Comparative study of equine bone marrow and adipose tissue-derived mesenchymal stromal cells

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Summary

Reasons for performing study: Mesenchymal stromal cells (MSCs) represent an attractive source for regenerative medicine. However, prior to their application, fundamental questions regarding molecular characterisation, growth and differentiation of MSCs must be resolved.

Objectives: To compare and better understand the behaviour of equine MSCs obtained from bone marrow (BM) and adipose tissue (AT) in culture. **Methods:** Five horses were included in this study. Proliferation rate was measured using MTT assay and cell viability; apoptosis, necrosis and late apoptosis and necrosis were evaluated by flow cytometry. The mRNA expression levels of 7 surface marker genes were quantified using RT-qPCR and CD90 was also analysed by flow cytometry. Differentiation was evaluated using specific staining, measurement of alkaline phosphatase activity and analysis of the mRNA expression. **Results:** High interindividual differences were observed in proliferation in both cell types, particularly during the final days. Statistically significant differences in viability and early apoptosis of cultured AT- and BM-MSCs were found. The highest values of early apoptosis were observed during the first days of culture, while the highest percentage of necrosis and late apoptosis and lowest viability was observed in the last days. Surface marker expression pattern observed is in accordance to other studies in horse and other species. Osteogenic differentiation was evident after 7 days, with an increasing of ALP activity and mRNA expression of osteogenic markers. Adipogenic differentiation was achieved in BM-MSCs from 2 donors with one of the 16 media tested. Chondrogenic differentiation was also observed.

Conclusions: Proliferation ability is different in AT-MSCs and BM-MSCs. Differences in viability and early apoptosis were observed between both sources and CD34 was only found in AT-MSCs. Differences in their osteogenic and adipogenic potential were detected by staining and quantification of specific tissue markers.

Potential relevance: To provide data to better understand AT-MSCs and BM-MSCs behaviour *in vitro*.

Keywords: horse; mesenchymal stromal cells; regenerative medicine; AT-MSCs; BM-MSCs

Introduction

The use of mesenchymal stem cells (MSCs) has been recently shown as a therapeutic alternative that

could help in the treatment of tendon and ligament lesions (Murphy *et al*. 2003; Smith *et al*. 2003; Angele *et al*. 2008).

Cell-based therapeutic options favour the use of autologous cells such as a population of bone marrow cells or adipose tissue cells that have been characterised as mesenchymal stromal cells (MSCs) (Herzog *et al*. 2003). Cultured MSCs exhibit good multipotency and they can differentiate both *in vitro* and *in vivo* into several tissues, including bone (Holtorf *et al*. 2005), cartilage (Bernardo *et al*. 2007), tendon, skeletal muscle (Dezawa *et al*. 2005), adipose tissue and cardiac muscle (Orlic *et al*. 2001). This multi-differentiation potential of MSCs provoked clinical interest in whether these cells could be used for regenerative medicine (Horwitz *et al*. 1999; Bobis *et al*. 2006). In horses, to date, several reports have analysed both bone marrow (BM) and adipose tissue (AT) derived MSCs as potential therapeutic agents for horse musculoskeletal diseases (Smith *et al*. 2003; Richardson *et al*. 2007) such as tendon lesions (Violini *et al*. 2009), suspensory ligament rupture (Smith and Webbon 2005) and bone and joint related conditions in the equine athlete (Wilke *et al*. 2007). These lesions result in high morbidity and often compromise full recovery (Smith *et al*. 2003).

However, the efficacy of MSC therapy in both animals and human patients is sometimes extremely low (Brooke *et al*. 2007; Granero-Molto *et al*. 2008). Indeed, in bone repair, utilising MSC subpopulations with poor osteogenic potential and a short lifespan decreases the therapeutic efficacy (Liu *et al*. 2009).

MSC specific markers have been characterised in man and some laboratory animals using techniques such as the phenotypic analysis of cell surface markers by flow cytometry and indirect immunofluorescence (Koch *et al*. 2009). Limited work about equine MSC membrane surface markers

has been done. A recent work describes the immunophenotype of equine AT-derived MSCs for CD13, CD44 and CD90 (de Mattos Carvalho *et al*. 2009).

Recently, several reports have focused on characterising equine MSCs obtained from different sources (Vidal *et al*. 2006, 2007; Colleoni *et al*. 2009; Mambelli *et al*. 2009). Further characterisation is necessary to predict the potential and suitability of a specific cell population for application in equine cellular therapy. The aim of our work was to analyse the proliferation capacity and cell viability of equine BM- and AT-MSCs. We examined the expression of specific cell surface markers and the tri-lineage differentiation ability of the MSCs into osteoblasts, adipocytes and chondrocytes by staining procedures and evaluated the utility of several molecular markers for monitoring MSC differentiation.

Materials and methods

Animals

Five horses (400–450 kg bwt, age: 8–12 years) were used to characterise equine MSCs. The horses were clinical patients suffering from varying types of tendon and ligament injuries and biological samples (bone marrow and adipose tissue) were obtained with owner consent and according to local animal welfare regulations. The study was approved by the ethics committee of the University of Zaragoza.

MSC isolation, culture and expansion

Bone marrow aspiration and isolation: Each BM aspirate, collected as previously described by Orsini and Divers (1998), was diluted 1:3 with PBS[1] and then layered over Lymphoprep[2] and centrifuged for 20 min at 1700 ***g***. The MSC-enriched cell population above the Lymphoprep layer was aspirated and washed twice with PBS. The pellet was resuspended in 10 ml basal medium, consisting of low glucose Dulbecco Modified Eagle's Medium (DMEM)[3] and supplemented with 10% fetal bovine serum, 1% glutamine[3] and 1% streptomycin/penicillin[3]. Cells were counted, plated at a density of 2×10^6 nucleated cells/cm^2 in 6-well plates and incubated at 37°C, 5% CO_2.

Adipose tissue harvest and isolation: Adipose tissue samples were collected, as previously described by Vidal *et al*. (2007), from the supragluteal subcutaneous area of the same 5 animals. The tissue was

digested with 0.01% of activated collagenase (*type I*)[3] for 30 min at 37°C with continuous shaking. Subsequently, samples were centrifuged, the supernatant removed and the cells washed with PBS by further centrifugation. Finally, the pellet was resuspended in 10 ml basal medium (DMEM), counted and plated at a density of 10^5 nucleated cells/cm^2 in 6 well plates and incubated at 37°C, 5% CO2.

