Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue

Beatriz Ranera[1*], Jaber Lyahyai[1*], Antonio Romero[1,2], Francisco José Vázquez[1,2], Ana Rosa Remacha[1], María Luisa Bernal[3], Pilar Zaragoza[1], Clementina Rodellar[1], Inmaculada Martín-Burriel[1#]

[1]Laboratorio de Genética Bioquímica (LAGENBIO). Facultad de Veterinaria. Universidad de Zaragoza. 50013, Zaragoza (Spain)

[2]Hospital Veterinario. Facultad de Veterinaria. Universidad de Zaragoza. 50013 Zaragoza (Spain)

[3]Departamento de Farmacología y Fisiología. Facultad de Medicina. Universidad de Zaragoza. 50009, Zaragoza (Spain)

* These authors contributed equally to this work.

 # Corresponding author

Laboratorio de Genética Bioquímica. Facultad de Veterinaria. Miguel Servet 177, 50013 Zaragoza

Fax: +34 976762949

Phone: +34 976 761622

Email: minma@unizar.es

Abstract

Bone marrow and adipose tissue are the two main sources of mesenchymal stem cell (MSC). The aim of this work was to analyse the immunophenotype of 7 surface markers and the expression of a panel of 13 genes coding for cell surface markers in equine bone marrow and adipose tissue-derived MSCs obtained from 9 horses at third passage. The tri-lineage differentiation was confirmed by specific staining. Equine MSCs from both sources were positive for the MSC markers CD29 and CD90, while were negative for CD44, CD73, CD105, CD45 and CD34. The gene expression of these molecules was also evaluated by reverse transcriptase real-time quantitative PCR along with the expression of 5 other MSC markers. Both populations of cells expressed *CD13*, *CD29*, *CD44*, *CD49d*, *CD73*, *CD90*, *CD105*, *CD106*, *CD146* and *CD166* transcripts. Significant differences in gene expression levels between BM- and AT-MSCs were observed for *CD44*, *CD90*, *CD29* and *CD34*. Both cell types were negative for *CD45* and *CD31*. The surface antigens tested revealed a similar phenotypic profile between horse and human MSCs, although specific differences in some surface antigens were noticed.

Keywords: Horse, MSC, cell surface markers, immunophenotype, gene expression

1. Introduction

The use of stem cell in therapy and tissue engineering in equine medicine is relatively new, but it is an exciting research field that is beginning to rapidly expand (Smith et al., 2003; Crovace et al., 2007; Richardson et al., 2007). Due to similarities in size, load and types of joint injuries suffered by horses and humans, a U.S. Food and Drug Administration (FDA) report concluded that the horse was the most appropriate model animal for testing the clinical effects of mesenchymal stem cell (MSC)-based therapies for certain types of injuries in humans, especially joint injuries (Cellular, Tissue and Gene Therapies Advisory Committee, 2005). In addition, the economic and welfare costs of performance-related injuries in horses have stimulated interest in stem cell-based regenerative medical techniques to accelerate and improve healing (Paris and Stout, 2010). Therefore, the horse can be considered not only as an animal model for human injuries and osteoarthritis (Goodrich et al., 2007) but also as a patient itself.

Bone marrow and adipose tissue are the main sources of MSCs for the treatment of equine orthopaedics (Smith et al., 2003; Koch et al., 2008), although alternative sources for MSC isolation, such as umbilical cord or peripheral blood, have been described (Koerner et al., 2006; Hoynowski et al., 2007). Recent studies have demonstrated that MSCs are very heterogeneous; there are subpopulations of cells that have different shapes and varying proliferation and differentiation abilities (Zhang and Chang, 2010). Clear characterisation of MSCs is extremely relevant for their identification before use in therapy (Tarnok et al., 2010).

Because MSCs are becoming tools utilised in equine regenerative medicine, it is important to define equine-specific markers to precisely characterise this cell

