Isolation and Culture of Mobilized Mesenchymal Stem Cells in Preclinical Models for Tissue Engineering: Basic Science

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ABSTRACT

Background: Mesenchymal Stem Cells **(MSC)** isolated from Bone Marrow **(BM)** have extensive plasticity to differentiate into different cellular lineages as adipocytes, chondrocytes, and osteocytes. Bone Marrow **(BM)** is considered the best source to isolate MSC with the disadvantages of requires a surgical procedure and anesthesia to obtain them and also involves the risk of different morbidities. The need to find less invasive sources of stem cells that offer high plasticity and that permit their expansion in sufficient numbers where novel technologies can be developed for the repair of damaged tissues and its safely transfer to human therapy, has promoted investigators to explore the mobilization process. This opens the possibility the use of MSC from Mobilized Peripheral Blood **(MPB)** for preclinical application or experimental models with applications in tissue engineering.

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Methods: Isolation of MSC by two methods, adherence to culture flasks and by Magnetic Activated Cell Sorting (MACS). Mobilization process was performed with the administration of Granulocyte-Colony Stimulating Factor (G-CSF) at low dose (300mcg/kg/day). Morphology and immunophenotype is evaluated at baseline and after three weeks in primary cultures.

Results: The mean of mononuclear cells obtained from the 20-mL MPB sample was $16x10^6$ while isolated CD90+ cells number was $8x10^6$. After 15 days under *in-vitro* differentiation culture, the CD90+ were stimulated and differentiated toward three mesenchymal lineages: osteoblastic, chondrogenic and adipogenic. Conclusions: Is possible to increase the number of CD90+ cells in peripheral blood though mobilization process with G-CSF at low doses. Mobilized CD90+ cells have the capacity to differentiate in-vitro into chondrogenic, osteogenic and adipogenic lineages with the appropriate stimulus.

Keywords: Mesenchymal Stem Cells; Chondral Differentiation; Cartilage Repair; Tissue Engineering; Mobilized Mesenchymal Stem Cells

Abbreviations: Bone Marrow **(BM)**; Granulocyte Colony Stimulating Factor **(G-CSF)**; Magnetic Activated Cell Sorting **(MACS)**; Mesenchymal Stem Cells **(MSCs)**; Mobilized Mesenchymal Stem Cells **(MMSCs)**; Mobilized Peripheral Blood **(MPB)**; Peripheral Blood **(PB)**; Tissue Engineering **(TE)**

INTRODUCTION

Mesenchymal Stem Cells **(MSC)** isolated from Bone Marrow **(BM)** have extensive plasticity to differentiate into different cellular lineages as adipocytes, chondrocytes, and osteocytes. Bone Marrow **(BM)** is considered the best source to isolate MSC with the disadvantages of requires a surgical procedure and anesthesia to obtain them and also involves the risk of different morbidities [1-4]. There are different studies that have evaluated the potential of bone marrow cells in sheep as an experimental model in Orthopedics. In these studies, monoclonal antibodies (CD90, CD44, and CD166) against human were used to determine the number of MSC obtained from sheep bone marrow [5]. In addition, there have been developed specific CD34 antibodies for HSCs identification in sheep [6].

The need to find less invasive sources of stem cells that offer high plasticity and that permit their expansion in sufficient numbers where novel technologies can be developed for the repair of damaged tissues and its safely transfer to human therapy. These have given rise to investigators turning toward the obtaining of Mobilized Mesenchymal Stem Cells **(MMSC)** from BM toward Peripheral Blood **(PB)** by means of treatment with the Granulocyte-Colony Stimulating Factor **(G-CSF)** [7,8]. The G-CSF this process is based on the negative modulation of Vascular adhesion surface molecule 1 (VCAM-1) and of nestin inhibition [9] in nestin-positive Mesenchymal Stem Cells **(MSC)** [10], localized in the vascular compartment of the BM niche, favoring their release from the niche and their migration through intramedullary space toward the peripheral circulation,

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where obtaining these is easily accessible, less invasive and minimally painful compared with iliac crest puncture for BM obtaining [11, 12].

