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RESEARCH ARTICLE

A novel method for the isolation of subpopulations of rat adipose stem cells with different proliferation and osteogenic differentiation potentials

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Abstract

Bone marrow has been the elected cell source of studies published so far concerning bone and cartilage tissue-engineering approaches. Recent studies indicate that adipose tissue presents significant advantages over bone marrow as a cell source for tissue engineering. Most of these studies report the use of adipose stem cells (ASCs) isolated by a method based on the enzymatic digestion of the adipose tissue and on the ability of stem cells to adhere to a cell culture plastic surface. Using this method, a heterogeneous population was obtained containing different cell types that have been shown to compromise the proliferation and differentiation potential of the stem cells. This paper reports the development and optimization of a new isolation method that enables purified cell populations to be obtained that exhibit higher osteogenic differentiation and/or proliferation potential. This method is based on the use of immunomagnetic beads coated with specific antibodies and it is compared with other methods described in the literature for the selection of stem cell populations, e.g. methods based on a gradient solution and enzymatic digestion. The results showed that the isolation method based on immunomagnetic beads allows distinct subpopulations of rat ASCs to be isolated, showing different stem cells marker expressions and different osteogenic differentiation potentials. Therefore, this method can be used to study niches in ASC populations and/or also allow adipose tissue to be used as a stem cell source in a more efficient manner, increasing the potential of this cell source in future clinical applications.

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Keywords **adult stem cells; mesenchymal stem cells; cell separation; cell isolation; adipose tissue; cell differentiation**

1. Introduction

Tissue-engineering approaches usually result from the combination of cells, biomaterials and bioactive factors to create surgically implantable products that promote the regeneration and functional restoration of the tissue (Guilak *et al*., 2006; Langer and Vacanti, 1993; Langer, 2000; Naughton, 2002; Tuan *et al*., 2003; Gomes and Reis, 2004). The ideal cells for tissue-engineering applications must be immunocompatible and have self-regenerative potential (Im *et al*., 2005); multilineage cells comply with both these requirements, also showing excellent therapeutic potential for tissue regeneration (Caplan and Goldberg, 1999; Griffith and Naughton, 2002; Muschler *et al*., 2004). Bone marrow has been the most widely studied source of adult stem cells for this purpose. Nevertheless, there are several drawbacks associated with the clinical use of bone marrow, mainly related to the harvesting of these cells, which consists of

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a significantly painful and invasive procedure and also to the low number of cells it is possible to obtain correlated to the amount of bone marrow harvested; this leads to the need for longer culture times to reach a relevant number of cells to use in a clinical application of the envisioned tissue-engineering approach (Strem *et al*., 2005).

Several recent reports have shown that MSCs are present in other tissues, such as synovium, periosteum, muscle and adipose tissue (Yoshimura *et al*., 2007).

Adipose tissue presents great potential as a cell source for tissue-engineering applications because it can be obtained as a 'waste' from a number of surgical procedures and/or harvested from the human patient via liposuction, which is considered a common and safe surgical procedure (Fraser *et al*., 2006a). Furthermore, adipose stem cells (ASCs) have great differentiation potential, demonstrated by several studies that show their ability to differentiate into osteoblasts, chondrocytes, endothelial cells and neuronal cells (Guilak *et al*., 2006; Qu *et al*., 2007; Hattori *et al*., 2004; Fraser *et al*., 2006b; Miranville *et al*., 2004; Gimble and Guilak, 2003; Romanov *et al*., 2006; Huang *et al*., 2007; Kang *et al*., 2004; Safford *et al*., 2002; Fujimura *et al*., 2005; Xu *et al*., 2005; Ogawa *et al*., 2004; Awad *et al*., 2004). Another important advantage of adipose tissue as a cell source is the high amount of stem cells that are found in this tissue compared to bone marrow. In fact, the ratio between 'colony-forming units fibroblast' (CFU-F) and 'colony-forming units expressing alkaline phosphatase' (CFU-AP) of the MSCs isolated from bone marrow is 1 : 100 000, while the same ratio for ASCs isolated from an equal amount of adipose tissue is 1 : 100 (Fraser *et al*., 2006a; Castro-Malaspina *et al*., 1984)[13,27].

Nevertheless, ASCs have obvious and profound similarities to bone marrow MSCs (BM-MSCs) and, probably for this reason, most of the research performed on ASCs has been based on previous studies on BM-MSCs (Tholpady *et al*., 2006). In fact, cell populations are delivered from the mesenchyma and both represent the stromal cell fraction and have similar morphology and surface proteins (Awad *et al*., 2004; Katz *et al*., 2005; Zuk *et al*., 2001).

The adipose stromal fraction is composed by an heterogeneous cell population composed of a variety of cells, such as immuno cells, haematopoietic stem cells, adipocytes, endothelial cells and fibroblasts (Tholpady *et al*., 2006), which can negatively affect the proliferation and/or differentiation potential of ASCs. In fact, the major drawback of adipose tissue as a source of stem cells is the contamination by other cell types that occurs upon the isolation of these cells, using a technique based on enzymatic digestion with collagenase followed by the seeding of the heterogeneous cell populations, as described in the majority of articles found in the literature regarding the use of ASCs. This method does not address the problem of 'contamination' by other cells, as the principle of this procedure is the selection of ASCs based on their ability to adhere to the surface of the culture flasks (Katz *et al*., 2005; Rodbell, 1964, 1966a, 1966b; Van and Roncari, 1977; Van *et al*., 1976; Marin *et al*., 1992; Dubois *et al*., 2008; Fraser *et al*., 2007; Gronthos *et al*., 2001; Mitchell *et al*., 2006). Moreover, while haematopoietic cells and adipocytes are easily dispensable from the cell suspension, other cell populations can adhere and remain in culture with the ASCs culture, since the culture medium used to expand these cells is Dulbecco's modified Eagle's medium (D-MEM), which is not a selective culture medium.

