Characterization of endothelial colony-forming cells from peripheral blood samples of adult horses

Margaret M. Salter MS
Wen J. Seeto PhD
Blake B. DeWitt BS
Sarah A. Hashimi BS
Dean D. Schwartz PhD
Elizabeth A. Lipke PhD
Anne A. Wooldridge DVM, PhD

OBJECTIVE
To isolate and characterize endothelial colony-forming cells (ECFCs; a subtype of endothelial progenitor cells) from peripheral blood samples of horses.

SAMPLE
Jugular venous blood samples from 24 adult horses.

PROCEDURES
Blood samples were cultured in endothelial cell growth medium. Isolated ECFCs were characterized by use of functional assays of fluorescence-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) uptake and vascular tubule formation in vitro. Expression of endothelial (CD34, CD105, vascular endothelial growth factor receptor 2, and von Willebrand factor) and hematopoietic (CD14) cell markers was assessed through indirect immunofluorescence assay and flow cytometry. The number of passages before senescence was determined through serial evaluation of DiI-Ac-LDL uptake, vascular tubule formation, and cell doubling rates.

RESULTS
Samples from 3 horses produced colonies at 12 ± 2.5 days with characteristic endothelial single layer cobblestone morphology and substantial outgrowth on expansion. Equine ECFCs formed vascular tubules in vitro and had uptake of DiI-Ac-LDL (74.9 ± 14.7% positive cells). Tubule formation and DiI-Ac-LDL uptake diminished by passage 5. Equine ECFCs tested positive for von Willebrand factor, vascular endothelial growth factor receptor 2, CD34, and CD105 with an immunofluorescence assay and for CD14 and CD105 via flow cytometry.

CONCLUSIONS AND CLINICAL RELEVANCE
ECFCs can be isolated from peripheral blood of horses and have characteristics similar to those described for other species. These cells may have potential therapeutic use in equine diseases associated with ischemia or delayed vascularization. (Am J Vet Res 2015;76:174–187)
Studies of other species indicate that EPCs have endothelial cells, ECFCs are considered to fit the true genesis and the terminally differentiated progeny are vascularized. Because the cells can undergo vasculogenesis and the terminally differentiated progeny are endothelial cells, ECFCs are considered to fit the true definition of an EPC.

The number of circulating EPCs has been shown to be low, and their function has been found to be impaired in human patients with diseases causing endothelial damage, such as cardiovascular disease and diabetes. Endothelial progenitor cells have been used therapeutically to treat nonhealing wounds, limb ischemia, and cardiovascular ischemia and are important for the vascularization of engineered tissues. Endothelial progenitor cells from humans, dogs, mice, pigs, sheep, and chickens have been isolated and investigated, but to our knowledge, similar studies of equine EPCs have not been performed. Stem cell-based treatments and regenerative medicine are of increasing interest in equine medicine. Results of studies of other species indicate that EPCs have the potential to increase the rate and quality of vascular repair when used alone or in conjunction with other stem cell sources. In addition, the functional properties of circulating EPCs from horses could be evaluated to identify differences between healthy and diseased animals, and EPC characteristics could potentially be used as a basis for diagnostic testing.

The objective of the study reported here was to isolate, culture, and characterize a specific subtype of EPCs, ECFCs, from healthy horses. We sought to characterize these cells by analysis of cell morphology, in vitro tube formation, uptake of Dil-Ac-LDL, and expression of endothelial and hematopoietic cell markers.

Materials and Methods

ISOLATION AND CULTURE OF EQUINE ECFCs

All procedures involving animals were approved by the Auburn University Animal Care and Use Committee. Blood samples (1 sample/horse) were obtained from 24 adult horses from the Auburn University College of Veterinary Medicine teaching and reproduction herds and from the Auburn University Equestrian Team herd. Peripheral blood was collected from a jugular vein into an evacuated tube containing lithium heparin and transported on ice to the university laboratory for processing.

An isolation protocol for human ECFCs was modified and used for the isolation of equine ECFCs. For each horse, 5 mL of the collected whole blood was transferred with a pipette into an uncoated 75-cm² (growth surface) tissue culture polystyrene flask containing 15 mL of prewarmed (37°C) endothelial cell growth medium supplied as a kit. Medium for growth of equine ECFCs consisted of the manufacturer-supplied culture medium containing growth factors and antimicrobials but not the provided FBS; instead, equine serum was added to the medium to a final concentration of 10%. An additional 5 mL of this supplemented culture medium was used to rinse the pipette of any residual blood and was added to the flask for a total of 25 mL of blood-medium mixture. The flask was incubated with standard cell culture conditions (37°C, 5% CO₂, and 95% humidity) for 24 hours to allow for cell adherence. After 24 hours, the blood-medium mixture was gently removed via pipette, with care taken to not disturb attached cells. The flask was rinsed with 30 mL of calcium-free, magnesium-free PBS solution, and then 15 mL of fresh supplemented medium was added. Adherent cells were maintained with standard cell culture conditions and observed daily for colony formation; 30% of the medium was changed twice each week.