In Mobilized Peripheral Blood (MPB), two stem cells populations have been identified: Peripheral Blood-Hematopoietic Stem Cells (PB-HSC) [13] and Peripheral-Blood-Mesenchymal Stem Cells (PB-MSC) [14]. PB-HSC have been characterized by means of surface markers such as CD133+, CD45+, CD34+, and CD38- [15,16], while PB-MSC have been characterized by their adherence capacity to culture flasks under standard conditions of *in-vitro* culture, the expression surface markers CD105+, CD90+, CD73+, CD34–, and CD45– [16-19], and their plasticity to differentiated in adipocytes, chondrocytes, and osteocytes [16,20,21].

Different studies have isolated BM cells obtained from sheep, where they have been employed CD29, CD44 and, CD166 markers obtaining positive cells to these antibodies [5]. However, subpopulations of mesenchymal stem cells (CD90+) obtained from MPB cells have not been either characterized, or expanded in preclinical models for cartilage repair [22].

The cell plasticity described for populations of PB-MSC possesses a good option for the development of new technology in pre-clinical animal models for Tissue Engineering **(TE)**. Sheep due to its anatomical similarity with the human knee joint [23], is the ideal model for development of novel technology based on PB-MSC to repair cartilage lesions. Thus, the characterization of PB-MSC is crucial for establishing a cellular differentiation in a preclinical models looking for their translation to humans.

One of our principal research line at the National Institute of Rehabilitation is cartilage repair, however, before clinical application cell therapies are firs table performed *in-vitro* to standardized isolation and cell culture, then we identify the risk, effects and benefits small and bigger preclinical models (mice and sheep). Because of animal models are limited in their ability to mimic the extremely complex process of human tissue repair, the safety and efficacy identified in those models have to be translated to human pilot studies and finally to human trials. Our objective in this research line is to support the development of cartilage repair therapies for human chondral lesions based on stem cells looking for new opportunities for young patients before joint replacement. we decided to isolate MSC by their adherence capacity to culture flasks and a subpopulation of CD90+ cells by magnetic pearls [24], from MPB by means of the administration of G-CSF, and evaluate their behavior in primary *in-vitro* culture.

STRATEGIES FOR ISOLATION AND CELL CULTURE

Methods

Sheep models

Our initial preclinical studies included male Suffolk sheep with body weight between 60 to 80 kilograms. Animals must hold in the animal facilities with a detailed clinical history describing

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their health status and general conditions before any experimental procedure. Humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the USA National Institutes of Health (National Institutes of Health Publication No. sed 1985) must be applied in any animal facility.

Cell Mobilization from Bone Marrow (BM) to Peripheral Blood (PB)

In the setting of autologous stem cell transplantation, the goal is to obtain the required number of cells with the lowest growth factor dose. The optimal dose of G-CSF to obtain an adequate amount of MSC is not well defined. Furthermore, the kinetics of MSC release induced by G-CSF has not been extensively evaluated in subjects with normal bone marrow function. For hematopoietic cell mobilization the G-CSF dose is 10 μg/kg/day administered subcutaneously every 24-hours during five to ten days. Those two factors, dose and duration, influencing in the magnitude of mobilization and also in the side effects. As there are no publications about the recommended dose to increase the MSC pool in the peripheral blood by mobilization process, we prefer to use a low standard dose of 300 μg/day of G-CSF every 24-hours during three consecutive days. Basal and consecutive blood tests are performed until the fourth day for evaluation of polymorphonuclear changes through time. At day four harvesting is performed in involved animals. An average of 20 ml of mobilized peripheral blood is taken from the jugular vein with a heparinized syringe.

ISOLATION OF MONONUCLEAR CELLS

To MSC isolation, the obtained pools of cells from peripheral mobilized blood are processed by continuous ficoll concentration gradient. Every sample is divided in polypropylene tubes and diluted 2:1 with phosphate saline solution (Invitrogen Co. Gibco. NY, USA). Thereafter, 15 ml of ficoll-Paque (Amersham Biosciences, Piscataway, NJ, USA) is added in new polypropylene tubes and then 20 ml of the diluted blood sample is carefully added to the ficoll taking care not to break the superficial tension. Samples are centrifuged at 400G for 30 min. After centrifugation cell count and viability assessment is performed. Cells are analyzed by flow cytometry to determine the surface markers.