Particular attention should be directed to the presence of the fibroblasts, which usually overgrow when cultured with D-MEM, causing the death of the other cells in culture. In addition, the medium usually used for osteogenic differentiation does not inhibit the proliferation of the fibroblasts that are able to produce fibroblast growth factor 2 (FGF2), which is known to inhibit the osteogenic differentiation of stem cells (Cowan *et al*., 2004; Solchaga *et al*., 2005).

Therefore, the main aim of this work was to develop and optimize an isolation/purification method to obtain ASC populations with lower amounts of other cell types, enhancing the proliferation and differentiation ability of stem cells isolated from the adipose tissue. For this purpose we have developed an innovative methodology based on the use of immunomagnetic beads coated with three different antibodies, viz. NGFr Ab, STRO-1 Ab and CD49d. The ASC populations obtained using this method were compared to populations obtained using other procedures described in the literature, viz. the method based on enzymatic digestion (Zuk *et al*., 2002) and that based on Percoll gradient separation (Haynesworth *et al*., 1992). The comparison was based on the ability of the cells to (a) form colony-forming units (CFU), (b) express typical markers of stem cells and (c) differentiate into the osteogenic lineage.

2. Materials and methods

2.1. Harvest and digestion of adipose tissue

Adult male Wistar rats (4 weeks old) were sacrificed by decapitation and their epididymal and perirenal fat were removed as described by Ailhaud (2001). Briefly, the abdominal cavity was opened, the testes were extracted and the surrounding adipose tissue was dissected and collected; the fat tissue in the perirenal region was then collected by cutting the innervations of the retroperitoneal fat pad. Finally, the controlateral pad were simply dissected and collected. For the isolation of rat ASCs, the tissue was first washed with phosphate-buffered saline (PBS; Sigma-Aldrich, Germany) containing 10% of an antibiotic–antimycotic mixture (Gibco, UK) and then minced with a blade into small fragments, which were then immersed in a solution of 0.2% collagenase type IA (Sigma-Aldrich) in PBS for 40 min at 37 °C under gentle stirring. The digested tissue was filtered using a 100 μm filter mesh (Sigma-Aldrich) and centrifuged at 1250 rpm for 5 min at 20 °C, after which the supernatant was eliminated.

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2.2. Cell isolation procedures

2.2.1. Enzymatic method

This method consisted in performing the enzymatic digestion of the adipose tissue as described above, after which the resulting cells were seeded in 75 cm^2 culture flasks with basal medium composed of D-MEM (Sigma-Aldrich), 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic–antimycotic (Gibco).

2.2.2. Percoll gradient

The Percoll gradient method was performed following the procedure described by Haynesworth *et al*. (1982) for the isolation of bone marrow MSCs (BM-MSCs). Briefly, a Percoll density gradient was prepared by mixing 22.05 ml Percoll (Fluka, Sweden), 2.45 ml 1.5 M sodium chloride and 10.5 ml Tyrode's balanced salt solution (TBSS; Sigma-Aldrich) in a 50 ml tube. The Percoll density gradient was then centrifuged at 20 000 $\times$ *g* for 15 min at room temperature. The cell pellet obtained after the enzymatic digestion was resuspended in 5 ml basal medium and gently mixed with the Percoll density gradient solution. The solution obtained was centrifuged at 480 $\times$ *g* for 15 min at room temperature. Subsequently, the upper 10 ml were collected and transferred to a 50 ml tube; the volume was then made up to 50 ml with D-MEM. After this, the solution was mixed and centrifuged again at 450 $\times$ *g* for 10 min to remove the Percoll. After removing the supernatant, the pellet was resuspended in basal medium and seeded in 75 cm^2 culture flasks.

2.2.3. Immunomagnetic beads isolation method

The immunomagnetic beads isolation method was based on coating the commercially available magnetic beads (Dynabeads; Dynal M-450 epoxy beads) with the antibodies selected for this study, viz. anti-CD49d Ab (Biogenesis, UK), anti STRO-1 Ab (Invitrogen, USA) or anti p75 NGFr Ab (Abcam, UK). The selection of STRO-1 and CD49d was determined by results from recent studies (Gronthos *et al*., 2001; Schaffler and Buchler, 2007; Wagner *et al*., 2005; Dicker *et al*., 2005; Lee *et al*., 2004; Dominici *et al*., 2006) that showed the presence of these markers on the cell surface of ASCs; NGFr was selected because different research groups have described the isolation of BM-MSCs using this marker (Yamamoto *et al*., 2007; Buhring *et al*., 2007).

The coated beads were prepared according to the instructions provided by the manufacturer. For that purpose, a mixture of each antibody with beads was prepared, using a concentration of 4 μg Ab/1 $\times$ 10^7 beads, which was incubated for 24 h under gentle stirring with sodium phosphate buffer.

