Temporal Analysis of Equine Bone Marrow Aspirate During Establishment of Putative Mesenchymal Progenitor Cell Populations

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Mesenchymal progenitor cells (MPCs) are often characterized using surface markers after expansion and treatment in culture. There are no studies directly comparing gene and protein markers in undifferentiated samples during the very early phases of culture. The goal of this study was to evaluate temporal gene and protein expression changes during establishment of equine MPC cultures. Bone marrow aspirate was obtained from 35 horses and processed by density gradient centrifugation. In freshly isolated bone marrow, mononuclear cells had variable expression of CD44, CD11a/CD18, CD90, and CD45RB cell surface molecules. After 2 h of culture, bone marrow mononuclear cells had a phenotype of CD44^{hi}, CD29^{hi}, CD90^{lo}, CD11a/CD18^{hi}, and CD45RB^{lo}. Isolated mononuclear cells were analyzed by flow cytometry and RT-qPCR at 2, 7, 14, 21, and 30 days of culture. At all culture time points, gene expression was in agreement with cell surface protein expression. In established cultures of MPCs, cells remained robustly positive for CD44 and CD29. The proportion of positive cells and the mean fluorescence intensity of positive cells increased in CD90 expression as MPC cultures became more homogeneous. Inversely, the population of cells in culture decreased expression of CD11a/CD18 and CD45RB molecules over time. The decreased expression of the latter molecules makes these useful negative markers of established MPC cultures under normal expansion conditions. The results of this study demonstrate numerous dynamic changes in cell surface molecule expression during early establishment of MPC populations, which may aid to improve MPC isolation methods for research or therapeutic applications.

Introduction

MESENCHYMAL PROGENITOR CELLS (MPC) have been studied extensively in many species since the first report by Friedenstein over 30 years ago [1]. Characterization studies of established human MPC cultures using differentiation assays, gene expression analysis, and cell surface protein markers have been performed for nearly a decade [2]. Most studies evaluate MPC cell surface markers and gene expression after expansion in culture in order to obtain sufficient cell numbers for analysis [3–6]. However, there are reports of conflicting results in MPC marker protein expression patterns when comparing phenotypes of freshly sorted MPCs to expanded MPCs [7,8]. These studies suggest that the phenotype of MPCs is dynamic during isolation and culture processes.

Temporal changes in cell surface protein expression during expansion in culture have been reported in only a few studies. In the original MPC description by Pittenger et al.

[2], population enrichment from Day 2 through 14 was described based on flow cytometric measurement of SH2 and SH3 expression, but full cell surface protein characterization was not reported until passage one or two using expanded cells. Another study reported no temporal changes in cell surface phenotype for bone marrow cells after they had reached confluence in culture compared to their next five passages [9]. Although these studies have added important information concerning cell expansion, early immunophenotype changes remain incompletely understood.

The use of gene expression data in most MPC studies has focused primarily on assessment of MPC differentiation capacity into terminally differentiated tissues (ie, collagen type II for cartilage; osteonectin for bone) [2,10]. When monoclonal antibodies are used to immunophenotype cells in a previously uncharacterized tissue type, gene expression data provides supporting evidence for protein expression in the tissue and helps to validate the reactivity of the antibody.

The advantage of dual protein/gene analysis is to confirm negative protein results and account for kinetic changes of transcription and translation.

Early bone marrow cultures contain a heterogeneous mixture of cell types, which become more homogeneous over the first 3 weeks of culture. There is no uniformly accepted definitive phenotype or surface markers for isolation of MPCs from uncultured samples [11]. In fresh bone marrow aspirate, cells of varying maturity in both hematopoietic and non-hematopoietic lineages are present, with varying levels of surface protein expression within each population, making separation of cells from distinct lineages difficult. During early culture, the proportion of hematopoietic cells committed to terminal differentiation is reduced via spontaneous apoptosis and removal due to nonadherence, leading to a more uniform population of mesenchymal cells. In the present study, our hypothesis was that the immunophenotypes of bone marrow cells were changed during the very early phases of MPC culture establishment as the cell population became more homogeneous. The goal of this study was to evaluate both gene and protein expression of cell surface markers to characterize MPCs using flow cytometry and RT quantitative PCR (RT-qPCR) throughout culture duration. The results of this study may aid to improve MPC selection and isolation methods for research or therapeutic uses.

Materials and Methods

Study design

Candidate antibodies were tested for reactivity and specificity with equine cell surface antigens. Subsequently, cell surface molecules of uncultured bone marrow cells were analyzed using flow cytometry. Bone marrow cells were cultured and harvested on 2, 7, 14, 21, and 30 days for analysis of cell surface proteins and gene expression. All procedures were performed in compliance with institutional guidelines for research on animals.

Antibody validation

To validate reactivity of antibodies with equine cells, peripheral blood cells were used as positive and negative controls. Whole blood (30 mL) was collected from five horses for antibody validation. Blood samples were drawn into preservative free heparin to a final concentration of 33 units/mL. Candidate equine and human monoclonal antibodies tested are listed in Table 1. Whole venous blood was processed prior to flow cytometry analysis using density gradient centrifugation to remove the majority of red blood cells as previously described [12].

Validation of antibody specificity

The CD44 and CD11a/CD18 antibodies have been previously validated as specific for their respective molecules in the horse [13,14]. The CD11a/CD18 antibody (clone CZ3.2) identifies a noncovalently linked heterodimer consisting of a 180 kDa α -chain (CD11a) and a 95 kDa β -chain (CD18) using immunoprecipitation under reducing conditions [13,14]. The CD44 antibody (clone CVS 18) identifies a heavily glycosylated molecule of 65–100 kDa [13,14]. On a 12% SDS-PAGE

analysis, a “smear” was produced approximately in the 100 kDa position, indicating that the precipitated molecule was heavily glycosylated. The analysis was repeated after endoglycosidase F treatment of the precipitate, and a single 76 kDa band was produced in both reducing and nonreducing conditions [13,14]. The CD44 antibody has also been shown to react with protein produced by a cDNA-encoding equine CD44 molecule in a COS cell expression system [15].