Cell culture, cryopreservation and thawing: For culture expansion, cells were maintained in basal medium at a density 5000 cells/cm^2 in T75 or T175 culture flask[4]. The medium was changed every 3 days until cells reached approximately 80% confluence. Subsequently, cells were detached by treating with 0.25% tryspin-EDTA[3] and counted with a haemocytometer Z2 Coulter particle count and size analyser[5]. Cells were passaged until passage 3. Then, they were frozen in 10^6 all quots in crystals[6] with freezing medium, consisting of 90% FBS and 10% DMSO. MSC characterisation assays were performed with cryopreserved cells at passage 3. Cells were thawed at 37°C and set in culture for 3 days to readjust the culture conditions prior to be used on the different experiments.

MSC proliferation, viability, apoptosis and necrosis

MTT assay: A MTT assay[3] was applied to analyse the proliferation characteristics of cultured BM-MSCs (n = 5) and AT-MSCs (n = 4). Eight 96 well plates were seeded on Day 0, and 8 replicas of each horse and source at a density of 5 x 10^3 cells/cm^2 in basal medium and allowed to proliferate over the course of 8 days. On each of these 8 days, one plate was used to measure proliferation using the MTT assay (Mosmann 1983). Optical density (570 nm) for each well was determined using a Biotek Synergy HT spectrophotometer. Linearity of the assay was tested for the range of cell densities expected for the experiment for both BM- and AT-MSCs. Briefly, 0–15,000 cells/cm^2 (8 replications of each density) from each source were seeded in a 96 well plate and incubated for 24 h in basal medium. The MTT assay was performed and average absorbance for each density used to construct a standard curve. Viable cell numbers for the different samples and days were determined using the calibration curve.

Cell doubling time at this passage was calculated according to the formulae:

$$CD = ln\frac{\frac{Nf}{Ni}}{ln2} DT = \frac{CT}{CD}$$

DT = cell doubling time; Nf = final number of cells; Ni = initial number of cells; CD = cell doubling number.

Flow cytometry: Flow cytometry was used to evaluate cell viability, apoptosis and necrosis of cultured MSCs. AT-MSCs and BM-MSCs from 2 animals were seeded at a density of 1500 cells/cm^2 and cultured in 6 well plates for 8 days. Cells were trypsinised daily for flow cytometry analysis. Apoptosis was measured using the FITC-conjugated Annexin V kit (Immunostep) according to the manufacturer's protocol. Briefly, Annexin V-FITC (AnV) bounded to cells which showed phosphatidylcholine and sphingomyeline in their membranes, which is associated with early apoptosis. Propidium iodide (PI) bounded to necrotic cells because of their membranes have lost the integrity. Fluorescence was read by flow cytometry using FAC Saria[4] in accordance with the manufacturer's protocol.

Expression of cell surface marker genes

The expression levels of 7 genes coding for 4 MSC surface markers (*CD73*, *CD90*, *CD105* and *CD166*), 2 haematopoietic markers (*CD34* and *CD45*) and an endothelial marker (CD31) were evaluated in cultured AT-MSCs and BM-MSCs from passage 3, using RT-qPCR. Total RNA was isolated from approximately 40,000 BM and AT-MSCs using Cell-to-cDNA II[7] according to the manufacturer's instructions. RNA obtained from equine peripheral blood and whole bone marrow was used as a positive control to validate the primers for the study of haematopoietic markers.

Primers were designed using Primer Express 2.0 software[8] based on known equine sequences. Primer

details, accession numbers for equine mRNA sequences and amplicon sizes are shown in Table 1.

Real-Time RT-PCR (RT-qPCR) was performed and monitored using the StepOne Real Time PCR System[8]. All reactions were carried out in a total volume of 10 ml with 2 ml of cDNA as a template and Fast SYBR Green Master Mix[8]. cDNA was amplified following the manufacturer's protocol: 20 s at 95°C of an initial activation and denaturation, 45 cycles consisting of 3 s at 95°C and 30 s at 60°C. Each cDNA sequence was tested in triplicate and a dissociation curve protocol run after every PCR reaction. The levels of gene expression were determined by the comparative Ct method. A normalisation factor (NF) was used to determine the expression level of each gene in each sample. The NF was calculated as the geometric mean of the quantity of 2 housekeeping genes (*GAPDH* and *B2M*). Primers used for housekeeping gene amplification were previously described (Kolm *et al*. 2006).

CD90 (Thy-1) was also analysed by flow cytometry. Briefly, 50,000 BM-MSCs and AT-MSCs in passage 3 were incubated at 4°C for 15 min with 5 ml of mouse anti-human CD90-PE monoclonal antibody[10] (BD Pharmingen). Cells were analysed by a fluorescence activated cell sorter (Facsaria)[4].

Osteogenic differentiation

Osteogenic differentiation was induced by culturing thawed AT- and BM-MSC from passage 3. Cells were plated at a density of 20,000 cells/cm^2 in triplicate (in 24 well plates) in differentiation medium containing basal medium supplemented with 10 nmol/l dexamethasone, 10 mmol/l b-glycerophosphate and 100 mmol/l ascorbate-2-phosphate (all from Sigma-Aldrich)[3] (Koerner *et al*. 2006). Differentiation culture was maintained for 7 days.

Alizarin red staining: To evaluate calcium deposition, cells were fixed on the last day of culture with 70% ethanol and stained with 2% Alizarin Red dye[3] pH 4.6 for 10 min and washed with PBS for 15 min.

Alkaline phosphatase assay: The activity of alkaline phophatase (ALP) was evaluated for every 2 days in culture. Cells were lysed with RIPA buffer (NaCl 150 mmol/l, TRIS-HCl 50 mmol/l, NP-40 1%, Sodium deoxicolate 0.25%, EDTA 1 mmol/l, PMSF 100 mmol/l and Sodium ortovanadate 100 mmol/l) for 45 min at 4°C. A fraction of the lysate was used for the ALP assay using p-nitrophenyl phosphate (pNPP)[3] as a substrate. Absorbance of p-nitrophenol (pNP) was measured at 405 nm in a Biotek Synergy HT spectrophotometer.

The ALP activity value was quantified by comparison with a standard curve of commercial pNP[3]. ALP activity, expressed as mmol/l pNP/mg total protein, was normalised using the remaining lysis fraction to calculate the total protein content by the BCA method[3].

Osteogenic marker gene expression: Osteogenic differentiation was monitored every 2 days by analysing the mRNA expression of osteogenic markers, alkaline phosphatase (*ALP*), osteocalcin (*BGLAP*), collagen *type I* (*COL1A1*), runt-related transcription factor 2 (*RUNX2*) and osteopontin (*SPP*). Briefly, mRNA was extracted from differentiated and control AT- and BM-MSC samples using the same protocol as described for the analysis of surface membrane markers (see above). Specific primers for the genes are shown in Table 1.

