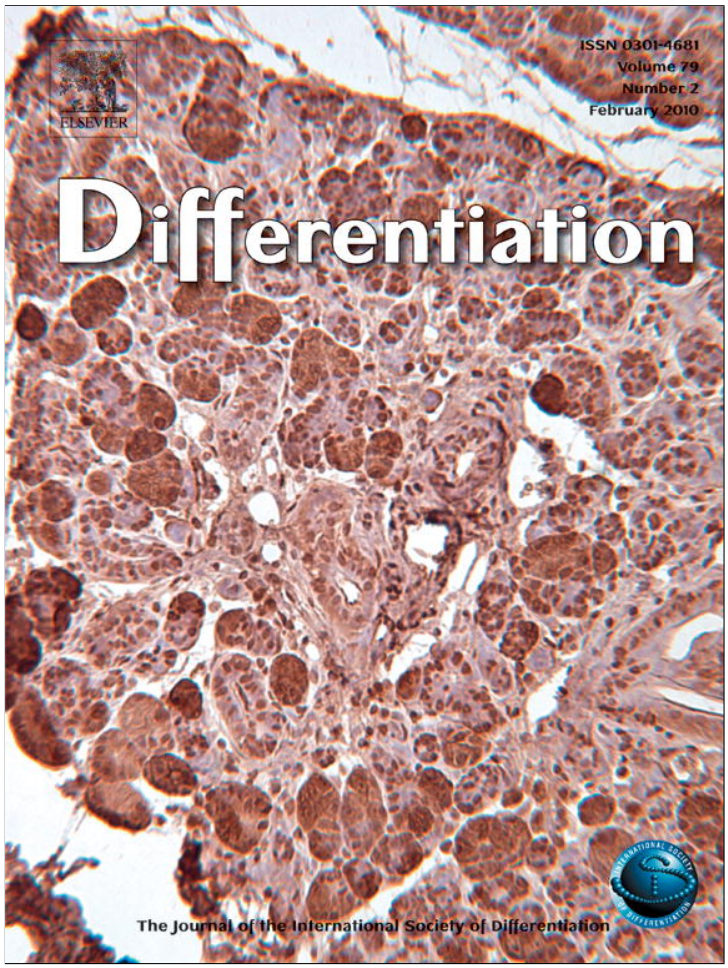

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Cardiomyogenic differentiation of human bone marrow mesenchymal cells: Role of cardiac extract from neonatal rat cardiomyocytes

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ABSTRACT

Bone marrow mesenchymal stromal cells (BM-MSCs) with regenerative potential have been identified in heart. Whether these cells become new cardiac lineage cells by phenomena of transdifferentiation or fusion is also being investigated. Although, these mechanisms give cardiomyocytes, it has to be considered that MSCs transplantation could carry out ossification and calcification processes. An alternative might be the use of myocytes; however, the problem is the arrythmia. For those reasons, is that we investigated how to obtain cardiomyocyte-like cells from human MSCs (hMSCs). The aim of the present work was to evaluate a nuclear reprogramming of the hMSCs by a neonatal rat cardiomyocytes extract (EX) using Streptolysin O (SLO) treatment. hMSCs treated with 57.5 ng/ml SLO presented ball-like, stick-like and myotube-like morphology. In the absence of cardiomyogenic stimuli, hMSCs expressed markers of cardiac phenotype-like sarcomeric α-actinin, connexin-43 and GATA-4. However, when hMSCs were treated with SLO+EX or 10 μM of 5-azacytidine (5-AZA), the expression of these markers were significantly increased and furthermore, expressed SERCA-2, cardiac Troponin I, β-MyHC, desmin, MLC-2a and MLC-2v thus showing the phenotype of mature cardiomyocytes. PCR analysis showed that cardiomyocyte-related genes, such as β1-adrenergic receptor (β1-AR), MLC-2a and cardiac Troponin T, were expressed after SLO+EX treatment like with 5-AZA. We concluded that the extract of neonatal rat cardiomyocytes could promote a nuclear modification of hMSCs to cardiomyogenic-like cells differentiation. Since the 5-AZA treatment appears to be genotoxic and taking into account the obtained results, the nuclear reprogramming by cell extract may be an approach leading to the identification of soluble factors that drives the reprogramming.

1. Introduction

Recent reports demonstrating the ability of bone marrow cells (BMC) in regenerating cardiomyocytes have prompted both clinical and basic studies for the treatment of ischemic and non-ischemic cardiomyopathies. However, the responsibility of BMC and the underlying mechanism of regeneration remain unclear (Prockop, 2007).

When cardiovascular diseases, such as myocardial infarction or ischemic condition occur, cardiomyocytes die, and necrotize. Human cardiomyocytes rarely regenerate after necrosis in infarcted ventricular tissues and are progressively replaced by fibroblasts to form scar tissues. The remaining cardiomyocytes are unable to reconstitute the necrotic tissue and cardiac dysfunction due to this massive loss of functional cardiomyocytes leads to chronic heart diseases or death (Kadivar et al., 2006; Fine et al., 2008). Although heart transplantation has been a therapy for some of these cases for several decades, it is much limited by the shortage of donors and the host immuno-rejective reaction to the grafts.

As a newly developed strategy, cell transplantation, which aims to introduce healthy myogenic cells into the myocardium, is showing a bright future in this field (Fine et al., 2008). Up to now, a number of cell types, including skeletal myoblasts, fetal

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cardiomyocytes, smooth muscle cells, embryonic stem cells and BM mesenchymal stromal cells (MSCs), have been employed for cell therapy, and have shown benefits on heart functions in various animal models of myocardium injury (Abraham et al., 2005; Fukuda and Yuasa, 2006; Breitbach et al., 2007; Beltrami et al., 2007; Nassiri et al., 2007). However, each all these cell sources have it own inherent problems (Fukuda and Yuasa, 2006).

hMSCs, which include mesenchymal stem cells and mesenchymal stromal progenitors, can be a useful source of cells for transplantation for several reasons: they have the ability to proliferate and differentiate *in vitro* into mesoderm, neuroectoderm as well as endoderm tissues, have immunomodulatory properties and entail no ethical or immunological problems (Nauta and Fibbe, 2007; Prockop, 2007).

