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Brazilian minipig as a large-animal model for basic research and stem cell-based tissue engineering. Characterization and *in vitro* differentiation of bone marrow-derived mesenchymal stem cells

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ABSTRACT

Stem cell-based regenerative medicine is one of the most intensively researched medical issues. Pre-clinical studies in a large-animal model, especially in swine or miniature pigs, are highly relevant to human applications. Mesenchymal stem cells (MSCs) have been isolated and expanded from different sources. Objective: This study aimed at isolating and characterizing, for the first time, bone marrow-derived MSCs (BM-MSCs) from a Brazilian minipig (BR1). Also, this aimed to validate a new large-animal model for stem cell-based tissue engineering. Material and Methods: Bone marrow (BM) was aspirated from the posterior iliac crest of twelve adult male BR1 under general anesthesia. MSCs were selected by plastic-adherence as originally described by Friedenstein. Cell morphology, surface marker expression, and cellular differentiation were examined. The immunophenotypic profile was determined by flow cytometry. The differentiation potential was assessed by cytological staining and by RT-PCR. Results: MSCs were present in all minipig BM samples. These cells showed fibroblastic morphology and were positive for the surface markers CD90 (88.6%), CD29 (89.8%), CD44 (86.9%) and negative for CD34 (1.61%), CD45 (1.83%), CD14 (1.77%) and MHC-II (2.69%). MSCs were differentiated into adipocytes, osteoblasts, and chondroblasts as demonstrated by the presence of lipidic-rich vacuoles, the mineralized extracellular matrix, and the great presence of glycosaminoglycans, respectively. The higher gene expression of adipocyte fatty-acid binding protein (AP2), alkaline phosphatase (ALP) and collagen type 2 (COLII) also confirmed the trilineage differentiation ($p<0.001$, $p<0.001$, $p=0.031$; respectively). Conclusions: The isolation, cultivation, and differentiation of BM-MSCs from BR1 makes this animal eligible as a useful large-animal model for stem cell-based studies in Brazil.

Keywords: Animal models. Miniature swine. Bone marrow. Stem cells. Tissue engineering.

INTRODUCTION

Although *in vivo* experiments using small animals provide the therapeutic potential of a treatment, there exist many fundamental differences between the small animal and the human[10]. Before applying the therapy to clinical patients, large animal studies, especially in swine or miniature pigs, are a prerequisite to validate the efficacy in an animal model more relevant to the human[10,17]. Previous studies have demonstrated that the minipig represents a suitable large animal

model for preclinical testing of different diseases and treatments[1,11].

Mesenchymal stem cells (MSCs) were first described by Fridenstein, et al. in 1976 as the clonal, plastic adherent cells, being a source of the osteoblastic, adipogenic and chondrogenic cell lines[5]. MSCs are non-hematopoietic cells, which are present in a variety of tissues, being more prevalent in the bone marrow (BM) compartment, playing a key role in the maintenance of BM homeostasis and regulate the maturation of both hematopoietic and non-hematopoietic cells[14].

The isolation and the expansion of porcine MSCs (pMSCs) from different tissues have been relatively easy due to their adherence to culture plastic, fibroblastic morphology, self-renewal, proliferation, and *in-vitro* tri-lineage differentiation (adipogenic, chondrogenic, and osteogenic)[3,4,17,20].

As reported previously, the techniques used for isolation, expansion, osteogenic, chondrogenic, and adipogenic differentiation of human MSCs can be adopted for analysis of pMSC, which may serve the increasing demand for stem and progenitor cells in tissue engineering[1,11,17].

In Brazil, the minipig BR-1 is the only Brazilian's miniature pig, developed exclusively for research. This paper aimed at isolating and characterizing BM-MSCs from BR1 to validate this new large-animal model for stem cell-based tissue engineering in Brazil.

MATERIAL AND METHODS

Animals

Twelve adult male Brazilian miniature pigs (BR-1) aged 18–24 months and weighing 30–40 kg (Figure 1) (MINIPIG Research and Development, Campina do Monte Alegre, SP, Brazil) were used in the present study. The Ethics and Research Committee at Positivo University, Curitiba, Paraná, Brazil, approved this study (protocol 001/2009). The experimental procedures and care of the animals were conducted in accordance with the Law 11794/2008 of the Brazilian Federal Constitution, which regulates and establishes the "Procedures for the scientific use of animals".

The animals were kept in the bioterium throughout the experiment in pre-cast bays, with enough space for two animals in each, at a temperature of 23±2°C, a relative humidity of 55±10%, a 12/12 h dark/light cycle, and at hygienic conditions. The animals were fed twice a day with appropriated food (Presuntina Pro, Nestlé Purina, São Paulo, SP, Brazil) and water *ad libitum*.