Adipogenesis was induced by culturing thawed, AT- and BM-MSC from passage 3. Cells were plated in triplicate at a density of 20,000 cells/cm^2 in 24 well plates. Sixteen adipocyte differentiation media were tested for differentiation of BM- and AT-MSCs. Five of these media were previously described for the differentiation of equine MSCs Table 2 the remainder were modifications based on them. Only basal medium supplemented with 1 mmol/l dexamethasone[3], 500 mmol/l 3-isobutyl-1-methylxanthine[3], 200 mmol/l indomethacin[3] and 15% rabbit serum[3] (highlighted medium) was able to induce adipogenic differentiation. Cultures were maintained for 15 days.

Oil red O staining: Adipogenic differentiation was examined by performing Oil Red O staining. On Day 15, cells were fixed with 10% formalin[3] for 15 min at room temperature, stained with 0.3% Oil Red O dye

(dissolved in 60:40; isopropanol : distilled water) and washed with distilled water.

Adipogenic marker expression: Adipogenic differentiation was monitored by analysing the expression of lipoprotein lipase (*LPL*) and peroxisome proliferator-activated receptor gamma (*PPAR*g) markers every other day throughout the differentiation process. Briefly, mRNA extraction, RT-qPCR and evaluation of results were conducted as described above. Specific primers for the adipogenic markers are shown in Table 1.

Chondrogenic differentiation

Chondrogenic differentiation was performed in pellet culture. Approximately 3 x10^5 cells were placed in a 15 ml conical polypropylene tube, centrifuged at 1700 ***g*** for 5 min and resuspended in chondrogenic medium, consisting of DMEM high glucose supplemented with 10% FBS, 10 ng/ml TGFb-3[9], ITS+ premix[4], 40 mg/ml proline[3], 50 mg/ml ascorbate-2-phosphate[3], 0.1 mmol/l dexamethasone[3]. Differentiation culture was maintained for 21 days.

Histology

Pellets were fixed in 10% formalin[3], embedded in paraffin and sectioned into 5 mm sections. The sections were hydrated with increasing amounts of alcohols, stained with Mayer's haematoxylin and 3% Alcian Blue dyes, rinsed with distilled water, dehydrated with decreasing amounts of alcohols and mounted.

Statistical analysis

Differences in viability, apoptosis and necrosis over time were evaluated by a multiple linear regression, which considers a specific slope for each combination of tissue (AT and BM) and individual:

$$y_{ijk} = \mu + b_i x_{ij} + e_{ijk}$$

where y_{ijk} is the percentage of necrotic, late apoptotic, viable or early apoptotic cells for the i^{th} combination of tissue and individual (AT-1, AT-2, BM-1, BM-2), the j^{th} recording date (Days 1–10) and the k^{th} replicate (13), bi is the specific slope for each tissue and individual combination, x_{ij} is the day after seeding and e_{ijk} is the residual. Thus, a general mean and 4 different regressionslopes were estimated for each process (necrosis, late apoptosis, viability and early apoptosis).

Differences in osteogenic and adipogenic marker gene expression and ALP activity between control and treated cultures were evaluated with the Student's *t* test. In addition, a 2-way ANOVA test was performed to identify the effect of time and animal in the expression or activity of these markers during the differentiation period. When significant main effects or an interaction between the main effects was found, specific comparisons were made with paired Student's *t* tests. P values <0.05 were considered significant. Finally, Pearson's correlation was used to analyse the association between the expression of every pair of osteogenesis differentiation markers and ALP activity. Statistics were assessed using SPSS 14.0 software.

Results

Isolation and proliferation of MSCs

Plastic-adherent fibroblast-like cells were observed in all donor samples obtained from both bone marrow aspirates and adipose tissue within the first days of culture. Cells were expanded until passage 3 and frozen. After thawing, cells maintained their morphology and proliferation capacity.

The MTT assay was applied to analyse the proliferation characteristics of cultured BM-MSCs and AT-MSCs. The total number of viable cells for the different samples and days were determined by extrapolation from the calibration curves: $y = 1.91 \cdot 10^{-5} x + 0.0384$, $r^2 = 0.978$ for BM-MSCs and $y =$

$1.77 \cdot 10^{-5}\, x + 0.0272$, $r^2 = 0.991$ for AT-MSCs, where *y* is the quantity of cells and *x* the optical density of the well. Mean values of the number of cells per well from BM- and AT-MSCs are presented in Figure 1. The lag phase extended until the second day post seeding. Afterwards,

AT-MSCs started growing at a consistent rate until Day 8. The BM-MSC growing slowed down on Days 4 and 5, showing differences within both types with a residual significance (Student's *t*, P = 0055 and P = 0.063, respectively). Since that moment, on average, AT-MSC culture had a higher cell number than BM-MSC culture, although great interindividual differences were observed in both cell types, particularly during the final days. The higher differences of the cell number of AT-MSCs vs. BM-MSCs were more evident when comparing cells of each origin from the same animal (data not shown).

Cell doubling time were also calculated being similar in both cell type, 2.47 ± 0.4 days for BM-MSC and 2.2 ± 0.2 days for AT-MSC.

Viability, apoptosis and necrosis of MSCs

The Annexin V assay was used to determine the percentage of viable, early apoptotic, necrotic + late apoptotic and necrotic cells during 8 days of culture. The highest percentage of viable cells, propidium iodide negative and annexin V negative (PI-, AnV-), was observed at Day 1 for both MSC types (61.23% in BM and 62.75% in AT) and the lowest percentages were found at Day 5 in BM-MSC (38.2%) and at Day 8 in AT-MSC (18.72%). The percentage of necrotic cells (PI+ AnV- cells) was very low, ranging from 0.48–2.37% in BM-MSC and from 0.75–2.12% in AT-MSC at Days 0 and 1, respectively. The highest percentage of early apoptotic cells (PI-, AnV+) was observed the second day of culture (22.75% in BM- and 21.73% in AT-MSC) and the lowest at Day 7 (9.62% for BM and 3.02% for AT). Finally, necrotic + late apoptotic cells (PI+, AnV+) displayed the lowest percentage the first day of culture (21.87% in BM and 21.4% in AT) and the highest value was observed at Day 5 in BM (48.03%) and Day 8 in AT (76.18%).

Differences in the kinetic of the viability changes between MSC sources and animals were assessed by multiple linear regressions. Slope values show the decrease or increase of each parameter as time goes by (Table 3). Whereas necrosis + late apoptosis was increasing during the culture period (positive slope), the other parameters were decreasing (negative slope). For necrosis + late apoptosis, we did not find any significant differences between regression slopes. Neither significant differences were found in necrosis between individuals or MSC source. Nevertheless, the percentage of early apoptosis along the course of the experiment decreased significantly faster in AT than in BM MSC ($P<0.05$). However, this result did not correspond with an increase of viability in AT-MSCs. As shown in Table 3, only BM-MSC from the second individual had a less marked slope in the decrease of viability along the course of culture.