Several clinical studies have shown safety and feasibility of autologous BM cell transplant in patients with myocardial infarction, refractory ischemia, or heart failure, and it has also been shown that these symptoms and clinical parameters improve after cell therapy (Charwat et al., 2008). However, the precise molecular mechanism of BM-hMSCs differentiation into mature and functional cardiomyocytes *in vitro* is still controversial. Direct transplantation of hMSCs into the body may be dangerous because of hMSCs' multipotentiality and transdifferentiation capacity (Breitbach et al., 2007; Li et al., 2007). Transplanted hMSCs can differentiate into endothelial cells, smooth muscle cells, and of course cardiomyocytes in the infarcted area, but may also induce calcification and ossification (Fukuda and Yuasa, 2006; Breitbach et al., 2007; Li et al., 2007). Nevertheless, if hMSCs were differentiated into cardiomyocyte-like cells before transplantation, side effects would be greatly decreased. On the other hand, the disadvantage to use transplantated myocytes is that they have spontaneous impulses which restrict the ability to establish proper electrical interaction with the host's cardiomyocytes. Thus, spontaneous impulse formation and impaired cell-to-cell coupling have been shown to be arrhythmogenic (Gillum and Sarvazyan, 2008; Menasché et al., 2008). It has to be considered that the differentiated cardiomyocyte-like cells, from hMSCs, for transplantation should not beat spontaneously to decrease the possibility of arrhythmias.

In view of the advantages of hMSCs, such as the ability to self-renew, potential to differentiate into cardiomyogenic cells (Fukuda and Yuasa, 2006), and immunomodulatory properties (Nauta and Fibbe, 2007), hMSCs have been considered as one of the most promising candidates for cell therapy in cardiac disease (Fine et al., 2008).

Previous researchers have proved that hMSCs treated with 5-azacytidine (5-AZA) can differentiate towards a cardiomyogenic lineage *in vitro* (Antonitsis et al., 2008). However, 5-AZA might have genotoxic effects (Stresemann et al., 2006; Li et al., 2007).

It was reported by Gaustad et al. (2004) that human adipose tissue stem cells take on cardiomyocyte properties following transient exposure to a neonatal rat cardiomyocytes extract (EX) by reversibly permeabilized with Streptolysin O (SLO). On the other hand, it is known that cellular extracts from one target cell type can elicit some reprogramming of nuclear function in a different cell type and the new phenotype persisted for at least >100 population doublings in culture (Håkelien et al., 2004). Taking into account that a cardiomyocyte nuclear reprogramming could be an alternative differentiation treatment, we decided to study how to obtain cardiac differentiation by EX into hMSCs permeabilized with SLO. This treatment was compared with the already known capacity of 5-AZA to differentiate hMSCs into cardiomyocytes.

Morphological change analysis, immunostaining techniques, cytochemistry techniques and RT-PCR analysis revealed that hMSCs isolated from normal BM are able to differentiate into cardiomyocytes under SLO+EX treatment.

2. Materials and methods

2.1. Isolation of human BM mononuclear cells (MNCs)

BMs were obtained from eight healthy volunteers, donors for allogenic BM transplantation, sex matched, and the age range selected was 9–38 years old. These volunteers gave consent to participate in this study, in accordance with the principles of Helsinki Declaration. This work was approved by the Ethical Committees of the Fundación Favaloro and Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Buenos Aires, Argentina.

BMs were aspirated from the posterior iliac crest with heparine (25 units/ml, Gibco). Aspirates were diluted 1/2 with PBS (pH=7.5) and the MNCs were isolated by centrifugation through 1.075 g/cm^3 Hystopaque (Sigma) density gradient and the enriched MNCs were collected from the interface, washed twice in PBS and resuspended in α-MEM medium with 100 IU/ml penicillin G, 100 μg/ml streptomycin sulfate, 25 μg/ml of amphotericin B and 2 mM of L-glutamine (supplemented α-medium) (Gibco). MNCs were counted with 3% acetic acid and 0.04% trypan blue solutions.

2.2. Fibroblast colony-forming units (CFU-F) assay

MNCs (2×10^6/25 cm^2) were cultured in supplemented α-MEM with 20% fetal bovine serum (FBS, Gibco) at 37 °C in 5% CO_2. After 7 days, the non-adherent cells were removed and adherent cells were cultured with fresh medium until day 14. Then, cultures were washed twice with PBS, fixed with 100% methanol (Merck) for 15 min and stained with Giemsa (Biopure) for 5 min. Aggregates of 50 cells or more were scored as CFU-F. The fibroblastic nature of the hMSCs that composed the CFU-F cultures was evaluated by immunocytochemistry using monoclonal antibodies (mAb) against human β-subunit of prolyl-4-hydroxylase (IgG1, M0877, 1:50, Dako), CD45 (monoclonal IgG1, M0701, 1:50, Dako), and CD34 (monoclonal IgG1, M7165, 1:50, Dako) and revealed with biotin labeled secondary Ab and streptavidine-peroxidase, following the manufacturer's instructions (kit K0673, Dako). Negative controls were performed using isotype control mAb (IgG1, X0931, Dako). Each sample was carried out by duplicate.

2.3. Preparation of BM-hMSC cultures for plasticity studies

BM-hMSCs (10×10^6/25 cm^2) were cultured in supplemented α-MEM with 20% FBS. After 24 h, the non-adherent cells were discarded and the primary cultures were incubated until confluence and the medium was renewed every 7 days. Confluent hMSCs were washed twice with PBS and harvested with a solution of trypsin–EDTA (0.05–0.02% in PBS, Gibco). hMSCs were induced to proliferate until a second confluence. After this period, the cells were plated at a low density (240 hMSCs/cm^2) and cultured in supplemented α-MEM plus 20% FBS for 12 days. Finally, hMSCs were isolated with trypsin–EDTA and counted with trypan blue and acetic acid. These hMSCs of a high grade of multipotentiality were used for plasticity protocols. Each sample was carried out by triplicate.

2.4. Phenotypical characterization of hMSCs from third passage cultured at low cell density (240 MSCs/cm^2, 12 days)

Cells were studied for the expression of hMSCs phenotypical markers by peroxidase-based immunocytochemistry staining method (K0673, Dako) using mAb against β-subunit of prolyl-4-hydroxylase, CD73 (monoclonal IgG 4G4, HM2215, 1:50 Cell Sciences), CD105 (polyclonal IgG, SC9048, 1:60, Santa Cruz), CD44 (monoclonal IgG1, M7082, 1:50, Dako), CD54 (monoclonal IgG1, M7063, 1:50 Dako), CD49b (monoclonal IgG1, M0603, 1:10, Dako), CD49e (monoclonal IgG3k, M0604, 1:25, Dako), CD68 (monoclonal IgG1, M0718, 1:150, Dako), CD45 and CD34. Negative controls (IgG1 isotype, X0931, Dako; goat IgGs, Bethil P 50 and mouse IgG, 086500, Zymed) were used for assessing non-specific staining.