Isolation of CD90+ Cells by Magnetic-Activated Cellular Separation

The subpopulation of CD90+ cells contained in mononuclear cells isolated from MPB, can be separated using the magnetic-activated cellular separation kit "MACS" (Miltenyi cat. 130-042-303) with the anti-CD90 monoclonal antibody (Miltenyi cat. 130-096-253) coupled with magnetic particles through LS cell separation columns (Miltenyi cat. 130-096-253). From the total number of CD90+ cells obtained, we recommend to separate aliquots of 1×105 cells for characterization by flow cytometry.

The MACS technique is a recommended methodology, widely used in research and therapy. Its objective is isolating specific cells from a mixed population, for further evaluation or culture. It enables study of targeted cell types, with exceptionally limited contamination or interference

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from the total mass of cells in a heterogeneous population. Positive separation techniques use primary antibodies, species-specific antibodies, enzymes, lectins or strepavidins to coat beads and attract cells. This procedure positively labels the cells targeted.

Isolate MSC by Their Adherence Capacity to Culture Flasks

Another way to isolate the MSC present in the fraction of mononuclear cells is using their ability to adhere in cell culture flasks. After ficoll gradient separation we took an aliquot of 5x105 cells, those are seeded in culture flasks and expanded with Dulbecco's Modified Eagle's Medium (DMEM, Gibco Life Technologies, USA), enriched with 10% of adult bovine serum (Biowest, cat. S4190-100). Cells are seeded in 75 mm2 flasks in a density of 2x105 cells per every 25 millimeters. Cultures are maintained in an incubator at 37oC, and 5% CO_2 for three weeks. When cultures formed proper confluence, aliquots of 5x105 cells are taken to establish their immunophenotype.

Immunophenotype Analysis by Flow Cytometry

To evaluate the presence of stem cells markers by flow cytometry, an aliquot of 1 mL with 5x105 cells of the sample separated by ficoll is used. This sample is divided in different polystyrene tubes (Falcon, Becton Dickinson) with $2.5x10^4$ cells. A suspension of 10 µl of antibodies is added in every tube and incubated for 30 minutes at 4°C. The monoclonal antibodies applied are: PE-conjugated CD34 (20 µg/ml, mouse IgG1κ, cat. 555822), FITC-conjugated CD90 (50 µg/ml, mouse IgG1κ, cat. 555595), PE-conjugated CD73 (20 µg/ml, mouse IgG1κ, cat. 561014), FITC-conjugated CD105 (5 µg/ml, mouse IgG1κ, cat. 561443), and FITC-conjugated CD45 (20 µg/ml, mouse IgG1κ, cat. 347463) all from BD Pharmigen [California, USA; [25]. The samples and unlabelled controls are included for each antibody and used to set the electronic gates on the flow cytometer. All data is obtained in a BD FACS Calibur flow cytometer and analyzed by Cell Quest PRO software (Becton Dickinson, USA) with a mean of 20,000 events.

In-Vitro Differentiation Of Mobilized CD90+ Into Mesenchymal Tissues

As reported by our group [24], in order to characterize MSC obtained from mobilized peripheral blood and demonstrate their functionality before and after cryopreservation, *in-vitro* differentiation into mesenchymal tissues like osteocytes, chondrocytes, and adipocytes was induced. Cells were cultured in six-well dishes at a plating density of 1.6×10^3 cells/cm^2.

For osteogenic differentiation, a 70% subconfluent culture of mobilized MSC from passage P2 was used. Cells were incubated in osteogenic medium with b-FGF (1:1000), BMP-2 (1:1000), β-glycerol phosphate (1:100), ascorbic acid (1:100), and dexamethasone. Cultures were growth during fifteen days with differentiation medium and cell colonies displayed bone-like nodular aggregates.

For cartilage differentiation same cell confluency was used, cultures were incubating for 21 days in chondrogenic medium with b-FGF (1:1000), kartogenin (1:1000), and ascorbic acid (1:100). Change medium was every third day.

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For adipogenic differentiation medium with adipogenic stimulatory supplements was used (b-FGF 1:1000, dexamethasone 1: 10,000, and insulin 1:1000). Cultures were incubated during 21 days and medium was changed every third day.

