Characterization and osteogenic potential of equine muscle tissue- and periosteal tissue-derived mesenchymal stem cells in comparison with bone marrow- and adipose tissue-derived mesenchymal stem cells

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Objective—To characterize equine muscle tissue- and periosteal tissue-derived cells as mesenchymal stem cells (MSCs) and assess their proliferation capacity and osteogenic potential in comparison with bone marrow- and adipose tissue-derived MSCs.

Sample—Tissues from 10 equine cadavers.

Procedures—Cells were isolated from left semitendinosus muscle tissue, periosteal tissue from the distomedial aspect of the right tibia, bone marrow aspirates from the fourth and fifth sternebrae, and adipose tissue from the left subcutaneous region. Mesenchymal stem cells were characterized on the basis of morphology, adherence to plastic, trilineage differentiation, and detection of stem cell surface markers via immunofluorescence and flow cytometry. Mesenchymal stem cells were tested for osteogenic potential with osteocalcin gene expression via real-time PCR assay. Mesenchymal stem cell cultures were counted at 24, 48, 72, and 96 hours to determine tissue-specific MSC proliferative capacity.

Results—Equine muscle tissue- and periosteal tissue-derived cells were characterized as MSCs on the basis of spindle-shaped morphology, adherence to plastic, trilineage differentiation, presence of CD44 and CD90 cell surface markers, and nearly complete absence of CD45 and CD34 cell surface markers. Muscle tissue-, periosteal tissue-, and adipose tissue-derived MSCs proliferated significantly faster than did bone marrow-derived MSCs at 72 and 96 hours.

Conclusions and Clinical Relevance—Equine muscle and periosteum are sources of MSCs. Equine muscle- and periosteal-derived MSCs have osteogenic potential comparable to that of equine adipose- and bone marrow-derived MSCs, which could make them useful for tissue engineering applications in equine medicine. (Am J Vet Res 2013;74:790–800)

Catastrophic breakdown is a major source of animal wastage in racehorses, with as many as 83% of deaths attributed to exercise-related injury. Fractures account for 71% of fatal musculoskeletal injuries in racing Thoroughbreds.1,2 Because of their size and temperament, horses must bear weight on all 4 limbs during the healing process. This leaves a surgical re-

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ments. Mesenchymal stem cells are an option for clinical application because they can be effectively isolated and expanded with high efficiency. Mesenchymal stem cells can be cryopreserved, and they will maintain their viability and later can be induced to differentiate along multiple lineages. Although MSCs are being used clinically for certain musculoskeletal injuries, there are many unknown factors associated with their use, such as the ideal number of cells for transplantation, cell yield per gram of donor tissue, and ideal tissue source. Despite encouraging results for studies of MSC treatments for horses with soft tissue and joint injuries, little is known about the efficacy of equine MSCs for treatments for horses with soft tissue and joint injuries, and the hemodermal aspect of the right tibia were aseptically prepared.

Identification of the optimal source of MSCs with the best osteogenic potential may prove critical for moving basic science research toward clinical cell-based treatments to promote bone healing. Ideal tissue sources may be identified for human and equine MSCs on the basis of their intended use in vitro and in vivo. There is evidence that equine MSCs from bone marrow and fat have a high osteogenic potential, compared with that for MSCs from umbilical cord tissue and umbilical cord blood.

Our laboratory group has identified periosteum and muscle tissues as sources of spindle-shaped, plastic-adherent cells able to undergo osteogenic differentiation. We hypothesized that equine periosteum and skeletal muscle are equivalent, if not superior, multipotent sources of MSCs with osteogenic potential, compared with results for the conventionally chosen donor tissues of fat and bone marrow.

The purpose of the study reported here was to characterize equine muscle tissue- and periosteal tissue-derived MSCs, assess proliferative capacity of equine muscle- and periosteum-derived MSCs, and determine osteogenic potential of equine muscle- and periosteum-derived MSCs in comparison with that of bone marrow- and adipose tissue-derived MSCs. We hypothesized that muscle- and periosteum-derived MSCs would have proliferative capacity and osteogenic potential equal to or greater than that of bone marrow- and adipose-derived MSCs.