The pellet obtained by the enzymatic digestion was resuspended in a solution of bovine serum albumin (BSA; Sigma-Aldrich, Germany) in PBS (0.1%). The beads coated with the antibodies were then mixed with the cell suspension and incubated for 30 min at 4 °C under gentle stirring. Subsequently, the cell suspension and beads mixture was washed with PBS and BSA and the cells bonded to the Ab-coated Dynabeads were separated from the remaining of the cell suspension using a magnet (Dynal MPC, Dynal Biotech, USA). Finally the cells bonded to the Ab-coated beads were seeded in 75 cm^2 cell culture flasks (patent of the isolation method submitted).

The cells were seeded and expanded in the culture flasks without performing any procedure to remove the beads from the cell membrane, at it had previously been observed that the beads tend to spontaneously lose the bonding with the cells during the expansion phase.

2.3. Expansion and characterization of the cells isolated using the different methods

All the cell populations obtained using the different methods described were cultured in 75 cm^2 cell culture flasks with basal medium until confluence. Subsequently, the cells were trypsinized and finally seeded/cultured into different types of flasks/well-plates according to the characterization to be performed using basal medium. To assess the capacity of colony-forming units (CFUs) using the CFU test (for 10 days), cells were cultured in six-well plates. To evaluate the expression of several markers (CD44, STRO-1, CD105 and CD90) using RT–PCR analysis, the cells were cultured in 25 cm^2 culture flasks for 10 days using basal medium. For assessment of the osteoblastic differentiation potential, cells isolated using the different procedures described were seeded in 25 cm^2 flasks and cultured for 21 days, using basal medium as control, and osteogenic medium, composed of α-modified Eagle's medium (Sigma-Aldrich), supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich), 10^{-8} M dexamethasone (Sigma-Aldrich), 10 mM β-glycerol phosphate (Sigma-Aldrich), 10% FBS (Gibco) and 1% antibiotic–antimycotic (Gibco), for 21 days.

2.4. CFU test

Following expansion, the cells were seeded in six-well plates at different densities (5, 50, 100, 500, 1000, 5000, 10 000 and 15 000 cells/10 cm^2. The cells were cultured for 10 days, changing the culture medium every 2 days. After this period, the cells were fixed with formalin (Sigma-Aldrich) and stained with eosin (Sigma-Aldrich). Each well was observed under an inverted microscope (Axivert 40 CFL; Zeiss; Germany) and photographed using a Canon PowerShot G6 camera.

2.5. RT–PCR

Each cell population isolated using the different method was cultured with basal medium for 10 days in 25 cm^2 culture flasks. The mRNA was extracted with TriZol reagent (Invitrogen), following the procedure provided

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by the supplier. Briefly, 800 µl TriZol were added for each sample containing 1×10^6 cells. After an incubation of 5 min, 160 µl chloroform (Sigma-Aldrich) was added to each sample; the samples were then incubated for 15 min at 4 °C and centrifuged at the same temperature and 13 000 rpm for 15 min. After the centrifugation, the aqueous part was collected and an equal part of isopropanol (Sigma-Aldrich) was added. After an incubation of 2 h at −20 °C the samples were washed in ethanol, centrifuged at 4 °C and 9000 rpm for 5 min and resuspended in 12 µl RNase/DNase-free water (Gibco). The samples were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). The samples selected for the cDNa synthesis were those presenting a 260 : 280 ratio in the range 1.7–2.0. The cDNA synthesis was performed using the iScript cDNA synthesis Kit (BioRad, USA) and the MiniOpticom Real-Time PCR Detection System (BioRad), using an initial amount of mRNA of 2 µg and a total volume of 20 µl. RNAse-free water (Gibco) was used as a negative control.

2.6. Real-time PCR

After the synthesis of cDNA, the cells were analysed regarding the expression of STRO-1, CD105, CD90 and CD44. The selection of the markers used for characterization of the ASCs was based on the characterization criteria described by the Mesenchymal and Stem Cells Committee of the International Society for Cellular Therapy (Dominici *et al*., 2006), which states CD105 and CD90 as markers for MSCs; CD44 was chosen because the presence of the primer was previously reported on ASCs (Katz *et al*., 2005); and STRO-1 was selected because it has been considered by several research groups a fundamental characteristic marker of bone marrow mesenchymal stem cells(BMSCs) (Gronthos and Zannettino, 2008; Nagamoto *et al*., 2006; Nasef *et al*., 2009; Rolf *et al*., 2008; Waddington *et al*., 2009; Zannettino *et al*., 2007). Evaluation of STRO-1 expression also allows the similarity between ASCs and BMSCs to be assessed.

For each sample, *GAPDH* was used as housekeeping gene. The primers were previously designed using Primer 3 software (v 0.4.0) and synthesized by MWG Biotech (Germany).

The RT–PCR was carried out using iQ Syber Green SuperMix equipment (BioRad, USA). The procedure followed to perform the reactions was based on the manufacturer's instructions. The data obtained were normalized using the $^{\Delta\Delta}$Ct method. For the stem cell markers, $^{\Delta\Delta}$Ct was calculated using the results obtained in the enzymatic method, in which the cells are derived directly from the stromal vascular fraction and thus have the highest amounts of other cell types. The results obtained were elaborated using Origin Pro 7.0 (OriginLab Corp., USA) and the correlation between the dataset from the same gene analysed was investigated using the ANOVA single factor method.