Isolation, expansion and culture of BM-pMSCs

For BM aspiration, animals received an intramuscular injection of a combination of ketamine hydrochloride 10% (10 mg/kg) and xylazine hydrochloride 2% (5 mg/kg), followed by intravenous administration of sodium pentobarbital 5% (5 mg/kg). From each animal, a total of 20 mL of fresh heparinized BM was aspirated from posterior iliac crest using a Jamishidi needle (Raiomedic, São José dos Pinhais, PR, Brazil). The samples were added into Dulbecco's modified Eagle medium (DMEM) (Gibco® Invitrogen, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco® Invitrogen, Grand Island, NY, USA), and 50 IU/mL heparin (Cristália, São Paulo, SP, Brazil). Mononuclear cells were isolated by Ficoll-Hypaque (Sigma-Aldrich Corp, St. Louis, MO, USA) density gradient centrifugation (d=1,077 g/cm^3)[20]. After centrifugation at 400 xg for 30 min at room temperature, mononuclear cells, which were situated in an opalescent layer between the gradient and blood plasma, were retrieved and washed in DMEM supplemented with 1% antibiotics. The number of mononuclear cells was counted and the cell viability was assessed.

Mononuclear cells were resuspended in DMEM supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin, plated on 75 cm^2 flasks (TPP, TRA, Switzerland) at a density of approximately 1x10^5 cells/cm$_2$ and

Figure 1- Adult male Brazilian miniature pigs (BR-1)

incubated in a humidified 5% CO_2 atmosphere at 37°C (Thermo Forma Series II, Marietta, Ohio, USA). After 48 hours, the non-adherent cells were discarded and the adherent cells were cultured at the same conditions until 80–90% of confluence. Cells were detached using 0.25% trypsin-ethylenediamine tetraacetic acid solution (Trypsin-EDTA) (Gibco® Invitrogen, Grand Island, NY, USA) for 4 min at 37°C, washed twice with phosphate-buffered saline (PBS) (0.01 M, pH 7.2) (Gibco® Invitrogen, Grand Island, NY, USA), centrifuged at 400 g for 10 min, and resuspended in the same culture conditions (passage 1). The cells were plated at a density of $4x10^3$ cells/cm^2. Media change was carried out every three days. The BM-pMSCs were expanded until passage 4, when the immunophenotypic profile was characterized by flow cytometry and the *in vitro* differentiation was performed. In each passage, the number of MSCs was counted and the cell viability was assessed. Cells were continuously observed until passage 4 by light microscopy to evaluate cell morphology, the lack of proliferation signs and the cell death ratio.

Cell viability

The cell number and the viability were evaluated in each passage. Briefly, 50 µL of cell suspension were added to 10 µL of the Trypan Blue 0.4% dye (Sigma Aldrich, St. Louis, MO, USA) during five minutes. Using a Neubauer chamber in a light microscope, the total numbers of live and dead cells (blue cells) were counted and the percentage of viable cells was calculated.

Flow cytometry

Immunophenotypic analyses were performed by staining approximately $5x10^5$ cells with unconjugated monoclonal antibodies against pig antigens. At passage 4, undifferentiated BM-pMSCs were detached using 0.25% trypsin-EDTA and labeled with unconjugated monoclonal antibodies against swine cluster of differentiation (CD) antigens CD14 (CAM36A), CD44 (PORC24A), CD45 (74-9-3A1), CD29 (FW4-101) and MHC-II (MSA3) (all from VMRD Inc., Pullman, WA, USA). Briefly, cells were detached, washed with PBS (Gibco® Invitrogen, Grand Island, NY, USA), incubated with antibodies for 15 minutes on ice, washed again for three times, and then further incubated with an isotype-specific phycoeritrin (PE) conjugated rat anti-mouse (BD Biosciences, Seattle, WA, USA) for another 15 minutes. The cells were washed with PBS and resuspended in 500 µL of PBS with 1% of formaldehyde (Gibco® Invitrogen, Grand Island, NY, USA). Mouse isotype IgG1 antibody was used as control (BD Pharmingen, San Jose, CA, USA). The cells were also stained with mouse anti-human CD90 and CD34 both PE conjugated (BD Biosciences, Seattle, WA, USA).

7-AAD viability dye solution was used to discriminate viable from non-viable cells (BD Biosciences, San Jose, CA, USA). Cells were washed with PBS, incubated with dye solution for 30 minutes, washed again, and resuspended in 500 µL of PBS with 1% of formaldehyde (Sigma-Aldrich Corp, St. Louis, MO, USA). Not all lineage phenotypes could be examined because of the limited availability of commercial antibodies working in pig immunostaining. For this reason, we used anti-human CD90 and CD34.

Approximately 100,000 events were acquired using a FACS Calibur flow cytometer (Becton & Dickinson Company, Franklin Lakes, NJ, USA) and analyzed with the FlowJo software (TreeStar Inc., Ashland, OR, USA).

In vitro pMSCs differentiation

The *in vitro* differentiations of BM-pMSCs were evaluated in triplicates by culturing cells from passage 4 in a commercial media. The control ones were cultured in DMEM with 15% FBS for the same period.