Materials and Methods

Samples—Cadavers of 10 young adult (2 to 5 years old) horses were used for postmortem collection of samples of bone marrow, periosteum, skeletal muscle, and adipose tissue. The horses were donated to the Atlantic Veterinary College for reasons other than this study and were euthanized in accordance with protocols approved by the University of Prince Edward Island Animal Care Committee. All horses were sedated with xylazine hydrochloride (1.1 mg/kg, IV) and then euthanized via pentobarbital sodium injection (108 mg/kg, IV). All samples were collected immediately after horses were euthanized.

Bone marrow collection—A 10-cm-wide band overlying the sternum was clipped of hair. Palpation was performed to identify the fourth and fifth sternebrae. The skin over the sternum was aseptically prepared, and a bone marrow biopsy needle was used to obtain a bone marrow aspirate from the fourth sternebra. The aspirate (9.5 mL) was collected into a 12-mL syringe that contained 2.5 mL of heparin (1,000 U/mL). Another aspirate was immediately obtained from the fifth sternebra in the same manner.

Fat, muscle, and periosteum collection—The area left lateral to the base of the tail, the area superficial to the left semitendinosus and semimembranosus muscles, and the distomedial aspect of the right tibia were aseptically prepared. Skin incisions were made, and underlying tissues were harvested. A 24-cm² (3-g) section of adipose tissue was harvested from the subcutaneous tissues over the gluteal muscles in the region of the base of the tail. A 9-cm² (6-g) section of muscle was dissected and harvested from the left semitendinosus muscle. A 4-cm² (0.5-g) section of periosteum from the medial surface of the proximal portion of the right tibia was elevated and harvested. The amount of sample collected was considered to represent a clinically feasible biopsy specimen that would not result in adverse effects for a donor. The tissues collected were placed in α-MEM and transported to our laboratory.

Cell isolation from bone marrow—Cells were isolated from bone marrow via a centrifugation gradient technique. The samples were centrifuged in 50-mL tubes at 1,500 × g for 10 minutes. The buffy coat then was collected and placed into standard medium, which was composed of α-MEM supplemented with 10% fetal bovine serum, l-glutamine (2mM), 10,000 U of penicillin, and 10 mg of streptomycin/mL, and amphotericin B (250 µg/mL). This standard medium was maintained the same for the bone marrow and the other 3 tissues.

Fat, muscle, and periosteum cryopreservation—Fat, muscle, and periosteum were collected from each equine cadaver and placed in separate vials of chilled α-MEM solution. Tissues were processed within 24 hours after collection. Tissues that were not processed immediately were kept on ice and refrigerated at 4°C for 12 hours and then processed. Cold, sterile PBS solution was placed in Petri dishes to provide a moist environment for tissues subsequently cut into 1-cm segments. Tissue segments were placed into 2-mL cryovials and submerged in freezing medium composed of 92.5% PBS solution and 7.5% dimethyl sulfoxide. The cryovials remained at room temperature (approx 20°C) for 30 minutes to allow the freezing medium to penetrate the tissue. The samples were then placed in closed-cell extruded polystyrene foam containers and stored in a –80°C freezer for a minimum of 24 hours. All samples were placed into a –196°C liquid nitrogen tank within 72 hours after processing.

Fat, muscle, and periosteum cell isolation—Cells were isolated from fat, muscle, and periosteum by means of an enzyme digestion technique. Cryopreserved adipose and muscle tissues were warmed in a water bath (37°C) for approximately 5 minutes until the liquid was thawed. Tissue handling was performed via sterile technique in a biosafety cabinet. Each tissue was removed from the cryovials and placed in a 50-mL centrifuge tube that contained 25 mL of sterile PBS solution. The tissue was rinsed with PBS solution, weighed, and
minced. Minc ed tissue was placed in centrifuge tubes that contained 10 mL of collagenase type I (2,000 U/mL); tubes then were mixed via a vortexer and placed in a 37°C incubator. The incubated tubes were mixed via a vortexer every 20 minutes for 60 minutes. Once the tissue was digested, 10 mL of standard medium was added to the mixture to inhibit further enzymatic digestion. The cell suspension was filtered through a 100-µm filter and then a 70-µm filter and then was centrifuged at 377 X g for 10 minutes. Supernatant was removed, and the cell pellet was resuspended in standard medium. Viable adipose tissue and muscle cells were plated in T-75 flasks at a cell density of 33 X 10³ cells/cm² in standard medium. Cell cultures were maintained in a humidified incubator with 5% CO₂ and 95% air at 37°C.