Once cell colonies developed, the day of appearance and colony number were recorded for each sample, and colony morphology was observed by means of light microscopy and recorded. Colonies were harvested 2 days after appearance by adding a trypsin-EDTA solution (0.25 mg/mL) to the flask and incubating for 1 minute. Trypsin was neutralized by the addition of approximately 7.5 mL of fresh ECFC culture medium, followed by centrifugation at 200 g for 5 minutes. Cells were resuspended onto a collagen-coated 75 cm² cell culture flask for cell expansion. To coat the flask with collagen, 7.5 mL of rat-tail type 1 collagen (50.0 µg/mL) in 0.02N acetic acid solution was added to each flask, followed by incubation for 30 minutes at room temperature (approx 25°C). The collagen solution was then removed by careful aspiration, and the flask was rinsed with 5 mL of PBS solution. When the seeded cells reached 80% to 90% confluence, they were subcultured at a cell density of 5,000 cells/cm² to be used for characterization assays, and remaining cells were cryopreserved at a concentration of 100,000 cells/mL in a freezing medium containing 95% equine serum and 5% dimethyl sulfoxide for future experiments.

CULTURE OF HUMAN ECFCs AND MOUSE EMBRYONIC FIBROBLAST CELLS

Human ECFCs (passages 11 through 14) and mouse embryonic fibroblast (3T3) cells were used as controls for cell characterization assays. Human umbilical cord blood-derived ECFCs (20,000 cells/mL) were expanded in 75-cm² collagen-coated tissue culture polystyrene flasks containing 15 mL of endothelial cell growth medium (37°C), which was prepared with the manufacturer-supplied growth factors, antimicrobials, and FBS (added to a final concentration of 10%). When the cells reached 80% to 90% confluence, they were subcultured as described for equine cells or used immediately for experiments.

Mouse embryonic fibroblasts (20,000 cells/mL) were expanded in uncoated 75-cm² tissue culture
polystyrene flasks containing 15 mL of Dulbecco modified Eagle medium, with FBS added to a final concentration of 5%. When the cells reached 80% to 90% confluency, they were subcultured as described for equine and human ECFCs, with the exception that a 0.5 mg/mL solution of trypsin-EDTA was used to harvest confluent cells.

**IN VITRO TUBULE FORMATION ASSAY**

Equine ECFCs collected at passages 1 through 3 were seeded into a 96-well cell culture dish containing solubilized basement membrane (75 µL/well), which had been incubated for 30 minutes at 37°C prior to cell seeding. A range of seeding densities (7,500, 10,000, 15,000, and 21,000 cells/cm²) at 2 wells/density) was evaluated to optimize tubule formation. Human ECFCs (passages 11 through 14) served as positive controls, and mouse embryonic fibroblast cells served as negative controls. Each cell type was seeded at the same cell density as the equine ECFCs. All cells were incubated with standard cell culture conditions for 48 hours. Vascular tubule formation for each cell type was assessed at 0, 5, 24, and 48 hours with light microscopy at 4X (7.09 mm²), 10X (1.1 mm²), and 20X (0.08 mm²) magnifications. Three replicates of duplicate assays were performed for samples from each horse with isolated ECFCs. The presence or absence of tubule formation was noted by 2 investigators (MMS and WJS), using photomicrographs at each time point.

**UPTAKE OF DII-Ac-LDL**

Equine ECFCs (passages 1 through 3), human ECFCs (passages 11 through 14), and mouse embryonic fibroblasts in their respective culture media each were seeded at a density of 8,000 cells/cm² into 24-well cell culture plates that had been precoated with collagen (50 µg/mL) as described for tissue culture flasks. All cells were incubated for 24 hours with standard cell culture conditions. After 24 hours, Dil-Ac-LDL, diluted in prewarmed supplemented medium appropriate for the cell type, was added to a final concentration of 50 µg/mL, and the cells were incubated for 4 hours with standard cell culture conditions. After incubation, the cells were washed with probe-free medium, fixed with 4% paraformaldehyde solution for 20 minutes, and counterstained with DAPI for 25 minutes. The cells within the 24-well plates were covered with mounting media and evaluated with fluorescence microscopy (MMS and WJS). Red fluorescence was detected with excitation and emission wavelengths of 562 and 576 nm, respectively. Images were analyzed to determine the percentage of Dil-Ac-LDL-positive cells per cell type (equine ECFC, human ECFC, and mouse embryonic fibroblast) by evaluating 2 wells (2 separate fields of view/well at 10X [1.26 mm²] magnification), for a total of 4 images/assay. The mean number of Dil-Ac-LDL-positive ECFCs from the 4 images was calculated and reported as the percentage of Dil-Ac-LDL-uptake-positive cells. Overlaid (merged) images showing DAPI staining and uptake of Dil-Ac-LDL at 10X (1.26 mm²) magnification were used for evaluation. The percentage of cells positive for Dil-Ac-LDL uptake was determined by dividing the total number of DAPI stained cells per field of view by the number of cells taking up Dil-Ac-LDL. Assays were repeated 3 times for each ECFC isolation from an individual horse, and all cell samples were assayed in duplicate.