For CD90, CD29, and CD45RB antibody validation analyses, whole cell lysates were prepared from fresh peripheral blood leukocytes and from red blood cells with platelets. Western blot analyses were performed to determine if the reactive candidate antibodies bound proteins of the expected size based on previous literature, protein size similarity to other species, or predicted equine sequences. The CD90 antibody was expected to detect a ~17 kDa protein, similar in size to the equivalent human protein. Similarly, the CD29 antibody was expected to detect an ~130 kDa protein based on the size of the human protein. The CD45RB antibody was expected to have one or more bands <150 kDa based on the multiple isotypes of the human protein. To test the CD45RB and CD29 antibodies, proteins from cell lysates were resolved on 7.5% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels, which were subsequently transferred to polyvinylidene fluoride (PVDF) membranes and probed with the relevant antibody. A 15% SDS-PAGE gel was used to resolve cell lysates for subsequent analysis of the CD90 antibody following protein transfer to a PVDF membrane.

An immunoprecipitation was performed in addition to western blot analysis for the CD29 antibody, using an unconjugated version of the antihuman CD29 (Beckman Coulter, clone 4B4LDC9LDH8) used in this study. A 7.5% SDS-PAGE gel was used to resolve the immunoprecipitated products. Following protein transfer, the PVDF membrane was probed with antibody known to recognize human β 1-integrin (Calbiochem, clone 4B7-CP26).

Bone marrow aspirate collection and cell isolation

Bone marrow aspirate was used to assess changes in cell surface markers over time and for tri-lineage (cartilage, bone, and adipose) differentiation. Bone marrow aspirate was collected from the sternabrae of 35 horses (11 males and 24 females, age range 6 months–20 years) under standing sedation with xylazine hydrochloride (0.55 mg/kg IV) and local anesthesia using 2% lidocaine hydrochloride (10 mL/site). Samples were collected in preservative-free heparin (American Pharmaceutical Partners Inc, Schaumburg, IL) to a final concentration of 33 units/mL.

Aspirate (60 mL) from each horse was diluted to 180 mL total volume using phosphate-buffered saline + 0.5% bovine serum albumin. The white blood cell fraction of the sample was enriched and the majority of red blood cells were removed by layering each 30 mL aliquot of dilute sample on Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) for density gradient centrifugation, as described for antibody validation. Samples were resuspended in 50 mL MPC culture media (see below) prior to cell counting using a hemocytometer. Approximately $2\text{--}9 \times 10^8$ bone marrow mononuclear cells (BMMNC) were obtained per sample using this method. A portion ($\sim 10 \times 10^6$ cells) of the uncultured bone marrow aspirate samples from all 35 horses were analyzed using flow cytometry. In a subpopulation of horses

TABLE 1. CANDIDATE ANTIBODIES TESTED TO DETERMINE THE CHANGES IN EQUINE MESENCHYMAL PROGENITOR CELLS SURFACE ANTIGENS IN UNCULTURED SAMPLES AND SUBSEQUENT PROPAGATION OF CELLS IN CULTURE

<i>Antibody tested</i>	<i>Species which antibody is produced against (company; catalog #)</i>	<i>Positive human cell type</i>	<i>Positive equine controls tested</i>	<i>Negative equine controls tested</i>
CD29 (Integrin β 1)	Human (BD Biosciences, San Jose, CA; cat # 6603177)	Lymphocytes, monocytes, granulocytes-low, platelets, fibroblasts, endothelial, NK cells, thymic/lymph node cells	Lymphocytes, monocytes, granulocytes, platelets	Red blood cells
CD44 (H-CAM)	Equine (Serotec, Raleigh, NC; cat # MCA 1082)	Hematopoietic and non-hematopoietic except platelets	Leukocytes	Red blood cells platelets
CD90 (Thy-1)	Canine (VMRD Inc, Pullman, WA; cat # DH24A)	Hematopoietic stem cell subset, neurons, fibroblasts, stromal cells	Granulocytes	Lymphocytes
CD45RB (LCA)	Bovine (VMRD Inc, Pullman, WA; cat # DH16A)	Granulocyte/lymphocyte subpopulations	Granulocytes, lymphocytes	Red blood cells, platelets
CD11a/CD18 (Integrin α L, LFA-1)	Equine (CZ 3.2 courtesy of Dr. D. Antczak, Cornell University; clone 116.2D11B10)	All leukocytes	Leukocytes	Red blood cells, platelets
Stro-1	Human (R&D Systems, Minneapolis MN; cat # MAB1038)	Human stromal cell precursors	None available	Leukocytes
CD13 (APN)	Equine (Serotec; cat # MCA 1084)	Granulocytes, monocytes and their precursors, endothelium, epithelium	Granulocytes, monocytes	Lymphocytes
SSEA 1	Mouse (DHSB University of Iowa; cat # MC-480)	Human ES Cells	None available	Leukocytes
SSEA 3	Rat (DHSB University of Iowa; cat # MC-631)	Human ES Cells	None available	Leukocytes
SSEA 4	Human (DHSB University of Iowa; cat # MC-813-70)	Human ES Cells	None available	Leukocytes
CD34 (Mucosalin)	Human (BD Biosciences; cat # 555824 & cat # 340666)	Human hematopoietic stem and endothelial cells	None available	Mature leukocytes
CD3 (T3)	Equine (courtesy of Myra Blanchard, UC Davis; clone UC F6G-3.3)	Mature T lymphocytes	T lymphocytes	Granulocytes, monocytes
MHC class I	Equine (CZ3 courtesy of Dr. D. Antczak; clone 117.1B12C11)	Leukocytes and almost all nucleated cells in the body	Leukocytes	Early allantochorion cells
MHC class II	Equine (CZ11 courtesy of Dr. D. Antczak; clone 130.8E8D9)	Mature lymphocytes	Lymphocytes	Granulocytes
Mouse α Parvo Virus	Canine (courtesy of Dr. D. Antczak; tissue culture supernatant)	Parvo virus	None available	All equine cells should label negatively

Alternate antigen names are listed in parentheses.

Abbreviations: CD, cluster of differentiation; H-CAM, homing-associated cell adhesion molecule; Thy-1, thymocyte-differentiation antigen-1; LCA, leukocyte common antigen; LFA-1, lymphocyte function-associated antigen-1; APN, aminopeptidase N; SSEA, stage-specific embryonic antigen; T3, T cell antigen receptor complex.

($n = 8$), samples of bone marrow aspirate before and following density gradient centrifugation were submitted for cytological analysis.

Samples from some horses ($n = 14$) were used only for antibody validation and were not cultured. The remaining samples ($n = 21$) were subsequently cultured as described below. Bone marrow samples from a subpopulation ($n = 6$) of horses were cultured for 14 days and then utilized for analysis of DNA content to determine the cell cycle state. Some samples ($n = 6$) did not have sufficient cell numbers to complete analysis at all time points; yet they were used for flow cytometry at one or more culture time points to check for repeatability or alterations in cell surface protein expression. Samples from three horses were cultured for 21 days and then subjected to tri-lineage differentiation assays. Sufficient cell numbers for protein and gene expression analysis at all time points were available from six horses.