2.7. Assessment of osteoblast differentiation potential

2.7.1. Von Kossa staining

The cells cultured in 25 cm^2 flasks were fixed with formalin and washed with distilled water; a 2% silver nitrate solution (Sigma-Aldrich) was added to the cell culture and the flasks were kept in the dark for 10 min; then the cells were washed twice with distilled water and exposed to bright light for 15 min. Finally, the cells were washed with distilled water and then photographed (after drying at room temperature) under an inverted microscope (Axivert 40 CFL; Zeiss; Germany).

2.7.2. Alizarin red staining

The cells were fixed with formalin and washed, first with PBS and then with distilled water. The cells were then stained with 2% alizarin red solution (Merck, Germany) in distilled water for 5 min and finally washed with distilled water. Stained cultures were observed under an inverted microscope (Axivert 40 CFL, Zeiss, Germany).

2.7.3. Osteopontin and osteocalcin expression

Osteopontin and osteocalcin expression was assessed on cells after mRNA extraction and RT–PCR synthesis, as described above for the synthesis of the cDNA. For each sample, *GAPDH* was used as housekeeping gene. As reported above, the primers were previously designed using the Primer 3 software (v 0.4.0) and synthesized by MWG Biotech (Germany). The equipment used for the RT–PCR analysis as well as the procedure to carry out the reactions are described in the previous section

Once again, the $^{\Delta\Delta}$Ct method was used to normalize the data, using the values for osteopontin and osteocalcin expression of cells cultured with basal medium. Again, the correlation between the dataset from the same gene analysed was investigated using the ANOVA single factor method.

2.8. Statistical analysis

Data are presented as arithmetic means ± SD. An ANOVA single factor test was used to compare the mean values of the RT–PCR results. Differences were considered to be significant at $p < 0.05$. All statistical analysis was performed using Origin Pro 7.0 (OriginLab Corp., USA).

3. Results

3.1. CFU test

All the cell populations isolated using the three different methods described and cultured with basal medium for 10 days showed the capacity of forming colony units, as

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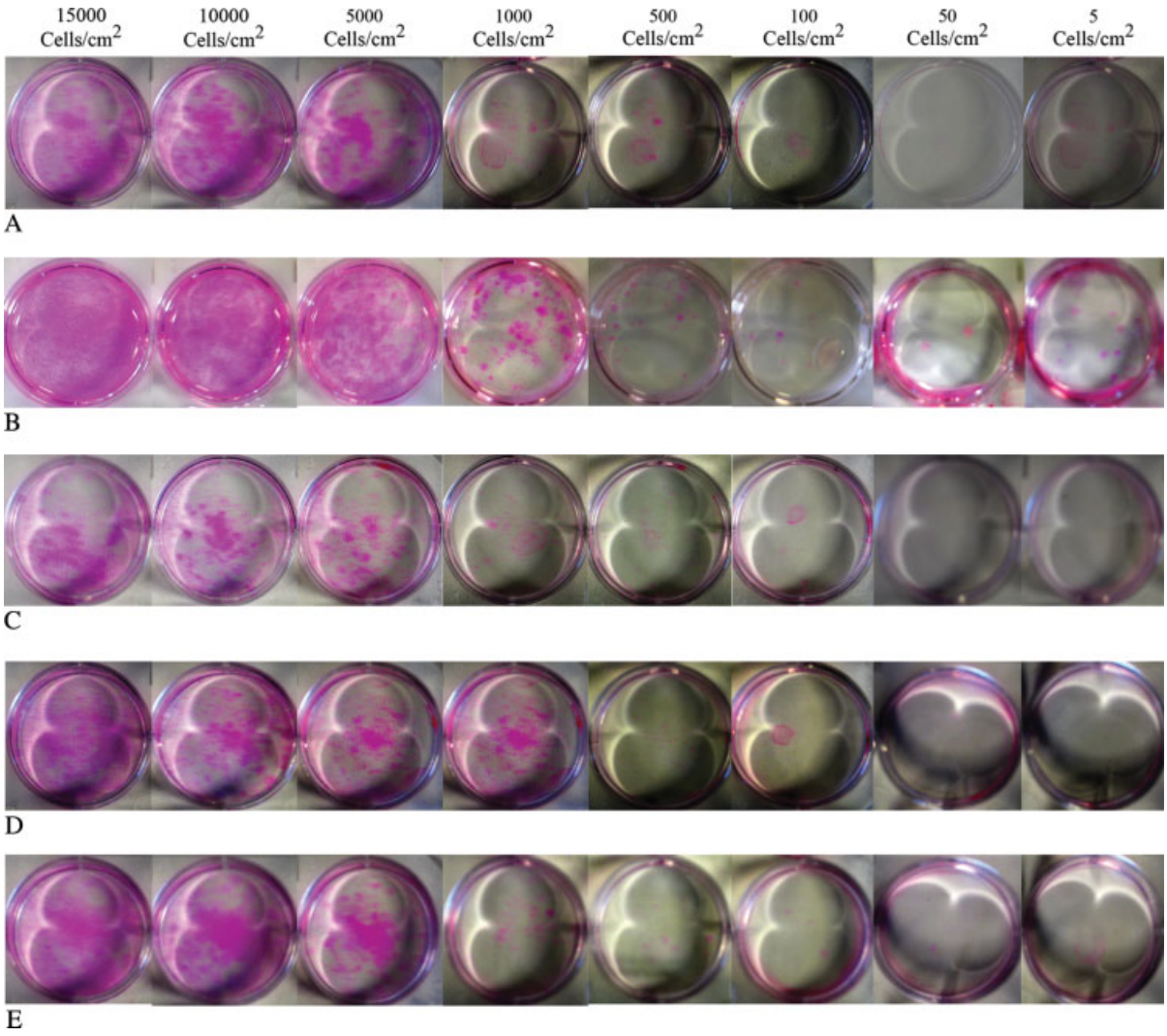

