin (Sigma), which was given intravenously at least 1 week before transplantation. This dose produces irreversible hyperglycemia, polyuria, and progressive weight loss. The injected mice were immediately placed in metabolic cages under good ventilation.

Diabetes was confirmed when the rat exhibited two successive nonfasting blood glucose levels more than 350 mg/dl, glycosuria, 24 h urine volume more than 15 ml, and loss of body weight.

For transplantation of differentiated islet-like clusters in mice, the recipient mouse was anesthetized with pentobarbital (50 mg/ml) at a dose of 0.1 ml/100 g of body weight intraperitoneally. A lateral abdominal incision was made. The kidney was exposed. The clusters (1000 clusters, equivalent to one million cells), in 20 µl of culture media, were implanted into each mouse through a 26 G needle under the renal capsule. The muscles was then closed by chromic stitches and the skin was closed with silk stitches.

The transplanted mice were then returned to the metabolic cages for further daily monitoring. Excess food and water were allowed as needed.

The biochemical profiles of the mice in both groups were periodically assessed. Blood samples were obtained from the tail and examined for blood glucose, serum mouse insulin, human insulin, and c-peptide by means of enzyme-linked immunosorbent assay.

Three months after transplantation, the cell-bearing kidneys underwent nephrectomy. Kidneys were prepared for histological studies to examine the grafts. Nine mice survived and were monitored for their blood sugar and human insulin levels. A confocal study with dual labeling of the cells under the renal capsule for insulin and glucagon was also carried out.

Further immunohistochemical studies were performed to define the nature of the whorls: the renal sections were stained for Congo red, Masson trichrome, silver, and factor VIII.

**Statistical analysis**

Nonparametric data were evaluated by means of the Friedman test. Post-hoc testing was performed using the Wilcoxon signed-rank test and the P values were corrected by Bonferroni adjustments. A P value less than 0.05 was considered significant.

**Experiment II**

The retrieval of HBM cells, isolation and expansion of HBM–MSCs, characterization of the isolated HBM–MSCs, multilineage differentiation potential, and differentiation of the HBM–MSCs into endocrine cells were carried out according to procedures detailed in experiment I.

**In-vivo transplantation studies in mice**

The ability of differentiated cells to induce normoglycemia in diabetic nude mice (Swiss Nu/Nu; Charles River Laboratories) was examined following implantation of these cells into the renal subcapsular space.

Diabetes was chemically induced with a single dose of 220 mg/kg of streptozotocin (Sigma). The mice were considered diabetic once the blood glucose levels exceeded 350 mg/dl for two consecutive readings.

Twenty-nine animals of an average age of 12 weeks were utilized. The diabetic mice were anesthetized through intraperitoneal injection of ketamine (100 mg/kg) and diazepam (5 mg/kg). A total of 1 x 10⁶ of cells obtained at the end of in-vitro differentiation were suspended in 20 µl of culture medium and implanted beneath the renal capsule of each mouse. The surviving animals were killed 1, 2, 4, or 12 weeks after transplantation. Before euthanization, blood samples were obtained from the tail vein and measured for blood glucose levels using glucometer strips (Accu-Chek; Roche Diagnostics, Basel, Switzerland), serum human insulin, serum human c-peptide, and serum mouse insulin levels by means of enzyme-linked immunosorbent assay (DRG Diagnostic, Germany).

The HBM–MSC-bearing kidneys of the euthanized animals were divided into halves. One-half was immunolabeled for histological analysis and for counting the insulin-positive cells. The expression of relevant endo-
crine genes was determined in the other half. The pancreas of these animals was also harvested and immunostained for insulin.

**Immunolabelling**

The antibodies used, flow cytometry, and immunocytochemistry were as followed in experiment I.

**Immunofluorescence**

The harvested organs were fixed in formalin and sectioned on coated positively charged adhesion slides (Citoglas; Citotest Labwaremanufacturing Co., Haimen, China). The slides were then deparaffinized using xylene and a decreasing ethanol gradient. The antigens were unmasked by boiling the slides in 10 mmol sodium citrate buffer (pH 6.0) and by maintaining the sub-boiling temperature for 10 min. The sections were blocked with 5% normal goat serum and incubated overnight with the primary antibody at 4°C. Thereafter, the slides were washed three times in PBS and incubated with the secondary antibody for 2 h at room temperature. The nuclei were counterstained using DAPI. ImageJ software (developed by National Institutes of Health) was used to determine the proportion of transplanted cells beneath the renal capsule that intracytoplasmically expressed insulin. To this end, 10 fields were randomly selected. The results from all fields were calculated and expressed as the mean proportion of insulin-positive cells out of the total transplanted cells. In all of the above studies, confocal images were captured using a Leica TCS SP8 microscope (Leica Microsystems, Mannheim, Germany).