**CELL SENESCENCE AND CELL GROWTH AFTER SUBCULTURE**

To evaluate cell senescence and cell growth, isolated cells showing characteristics of ECFCs such as colony formation, cobblestone morphology, Dil-Ac-LDL uptake, and tubule formation were continually subcultured, and in vitro tubule formation and cell proliferation calculations were performed at each passage. The Dil-Ac-LDL uptake assays were performed at passages 1, 2, 3, 4, 6, 8, and 10. The tubule formation assay was performed with light microscopy as described for each cell passage, with a seeding density of 10,000 cells/cm². The time until tubule appearance and tubule quality score were recorded and subjectively scored (1 = no tubule formation, 2 = projecting tubules from cells but no connections between any cells, 3 = vascular tubule formation with connecting tubules in ≤ 50% of the field, and 4 = vascular tubule formation with connecting tubules in > 50% of the field; Figure 1). At the 24-hour time point, photomicrographs of 3 fields of view at 10X (1.1 mm²) magnification were obtained for each passage. The images were scored twice by 1 investigator (MMS) who was blinded to sample identification, and if discrepancies were detected, the mean value of the 2 scores was determined. The median score of the 3 fields for ECFCs from individual horses was determined and compared among passages.

The Dil-Ac-LDL uptake assay was performed in duplicate as previously described for ECFCs at passages 1, 2, 3, 4, 6, 8, and 10. Photomicrographs of 4 fields of view at 10X (1.26 mm²) magnification were acquired for a total of 4 images/passage/horse, and the percentage of cells positive for Dil-Ac-LDL uptake was determined. The mean percentage for all images from an individual horse was used for statistical analysis at each passage.

Cell seeding density after each subculture, cell number at the time of subculture, and time (hours) between subcultures were recorded and used to determine the number of cell doublings in each 24-hour period as well as the population doubling time. These were calculated by use of the following equations:

$$\text{PDT} = \frac{\text{NCD}}{\text{Total number of hours}}$$

$$\text{NCD} = \frac{\log_2 \left( \frac{C_H}{C_S} \right)}{\text{Number of days}}$$

where NCD is the number of cell doublings, $C_H$ is the...
number of cells at the time of subculture, $C_s$ is the number of cells at seeding, and PDT is the total time (in hours) required for the cell population to double.

**ISOLATION OF EQUINE CAROTID ENDOTHELIAL CELLS**

For use as a positive control for flow cytometry and indirect immunofluorescence staining, carotid endothelial cells were collected from an adult horse that was submitted for necropsy according to previous protocols. Briefly, isolated carotid arteries were digested with type II collagenase and cultured with standard conditions in Dulbecco modified Eagle medium supplemented with 25mM HEPES and 1% penicillin-streptomycin solution, with FBS and calf serum each added to a final concentration of 10%. When cells reached 80% confluency, they were subcultured into collagen-coated flasks with the same culture medium used for equine ECFCs. When these cells reached 70% confluency, they were harvested as previously described and sorted by flow cytometry on the basis of DiI-Ac-LDL uptake to eliminate any contaminating smooth muscle cells and obtain a more pure endothelial cell population. Cells were incubated with DiI-Ac-LDL according to the same protocol described for ECFCs. The brightest 22% of cells were gated, sorted at 65 lb/inch^2 into medium, and then maintained with standard culture conditions until they were subcultured or cryopreserved. The endothelial cells were further characterized by light microscopic examination of phenotypic appearance and vascular tubule formation in vitro.