Figure 1. Optical microscopy pictures showing the results obtained in the CFU test performed on ASCs isolated using the different methods: (A) enzymatic; (B) Percoll gradient; (C) immunomagnetic beads coated with CD49d Ab; (D) immunomagnetic beads coated with NGFr Ab; (E) immunomagnetic beads coated with STRO-1 Ab

it can be observed in Figure 1. However, the different isolation methods result in different abilities to form fibroblast colony units. In fact, rat ASCs isolated with Percoll gradient were able to form colony-forming units (CFUs) up to a concentration of 5 cells/cm^2, while cells isolated with immunomagnetic beads coated with STRO-1 Ab were able to form CFUs up to a concentration of 50 cells/cm^2 and rat ASCs isolated using the enzymatic method combined with immunomagnetic beads coated with the NGFr Ab and with CD49d Ab were able to form CFUs up to a concentration of 100 cells/cm^2.

Table 1. Summary of the results obtained from real-time RT–PCR analysis regarding the gene expression of the cells isolated with the different methods compared to the reference sample (cells isolated with the enzymatic method)

	Gene expression			
Isolation methods	CD105	CD44	CD90	STRO-1
STRO-1	+	+	+	+
CD49d	−	+	+	+
Percoll	−	−	+	+
NGFr	−	−	+	−

Data calculated using the $^{\Delta\Delta}$Ct method; +, expression levels higher than the reference sample (>1); −, expression levels lower than the reference sample (<1).

3.2. Expression of stem cell markers, real-time RT–PCR

After being cultured for 10 days in basal medium, the cells isolated using the different methods were evaluated for the expression of CD44, CD105, CD90 and STRO-1 by RT–PCR. The results obtained are summarized in Table 1. As previously mentioned, CD105 and CD90 are well-established markers of stem cells and STRO-1 is a typical marker for bone marrow stem cells, while CD44 has been recently found present in ASC cultures.

The rat ASC populations isolated using the different methods described expressed CD44, CD105, CD90 and STRO-1, as shown in Figure 2, although the levels

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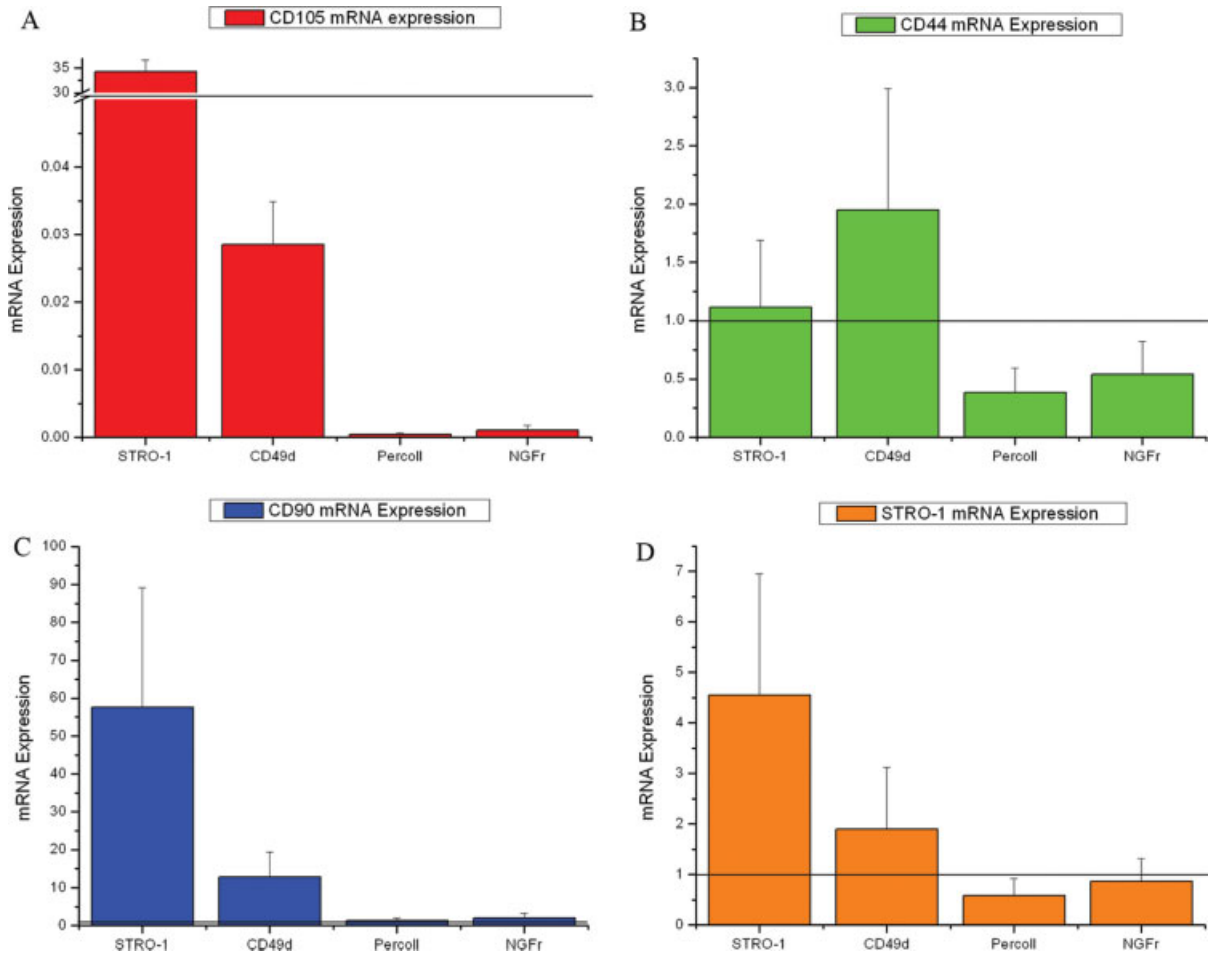