Approximately $15x10^3$ pMSCs were plated on glass coverslips (Sarstedt Group, Newton, NC, USA) in 24-well plates (TPP Techno Plastic Products, Trasadingen, Switzerland) in a DMEM with 15% FBS. When the cell confluence was approximately 80%, the medium was replaced with the osteogenic medium (StemPro® Osteogenic Differentiation Basal Medium, Gibco® Invitrogen, Grand Island, NY, USA) or the adipogenic medium (StemPro® Adipogenic Differentiation Basal Medium, Gibco® Invitrogen, Grand Island, NY, USA). Cells were fed with the fresh differentiation medium every 3 days for 21 days[16].

For chondrogenic differentiation, cells were grown in micromass culture[4,6]. Briefly, $2x10^5$ cells in 0.5 mL of DMEM were centrifuged at 300 g for 10 min in a 15 mL polypropylene tube to form a pellet. Without disturbing the pellet, cells were cultured for 21 days in an induced medium (StemPro® Chondrogenic Differentiation Basal Medium, Gibco® Invitrogen, Grand Island, NY, USA). Cells were fed with the fresh differentiation medium every 3 days for 21 days.

Alizarin red staining for mineralized matrix

Cells were fixed with 4% paraformaldehyde for 5 min and washed with water three times, then the cells were incubated with alizarin red S at pH 4.1 (Sigma-Aldrich Corp, St. Louis, MO, USA) for 15 min at room temperature. Cells were washed with water several times prior to imaging.

Oil red staining for cytoplasmic fat globules

Cells were fixed with 4% paraformaldehyde for 5 min and washed with water three times, then the cells were incubated for 10 min with a filtered 0.5% Oil red (Sigma-Aldrich Corp, St. Louis, MO, USA) solution prepared in 60% isopropanol. Cells were washed with water several times prior to photographing.

Alcian blue staining for mucosubstances

Cell aggregates were fixed in 10% paraformaldehyde for 1 h at room temperature, dehydrated in serial ethanol dilutions, and embedded in paraffin blocks. Paraffin sections of 4 µm of thickness were stained for histological analysis with Alcian Blue 8GX (Sigma-Aldrich Corp, St. Louis, MO, USA) solution prepared in 3% acetic acid. The samples were washed with water several times prior to imaging.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Osteogenic, adipogenic and chondrogenic differentiation were further confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). BM-pMSCs cultured in DMEM with 15% FBS were used as control.

Total RNA was obtained with the RNeasy purification kit (QIAGEN, Austin, TX, USA) and treated in column with DNAse I (QIAGEN, Austin, TX, USA). Concentrations were determined by spectrophotometry (Gene-Quant, Amersham Biosciences, Sunnyvale, CA, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA by using 1 µl of 10 µM oligo-dT primer (USB Corporation, Cleveland, OH, USA) and 1 µl of reverse transcriptase (IMPROM II, Promega, Fitchburg, WI, USA) according to the manufacturers' instructions.

RT-PCR was carried out with 20 ng of cDNA as template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 pmol of primers (10 pmol for each gene), 2.5 mM MgCl2, 0.0625 mM dNTPs, and 1 unit Taq polymerase (Gibco® Invitrogen, Grand Island, NY, USA). The primer sets used for PCR and the amplicon size are depicted in Figure 2. PCR included denature at 94°C for 2 minutes, and the heating was followed by 30 cycles of denature at 94°C for 15 seconds, annealing for 30 seconds, extension at 72°C for 40 seconds, and a final extension of 72°C for 3 minutes by using a Bio-Cycler II thermocycler (Peltier Thermal Cycler; Bio-Rad, Hercules, CA, USA). Ten microliters of RT-PCR products were analyzed by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed under ultraviolet illumination (UV White Darkroom, UVP Bioimaging Systems, Upland, CA, USA). GAPDH was used as a housekeeping control. This experiment was performed with material from at least three animals, with technical triplicates. RT-PCR bands were analyzed by using ImageJ version 1.45d.

Target gene	Primer sequence		Annealing	Predicted
	Forward (5'- 3')	Reverse (3'- 5')	temperature (°C)	size (bp)
GAPDH	CTG CCC CTT CTG CTG ATG C	GAC AAC TTC GGC ATC GTG GA	60	151
ALP	ATG AGC TCA ACC GGA ACA A	GTG CCC ATG GTC AAT CCT	56	131
AP2	GGC CAA ACC CAA CCT GA	GGG CGC CTC CAT CTA AG	58	167
Col2	CTG GAG CTC CTG GCC TCG TG	CAG ATG CGC CTT TGG GAC CAT	67.1	180

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; ALP: alkaline phosphatase; AP2: adipocyte protein 2; Col2: type 2 collagen

Figure 2- Primer sequences and conditions used for reverse transcriptase-polymerase chain reaction (RT-PCR)