MPC expansion in culture

BMMNCs were plated onto 10 cm diameter tissue culture plates at a density of $\sim 300,000$ cells/cm² (20×10^6 cells/plate). Cells were cultured at 37°C in a 5% CO₂, 95% air atmosphere at 5% humidity. Cells were cultured in media containing Dulbecco's modified Eagle's medium (DMEM, glucose at 1,000 mg/L), 2 mM L-glutamine, penicillin (100 units/mL), streptomycin (100 units/mL), basic fibroblastic growth factor (bFGF, 1 ng/mL), and 10% fetal bovine serum. One-half of the media (5 mL) was removed at 24 h of culture and replaced with fresh media. Subsequently, media were exchanged every 72–96 h. At subconfluence of 70%–90%, cells were passaged 1:3 using Accumax® cell dissociation solution (Innovative Cell Technologies Inc, San Diego, CA) and plated at a density of about 10,000 cells/cm². Approximately 10×10^6 cells from each sample were analyzed by flow cytometry for cell surface protein expression at 2 h and on days 2, 5, 7, 14, 21, and 30 of culture. Cells were analyzed at these time points to evaluate the changes in cell surface proteins over time, and to characterize the cells prior to performing differentiation assays.

Flow cytometry analysis

Cell surface markers of putative stemness were assessed using flow cytometry. Cells were pelleted in aliquots containing 1×10^6 cells and labeled for cell surface molecules selected from a panel of monoclonal antibodies known to define human MPCs (Table 1). Cells were treated with a 20-min blocking step using 10% normal goat serum in FACS-Buffer (phosphate-buffered saline containing 2.5% fetal bovine serum). The cells were pelleted, washed with FACS-Buffer, and pelleted again. Cell pellets were resuspended in fluorescent-conjugated or -unconjugated primary monoclonal antibody and incubated for 45 min at 4°C. Cells were then washed, a second fluorescent-conjugated goat anti-mouse IgG or IgM antibody (fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) or IgM μ -Chain-Specific, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied to the unconjugated antibodies, and the samples were incubated for an additional 45 min at 4°C. The CD29 antibody was directly conjugated with PE (read at FL2); all others were labeled with FITC-conjugated secondary

antibody (read at FL1). Cells were resuspended in FACS-Buffer and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with a 488 μ m argon laser and BD Cell Quest™ analysis software (BD Biosciences, San Jose, CA). Cells not treated with antibody, and cells exposed to mouse anti-parvovirus antibody and FITC or PE-conjugated secondary antibodies were used as negative controls. The settings for the flow cytometric analyses determined $<2\%$ positive cells for the control antibodies. Data were collected on 1×10^5 cells for each sample regardless of size and granularity to prevent bias in gating.

For culture-expanded cells, flow cytometry analysis was performed on days 2, 7, 14, 21, and 30 following isolation. Supernatant was removed and adherent cells were lifted from the plate using Accumax® solution (1 mL/15 cm²) to prevent damage to cell surface proteins and avoid cellular clumping. Cells were processed and analyzed by flow cytometry as described earlier, except dot plot settings were adjusted to a logarithmic scale in the cultured cells to include large, granular cells. Flow cytometric analysis of cell surface molecule expression was performed in the gate determined to contain dividing cells based on the results from the propidium iodide DNA staining assay described later.

Propidium iodide DNA staining assay for cell cycle analysis

Propidium iodide can be used to determine DNA content in cells and identify populations of cells undergoing division. A reported feature of MPCs is their ability to proliferate [2]. To determine the region of cell division, samples (0.5×10^6 cells) from six cultures were collected on Day 14 and resuspended in 500 μ L hypotonic propidium iodide solution containing 0.05 mg/mL propidium iodide, 1 mg/L sodium citrate, and 0.1% Triton X-100 [16]. Samples were protected from light and incubated at 4°C until analysis. Samples were analyzed by flow cytometry on FL2. Histograms were plotted for each cell population on a linear scale. The DNA content is proportional to the mean fluorescence intensity, and consequently indicates the stage of cell division (G0/G1, S, G2, M; cells in subG0 are dead) [17].

RNA extraction and one-step reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Gene expression analysis was included to confirm negative protein results and account for kinetic changes in transcription and translation. At the same time points when cells were analyzed by flow cytometry, RNA was extracted from $\sim 1\text{--}3 \times 10^6$ cells of the corresponding samples using Trizol® (Life Technologies, Invitrogen, Carlsbad, CA) according to the manufacture's directions. RT-qPCR was performed to provide supporting evidence that gene expression levels were consistent with cell surface protein expression levels. RNA quantity and quality were determined using a Nanodrop® spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE), and visualization of 18 and 28S bands on 0.8% agarose gels. Gene segments were cloned and novel sequence data files were submitted to Genbank (accession numbers, EF442070 for CD13; EF442071 for CD29; EF576851

for *CD45*; EU881920 for *CD90*; and EU881921 for *CD11a*). A portion of the *CD44* gene was also cloned and agreed with previously reported data (X66862).

Total RNA was reverse-transcribed and amplified using the one-step RT-PCR technique and the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and dual-labeled fluorescent probe [6-FAM as the 5' label (reporter dye) and TAMRA as the 3' label (quenching dye)] were designed using Primer Express Software Version 2.0b8a (Applied Biosystems, Foster City, CA). All probes and primers were designed using equine-specific sequences published in Genbank, or sequenced in our laboratory (Table 2). Since several isoforms (five in humans) of *CD45* exist, primers and probes were designed to detect as many equine isoforms as possible (equivalent to four of the five human isotypes). Two genes (*CD45* and *CD11a*) did not reach a C_T value in later time point samples. Therefore, normalized copy numbers per nanogram of RNA values for each gene were calculated. A quantity value of 1 was assigned to samples that did not reach a C_T .

MPC differentiation assays

To verify that cultured bone marrow cells were capable of tri-lineage differentiation, 10×10^6 culture-expanded cells from three horses were used for adipogenic, osteogenic, and chondrogenic induction assays.

