Research article

DIFFERENTIATION OF MESENCHYMAL STEM CELLS DERIVED FROM HUMAN BONE MARROW AND SUBCUTANEOUS ADIPOSE TISSUE INTO PANCREATIC ISLET-LIKE CLUSTERS *in vitro*

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Abstract: Although stem cells are present in various adult tissues and body fluids, bone marrow has been the most popular source of stem cells for treatment of a wide range of diseases. Recent results for stem cells from adipose tissue have put it in a position to compete for being the leading therapeutic source. The major advantage of these stem cells over their counterparts is their amazing proliferative and differentiation potency. However, their pancreatic lineage transdifferentiation competence was not compared to that for bone marrow-derived stem cells. This study aims to identify an efficient source for transdifferention into pancreatic islet-like clusters, which would increase potential application in curative diabetic therapy. The results reveal that mesenchymal stem cells (MSC) derived from bone marrow and subcutaneous adipose tissue can differentiate into pancreatic islet-like clusters, as evidenced by their islet-like morphology, positive dithizone staining and expression of genes

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such as Nestin, PDX1, Isl 1, Ngn 3, Pax 4 and Insulin. The pancreatic lineage differentiation was further corroborated by positive results in the glucose challenge assay. However, the results indicate that bone marrow-derived MSCs are superior to those from subcutaneous adipose tissue in terms of differentiation into pancreatic islet-like clusters. In conclusion, bone marrow-derived MSC might serve as a better alternative in the treatment of diabetes mellitus than those from adipose tissue.

INTRODUCTION

Cell therapy using autologous adult stem cells has been recognized for its potential to revolutionize the field of regenerative medicine. Of the various sources of adult stem cells, bone marrow has been the most frequently chosen source and it is widely used for clinical transplantation in the treatment of a wide range of diseases [1, 2]. In recent years, adipose tissue has emerged as another potent, accessible and reliable source of stem cells. Evidence is accumulating on the applicability of adipose tissue as a leading source of stem cells for use in tissue repair and regeneration for the treatment of various disorders [3-6].

Diabetes mellitus is one such debilitating disorder, characterized either by absolute insulin deficiency due to the destruction of beta cells (Type 1) or by relative insulin deficiency associated with the reduced insulin sensitivity that is common with obesity (Type 2) [7]. Transplantation of islet cells is a potent treatment for patients with type I diabetes as it can restore normoglycemic levels [8]. The major shortcomings of this therapeutic approach are the shortage of donors and immune rejection, which limit its large-scale application [9]. In the light of the widespread diabetes epidemic, transplantation of autologous adult stem cells derived from bone marrow and adipose tissue [10-12] has shown potential as an alternative therapeutic approach that could obviate the aforementioned limitations associated with diabetic treatment.

Recent evidence showed that bone marrow and subcutaneous adipose tissue are both endowed with the capacity for multilineage differentiation, including the possibility of becoming a pancreatic beta cell-like phenotype. However, bone marrow-derived stem cells have a lower proliferative potency and have not proven promising candidates for the treatment of widespread diseases [13-18]. Despite this limitation, there have been reports on the ability of bone marrow-derived mesenchymal stem cells (BMSC) to successfully transdifferentiate into pancreatic beta cells [12, 19], thereby indicating the potential of BMSC as a promising alternative in the treatment of diabetes. There have also been reports indicating the successful transdifferentiation of adipose-derived stem cells (ADSC) into pancreatic beta cells [11, 20].

From the published evidence, it is clear that both bone marrow and subcutaneous adipose tissue can differentiate into pancreatic beta cells and could be of greater therapeutic value in treatment of diabetes mellitus. Although there are reports to substantiate the plasticity of BMSC and ADSC with regards to the generation of islet-like clusters, the paucity of evidence creates an uncertainty as to which tissue would be the better source for diabetic therapy. Furthermore, a comparison of the potency of BMSC and ADSC in terms of transdifferentiation into pancreatic islet-like clusters has not yet been reported. Our study investigated the comparative transdifferentiation ability of BMSC and ADSC into pancreatic islet-like clusters with a view to determining which would be the better stem cell type for curative therapy in the treatment of diabetes mellitus.