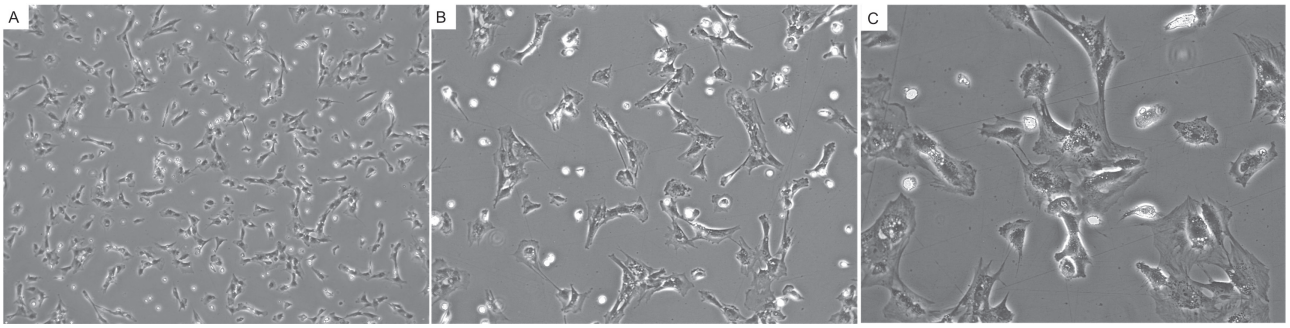

Figure 3- Morphology of MSCs-BM of Minipig BR-1: (A) 40x (B) 100x (C) 200x

Statistical analysis

Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as mean and standard deviation. Student's t test was used to assess the significance of differences between the cell populations that were analyzed. P values ≤0.05 were considered to be statistically significant.

RESULTS

Porcine MSCs were successfully isolated from BM of all 12 animals. By day 3, cells adhered, and nonadherent hematopoietic cells present in the culture after initial plating were removed with sequential exchange of culture medium. After 4–5 days of culturing the first colonies of mononuclear cells were observed. Morphologically, pMSC appeared as single, stretched cells leading to large clusters of stellate cells as they multiplied. Around the 10th day, 80–90% of cell confluence was observed. MSCs attached to the culture flasks adopted a fibroblast-like morphology (Figure 3). These cells appeared to become more flattened and enlarge in size after several sequential passages. Until passage 4, an average of 25 days (up to 30 days) of culturing was necessary. Considerable individual differences were observed in the number of isolated mononuclear cells and in the BM-pMSCs in each passage (Table 1).

The cells expressed the mesenchymal surface markers, CD29, CD44 and CD90, and did not express the hematopoietic markers, CD45, CD34 and CD14. The cells also did not express MHC-II. The cell viability evaluated by 7-AAD was 97.3% (Figure 4).

The BM-pMSCs exposed to medium supplemented with differentiation factors were capable of differentiating into mesenchymal lineages, such as osteogenic, adipogenic, and chondrogenic. During osteogenic stimulation, BM-pMSC continued to proliferate and readily formed multilayers showing differentiation along the osteogenic lineage. At day 21, after alizarin red staining, almost the whole cell layer was heavily covered with mineralized matrix. The adipogenic differentiation was confirmed by staining with oil red O, since the adipocytes were easily identified morphologically, by intracellular accumulation of neutral lipids. The chondrogenic potential of BM-pMSC was also characterized by alcian blue staining, after 21 of culturing, which revealed a homogeneous deposition of glycosaminoglycans. The control cells cultured during the same period in DMEM supplemented with 15% FBS did not exhibit spontaneous differentiation (Figure 5).

The higher gene expression of AP2, ALP and COLII in induced cells compared with non-induced cells also confirmed their potential to differentiate into adipocytes, osteoblasts and chondroblasts, with statistical significance ($p<0.001$, $p<0.001$ and $p=0.031$, respectively). The relative quantification of mRNA levels normalized to GAPDH was respectively for control and induced cells: 0.1

Table 1- Total number and viability of mononuclear cells isolated from bone marrow of minipig BR-1 and number and viability of mesenchymal stem cells in each passage

Sample	Isolated Mononuclear Cells		Passage 1		Passage 2		Passage 3		Passage 4	
	N cells	Viability (%)	N cells	Viability (%)	N cells	Viability (%)	N cells	Viability (%)	N cells	Viability (%)
1	183.5x10^6	87.0	30.5x10^6	97.0	36.5x10^6	96.4	32.5x10^6	88.0	50.0x10^6	87.5
2	122.5x10^6	95.2	47.5x10^6	97.0	40.5x10^6	97.0	55.5x10^6	91.0	70.0x10^6	93.0
3	181.7x10^6	94.0	37.0x10^6	97.3	31.0x10^6	95.5	65.5x10^6	90.0	154.0x10^6	98.0
4	176.2x10^6	83.0	48.3x10^6	92.1	45.0x10^6	91.0	87.0x10^6	95.2	120.0x10^6	96.3
5	122.5x10^6	93.5	50.7x10^6	93.0	59.7x10^6	97.0	63.5x10^6	90.0	72.5x10^6	97.0
6	211.5x10^6	93.6	43.5x10^6	95.0	47.5x10^6	96.0	39.5x10^6	92.0	45.5x10^6	96.0
7	127.5x10^6	94.7	29.7x10^6	92.0	32.0x10^6	97.0	43.5x10^6	95.0	50.0x10^6	99.0
8	218.2x10^6	94.0	70.7x10^6	95.0	73.5x10^6	95.0	68.5x10^6	96.0	64.0x10^6	96.0
9	244.0x10^6	94.0	90.5x10^6	97.0	85.3x10^6	97.0	92.7x10^6	91.0	111.0x10^6	97.0
10	154.0x10^6	90.0	78.7x10^6	92.0	81.2x10^6	92.0	88.5x10^6	97.5	95.5x10^6	100.0
11	220.0x10^6	89.0	92.5x10^6	94.0	88.0x10^6	94.0	95.5x10^6	93.5	100.5x10^6	93.0
12	140.0x10^6	92.0	60.5x10^6	97.0	75.7x10^6	96.0	70.5x10^6	92.0	78.5x10^6	94.0
Average	183.5x10^6	91.7	56.7x10^6	94.9	58.0x10^6	95.3	66.9x10^6	92.6	84.3x10^6	95.5
SD	38.9x10^6	3.7	21.9x10^6	2.2	21.7x10^6	2.0	21.3x10^6	2.8	32.8x10^6	3.3