Isolation of cells from the periosteum was similar to that of adipose tissue and muscle, except that the minced tissue was pretreated by incubation with type I collagenase (2,000 U/mL) for 10 minutes. The partially digested tissue was rinsed and treated by incubation with type I collagenase (2,000 U/mL) for an additional 160 minutes. Viable periosteal cells were plated in T-75 flasks at a cell density of 33 X 10³ cells/cm² in standard medium.

**Cell culture**—Primary cultures from each tissue (bone marrow, fat, muscle, and periosteum) were maintained in a humidified incubator with 5% CO₂ and 95% air at 37°C. Cells adhered to the flasks by 24 to 48 hours (start of culture was designated as time 0); unattached cells were washed from the flasks with PBS solution. Medium was changed 3 times/wk. Cells were maintained in this environment until confluence, but culture did not exceed 9 days.

**Cell passage**—At each passage, cells were detached with 0.25% trypsin-EDTA. Cells were counted and viability determined via 0.4% trypan blue dye exclusion. First-passage cells from each tissue of each horse were used for a proliferation assay and to culture a second passage with higher cell counts. Second-passage cells from each tissue of each horse were used for a second proliferation assay and characterization assays at the various cell densities.

**Characterization of MSCs**—Cells from each tissue were maintained for 1 week in standard medium. After this period, adherence to the flask and spindle-shaped morphology were confirmed.

**Cell differentiation**—Cells from each tissue from each of 3 horses were induced to differentiate into adipocytes, chondrocytes, and osteoblasts. Each of the 3 lineages was cultured in parallel with the same cells in standard medium. Light microscopy digital images were obtained at various times representative of the different morphologies. Histochemical analysis and morphology were used to confirm differentiation into the 3 lineages.

**Adipogenic differentiation**—Cells were seeded at a density of 12,000 cells/cm² into chamber slides. Cells initially were cultured in standard medium for 3 days. Thereafter, cells were incubated with an adipogenic induction medium (Dulbecco modified Eagle medium and F12, 3% fetal bovine serum, 10,000 U of penicillin and 10 mg of streptomycin/mL, amphotericin B [250 µg/mL], biotin [33 µmol/L], pantothenate [17 µmol/L], insulin [1 µmol/L], dexamethasone [1 µmol/L], isobutylmethylxanthine [0.5 mmol/L], rosiglitazone [5 µmol/L], and 5% rabbit serum) for 2 days. The same medium without isobutylmethylxanthine and rosiglitazone then was used to maintain the adipocyte cell culture until day 7, when the cells were fixed for 20 minutes in 10% formalin at room temperature and stained for neutral lipid accumulation with oil red O to indicate adipogenic differentiation.

**Chondrogenic differentiation**—Cells were seeded at a density of 500,000 cells/15 mL in polypropylene conical tubes and were then centrifuged (500 X g for 5 minutes) into pellets, which were supplemented with a chondrogenic differentiation medium (Hams 12; dexamethasone [10⁻⁴ M]; culture supplement containing bovine insulin, transferrin, selenium, acid, linoleic acid, and BSA, 5% fetal calf serum, 10,000 U of penicillin and 10 mg of streptomycin/mL, amphotericin B [250 µg/mL], ascorbic acid [50 µg/mL], and recombinant human transforming growth factor-β 1 [11 ng/mL]). Pellet cultures were maintained for 21 days. Pellet cultures were performed in parallel with standard medium and chondrogenic medium, with no growth factor as a control culture. After culture for 21 days, differentiated pellets were fixed in 10% formalin for 24 hours, dehydrated in a graded series of ethanols, and embedded in paraffin. A microtome was used to make sections (thickness, 5 µm) that were then stained with Alcian blue (pH, 1.0) for the detection of cartilage-specific proteoglycans to confirm chondrogenic differentiation.

**Osteoblastic differentiation**—Cells were seeded at a density of 12,000 cells/cm² into chamber slides. Cells were supplemented with an osteogenic induction medium (α-MEM, 5% fetal cell serum, 10,000 U of penicillin, and 10 mg of streptomycin/mL, amphotericin B [250 µg/mL], ascorbic acid [50 µg/mL], dexamethasone [10⁻⁴ M], and β-glycerophosphate [10 mM]). Cultures were maintained for 7 days and then fixed for 20 minutes in 10% formalin at room temperature. Cultures were then stained with von Kossa stain for the detection of calcium and with the substrate naphthol AS MX-PO₄ and red violet LB salt for the detection of alkaline phosphatase to confirm mineralization and osteoblastic differentiation.

