Isolation and characterization of bone marrow–derived equine mesenchymal stem cells

Stefan J. Arnhold, Dr med vet habil; Iris Goletz, DVM; Helmut Klein, DVM; Gerald Stumpf, DVM; Lisa A. Beluche, DVM; Carsten Rohde, DVM; Klaus Addicks, Dr med habil; Lutz F. Litzke, Dr med vet habil

Objective—To isolate and characterize bone marrow–derived equine mesenchymal stem cells (MSCs) for possible future therapeutic applications in horses.

Sample Population—Equine MSCs were isolated from bone marrow aspirates obtained from the sternum of 30 donor horses.

Procedures—Cells were cultured in medium (alpha-minimum essential medium) with a fetal calf serum content of 20%. Equine MSC features were analyzed to determine self-renewing and differentiation capacity. For potential therapeutic applications, the migratory potential of equine MSCs was determined. An adenoviral vector was used to determine the transduction rate of equine MSCs.

Results—Equine MSCs can be culture-expanded. Equine MSCs undergo cryopreservation in liquid nitrogen without altering morphologic characteristics. Furthermore, equine MSCs maintain their ability to proliferate and differentiate after thawing. Immunocytochemically, the expression of the stem cell marker CD90 can be detected on equine MSCs. The multilineage differentiation potential of equine MSCs was revealed by their ability to undergo adipogenic, osteogenic, and chondrogenic differentiation.

Conclusions and Clinical Relevance—Our data indicate that bone marrow–derived stromal cells of horses can be characterized as MSCs. Equine MSCs have a high transduction rate and migratory potential and adapt to scaffold material in culture. As an autologous cell population, equine MSCs can be regarded as a promising cell population for tissue engineering in lesions of the musculoskeletal system in horses. (Am J Vet Res 2007;23:1095–1105)

Diseases of the musculoskeletal system are a main reason for early retirement and euthanasia of race, pleasure, and working horses.1 Young horses starting active training are also affected with musculoskeletal diseases. Osteoarthritis and tendinopathy are the most common disorders of the locomotor system observed in working horses.2,3

A variety of treatment modalities exist for osteoarthritis and tendinopathy in horses. Therapeutic options are dependent on the cause and degree of the lesion. Stall rest and physiotherapy are often combined with the use of steroidal and nonsteroidal anti-inflammatory drugs.4 Furthermore, hyaluronic acid or glycosaminoglycans can be administered prophylactically to prevent progressive degeneration.5 Finally, the possibility of surgical treatment for both disorders exists. Arthroscopic abrasion arthroplasty can stimulate an intrinsic regeneration of cartilage for the treatment of osteoarthritis.6 For treatment of osteoarthritis and tendon injury, autologous tissue or cellular transplantation is used. Transplantation of autologous chondrocytes is used to treat osteoarthritis,7,8 and transplantation of autologous tendon is used to treat tendinopathy.9,10 Cell-based therapeutic options favor the use of autologous cells such as bone marrow stromal cells, which have been characterized as MSCs.11 Recently, the use of autologous bone marrow–derived MSCs to treat superficial flexor tendon injuries has been reported.12 Mesenchymal stem cells have recently received increased attention because these cells are easy to acquire, isolate, and expand.13,14 Mesenchymal stem cells can be culture-expanded through as many as 20 to 50 population doublings in approximately 10 weeks.14 Findings in recent studies indicate that bone marrow cells differentiate into various cell types, including hepatocytes,15 endothelial cells of the blood vessels,16 cardiac muscle,17 and skeletal muscle.18 It has also been reported that bone marrow–derived MSCs differentiate...
into neural cells and astrocytes in vitro and in vivo.\textsuperscript{19,20} Furthermore, culture-expanded MSCs from rats and mice induce therapeutic effects in experimental mice with retinal degenerations.\textsuperscript{21} In addition to human or rat MSCs, reports\textsuperscript{22,23} exist on isolation of equine MSCs and their potential to differentiate into chondrocytes. Furthermore, the comparison of equine peripheral blood-derived progenitors with bone marrow–derived MSCs in regard to the proliferation and differentiation capacity of these cells has been made.\textsuperscript{24} In a recent study,\textsuperscript{25} frequency of cell division, in vitro growth rate, and the adipogenic and osteogenic differentiation potential of MSCs of foals and that of young adult horses were compared with those of other mammalian species.