62 population (Koch et al., 2009). Mesenchymal stem cells and other stem cell lineages
63 can be identified by the expression of specific “stemness” marker proteins and other
64 stem cell epitopes that are not expressed by somatic cells. A unique MSC marker has
65 not yet been identified, in contrast to the antigen CD34 that is used for positive
66 immunoselection of haematopoietic stem cells (Tuan et al., 2003). The different
67 subpopulations of adipose tissue are difficult to characterise due to the rapid nature of
68 adipose stromal vascular cells to adopt a mesenchymal phenotype *in vitro* and the
69 complex organisation of stromal cells surrounding the small vessels (Zimmerlin et al.,
70 2010). Moreover, it has been proposed that the expression level of stem cell markers is
71 also related to the method of cell isolation (Deans and Moseley, 2000; Panchision et
72 al., 2007; Martinez-Lorenzo et al., 2009). Human MSCs are the best characterised, and
73 the International Society for Cellular Therapy has established a minimal criteria for
74 defining this type of cells (Dominici et al., 2006). First, MSCs should adhere to plastic;
75 second, MSCs should express CD105, CD73 and CD90 and should not express CD45,
76 CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR; and finally, MSCs should be able to
77 differentiate into osteoblasts, adipocytes and chrondoblasts *in vitro*. There is also
78 general agreement that MSCs lack CD31 and CD14 expression (Kern et al., 2006) and
79 express the antigens CD44 (Tarnok et al., 2010), CD106 (Pittenger et al., 1999) and
80 CD166 (Mitchell et al., 2006) and other cell adhesion molecules, such as CD13, CD29,
81 CD49f, CD54, CD59, CD63 and CD146 (Deans and Moseley, 2000; Kern et al., 2006).
82 These antigens can be detected by flow cytometry, although this method has not been
83 used to validate most of these molecules in equine MSCs. In addition to flow

cytometry, the gene expression of these antigens can be detected quantitatively using reverse transcriptase real-time quantitative PCR (RT-qPCR) (Radcliffe et al., 2010).

Although MSCs derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) seem to be closely related, differences in their biological characteristics have been reported (Im et al., 2005; Puissant et al., 2005). Both AT-MSCs and BM-MSCs share the expression of most surface antigens, but some markers are expressed differentially. For example, in humans, AT-MSCs express CD34, whereas no expression is detected in BM-MSCs; conversely, the expression of the CD106 marker has been found in BM-MSCs but not in AT-MSCs (De Ugarte et al., 2003; Gangenahalli et al., 2006).

Although there are several groups working on the characterisation of adult equine MSCs (Koerner et al., 2006; Vidal et al., 2006; Arnhold et al., 2007; Vidal et al., 2007; Vidal et al., 2008; Colleoni et al., 2009; Violini et al., 2009), only three recent studies (de Mattos Carvalho et al., 2009; Mambelli et al., 2009; Radcliffe et al., 2010) have reported the analysis of surface antigens by flow cytometry.

The lack of reactivity between commercial monoclonal antibodies and epitopes on equine cells makes it difficult to establish the phenotype of equine MSCs (Taylor et al., 2007) using flow cytometry as the sole technique. However, RT-qPCR allows monitoring of the expression levels of these markers in a limited number of cells, so the combined use of both techniques could facilitate the study of the phenotype of horse MSCs. The purposes of this work are to analyse the phenotype of both BM-MSCs and AT-MSCs with regard to 7 membrane cell surface markers using flow cytometry and also to extend the analysis to 6 more markers by RT-qPCR.

2. Materials and methods

2.1 Animals and cell isolation and expansion

Aspirates from bone marrow of nine horses were harvested using a 4'' 11G Jameshdi needle with 2500 UI of sodium heparin. Mononuclear cells were isolated by gradient centrifugation on Lymphoprep (Atom) for 20 minutes at 1700 rpm. The cells were rinsed twice with PBS (Gibco), counted, and plated at $2x10^6$ nucleated cells/cm^2 in 6-well plates (Becton Dickinson) in growth medium consisting of low glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% Foetal Bovine Serum, 1% Glutamine (Sigma) and 1% Streptomycin/Penicillin.

Samples of subcutaneous adipose tissue from near to the dorsal gluteal muscle below the tail were collected from eight horses. The stromal vascular fraction (SVF) was isolated by digestion with 0.01% collagenase (Type I, Sigma-Aldrich) for 30 minutes at 37 °C with continuous shaking, followed by centrifugation at 1700 rpm for 5 minutes. The cells were washed twice with PBS, counted, and seeded in growth medium at 10^5 nucleated cells/cm^2 in 6-well plates.

Both mononuclear and SVF cells were washed twice with PBS after 24, 48 and 72 hours of incubation at 37ºC at 5% CO_2 and were maintained in growth medium until reaching approximately 80% confluence. The cells were then treated with trypsin (Sigma Aldrich) and plated in T75 or T175 flasks (Becton Dickinson) at 5000 cells/cm^2. The cells were trypsinised repeatedly until the third passage and then were cryopreserved in FBS with 10% DMSO. Approximately 10^6 cells from passage three were thawed at 37 °C and plated in a T75 flask for three days to re-adjust prior to being used for the different analyses.