**Immunofluorescent analysis for MSC surface markers**—Cells from each tissue from each of 3 horses were plated at 2,500 cells/cm² in standard medium. Cells were then incubated at 37°C for 24 hours in a humidified incubator at 5% CO₂ and 95% air. Culture medium was removed, and cells were washed twice with PBS solution. Cells were fixed in 4% paraformaldehyde (pH, 7.4) for 15 minutes. Paraformaldehyde was removed, and cells were washed twice with PBS solution. Cells were blocked by incubation with 1% BSA in PBS solution at room temperature for 1 hour. The block-
ing solution was then removed and the remaining steps were conducted in a dark room. One microliter of antibodies (CD34, CD44, CD90, and CD146) diluted in 1% BSA in PBS solution was added to the cells, and plates were incubated overnight at 4°C. The next day, cells marked with the CD90 antibody were washed twice with PBS solution, and FITC-labeled secondary antibody diluted in 1% BSA in PBS solution was added. Cells were then maintained at room temperature for 1 hour. All cells were covered with PBS solution and allowed to sit for 5 minutes at room temperature in dark conditions; this process was repeated with fresh PBS solution 3 times. A nucleic acid stain (1 µM of stock solution/10 mL of distilled water) was added to all cells; cells were incubated for 1 minute and then washed once with PBS solution. Cells remained in PBS solution, and digital images were obtained immediately. Cells from each of the tissues were evaluated for staining indicative of MSC surface markers.

Flow cytometric analysis of MSC surface markers—Cultured and expanded cells from the second passage of each of the 4 tissues (bone marrow, fat, periosteum, and muscle) from 1 horse were used for the flow cytometric analysis. The amount of antibody was optimized with a cytometer.

Cell preparation—Cells were washed with PBS solution and then incubated for 15 minutes in a humidified incubator at 5% CO₂ and 95% air at 37°C with a mixture of versene4 and trypsin (5:1). This detachment method yielded the highest values for viability (up to 95% after 8 hours). The reaction was stopped with an equal amount of standard medium. The cell suspension was centrifuged (377 X g for 10 minutes), and the pellet was resuspended in ice-cold 1% BSA in PBS solution. The cell suspension was again centrifuged (377 X g for 10 minutes), and the resulting pellet was resuspended in ice-cold 1% BSA in PBS solution, stained with trypan blue to determine viability, and counted via flow cytometric analysis.

Cell labeling—One million cells per sample were labeled. Sample 1 was unstained and served as a negative control sample. Samples 2 through 5 were labeled with validated25,26 antibodies (CD45, CD44, CD90, and CD34, respectively). Sample 6 was labeled with a combination of 45 allophycocyanin, R-phycocyanin, and 90 FITC. Sample 7 was labeled with validated25,26 antibodies (CD45, CD44, CD90, and CD34, respectively). One million cells were collected and centrifuged (377 X g for 10 minutes), and primary antibodies were added in 1% BSA in PBS solution (Appendix). Samples were placed on ice and incubated for 45 minutes; samples then were washed in ice-cold 1% BSA in PBS solution and centrifuged (377 X g for 10 minutes). The washing and centrifugation steps were repeated 3 times. Cells were stored at 4°C until flow cytometric analysis. The secondary antibody for CD90 was diluted in 1% BSA in PBS solution and incubated on ice for 30 minutes and then washed in ice-cold 1% BSA in PBS solution and centrifuged (377 X g for 10 minutes). The washing and centrifugation steps were repeated 3 times. Cells were stored at 4°C until flow cytometric analysis.

Proliferation assay—Cells from each tissue from each of the 10 equine cadavers were plated (in triplicate) in 35-mm wells at 3,000 cells/cm². The cells were detached with trypsin and counted at 24, 48, 72, and 96 hours with a hemacytometer to determine the proliferation rate and doubling time. The procedure was repeated for the first and second passage of each tissue.