MATERIALS AND METHODS

Collection of samples
Samples of human adipose tissue were collected from patients undergoing abdominoplasty and samples of bone marrow from patients undergoing stem cell therapy for spinal cord injury. Written informed consent was obtained prior to sample collection and all protocols followed ICMR guidelines and were reviewed and approved by the ethical committee of Lifeline Multispecialty Hospitals, Chennai, India. Adipose tissue was obtained from 4 patients in an age range 35 to 55 with a BMI of 28.7 ± 4.8 kg/m^2. Bone marrow (BM) was obtained from 4 patients in an age range from 23 to 50 with a BMI of 24.7 ± 2.6 kg/m^2. The tissue samples were quantified and processed within 2 h of collection.

Isolation and culture
The isolations of the stromal vascular fraction (SVF) from the adipose tissue and the mononuclear cells from the bone marrow were performed using our previously reported protocol [21]. The isolated SVF and mononuclear cells were seeded in a culture flask at a seeding density of 3×10^4 cell/cm^2 in DMEM - low glucose (DMEM-LG, Invitrogen) with 10% FBS (Invitrogen) and 1% antibiotics (Invitrogen) and incubated at 37ºC, 5% CO_2 in 95% humidity with the media replaced every 3 days until confluence. At 80-90% confluence, the adherent cells were trypsinized using 0.25% trypsin-EDTA and re-seeded with same cell density. The procedure was repeated until passage 3.

Phenotypic characterization by flow cytometry
Approximately 1×10^5 cells in each sample were phenotypically characterized for the expression of the following cell surface markers: CD 90-PERCP (e-Biosciences); CD 105-APC (e-Biosciences); CD 73-PE (BD Biosciences); CD 34-PE (BD Biosciences); CD 45-APC-CY7 (BD Biosciences); CD 54-PERCP (BD Biosciences); CD 49d-PE (e-Biosciences); CD 166-PE (BD Biosciences); and CD 106-FITC (BD Biosciences). The cells were incubated with the corresponding concentration of flourochrome-conjugated antibodies at room temperature for 20 min. The incubated cells were then washed with wash flow

buffer and pelleted by centrifugation. The pellets were resuspended in BD FACS flow and analyzed using a flow cytometer (BD FACS Aria). Data acquisition and analysis was performed using BD FACS-Diva software.

Multilineage differentiation of MSCs
The multilineage differentiation capacities of the two types of MSC were assessed by inducing them to differentiate into osteoblasts and adipocytes at passage 3. Osteogenic and adipogenic differentiation was performed using the appropriate induction media (Table 1). The extent of osteogenic differentiation was verified by von Kossa and Alizarin Red staining.
Von Kossa staining was done by treating 10% formalin-fixed cells with 1% silver nitrate (Sigma-Aldrich) and UV exposure for 45 min. UV-exposed cells were further treated with 5% sodium thiophosphate (Sigma-Aldrich) for 2 min and nuclear fast red (Sigma-Aldrich) staining for 1 min. Each step was done with intermittent repeated washing with distilled water.
Alizarin Red staining was performed by treating 70% alcohol-fixed cells with 5 ml of Alizarin Red (Sigma-Aldrich). The cells were incubated for 30 min, washed, and viewed under a microscope. The extent of adipogenic differentiation was confirmed by Oil Red O staining on formalin-fixed cells. The cells were washed with distilled water and 60% isopropanol prior to incubation with 1 ml of Oil Red O working solution (Sigma-Aldrich). Stained cells were incubated for 10 min, washed, and imaged under a microscope.

Table 1. Composition of induction media for differentiation.