The purpose of the study reported here was to prepare bone marrow–derived equine MSCs for future therapeutic applications in the treatment of diseases of the musculoskeletal system in horses. As a first step, we isolated and culture-expanded equine MSCs obtained from bone marrow aspirates of the sternum, identified the stem cell nature of equine MSCs by use of the stem cell marker CD90, and characterized the proliferative capacity of these cells. Furthermore, we studied the pluripotency of equine MSCs before and after cryopreservation based on their capacity to differentiate into osteocytes, adipocytes, and chondrocytes. To establish equine MSCs as a cell source for tissue regeneration, we studied the ability of equine MSCs to synthesizesize extracellular matrix proteins, their migratory potential, and their transduction potential by use of an adenoviral vector. Finally, we examined the ability of equine MSCs to adhere and grow on a collagen matrix to test their applicability in tissue engineering, especially for tendon defects.

To our knowledge, results of our study are the first to provide evidence of the proliferation and differentiation capacity of equine MSCs before and after cryopreservation and to extend the already existing data on basic differentiation potential of these cells. Furthermore, new details are provided about the use of MSCs as a potential source of autologous adult stem cells for tissue engineering applications in equine veterinary medicine.

Materials and Methods

Isolation and culture of MSCs—Equine MSCs were isolated from bone marrow aspirates of the sternum of 30 horses (18 males and 12 females) that ranged in age from 6 to 15 years. All horses were clinical patients. Bone marrow aspirates were obtained with owner consent and according to local animal welfare regulations. Bone marrow aspirates were collected with the aid of ultrasonography from the sternum at the level of the third or fourth intercostal space by use of a 10-gauge needle.\textsuperscript{2} Horses were standing and under sedation with detomidine hydrochloride (20 to 40 \( \mu \)g/kg, IV); local anesthesia was provided with 2% lidocaine (1 to 2 mL, SC). Bone marrow aspirates were then transferred to two 50-mL sterile plastic tubes. Prophylactically, horses received gentamycin (3 mg/kg, IV) and flunixin meglumine (1.1 mg/kg, IV).

Aspirates were diluted 1:1 with alpha-MEM\textsuperscript{8} and filtered through a cell strainer made of a nylon mesh with 70-\( \mu \)m pores.\textsuperscript{4} Further treatment was performed as described previously for human MSCs.\textsuperscript{20} In brief, cells were incubated for 3 days, and nonadherent cells were removed by replacing the medium (alpha-MEM supplemented with L-glutamine [2 mg/mL], streptomycin [30 \( \mu \)g/mL], and 20% FCS [vol/vol]) in 3 washing steps. After cultures reached 80% confluence, cells were lifted by incubation with an enzyme cell detachment medium\textsuperscript{2} at 37\( ^\circ \)C for 3 to 4 minutes. Cells were diluted and plated at a density of 2,000 cells/cm\(^2\) in 100-mm culture dishes.

Cells generally underwent passage when cells reached a confluence of 80% in the culture dishes. For passage, cells were again lifted with the enzyme cell detachment medium\textsuperscript{2} and centrifuged. Resuspended cells were plated at a density of 500 cells/cm\(^2\). Cell counts were performed at the time of each passage. Cells of bone marrow aspirates from all 30 horses were subjected to this procedure.

CFUs—Following expansion, cells were plated at 10, 50, 100, 500, 1,000, 10,000, and 50,000 cells/100-mm-diameter culture dish. After 12 days of culture, cells were fixed and stained with 1% cresyl violet in 100% methanol. The number of colonies with > 50 cells was counted.

Cryopreservation and thawing—After 80% confluence, cells were lifted by incubation with the enzyme cell detachment medium\textsuperscript{2} and rinsed in 0.1M PBS solution. Finally, cells were resuspended in 1 mL of the freezing medium and transferred to a cryotube. Cryotubes were then frozen in a container filled with isopropanol, resulting in a reduction of temperature within the tubes for 1\( ^\circ \)C/min, until the final temperature of \( \sim \)80\( ^\circ \)C was attained.

After cryopreservation of cells for 1 to 6 months, thawing was performed at 37\( ^\circ \)C by transferring the cell suspension to 10 mL of medium (alpha-MEM supplemented with 20% FCS). Cells were then centrifuged at 800 \( \times \)g for 5 minutes. The supernatant was discarded and the pellet resuspended in the culture medium; cells were plated in a cell culture dish.

Proliferation assay—Cell proliferation was assessed before and after cryopreservation of samples from bone marrow aspirates of 6 horses. For the assay, cells were cultured for 48 hours in alpha-MEM and were then incubated with 10\( \mu \)M BrdU for 20 hours in alpha-MEM. Cells were then washed twice in PBS solution (0.1M) and fixed in 4% paraformaldehyde for \( \geq \) 20 minutes. Cells were then made permeable with 1M hydrochloric acid for 30 minutes at 20\( ^\circ \)C followed by subsequent washes with 0.1M sodium tetraborate and PBS solution. Incorporation of BrdU was demonstrated by incubation with a mouse anti-BrdU monoclonal antibody followed by incubation with a Cy3 dye-conjugated affinity-purified goat anti-mouse IgG\textsuperscript{8} at a ratio of 1:1,000. The percentage of BrdU-positive nuclei was determined in comparison to the total cell population as marked by a nuclear stain.\textsuperscript{7} The percentage of BrdU-positive nuclei per sample was determined by use of a digital camera and a software program. In each sample,
5 consecutive random fields were analyzed for BrdU-positive cells under a fluorescence microscope.