2.5. Differentiation of hMSCs into osteogenic, adipogenic and chondrogenic lineages

2.5.1. Osteogenic potential

hMSCs (3000/cm^2) from third passage (12 days) were cultured in osteogenic differentiation medium [supplemented α-MEM with 10% FBS, 10^{-8} M dexamethasone (Sigma), 0.2 mM ascorbic acid (Sigma), 10 mM β-glycerol phosphate (Sigma)] for 3 weeks. After this, cultures were washed with PBS and stained with Giemsa (Biopure), Von Kossa (5% silver nitrate and 5% sodium thiosulphate; Sigma) and Alizarin Red-S (40 mM, pH=4.1, Sigma). Immunocytochemistry for human osteocalcin (goat polyclonal IgG, SC18319, 1:50, Santa Cruz) was also performed and developed with the same immunocytochemistry kit previously described. Each sample was carried out by quadruplicate.

2.5.2. Adipogenic potential

hMSCs (3000/cm^2) from third passage (12 days) were cultured in supplemented α-MEM containing 20% FBS until confluence. Adipogenic differentiation was carried out by three cycles of induction/maintenance (medium of CAMBREX, PT3004) during 3 weeks. Each cycle consists of 3 days of culture in the induction medium (supplemented α-MEM with 10% FBS, 0.5 μM dexamethasone, 50 μg/ml 3-isobuthyl 1-methylxanthine and 50 μg/ml indomethacin) followed by 3 days of culture in the maintenance medium (same medium without dexamethasone).

For adipocyte identification, intracellular lipid accumulation was visualized using Oil Red-O-staining (Sigma). Briefly, cells were fixed in 10% formaldehyde/PBS (Merck) for 1 h and stained with Oil Red-O (0.12%) and Giemsa (Biopure). Each sample was carried out by quadruplicate.

2.5.3. Chondrogenic potential

hMSCs (2.5×10^5) from third passage (12 days) were centrifuged at 450*g* for 10 min to generate a micromass pellet culture. The medium was changed every 3 days. Pellet was cultured for 21 days in supplemented α-MEM media containing 10% FBS plus chondrogenic factors (500 ng/ml BMP-6, 25 mM glucose, 10 ng/ml of TGFβ-3 (Genzyme), 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate and 50 mg/ml ITS+Premix; CAMBREX, PT 3003). Pellets were processed for cryosectioning, as follows: sections (5 μm) of the pellet were stained for glycosaminoglycane with 1% the Toluidine blue (Richard Allan Scientific) and 1% Borate sodium (Sigma) for 5 min. Chondrogenic differentiation was confirmed by staining the slides with anti-human Ab against collagen II (polyclonal IgG, SC7763, 1:50, Santa Cruz). To develop the immunocytochemistry, we followed the manufacturer's instructions (K0690, Dako). Negative controls were carried out by assessing non-specific staining with rabbit IgG (X0936, Dako).

As controls of osteogenic, adipogenic and chondrogenic differentiation, hMSCs were incubated in α-MEM medium with 20% FBS. Each sample was carried out by quadruplicate.

2.6. Cardiomyogenic differentiation protocols

2.6.1. 5-AZA (DNA demethylation agent) treatment

To confirm if a chemical induction can trigger the differentiation of hMSCs into cardiomyocytes, hMSCs from the second to the sixth passage were exposed to different concentrations of 5-AZA (Sigma) at 3, 5 or 10 μM in supplemented α-MEM with 10% FBS for 24 h. The medium was changed every 3 days with supplemented α-MEM with 20% FBS until the experiment was finished after 1 month. Based on the morphological changes of hMSCs, 10 μM of 5-AZA and hMSCs (3000/cm^2) from third passage (12 days) appeared to be the optimal conditions for inducing differentiation, and were thus used in subsequent experiments as positive control.

2.6.2. SLO+EX

Neonatal rat cardiomyocyte extract was obtained from 1-3-day-old Sprague Dawley female rat hearts by enzymatic methodology (Gaustad et al., 2004; Mahler et al., 2004). Briefly, the hearts were minced and the pieces were washed twice with 10 ml PBS-1% glucose. After that, were digested by eight cycles by 8 ml enzymatic solution (0.16% p/v trypsin, 0.016% (P/V) collagenase and 0.002% (P/V) DNAse I) with gently agitation at 37 °C for 8 min. Then, the tube was put immediately in ice and 4 ml of pipetting solution (0.004% (P/V) DNAse I, dissolve in 24 ml PBS-1% glucose and 4% (V/V) FBS) was added. It was pipeted carefully for 2 min up and down and the supernatant was transferred to a new tube and was centrifuged for 5 min to 1200 rpm. The pellet was resuspended in 1 ml of DMEM-F12 media containing 10% FBS with 0.357% (P/V) HEPES, 0.12% (P/V) sodium bicarbonate, 0.1% (P/V) glucose and 100 IU/ml penicillin G, 100 μg/ml streptomycin sulfate and stored in ice. Ended the cycles, the myocytes were cultured 1×10^6 cells on gelatinized hexawell plates (adding the calculated volume of cell pool to 1.5 ml of DMEM-F12 with 10% FBS). Twenty-four hours after, the medium was changed and cultured for 4 days at 37 °C in 5% CO_2.

To prepare cardiomyocyte extracts (CEs), cardiomyocytes were harvested with a solution of trypsin–EDTA and cells were thawed on ice and washed twice in cold PBS and once in cold cell lysis buffer (20 mM Hepes, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT, and protease inhibitor cocktail). Cells were packed at 800*g*, and resuspended in 1.5 volumes of cell lysis buffer, and allowed to swell on ice for 45 min. Cells were homogenized by pulse-sonication until complete lysis of cells and nuclei was achieved (monitored by phase contrast microscopy) and the lysate was sedimented at 15,000*g* for 15 min at 4 °C and the supernatant was aliquoted, frozen in liquid nitrogen, and stored at −80 °C. Protein concentration of the extract was usually ~5 mg/ml.