2.2. Differentiation assays

Osteogenic differentiation. Cells were plated at 20,000 cells/cm^2 in 12-well plates and cultured under osteogenic conditions for 9 days. Differentiation medium consisted of growth medium supplemented with 10 nM dexamethasone, 10 mM β-glycerophosphate and 100 µM ascorbate-2-phosphate. To assess their osteogenic differentiation, cells were fixed in 70% ethanol for 1 hour and stained with 2% alizarin red S (Sigma-Aldrich) for 10 minutes.

Adipogenic differentiation. Cells were plated at 2,500 cells/cm^2 in 12-well plates and cultured for 14 days with adipogenic medium, consisting of growth medium supplemented with 1 µM dexamethasone, 500 µM IBMX, 200 µM indomethacin and 15% rabbit serum. To examine their adipogenic differentiation, the cells were fixed with 10% formalin (Sigma) for 15 minutes and stained with 0.3% oil red O for 30 minutes at 37 °C.

Chondrogenic differentiation. Approximately 500,000 cells were pelleted and placed into 15 mL conical polypropylene tubes with chondrogenic medium, consisting of high glucose DMEM supplemented with 10% FBS, 10 ng/mL TGFβ-3 (R&D Systems), ITS+ premix (BD), 40 µg/mL proline (Sigma), 50 µg/mL ascorbate-2-phosphate and 0.1 µM dexamethasone. The culture was maintained for 21 days. To assess the chondrogenic differentiation of these cells, pellets were fixed with 10% formalin, embedded in paraffin and cut into 5 µm sections. Finally, sections were stained with haematoxylin and alcian blue dyes.

153 **2.3 Immunophenotyping**

154 A total of 7 surface markers were analysed by flow cytometry, the mesenchymal cell
155 markers CD29 (Integrin β-1), CD44 (H-CAM), CD73 (ecto-5'-nucleotidase), CD90 (Thy-1)
156 and CD105 (Endoglin), and the haematopoietic markers CD34 and CD45 (LCA).

157 Cells were suspended in PBS/ 2 mM EDTA at 10^6 cells/mL. Fifty microliter aliquots of
158 cells were transferred to flow cytometry tubes and incubated for 15 minutes at 4 °C
159 with mouse anti-human CD29-FITC (Caltag Laboratories), CD34-PE (Becton Dickinson),
160 CD44-FITC (Immunostep Research), CD45-APC (Becton Dickinson), CD73-PE (BD
161 Pharmingen), CD90-PE (BD Pharmingen) or CD105-FITC (R&D Systems) monoclonal
162 antibodies. Negative control staining was performed using a FITC-conjugated mouse
163 IgG1 κ isotype, a PE-conjugated mouse IgG1 κ isotype, a PERCP-Cy 5.5-conjugated
164 mouse IgG1 κ isotype and an APC-conjugated mouse IgG1 isotype antibody (all from
165 BD Biosciences). Subsequently, cells were washed with PBS and diluted in 500 μL of
166 PBS/ 2 mM EDTA.

167 Before the analysis with the fluorescence–activated cell sorter (FACSARIA, BD
168 Biosciences), 0.5 μL of SYTOX® Blue dead cell stain (Molecular Probes™) was added to
169 the cell dilution to get a 1 μM final concentration of dye. Samples were analysed after
170 5 minutes of incubation at room temperature. This staining discriminates between
171 viable and non-viable cells. Living cells were gated in a dot-plot of side scatter signals
172 versus SYTOX staining. At least, 3000 gated events were acquired on a biexponential
173 fluorescence scale. Positive staining for the CD markers was defined as the emission of
174 a fluorescence signal that exceeded levels obtained by >95% of cells from the control

population stained with matched isotype antibodies. Dot-plots were generated using the software FACSDIVA 5.0.1 (BD Biosciences).

2.4 Gene expression analysis of cell surface markers

The expression of 13 genes coding for cell surface antigens was analysed by RT-qPCR on both types of equine MSCs. These markers included the 7 molecules analysed by flow cytometry (see above) as well as *CD13* (aminopeptidase), *CD31* (PECAM), *CD49d* (α-4 integrin), *CD106* (VCAM-1), *CD146* (MCAM) and *CD166* (ALCAM).