Figure 2. Graphical representation of the results obtained from RT–PCR analysis of the different ASC populations, obtained using various isolation procedures regarding the expression of different genes: (A) *CD105* mRNA expression; (B) *CD44* mRNA expression; (C) *CD90* mRNA expression; (D) *STRO-1* mRNA expression. ANOVA, single factor, $p < 0.05$

of expression of the same marker differ significantly depending on the isolation procedure used. Regarding CD105, CD90 and STRO-1, the highest expression has been detected in rat ASCs isolated with immunomagnetic beads coated with STRO-1 Ab, followed by the cell population isolated with immunomagnetic beads coated with CD49d Ab, while the population isolated with immunomagnetic beads coated with NGFr Ab and that isolated using Percoll gradient (see Figure 1A–C) exhibited the lowest expression of these markers. Regarding CD44, the highest expression level was detected in cells isolated with immunomagnetic beads coated with CD49d Ab, and the lowest was found for cells isolated with Percoll gradient (Figure 1B). Considering the methods used to calculate $^{\Delta\Delta}$Ct, cells isolated with immunomagnetic beads coated with the STRO-1 Ab have the highest expression of all the genes investigated, compared to the reference sample (cell population isolated using the enzymatic method). Cells isolated with immunomagnetic beads coated with CD49d Ab showed a lower expression of *CD105* genes when compared with the reference sample. Finally, cells isolated with Percoll gradient methods and immunomagnetic beads coated with the NGFr Ab exhibited a higher expression of the *CD90* gene when compared with the results obtained from cells isolated with the enzymatic method. In general, considering the positive results obtained in the CFU test and in the expression of all the genes considered as markers of stem cells, it is possible to conclude that all the methods used allowed stem cells the be isolated from adipose tissue.

3.3. Von Kossa and alizarin red staining

After 4 weeks of culture with osteogenic medium the cells were stained with alizarin red and Von Kossa. All the cell populations showed positive staining for both (as can be seen in Figures 3 and 4, respectively), indicating the occurrence of the mineralization process. However, these same pictures also suggested the occurrence of a higher mineralization in cells isolated using the Percoll gradient and the enzymatic methods.

3.4. Real-time PCR for osteocalcin and osteopontin

After 4 weeks of culture, real-time RT–PCR analysis was performed to study the expression of two well-known

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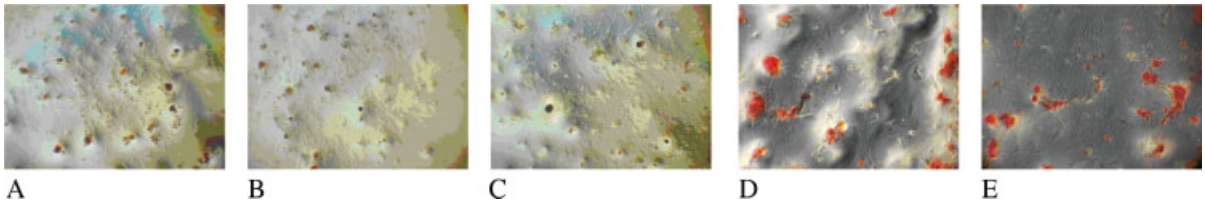

Figure 3. Optical microscopy pictures showing the cultures stained with alizarin red corresponding to rat ASCs isolated using the various isolation procedures: (A) immunomagnetic beads coated with CD49d (×100); (B) immunomagnetic beads coated with NGFr Ab (×100); (C) immunomagnetic beads coated with STRO-1 (×100); (D) Percoll gradient method (×100); (E) enzymatic method (×100)