**INDIRECT IMMUNOFLUORESCENCE LABELING**

Equine ECFCs (passages 3 through 5) and carotid endothelial cells (passages 4 through 6) were evaluated for expression of the cell markers vWF, VEGFR-2, CD34, CD105, and CD14 through IFA. Equine ECFCs or carotid endothelial cells were seeded onto collagen-coated glass coverslips at a density of 6,700 cells/cm^2 and allowed to expand at 37°C with 5% CO₂ until 80% to 90% confluency was reached. Cells were then fixed with 4% paraformaldehyde solution, rinsed with PBS solution, and blocked by application of 3% FBS solution for 30 minutes. To detect the intracellular protein vWF, cells were permeabilized with a 0.1% nonionic surfac-
tant solution" for 30 minutes prior to blocking. Excess blocking solution was removed, and ECFCs or carotid endothelial cells were incubated at room temperature with primary antibodies diluted in 3% FBS solution as follows: rabbit anti-human vWF at 1:200 for 3 hours, mouse anti-human VEGFR-2 at 1:200 for 3 hours, biotinylated anti-mouse CD34 at 1:100 for 3 hours, mouse anti-human CD105 at 1:100 for 3 hours, and mouse anti-horse CD14 at 1:100 for 1 hour. After incubation, cells were rinsed with PBS solution. Appropriate secondary antibodies (fluorophore 488-conjugated goat anti-rabbit IgG, fluorophore 488-conjugated goat anti-mouse IgG, or streptavidin-conjugated fluorophore-550; diluted 1:400 in 3% FBS solution) were applied, and cells were incubated at 4°C for 12 to 18 hours. Because many of the antibodies used were not specific for equine proteins, all cells were incubated with the secondary antibody only to verify specific versus background staining for IFA. Coverslips bearing the labeled cells were mounted on slides, and photomicrographs were obtained with fluorescence microscopy.

Red fluorescence (fluorophore 550) was detected with excitation and emission wavelengths of 562 and 576 nm, respectively; for green fluorescence (fluorophore 488), excitation and emission wavelengths were 495 and 519 nm, respectively. The DAPI signal was imaged with excitation at 340 to 380 nm and emission at 435 to 485 nm.

FLOW CYTOMETRY

Expression of CD105 and CD14 by equine ECFCs (passages 3 through 5) was characterized by means of flow cytometry. Cultured equine endothelial cells served as the positive control for evaluation of CD105 expression. Monocytes from equine whole blood served as the positive control for evaluation of CD14 expression, with equine carotid endothelial cells used as a negative control. Cultured cells were trypsinized as previously described, centrifuged, resuspended in ECFC culture medium, and allowed to incubate for 30 minutes at room temperature for cell surface marker regeneration. For labeling, $5 \times 10^5$ cultured cells in 100 µL of medium were used for each condition. Whole blood from a healthy horse was collected in lithium-heparin tubes, and aliquots of 100 µL were used for the labeling protocol. Following the 30-minute incubation period, equine ECFCs and endothelial cells were blocked with 10% horse serum for 30 minutes. Serum present in whole blood provided natural blocking for the monocytes prior to the addition of the primary antibody. The previously described antibodies against CD105 or CD14 were added to cultured cells and whole blood aliquots, and samples were again incubated at room temperature. The anti-CD105 antibody was used at a concentration of 0.02 µg/µL with an incubation period of 1 hour; anti-CD14 was used at 0.01 µg/µL with a 30-minute incubation period. After incubation with the primary antibody, the RBCs were lysed with 3 mL of lysis buffer (200mM NH₄Cl, 3mM KHCO₃, and 0.002mM EDTA; pH 7.3). All cells were washed with 1 mL of PBS solution and then stained with the appropriate secondary antibody at a 1:400 dilution. All cells were filtered through 35-µm mesh to prepare for flow analysis.
A total of 10,000 events were collected for each sample, with forward scatter versus side scatter plots used for imaging. Gates were set to select for live cultured cells, with elimination of doubled cells, dead cells, and debris. Whole blood samples were gated to include only monocytes. The percentage of cells positive for expression of CD14 and CD105 was determined for 2 separate samples of cultured ECFCs from each horse that had ECFCs isolated successfully.

**STATISTICAL ANALYSIS**

All continuous data were normally distributed on the basis of a D’Agostino and Pearson omnibus test and are reported as mean ± SD. Ordinal score data are reported as median and interquartile range. The percentage of LDL positive cells, number of cell doublings, and population doubling times after serial subculture were analyzed by means of a 1-way ANOVA with Tukey post hoc comparison. The tubule formation of equine ECFCs was seen at 24 hours and persisted up to 48 hours; tubule formation of human ECFCs (positive control) was seen at 5 hours and also persisted up to 48 hours. Mouse embryo fibroblasts (3T3 cells; negative control) did not form tubules. Bar = 100 µm. B—Representative images of equine ECFCs obtained 24 hours after seeding (21,000 cells/well) from a 24-year-old Quarter Horse (left), an 18-year-old warmblood (center), and a 10-year-old warmblood (right). Vascular tubule formation is evident in all 3 samples. Bar = 200 µm.