Adipogenic induction. Aliquots of MPCs (0.2×10^4 cells/well) were treated with 5% rabbit serum (lot 24129, Innovative Research, Novi, MI) in culture media to induce adipogenesis [18]. Media was changed at Day 4 following induction. Samples were collected on days 1, 3, and 7 post-induction. To assess adipogenic differentiation, cells were fixed in 4% paraformaldehyde, incubated in a solution containing Oil-Red-O for 10 min to stain for lipid inclusions, and counterstained with hematoxylin. Stained samples were imaged using standard microscopy and graded positive or negative for Oil-Red-O staining.

Osteogenic induction. Aliquots of MPC (0.2×10^4 cells/well) were treated with 100 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ M ascorbic acid (all Sigma-Aldrich, Inc., St. Louis, MO) in low glucose DMEM/10% fetal bovine serum media. Media was changed at Day 4 for the 7-day culture samples. Samples were collected on days 1, 3, and 7 following induction to assess early osteogenic differentiation. To assess calcium accumulation, cells were fixed in 4% paraformaldehyde and incubated in 2% aqueous Alizarin Red S (Sigma) for 3 min, followed by counterstaining with hematoxylin. Stained samples were imaged using standard microscopy and assessed for Alizarin Red staining. Intracellular calcium concentration was measured using a commercially available kit (QuantiChrom™ Calcium

Assay Kit, BioAssay Systems, Hayward, CA) in cell extracts collected on days 2, 3, 4, and 7 following induction. Protein content in the same cell extracts was determined using the Bradford protein assay kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. Calcium concentration was expressed as μ g Ca^{2+}/μ g of total protein.

Chondrogenic induction. Pellet cultures were generated using 5×10^5 cells/pellet with processing as previously described [19]. Pellet cultures were maintained in medium consisting of high glucose DMEM containing 100 μ g/mL sodium pyruvate, 10 ng/mL TGF- β 3, 100 nM dexamethasone, $1 \times$ insulin/transferrin/selenium (ITS+1) premix, 40 μ g/mL proline, and 25 μ g/mL ascorbate-2-phosphate (all Sigma-Aldrich, Inc., St. Louis, MO). Medium was replaced twice weekly. Samples were collected on days 1, 3, 7, 14, 21, and 28 of culture. Pellets were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 4 mm sections. Matrix metachromasia was assessed with Safranin-O/fast green staining.

Statistical analysis

Gene expression data were categorized into four groups by culture duration: 1 = <1 week; 2 = 1 week; 3 = 2 weeks; 4 = 3 weeks or more. Calcium/protein ratio data were categorized into one of four groups by induction duration: 1 = control (no osteogenic induction); 2 = 48 h of induction; 3 = 72 h of induction; 4 = 1 week of induction. Groups were compared using a one-way ANOVA with a Tukey all-pairwise comparisons post hoc test. A *P* value of <0.05 was considered significant.

Results

Cytology of bone marrow aspirate

Cellular counts and distribution were compared between whole bone marrow aspirate and following gradient density centrifugation. Density gradient cell isolation was highly effective in removing RBC from the bone marrow aspirate with only 0.07% of original RBC remaining after isolation (Table 3). Approximately 70% of nucleated cells were also lost during processing. The monocytic lineage had the highest postprocessing recovery at 47%, while the eosinophil lineage had the lowest recovery with only 5% of preprocessing numbers.

Validation of antibodies against equine peripheral blood and bone marrow cells

Equine CD44, human CD29, canine CD90, and equine CD11a/CD18 antibodies were confirmed reactive to equine

TABLE 2. PRIMERS AND PROBES UTILIZED IN RT-qPCR OF MESENCHYMAL PROGENITOR CELL MARKER GENES

Gene	Forward Primer	Reverse Primer	Probe
18S	5'-CGGCTTTGGTGACTCTAGATAACC-3'	5'-CATGGTAGGCACAGCGACTA-3'	5'-TCGAACGTCGTGCCCTATCAACTTTCGAT-3'
CD44	5'-TCCACCCCACTCCATCTGT-3'	5'-TGAAGCAATAGGTGTCGTACTGAGA-3'	5'-CCGCCAACAACACGGGGCG-3'
CD29	5'-GACAAGGTGAGCAATAGAAGGATAATC-3'	5'-TTGGTGCCATTGTTTTACAAA-3'	5'-CACATCATTTTCCAAGTGACACTGTCCATCA-3'
CD90	5'-CCGTGAGACAAAGAAGCATGTG-3'	5'-CCTGATGTTGACTGTGCTGGTGAAG-3'	5'-TGAGCACACATACCGTCCCGGAC-3'
CD11a	5'-GCCGAGATCCAGTACATGAA-3'	5'-ACTGTGATGTTGACTCCTTCCTTCT-3'	5'-AGTGCTCCCTTCAGCCAGCAACAA-3'
CD45	5'-TGATGATTCTGGAGGATGATCTG-3'	5'-CACTGTTCCTATTTCCTTCTTACA-3'	5'-AAGCCACAGTCATTGTCATGTCACCTCG-3'

The 18S ribosomal subunit was used to normalize gene expression.

TABLE 3. BONE MARROW ASPIRATE CELL COUNT AND DIFFERENTIAL BEFORE AND AFTER DENSITY CENTRIFUGATION CELL ISOLATION

Cell type	Pre-isolation ($\times 10^6$) cells	Post-isolation ($\times 10^6$) cells	Percentage of original post-isolation
Red blood cells	375,000 (299,000/427,000)	264 (45/565)	0.07
Red cell precursors	754 (77/1,660)	181 (24/438)	24.00
White cells and their precursors	847 (463/1,340)	310 (106/747)	36.60
Neutrophils	590 (211/989)	216 (44/558)	36.61
Monocytes	42 (19/74)	20 (6/41)	47.28
Mitotic myeloid cells (lineage was not classified neutrophil/monocyte)	23 (2/63)	7 (2/15)	28.95
Lymphocytes (and precursors)	191 (106/266)	61 (27/112)	31.99
Eosinophils	15 (6/33)	1 (0/2)	5.16

Cell count is calculated to reflect a 60 mL BMA sample. Mean (Min/Max) $n = 8$.

molecules using flow cytometry analysis (Fig. 1 and Supplementary Fig. 1; Supplementary materials are available online at <http://www.liebertpub.com/>). The bovine CD45RB antibody had questionable reactivity with equine molecules. CD45RB data is included to demonstrate the importance of antibody validation, and the value of gene expression data in providing supporting evidence when protein expression detection is questionable.