Medium	Composition
Osteogenic induction medium	DMEM-LG (Invitrogen), 10% FBS (invitrogen), 1% antibiotic (Invitrogen), 0.1 µmol/l dexamethasone, 10 mM β-glycerophosphate, 2 mmol/l ascorbic acid
Adipogenic induction medium	DMEM-LG (Invitrogen), 10% FBS (invitrogen), 1% antibiotic (Invitrogen), 1 µmol/l dexamethasone, 0.5 mmol/l isobutyl methyl xanthine, 10 ng insulin, 200 µmol/l indomethacin
Beta cell induction medium	Step 1: DMEM-HG (Invitrogen) supplemented with 10% FBS (Invitrogen) and 10^{-6} mol/l retinoic acid (Sigma-Aldrich)
	Step 2: serum-free DMEM-LG supplemented with 1% N_2 (Invitrogen), 1% B27 (Invitrogen), 10 mmol/l nicotinamide (Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF; Sigma-Aldrich) and 2 nmol/l activin A (Sigma-Aldrich)
	Step 3: serum-free DMEM-LG supplemented with 1% N_2, 1% B27, 10 mmol/l nicotinamide, 10 ng/ml EGF, 2 nmol/l activin A and 10 nmol/l exendin-4 (Sigma-Aldrich)

Transdifferentiation of MSCs
The bone marrow and adipose tissue-derived MSCs were induced to transdifferentiate into pancreatic β-cell islet-like clusters in a three-step protocol

using beta cell induction medium (Table 1). The cells were grown to 70-80% confluence and incubated with step 1 induction medium for 2 days. The incubated cells were then trypsinized (0.25% trypsin-EDTA; Sigma-Aldrich) and seeded into 6-well extra cellular matrix-coated plates (Nunc). The cells were then treated with step 2 induction medium for 6 days. Then the medium was replaced with step 3 induction medium and incubated for 4 days. The transdifferentiation was confirmed by assessing the morphological appearance of the islet-like clusters and by dithizone staining, intracellular staining for nestin and PDX1 using FACS, and gene expression profiles of insulin, Isl1, PDX1, Pax4 and Ngn3 using real-time PCR. The transdifferentiated cells were further subjected to the glucose challenge test to determine their insulin-secretion capacity.

Dithizone staining
The transdifferentiated cells in the culture plate were incubated with dithizone (DTZ) (Sigma Aldrich) solution (10 µl of DTZ working solution in 1 ml of the culture medium) for 30 min at room temperature (37ºC). After incubation, the cells were washed in PBS and imaged under the microscope. Crimson-red stained clusters were documented.

Intracellular staining using FACS
The cells were harvested and washed with cold DPBS (Dulbecco's Phosphate Buffer Saline; Invitrogen) by centrifugation at 200 g for 7 min. The supernatant was discarded and the cells were mixed well with 500 µl of cytofix/cytoperm (BD Biosciences) solution to form a single cell suspension and incubated at 4ºC for 20 min. The fixed cells were then washed with 4 ml of freshly prepared 1x wash buffer (BD Biosciences) at 200 g for 7 min. The supernatant was discarded and the cells were then resuspended with 500 µl of perm wash buffer (BD Biosciences). 1×10^5 differentiated cells were treated with anti-PDX1 or anti-nestin. All of the tubes were vortexed well and incubated for 45 min at 18-24ºC in the dark. The cells were then washed twice with perm wash buffer at 200 g for 7 min. The pellet was then resuspended in 400 µl DPBS for final flow cytometric analysis.

Real-time PCR
Total RNA from both pre- and post-treated cells was isolated using TRIzol reagent including a DNase treatment. Qualitative and quantitative analysis of the extracted RNA was done using a Thermo Scientific NanoDrop with a concentration of 260/280 nm. cDNA synthesis was performed using a Verso cDNA Kit based on the manufacturer's instructions. The quantitative real-time PCR was carried out using a USB VeriQuest SYBR Green qPCR Master Mix (2x) on an Applied Biosystems 7500 Fast Real-Time PCR System with the appropriate primers for Pax-4, Isl-1, Ngn-3, PDX1 and insulin designed suitably for SYBR Green detection using specific software called Integrated DNA Technologies (IDT; Table 2). β-actin was used as an internal control and the products were tested by gel electrophoresis.