This treatment consists of two steps (Gaustad et al., 2004; Håkelien et al., 2004): (**a) Cell permeabilization:** hMSCs from third passage (12 days) were washed in cold PBS and resuspended in aliquots of 20,000 cells/100 μl in cold Ca^{2+}- and Mg^{2+}-free HBSS (Gibco). Aliquots were placed in 1.5 ml tubes and centrifuged for 5 min at 400*g* at 4 °C in a swing-out rotor. Sedimented cells were suspended in 15.5 μl cold HBSS, then tubes were placed in a H_2O bath at 37 °C for 2 min, and 4.5 μl SLO (Sigma; 100 μg/ml stock, diluted 1:100 in ice cold HBSS) was added to yield a final SLO concentration of 57.5, 115 and 230 ng/ml. Samples were

incubated for 50 min at 37 °C with occasional agitation and placed on ice. SLO was diluted with 200 μl cold HBSS, and cells were sedimented for 5 min at 4 °C in a swing-out rotor. Permeabilization was assessed by monitoring uptake of a 70,000 Mr Texas red-conjugated dextran (Molecular Probes; 50 μg/ml) in a separate sample 24 h after resealing and replating cells. (**b) Incubation of hMSCs with EX:** The supernatant was removed and cells were suspended in 20 μl of EX (1 mg/ml) containing an ATP-regenerating system (1 mM ATP, 10 mM creatine phosphate, and 25 μg/ml creatine kinase), 100 μM GTP, and 1 mM of each NTP. Cells were incubated for 1 h at 37 °C in a water bath with occasional agitation. To reseal plasma membranes, the extract was diluted with RPMI 1640 medium (Gibco) containing 10% FBS, antibiotics, and 2 mM $CaCl_2$. Cells were transferred to culture flasks (25 cm^2, 3000 hMSCs/cm^2) cells per well. After 2 h, floating cells were removed and adherent cells were cultured. The medium was changed to remove $CaCl_2$, and cells were cultured 1 month in supplemented α-MEM with 20% FBS. The medium was changed every 3 days.

Finally, dynamic changes in morphology and growth properties were compared with the control cultures incubated in supplemented α-MEM with 20% FBS. Moreover, cell aliquots were prepared for immunofluorescence and RT-PCR techniques. Experiment carried out in triplicate. Based on the morphological changes of hMSCs, 57.5 ng/ml of SLO appeared to be the optimal conditions for inducing cardiomyogenic differentiation, and were thus used in subsequent experiments.

2.7. Evaluation of cardiomyogenic markers expression in hMSC cultures incubated with cardiomyogenic differentiation treatments

To evaluate whether SLO+EX or 5-AZA treatments can induce hMSCs to express cardiomyogenic-specific proteins, immunofluorescence assay was performed. One month after treatments, cells that grown on labteck chamber slides (50×10^4 cells/well) (Nunc) were washed with PBS, fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100/PBS (Sigma) for 15 min. Each manipulation was preceded by washing the cells three times in PBS with 0.05% Tween 20 (Sigma). After blocking in PBS-BSA 5% (Sigma) for 1 h, the cells were incubated overnight at 4 °C with the anti-human primary Abs: sarcomeric α-actinin (monoclonal, EA-53, 1:100, Sigma), connexin-43 (monoclonal, 610061, 1:100; BD Transduction Laboratories), SERCA-2 (goat polyclonal, SC8095, 1:100, Santa Cruz Biotech, CA, USA), cardiac Troponin I (rabbit polyclonal, SC15368, 1:100; Santa Cruz Biotech, CA, USA), β-MyHC (monoclonal, 1548, 1:10; Chemicon), and transcription factors GATA-4 (monoclonal, SC25310, 1:100, Santa Cruz Biotech, CA, USA), Nkx2.5 (rabbit polyclonal, SC14033, 1:100, Santa Cruz Biotech, CA, USA), desmin (monoclonal, DE-U-10,1:40, Sigma), MLC-2a (goat polyclonal, SC344, 1:100, Santa Cruz Biotech, CA, USA) and MLC-2v (goat polyclonal, SC8095, 1:100, Santa Cruz Biotech, CA, USA). Bound Abs were visualized by fluorescence using texas red-labeled goat anti-rabbit and mouse anti-goat IgG (H+L) or with FITC-labeled goat anti-mouse IgG (H+L) from Jackson ImmunoResearch (Baltimore, USA). Images were obtained using Confocal Scanning Laser Microscope (Zeiss, LSM-510, Carl Zeiss, Oberkoken, Germany) with a highly corrected objective (C-Apochromat ×40 numerical aperture 1.2 under water) and processed by Image Browser 3.0. Cells were incubated either with an irrelevant Ab as a negative isotype control or with rabbit or goat negative controls to exclude non-specific labeled cells from the examination. The same protocols were followed for staining of control hMSC cultures.

Table 1
Six target primers

Target/control gene	Primer sequence (5′–3′)	Amplicon size (bp)
MLC-2a	For: GAGGAGAATGGCCAGCAGGAA Rev: GCGAACATCTGCTCCACCTCA	450
Hemi-nested MLC-2a	For: GGCATCATCTGCAAGGCAGA Rev: GCGAACATCTGCTCCACCTCA	296
β-actin	For: CCTGAACCCCAAGGCCAACCG Rev: GCTCATAGCTCTTCTCCAGGG	398
Cardiac Troponin T	For: AGGCGCTGATTGAGGCTCAC Rev: ATAGATGCTCTGCCACAGC	407
β1-adrenergic receptor 1	For: ACGCTCACCAACCTCTTCAT Rev: AGGGGCACGTAGAAGGAGAC	440
Hemi-nested β1-adrenergic receptor 2	For: CCTTCTTCTGCGAGCTGTGG Rev: AGGGGCACGTAGAAGGAGAC	328

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from hMSCs using Trizol reagent (Gibco-BRL, USA). cDNA was synthesized in 20 μl reaction volume containing 4 μg of total RNA and SuperScript II RT, according to the manufacturer's instructions. The endogenous "housekeeping" gene β-actin was also quantified to normalize differences in the added RNA and efficiency of reverse transcription.

The thermal profile for PCR was 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, at 55 °C for 1 min, and at 72 °C for 1 min. The PCR products (1 μl) of the first amplification with β1-adrenergic receptor (β1-AR) or MLC-2a primers were subjected to a hemi-nested PCR with a new inner sense primer and with the same reverse primer. The hemi-nested PCR cycles were carried out as the first PCR cycles. The PCR products were size fractioned by electrophoresis on 2% agarose gel.

RNA of non-induced hMSCs was used as a negative control, and RNA of neonatal rat cardiomyocytes and HL-1 (an immortalized atrial cardiomyocyte murine cell line which retains the characteristics of adult atrial cardiac muscle cells) were used as positive controls. The six specific primers used are illustrated in Table 1.

3. Results

3.1. CFU-F assay

Each BM sample ($n=8$) yielded similar results in terms of CFU-F formation ($\overline{X} \pm \text{SE} = 15.33 \pm 1.59$, CFU-F/$2 \times 10^6$) and cellular morphology (spindle shape) (Fig. 1A, a). Immunocytochemistry analysis showed 95% of stromal cells from CFU-F cultures with positive staining for β-subunit of prolyl-4-hydroxylase (fibroblast marker) and negative staining for CD45 and CD34 (hematopoietic markers, data not shown).