To confirm reactivity of the antibodies to equine molecules, isolated peripheral blood neutrophils (Supplementary Fig. 1B), lymphocytes (Supplementary Fig. 1C), and monocytes (Supplementary Fig. 1D) were tested. Results could then be used for comparison with fresh bone marrow cells of the same size and granularity (Fig. 1). In addition, for cells of the monocytic lineage, bone marrow was cultured for 2 h and the adherent cells were collected and compared to uncultured bone marrow cells of the same size and granularity (see Fig. 2, 2-h sample). There was increased mean fluorescence intensity for the CD44 and CD11a/CD18 molecules in both lymphocyte and monocyte lineage cells compared to uncultured bone marrow cells of the same size and granularity, confirming these antibodies were reactive to mononuclear cells. The monoclonal antibodies against equine MHC class I, equine MHC class II, and equine CD3 labeled the expected cell populations, and were used as internal controls for subsequent flow cytometry assays.

By analogy to peripheral blood cells, Region 1 included cells of the neutrophil lineage, Region 2 cells of the lymphocyte lineage, and Region 3 cells of the monocytic lineage. Interestingly, CD44, and CD11a/CD18 antibodies were variably immunoreactive in freshly isolated bone marrow cells. Cells in Region 1 were CD44^{hi}, CD29^{hi}, CD90^{hi}, CD11a/CD18^{hi}, and CD45RB^{hi} (see Fig. 1B). Regions 2 and 3 contained bone marrow cells that were CD44^{lo}, CD29^{hi}, CD90^{lo}, CD11a/CD18^{lo}, and CD45RB^{lo} (Fig. 1C and 1D), suggesting these regions contained cell types other than mature mononuclear cells.

Other antibodies tested were found to be nonreactive to equine molecules or differed in expression from MPCs of other species. The specific lot of monoclonal antibody against equine CD13 and several human antibodies tested, including Stro-1, SSEA 1, SSEA 3, SSEA 4, and CD34, did not label equine cells using flow cytometry. No equine-specific positive controls, such as embryonic stem cells, were available to confirm the negative findings.

Validation of protein specificity

Western blot analysis alone did not clearly indicate specificity of the CD29 antibody with equine blood cells. No distinct band was detected on multiple attempts to analyze CD29 antibody binding. It was proposed that this antibody did not recognize the denatured protein. The Beckman Coulter CD29 antibody, clone 4B4LDC9LDH8, has been extensively used for immunoprecipitation in human cells [20]. Immunoprecipitation using clone 4B4LDC9LDH8 antibody followed by western blot analysis with the Calbiochem clone 4B7-CP26 antibody confirmed the CD29 antibody (4B4LDC9LDH8) reacted with a single protein of ~130 kDa (Supplementary Fig. 2A1). Western blot analysis was successful in demonstrating that the CD90 antibody reacted with a protein of appropriate size of 17 kDa (Supplementary Fig. 2B1) in equine peripheral blood leukocytes and not in red blood cells or platelets. The CD45RB antibody did not react with a protein of expected size of ~150 kDa on western blot analysis. Instead, multiple poorly defined protein bands were noted of varying sizes (data not shown).

DNA content analysis to determine cell cycle stage of cultured bone marrow cells

DNA content analysis was useful for identification of the region of interest (Gate 3) on the dot plot of cultured MPC, and subsequent flow cytometry analysis was restricted to this cell population (Fig. 3). Proliferating cultured bone marrow cells were identified in Gate 3 by the high number of cells in the S/G2/M phase based on their increased DNA content [17]. Cells in S/G2/M were consistently located only in this gate. In contrast, in Gate 1 (R1) and Gate 2 (R2), the majority of cells are presumed to be dying (subG0).

Cell surface marker expression in cultured bone marrow cells changes over time in culture

After 2 h of culture, adherent mononuclear cells displayed an antibody labeling pattern of CD44^{hi}, CD29^{hi}, CD90^{lo}, CD11a/CD18^{hi}, and CD45RB^{lo} (Fig. 2). By 2 days of culture, expression of CD11a/CD18 began to decrease, while CD90 expression increased. In Gate 3, there appeared to be a heterogeneous cell population as evidenced by multiple

FIG. 1. Flow cytometric analyses of surface molecule expression in uncultured bone marrow cells. **(A)** Dot plot distribution of uncultured bone marrow cells isolated using gradient density centrifugation. **(B–E)** Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated areas (Regions 1, 2, 3, and 4, respectively). The shaded curves represent negative isotype control staining; open lines represent the labeling for the cell surface markers indicated in the left-hand side. The expected labeling pattern for each marker is indicated in parenthesis. Note the variation in the CD44 and CD11a/CD18 expression in Region 2-gated cells between uncultured bone marrow **(C)** and peripheral blood isolated cells (see Supplementary Fig. 1C). Also, the expression of these molecules in Region 3 is different between uncultured **(D)** and isolated peripheral blood cells (see Supplementary Fig. 1D) or 2-h cultured bone marrow cells (Fig. 2). Abbreviations: WBC, white blood cell; RBC, red blood cell.

FIG. 2. Flow cytometric analyses of cell surface molecule expression in bone marrow cells over increasing culture duration. (A) Dot plot distribution of bone marrow cells isolated using gradient density centrifugation followed by culture of indicated duration. (B) Histogram analysis of mean fluorescence intensity of cell surface molecule expression in the gated area (Region 3). The shaded curves represent negative isotype control staining; open lines represent the labeling for the cell surface markers indicated in the left-hand side. At 2 h, adherent cells gated in Region 3 displayed a molecular profile consistent with monocyte lineage, while later time points revealed changes, particularly in CD90 and CD11a expression. At 48 h, the presence of multiple cell types was evidenced by several peaks in antibody labeling for CD44, CD29, and CD90. By 14 days, when cells display a fibroblastic morphology in culture, putative mesenchymal progenitor cells were positive for CD44, CD29, and CD90, while negative for CD45 and CD11a. This staining pattern is retained throughout long-term culture.

peaks in CD44, CD29, and CD90 mean fluorescence intensity. On Day 7, CD11a/CD18 cell surface expression further decreased, while CD90 expression began to increase. By 14 days, adherent cells in culture were CD44^{hi}, CD29^{hi}, and CD90^{hi}, CD11a/CD18^{neg}, and CD45RB^{neg}; these cells displayed a fibroblastic morphology characteristic of MPC (data not shown). This pattern of molecule expression was retained at 21 and 30 days for all MPC samples. At all time points (2 h, 2, 7, 14, 21, and 30 days), adherent bone marrow cells in culture showed a consistent labeling pattern with the results described earlier in all samples analyzed.