Table 2. Primers for real-time PCR.

Gene		Primer sequence
Insulin	Sense	CCTGTGCGGCTCACACCTGG
	Antisense	AGCTCCACCTGCCCCACCTG
Isl 1	Sense	TCTGTGGGCTGTTCACCAACTGTA
	Antisense	GCCGCAACCAACACATAGGGAAAT
Ngn 3	Sense	GCGCAATCGAATGCACAACCTCAA
	Antisense	TTGAGTCAGCGCCCAGATGTAGTT
Pax 4	Sense	GAGGACACGTGAGGGTCTGGT
	Antisense	ACAGTCAGCCCCTGGGAAGCA
Pdx 1	Sense	GTCCTGGAGGAGCCCAAC
	Antisense	GCACTCCTGCTCAGGCTC
β-actin	Sense	CCAAGGCCAACCGCGAGAAGATGAC
	Antisense	AGGGTACATGGTGGTGCCGCCAGAC

Abbreviations: Isl 1 – islet-1 transcription factor; Ngn 3 – neurogenin 3; Pax 4 – paired box gene 4; Pdx 1 – pancreatic duodenal homeobox 1

Glucose challenge assay
The capacities of the transdifferentiated cells for insulin secretion were assessed using the glucose challenge assay. The islet-like cells were tested for insulin secretion at both basal (5.5 mmol/l) and stimulated (25 mmol/l) glucose concentrations. The differentiated cells in the plates were washed with PBS and incubated in 1 ml of serum-free DMEM containing 5.5 mmol/l glucose for 5 h at 37ºC. The media were collected and stored at -20ºC, and then fresh media with 25 mmol/l glucose were added. After 5 h of incubation at 37ºC, the media were collected and stored. The stored media were then analyzed for insulin content using a direct human insulin ELISA kit (Invitrogen). Non-induced MSCs were used as a control.

RESULTS

Phenotypic characterization of BMSC and ADSC
From the expression profile analysis using flow cytometry, we identified that BMSC and ADSC showed remarkable expression of the MSC markers CD 90, CD 105 and CD 73. They showed a negative expression of CD 34, CD 45, HLADR and CD 31, thus indicating a good level of the homogeneity of the MSCs upon culture. However, CD 54 and CD 49d showed variable expression in the two cell types. BMSC showed a lower expression of CD 49d than ADSC whereas CD 54 expression was minimally higher in ADSC (Fig. 1 and 2).

Culturing and differentiation
The cells cultured in DMEM-LG had a fibroblastic-mesenchymal phenotype at passage 3 for both ADSC (Fig. 3A) and BMSC (Fig. 3B). ADSC (Fig. 3C, E and G) and BMSC (Fig. 3D, F and H) were proficient enough to differentiate into osteogenic and adipogenic lineages at passage 3 when exposed to specific