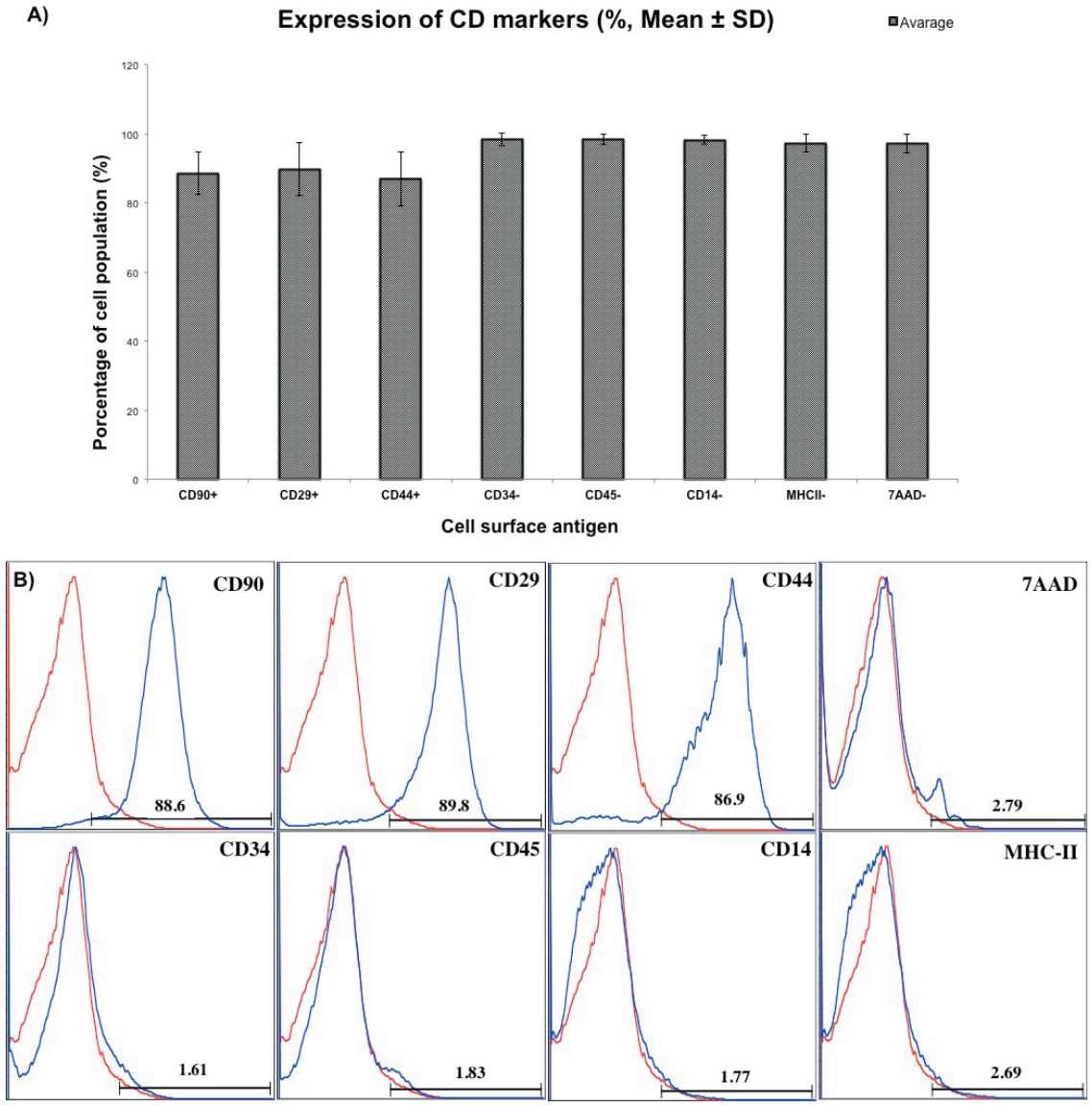

Figure 4- A) Immunophenotypic analysis. Each value is the mean percentage of the twelve samples ± standard deviation of BM-pMSCs, which were tested for each cell surface antigen expression. B) BM-pMSCs were labeled with antibodies against the indicated antigens and analyzed by flow cytometry. Representative histograms are displayed. On the y axis, there is the % max (the cell count in each bin divided by the cell count in the bin that contained the largest number of cells), and the x axis is the fluorescence intensity in a log (100–104) scale. The isotype control is showed in red, and blue histogram indicates reactivity with the antibody indicated