RNA Extraction and Reverse Transcription

Cells were seeded on 24-well plates at 76,000 cells/well for 24 hours in triplicate. Afterwards, the cells were washed with PBS and stored at -80 °C. Cell to cDNA II kit (Ambion) was used for total RNA isolation according to the manufacturer's instructions. Briefly, the cells were thawed at 0 °C, rinsed with ice-cold PBS, and 100 µL of ice-cold Cell Lysis II Buffer was added to each well. Samples were transferred to a 96-well plate and incubated at 75ºC for 15 minutes. Then 2 µL of DNAse I was added, and the reaction was incubated at 37ºC for 15 minutes and 75ºC for 5 minutes. Afterwards, the reverse transcriptase master mix reaction, consisting of buffer x10, dNTPs, random decamers, RNAse inhibitor and M-MLV retrotranscriptase, was added to 10 µL of the samples. Finally, the samples were maintained for 60 minutes at 42 °C for cDNA synthesis and heated for 10 minutes at 95ºC.

Real-Time Quantitative PCR Analysis

The cDNA generated was analysed by real-time PCR. The primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Primer information, accession numbers for equine mRNA sequences and amplicon sizes are

198 shown in Table 1. The amplification reaction was performed in triplicate using the Fast
199 SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and the StepOne™
200 Real Time PCR System device (Applied Biosystems, Foster City, CA, USA). All of the
201 reactions were performed in a total volume of 10 µL with 2 µL of cDNA as template
202 and 300 nM of forward and reverse primers. Amplification of the cDNA was achieved
203 following the manufacturer's conditions: an initial activation and denaturation step of
204 20 s at 95 °C followed by 45 cycles consisting of 3 s at 95 °C and 30 s at 60 °C. A
205 dissociation curve protocol was run after every reaction to identify the presence of
206 spurious PCR bands or high levels of primer dimers. The levels of gene expression were
207 determined by the comparative Ct method. A normalisation factor (NF) calculated as
208 the geometric mean of the quantity of two housekeeping genes (*GAPDH* and *B2M*) was
209 used to normalise the expression of each gene. The primers, probes and PCR
210 conditions for the amplification of housekeeping genes was described previously (Kolm
211 et al., 2006) .

212 **Statistic analysis**

213 The software SPSS 15.0 was used for the statistical analysis. Data obtained from flow
214 cytometry and RT-qPCR were analysed for normality with the Shapiro-Wilk test
215 Differences in gene expression and reactivity levels between BM- and AT-MSCs were
216 determined using the unpaired non-parametric Mann–Whitney test. For both test,
217 $p<0.05$ was considered statistically significant.

218 ## 3. Results and Discussion

219 ### 3.1. MSC isolation and differentiation

The minimal criteria established to define the human MSCs (Dominici et al., 2006) are: the capacity for attachment to plastic, the expression of certain markers in their cell surface and the ability of differentiation into osteoblast, adipocyte and chondrocyte.

In this study, **c**olonies of fibroblast-like cells were observed in all of the cultures the day following isolation and plating (Figure 1a). These cells were expanded until reaching passage three, and then they were frozen. The tri-lineage differentiation ability was confirmed in equine MSCs (Figure 1). Specific Haematoxylin and alcian blue staining of sections obtained from pellets of cells undergoing chondrogenic differentiation showed lacunae formation, which is a typical characteristic of the chondrogenic phenotype; moreover the proteoglycans produced in the extracellular matrix during cartilage differentiation were stained in blue. Calcium deposits formed during osteogenic differentiation were stained in red by alizarin red, whereas control cells did not display any deposit. Finally, oil red O-stained lipid droplets appeared inside of the cells under adipogenic induction while the control cultures did not show any change. Therefore, the equine cells used for further analysis met the minimal criteria concerning the plastic attachment and pluripotency.

3.2. Immunophenotype

Most of the cell surface markers used to sort subpopulations of human mesenchymal stem cells using flow cytometry have not been validated in horses, and there is evidence that some of these markers do not cross-react with horse antigens. In this work, we have analysed 7 surface markers by flow cytometry.

Equine MSCs displayed large size and complexity (Figure 2a) and a lack of immunoreactivity was observed for the isotype controls for each mouse monoclonal