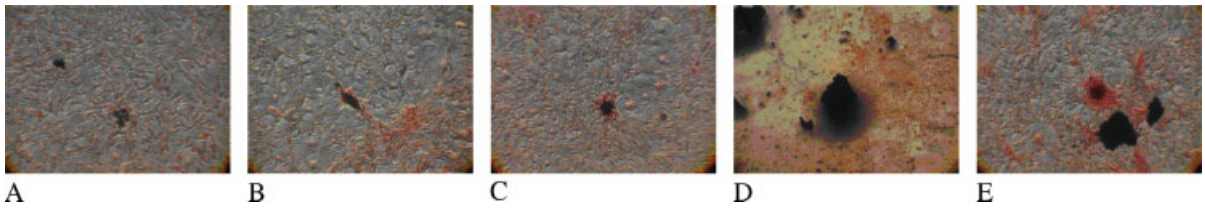

Figure 4. Optical microscopy picture showing cultures stained with Von Kossa corresponding to rat ASCs isolated using the various isolation procedures: (A) immunomagnetic beads coated with CD49d (×100); (B) immunomagnetic beads coated with NGFr Ab (×100); (C) immunomagnetic beads coated with STRO-1 (×100); (D) Percoll gradient (×100); (E) enzymatic method (×100)

Table 2. Summary of the results obtained from real-time RT–PCR analysis regarding the gene expression for osteopontin and osteocalcin of the cells isolated with the different methods compared to the reference sample (cells cultured with basal medium)

	Gene expression	
Isolation methods	Osteocalcin	Osteopontin
CD49d	−	+
NGFr	−	+
STRO-1	+	+
Percoll	+	+
Enzymatic method	−	+

Data calculated using the $^{\Delta\Delta}$Ct method; +, expression levels higher than the reference sample (>1); −, expression levels lower than the reference sample (<1).

markers of osteogenic differentiation, viz. osteopontin and osteocalcin. Osteopontin is usually expressed by cells at on early stage of the osteogenic differentiation process, while osteocalcin is expressed at a later stage of this process (Beck *et al.*, 2000). The results obtained are summarized in Table 2. All the cells isolated using the different methods described showed expression of the osteopontin gene, indicating the occurrence of an initial osteogenic differentiation process (Figure 5A). Nevertheless, some differences were found in the expression levels of these markers for cells isolated using the different methods. In fact cells isolated with immunomagnetic beads coated with STRO-1 Ab exhibited the highest expression of osteocalcin when compared to the remaining cell populations (Figure 5B), while rat ASCs isolated with immunomagnetic beads coated with NGFr Ab show the highest expression of osteopontin (Figure 5A). In both cases, the differences found in the expression levels are >10 times. All the results obtained were statistically relevant.

4. Discussion

The present work allowed the development and optimization of a range of different isolation methods that enabled different populations of adult stem cells to be obtained from adipose tissue. In order to confirm that the cells isolated were adipose stem cells, the CFU test was used to evaluate the ability of the cells to form CFUs and real-time RT–PCR analysis was used to assess the expression of CD44, CD105, CD90 and STRO-1. CD44 encoded by this gene is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid and can also interact with other ligands, such as osteopontin, collagens and matrix metalloproteinases. This protein participates in a wide variety of cellular functions, including lymphocyte activation, recirculation and homing, haematopoiesis and tumour metastasis. CD105 is a transmembrane protein, which is a major glycoprotein of the vascular endothelium. This protein is a component of the transforming growth factor-β receptor complex and it binds TGFβ1 and TGFβ3 with high affinity. CD90 is a cell surface antigen and STRO-1 is a cell surface protein expressed by mesenchymal stem cells and erythroid precursors. The results obtained showed that all the methods enable to isolate cells with the capacity to form colony units and that these cells expressed the genes investigated (usually used to characterize MSCs). Nevertheless, the expression levels differ among the different cell populations isolated. Furthermore, the levels of gene expression for some methods were lower than those obtained for the reference sample (cells isolated with the enzymatic method) (Table 1). Cells isolated with immunomagnetic beads coated with STRO-1 exhibited the highest gene expression for all the markers considered, compared with the reference sample, and the highest expression of CD105, CD90 and STRO-1. Cells

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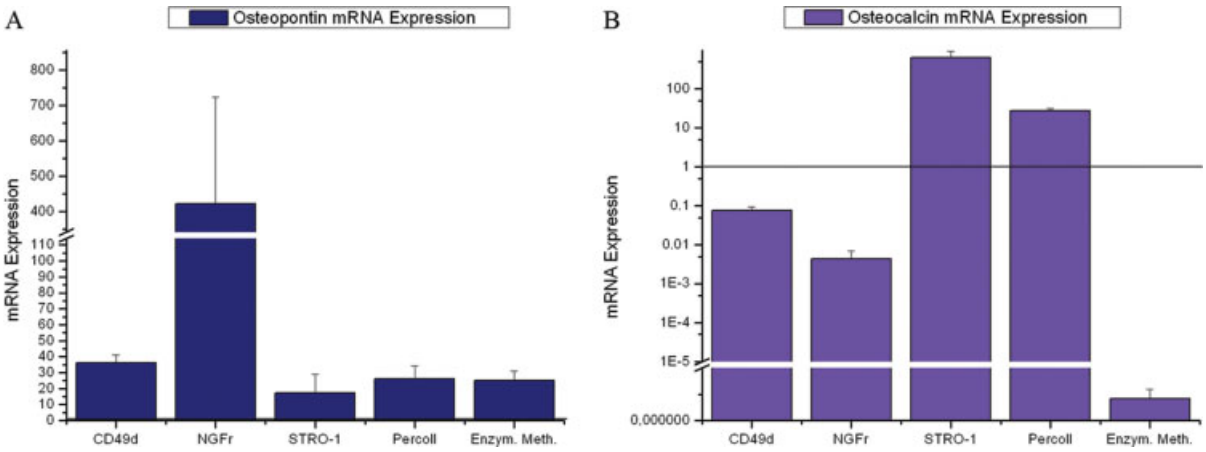

Figure 5. Graphical representation of the results obtained from RT–PCR analysis of the ASCs population obtained using different isolation procedures, regarding the expression of different genes: (A) osteopontin gene expression; (B) osteocalcin gene expression. Results treated using ANOVA, single factor; $p < 0.05$

isolated with immunomagnetic beads coated with CD49d Ab exhibited the highest expression of CD44 and higher expression of STRO-1 and CD90 but lower expression of CD105, when compared with the reference sample.