Cell Layer and Tissue Staining

Cells were cultured in six-well dishes for alkaline phosphatase, Alcian blue, and oil red staining assays. Cartilage matrix deposition in cells cultured with chondrogenic medium before and after thaw was assessed by Alcian blue staining. Cell layers were stained with Alcian blue (1% in 3% acetic acid) for 30 min, washed three times for 2 min in 3% acetic acid, rinsed once with water, and solubilized in 1% SDS.

To assess osteocyte differentiation, alkaline phosphatase staining was used. Cell layers were extracted in 2 ml of lysis buffer (100 mM Tris-HCl, pH 9.0, 200 mM NaCl, 0.2% Nonidet P-40, 0.2% Triton X-100, 1 mM MgSO4, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin) by rotating plates for 30 min at 4^0C. Reaction mixtures, containing 50 µl of extract, 200 µl buffer, and 250 µl of phosphatase substrate (1 mg/ml in 20% diethanolamine-HCl, pH 9.8), were incubated for 30 min at 37^oC.

Oil red staining assay was used to measure adipogenic differentiation. Medium was aspirated from plate dishes and cells were washed once in PBS. Culture layers were fixed in 4% PFA for 15 min and washed twice with bdH2O for 2 to 5 min. Oil red working solution to stain was applied and leave for 15 min, after that cells were washed three times with 1x PBS for 5 min; finally, cells were rinsed with 50% isopropanol once and 1x PBS once.

STATISTICAL ANALYSIS

All data are expressed as mean ± Standard Deviation of the Mean **(±SD)**. Statistical analysis was performed with the STATISTICA software (StatSoft v7, OK, USA). We compared MPB cell populations positive to surface markers using Student's t-Test. Analysis was done in cells recently isolated and three weeks after culture. Significant difference was considered when P value was < 0.05.

RESULTS

The mean number of mononuclear cells obtained from 20 mL of MPB was $16.1x10^6$ cells. Their immunophenotype was: CD34 3.12% ± 0.55; CD45 1.91% ± 0.77; CD73 4.19% ± 3.62; CD90 3.04% ± 1.23 and CD105 1.91% ± 1.03. After three weeks in primary culture, their immunophenotype was: CD34 8.47% ± 3.34; CD45 6.05% ± 2.30; CD73 12.42% ± 5.26; CD90 7.49% ± 0.23 and CD105 33.34% ± 3.93 (Figure 1) and cells had an elongated, fibroblast-like shape (Figure 2).

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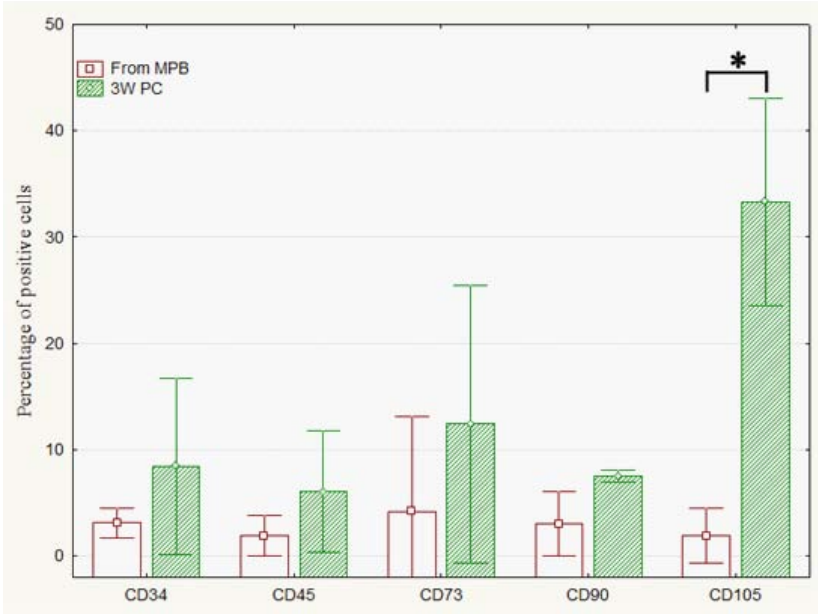


Figure 1: Comparison of immunophenotype obtained by Flow cytometry of mononuclear cells present in Mobilized peripheral blood cells after isolated by a concentration gradient with Ficoll (From MPB), and after primary culture for 3 weeks(3W PC). Statistically significant differences where established by Student's t-test for independent samples ($p < 0.05$). *: Significant p value.