In vitro differentiation assays—Adipogenic, osteogenic, and chondrogenic in vitro differentiation were assessed before and after cryopreservation. Thus, for the differentiation assays, paired samples of equine MSCs before and after cryopreservation were analyzed. For each differentiation, samples from bone marrow aspirates of 6 horses were investigated.

Adipogenesis assay—To induce adipogenic differentiation, cells derived from the bone marrow were plated in alpha-MEM containing 20% FCS and grown until confluence was established. The medium was then replaced with high-glucose Dulbecco MEM containing 10% FCS, 1µM dexamethasone, 10µM insulin, 0.5mM 3-isobutyl-1-methylxanthine, and 100µM indomethacin (induction medium) or alpha-MEM containing 20% FCS and 10µM insulin (maintenance medium). At confluence, cells assigned to the adipogenic-differentiated group were grown in the induction medium for 3 days followed by 3 days of growth in the maintenance medium (1 cycle of induction and maintenance). Three cycles of induction and maintenance were performed before cells were harvested. Cells assigned to the adipogenic control group were grown in maintenance medium for the entire period of differentiation. At the end of the assay, cells were fixed in 10% formalin for 5 minutes. The formalin was then discarded, and cells were rinsed with 60% isopropanol. Cells were then stained by incubation with the oil red O dye (6 parts oil red O dye stock solution and 4 parts deionized water) for 15 minutes at room temperature (approx 21°C) to confirm adipogenic differentiation. Cells were then rinsed in deionized water. Adipogenic differentiation was analyzed by microscopically assessing the mean number of lipid droplets in 30 cells/preparation before and after cryopreservation.

Osteogenesis assay—To induce osteogenic differentiation in vitro and after cryopreservation, equine MSCs were plated at a density of 3,000 cells/cm² in alpha-MEM containing 20% FCS (maintenance medium) and in an induction medium consisting of alpha-MEM containing 10% FCS, 0.05mM ascorbic acid-2-phosphate, 10mM β-glycerophosphate, and 0.1µM dexamethasone. Osteogenic differentiation was examined by performing alkaline phosphatase staining by use of 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium tablets. After the 3-week differentiation period, increased concentrations of alkaline phosphatase were detectable by an increased staining intensity. After fixation in 0.1M paraformaldehyde, cells were rinsed in deionized water. The 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium tablets were dissolved in 10 mL of deionized water, and the staining solution was layered on top of the cells. After 2 minutes, the reaction was stopped by rinsing cells in deionized water. For the enhancement of contrast and improved cell observation, cells were counterstained with nuclear fast red. The osteogenic differentiation potential was quantified by densitometry measurements analyzing different gray units and thus different staining intensities with and without osteogenic differentiation by image analysis of gray units. Staining intensity was presented as mean of the measured gray unit minus the mean of the measured background gray unit. For staining intensity detection, a microscope coupled to a 3-chip charge-couple device camera was used and the analysis was performed by means of an image analysis program.

Chondrogenesis assay—To induce chondrogenic differentiation, aliquots of 2.5 × 10⁶ equine MSCs derived from bone marrow were centrifuged in 15-mL conical centrifuge tubes and incubated overnight in alpha-MEM containing 20% FCS. One day later, in chondrogenic control cultures, this medium was replaced with medium containing 2% FCS, an insulin-transferrin-selenium supplement (6.25 µg/mL), 50nM ascorbic acid, and 0.1µM dexamethasone (maintenance medium). In chondrogenic differentiated cell cultures, the original medium was replaced with an induction medium containing transforming growth factor β1 (10 ng/mL). Cell pellets in each group were kept in conical tubes in the incubator for 3 weeks, and then pellets were fixed with 0.1M paraformaldehyde embedded in paraffin. Paraffin sections (10 µm thick) of these pellets were subjected to staining with toluidine blue, which is specific for the detection of highly sulfated proteoglycans to confirm chondrogenic differentiation. Cells were stained for 20 minutes in toluidine blue solution (0.8%, pH, 4) and were then differentiated by use of 0.5% acetic acid. The chondrogenic differentiation potential was also quantified by densitometry measurements analyzing different gray units and thus different staining intensities with and without osteogenic differentiation, before and after cryopreservation. The mean densitometry measurement value was assessed from 10 measurements in each sample by use of a microscope, and the analysis was performed by means of an image analysis program.