and 1.2 (AP2), 1.0 and 13.7 (ALP), 1.0 and 1.7 (COLII). The RT-PCR of each gene also showed a strong band in induced cells. The overall RT-PCR profile was very similar for the technical triplicates (Figure 6).

DISCUSSION

Great advances in tissue engineering have been obtained in large-animal models. The development of preclinical models in large animals requires the use of well-characterized animal cell lines similar to their human counterparts[2]. The use of miniature pigs in experimental research has advantages because this animal has characteristics and dimensions similar to the human body, bringing the experiences of clinical trials in human medicine[15]. Furthermore, minipigs have close similarities to humans in terms of platelet count, clotting parameters, metabolic rate, bone structure and MSCs characteristics[2,10,11,15,17,20], providing good representation of individual patient-based investigations. The BR1 still has larger dimensions compared to Göttingen minipigs, but in Brazil the BR1 is the only breed developed exclusively for research. The Gottingen minipigs here in Brazil are rare to find and they are more expensive than the BR1. The advantage of using this breed

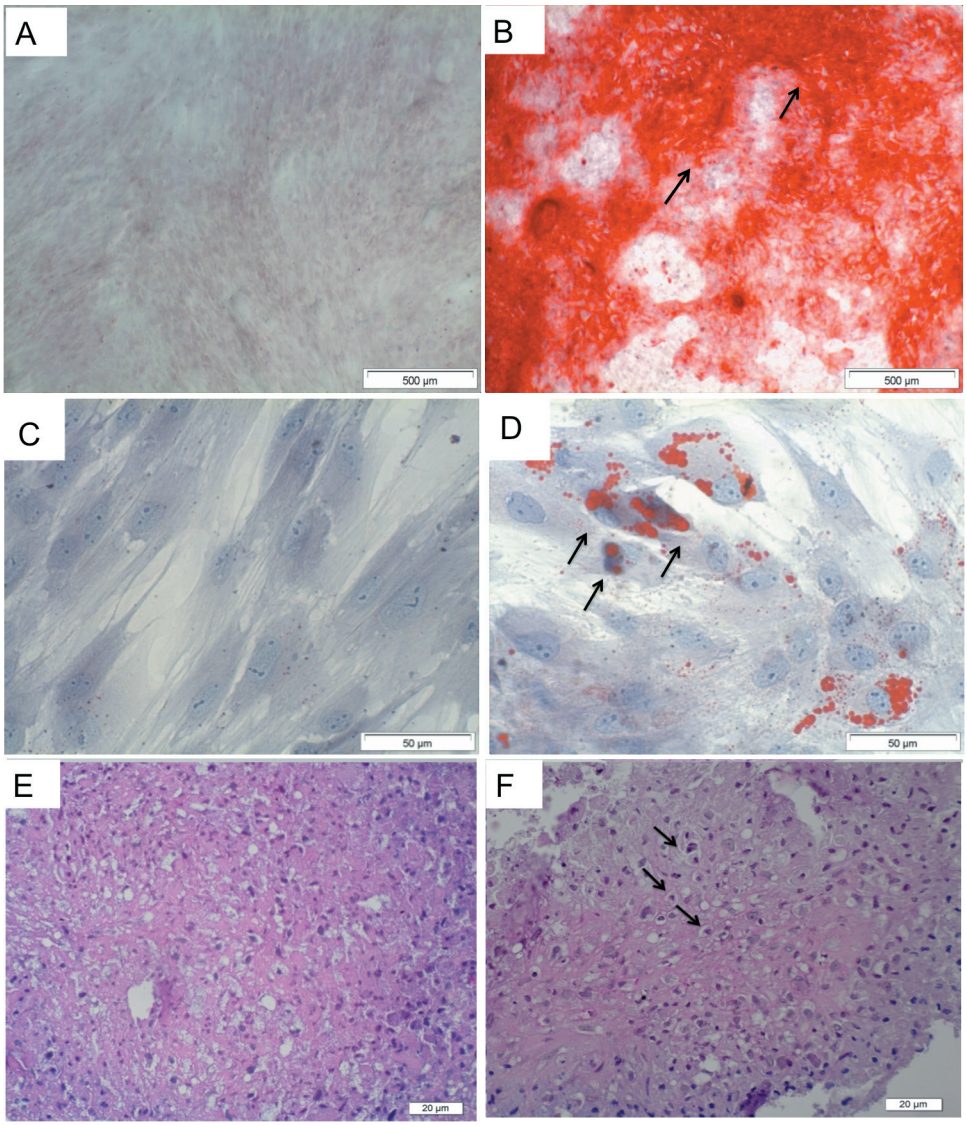

Figure 5- Differentiation of BM-pMSCs into osteoblasts (B), adipocytes (D) and chondrocytes (F) after 21 days of culturing in an induced medium, showing by Alizarin Red S, Oil Red O and Alcian Blue 8GX staining, respectively. Control cells were grown during the same period in a non-induced medium (A, C, E)

in comparison with conventional pigs is that they can weight 150 kg at 6 months. This makes them easier to handle during experimental work.