243 antibody (Figures 2b-e). Cells negative for SYTOX® staining were then included in the
244 cytometry analysis as viable cells (Figure 2f). In accordance with the
245 immunophenotype described for human MSCs (De Ugarte et al., 2003; Kern et al.,
246 2006; Liu et al., 2008), our flow cytometry results revealed that horse MSCs derived
247 from the two sources were robustly positive for the typical MSC markers CD29 and
248 CD90 (Figure 2g and 2h), with more than 90 percent of positive cells and no statistically
249 significant differences between cell sources (Figure 2). Despite using a different
250 antibody, our results confirmed the expression of CD90 by equine AT- and BM-MSCs as
251 reported recently (de Mattos Carvalho et al., 2009; Radcliffe et al., 2010). There are
252 contradictory findings with respect to the CD29 immunophenotype of equine MSCs,
253 the immunoreactivity observed in our work is in agreement with the results obtained
254 for equine BM-MSCs (Radcliffe et al., 2010), but not with those reported previously for
255 equine AT-MSCs (Mambelli et al., 2009). Although we must bear in mind that the
256 antibodies used in this and other studies are not specific to the equine species,
257 confirming the immunoreactivity for CD29 and CD90 in different works using different
258 antibodies gives more reliability to these results.
259 Unfortunately, we could not expand the putative markers set with other antibodies as
260 both BM- and AT-MSCs were negative for CD44, CD73 and CD105 (data not shown).
261 Negative results for CD73 have also been reported in rabbit and sheep, although these
262 species displayed cross-reactivity with anti-human CD44 and CD105 antibodies
263 (Martinez-Lorenzo et al., 2009).
264 Reactivity against the haematopoietic antigens CD45 and CD34 (Figure 2h and 2i),
265 considered negative markers for human MSC (De Ugarte et al., 2003), was not

detected for either of the two cell types, which is consistent with the results obtained in other species such as sheep or rabbit (Martinez-Lorenzo et al., 2009). Nevertheless, this result should be interpreted with caution, as the negative reactions for these markers are not truly negative because cross-reactivity between human and horse marker antibodies has not been confirmed. The expression of these antigens could be different between species and between cells with different origins. For example, haematopoietic stem cells (HSCs) from murine bone marrow and from human liver express CD45 but differ in the expression of CD34 and CD133 (Tarnok et al., 2010).

In order to clarify if the absence of immunostaining for the markers analysed was due to dissimilarities in the epitope sites between horse and human, we compared the public protein sequences of these two species using the BLAST tool. Percentages of identities ranged between 69% with a 100% query coverage (QC) for CD34 and 95% with a 97% QC for CD29. The remaining markers displayed the following identity percentages: 72% with 100% QC for CD105, 84% with 77% QC for CD90, 85% with 55% QC for CD44, and 89% with 90% QC for CD73. It was not possible to complete this analysis for CD45 as only a small sequence of the equine marker was found. These BLAST comparisons did not revealed a clear relationship between identity and immunoreactivy, although we might expect that if homology between human and horse molecules is 100% it is very likely that the anti-human antibody detects the corresponding horse protein, and that the staining pattern of the other antibodies remain questionable as long as no proof of specificity for the horse molecules exists.

289 **3.3. Gene expression of cell surface markers**

290 Because there are few specific markers for equine MSCs and many of the positive stem
291 cell markers described for other species show little or no cross-reactivity with horses
292 (Ibrahim and Steinbach, 2007; Smith, 2008), gene expression-based technologies can
293 help in the identification of other possible molecules described as MSC markers
294 (Rallapalli et al., 2009; Radcliffe et al., 2010). In our study, reverse transcriptase RT-
295 qPCR was performed to quantify the mRNA expression of the cell surface antigen
296 genes analysed by flow cytometry and other 6 molecules considered as positive or
297 negative MSC markers in human.

298 These markers analysed by flow cytometry in our study are commonly used as markers
299 to define human MSCs (Dominici et al., 2006; Mitchell et al., 2006). In accordance with
300 our cytometry results, equine cells expressed *CD29* and *CD90* (Figure 3). Although both
301 AT and BM-MSCs expressed these markers, their mRNA expression was significantly
302 higher in AT-MSCs than in BM-MSCs ($p < 0.05$ and $p<0.01$, respectively). However, in
303 contrast to the negative results obtained by flow cytometry, the real time PCR study
304 revealed the amplification of *CD73*, *CD105* and *CD44* transcripts in both types of cells.
305 Whereas AT and BM-MSCs displayed similar expression levels for *CD73* and *CD105*,
306 *CD44* expression was significantly higher in AT-MSC than in BM-MSCs ($p<0.001$).
307 Therefore, the negative immunophenotyping observed in this work might be due to a
308 lack of cross-reactivity of the antibodies used to the equine antigens rather than to the
309 absence of these antigens in the cell surface. As in human MSCs, CD73, CD105 and
310 CD44 molecules might also be considered as markers to identify equine MSCs.