For immunolabelling of the native pancreas by immunohistochemistry, the primary antibody used was mouse monoclonal anti-insulin (L6B10) (Cell Signaling Technology) and the secondary antibody was the poly-HRP and DAB Kit for mouse (Genemed Biotechnologies, San Francisco, California, USA). The sections were examined under light microscopy.

**Statistical analysis**

Nonparametric data were evaluated using Friedman’s test. Post-hoc analysis was performed using the Wilcoxon signed-rank test, and the $P$ values were corrected using Bonferroni adjustments. A $P$ value less than 0.05 was considered significant. The mean values were used as a measure of variation. The median values were utilized only if there were extreme observations.

**Results**

**Experiment I**

**Morphological and phenotypical characterization of the cultured mesenchymal stem cells**

At the end of the expansion phase, the cells became homogenous, spindle shaped, fibroblast like, and arranged in monolayers. Flow cytometric analysis showed that they expressed high levels of CD73 (99.8%), CD90 (99.4%), and CD105 (96.4%) but negligible levels of CD14 (2.6%), CD34 (0.2%), and CD45 (0.1%) and did not express class II HLA antigens (Table 1). These results indicate that the majority of bone marrow-derived cells were MSCs. From a starting culture of $3 \times 10^6$ MSCs, $\sim 5000$ clusters (five million cells approximately) were present at the end of the differentiation procedure. During the differentiation phase, the cells gathered gradually in groups with the formation of three-dimensional aggregates. At the end of the differentiation protocol the cells formed clusters with a spheroidal morphology. No apparent differences in the rate of growth or differentiation was observed between cells from different donors or between those with and without diabetes. These cells could be differentiated to form adipocytes, chondrocytes, and osteocytes when the appropriate growth factors were added. Accordingly, evidence for their multilineage potential was confirmed.

**Immunofluorescence labeling**

It demonstrated that $\sim 3\%$ of cells at the end of differentiation were hormone-positive both in the mono- and in the aggregates. Insulin and glucagon appeared as punctuate labeling within the cytoplasm. Furthermore, insulin and c-peptide were coexpressed by the same cells (Fig. 1a–c).

**Immunohistochemistry for Ki-67**

In contrast to the undifferentiated MSCs, differentiated cells were Ki-67 negative.

**Electron microscopic studies**

Nanogold immunostaining showed the presence of c-peptide in the cytoplasm of the IPCs by electron microscopy. Ultrastructural analysis demonstrated that the c-peptide is localized at the rough endoplasmic reticulum (Fig. 2a). The cytoplasm of undifferentiated cells was unlabeled (Fig. 2b), whereas that of islet cells was labeled in the vicinity of the rough endoplasmic reticulum (Fig. 2c).

**Outcomes of in-vivo transplantation experiments**

The blood glucose levels of the diabetic mice implanted with the differentiated clusters of cells were normalized within a few days, whereas those receiving no cells or undifferentiated cells remained hyperglycemic. Three mice that received differentiated cells died 2, 3, and 8 weeks after transplantation. The remaining 17 animals remained euglycemic throughout the observation period of 3 months. Serum levels of human insulin and human c-peptide were measurable and maintained during the same period. Serum levels of mouse insulin were negligible in comparison with that of normal mice.

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Table 1. Flow cytometric quantitation of surface markers of the undifferentiated MSCs