*Figure 3*—Photomicrographs depicting results of vascular tubule formation assays. A—Representative images of equine ECFCs, human ECFCs, and mouse fibroblasts cultured with solubilized basement membrane at 0, 5, and 24 hours after seeding at 10,000 cells/well. Tubule formation of equine ECFCs was seen at 24 hours and persisted up to 48 hours; tubule formation of human ECFCs (positive control) was seen at 5 hours and also persisted up to 48 hours. Mouse embryo fibroblasts (3T3 cells; negative control) did not form tubules. Bar = 100 µm. B—Representative images of equine ECFCs obtained 24 hours after seeding (21,000 cells/well) from a 24-year-old Quarter Horse (left), an 18-year-old warmblood (center), and a 10-year-old warmblood (right). Vascular tubule formation is evident in all 3 samples. Bar = 200 µm.
formation assay scores at each passage were analyzed with a Kruskal-Wallis test and Dunn post hoc comparison, and scores for early (1 through 4) and late (5 through 10) passages were compared with a 2-tailed Mann-Whitney U test. All analyses were performed with a commercial statistics package, and values of \( P < 0.05 \) were considered significant.

**Results**

**ISOLATION OF EQUINE ECFCs FROM PERIPHERAL BLOOD OF HORSES**

Of 24 samples collected from 24 horses, 3 produced colonies exhibiting the characteristic phenotypic appearance of cobblestone morphology and substantial outgrowth in a monolayer after subculture ([Figure 2](#)). Breeds of the 24 horses included 14 warm-blood type, 8 Quarter Horses, 1 American Paint Horse, and 1 Arabian. Horses ranged in age from 4 to 23 years (mean, 10.9 ± 4.4 years). There were 20 geldings and 4 mares. Blood samples from the 3 horses that successfully produced colonies had a colony appearance at 12 ± 2.5 days with 2.8 ± 1.5 colonies/mL of blood. The horses from which cell colonies were produced (a 23-year-old Quarter Horse, an 18-year-old warmblood, and a 10-year-old warmblood) were all geldings.

**IN VITRO TUBULE FORMATION ASSAY**

Equine ECFCs (passages 1 through 3) from all 3 horses formed vascular tubules in vitro ≤ 24 hours after cell seeding onto basement membrane, and the tubule structures were maintained until 48 hours after seeding ([Figure 3](#)). The positive control for assay conditions, human ECFCs, had vascular tubule formation ≤ 5 hours after cell seeding and maintained tubule structures until 48 hours after seeding. The negative control for the assay, mouse embryonic fibroblasts, failed to form vascular tubules at any time point and aided validation that the isolated equine cells had phenotypic and functional characteristics of true ECFCs. At 4X magnification, the branching networks that formed by 24 hours after seeding were detectable throughout the entire well. Formation of vascular tubule structures in vitro confirmed that the cultured equine cells were phenotypically and functionally similar to ECFCs described for other species and were not early EPCs or other circulating progenitor cells.

**UPTAKE OF DiI-Ac-LDL**

Isolated equine ECFCs (passages 1 through 3) from all 3 horses had uptake of DiI-Ac-LDL, with positive results in 74.9 ± 14.7% cells ([Figure 4](#)). The positive control, human ECFCs, had uptake of DiI-Ac-LDL in 100% of cells. The negative control, mouse embryonic fibroblasts, had no DiI-Ac-LDL uptake, supporting the specificity of the assay for endothelial-type cells.

**SENESCENCE AND GROWTH OF EQUINE ECFCs**

Median tubule quality scores were significantly \( P = 0.010 \) higher for earlier passages (1 through 4) than
Subjectively, cells lost their characteristic cobblestone morphology and became more spindle-shaped after multiple cell subcultures, and an increase in the presence of cytoplasmic vacuoles in the cytoplasm of cells was observed. No tested passage of ECFCs from any of the 3 horses completely lost the ability to uptake DiI-Ac-LDL; however, a significant decrease in the percentage of cells with positive results was identified for passages 6, 8, and 10, compared with passage 1. As passage number increased from 3 to 9, the cell doubling rate appeared to decrease and population doubling time appeared to increase; however, differences among passages were nonsignificant (Figure 7).

ANALYSIS OF EQUINE ECFC MARKER EXPRESSION

The ECFCs from all 3 horses tested positive via IFA for expression of vWF, VEGFR-2, CD34, and CD105 (Figure 8). Lack of nonspecific binding of the secondary antibodies was confirmed (data not shown). Equine endothelial cells, the positive control, were positive for vWF, VEGFR-2, CD34, and CD105 expression by this method. Testing for CD14 expression via IFA was unsuccessful; conclusive evidence could not be obtained because the only anti-equine CD14 antibody available to our laboratory was optimized for flow cytometry and not for IFA.