Investigation of the migratory potential—For studying the migratory potential of cryopreserved equine MSCs in a wound-healing assay, cells were cultured in a 60-mm-diameter dish until cell confluency of 60% to 80% was accomplished. With a 200-µL Eppendorf pipette tip, a scratch of approximately 0.5 mm in diameter was created, destroying the monolayer of cells (n = 5). Cells either were treated with interleukin-6 (10 ng/mL) or remained untreated. Culture dishes were then transferred to a heated incubator chamber (5% CO₂) mounted on the stage of an inverted phase-contrast microscope. By use of the life cell imaging system in conjunction with a digital camera and a software program, migration of cells into the cell-free area of the scratch was continuously analyzed for up to 24
hours. After that, photographs of defined time points were selected to determine the time when the scratch was completely covered again. For the detection of rearrangement of actin filaments into the direction of migration, the phalloidin staining procedure was performed with a phalloidin staining kit.

**Culture of equine MSCs on a collagen scaffold**—After lifting from culture dishes with enzyme cell detachment medium, cells were transferred to a rat tail collagen I gel. For preparation, the rat tail collagen was dissolved in 200 µL of 10X medium 199, 55 µL of distilled water, 100 µL of sodium bicarbonate, 20 µL of L-glutamine, and 125 µL of NaOH. Cells were evenly distributed within the liquid collagen. The cell suspension was then transferred to a 35-mm-diameter culture dish, which was placed at 37°C in an incubator for 30 minutes. After 30 minutes, the gel was hardened and culture medium was applied on top of the collagen matrix. The culture dish with equine MSCs captivated in the collagen matrix was placed in the incubator of the life cell imaging system. Migration and network formation of equine MSCs within the collagen matrix were observed and recorded for 24 hours.

**Construction of a high-capacity adenoviral vector HC-AdFK7**—The high-capacity adenoviral vector HC-AdFK7 was generated as previously described to express the increased green fluorescent protein under the control of the human cytomegalovirus promoter. After replating of thawed equine MSCs, cells were infected with a stock of adenoviral vector infectious concentrations (U/µL), corresponding to 10, 50, 100, and 200 MOI for a single cell. Transduction rate was quantified 48 hours after transduction by determining the population of cells with positive results for increased green fluorescent protein expression within the total cell population labelled by the nuclear stain. In each sample, 5 consecutive random fields were analyzed for green fluorescent protein-positive cells by use of a fluorescence microscope.

**Reverse transcriptase–PCR**—Total RNA was isolated from the 3-dimensional MSC pellet cultures of unstimulated cells (n = 3) and from cells subjected to chondrogenic differentiation (3) by use of a commercial kit. The RNA was quantified, and 800 ng of RNA was loaded. The RNA was incubated with DNase I to remove DNA contamination. Procedures were performed according to the protocol of the manufacturer. The RNA was reverse transcribed to first-strand cDNA by Moloney murine leukemia virus reverse transcriptase in a 50-µL reaction mixture with an Oligo(dT)₁₈ primer. The PCR was performed with a thermocycler in a volume of 50 µL containing 4 µL of reverse transcribed product by use of the following specific primers: equine β-actin (forward primer 5'-ATC CAC GAA ACT ACC TTC AAC-3’; reverse primer 5’-CGC AAT GAT CTT GAT CTT CAT C-3’), equine aggrecan (forward primer 5’-ACA ACA ATG CCC AAG ACT AC; reverse primer 5’-GCC AGT TCT CAA ATT GCA AG), and equine collagen II (forward primer 5’-TGA AAC TCT GCC ACC TTC GAT G-3’; reverse primer 5’-TTG TCC TTG TCC TCT TG T GT CTC CTC-3’). β-Actin was used as the housekeeping gene. As a DNA marker, a 100-bp ladder was used.

![Figure 1](image-url) —Morphologic characteristics of equine MSCs before and after cryopreservation and immunocytochemical evaluation of cell proliferation. A—Phase-contrast photomicrograph of noncryopreserved equine MSCs. Notice the typical spindle-like morphologic characteristics of confluent growing adherent cells on the bottom of an uncoated culture dish in a serum-enriched (20% FCS) culture medium. B—Phase-contrast photomicrograph of cryopreserved equine MSCs after thawing and plating. Notice that the morphologic characteristics are not altered, compared with the noncryopreserved cells. C—Immunofluorescent photomicrograph of cryopreserved equine MSCs. Immunocytochemical testing with the BrdU assay was used to detect cell proliferation. Bar = 60 µm for panels A and B and 30 µm for panel C.
**Immunocytochemical analysis**—For the immunocytochemical characterization of equine MSCs, cells after cryopreservation were generally used. For these investigations, samples from bone marrow aspirates of 6 horses were analyzed. After rinsing in 0.1M PBS solution, cells were fixed in 4% paraformaldehyde. Immunocytochemistry was performed at room temperature.