Cell surface antigen gene expression

BM-MSCs and AT-MSCs expressed transcripts for *CD73*, *CD90*, *CD105* and *CD166* (Fig 2). The levels for these MSCs marker expressions were similar for both sources of MSCs. *CD34* was significantly enriched in AT-MSCs compared to BM-MSCs ($P<0.01$), which did not express this cell marker. Equine MSCs did not express haematopoietic (CD45) and endothelial (CD31). In the same RT-qPCR assays, *CD34* in bone marrow and *CD31* and *CD45* in peripheral blood displayed a positive amplification with a relative expression value of 1.

CD90 flow cytometry analysis showed the presence of this molecule in a high percentage in BM-MSCs (93.05 ± 2.30) and AT-MSCs (96.07 ± 0.76) (data not shown).

Osteogenic differentiation

Osteogenic differentiation was achieved for both sources of MSCs, which showed multiple Alizarin Red-stained calcium deposits (Fig 3). During osteogenic induction, ALP activity was measured on Days 0, 3, 5 and 7 of culture. ALP activity increased at Day 5 for both BM-MSCs and AT-MSCs and increased further still by Day 7 (Fig 4). Differences in ALP activity between Days 0 and 7 were significant for BM-MSCs ($P<0.001$) and AT-MSCs

Transcript levels of 4 osteogenic markers were analysed on Days 0, 3, 5 and 7 of culture (Fig 4). *RUNX2*

expression levels started to increase at Day 3 in osteogenic cell cultures, and were significantly compared to controls at Day 7 in both cell types. *SPP1* mRNA expression reached its maximum at Day 5 for BM-MSCs (146-fold higher than in control cultures) and decreased afterwards, although at Day 7 its expression was still significantly higher in differentiated cells than in control cultures. The highest level of *SPP1* expression in treated AT-MSCs was found on Day 7 (19.4-fold higher than in controls), although not significant difference was observed.

BGLAP mRNA expression increased slightly in treated AT-MSCs. At Day 5 in differentiated BM-MSCs, significant differences were observed with respect to control cells and the highest expression (13.4-fold higher) was on Day 7. *ALP* mRNA increased during the early stage of osteogenesis for both types of cells. In addition, a correlation between *ALP* gene expression level and activity was observed for BM-MSCs (r = 0.429, P□0.01). Finally, *COL1A1* expression levels in osteogenic cultures were similar or lower than controls for both cell types. The expression of *RUNX2* correlated with the expression of *ALP* and *SSP1* in both MSC sources. Table 4 shows these and other significant Pearson correlation coefficients that were obtained. *COL1A1* expression did not positively correlate with any of the other osteogenic markers.

Adipogenic differentiation

Sixteen different media and several seeding conditions were tested for their ability to induce the adipogenic differentiation of equine MSCs (Table 2). Under the conditions tested, only one medium could induce differentiation in BM-MSCs from 2 donors. No differentiation was observed in any of AT-MSCs. Lipid droplets appeared at Day 4 and increased in size over time. On Day 15 of culture, the oil red O staining showed intracellular accumulation of red-stained lipid droplets (Fig 3). The remaining samples were subjected to the same culture conditions, but their morphology did not change during the differentiation process.

Expression of adipogenic markers was analysed every other day for 15 days. *PPAR*g and *LPL* expression profiles in BM-MSCs are shown in Figure 5. *PPAR*g and *LPL* mRNA expression levels increased 64- and 354-fold, respectively, on the last day of culture with respect to control cells. These markers were also analysed for cells that did not exhibit morphological signs of differentiation. RT-qPCR results confirmed the lack of differentiation because no significant changes were observed in their expression (data not shown).

Chondrogenic differentiation

Equine MSCs were able to differentiate into chondrocytes (Fig 3). Haematoxylin and Alcian blue stainings were performed on Day 21 of culture showed the characteristic chondrogenic phenotype with lacunae formation throughout the pellet and the glycosaminoglycans in the extracelullar matrix appeared stained in blue.

Discussion

Characteristics of marker expression, proliferation and differentiation show significant variations between analogous cell populations (Conget and Minguell 1999; Larson *et al*. 2008). Prior to the application of stromal cell populations in regenerative medicine, fundamental questions about *in vitro* growth and differentiation of mesenchymal stromal cell populations must be resolved (Ciba *et al*. 2009). In this work, we extensively characterise 2 types of MSCs (AT-derived MSCs and BM-derived MSCs), evaluating their proliferation potential, viability, gene expression of membrane surface markers and ability to differentiate into osteogenic, adipogenic and chondrogenic cells.

Different attempts to monitor the growth of equine BM- and AT-MSCs have been reported (Vidal *et al*. 2006, 2007; Mambelli *et al*. 2009). Our work compares the proliferation capacity and viability of both sources of MSCs using the MTT approach at passage 3. Vidal *et al*. (2006, 2007) reported a cell doubling time for AT-MSCs that was higher than BM-MSCs for at passage 3. Under our conditions, AT-MSCs had a slightly lower cell doubling time than BM-MSCs, but the differences were not significant. A slowing down in the cell proliferation of BM-MSC was observed at Days 4 and 5. For this reason the

number of cells in the AT-MSC was higher than BM-MSC culture since these days. This result was more evident for cells from the same animal, indicates the existence of interindividual differences and confirms the results of other studies that demonstrate a large variation between MSC populations with regard to growth rate (Sen *et al*. 2001; Izadpanah *et al*. 2006).

In agreement with MTT results, viability and necrosis + late apoptosis percentage displayed the lowest and highest values, respectively on Day 5. To our knowledge, this is the first study that analyses the viability, apoptosis and necrosis of equine MSCs in culture. Given the limited size of the experiment, we cannot infer a general pattern of evolution over time, but we detected evidence of variability in viability and early apoptosis between cells from different sources. The highest values of early apoptosis were observed during the first days of culture that could correspond with the lag phase, when cells are readjusting to the culture. In AT-MSCs, early apoptosis decreased faster than in BM-MSCs while necrosis + late apoptosis increased following the same tendency (faster in AT-MSC than in BM-MSC); these 2 findings could explain that not significant differences in viability were found between cells from the 2 sources. Future research must be done to clarify the sources of this variability and improve the ability to predict the specific pattern of evolution of viability, apoptosis and necrosis over time. Nevertheless, for their use in cell therapy, the culture of AT-MSC should be stopped before the culture is close to confluence, as the highest percentage of necrotic + late apoptotic cells and lowest viability was observed on the last day of culture.