311 The expression of the haematopoietic *CD34* marker was only observed in AT-MSCs, in
312 contrast with the lack of immunoreactivity detected by flow cytometry but in
313 agreement with human AT-MSC phenotyping (Noel et al., 2008). As the expression of
314 this marker tends to decrease with the number of passages (Mitchell et al., 2006), our
315 expression results indicate that equine AT-MSCs at passage three still express *CD34*.
316 Both cell types were negative for the haematopoietic marker *CD45*, which confirmed
317 the negative result observed in flow cytometry. Therefore, as in humans (De Ugarte et
318 al., 2003; Kern et al., 2006), equine MSCs do not expresses CD45 in established MSC
319 cultures. Our results confirmed those previously reported (Radcliffe et al., 2010).
320 Similarly, the endothelial marker *CD31* was not expressed by equine MSCs in
321 agreement with the findings reported for human MSCs (Noel et al., 2008).
322 The antigens *CD166, CD13,* and *CD146* are considered positive markers for human
323 MSCs from both origins (De Ugarte et al., 2003), in agreement with this both equine
324 MSC types expressed these genes without significant differences between both cell
325 sources.
326 *CD49d* and *CD106* have been reported as differential markers for human BM- and AT-
327 MSCs (De Ugarte et al., 2003; Kern et al., 2006; Noel et al., 2008). In our real time PCR
328 analysis both cell sources displayed similar transcript levels for these markers.
329 Consequently, neither *CD49d* nor *CD106* would be considered key markers to
330 differentiate MSCs from different sources in the horse, suggesting that the pattern of
331 marker expression established for human MSC may not always be followed by MSCs of
332 other species.

In summary, despite the lack of antibodies that cross-react with horse epitopes to identify markers that allow one to define MSCs by flow cytometry, it is possible to establish a profile of markers through gene expression analysis using RT-qPCR. This technique detects the expression of markers in the cells without the problem of specificity. And it also makes possible to quantify the expression levels between the different types of cells, which cannot be assessed by flow cytometry because this methodology only reflects the percentage of cells expressing antigens without quantifying the intensity of signal. The panel of surface antigens tested revealed a similar phenotypic profile between horse and human MSCs, although specific differences in some surface antigens were noticed. A similar cell surface profile was also observed between BM-MSCs and AT-MSCs, with CD34 emerging as a key molecule to differentiate cells derived from bone marrow and adipose tissue. The present study could help researchers identify these cells more quickly before using them for cellular-based therapies in equine medicine. However, many questions still remain, and further investigation will be necessary to clarify the mechanisms and functions of stem cell epitopes, such as the effect of marker expression variation on the pluripotency of MSCs or the study of their expression by cells from different passages.

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FIGURES

Figure 1. Isolation and differentiation of equine MSCs. a) A colony of MSCs after eight days of culture post-isolation (x4 magnification). b) Chondrogenic differentiation. A pellet section stained with haematoxylin and alcian blue (x20 magnification). c) Osteogenic differentiation. Lack of alizarin red staining in control cultures (x4 magnification) and d) calcium deposits stained in red in differentiated cells (x4 magnification). e) Adipogenic differentiation. Control cultures showing the absence of staining (x20 magnification) and f) oil red O dye-stained lipid droplets produced inside differentiated cells (x20 magnification).

Figure 2. Flow cytometry representative plots *n=1*. a) Plot showing equine MSCs morphology. b-e) Isotype controls of mouse monoclonal antibodies for FITC, PE, PE-Cy7 and APC, respectively. f) Plot showing SYTOX® Blue staining for dead cells. g- j) Plots showing flow cytometry results for the markers CD29, CD90, CD34 and CD45, respectively. Below the plots are shown the means ± s.e. of percentage of positive cells for these markers in BM-MSC (*n=9)* and AT-MSC *(n=8)*.

Figure 3. mRNA expression of the *CD13, CD29, CD34, CD44, CD49d, CD73, CD90, CD105, CD106, CD146, CD166, CD45* and *CD31* cell surface markers. Relative mRNA expression levels are expressed as mean ± s.e. White bars correspond with BM-MSCs (*n*=9) and grey bars with AT-MSC (*n*=8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Genes analysed, GenBank accession numbers, primer sequences for reverse transcriptase RT-PCR (forward and reverse) and amplicon sizes in base pairs (bp)