3.2. Morphology and growth properties of BM-hMSCs

hMSCs from eight healthy volunteers were successfully expanded and used for subsequent analyses. On the third day of primary culture, hMSCs displayed a large spindle (fibroblast-like) or a small round shape with one nucleus (Fig. 1A, b). These cells began to proliferate at about day 3–4, and gradually grew to form small colonies on day 7 (Fig. 1A, c). As growth of cells continued, colonies expanded in size with the adjacent ones interconnected with each other reaching confluence on day 14–28 ($\overline{X} \pm \text{S.E} = 19.07 \pm 0.75$ days) (Fig. 1A, d). Confluent stromal cells from the primary cultures mainly consisted of homogeneously

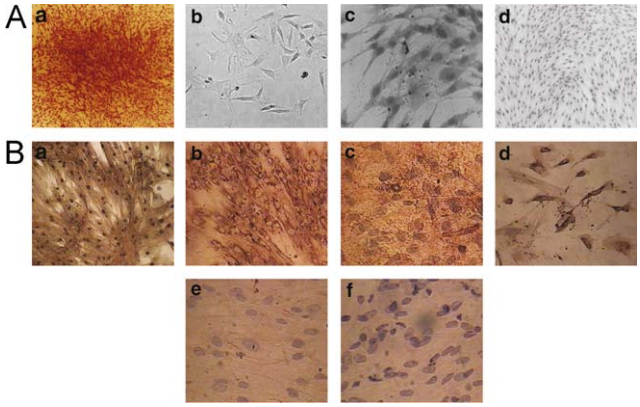

Fig. 1. *In vitro* growth and characterization of hMSCs. (A) Growth morphology of hMSCs in primary cultures. CFU-F assay, ×100 (a); attachment of single spindle cells at 3–4 days, ×400 (b); expansion of spindle cells into colonies at 7 days, ×400 (c) and confluent hMSCs culture for 14–28 days, ×100 (d). (B) Characterization of cell-surface antigens for hMSCs (third passage) by immunocytochemistry staining. hMSCs showing positive for β-subunit of prolyl-4-hydroxylase (a), CD73 (b), CD105 (SH2) (d) and CD44 (e) and negative for the control without the first Ab (f) and the isotype control (g); ×400.

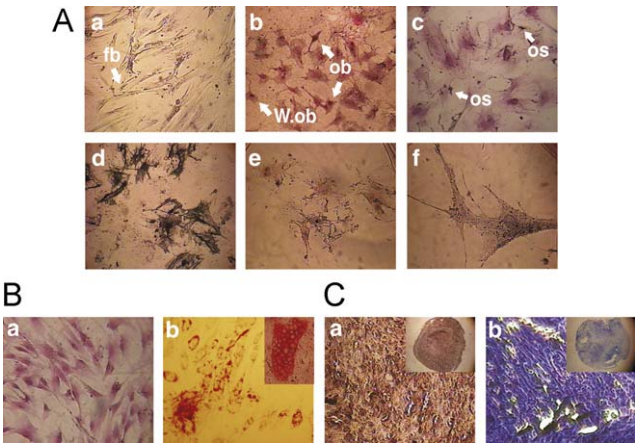

Fig. 2. Multilinage differentiation of hMSCs. Cells were cultured under osteogenic (A), adipogenic (B) and chondrogenic (C) differentiation mediums. Differentiation into respective lineages was identified using cytochemical and immunocytochemical staining. (A) hMSCs cultured with control medium showed fibroblast-like cells morphology (fb) and was negative for Von Kossa–Giemsa staining (a). hMSCs cultured with osteogenic differentiation medium, cells in the way to osteoblasts (W.ob) and osteoblasts (ob) (b) and hMSCs cultured with osteogenic differentiation medium, osteocytes (os) (c) both positive Von Kossa–Giemsa staining, ×400. hMSCs cultured with osteogenic differentiation medium showed positive Von Kossa (d), Alizarin Red-S (e) (×400); and Osteocalcin (f) (×600) staining. These results were accompanied by morphological changes from spindle-shaped to cuboids- or polygonal-shaped cells. (B) hMSCs with control medium (α-medium +20% SBF), Giemsa and Oil Red-O-negative staining (×400) (a); hMSCs with adipogenic differentiation medium, Oil Red-O-positive staining revealed the presence of numerous oil droplets in the cytoplasm (×400), on the top right corner (×600) (b); (C) Typical differentiation to chondrocytes was shown by type II Collagen staining (×400) (a) and Toluidine blue staining (×400) (b) when hMSCs were cultured in chondrogenic differentiation medium. Stain revealed evidence of matrix production. This was not present in hMSCs cultured in control medium.

spindle shape typical of fibroblastic morphology, but 5% of them were polygonal. Moreover, these stromal cells had the capacity to proliferate and retain their spindle morphology for over 10 passages (data not shown).

3.3. Phenotypical characterization of hMSCs from third passage cultured at low cell density (240 cells/cm^2, 12 days)

Immunocytochemistry analysis showed positive staining for β-subunit of prolyl-4-hydroxylase, CD73, CD105 and CD44 (Fig. 1B, a–d). Staining was not observed when cells were incubated without first Ab or with isotype control (Fig. 1B, e and f). These cells had also positive expression for CD54, CD49b, CD49e, CD68 (low expression) and negative for CD45 and CD34 (data not shown). These results indicated that these cells had mesenchymal stromal markers.

3.4. Osteogenic, adipogenic and chondrogenic differentiation of hMSCs

3.4.1. Osteogenic potential

When hMSCs were incubated in the osteogenic medium, some of them differentiated into the osteoblastic/osteocytic lineage, suffering changes in the nucleus–cytoplasm relation: the cytoplasm became condensed and the nucleus acquired a rounded shape and moved becoming slightly off-centered (Fig. 2A, b). In the more differentiated stages, osteocytes, cells became smaller (Fig. 2A, c). The results also showed that 100% of stimulated cultures developed colonies with osteogenic cells and presented 24 ± 4% of Petri dish surface with differentiated cells. These cultures developed calcium deposits and mineralization matrix with homogeneous distribution over the complete surface detected by Von Kossa and Alizarin Red-S staining (Fig. 2A, d–e). Osteocalcin staining was also observed (Fig. 2A, f). In the control cultures, the stromal cell morphology observed was the classic spindle shape (prolyl 4-hydroxylase positive) (Fig. 2A, a) and showed negative staining for Von Kossa, Alizarin Red-S and Osteocalcin (data not shown).