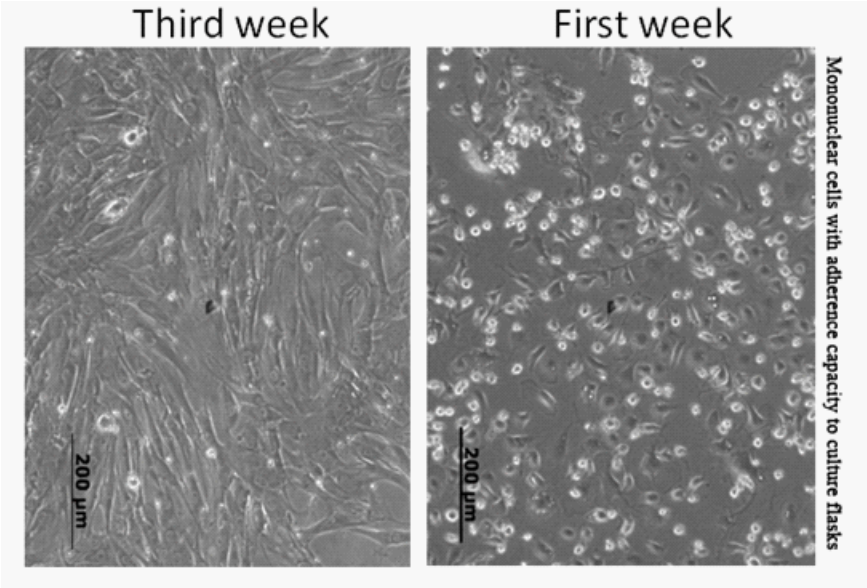


Figure 2: Representative visible-light microscopy; photomicrographs showing mononuclear cells isolated from mobilized peripheral blood in primary cultures at one week (top row) and after three weeks (bottom row).

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Isolation of CD90+ cells from the 20-mL sample of MPB after separation of the concentration gradient with the Ficoll the mean number of CD90+ cells isolated from mononuclear cells by means of magnetic pearls was 8.2×106 ± 0.58×106 cells. Their immunophenotype was: CD34 5.87% ± 1.69; CD45 5.61% ± 1.53; CD73 3.97% ± 1.04; CD90 25.3% ± 4.60 and CD105 4.37% ± 1.57. After three weeks in primary culture, their immunophenotype was: CD34 135% ± 0.69; CD45 0.92% ± 0.35; CD73 45.1 ± 3.48; CD90 84.38% ± 10.23 and CD105 68.21% ± 9.20 (Figure 3) and cells had an elongated, fibroblast-like shape (Figure 4).

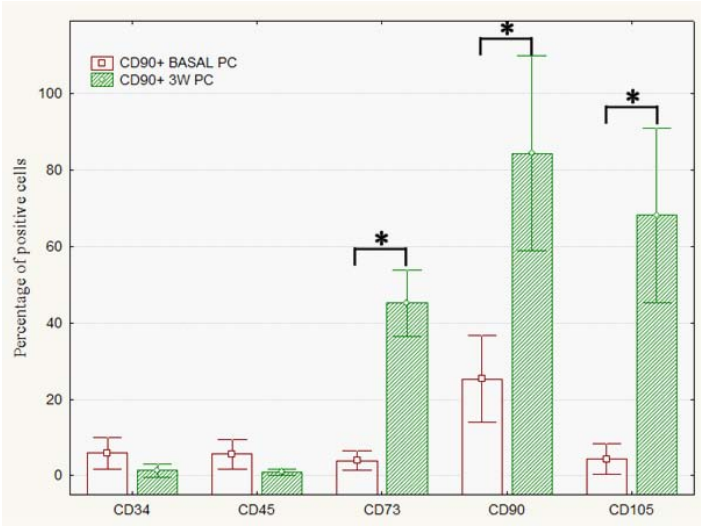


Figure 3: Comparison of immunophenotype obtained by Flow cytometry of CD90+ cells present in mobilized peripheral blood cells after isolated by MACS (CD90+ BASAL PC), and after Primary culture for 3 weeks(CD90+ 3W PC). Statistically significant differences where established by Student's t-test for independent samples ($p < 0.05$). *: Significant p value.