MSC yield—At the first passage, data were collected from the 10 horses to determine the mean MSC yield (MSCs obtained per gram of tissue) for bone marrow, adipose tissue, muscle, and periostum. The volume of bone marrow and weight of the 3 other tissues were recorded during the isolation and culture procedures. Equine bone marrow has a mass density of 1 g/mL. Therefore, the bone marrow volumes were converted to weights for ease of comparison with results for the other 3 tissues.

Osteogenic assay—Cells from the second passage of all 10 horses and all 4 tissues were seeded in 6-well plates at 200 cells/cm². Half of the wells were induced with osteogenic medium, and the other half were maintained in standard medium to serve as control cultures. Growth of one group of the paired cultures was stopped on day 7, and growth of the other was stopped on day 10. Total RNA was extracted from the cells. The cDNA was synthesized from total RNA via a cDNA synthesis kit.29 Primers derived from the coding regions of osteocalcin were as follows: forward, 5′-CTGGGCCCCAGGACTCCGCACTCT-3′; and reverse, 5′-AGCAGCTGTCACAGTCTGCGTTG-3′. Expression of the osteocalcin gene was quantified via real-time PCR assay with a mix.30 The PCR assay was performed on a thermal cycler.31 Cycling conditions were as follows: 95°C for 5 minutes; 35 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 45 seconds; and melting from 55° to 99°C. Nuclease-free water instead of cDNA was used as a negative control sample. Gene expression was determined via the comparative cycle threshold (ΔΔCT) method.32 The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used to normalize the expression of each osteocalcin gene.

Statistical analysis—Statistical analysis of osteocalcin gene expression measured with a real-time PCR assay was conducted via a 2-way ANOVA with effects of tissues and horses, after square root transformation of the normalized cycle threshold values to comply with model assumptions. Data for days 7 and 10 were analyzed separately. Tissues were compared for their expression of osteocalcin with that of the standard medium via t tests on the basis of least squares means. Pairwise comparisons among tissues were conducted with the Tukey method.

Statistical analysis of proliferation data was conducted with a linear mixed model on logarithmically transformed outcomes with fixed effects of tissues, passages (first and second), and time (24, 48, 72, and 96 hours) as well as all interactions and random effects of horses, tissues within horses, passages within tissues,
and sets of triplicates for each of the time points. Pairwise comparisons between tissues within time points underwent a Holm adjustment for multiple testing. An additional analysis with an assumed linear effect of time was used to estimate doubling times for each of the tissues.

Statistical software was used for the analysis of osteocalcin data and proliferation data. Significance was set at values of \( P < 0.05. \)

**Results**

**Characterization of MSC morphology, adherence, and confluence**—Cells isolated from all 10 horses and all 4 tissues developed a spindle-shaped morphology and readily adhered to plastic. The MSC cultures derived from muscle, periosteum, and adipose tissue became 80% to 100% confluent within 6 to 8 days after initial seeding of flasks in the first passage. However, MSC cultures derived from bone marrow consistently required longer to achieve confluence and only achieved 45% to 75% confluence during this time.

**Differentiation**—Cells isolated from 3 horses and all 4 tissues were capable of trilineage differentiation (Figure 1). Cells cultured in adipogenic differentiation medium for 4 days had positive results for oil red O staining of lipid droplets. Cells cultured in standard medium did not develop lipid droplets and lacked staining with oil red O. Pelleted MSCs cultured in chondrogenic differentiation medium for 21 days had cells within lacunae in Alcian blue–stained material. Cells cultured in standard medium did not have lacunae and lacked staining with Alcian blue.

Cells cultured in osteogenic differentiation medium for 7 to 10 days formed bone nodules as determined on the basis of positive results for alkaline phosphatase– and calcium-specific stains. Cells cultured in standard medium did not develop nodules and lacked staining for alkaline phosphatase and calcium.

**Immunofluorescent analysis of CD markers**—Cells isolated from 4 horses and all 4 tissues cultured via standard conditions in the first passage strongly expressed the cell surface antigen CD90 and weak-
ly expressed CD44, as determined on the basis of immunofluorescence. None of the isolated cells had immunofluorescence for CD45, CD34, CD146, or CD105 (Figure 2).

Flow cytometric analysis—Cells isolated from 1 horse and all 4 tissues that were cultured via standard conditions in the second passage strongly expressed the cell surface antigens CD90 and CD44 and weakly expressed CD45 and CD34, as determined on the basis of flow cytometric data (Figure 3; Table 1).