induction factors. The mineralization of calcium deposits was observed over a period of 15 days. Positive von Kossa and Alizarin Red staining on day 21 confirmed the differentiation into osteogenic lineage. However, an accumulation of lipid vacuoles occurred between days 10 and 12 and lipid droplets were identified on day 15 using Oil Red O stain. The results indicate that there was no difference in the differentiation capacity of BMSC and ADSC with regards to osteogenic and adipogenic lineage.
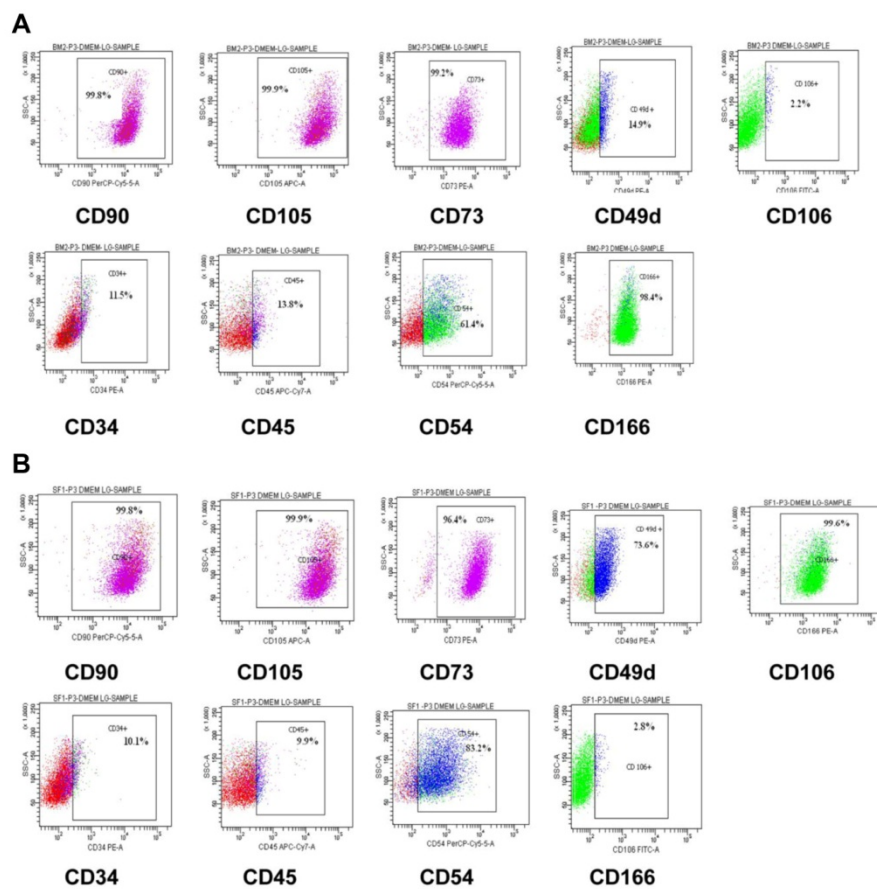

Fig. 1. Cell surface antigenic expression profiles of BMSC and ADSC at passage 3. Profiles created using flow cytometry. A – Expression profile for BMSC. B – Expression profile for ADSC.