3.4.2. Adipogenic potential

When hMSCs were cultured in adipogenic medium, 100% of the samples developed lipidladen intracellular vacuoles with Oil Red-O-positive staining (Fig. 2B, b). By day 2 of stimulation, the first cells filled with lipid droplets appeared, and increased in number, covering almost 40% of the cell layer on day 21. In non-treated hMSC cultures, cells showed no detectable cytoplasmic lipid vacuoles (Fig. 2B, a).

3.4.3. Chondrogenic potential

All hMSC cultures treated with chondrogenic medium developed chondrogenic pellet after 3 weeks, with a pellet weight of $\overline{X} \pm SE = 0.015 \pm 0.008$ g. The pellet slide of hMSCs treated with chondrogenic medium presented collagen type II deposits (Fig. 2C, a) and sulfated proteoglycan accumulation by toluidine blue staining (Fig. 2C, b). Differentiation of hMSCs until mature chondrocytes was observed in all chondrogenic pellets. Non-treated cultures did not develop chondrogenic pellet after 3 weeks.

3.5. Cardiomyogenic differentiation of hMSCs

3.5.1. Characteristics of hMSCs committed with cardiomyocyte-like cells during SLO+EX treatment

The capacity to give CFU-F and differentiate into adipogenic, osteogenic and chondrogenic lineages, after appropriate stimulation, confirms that these adherent cells were hMSCs. Therefore we decided to investigate their cardiomyogenic potential.

One week after SLO+EX or 5-AZA treatments, 27.58±0.68 (x±ES, #8) of all the hMSCs with spindle shape, gradually increased in size and formed a ball-like appearance (Fig. 3). At the second week these cells lengthened in one direction and formed a stick-like morphology. Within 3–4 weeks, they

connected with adjoining cells and formed myotube-like structures. hMSC cultures without treatment had a spindle shape (0 week).

3.5.2. *Effect of SLO+EX treatment on the expression of cardiomyogenic markers*

Sarcomeric α-actinin, connexin-43 and nuclear transcription factor GATA-4 were expressed in non-stimulated and stimulated (SLO+EX or 5-AZA) hMSC cultures (Fig. 4). However, the expression of sarcomeric α-actinin and connexin-43 was stronger in differentiated hMSCs treated with SLO+EX or with 5-AZA compared to the non-treated hMSCs. Analysis of cardiac transcription factor GATA-4 showed strong nuclear staining in both treated and non-treated hMSCs. However, the strongest expression of this factor was found in cells treated with SLO+EX.

After treatments, the hMSCs were consistently positive stained for SERCA-2, cardiac Troponin I, β-MyHC and Nkx2.5, while non-treated hMSCs were negative for these specific cardiac markers (Fig. 5A). These results demonstrated a development of the cardiomyocyte phenotype with a mature contractile apparatus.

hMSCs differentiated with SLO+EX or 5-AZA presented expression of desmin through the cytoplasm (Fig. 5B). MCL-2a and MLC-2v showed a diffuse cytoplasmic expression, with faint sarcomeric pattern in both cases (Fig. 5B). In contrast, no staining was observed in non-treated hMSC cultures.

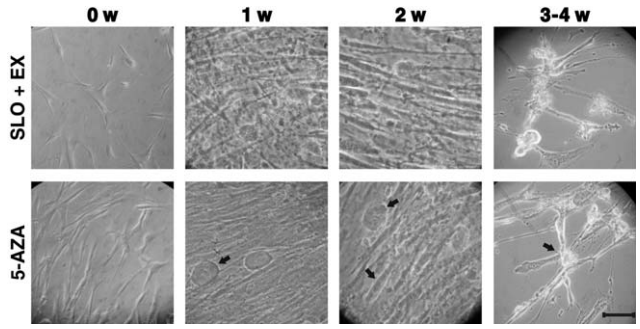

Fig. 3. Morphological changes of hMSCs during SLO+EX or 10 μM 5-AZA treatments. hMSCs before any treatments (0 weeks, 0w). One week (1w) after treatments, some cells gradually increased in size and formed a ball-like shape (*arrowheads*). Two weeks (2w) after treatment, cells developed a stick-like appearance. Three–four weeks (3–4w) after treatment, most cells connected and formed myotube-like structures. Scale bar: 50 μm.

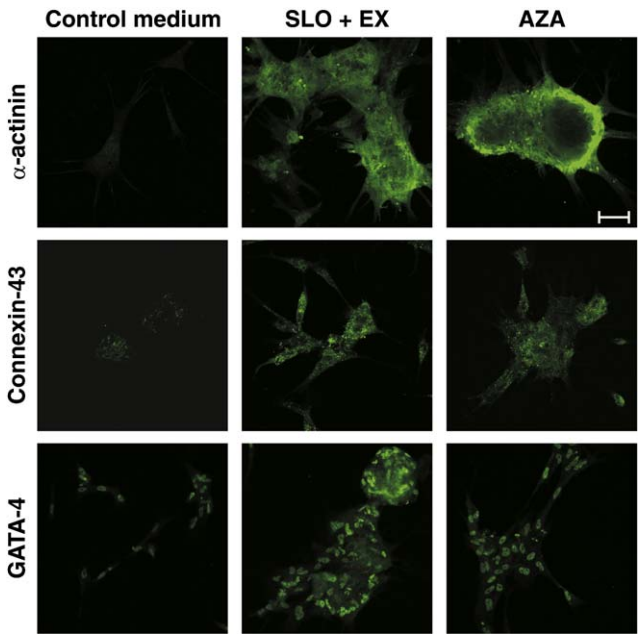

Fig. 4. Expression of early markers of cardiac lineage in treated and non-treated hMSCs. Immunofluorescence staining for sarcomeric α-actinin, connexin-43 and GATA-4 in hMSCs treated with or without SLO+EX or 10 μM 5-AZA. The scale bar represents: 50 μm.