Proliferation rate—Analysis of the logarithmically transformed MSC counts revealed a significant \( P < 0.001 \) interaction between tissues and times, whereas no significant effects were detected for passages (including interactions). At 72 and 96 hours, muscle-, periosteum-, and adipose-derived MSCs proliferated significantly faster than did bone marrow–derived MSCs (Figure 4). Assuming a log-linear effect of time (equivalent to assuming a constant doubling rate) yielded estimated doubling times for MSCs derived from bone marrow (27.3 hours), periosteum (15.0 hours), muscle...
Table 1—Expression and coexpression of CD markers for each tissue obtained from 1 representative horse immediately after the horse was euthanized.

<table>
<thead>
<tr>
<th>CD marker</th>
<th>Fat</th>
<th>Bone marrow</th>
<th>Periosteum</th>
<th>Muscle</th>
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<tr>
<td>CD45+</td>
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<td>97.1</td>
<td>99.2</td>
<td>99.8</td>
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<tr>
<td>CD44+</td>
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<td>97.1</td>
<td>99.2</td>
<td>99.1</td>
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<td>2.8</td>
<td>3</td>
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<td>1.6</td>
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<tr>
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<td>0.4</td>
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<tr>
<td>CD90-</td>
<td>0.5</td>
<td>0</td>
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Values reported are percentages. + = Positive results for the indicated marker. – = Negative results for the indicated marker.

Figure 4—Graph of model-based estimated median cell counts of MSCs versus time after start of culture for MSCs derived from equine muscle (triangles), periosteum (squares), adipose tissue (diamonds), and bone marrow (circles). Start of MSC culture was designated as time 0. *Within a time point, value for bone marrow differs significantly (P = 0.01) from the values for all other tissues.

Figure 5—Mean ± SD results of nontransformed data for real-time PCR assay of osteocalcin gene expression for MSCs derived from 4 tissues obtained from 10 horses (immediately after the horses were euthanized) and cultured for 7 (gray bars) and 10 (black bars) days. *Within a tissue, value differs significantly (P < 0.05) from the baseline number of MSCs cultured in standard medium (horizontal black line). BM = Bone marrow. F = Fat. GADPH = Glyceraldehyde 3-phosphate dehydrogenase. M = Muscle. P = Periosteum.

Discussion

Analysis of results of the present study confirmed that cells derived from equine muscle and periosteal tissues can be characterized as MSCs, equine muscle- and periosteum-derived MSCs had superior proliferative capacity to that of bone marrow-derived MSCs, and equine muscle- and periosteum-derived MSCs had osteogenic potential comparable to that of equine adipose- and bone marrow-derived MSCs. In this study, equine muscle and periosteal tissues were sources of MSCs, as determined by morphology, adherence to plastic, trilineage differentiation, and detection of stem cell surface markers with immunofluorescent and flow cytometric analyses.

Muscle and periosteum are good sources of MSCs in rats and dogs, only muscle has been validated as a source of MSCs in horses. In humans, the importance of muscle-derived MSCs and periosteum-derived MSCs in bone repair has been reported. Therefore, it appears reasonable that muscle and periosteum may be useful sources of MSCs in horses as well. Muscle-derived MSCs have been isolated from horses and evaluated for potential tendon differentiation, and periosteum-derived MSCs have been isolated from horses and evaluated for their potential osteogenic differentiation. However, muscle- or periosteum-derived MSCs have not been thoroughly characterized as MSCs.

Authors of a previous study suggested that postmortem collection of tissues did not alter MSC attainment, proliferation, or phenotyping in dogs. The present study had similar findings for equine tissues. The rationale for cryopreserved whole tissue sections was to preserve samples for later stem cell recovery. Immediate cryopreservation of tissues was considered more practical than direct primary isolation of stem cells, which requires additional equipment and personnel. Cryopreservation techniques may be advantageous for banking of specimens from which MSC cultures are not immediately available.
needed. This is supported by a study in humans in which it was found that cells isolated from tissue processed and frozen with cryopreservation medium and subsequently thawed maintained morphological and developmental competence and had MSC-hallmark trilineage differentiation with appropriate culture conditions. The buffy coat of the bone marrow does not survive cryopreservation procedures well and was therefore cultured immediately after collection from the horses of the present study.