Primary antibodies were used at the following dilutions: mouse anti-CD90 at 1:800, mouse anti-perlecan at 1:200, mouse anti-fibronectin at 1:500, mouse anti-collagen IV at 1:100, and mouse anti-β integrin at 1:100. Incubations were performed overnight at 4°C. Secondary antibodies were used for single immunofluorescence analysis at the following dilution: CyTM3 dye-conjugated affinity-purified goat anti-mouse or anti-rabbit IgG at 1:1,000. For the immunocytochemical characterization, cells of 6 horses were investigated before cryopreservation and cells of 5 horses were investigated after cryopreservation. After incubation with antibody, DNA was stained with a nuclear stain. Preparations underwent cover glass placement by use of a synthetic medium. The percentage of equine MSCs that were positive for CD90 after cryopreservation was assessed with the fluorescence microscope.

In each sample from bone marrow aspirated from 6 horses, 5 consecutive random fields were analyzed for the percentage of immunoreactive cells in the total cell population.

Equine MSCs served as negative controls by omitting the primary antibodies. Human MSCs served as positive controls to test the CD90 antibody; appropriate equine and human tissues such as cartilage and connective tissue were used for testing antibodies to extracellular matrix markers.

**Transmission electron microscopy of MSCs**—For light (semithin sections) and electron microscopic investigations, only cryopreserved cells were used. Equine MSCs were processed according to standard protocols. In brief, cells were fixed overnight at 4°C in glutaraldehyde (4% solution in 0.1M cacodylate buffer; pH, 7.4). Equine MSCs were postfixed with 1% osmium tetroxide at room temperature in 0.1M cacodylate buffer; pH, 7.4. Equine MSCs were postfixed with 1% osmium tetroxide at room temperature in 0.1M cacodylate buffer and stained en bloc with uranyl acetate. After dehydration, MSCs were embedded in an epoxy resin. Blocks were either sectioned semiserially in 0.7-µm-thick sections or prepared as ultrathin sections (70 nm thick). The latter were collected on copper grids and examined by transmission electron microscopy.

**Cell quantification and statistical analysis**—Data are presented as mean ± SD values from 3 to 10 horses/group. Analysis was performed by applying the paired t test by use of a software program followed, where necessary (adenoviral transfection rate, MOI), by a Bonferroni adjustment. The adenoviral transfection rate was additionally analyzed by means of a 1-way ANOVA. Values of P < 0.05 were considered significant.

**Results**

Isolation, culture, and in vitro characterization of equine MSCs—Bone marrow aspirates were obtained from 30 donor horses to isolate MSCs by cell adherence to plastic dishes. Populations of adherent equine MSCs that had similar morphologic characteristics to rodent and human MSCs were successfully isolated. Cell counts were performed with each passage from each bone marrow sample obtained. Upon culture, an initial lag phase of 7 to 10 days was noticed in cultures, after which cells entered the log phase of growth and increased in number rapidly.

Phase-contrast microscopy of equine MSCs in culture before and after cryopreservation revealed a homogenous population of adherent cells (95%), which was composed predominantly of elongated cells and some smaller cells. These morphologic characteristics of equine MSCs were similar to that of MSCs from other species, including human bone marrow–derived MSCs and rodent and human MSCs were successfully isolated. Cell counts were performed with each passage from each bone marrow sample obtained. Upon culture, an initial lag phase of 7 to 10 days was noticed in cultures, after which cells entered the log phase of growth and increased in number rapidly.

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**CFUs**—The potential of cell self-renewal was analyzed by use of the CFU assay. Cells were plated at a density of 10, 50, 100, 1,000, 5,000, and 10,000 cells/100-mm culture dish and were expanded for 12
days. Individual colonies were generated even when equine MSCs were plated at low densities such as 5 and 10 cells/culture dish (not shown). The mean rate of colony formation of equine MSCs was 27% of the starting MSC population, indicating a subpopulation of cells with self-renewing capacity in vitro.

Cryopreservation and thawing—Cryopreservation was performed to keep cells for longer periods and to provide the possibility of performing different experiments on cells from 1 bone marrow donor horse. After thawing of cryopreserved equine MSCs, cell viability of 83 ± 11% (n = 15) was determined from trypan blue staining. After plating, cells appeared to be vital and their morphologic appearance was similar to cells that were not subjected to cryopreservation.