Gene expression in cultured bone marrow cells

Gene expression data was consistent with cell surface protein expression at all culture time points. *CD44* gene expression was present in all samples but changed over time (Fig. 4). Early samples (≤ 7 days) had significantly more *CD44* expression compared to samples cultured for 14 days or more ($P < 0.001$). *CD29* gene expression was also high and present in all samples, but no significant differences were detected based on culture duration ($P = 0.19$). Expression of *CD90* varied with culture duration and was significantly

FIG. 3. Cell cycle analysis of bone marrow cells in culture. (A) Flow cytometry dot plot (side scatter, SSC $\times$ forward scatter, FSC) demonstrates the distribution of three cell populations (R1, R2, R3) in bone marrow aspirate cultured for 14 days. (B) Flow cytometry histogram analyses of each gated area in Figure 3A reveals the cell cycle status according to their DNA content (stained with propidium iodide). In Gates 1 (R1) and 2 (R2), the majority of cells are within sub-G0 and G0/G1, consistent with cell death. In Gate 3 (R3), cells are undergoing division, and one-third of cells are in S/G2/M phase. Therefore, the flow cytometric cell surface molecular analysis reported in Figure 2 was performed with cells gated in Region 3 (R3).

greater in cells cultured for 21 days or more compared to all earlier samples ($P < 0.001$). Gene expression of *CD11a* and *CD45* was present in all samples cultured 7 days or less. In

contrast, samples cultured 14 days or longer failed to reach a C_T value with a threshold of 40 cycles in 60% of samples analyzed for *CD11a*, and 84% for *CD45*. For *CD11a*, early duration culture samples had significantly more expression than samples cultured 14 days or more ($P < 0.001$). *CD45* gene expression levels also significantly decreased with increasing culture duration ($P < 0.001$).

Differentiation assays

Tri-lineage differentiation capacity of cultured bone marrow cells was confirmed through in vitro adipogenic, osteogenic, and chondrogenic assays. Following induction, histochemical stainings for adipogenesis and osteogenesis in short-term (≤ 7 days) assays and longer duration chondrogenic induction (up to 4 weeks) were consistent with previous reports of MPC differentiation potential [2]. In the description by Pittenger et al., lipid vacuoles were detected within 48 h of induction under adipogenic conditions, calcium accumulation continued for at least 3 weeks, and Safranin-O staining increased over the 4-week time period under chondrogenic conditions.

Under adipogenic culture conditions, MPCs accumulated a large amount of lipid vacuoles in their cytoplasm, while control samples had no appreciable staining for lipid (Fig. 5). Osteogenic culture conditions induced a change in cell morphology from fibroblastic to a stellate or cuboidal shape within 7 days (Fig. 5). Early calcium accumulation was evidenced by Alizarin staining in the first 7 days of culture. Calcium accumulation in MPCs following osteogenic induction was significantly increased by Day 7 compared to earlier time points and control samples as assessed using calcium/protein ratios of the samples ($P = 0.003$). Control cells had $0.347 \pm 0.038 \mu\text{g Ca}^{2+}/\mu\text{g total protein}$, which increased to $0.490 \pm 0.120 \mu\text{g Ca}^{2+}/\mu\text{g total protein}$ within 24 h of osteogenic induction. By 72 h, content was $0.756 \pm 0.226 \mu\text{g Ca}^{2+}/\mu\text{g total protein}$ and by 7 days the ratio was $1.502 \pm 0.467 \mu\text{g Ca}^{2+}/\mu\text{g total protein}$, a 3- to 6-fold increase in relative calcium compared to control conditions. During chondrogenic induction, an increase in pellet size was noted with increasing culture duration over the 28-day culture period. The pellets also had progressively more matrix metachromasia as evidenced by enhanced staining with Safranin-O stain with increased culture duration (Fig. 5).

Discussion

In this study, temporal changes in cell surface protein and gene expression in MPC in culture were demonstrated. Based on the literature, it was anticipated that established equine MPC cultures would be negative for *CD45RB* and *CD11a/CD18*, and positive for *CD44*, *CD29*, and *CD90* (Thy-1) [2,21]. Protein expression data in early cultured (2 h) bone marrow mononuclear cells comprised *CD44^{hi}*, *CD29^{hi}*, and *CD11a/CD18^{hi}*-positive cells, with a smaller population of *CD90^{lo}* and *CD45RB^{lo}*-positive cells. Established cultures of MPC were robustly positive for *CD44*, *CD29*, and *CD90*, becoming negative for *CD11a/CD18* and *CD45RB*. Gene expression data followed the same pattern, in which established cultures retained expression of *CD44*, *CD29*, and *CD90*, whereas levels of *CD11a* and *CD45* dropped below the level of detection by 14 days of culture. The molecules detected by these antibodies were found to have protein and

FIG. 4. Gene expression kinetics in bone marrow-cultured cells. The gene expression of selected markers over increasing culture durations was tested by one-way ANOVA and Tukey's all-pairwise comparisons ($n = 6 \pm \text{SE}$). There were significant ($P \leq 0.05$) differences between gene expression (copy number) over time for most genes [CD44 (A), CD90 (C), CD11a (D), and CD45 (E)], but no significant difference for CD29 (B) gene. For CD11a and CD45, the majority of samples cultured 14 days or longer did not reach a C_T value. Samples with gene expression below the detectable limit (*) were assigned a quantity of one to permit statistical analysis. Schematic relative gene expression changes during the establishment of mesenchymal progenitor cells cultures are shown in F.

gene expression patterns consistent with results of protein expression in cultured MPCs of other species [2,4,21,22]. Equine MPCs had positive expression of CD44, CD29, and CD90 and negative expression of CD11a and CD45RB similar to flow cytometry analysis of expanded human bone marrow cells by Pittenger et al. Sung et al. noted CD44 and CD29 were present in expanded mouse MPCs and CD45 was

absent. Although other studies have found similar antibody labeling in established cultures of 14 days or more [2], none has specifically documented changes in gene and protein expression over time from isolation through 2 weeks and beyond.