Transdifferentiation into islet-like clusters
Pancreatic islet-like cluster formation was carried in passage 3 cells derived from bone marrow and adipose tissue. The morphological appearance of the islet-like clusters seen under the microscope after day 12 of induction period revealed that both ADSC and BMSC could differentiate into pancreatic islet-like
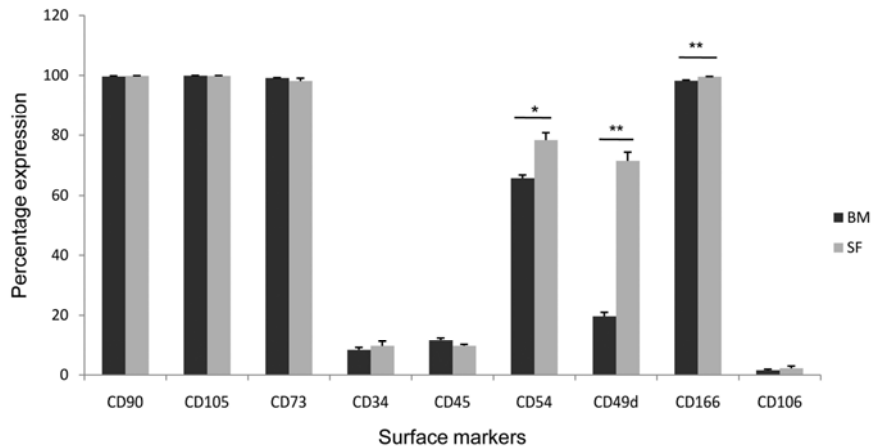
Fig. 2. Expressed percentages of cell surface markers of BMSC and ADSC. It depicts the percentage expression of markers in the form of means ± SEM. Statistical significance: * $p < 0.05$; ** $p < 0.01$.
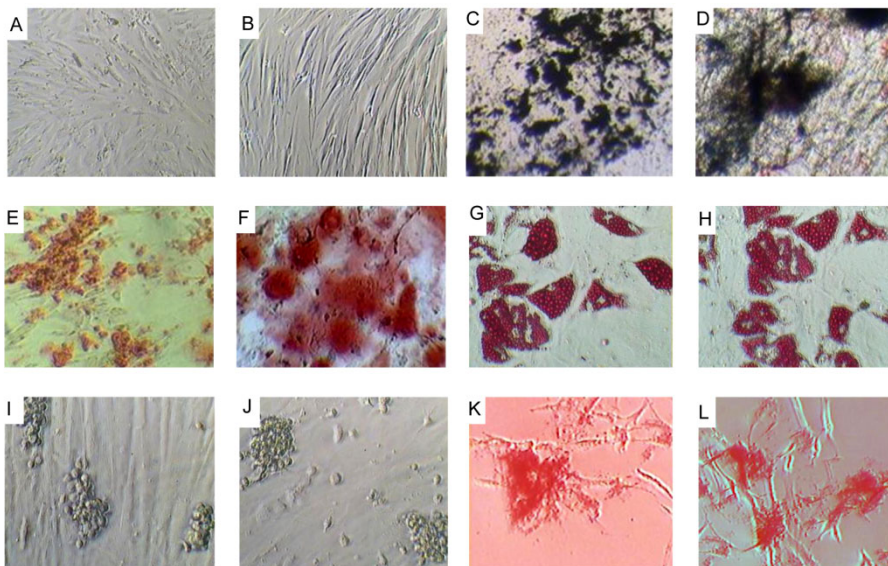

Fig. 3. Morphological appearance of cultured and differentiated cells from BMSC and ADSC. Morphology of cultured human ADSC (A) and BMSC (B). Osteogenesis was detected on day 21 using von Kossa staining in ADSC (C) and BMSC (D); and using Alizarin Red staining from ADSC (E) and BMSC (F). Adipogenesis was detected on day 18 using Oil Red O staining from ADSC (G) and BMSC (H). Islet-like cluster morphology of differentiated ADSC (I) and BMSC (J). Dithizone staining of islet-like clusters derived from ADSC (K) and BMSC (L).

clusters (Fig. 3I and J). However, it was observed that BMSC had more islet-like clusters than ADSC. Further substantiation of its potency to produce beta cells was confirmed using dithizone staining (Fig. 3K and L), intracellular staining by FACS, gene expression study using real-time PCR and the insulin assay. It became evident from the intracellular staining using FACS that bone marrow and subcutaneous adipose tissue are similar with regard to their expression profiles for nestin and PDX1 (Fig. 4).
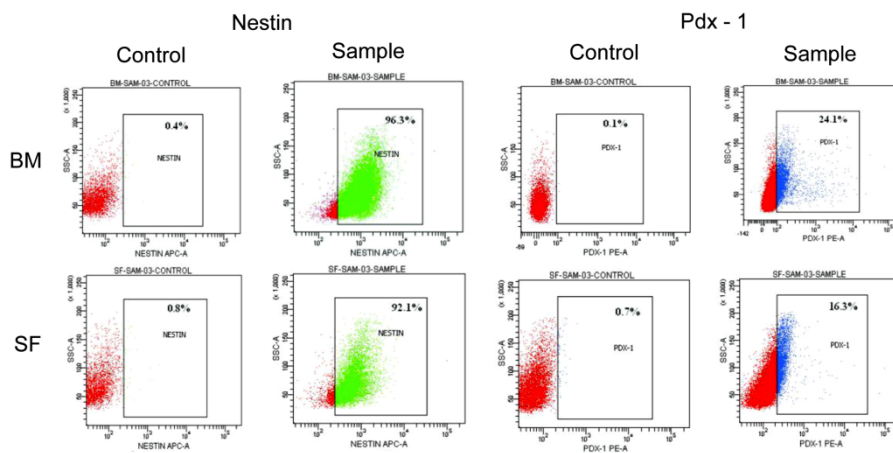