Proliferation assay—Cells were culture-expanded for > 10 passages. The culture doubling time was evaluated as 2 days, which did not vary between donor horses. By use of the BrdU proliferation assay (Figure 1), the proliferative state of MSCs as detected by immunofluorescence analysis was 30 ± 5.8% in the noncryopreserved cells. After thawing of cryopreserved cells, the percentage of proliferative cells was 26.5 ± 6.5% and thus not significantly altered, compared with cells before cryopreservation. Furthermore, the proliferation index was independent of the age of bone marrow donor horses.

Adipogenic differentiation—After 12 to 14 days of culture in adipogenic differentiation medium, the adipogenic phenotype of equine MSCs before and after cryopreservation was evaluated on the basis of intracellular accumulation of lipid droplets as detected by positive stain results with oil red O dye (Figure 2). During control conditions before and after cryopreservation, the mean number of lipid droplets was 2.2 ± 1.8 and 2.4 ± 1.7, respectively; the number of lipid droplets significantly increased after adipogenic differentiation to 20.1 ± 6.1 before cryopreservation (P < 0.001) and 21.4 ± 7.8 after cryopreservation (P < 0.001). However, no significant (P = 0.100) alteration was found in the accumulation of lipid droplets between equine MSCs before and after cryopreservation.

Osteogenic differentiation—Under conditions supporting osteogenic differentiation, cells proliferated rapidly and formed densely packed colonies. In culture, dense granular areas appeared within individual colonies, and over time, multiple layers of cells formed. Under conditions supporting osteogenic differentiation, a significant increase was found in alka-
line phosphatase staining intensity before and after cryopreservation, compared with cells cultured in the maintenance medium before and after cryopreservation (Figure 2). The increase in the alkaline phosphatase staining intensity was quantitatively determined by use of densitometry (in gray units). During control conditions, alkaline phosphatase staining intensity was 19.8 ± 4.3 gray units before and 18.1 ± 3.5 gray units after cryopreservation; alkaline phosphatase staining intensity significantly increased as a result of osteogenic differentiation to 63.8 ± 8.5 gray units before (P < 0.001) and 57.8 ± 8.7 gray units after (P < 0.001) cryopreservation. No significant (P = 0.1) difference was found in the osteogenic differentiation potential of equine MSCs before and after cryopreservation. Similar to the alkaline phosphatase activity, a continuous increase in calcium deposits, as demonstrated by von Kossa staining, was observed.

**Chondrogenic differentiation**—Under 3-dimensional culture conditions suitable to promote chondrogenic differentiation, cell aggregates were detected at 2 days after chondrogenic induction of cells before and after cryopreservation. The aggregates gradually increased in size over the 3-week culture period. After that time, aggregates were fixed and paraffin sections were stained with toluidine blue, which is specific for highly sulfated proteoglycans (Figure 2).

Quantitative determination of the staining intensity with toluidine blue revealed significant increases in gray units from 24.2 ± 4.4 (control conditions) to 39 ± 2.5 (stimulated conditions) before cryopreservation (P < 0.001) and from 23.5 ± 6.5 gray units to 41.6 ± 7.9 gray units after cryopreservation (P < 0.001), indicating an unaltered chondrogenic differentiation potential (P = 0.311) after cryopreservation. Furthermore, chondrogenic differentiation was also shown by reverse transcriptase-PCR by use of specific primers for collagen II and aggrecan (Figure 3).

**Immunocytochemical characterization of equine MSCs**—Immunocytochemical staining revealed that most cells (85%) were immunoreactive for the stem cell marker CD90 (Figure 4). Furthermore, expression of extracellular matrix proteins fibronectin, perlecan, and collagen IV was evaluated. Even under nonstimulated conditions, a high expression of fibronectin, perlecan, and collagen IV was found. All 3 proteins were expressed abundantly and were organized into distinct fibrils. Immunocytochemical analysis further revealed that in parallel with expression of fibronectin, perlecan, and collagen IV, a high expression rate of β1 integrin was also found, indicating that β1 integrins appear to be the predominant adhesion receptor subfamily used by stromal cells to adhere to extracellular matrix components. In the negative controls that had primary antibodies omitted, no positive staining results were detected for any of the antibodies used (not shown).