The differences found in the expression levels of the studied markers show that cells isolated with immunomagnetic beads coated with STRO-1 Ab and CD49d Ab exhibited a higher expression level than the basal levels considered (corresponding to cells isolated using the enzymatic method). The results obtained regarding the expression of CD90 showed that all cells isolated using the different methods express this gene, although with different levels of expression.

In general, the results obtained from analysing the expression of markers of ASCs suggest two important findings: the first is that cells isolated with immunomagnetic beads coated with STRO-1 Ab followed by cells isolated with immunomagnetic beads coated with CD49d Ab are the cell subpopulation with the highest number of cells expressing the stem cell markers considered; the second finding refers to the variability of gene expression inside the same population of cells, indicating the presence of several subpopulations within the ASCs stem cells population. The cell subpopulation isolated with STRO-1 Ab shows the highest 'stemness' character, i.e. the highest expression of the stem cells markers assessed.

The presence of subpopulations is not a new finding regarding MSCs, as this has been widely reported for BM-MSCs (Nasef *et al*., 2009; Hatzfeld *et al*., 2007; Liu *et al*., 2006). However, to our knowledge, the present study reports for the first time results demonstrating that adipose stem cells are composed of subpopulations.

It is also important to note that the gene expression of the proteins considered as markers for ASCs are not strictly correlated to the CFU capacity of the cells. In fact, cells isolated with the Percoll gradient were able to form CFUs with the lowest concentration studied (5 cells/cm^2), but the expression of the gene investigated is the lowest compared with the other cell populations studied. In contrast, cells isolated using immunomagnetic beads coated with CD49d Ab did not show a very high CFU capacity (CFUs not present for concentrations $<$100 cells/cm^2) but this is the second cell population with the highest expression of CD105, CD90 and STRO-1 and the population with the highest expression of CD44. Regarding the osteogenic potential of the ASCs population isolated using different methods, it was observed that all the ASCs populations were positively stained with alizarin red and Von Kossa. However, only rat ASCs that were isolated with immunomagnetic beads coated with STRO-1 Ab and those isolated with the Percoll gradient method exhibited expression levels of osteocalcin higher than the basal level.

The results obtained from the analysis of osteocalcin and osteopontin expression demonstrate that the cell populations exhibiting higher levels of osteocalcin expression are those exhibiting lower osteopontin expression levels. Considering that osteopontin and osteocalcin are known as markers of early and late osteogenesis, respectively, these results indicate that the osteogenic differentiation process in these populations is in a more advanced stage than that observed for other cell subpopulations (Beck *et al*., 2000). Furthermore, as shown in Table 2, the rat ASCs isolated with the enzymatic method expressed the lowest levels of osteocalcin, indicating that this might be due to a delay in osteogenic differentiation, with respect to the other rat ASCs isolated with the other methods, or due to an inhibitory effect of differentiation by the presence of FGF2 released by fibroblasts likely to be present in this cell culture population.

The higher mineralization of cells isolated with the Percoll gradient method, shown by Von Kossa and alizarin red staining and the high expression of osteocalcin, indicate that these cells are in an advanced stage of osteogenic differentiation, particularly when compared with rat ASCs isolated with immunomagnetic beads coated with anti STRO-1 ab. This enhanced mineralization may result from the presence of other cell types, e.g. endothelial cells, that produce VEGF, which affects the differentiation of progenitor cells into hypertrophic

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osteoblasts (Zeng *et al*., 2007; Maes *et al*., 2004) or adipocytes that can secrete leptin, an 'adipokine' that seems to block *in vitro* adipogenesis while enhancing osteogenesis (Gimble *et al*., 2006).

Again, as for the expression of the stem cell markers, the results obtained suggest the presence of several subpopulations within the stromal cell fraction of adipose tissue. The subpopulation isolated with immunomagnetic beads coated with STRO-1 Ab seems to undergo an enhanced osteogenic differentiation process, expressing the highest levels of osteocalcin, for the same culture period. With the other methods used, the subpopulations obtained are not able to undergo a complete osteogenic differentiation, as they express osteopontin but not osteocalcin. Considering both results regarding stem cell markers expression and the results obtained from osteogenic differentiation, it is possible to conclude that rat ASCs isolated with immunomagnetic beads coated with STRO-1 Ab are the best candidates for bone tissue-engineering applications. In fact, this rat ASC subpopulation expressed all the markers investigated and the cells showed an enhanced osteogenic differentiation process as compared to the remaining population. Nevertheless, further studies should address the behaviour of human ASCs in order to evaluate the validity of the rat model.

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