Gene	Accession number	Primer sequence (5'-3')	Amplicon size
CD13	XM_001487923	F: TGACCCTGACCCCAACGT R: CGCCCTGCAGCCAGTATT	110
CD29	XM_001492665	F: GTAAAAAGTCTTGGAACCGATCTGAT R: CACAAATGAGCCAAACCCAATT	81
CD31	NM_001101655	F: TCTAGAACGGAAGGCTCCCT R: TGGGAGCAGGGCAGGTTCA	145
CD34	XM_001491596	F: CACTAAACCCTCTACATCATTTTCTCCTA R: GGCAGATACCTTGAGTCAATTTCA	150
CD44	NM_001085435	F: CCCACGGATCTGAAACAAGTG R: TTCTGGAATTTGAGGTCTCCGTAT	95
CD45	AY114350	F: TGATTCCCAGAAATGACCATGTA R: ACATTTTGGGCTTGTCCTGTAAC	100
CD49d	XM_001917601	F: CATCGGCCTTCTCACAGAGAA R: GCCATTATTGTCTGCATCAATTTG	101
CD73	XM_001500115	F: GGGATTGTTGGATACACTTCAAAAG R: GCTGCAACGCAGTGATTTCA	90
CD90	EU881920	F: TGCGAACTCCGCCTCTCT R: GCTTATGCCCTCGCACTTG	93
CD105	XM_001500078	F: GACGGAAAATGTGGTCAGTAATGA R: GCGAGAGGCTCTCCGTGTT	100
CD106	NM_001101650	F: CATCGTGACCTGTGGGCATA R: TGGGTTTCCCTCCACTAGCA	111
CD146	XM_001917594	F: CTGGACTTGGAAACCACAACATC	85

		R: CAGGTCTCACTCGGACATCAGA	
CD166	XM_001503380	F: GTCTGGTCTTCTGCCTCTTGATC R: TCGGCAAGGCATGATAATAGTG	103

Figure 1

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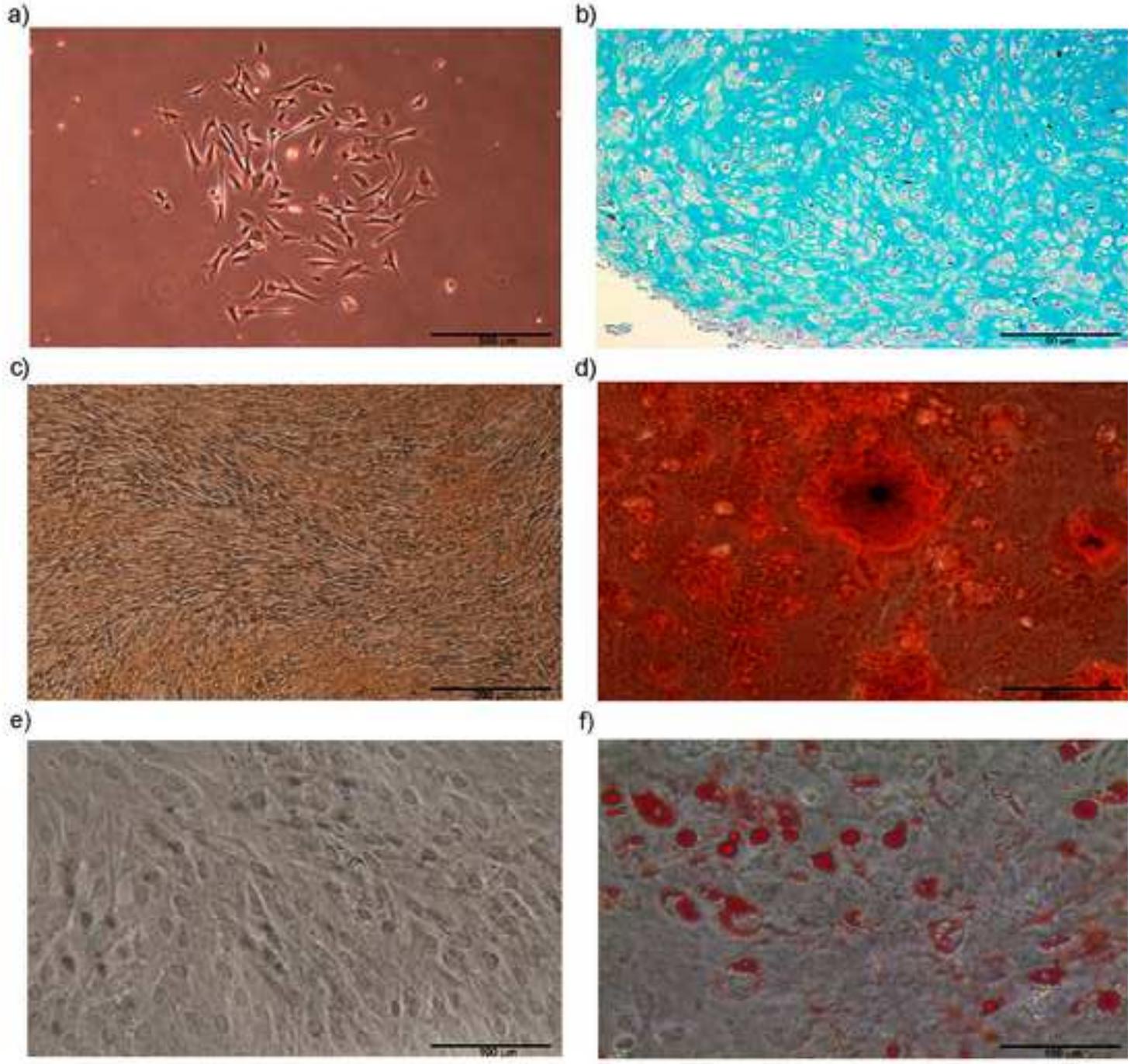