In bone marrow samples cultured for 48 h, a mixed population of cells was still present despite selecting for strongly

FIG. 5. Differentiation assays using cultured equine mesenchymal progenitor cells. (A–D) lipid induction with Oil-Lipid-O staining, (E–H) osteogenic induction with Alizarin staining, (I–L) chondrogenic induction with Safranin-O/fast green staining. (A, E) control MPC; (B, F) 24-h induction; (C, G) 72-h induction; (D, H) 7-day induction. (I) 3-day induction; (J) 1-week induction; (K) 2-week induction; (L) 4-week induction.

adherent cells by vigorously washing the plates prior to sample collection. This was evidenced by multiple peaks in CD44, CD29, and CD90 mean fluorescence intensity and side scatter distribution (Fig. 2; 48 h analysis) of cells in flow cytometry. At least two populations of cells expressing CD90 in different mean fluorescence intensities were detected at the protein level. This molecule is highly expressed in equine neutrophils. Neutrophils are known to enter apoptosis spontaneously within 24 to 36 h of culture [23], so they would be progressively removed during subsequent media changes, and the relative levels of *CD90* gene and protein expression of remaining cells should decrease proportionally during early culture. Over time, the cells expressing CD44, CD29, and CD90 became more homogeneous (see Fig. 2, 48 h versus 14 days) based on the flow cytometric histogram analyses. Expression of CD44 or CD29 molecule alone is not useful to determine putative MPCs because they

are not unique to MPCs. Although these molecules are not included in the minimal criteria for defining human multipotent mesenchymal stromal cells, they may play a role in equine characterization studies as part of a combination of positive and negative markers, since expression of the molecules are sustained in long-term culture [21].

Gene expression data was especially useful to confirm negative results when analyzing CD45RB cell surface protein expression, as CD45RB expression was weakly/inconsistently detected in isolated peripheral blood leukocytes and bone marrow cells when tested by flow cytometry. The bovine CD45RB antibody used in this study had previously been validated for the horse [24,25]. Nevertheless, protein detection using flow cytometry can be affected by variability in the reagents (monoclonal antibody product lot or secondary conjugated antibodies) or instrument settings used. Therefore, validation of reagents and standardized data collection and analysis are important for consistency in results. We have included the CD45RB data specifically to demonstrate the importance of reagent validation. Gene expression data can be a useful tool for cellular immunophenotyping when protein expression data is inconsistent, or reagents are not readily available, as it was for CD45RB. In the future, with adequate reagents to the equine species, protein expression for CD45 can be confirmed.

An interesting observation of this study was the difference in cell surface molecule expression between fresh bone marrow mononuclear cells and peripheral blood leukocytes, or short-term (2 h) cultured bone marrow mononuclear cells for CD44, and CD11a/CD18. To our knowledge, other studies that have analyzed and/or antibody-sorted fresh marrow did not account for this difference in protein expression [26]. Mitchell et al. [27] reported initially low levels of CD44 protein expression in uncultured cells, which increased during successive passages. The lack of antigen identification on freshly isolated cells from the bone marrow can be misleading since cells may not be sufficiently mature to express proteins characteristic of their lineage especially when trying to classify hematopoietic versus non-hematopoietic cells. Therefore, we suggest a short duration culture (ie, 2 h) to better classify adherent bone marrow cells based on their molecular expression of hematopoietic markers (ie, CD11a/CD18).

Lack of expression of Stro-1, SSEA1, SSEA3, SSEA4, and CD34 in isolated bone marrow and cultured cells could not be verified due to unavailability of equine-specific reagents, and the lack of reactivity of human reagents to horse molecules. Lack of positive controls prevents the validation of these reagents for equine molecules. The lack of cross-reactivity of several human antibodies to equine molecules is not surprising given the recent work by Ibrahim et al. [28] who reported only 14 out of 379 monoclonal human antibodies tested cross-reacted in a cell-type-specific manner with equine leukocytes. The findings of these studies emphasize the importance of rigorous testing with controls when using xenogenic antibodies.

Density gradient centrifugation of equine bone marrow aspirate was successful in removing nearly all of the red blood cells and allowed for analysis of the mixed mononuclear cell fraction in fresh samples using flow cytometry. Approximately 30% of nucleated cells were retained for analysis, which is identical to that of human bone marrow aspirate using the same technique [2]. The number of mononuclear cells in equine aspirates following isolation

was also proportionate to human bone marrow mononuclear cell counts on a per ml of aspirate basis, and 3–5 times more total mononuclear cells were harvested/isolated from equine since 3–5 times the volume of aspirates were collected. Equine bone marrow samples provided sufficient cell numbers to allow analysis at multiple early time points.

An accepted characteristic of MPC is the highly proliferative nature of this cell type [2]. Previous studies have not attempted to target the dividing cell population when analyzing MPC markers. Cell cycle analysis of cultured bone marrow cells was a simple way to identify the dividing population. Characterization of surface protein expression for this specific population may be more accurate than ungated analysis, as cells from the nondividing populations are removed from analysis. In actively dividing cultures, we have noted ~40%–65% of the total cells are located in the region of interest (Region 3). As cultures senesce, we have noted a drop in the percentage of cells located in Region 3 to 10%–30% (data not shown) with a shift in cellular distribution toward Regions 1 and 2 of the dot plot, and decreases in mean fluorescence intensity of CD90 and CD44 expression in Region 3. Not only does analysis of cellular markers in Region 3 focus on the dividing cell population, relative cell distribution between the three regions also reflects the overall proliferative activity of the culture.

Much work remains to be done in the full characterization of the equine MPC. This study presents a preliminary molecular profile using both gene and protein expression levels of bone marrow-nucleated cells from isolation to 1 month in culture. Understanding the early changes in cultured bone marrow cells may promote identification of cellular markers unique to early MPCs and help distinguish them from hematopoietic and other cell types. Our results suggest that freshly isolated cells from bone marrow aspirate do not express surface proteins uniformly due to varying stages of cellular maturity. This may lead to less accurate cell sorting when using freshly isolated bone marrow cells. For example, use of the cell surface markers CD44 or CD11a/CD18 to sort freshly isolated bone marrow cells may incorrectly select immature hematopoietic cells expressing low levels of these proteins. Short-term culture allows selection of adherent, more mature cells, leading to more accurate classification of cell lineage and the potential for mesenchymal differentiation. Taking advantage of changes in marker expression during culture establishment may be beneficial for enhanced isolation of MPC from bone marrow aspirate or other tissue sources.