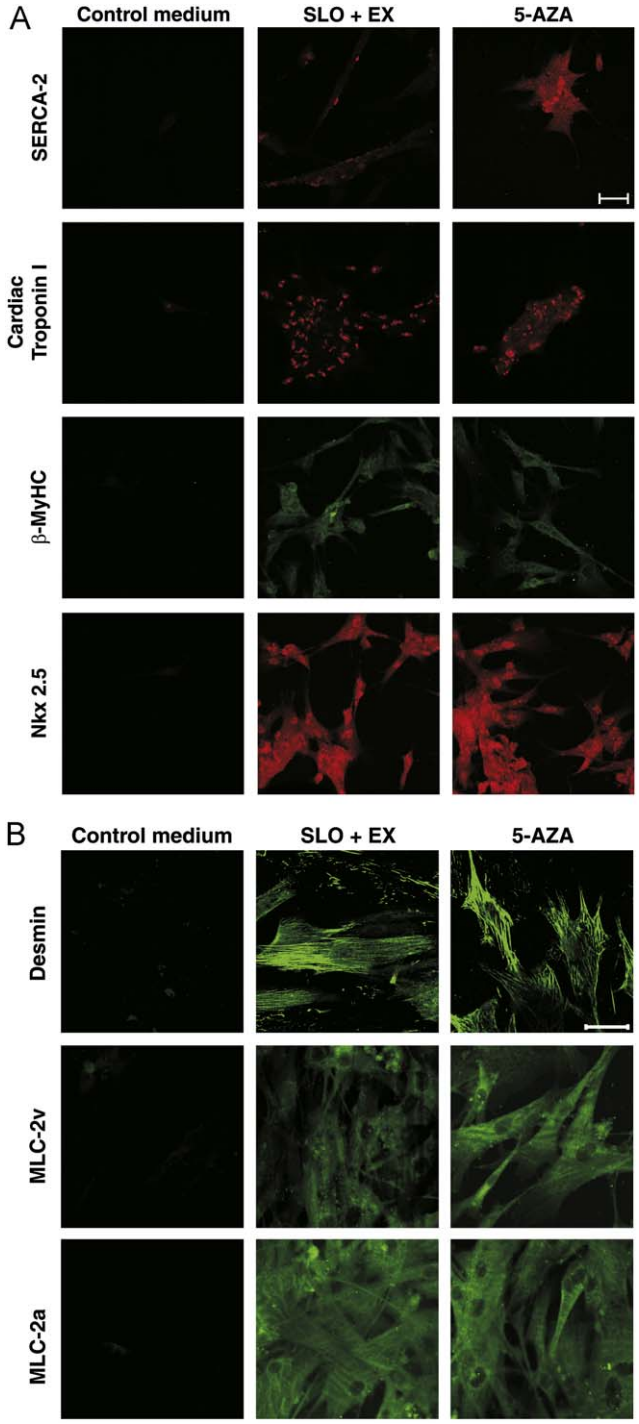

Fig. 5. Expression of specific cardiomyogenic markers in treated hMSCs. (A) Immunofluorescence staining for SERCA-2, cardiac Troponin I, β-MyHC and Nkx2.5 was positive in human adult cardiomyocyte-like cells and was negative in non-treated hMSC cells. (B) Immunofluorescence detection for cardiac desmin, MLC-2v and MLC-2a were also positive in treated hMSCs. No staining was present in hMSCs cultured in control medium. The scale bar represents 50 μm.

In the negative immunofluorescence controls, in which the primary Ab was omitted or isotype control was used, negligible immunofluorescence was observed (data not shown).

3.6. RT-PCR analysis

Fig. 6 shows that expression of β-actin (a), used as an internal control, was the same in undifferentiated and differentiated cells, whereas β1-AR (b,b1), MLC-2a (c,c1) and cardiac Troponin T (d) cardiomyogenic-specific genes were expressed in hMSCs after SLO+EX or 5-AZA treatments. β1-AR and MLC-2a expression was confirmed by highly sensitive hemi-nested PCR reaction. Total RNAs obtained from HL-1 cell line and neonatal rat cardiomyocytes (CAR) cultures were used as positive controls.

4. Discussion

In agreement with previous reports, the present study demonstrates that normal BM-hMSCs were able to develop CFU-F and differentiate into osteocytes, adipocytes and chondrocytes (Minguell and Erices, 2006; He et al., 2007; Phinney and Prockop, 2007), both characteristics (cloning capacity and plasticity) are exclusive and essential to define hMSCs and show its potential capacity to give cardiomyocyte linage.

When hMSCs were expanded *in vitro*, they presented splindle morphology and expressed a number of membrane molecules including the β-subunit prolyl-4-hydroxylase, CD73, CD105, CD44, CD54, CD49b, CD49e and they also presented a low expression of CD68 and no expression of hematopoietic linage markers, like CD45 and CD34. Results were in agreement with other authors (Nauta and Fibbe, 2007; Phinney and Prockop, 2007).

In addition hMSCs have been demonstrated to have pleiotropic immunomodulatory effects *in vitro* and have been advocated for therapeutic use in human (Haniffa et al., 2007). Since BM-MSCs have shown low expression of surface-associated HLA class I but not HLA class II antigens, they do not appear to elicit alloreactive lymphocyte proliferative responses and appear to modulate immune responses (Di Nicola et al., 2002; Le Blanc et al., 2003). Thus, they are able to survive in a xenogeneic environment.