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<th>CD14</th>
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<td>96.4</td>
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<tr>
<td>3</td>
<td>1.9</td>
<td>1.4</td>
<td>0.1</td>
<td>92</td>
<td>99.6</td>
<td>94</td>
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<tr>
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<td>93</td>
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<td>99.5</td>
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<tr>
<td>6</td>
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<td>Mean ± SD</td>
<td>1.9 ± 0.5</td>
<td>1.3 ± 0.7</td>
<td>0.2 ± 0.1</td>
<td>96 ± 4</td>
<td>90 ± 0.7</td>
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MSC, mesenchymal stem cell.
Eight animals died during nephrectomy of the clusters-bearing kidneys. The surviving nine mice became hyperglycemic and the human insulin levels in their sera dropped to 0. These animals died a few days after the procedure. The islets of their native pancreata were negative for insulin by immunohistochemistry. No difference in results was observed relative to the sex of the recipient mice. Histology of the removed kidneys revealed the presence of cell clusters under the renal capsule. These clusters contained some cells coexpressing insulin and c-peptide or were glucagon positive. These immunoreactive cells were largely situated at the periphery of the clusters. Confocal microscopy with dual labeling for insulin and glucagon demonstrated that these hormones were in different cell types.

The whorls showed no apple green birefringence when stained with Congo red. They were Masson trichrome positive, indicating the presence of collagen fibers. They also had reticular fibers as indicated by reticulin staining. As for the distribution of the blood supply, collagen deposits were seen occupying the central part of the clusters, indicating a poor blood supply, which was confirmed by factor VIII. This has led to the replacement of high-density cell clusters with low-density ones in further experiments.

**Experiment II**

**Functional evaluation of differentiated HBM–MSCs**

At the end of differentiation, flow cytometric analysis indicated that the percentage of generated IPCs was modest, ranging between 0.12 and 3.4%. The presence of insulin granules within the cytoplasm of the IPCs was detected via immunocytochemistry.
Immunostaining for c-peptide was also positive in the IPCs. Coexpression of insulin and c-peptide within the same cells was detected by means of electronic merging. Glucagon staining was detected in 30% of the examined samples. However, positive staining for somatostatin was not detected.

Outcomes of the in-vivo transplantation experiments for further differentiation

Out of the 29 transplanted animals, five mice did not tolerate the surgical procedure. The blood glucose levels of the surviving animals normalized within a few days after transplantation (4 ± 1.6 days). Thereafter, the animals remained euglycemic throughout the observation period.

The serum levels of human insulin and human c-peptide were measurable 1 week after transplantation, and these values also remained unchanging throughout the observation period. Serum levels of mouse insulin became negligible after induction of diabetes.

Immunofluorescence of the HBM–MSC-bearing kidneys revealed that the percentage of IPCs increased gradually, peaking at 4 weeks after transplantation (about 18%) without any substantial change thereafter. Again, the coexpression of insulin and c-peptide within the cytoplasm of these cells was confirmed. Positive staining for glucagon and somatostatin was detected in some cells that were not insulin positive (Fig. 3a–e).

Discussion

Bone marrow-derived MSCs carry the more important implications for possible clinical development, because they are easily accessible for an autograft and routinely collected from adults without the ethical concern inherent to fetal embryonic tissues (Krause et al., 2001). Some studies suggested that bone marrow-derived MSCs can differentiate into IPCs both in vitro and in vivo (Tang et al., 2004).

The current study reports a potential way to generate IPCs from bone marrow-derived adult human MSCs by directed differentiation using certain culture media. We have used MSCs for several reasons. We have shown that the HBM–MSCs utilized in this study meet the minimal criteria proposed by the International Society for Cellular Therapy (Dominici et al., 2006). We chose to perform a TSA-based protocol because of its simplicity and the short duration required for differentiation (Gabr et al., 2014).

MSCs residing in the bone marrow are multipotent and can differentiate into lineages of mesenchymal, endodermal, and epidermal cells. Evidence has shown that MSCs can be expanded ex vivo for more than 50 population doublings without obvious signs of differentiation. Accordingly, they can provide a rich source of autologous stem cells. An additional feature of bone marrow MSCs is their lack of expression of class II antigens and their ability to exert immunosuppressive activity on T cells. These properties suggest their possible use in the
treatment of type I diabetes mellitus by abrogation of the associated autoimmunity, and also allow the use of allogenic MSCs if autologous ones cannot be used (Reyes et al., 2001; Jiang et al., 2002; Efrat 2008; Ding et al., 2010).

In this study, HBM–MSCs were isolated on the basis of their ability to adhere to plastic. At the end of expansion, these cells assumed a spindle-shaped morphology and were negative for hematopoietic cell markers. Furthermore, their differentiation into adipocytes, chondrocytes, and osteocytes confirmed their multilineage potential, similar to the work done by Dominici et al. (2006).