Flow cytometry was performed for detection of CD14 and CD105 expression (Figure 9). This method was unsuccessful for other markers (no fluorescently labeled cells were detected with flow cytometry for the positive control or the equine ECFCs). The ECFCs from all 3 horses tested positive for CD105 expression with flow cytometry (83.1 ± 13.6% positive cells). The ECFCs from 1 horse, an 18-year-old warmblood, had the lowest percentage of cells with a positive result (67.4%), whereas 90.7% and 91.2% of cells from the remaining 2 horses tested positive; the positive control, equine endothelial cells, had expression of CD105 in 99.6% of cells. The ECFCs from all 3 horses also expressed CD14, with 83.3 ± 1.5% of cells testing positive. The CD14 marker was detected in 24.6% of whole blood monocytes (the positive control), with no detectable labeling of equine endothelial cells (the negative control).

Discussion

In the present study, equine ECFCs, a subtype of EPCs, were successfully isolated from the peripheral blood of adult horses and characterized. The potential relevance of EPCs in the clinical treatment of diseases associated with decreased vascularization, such as complicated wounds, nonunion fractures, tendon and ligament repair, infectious corneal disease, chronic laminitis, and intestinal ischemia, is evident. Results of the present study demonstrated that ECFCs can be isolated noninvasively from the peripheral blood of healthy horses, and the ability to isolate and accurately characterize these cells will allow for further research, including in vivo characterization and investigation of the use of equine ECFCs to treat specific diseases.

Although ECFCs were successfully isolated, cell colonies were produced from samples obtained from only 3 of 24 horses. The protocol used for the isolation of the equine ECFCs was selected on the basis of 1 author’s (EAL) experience and had been optimized for the isolation of human ECFCs. This protocol was specifically developed to reduce the need for foreign animal proteins, which could interfere with future therapeutic applications in human medicine (e.g., FBS and collagen). The protocol also only called for 5 mL of blood to be used for cell isolation. The larger cross-sec-

Figure 5—Representative photomicrographs showing light microscopic features (A and B) and Dil-Ac-LDL uptake (C) of equine ECFCs at passage 3 (left) versus passage 10 (right). A—Notice the decrease in tubule formation in the later passage; the passage 3 sample has a vascular tubule formation score of 3, whereas the passage 10 sample has a score of 1 (maximum value, 4). Bar = 100 μm. B—Notice the loss of cobblestone morphology and increase in vacuolated, spindle-shaped cells in the later passage. Bar = 300 μm. C—Uptake of Dil-Ac-LDL (red) in DAPI (blue)-counterstained ECFCs is evident in a smaller proportion of cells at the higher passage. Bar = 200 μm.
tional size of a horse’s jugular vein, compared with a typical human vein, could account for the low yield of equine ECFCs seen in this study; this difference in vessel size could also have potentially affected the uniformity of cell samples obtained during blood collection, considering that circulating cells are not evenly distributed between the vessel wall and the center. A proportionally larger blood sample may be needed from horses to obtain cell populations that would be similar to those obtained from human donors. Although 1 modification to the established human EPC isolation protocol was to supplement the culture medium with horse serum, it is important to note that the growth factors added to the medium had been optimized for use with human cells. Endothelial progenitor cells are known to be rare in circulation, and results from studies in other species indicate various cell yields ranging from 4 to 6 colonies/mL. Modifications of the isolation protocol, including use of a larger sample volume, concentration of mononuclear cells with density gradient centrifugation, and collection from smaller-diameter veins, are potential methods for improving the yield of these cells from horses. Collection of bone marrow samples, although more invasive, is a standard method of obtaining MSCs, and EPCs and EPC subtypes could also be isolated from this source or may potentially be differentiated from MSCs as was recently reported in a study of dogs.

Equine ECFCs isolated in our study had a characteristic cobblestone cell morphology with a single layer of growth, which are key features used to distinguish ECFCs from other cell types. The equine ECFCs formed colonies after 7 days in culture, and collected colonies had substantial outgrowth when subcultured. These observations of colony appearance, cell morphology, and expansion further aided in the determination that the isolated cells were ECFCs and not early EPCs, circulating endothelial cells, or other types of adult stem cells found in peripheral blood. The ECFCs from all 3 horses that had successful cell isolation formed vascular tubules when cultured with solubilized basement membrane. The appearance of these tubules in equine samples was quite similar to those described for other species. The ECFCs successfully cultured from peripheral blood of 3 horses formed tubules ≤24 hours after cell seeding, whereas human ECFCs had tubule formation ≤5 hours after seeding. Thus, the timing of tubule formation by equine ECFCs in vitro may vary slightly from that of human cells or could potentially vary among horses. Samples from a larger number of horses should be analyzed to determine typical tubule formation times. The fact that the growth medium used in this study was optimized for use with human cells may have influenced the timing of tubule formation by equine versus human ECFCs. Vascularization potential may also differ between equine and human EPCs; however, more direct comparisons between multiple horses and humans would need to be investigated to address this. Proof of vascular formation from ECFCs would require demonstration of vascularization in vivo derived from the implanted ECFCs, which was beyond the scope of this initial characterization. Uptake of acetylated low-density lipoprotein is a common method used to characterize EPCs because it is a trait of endothelial cells. The ECFC samples from