Equine MSCs derived from bone marrow and adipose tissue MSCs have been characterized on the basis of morphology, adherence to plastic, trilineage differentiation, and cell surface markers, and results of the present study confirmed those findings. In addition, cells isolated and expanded from muscle and periosteal tissues were characterized as MSCs on the basis of the identical criteria accepted for equine MSC characterization. Characterization with immunofluorescent staining revealed strong expression of the cell surface antigen CD90 for cells of all tissues, which indicated the cells were MSCs. Slight weakness of CD44 expression could have been attributable to sensitivity to the proteolytic action of trypsin, which is the agent most commonly used to detach cells during cell culture. Another explanation of the slightly weaker staining for CD44 is that direct immunolabeling was used to detect CD44, but an indirect immunolabeling method, which is more sensitive, was used to detect CD90. In addition, there can be variations in expression of cell surface markers on the basis of differences in culture times and isolation techniques. None of the isolated cells stained for CD45, CD34, CD146, or CD105, which is consistent with findings for equine MSC surface markers in another study.

Characterization with immunophenotyping also revealed that cells from periosteum and muscle as well as those from fat and bone marrow could be characterized as MSCs. Although there is currently no definitive consensus for expression of CD markers of equine MSCs, findings in the present study match those of other studies in that there was positive expression of cell surface markers CD90 and CD44 and low or no expression of CD34 and CD45.

To our knowledge, this is the first study conducted to characterize equine muscle- and periosteum-derived MSCs as defined on the basis of dual expression of CD44 and CD90 and extremely low expression of CD34 and CD45. Interestingly, for each of the 4 tissues, the percentages of cells with dual expression of CD90 and CD44 were extremely close to those with single staining for each cell surface marker, and the relatively high percentages indicated a reasonably pure population of MSCs from each tissue. Bone marrow–derived MSCs had a lower percentage of dual-staining cells in all 4 tissues, which confirmed that the concentration of MSCs was lower in bone marrow than in the other 3 tissues.

Analysis of data from the present study revealed that bone marrow–derived MSCs proliferated more slowly than did muscle-, periosteum-, and adipose-derived MSCs. This is consistent with previous findings that muscle-derived cells yield greater cell culture numbers in a shorter time than do bone marrow–derived cells.

Bone marrow may not be the optimum tissue for use in bone healing when the amount of time needed to culture clinically useful numbers of autogenous cells is considered. Bone marrow–derived MSCs also senesce much earlier than do other MSCs of horses. The slow proliferation of bone marrow–derived MSCs could have been attributable to the low number of proliferative cells in bone marrow aspirates. On the basis of the cell fibroblast assay, the frequency of MSCs in the mononuclear cell fraction of equine marrow is reported to be 1 in 4.2 × 10^7 cells and to differ among horses by 10-fold. The small fraction of proliferative cells among the total cell isolates could be to blame for the 1 to 2 weeks of extra expansion time needed for bone marrow–derived MSCs over the expansion time needed for other sources of MSCs. This indicates the need for a cell-sorting method to isolate this small proliferative fraction of cells before culture, which would aid in the removal of contaminant cells that physically impede MSC adherence and thereby hasten the expansion process.

Periosteum provided a higher MSC yield than did the other 3 tissues. Periosteum yielded a mean of 365 times as many MSCs per gram of tissue as did bone marrow at the end of passage 0. Although the exact dose of MSCs for various injuries has yet to be determined, there is evidence that the MSC effect is a dose-dependent phenomenon. It is clinically important to use stem cell sources that are extremely proliferative because treatment is dependent on the number of cells in culture, and up to 70 million osteoblasts may be required to generate 1 cm^2 of bone.

In addition, ease of harvest, quantity of donor tissue available, and morbidity at the donor site are clinically important issues. The general requirements for treatment with stem cells are that the cells be from a readily available source and that there is low morbidity associated with donor harvest. Muscle tissue, which can be readily harvested (similar to adipose tissue), meets these criteria. Harvest of bone marrow from the sternum is more difficult and can be associated with the risk of entering the thoracic cavity and potentially puncturing the heart when attempting aspiration from the sternum. Reports exist of iatrogenic pneumopericardium during bone marrow harvest. The temperament of the horses as well as the expertise of the veterinarians harvesting the bone marrow are risk factors associated with this method of harvest. Attempts at improving safety and optimizing bone marrow harvest from the sternum have recently been made. Bone marrow can also be harvested from the tuber coxae to avoid the risk associated with aspiration from the sternum; however, a recent study found reduced numbers of MSCs for aspiration of the tuber coxae, compared with results for aspiration of the sternum in middle-aged horses. Periosteum was relatively easy to harvest as well, but horses would likely need to be anesthetized. Harvest would be clinically feasible at the time of fracture repair because it only requires removal of a 4-cm^2 section of periosteum.