BM-MSCs have been isolated from many animal species and amplified in culture for various *in vitro* and *in vivo* applications[1,4,20]. The isolation and expansion of BM-pMSCs from BR-1 was feasible, using selected culture medium and protocols, as described for the isolation of human MSCs, as previously described by other authors[16,17]. Here, pMSC presented similar morphological features known from MSCs derived from other species[14,16,17,20]. Recently, other authors compared BM-MSCs from Göttingen minipigs and humans. MSCs were found in all minipig BM samples, but no circulating MSCs could be detected. Minipig BM-MSCs had morphology, proliferation, and colony formation capacities similar to humans[9].

As previously described by others, the techniques used for aspiration, isolation and osteogenic/chondrogenic/adipogenic differentiation of human MSCs can be adopted for analysis of pMSC, which may serve the increasing demand for stem and progenitor cells in tissue engineering[11,17]. As a result of the presented similarities between porcine and human MSC, pMSCs are likely to be considered as a valuable model system for skeletal research.

One of the remaining major problems in characterizing MSCs from distinct animal models such as pigs is the lack of appropriate species-specific MSCs marker molecules[17]. The cells had positive expression of known MSCs surface markers (CD90, CD29 and CD44) and negative expression of others (CD45, CD34 and CD14). The selection of markers for animal MSCs is challenging, since commercial markers are not available and not all human and murine antibodies cross-react with other species[19]. Because of the difficulty to obtain

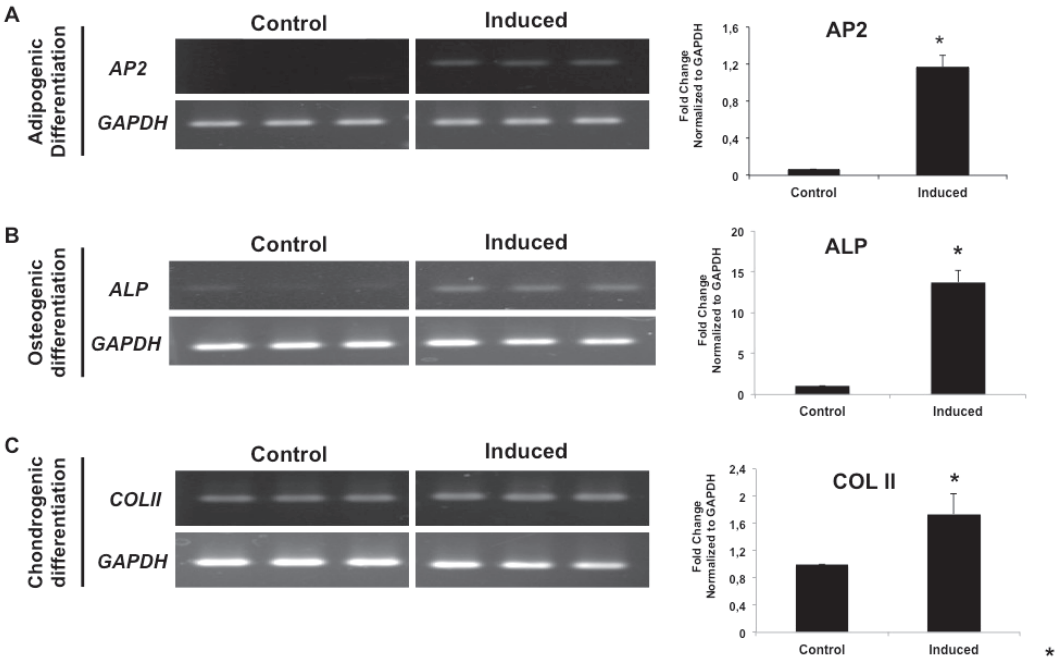

Figure 6- Expression analyses of three markers of mesenchymal stem cells from minipig BR-1 differentiated to: adipocytes (AP2), osteoblasts (ALP) and chondroblasts (COLII). Differentiation was induced over a period of 21 days, RNA was purified and the levels of the specific mRNAs were assayed by RT-PCR. The expression of GAPDH was used as a control. Data were collected from cell cultures of 3 independent animals, and reverse transcriptase-polymerase chain reaction (RT-PCR) reactions were carried out in technical triplicates, normalized to GAPDH. *p<0.05