There have been recent studies on the immunophenotype of equine AT-derived MSCs for CD13, CD44 and CD90 (de Mattos Carvalho *et al*. 2009) and equine BM-derived MSCs for CD29, CD44, CD90 and CD45 (Radcliffe *et al*. 2010). The determination of equine CD markers using anti-human antibodies is difficult because of the small reactivity of available antibodies with equine epitopes (Ibrahim and Steinbach 2007). In an attempt to determine the expression of well known MSC markers in man (CD73, CD105, CD90 and CD166) and haematopoietic and endothelial markers (CD34, CD35 and CD431), we used RT-qPCR. Although this technique shows the mRNA expression of different markers, this expression is not always correlated with the presence of protein. Protein detection by other methods may possibly complement the results we have obtained. Data obtained by flow cytometry for the CD90 antigen supported data from RT-qPCR. In agreement with previous reports in horses (Arnhold *et al*. 2007; de Mattos Carvalho *et al*. 2009; Radcliffe *et al*. 2010) CD90 is an antigen present in established culture of equine MSC. The expression profile of these markers in equine BM- and AT-MSCs in passage 3, was in accordance with the immunophenotype reported for human MSCs (Dominici *et al*. 2006; Kern *et al*. 2006; Jarocha *et al*. 2008; Liu *et al*. 2008), including the differences observed for the expression of CD34 between BM- and AT-MSCs. Because the immunoreactivity for CD34 in human AT-MSCs declines with passages (Mitchell *et al*. 2006; Noel *et al*. 2008), further analysis is necessary to confirm that the loss of CD34 in equine AT-MSCs is similar to that in human cells.

One of the main characteristics of MSCs is their capacity to differentiate into multiple lineages. We have analysed the osteogenic, adipogenic and chondrogenic potential of equine BM- and AT-MSCs. MSC differentiation into osteoblasts was confirmed by staining calcium deposits with Alizarin red and measuring the increase of ALP activity and the expression of osteogenic markers over time. The osteogenic differentiation in equine MSCs was faster than in other species, including human (Liu *et al*. 2009), porcine (Zou *et al*. 2008) and bovine (Bosnakovski *et al*. 2005) and similar to previous reports in horses (Vidal *et al*. 2006).

The use of molecular markers allows osteogenic differentiation to be monitored and indicates which genes are involved in the process. Here, we present for the first time the expression profiles of a battery of osteogenic markers in both types of equine MSCs. In agreement with data found in other species (Bosnakovski *et al*. 2005; Zou *et al*. 2008), 4 genes (*BGLAP, SPP1, ALP* and *RUNX2*) were upregulated during osteogenesis. Moreover, the expression of some these genes was correlated with ALP activity, indicating that they are involved in the differentiation process. Our findings suggest that osteogenic differentiation of equine BM- and AT-MSCs occurs by upregulating a cascade of specific genes, including *RUNX2*, *BGLAP*, *SSP1* and *ALP* but not *COL1A1*. While this osteogenic marker seems to be upregulated in other species (Bosnakovski *et al*. 2005) and is commonly used in human MSC characterisation (De Bari *et al*. 2008), other authors have reported no remarkable increase of *COL1A1*

mRNA after osteogenic differentiation in human (Liu *et al*. 2008) and porcine (Zou *et al*. 2008) MSCs.

We cultured BM- and AT-MSCs in different adipogenic media. Previous reports found that rabbit serum enhanced adipogenesis *in vitro* for human (Janderova *et al*. 2003), rat and mouse (Diascro *et al*. 1998) MSCs. Rabbit serum has a high content of free fatty acids, which are putative ligands of PPAR*y* and may thus enhance adipogenesis. When we compared 16 different kinds of adipogenic induction media, only the medium with a similar composition to the medium used for human BM- and AT-derived MSCs (Pittenger *et al*. 1999) and supplemented with 15% rabbit serum was able to induce adipogenic differentiation. Other authors did not find it necessary to add rabbit serum to achieve any reliable adipogenesis (Mambelli *et al*. 2009). The intracellular accumulation of red-stained lipid droplets on Day 15 of culture was indicative of adipogenic differentiation. However, cytoplasmic droplets were already visible within the first few days of culture. This characteristic was in agreement with findings observed by other authors (Vidal *et al*. 2007; Mambelli *et al*. 2009). Our results indicate that equine MSCs display an adipogenic potential lower than other species (Bosnakovski *et al*. 2005; Zou *et al*. 2008). Cells from one of the animals whose adipogenic differentiation was possible were used to monitor the process with molecular markers. Both PPAR*y* and *LPL* genes were upregulated under adipogenic conditions compared to controls, indicating that these markers are involved in the differentiation process. The increase was more evident on the last day of culture.

As a final step in the characterisation process, we demonstrated equine MSCs had tri-lineage potential since cells were able to differentiate into chondrocytes.

In summary, here we present the characterisation of equine BM- and AT-MSCs, comparing their proliferation activity, viability, apoptosis, necrosis and differentiation. We also describe a battery of molecular markers for phenotype characterisation and monitoring osteogenic and adipogenic differentiation, which will contribute to a more extensive characterisation of equine MSCs prior to their use in cell therapy.

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Author contributions B. Ranera carried out the isolation and expansion of the MSCs, the differentiation assays and histology and the writing of the manuscript. L. Ordovas participated in the expansion of the MSCs, and carried out the proliferation and visibility, apoptosis and necrosis assays. J. Lyahyai performed the RT-qPCRs. M.L. Bernal participated in the writing of the manuscript. F. Fernandes participated in the expansion of MSCs. A.R. Remacha participated in the differentiation assays and histology. A. Romero carried out the aspiration of the bone marrow and the harvesting of the adipose tissue. F.J. Vázquez participated in the aspiration of the bone marrow and the harvesting of the adipose tissue. R. Osta participated in the design of the study. C. Cong participated in the isolation and expansion of MSCs. L. Varona performed the statistical analysis. P. Zaragoza participated in the design of the study and the writing of the manuscript. I. Martin-Burriel participated in the design of the study and in the writing of the manuscript. C. Rodellar coordinated the design of the study, supervised the work and participated in the writing of the manuscript.

Manufacturers' addresses

[1]Gibco Invitrogen Corporation California, USA.

[2]Atom, Barcelona, Spain.

[3]Sigma-Aldrich, St. Louis, Missouri, USA.

[4]Becton Dickinson, Franklin Lakes, New Jersey, USA.

[5]Beckman Coulter, Inc., Brea, California, USA.

[6]Nalgene Thermo Fisher Scientific, Roskilde, Denmark.

[7]Ambion, Foster City, California, USA.

[8]Applied Biosystems, Foster City, California, USA.

[9]R&D Systems, Minneapolis, USA.