In the present study, we determined that equine muscle and periosteal tissues are donor sources of...
MSCs that have osteogenic potential for bone healing. It is unlikely that a single donor source of MSCs will be superior for regeneration of tissue from all different germ layers. In one in vitro study, adipose-derived MSCs required longer than did bone marrow–derived MSCs to undergo osteogenic induction, and investigators in another in vitro study found that bone marrow–derived MSCs required longer to undergo osteogenic induction and had more osteogenic potential than adipocyte-derived MSCs. Therefore, there is a need for research on which donor tissues are most suitable for use in bone healing.

Real-time PCR assay for osteocalcin expression was used in the present study to confirm osteoblastic differentiation. Osteocalcin is an abundant non-collagenous, hydroxyapatite-binding protein found in bone that is commonly measured and is a specific marker for the osteoblastic stage of osteogenesis. Mesenchymal stem cells from all 4 tissues could be induced to differentiate into the osteoblastic lineage, as indicated by an increase in osteocalcin expression measured on day 7. Mesenchymal stem cells from day 10 had no significant differences between differentiated and nondifferentiated cultures, which indicated a decrease in osteocalcin expression between days 7 and 10, which is consistent with a temporal sequence of osteogenic differentiation.

One of the limitations of the present study was the use of a hemacytometer for cell counts. Automated cell counters may have a lower error margin, but we adhered to research protocols that involved the use of hemacytometers. All counts were performed in triplicate for each sample to improve accuracy. Another limitation of the study was that counts of MSCs per gram of tissue did not address heterogeneity of the tissue. However, it is a repeatable and acceptable method to measure and compare cell yield from tissues because a weight measurement is more easily made and more accurate than is a size measurement. Finally, these measurements were paired with clinically feasible sizes of biopsy specimens for practical application. Another limitation of this study was the use of a single osteoblastic marker. Evaluation of the expression of additional genes as osteoblast markers may have highlighted differences in osteogenic potential among tissues, considering that no significant differences were detected with use of 1 marker. Because the focus of this study was to confirm osteogenic differentiation as part of trilineage differentiation, osteocalcin was used as the sole marker. Finally, although there was no significant (P = 0.17) difference in osteocalcin expression among the 4 tissues of the 10 horses, we believe that an increased number of horses may yield data in which the differences are significant.

In humans, periosteum and muscle clearly are potent sources of bone-forming cells for use in orthopedic repair. To our knowledge, the study reported here is the first to confirm osteocalcin expression in equine muscle- and periosteum-derived MSCs, which indicates their osteogenic potential. The characterization of muscle- and periosteum-derived MSCs broadens the choices available to clinicians who use MSCs in cell-based treatments, and MSCs from these tissues show much promise for future application in cell-based treatment for use in bone healing in horses.

References

10. Koch TG, Berg LC, Betts DH. Current and future regenerative


derived stromal cells as potential cell sources for cartilage repair in the horse. Vet J 2012;192:345–351.


### Appendix

Characteristics of antibodies used for flow cytometric analysis of MSC surface markers.

<table>
<thead>
<tr>
<th>CD marker</th>
<th>Fluorochrome</th>
<th>Emission wavelength (nm)</th>
<th>Excitation wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>Allophycocyanin</td>
<td>660</td>
<td>650</td>
</tr>
<tr>
<td>CD44</td>
<td>Phycoerythrin</td>
<td>667</td>
<td>496</td>
</tr>
<tr>
<td>CD45</td>
<td>Allophycocyanin</td>
<td>660</td>
<td>650</td>
</tr>
<tr>
<td>CD90</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Goat anti-mouse IgM</td>
<td>FITC</td>
<td>520</td>
<td>494</td>
</tr>
</tbody>
</table>

NA = Not applicable.