commercial antibodies specific for minipigs, we used antibodies CD90 and CD34 against human antigens. The reactivity observed in BM-pMSCs with anti-human CD90 and CD34 antibodies was probably due to the cross-reaction with human antigens. The other antibodies used were specific for swine antigens CD14, CD44, CD45, CD29 and MHC-II. Our results are in agreement with an earlier study with BM-pMSCs, which reported a cellular viability of 90.0% and over 95.0% of cells positive for CD29, CD44 and CD90[15]. In a previous study, the authors isolated and characterized BM-pMSCs from Chinese miniature pigs and determined, by flow cytometry, the mean percentage of CD29 (98.0%), CD44 (97.4%), CD34 (1.2%) and CD45 (3.5%)[8]. These data corroborate our results, since the averages for the same antibodies were 89.8%, 86.9%, 1.61% and 1.83%, respectively. In addition to MSCs related markers, in order to determine the potential immunogenicity of BM-pMSCs, we performed the analysis of class II histocompatibility molecule. Similarly to humans, the BM-pMSCs showed a negative expression of MHC-II[2].

Although the main source of MSCs is the BM, these cells constitute, however, only a small percentage of the total number of BM-populating cells. It is known that only 0.001% to 0.01% of mononuclear cells isolated on density gradient give rise to plastic adherent fibroblast-like colonies[14]. The number of MSCs isolated from this tissue may vary in terms of yield and quality, even when the cells are obtained from the same donor[13]. Regarding the volume of BM aspirate, the collection range is very large in the literature, varying from 5 ml to more than 20 ml[1,12,15,20]. Thus, it is not necessary to collect a large amount of BM to acquire great numbers of MSCs. In the current study, an aspirate of 20 mL of BM allowed an average isolation of $9.2x10^6$ mononuclear cells/ml of BM aspirate. After 3–4 weeks of culture, the average quantity of pMSC was $84.3x10^6$ with 95.5% of cell viability. A similar study in minipigs described that 5 mL of BM aspirate is sufficient to obtain two million MSCs in 3 weeks of culture with a cell viability of 90%[15]. In another previous study with Japanese minipigs, the authors collected 20 ml of BM from which pMSCs were isolated and expanded to a density of about $300x10^6$ cells in one month of culture[12]. The proliferation of pMSCs during culture, within approximately 3–4 weeks until passage 4, indicates that this animal model is very attractive for preclinical trials.

Regarding the differentiation potential, it was demonstrated in our study that BM-pMSCs from BR1 differentiated into adipocytes, osteoblasts and chondroblasts after 21 days using protocols and commercial media for human MSCs differentiation[16]. Although we have not performed any comparison with human cells, the results indicate that the commercial media developed for the differentiation of human cells can be used for BR1 without

jeopardizing the outcome. The induced cells also showed higher gene expression of AP2, ALP and COLII, confirming their differentiation ability. These results support that BR1 can be used in preclinical trials in regenerative medicine like other breeds of minipigs[8,17,20]. A previous study showed that BM-MSCs from Göttingen minipigs had a significantly lower ability than human BM-MSCs to differentiate into functional osteoblasts as demonstrated by a lower percentage von Kossa-positive staining and lower ALP activity[9]. This observation emphasizes the need for species-specific optimization of MSC culture protocol before direct comparisons of MSCs between human and various preclinical large animal models can be made.

Regarding the osteogenic differentiation, commercial medium containing dexamethasone, ß-glycerolphosphate and ascorbate was able to promote differentiation[17]. To promote adipogenic differentiation, pMSCs were treated with defined medium containing dexamethasone, insulin and indomethacin. Adipogenic induction was demonstrated by the accumulation of vacuoles filled with neutral lipids and by the induction of distinct adipogenic marker genes, as aP2, which is directly related to lipid metabolism[7]. Regarding the chondrogenic differentiation, pMSCs that were cultured with chondrogenic medium with TGF-ß developed typical morphologic features of mucopolysaccharide producing chondroblasts. Although the extracellular matrix protein collagen type ll was also expressed by pMSCs without differentiation induction, RT-PCR showed that its mRNA levels were higher in induced cells than in non-induced ones.

As *ex vivo* expansion of stem cells could lead to tumorigenic transformation, it has been recommended the monitoring of genetic stability during the culture, although the occurrence of aneuploid cells (with numerical chromosomal anomalies) is not necessarily associated with the transformation[18]. In this study, we did not perform karyotyping, but cells were continuously observed until passage 4 and had no sign of loss of proliferation potential, death or changes in morphology that could indicate apoptosis. A previous study recommended less than eight passages culture schedule to avoid senescence[1]. Early passage pMSCs retained the multipotentiality, whereas the late passage MSCs retained only the adipogenic potential[20].

CONCLUSIONS

The results show that the BM-MSCs of BR-1, obtained with the protocol detailed in this manuscript, are MSCs, since they showed plastic-adherence capacity, fibroblastic morphology, positive surface markers characteristic of MSCs, and potential to differentiate into adipocytes, osteoblasts and chondroblasts.

The establishment of isolation, cultivation and differentiation of BM-MSCs from BR-1 is a step forward to further preclinical research in Brazil. This breed has been proven to be a useful large-animal model for stem cell-based tissue engineering.

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