![Figure 6](https://example.com/figure6.png)

**Figure 6**—Qualitative vascular tubule formation scores (A) and percentage of cells with Dil-Ac-LDL uptake (B) at various passage numbers for ECFCs obtained from 3 horses. Bars represent the median, and whiskers show the interquartile range. A—Median tubule formation scores for passages 1 through 4 were significantly higher than those for passages 5 through 10. B—The percentage of cells with Dil-Ac-LDL uptake was significantly lower for passages 6, 8, and 10, compared with that for passage 1.

![Figure 7](https://example.com/figure7.png)

**Figure 7**—Number of cell doublings per 24 hours (circles) versus population doubling time (squares) for various passages of ECFCs from 3 horses.

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all 3 horses in the study with successful culture results reliably had Dil-Ac-LDL uptake, with a mean of 85% of cells testing positive.

Results of the IFA showed that equine ECFCs expressed the endothelial markers vWF, VEGFR-2, CD34, and CD105. Characterization of stem and progenitor cells with cell surface markers is a standard approach, but it presents specific challenges in horses because of a lack of available antibodies. The antibodies chosen for the present study had potential cross-reactivity as indicated in the literature and suggested by the manufacturer or were directed against a specific equine protein (eg, CD14). Cells were incubated with the secondary antibody only as a control to account for any background staining. Equine endothelial cells used as a positive control had positive results for the same assay, leading to the conclusion that the equine ECFCs tested positive for endothelial markers, which characterized them as ECFCs. The selected proteins are not only important endothelial cell markers but also have important roles in the functions of EPCs and endothelial cells. The glycoprotein vWF functions in hemostasis and endothelial cell adhesion. The key receptor vascular endothelial growth factor, VEGFR-2, is critical for endothelial cell development. Intracellular signaling by VEGFR-2 effectively promotes the survival, proliferation, permeability, and migration of EPCs and endothelial cells. Expression of VEGFR-2 by equine ECFCs is critical for their successful isolation and sustained culture. The CD34 marker is a glycoprophosphoprotein found on lymphohematopoietic and early stem cells or progenitor cells, endothelial cells, and embryonic fibroblasts. Investigators have shown that human cells positive for CD34 and VEGFR-2 expression isolated from umbilical cord blood, bone marrow, or peripheral blood differentiated into endothelial cells in culture; thus, the presence of CD34 is commonly seen as a way to distinguish EPCs from non-EPCs and stem cell progenitors. The CD105 marker is a homodimeric transmembrane protein associated with proliferation through transforming growth factor-β signaling and is predominantly found on endothelial cells. The positive IFA results for vWF, VEGFR-2, CD34, and CD105 provided additional evidence that equine cells isolated in the present study were true ECFCs.

One unexpected result in the present study was that most of the isolated equine cells expressed CD14. A receptor for the binding of lipopolysaccharide complex, CD14, allows for initiation of the immune response to bacteria and is a cell surface marker for macrophages and hematopoietic cells. Expression of CD14 is not limited to cells of myeloid lineage, and it is expressed on early EPCs in humans and at low concentrations in vascular endothelial cells. Human EPCs have been extensively characterized, and much controversy has existed regarding nomenclature and marker expression. To the authors’ knowledge, there is no single marker or group of markers that can distinguish ECFCs from terminally differentiated endothelial cells, which causes specific identification to rely on characteristics such as growth and function as well as marker identification. One difference between human early EPCs and ECFCs is that early EPCs express CD14 and CD45, and ECFCs do not. This suggests a hematopoietic origin of early EPCs. We do not believe, however, that the cells isolated in the present study were early EPCs, considering that early EPCs isolated from humans do not form vascular tubules in vitro, they appear in culture at <7 days, and their morphology differs from that of ECFCs. We do not know whether that cell type circulates in horses or whether the lineages for ECFCs are similar in horses and humans. It is unlikely that cross-reactivity of the