Fig. 4. Flow cytometric analysis of expression of differentiated Beta cells. BM – bone marrow; SF – subctaneous fat; PDX 1 – pancreatic and duodenal homeobox 1
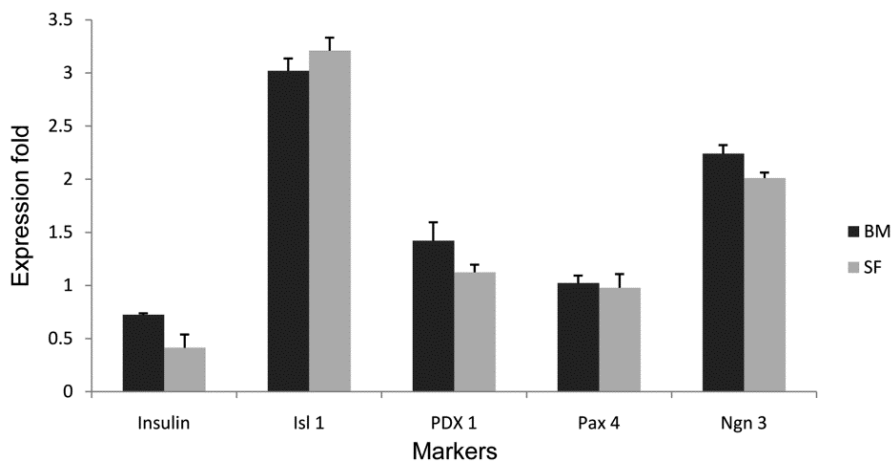

Fig. 5. Real-time PCR analysis of differentiated islet-like clusters. The real-time analysis of differentiated beta cells shows the fold increase in the expressions of the pancreatic islet-like cell-specific genes Insulin, Isl1, PDX 1, Pax 4 and Ngn 3. The values are represented graphically as means ± SEM. No statistically significant differences were found.
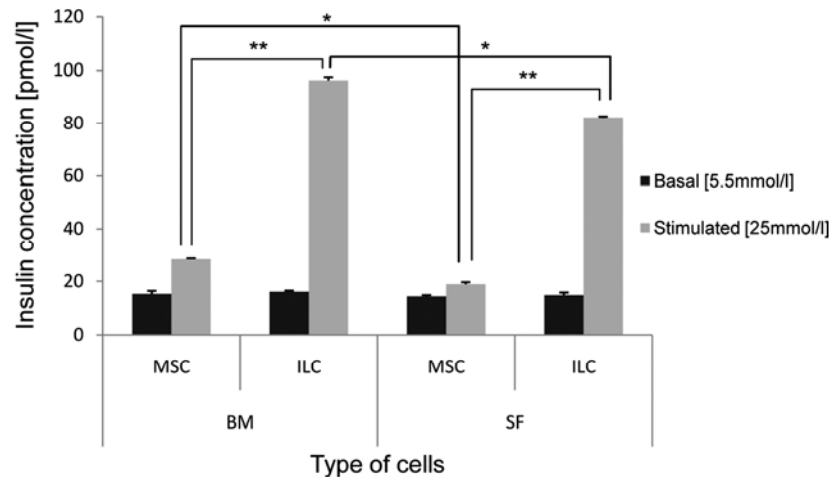
Fig. 6. Glucose challenge assay. The insulin secretion from differentiated islet-like clusters (ILC) and undifferentiated mesenchymal stem cells (MSC) from bone marrow (BM) and subcutaneous adipose tissue (SF) at both basal (5.5 mmol/l) and stimulated (25 mmol/l) concentrations of glucose are illustrated.