**Investigation of the migratory potential**—The migratory potential of equine MSCs was tested by use of a wound-healing assay. Time lapse investigations over 24 hours with phase-contrast microscopy revealed that cells migrated into the cell-free zone of the created scratch so that the lesion was completely covered by a confluent cell monolayer again after 20 ± 2.1 hours. In the presence of interleukin-6 (10 ng/mL), the time that was needed until the scratch was completely covered was significantly (P < 0.001) less (10 ± 2.6 hours) than under control conditions. In the course of cell migration into the cell-free area of the scratch, an organization of the cytoskeleton into the direction of migration was shown by the phallolidin staining method for the detection of actin filaments (Figure 5).

**Culture of equine MSCs on a collagen scaffold**—Time lapse investigations of equine MSCs cultured for 24 hours in a collagen matrix reveal that initially,
single rounded cells or cells organized in small aggregates after approximately 1 hour start to extend processes to establish cell-to-cell contacts with neighboring cells within the matrix. Finally, at the end of the observation period, a complex 3-dimensional network was formed. Network formation affected all layers of the collagen scaffold. Furthermore, cell proliferation was also detectable within the gel, which consequently gave rise to an even denser network of equine MSCs within the matrix (Figure 6).

**Adenoviral transduction of equine MSCs**—The transduction rate of equine MSCs with a third generation adenoviral vector was analyzed in the presence of 10, 50, 100, and 200 MOI by the expression of the green fluorescent protein. In the presence of 10 and 50 MOI, only moderate transduction rates of 13 ± 6.6% and 17 ± 4.2%, respectively, were achieved. However, the transduction rate was significantly increased, compared with 10 (P < 0.001) and 50 (P = 0.004) MOI, in the presence of 100 and 200 MOI as 61.7 ± 12.5% and 85 ± 6.2%, respectively (n = 6; Figure 7). Even with a vector concentration as high as 200 MOI, no signs of cytotoxicity were detectable.

**Histologic and ultrastructural investigation of MSCs**—One hour after initiation of micromass cultures, investigation of semithin sections of 3-dimensional pellets revealed that cells were mainly round with or without short processes. However, at this time, most cells were detected as single cells (Figure 8). At 4 hours in micromass culture, cells formed 3-dimensional aggregates. Although cells in the superficial zone had an elongated spindle-like appearance, cells within the aggregates had a rather polygonal appearance.

Results of ultrastructural examination of the micromass cultures by use of transmission electron microscopy revealed that at 1 hour after culture initiation, equine MSCs had a round appearance with a euchromatic and notched nucleus and a prominent nucleolus. Numerous cell processes were observed on the cell surface. Cells contained well-developed organelles, including mitochondria, endoplasm reticulum, Golgi apparatus, and large quantities of free ribosomes. Frequently, lipid droplets were observed throughout the cytoplasm. After 4 hours of culture, equine MSCs appeared to be densely packed; equine MSCs had lost their processes at the surface and close cell-to-cell contacts were observed (Figure 8).

**Discussion**

We have characterized bone marrow–derived equine MSCs after isolation from sternal aspirates of different donor horses to evaluate cells suitable for cell replacement strategies as well as in tissue engineering for lesions of the musculoskeletal system in horses. The proliferation capacity under different culture conditions and the expansion properties of these cells in vitro were evaluated. From our cell characterization studies, equine MSCs after expansion in the cell culture are characteristically similar to those of rats, mice, and humans. Equine MSCs exhibit the elongated morphologic characteristics generally seen in bone marrow stromal cells. In addition, adhesion properties and expression of the stem cell marker CD90 in isolated equine MSCs are considered properties of other MSCs. The potential of equine MSC for self-renewal, the most typical property of a stem cell, was determined by the quantification of CFUs after plating cells at low densities. Histologic investigation by use of light and electron microscopic techniques further revealed typical properties similar to human MSCs. Although as single cells, equine MSCs exhibit characteristic cellular processes, growth in a micromass culture for 4 hours leads to the formation of a hexagonal appearance with the establishment of close contacts to neighboring cells. Similar to MSCs from other species, pluripotency of equine MSCs can be determined from their differentiation capacity into the

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Figure 6—Phase-contrast photomicrographs of cryopreserved equine MSCs cultured in a collagen I matrix as a possible scaffold material. Notice the establishment of elongated cell processes and increasing intercellular network formation by equine MSCs within the collagen scaffold after 7 (A) and 14 (B) hours in culture. Bar = 70 μm for both panels.

Figure 7—Immunofluorescent photomicrographs of equine MSCs after cryopreservation and vector transduction. Green fluorescent protein expression was observed after infection with 10 (A) and 200 (B) MOI of the high-capacity adenoviral vector (green). A nuclear marker counterstain was used (blue). Notice the concentration-dependent increase in the transduction rate. Bar = 40 μm for both panels.
adipogenic, osteogenic, and chondrogenic lineages. These differentiation properties are important for therapeutic applications of equine MSCs in lesions of the musculoskeletal system including osteoarthritis and in tendinopathy. For potential clinical uses, we also investigated the capacity of equine MSCs to undergo cryopreservation. Fortunately, after thawing, equine MSCs retained an ability to proliferate and differentiate. Thus, it can be concluded that after isolation and expansion, MSCs can be cryopreserved for as long as 6 months until a demand to use them in cell-based treatments exists. Thus, it may be possible to isolate and collect cells from a horse at a younger age and store them for future applications. Successful cryopreservation is found for neural stem cells and MSCs of other species.