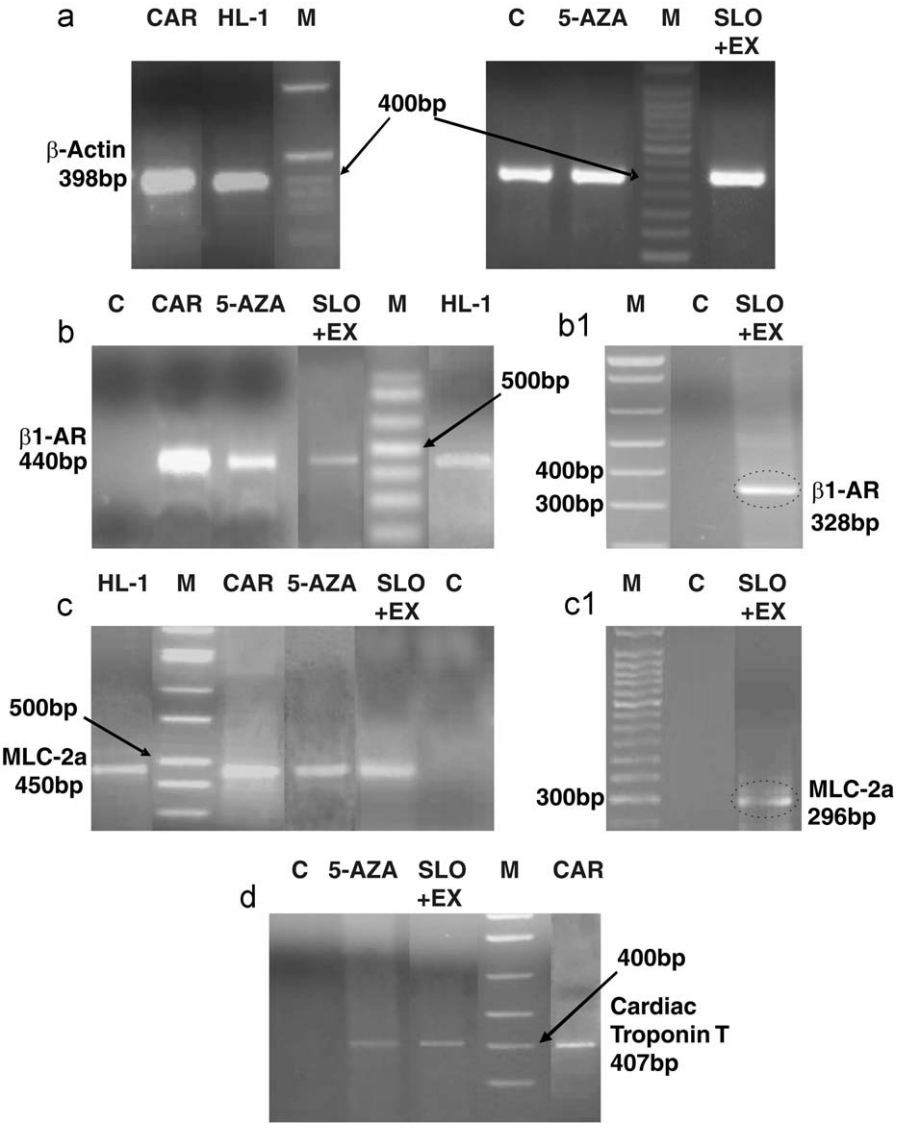

Fig. 6. RT-PCR analysis of expression of specific cardiomyocyte markers in treated hMSCs. Expression of β-actin (a), β1-adrenergic receptor (β1-AR) (b), hemi-nested β1-AR (b1), MLC-2a (c), hemi-nested MLC-2a (c1) and cardiac Troponin T (d). *HL-1*: positive control from immortalized atrial cardiomyocyte murine cell line RNA; *CAR*: positive control from culture of neonatal rat cardiomyocytes RNA; *5-AZA*: hMSCs induced with 10 μM 5-AZA RNA; *SLO+EX*: hMSCs induced with SLO+EX RNA and *C*: non-treated hMSC RNA. M: molecular weight marker.

As we mentioned above hMSCs are attractive candidates for cell therapy or cellular vehicles in molecular therapy to deliver genes (He et al., 2007; Phinney and Prockop, 2007). The study of hMSCs has been extended from bench to bedside with many clinical applications such as improving hematopoietic engraftment and correcting genetic disorders (He et al., 2007).

Various cell lineages have been used to generate evidence that BM-stem cells differentiate into cardiomyocytes, endothelium, and smooth muscle cells (Silva et al., 2005). However, there is much controversy regarding which stem cells subtype might be responsible for the therapeutic benefit of BM-MNC transplantation into ischemic myocardium (Silva et al., 2005). In theory, the ideal cell type for cellular therapy is likely to be a less committed one that can undergo full cardiomyocyte differentiation, augment angiogenesis, and trigger vasculogenesis. In that regard, hMSCs may have the necessary combination of plasticity and viability.

Until now, hMSCs therapy has been able to lead to successful cardiac regeneration or repair by any of three general mechanisms depending on the condition used: differentiation of the administered cells into all of the cellular constituents of the heart, release of soluble factors capable of paracrine signaling from the administered cells, and fusion of the administered cells with the existing constituents of the heart (Minguell and Erices, 2006; Iso et al., 2007).

Having demonstrated the ability and plasticity of hMSCs to give CFU-F and adipogenic, osteogenic and chondrogenic lineages, we decided to investigate the *in vitro* potential of these cells to differentiate into cardiomyocytes by using cardiomyocyte nuclear reprogramming treatment.

Several authors have demonstrated that MSCs from rat, mice and human treated with 5-AZA could differentiated into cardiomyocyte cells (Makino et al., 1999; Tomita et al., 1999; Antonitsis et al., 2008). We found that the better results for promoting *in vitro* cardiomyogenic differentiation of hMSCs was obtained in the third passage with 10 μM 5-AZA. According to this, we used hMSCs from the third passage treated SLO+EX to obtain cardiomyogenic differentiation and compare these results with the traditional 10 μM 5-AZA differentiation treatment.

We observed that SLO+EX treatment induced changes into cardiomyocyte-like morphology (ball, stick and myotube-like morphologies) in the same way that 10 μM 5-AZA treatment did.

hMSCs spontaneously expressed markers of cardiac phenotype-like sarcomeric α-actinin, connexin-43 and GATA-4. However, when these cells were exposed to SLO+EX or 10 μM 5-AZA treatments, the expression of sarcomeric α-actinin, connexin-43 and GATA-4 were significantly increased and furthermore, expressed SERCA-2, cardiac Troponin I, β-MyHC, desmin, MLC-2v and MLC-2a thus showing the phenotype of mature cardiomyocytes (Bayes-Genis et al., 2005). It is well known that the expression of sarcomeric α-actinin, cardiac Troponin I and β-MyHC indicate complete sarcomeric organization (Bayes-Genis et al., 2005). Moreover, the expression of connexin-43 may allow early intercellular contacts and facilitate electromechanical coupling between myocytes and adjacent myocytes, decreasing the possibility of arrythmias, after *in vivo* cell delivery (Abraham et al., 2005). The expression of SERCA-2 is responsible for transport of Ca^{2+} back into the sarcoplasmic reticulum and plays a key role in the contraction–relaxation cycle of the myocardium (Aoyagi et al., 1999; Li et al., 2007). In addition, the expression of GATA-4 and Nkx2.5 has also been identified in these differentiated hMSCs and it is well known that both molecules are potential regulators of the early and late steps of cardiac development, respectively. Furthermore, GATA-4 and 6 over expression at the time of Nkx2.5 induction results in a significant up-regulation of endogenous Nkx2.5 transcription (Shiojima et al., 1999; Brewer et al., 2005).

In relation with the expression of β1-AR, our results are similar to those of Hakuno et al. (2002). PCR techniques revealed that differentiated hMSCs with SLO+EX or 10 μM 5-AZA presented positive β1-AR while non-treated hMSCs were negative for this marker.

RT-PCR analysis also showed that other cardiomyocyte-related genes, such as MLC-2a and cardiac Troponin T, were expressed after SLO+EX treatment as was observed with 10 μM 5-AZA treatment. In contrast, non-treated hMSC was negative for these markers.

In summary, we showed that hMSCs isolated from BM from healthy volunteers achieved similar cardiomyogenic differentiation *in vitro* after both treatments. However, the SLO+EX treatment appear to be riskless in view of the genotoxic effects of the 5-AZA (Guttenbach and Schmid, 1994; Li et al., 2007; Stopper et al., 1995).

Finally, the nuclear reprogramming by cell extract may be an approach leading to the identification of soluble factors that drives the reprogramming. The soluble factors may then be produced as safe recombinant proteins.

Author disclosure statement

No competing financial interests exist.

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