Data obtained from the real-time PCR analysis revealed a significant difference between the two sources. It was observed that islet-like clusters obtained from BMSC were highly positive for genes such as Insulin, PDX 1 and Ngn 3 when compared to ADSC, which showed a slightly higher expression of Isl 1, proving the presence of immature islet clusters (Fig. 5). The gene expression profile of the islet-like clusters was further corroborated by the positive results in the glucose challenge assay (Fig. 6), which showed a higher amount of insulin secretion by BMSC-derived islet-like clusters than by those derived from ADSC. This indicates that BMSC have a greater potential than ADSC for diabetic treatment.

DISCUSSION

Transplantation of islet-like cells obtained from bone marrow- or subcutaneous adipose tissue-derived MSC materialized as a promising method for the treatment of diabetes mellitus [11, 12, 19, 22-25]. The existence of scant evidence on the transdifferentiation ability of BMSC and ADSC into beta cells meant that it was unclear which tissue, if either, would be the better source of transplantable material [11, 12, 19, 20]. Furthermore, there were no reports comparing the transdifferentiation ability of BMSC and ADSC. To obviate these limitations and uncertainty, our study concentrated on comparing the phenotype, differentiation and transdifferentiation ability of BMSC and ADSC. The results revealed a similar expression profile for BMSC and ADSC with regard to certain markers, except for CD 49d, which showed a higher expression in ADSC than BMSC. Both stem cell types exhibited a similar differentiation ability into osteogenic and adipogenic lineages as reported in other literature [1, 6, 13, 15, 17, 26, 27]. This is evidence for

the use of either bone marrow or adipose tissue as an alternative source of curative therapeutics for the treatment of a wide range of diseases.

Not much evidence exists to support the idea that BMSC or ADSC are appropriate for use in clinical transplantation for diabetic therapy. There were some reports indicating their possible transdifferentiation capacity into beta cells [11, 12, 17, 19, 20, 28]. There was also a report that provided no evidence of differentiation into beta cells [29]. This lack of clarity leads us to focus on the identification of an ideal therapeutic source for beta cell replacement. From our study, it is visible that both BMSC and ADSC could differentiate into pancreatic islet-like clusters, which is evident from the morphology, dithizone staining results, positive gene expression of nestin and PDX1, positive expression of the pancreatic developmental genes Insulin, Isl 1, PDX 1, Pax 4 and Ngn 3, and the results of the insulin secretion assay. This is consistent with the transdifferentiation capacity proven in other literature [11, 12, 19, 20, 28]. Our results for BMSC were contradictory to the report published by Lechner *et al.* [29] but comparable to the studies of Timper *et al*. The pancreatic development transcription factors Isl 1, PDX 1 and Ngn 3 were identified to be similarly highly expressed in their study. Furthermore, our study also identified a higher expression of Isl 1 than for the other genes, supporting their hypothesis that Isl 1-positive cells could be used as a human model to develop stem cell-based therapies for diabetes mellitus [11]. Similarly, our glucose challenge assay results were similar to those obtained by Sun Yu *et al.* [19]. We also demonstrated for the first time that bone marrow-derived stem cells are more promising at transdifferentiating into pancreatic islet-like clusters than those from adipose tissue. This is evident from the predominant expression of the genes Insulin, PDX l and Ngn3, and from the results of the glucose challenge assay, which support BMSC as a good source for beta cell replacement therapy.

In conclusion, from the results obtained, although both BMSC and ADSC were proven to have differentiated into pancreatic islet-like clusters, BMSC served as a much better source of transplantation material the treatment of diabetes mellitus. These findings are a breakthrough in the exploration of the potential of BMSC for treating diabetes mellitus. However, the results also indicate the need for further investigations on ADSC to explore their potential in beta cell replacement therapy.

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