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Author Disclosure Statement

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References

1. Friedenstein AJ, JF Gorskaja and NN Kulagina. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 4:267–274.
2. Pittenger MF, AM Mackay, SC Beck, RK Jaiswal, R Douglas, JD Mosca, MA Moorman, DW Simonetti, S Craig and DR Marshak. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147.
3. Ringe J, I Leinhase, S Stich, A Loch, K Neumann, A Haisch, T Haup, R Manz, C Kaps and M Sitter. (2008). Human mastoid periosteum-derived stem cells: promising candidates for skeletal tissue engineering. *J Tissue Eng Regen Med* 2:136–146.
4. Sung JH, HM Yang, JB Park, GS Choi, JW Joh, CH Kwon, JM Chun, SK Lee and SJ Kim. (2008). Isolation and characterization of mouse mesenchymal stem cells. *Transplant Proc* 40:2649–2654.
5. Han K, JE Lee, SJ Kwon, SY Park, SH Shim, H Kim, JH Moon, CS Suh and HJ Lim. (2008). Human amnion-derived mesenchymal stem cells are a potential source for uterine stem cell therapy. *Cell Prolif* 41:709–725.
6. You Q, L Cai, J Zheng, X Tong, D Zhang and Y Zhang. (2008). Isolation of human mesenchymal stem cells from third-trimester amniotic fluid. *Int J Gynaecol Obstet* 103:149–152.
7. Boiret N, C Rapatel, R Veyrat-Masson, L Guillaud, JJ Guerin, P Pigeon, S Descamps, S Boisgard and MG Berger. (2005). Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow. *Exp Hematol* 33:219–225.
8. Jo CH, HJ Ahn, HJ Kim, SC Seong and MC Lee. (2007). Surface characterization and chondrogenic differentiation of mesenchymal stromal cells derived from synovium. *Cytotherapy* 9:316–327.
9. Cournil-Henrionnet C, C Huselstein, Y Wang, L Galois, D Mainard, V Decot, P Netter, JF Stoltz, S Muller, P Gillet and A Watrin-Pinzano. (2008). Phenotypic analysis of cell surface markers and gene expression of human mesenchymal stem cells and chondrocytes during monolayer expansion. *Biorheology* 45:513–526.
10. Liu F, Y Akiyama, S Tai, K Maruyama, Y Kawaguchi, K Muramatsu and K Yamaguchi. (2008). Changes in the expression of CD106, osteogenic genes, and transcription factors involved in the osteogenic differentiation of human bone marrow mesenchymal stem cells. *J Bone Miner Metab* 26:312–320.
11. Liu ZJ, Y Zhuge and OC Velazquez. (2009). Trafficking and differentiation of mesenchymal stem cells. *J Cell Biochem* 106:984–991.
12. Flaminio MJ, BR Rush and W Shuman. (1999). Peripheral blood lymphocyte subpopulations and immunoglobulin concentrations in healthy foals and foals with rhodococcus equi pneumonia. *J Vet Intern Med* 13:206–212.
13. Kydd J, DF Antczak, WR Allen, D Barbis, G Butcher, W Davis, WPH Duffus, N Edington, G Grünig, MA Holmes, DP Lunn, J McCulloch, A O'Brien, LE Perryman, A Tavernor, S Williamson and C Zhang. (1994). Report of the first international workshop on equine leucocyte antigens, Cambridge, UK, July 1991. *Vet Immunol Immunopathol* 42:3–60.
14. Lunn DP, MA Holmes, DF Antczak, N Agerwal, J Baker, S Bendali-Ahcene, M Blanchard-Channell, KM Byrne, K Cannizzo, W Davis, MJ Hamilton, D Hannant, T Kondo, JH Kydd, MC Monier, PF Moore, T O'Neil, BR Schram, A Sheoran, JL Stott, T Sugiura and KE Vagnoni. (1998). Report of the second equine leucocyte antigen workshop, Squaw Valley, California, July 1995. *Vet Immunol Immunopathol* 62:101–143.
15. Tavernor AS, EV Deverson, WJ Coadwell, DP Lunn, C Zhang, W Davis and GW Butcher. (1993). Molecular cloning of equine CD44 cDNA by a COS cell expression system. *Immunogenetics* 37:474–477.

16. Flaminio MJ, A Yen and DF Antczak. (2004). The proliferation inhibitory proteins p27(Kip1) and retinoblastoma are involved in the control of equine lymphocyte proliferation. *Vet Immunol Immunopathol* 102:363–377.
17. Darzynkiewicz Z and E Bedner. (2000). Analysis of apoptotic cells by flow and laser scanning cytometry. *Methods Enzymol* 322:18–39.
18. Giovannini S, W Brehm, P Mainil-Varlet and D Nesic. (2008). Multilineage differentiation potential of equine blood-derived fibroblast-like cells. *Differentiation* 76:118–129.
19. Mackay AM, SC Beck, JM Murphy, FP Barry, CO Chichester and MF Pittenger. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* 4:415–428.
20. McMichael AJ, ed. (1987). *Leucocyte Typing III: White Cell Differentiation Antigens*. Oxford University Press, Oxford, England.
21. Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, D Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 8:315–317.
22. Harting M, F Jimenez, S Pati, J Baumgartner and C Cox Jr. (2008). Immunophenotype characterization of rat mesenchymal stromal cells. *Cytotherapy* 10:243–253.
23. Scheel-Toellner D, K Wang, R Craddock, PR Webb, HM McGettrick, LK Assi, N Parkes, LE Clough, E Gulbins, M Salmon and JM Lord. (2004). Reactive oxygen species limit neutrophil life span by activating death receptor signaling. *Blood* 104:2557–2564.
24. Ibrahim S and F Steinbach. (2007). Non-HLDA8 animal homologue section anti-leukocyte mAbs tested for reactivity with equine leukocytes. *Vet Immunol Immunopathol* 119:81–91.
25. Flaminio MJ, S Ibrahim, DP Lunn, R Stark and F Steinbach. (2007). Further analysis of anti-human leukocyte mAbs with reactivity to equine leukocytes by two-colour flow cytometry and immunohistochemistry. *Vet Immunol Immunopathol* 119:92–99.
26. Veyrat-Masson R, N Boiret-Dupre, C Rapatel, S Descamps, L Guillaud, JJ Guerin, P Pigeon, S Boisgard, J Chassagne and MG Berger. (2007). Mesenchymal content of fresh bone marrow: a proposed quality control method for cell therapy. *Br J Haematol* 139:312–320.
27. Mitchell JB, K McIntosh, S Zvonic, S Garrett, ZE Floyd, A Kloster, Y Di Halvorsen, RW Storms, B Goh, G Kilroy, X Wu and JM Gimble. (2006). Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 24:376–385.
28. Ibrahim S, K Saunders, JH Kydd, DP Lunn and F Steinbach. (2007). Screening of anti-human leukocyte monoclonal antibodies for reactivity with equine leukocytes. *Vet Immunol Immunopathol* 119:63–80.

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