Figure 2

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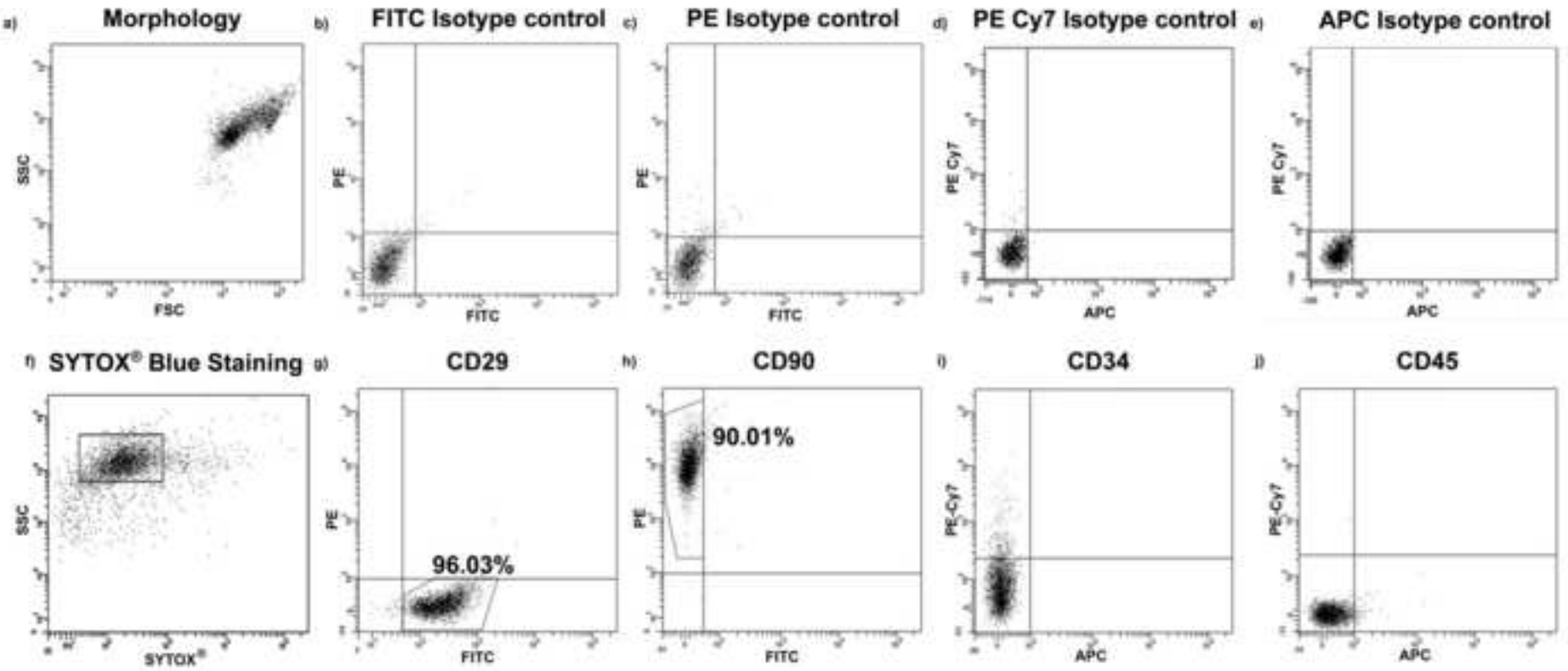

BM-MSC (*n=9*)	96.03 ± 0.40	90.01 ± 3.65	0.30 ± 0.02	1.50 ± 0.09
AT-MSC (*n=8*)	97.22 ± 1.09	95.12 ± 1.13	3.07 ± 0.12	0.07 ± 0.03

Figure 3

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