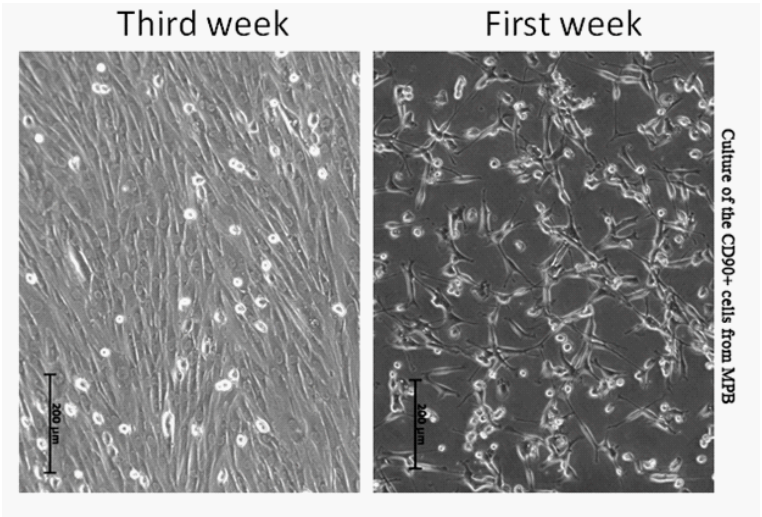


Figure 4: Representative visible-light microscopy; photomicrographs showing CD90+ cells isolated from mobilized peripheral blood in primary cultures at one week (top row) and after three weeks (bottom row).

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After 15 days under *in vitro* cell differentiation conductions the CD90+ showed switch towards 3 lineages: osteoblastic, chondrogenic and adipogenic, positive for their corresponding staining techniques (Figure 5).

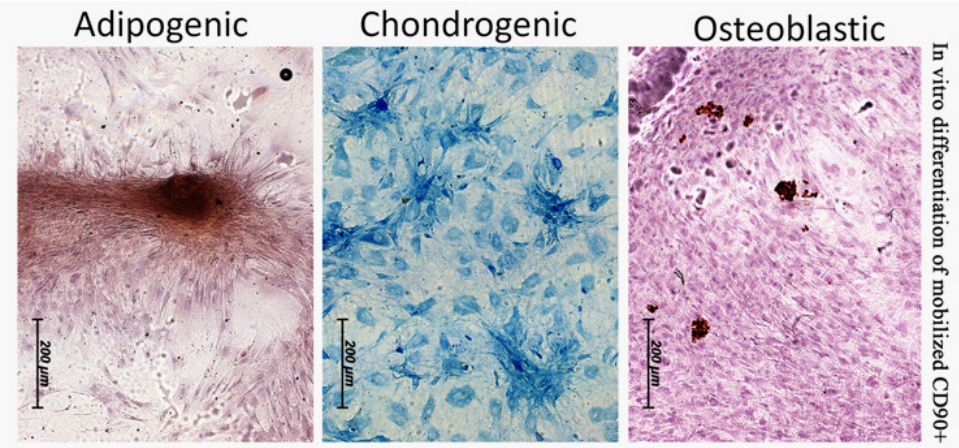


Figure 5: In vitro differentiation of CD90+ cells from MPB into mesenchymal tissues. Positive cells for alkaline phosphatase (osteoblasts, first row), Alcian blue positive staining for cartilage differentiation (middle row) and oil red O for adipocyte differentiation (last row). Figure adaptedfrom Landa-Solis et al., 2015.

SUPPLEMENTARY INFORMATION

Our results indicate that it is possible to isolate a PB-MSC population employing a standard culture medium supplemented only with adult sheep serum, and that PB-MSC obtained possess, according with the expressed immunophenotype, the potential to be employed in hyaline-like cartilage differentiation, bone and adipogenic models with applications in tissue engineering.

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