[10]BD Pharmingen, San Diego, California, USA.

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Tables:

TABLE 1: Cell surface, osteogenic and adipogenic markers analysed by RT-qPCR. GenBank accession numbers of the sequences used for primers design. Primers (F: Forward and R: Reverse) and length of the amplicon in base pair (bp).

Genes	Accesion number	Primer sequence	Amplicon size
CD31	NM_001101655	F:TCTAGAACGGAAGGCTCCCT R:TGGGAGCAGGGCAGGTTCA	145
CD34	XM_001491596	F:CACTAAACCCTCTACATCATTTTCTCCTA R:GGCAGATACCTTGAGTCAATTTCA	150
CD45	AY_114350	F:TGATTCCCAGAAATGACCATGTA R:ACATTTTGGGCTTGTCCTGTAAC	100
CD73	XM_001500115	F:GGGATTGTTGGATACACTTCAAAAG R:GCTGCAACGCAGTGATTTCA	90
CD90	EU881920	F:TGCGAACTCCGCCTCTCT R:GCTTATGCCCTCGCACTTG	93
CD105	XM_001500078	F:GACGGAAAATGTGGTCAGTAATGA R:GCGAGAGGCTCTCCGTGTT	100
CD166	XM_001503380	F:GTCTGGTCTTCTGCCTCTTGATC R:TCGGCAAGGCATGATAATAGTG	103
ALP	XM_001504312	F:GATGGCCTGAACCTCATCGA R:AGTTCGGTCCGGTTCCAGAT	92
BGLAP	DQ_007079	F:GGCAGAGGTGCAGCCTTC R:CTCCAGAGGGTCCGGGTAG	114
COL1A1	AF_034691	F:ACACAGAGGTTTCAGTGGTTTGG R:CACCATGGCTACCAGGTTCAC	89
RUNX2	XM_001502519	F:CTCCAACCCACGAATGCACTA R:CGGACATACCGAGGGACATG	80
SPP1	XM_001496152	F:CTCACATCACCTGTGGAAAGCA R:CACGGCTGTCCCAATCAGA	104
LPL	XM_001489577	F:TGTATGAGAGTTGGGTGCCAAA R:GCCAGTCCACCACAATGACAT	70
PPARg	XM_001492411	F:TGCAAGGGTTTCTTCCGGA R:GCAAGGCATTTCTGAAACCG	104
GAPDH	NM_001163856	F:GGCAAGTTCCATGGCACAGT R:CACAACATATTCAGCACCAGCAT	128
B-2M	NM_001082502	F:TCGTCCTGCTCGGGCTACT R:ATTCTCTGCTGGGTGACGTGA	102

TABLE 2: Media used for adipogenic differentiation including basal media, supplements and sera.

	DMEM	Dexamethasone	IBMX	Indomethacin	Insuline	Pantothenate	Biotin	Rosiglitazone	Sera	Human
1	LG	1 mmol/l	500 mmol/l	200 mmol/l					10% FBS	+
2	LG	1 mmol/l	500 mmol/l	200 mmol/l					5% RS 10% FBS	
3	LG	1 mmol/l	500 mmol/l	200 mmol/l					15% RS 10% FBS	
4	LG	1 mmol/l	500 mmol/l	200 mmol/l					10% HS 10% FBS	
5[1]	LG	1 mmol/l	500 mmol/l	200 mmol/l	10 mg/ml				15% RS 10% FBS	
6	LG	1 mmol/l	500 mmol/l	200 mmol/l	10 mg/ml				10% FBS	+
7[2]	DMEM/F12	1 mmol/l	500 mmol/l	200 mmol/l	10 mg/ml				10% FBS	
8[3]	DMEM/F12	1 mmol/l	500 mmol/l		100 nmol/l	17 mmol/l	33 mmol/l	5 mmol/l	5% RS 3% FBS	+
9	LG	1 mmol/l	500 mmol/l		100 nmol/l	17 mmol/l	33 mmol/l	5 mmol/l	5% RS 3% FBS	+
10	LG	1 mmol/l	500 mmol/l		100 nmol/l	17 mmol/l	33 mmol/l	5 mmol/l	5% RS 10% FBS	
11	LG	1 mmol/l	500 mmol/l		100 nmol/l	17 mmol/l	33 mmol/l	5 mmol/l	15% RS 10% FBS	
12	LG	1 mmol/l	500 mmol/l		100 nmol/l	17 mmol/l	33 mmol/l	5 mmol/l	10% HS 10% FBS	
13	HG	1 mmol/l	500 mmol/l	100 mmol/l	10 mmol/l				10% FBS	+
14[4]	DMEM/F12	1 mmol/l	500 mmol/l	100 mmol/l	10 mg/ml				10% FBS	+
15[5]	HG	1 mmol/l	500 mmol/l	100 mmol/l	10 mg/ml				2% KSR	
16	HG	1 mmol/l			5 mg/ml				20% FBS	

(+) Indicates media tested in human MSCs. Highlighted medium is the final medium used in the present work; [1]Koch *et al*. 2007; [2]Koerner *et al*. 2006; [3]Vidal *et al*. 2006; [4]Arnhold *et al*. 2007; [5]Mambelli *et al*. 2009; FBS, Fetal calf serum; HG = high glucose DMEM; HS = horse serum; KSR = knockout serum replacement; LG = low glucose DMEM; RS, Rabbit serum.

TABLE 3: Behaviour of viability, necrosis + late apoptosis, early apoptosis and necrosis along time for each sample origin (BM or AT) and individual (1 or 2)

Traits	Slope	Estimate	s.e.	Conf. interval
Necrosis	b	-0.031	0.027	[-0.084, 0.022]^{a}
	b_{BM1}			
	b_{AT1}	0.012	0.027	[-0.041, 0.065]^{a}
	b_{BM2}	-0.071	0.027	[-0.124, -0.018]^{a}
	b_{AT2}	-0.038	0.027	[-0.091,0.015]^{a}
Necrosis + late apoptosis	b_{BM1}	3.801	1.294	[1.265, 6.337]^{a}
	b_{AT1}	3.869	1.259	[1.401,6.337]^{a}
	b_{BM2}	2.804	1.259	[0.336,5.272]^{a}
	b_{AT2}	5.123	1.259	[2.655,7.591]^{a}
Viable cells	b_{BM1}	-2.989	0.385	[-3.744, -2.234]^{a}
	b_{AT1}	-2.382	0.385	[-3.137, -1.627]^{a}
	b_{BM2}	-0.637	0.385	[-1.392,0.118]^{b}
	b_{AT2}	-3.626	0.385	[-2.871, -4.381]^{a}
Early apoptosis	b_{BM1}	-0.677	0.194	[-1.057, -0.297]^{a}
	b_{AT1}	-1.488	0.194	[-1.868, -1.108]^{b}
	b_{BM2}	-0.149	0.194	[-0.529, 0.231]^{a}
	b	-1.445	0.194	[-1.825, -1.065]^{b}
	$_{AT2}$			

Slope estimates, standard error (s.e.) and confidential interval of the slopes. Same letter (^{a} or ^{b}) indicates overlapping intervals (no significant differences).