Several investigators have argued that the presence of insulin in such cells does not indicate intrinsic insulin production. They suggest that insulin from the utilized culture media can be absorbed by and sequestrated in these cells (Matsuoka et al., 2007). However, immunofluorescence staining was positive for insulin, c-peptide, somatostatin, and glucagon with coexpression of insulin and c-peptide by the same cells, confirming that proinsulin synthesis was occurring in the cells and not being derived from any insulin in the culture media. Moreover, the c-peptide-positive cells were not dividing (Ki-67 negative). Localization of c-peptide at the rough endoplasmic reticulum as revealed by immunogold staining provides an additional evidence of intrinsic synthesis of insulin.

The number of differentiated cells under the present conditions was modest. Microscopic examination suggests that less than or equal to 5% of cells were insulin positive. Evidence of glucose-sensitive insulin secretion suggests that the cellular machinery for glucose sensing is in place and that insulin release could be regulated by similar mechanisms as found in islet β-cells, as seen in a similar work done by Rorsman and Renstrom (2003).

This study compares cells from diabetic and nondiabetic donors. The diabetic status of the donors could be important. Long-term exposure of MSCs to high-glucose concentrations in vivo could influence their sensitivity for growth and differentiation. There were no significant differences in the growth, differentiation, or gene profile of the cells obtained from different diabetic and nondiabetic donors. However, the insulin c-peptide content of cells obtained from nondiabetic donors was significantly less than that of diabetic ones.

We and others have found that the proportion of IPCs at the end of in-vitro differentiation is small, irrespective of the protocol adopted. Despite this modest yield, we found that transplantation of a large number of cells (one million/mouse) was capable of correcting hyperglycemia in vivo. One may also speculate that an additional number of cells could have completed their differentiation in vivo. This was associated with the appearance of human insulin and c-peptide in the blood of these transplanted animals associated with a reduction in mouse insulin in streptozotocin-induced diabetes. No evidence was found to suggest regrowth of the islet β-cells, which would have been reflected by an increase in mouse insulin in the blood samples. Such a possibility was firmly excluded by our experiments because histopathologic examination of the harvested pancreas did not reveal any signs of regeneration. All these findings strongly suggest that the donor cells had a central role in controlling blood glucose levels.

In the second experiment the percentage of IPCs among the cells transplanted beneath the renal capsule increased over time, peaking at about 18% after 4 weeks.

We have provided evidence that the ability of these transplanted cells to cure the diabetic animals was due to an increase in the number of functional IPCs. Directed differentiation in vitro served as an initial step that induced expression of relevant endocrine genes. Subsequently, further maturation of these cells occurred after transplantation under the influence of favorable microenvironmental conditions. The maximal yield of functional IPCs was around 18% at 4 weeks after transplantation, with no further increase thereafter. This result suggests that only a subset of MSCs are capable of transdifferentiation into the pancreatic endocrine lineage. The identification, sorting, expansion, and subsequent differentiation of this cellular component would result in the production of sufficient IPCs displaying adequate functional capacity.

Conclusion
Taken together, all these experimental findings support the potential of adult human bone marrow-derived MSCs to differentiate into IPCs. Differentiation of autologous MSCs into IPCs would provide potential clinical benefits for diabetic patients. The donor could receive his or her own cells after differentiation.

Transplanting these cells results in the normalization of blood glucose levels in diabetic animals. Evidence was provided for the further maturation of these cells in vivo. The proportion of IPCs increased by 10-fold 4 weeks after transplantation. The glucose tolerance curves and the simultaneously measured c-peptide levels demonstrated that these cells are glucose-responsive and insulin-secreting.

Our experiments indicated that only a subpopulation of MSCs are capable of differentiation into the pancreatic lineage. The challenge is to demonstrate that enough insulin-positive cells with adequate functional capacity can be produced in culture without the requirement for molecular biological engineering. In addition, the duration for which these cells can maintain their active function in vivo, the needed numbers, and the optimal site for their implantation should also be studied.

Acknowledgements
Conflicts of interest
None declared.

References


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<td>Please provide the details of ‘the conflicts of interest disclosure’. If there is nothing to declare, please provide a statement to that effect.</td>
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<tr>
<td>Q16</td>
<td>If there are fewer than 8 authors for references, ‘Dominici et al, 2006; Gabr et al, 2014’ please supply all of their names. If there are 8 or more authors, please supply the first 6 authors’ names then et al.</td>
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