Extracellular matrix proteins are important components of tissue; their expression and organization are critical for proper tissue function and engineered tissue constructs. For bone, cartilage, and tendon repair, the formation of certain extracellular matrix components is an essential prerequisite. The potential of equine MSCs to synthesize perlecan, fibronectin, and collagen IV after culture was particularly important. Immunocytochemically, our results indicate that equine MSCs have the ability to synthesize these matrix molecules, even under nonstimulated conditions. Similar expression patterns are found for human MSCs. The bidirectional signaling interaction between cells and surrounding matrix is regulated by integrins; these transmembrane molecules also mediate cell cycle and tissue development. The cell adhesion apparatus, formed by integrins, is a class of heterodimeric cell surface receptors that form a direct physical linkage between the extracellular matrix and the cytoskeleton and activate biochemical-signaling proteins on the cytoplasmic side of the plasma membrane proteins. Integrins are a powerful regulator of tissue morphogenesis. In fact, within equine MSCs, we were also able to show the expression of the transmembrane proteins β1 integrin, validating that cell-matrix interaction is regulated by integrins in cells of equine origin similar to other species.

The transduction rate of a third generation, high capacity adenoviral vector was evaluated to determine the suitability of equine MSCs for gene therapy in future applications. Our findings indicate that equine MSCs have the potential to be used for transducing sequences of appropriate trophic factors and factors supporting differentiation. Similar options are proposed for human MSCs in a diversity of orthopaedic diseases and have been shown for equine cells including bone marrow–derived MSCs by use of a first-generation E1 deleted adenoviral vector. The E1 region, located at the left end of the 36-kb adenovirus genome, encodes proteins necessary for the expression of other early and late genes.

The autologous availability of MSCs and their good transduction properties without cytotoxicity provide the opportunity to use them as carrier cells for a combined cell and gene therapy in musculoskeletal diseases. Equine MSCs may be potentially used to produce trophic factors in therapeutically relevant concentrations to protect host cells from degeneration or to substitute other deficient components. Furthermore, it can be speculated that enhancement of cells’ paracrine potential by use of an adenoviral vector could provide an intrinsic stimulation of cell differentiation.

The migratory potential of MSCs is also important for clinical treatment where cells might be stimulated. Our data from the wound-healing assay clearly indicate that equine MSCs have a developed migratory potential. This potential can be effectively stimulated under the influence of the inflammation mediator interleukin-6. A comparable migratory potential under nonstimulated and stimulated conditions is also found for human MSCs with a similar experimental approach. In addition, cultured equine MSCs were placed in a collagen gel to determine their potential to grow on a scaffold material over different periods. In our study, the equine MSCs maintained their proliferative capacity within the collagen gel and formed extensive networks by establishing cell-to-cell contacts with their processes. Based on this data, a graft of MSCs embedded in a collagen matrix may maintain cells at the injection site and avoid cell loss as a result of unwanted diffusion and migration. A stable graft might be able to initiate regeneration of the lesioned tissue.

Figure 8—Morphologic investigation of cryopreserved equine MSCs cultured in a micromass culture. Photomicrograph of methylene blue–stained semithin sections of equine MSCs (A) at 1 hour after culture initiation reveals that most cells are round with short processes and cells appear to be loosely packed. A corresponding transmission electron micrograph (stained en bloc with uranyl acetate; B) confirms the round appearance. Photomicrograph of methylene blue–stained semithin sections of equine MSCs (C) at 4 hours after culture initiation reveals that cells are arranged in a densely packed manner with an epithelioid appearance. A corresponding transmission electron micrograph (stained en bloc with uranyl acetate; D) reveals a loss of processes and establishment of close cell-to-cell contacts without actually differentiating to true epithelial-like cells. Bar = 30 μm for panels A and C and 1 μm for panels B and D.
In conclusion, our data indicate that bone marrow–derived stromal cells can be characterized as MSCs. Equine MSCs can be culture-expanded in almost unlimited numbers and reveal a multilineage differentiation potential. Equine MSCs are a promising cell source for tissue engineering. Our results support the potential of equine MSCs for future use with an ex vivo gene therapy for diseases of the musculoskeletal system in horses.

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