TABLE 4: Correlation between osteogenic marker expression and ALP activity. No significant correlations are not shown

		ALP	BGLAP	COL1A1	RUNX2	SPP1	ALP activity
BM-MSC	ALP				0.527**	0.448**	0.429**
	BGLAP						0.559**
	COL1A1						-0.320*
	RUNX2	0.527**				0.584**	0.702**
	SPP1	0.448**			0.584**		
	ALP Activity	0.429**	0.559**	-0.320*	0.702**		
AT-MSC	ALP		0.472**		0.332*		
	BGLAP	0.472**					
	COL1A1						
	RUNX2	0.332*				0.609**	0.727**
	SPP1				0.609**		0.861**
	ALP Activity				0.727**	0.861**	

*P<0.05; **P<0.01.

Figures:

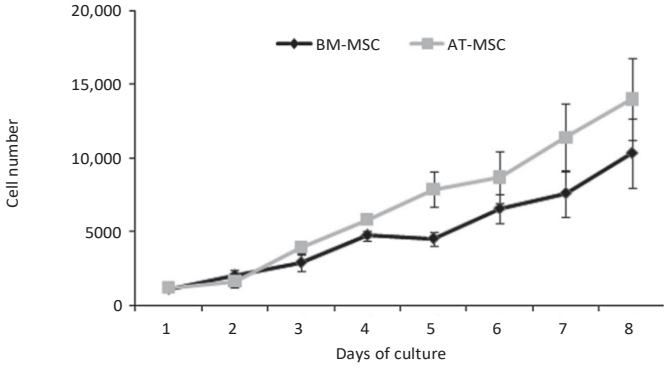

Fig 1: BM and AT-MSC proliferation along time evaluated with the MTT assay. Mean ± s.e. of cell number are shown.

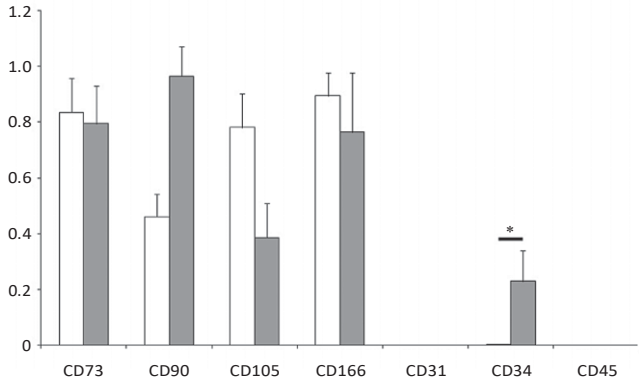

Fig 2: Mean ± s.e. of relative mRNA expression (y axis) of 6 antigens surface markers (x axis). White bars represent BM-MSC and grey bars are AT-MSC. *P<0.05, n = 5.

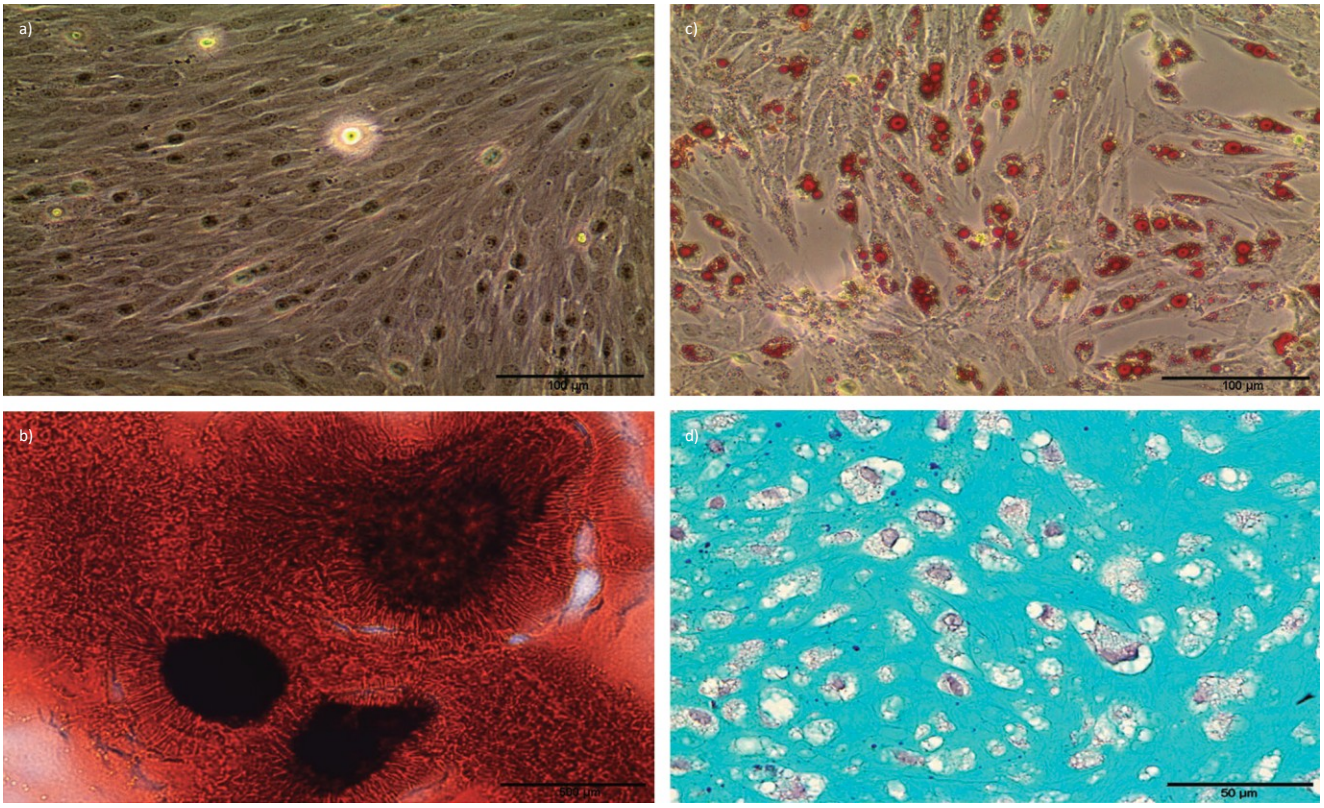

Fig 3: Staining for osteogenic, adipogenic and chondrogenic differentiation in BM-MSC. a) Control MSCs maintained in basal medium (magnification 10x). b) Alizarin red staining of cells cultured for 7 days in osteogenic differentation medium (magnification 4x). c) Oil red O staining of cells cultured for 15 days in adipogenic differentiation medium (magnification 10x). d) Haematoxylin Mayer's and Alcian Blue stainings of pellets cultured for 21 days in chondrogenic medium (magnification 40x).

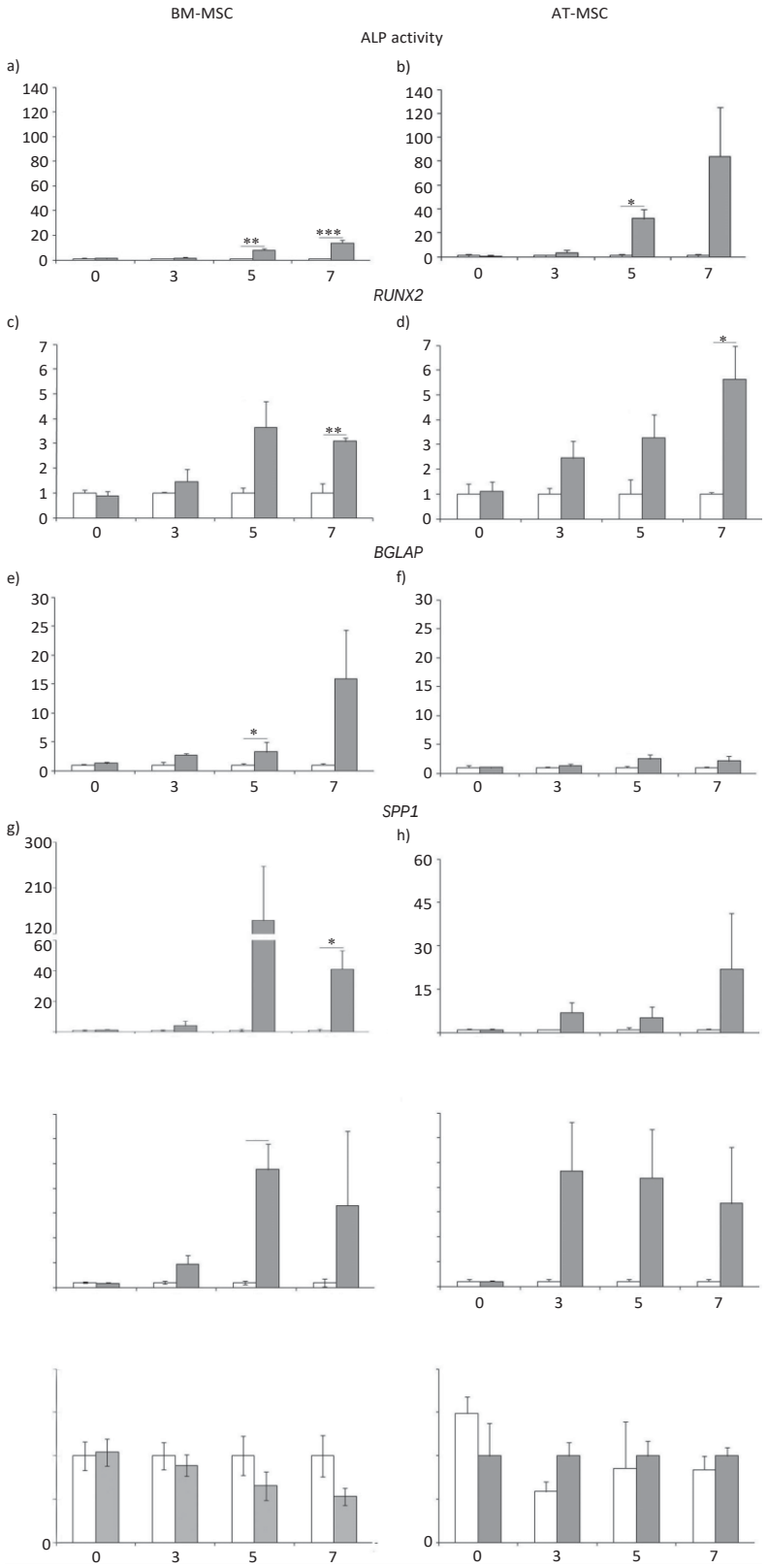

Fig 4: Alkaline phosphatase activity (a,b) and osteogenic marker expression levels (c–l) in controls (white bars) and differentiated (grey bars). BM-MSC (left) and AT-MSC (right). X axis represents culture days. Y axis represents the mean ± s.e. of relative mRNA expression data for RUNX2 (c,d), BGLAP (e,f) SPP1 (g,h) ALP (i,j) and COL1A1 (k,l) genes. *P<0.05 **P<0.01 ***P<0.001, n = 5

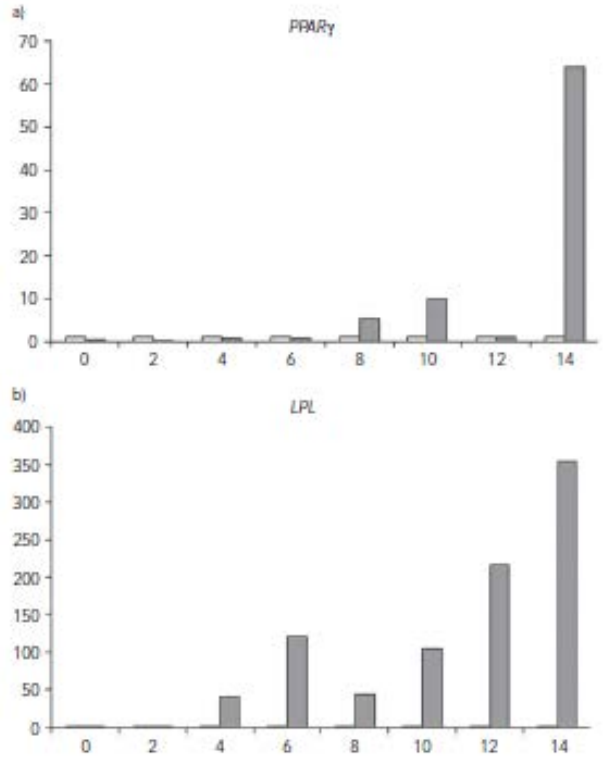

Fig 5: mRNA expression levels of adipogenic marker in controls (white bars) and differentiated (grey bars) BM-MSC evaluated by RT-qPCR. X axis represents culture days and Y axis represents realtive mRNA expression of the PPARg (a) LPL (b) genes.