Figure 8—Representative photomicrographs (merged images) showing endothelial marker expression in equine ECFCs and equine carotid endothelial cells (EC; positive control). Equine ECFCs and ECs tested positive for expression of CD34 (red), CD105 (green), vWF (green), and VEGFR-2 (green) via IFA. Nuclei are stained with DAPI (blue). Bar = 50 µm.
anti-CD14 antibody caused a false-positive result because equine endothelial cells were CD14 negative, and the peripheral blood samples tested positive in a manner similar to that previously shown for equine monocytes. The detection of CD14 on equine ECFCs is likely attributable to a species difference or may suggest the presence of > 1 cell type in the isolated population. The expression of cell surface markers of ECFCs in species other than humans or rodents is not as fully characterized, which may be caused in part by a lack of species-specific antibodies. For example, protein expression of CD34, vWF, CD146, VEGFR-2, CD31, and endothelial nitric oxide synthase has been evaluated in EPCs from dogs, but to the author's knowledge, CD14 and CD45 protein expression have not. Other types of equine cells (such as MSCs) have also been shown to vary slightly in gene expression from their human counterparts. Previous research with equine MSCs has shown that these cells test positive for CD14 expression, suggesting that equine MSCs derive from CD14-positive cells, unlike MSCs described for other species. Those investigators used the same anti-equine CD14 antibody as was used in the present study, and their results disputed the definition of MSCs as being derived from nonhematopoietic precursor cells in horses. Interestingly, the anti-CD14 antibody

Figure 9—Dot plots generated by flow cytometric analysis of cell surface marker expression for ECFCs isolated from 3 horses and for control samples. A — The percentage of ECFCs that were CD14-positive (lower right quadrant) in each of 3 samples (1 sample/horse) is shown; expression of CD14 in equine whole blood gated for monocyte size was used as a positive control. Equine carotid endothelial cells (EC; negative control) were CD14-negative. Gating for equine whole blood monocytes on the basis of forward scatter (FSC) and side scatter (SSC) characteristics is also shown. B — The percentage of ECFCs that were CD105-positive (upper right quadrant) in each of the 3 samples is shown. Equine carotid endothelial cells were used as a positive control. Unstained cells were incubated with the secondary antibody only (fluorophore 488–conjugated goat-anti mouse IgG) to evaluate background staining. PE-H = Phycoerythrin height.
in that study, was found to be trypsin labile, which was not the case in the present study. Further investigation of cell surface marker characterization over a range of passages, with isolation of single colonies or separation and characterization of cells on the basis of CD14 expression, would answer these questions more specifically, but the ability to form vascular tubules is a characteristic that many other cell types would not have. Results of 1 study showed that ECFCs from peripheral blood of human subjects tested positive for CD14 expression. This supports the conclusion that no single marker is capable of identifying EPCs and their subtypes and that a combination of characterization methods is needed for these distinctions.

Clinical evidence indicating that stem cell–based treatment improves clinical outcomes in horses with tendon and ligament injuries has been described, but questions remain concerning whether the observed effects are attributable to stem cell engraftment and tissue regeneration or to paracrine mechanisms. The use of biomaterial scaffolds to optimize the function of stem cells is becoming increasingly common. Current research in this area for horses has included the investigation of equine MSC migration out of fibrin hydrogels, treatment of meniscal lacerations with MSCs and fibrin glue, 3-D culture conditions and chondrogenesis, and creation of a decellularized tendon as scaffold for regenerative treatment. Promoting rapid vascularization is one of the biggest challenges in the potential application of engineered tissues for enhancement of wound healing and treatment of disease. Combining EPCs with engineered biomaterials could allow the direct delivery and retention of EPCs as well as the appropriate growth factors and signaling molecules needed in the area of interest. Although engineering complex tissues may not be an immediate clinical goal in equine research, to continue to advance the field of equine regenerative medicine, understanding and developing methods to promote equine vascularization are essential, and ready access to equine EPCs is a key part of that process.

In standard 2-D cell culture conditions, cells are not in their native environment; they are exposed to trypsinization, grown on surfaces not necessarily optimized for cell performance, and lack the same environmental stimuli that are present in vivo (eg, shear stress). When investigating cells for use in therapeutic applications, it is imperative to evaluate the duration for which cells can remain in culture before losing their functional properties. The subjectively scored quality of equine ECFC tubules in the present study was significantly lower for passages 5 through 10 than for passages 1 through 4, and ECFCs had a significant decrease in uptake of Dil-Ac-LDL for passages 6, 8, and 10, compared with passages 1 through 4. On the basis of results of this study, we recommend that equine ECFCs be used for research at or before passage 5. This lifespan of the cells’ performance is similar to that reported for human ECFCs in that cells can undergo about 100 cell doublings before reaching cell senescence. Given the importance of EPCs in human tissue engineering and regenerative medicine applications and the advances being made in equine regenerative medicine, equine EPCs will be a valuable resource. Future studies to validate these findings in vivo will be required prior to clinical use.

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The first 2 authors contributed equally to the scientific content of the manuscript.

Footnotes

a. EGM-2 with Bullet Kit, Lonza, Visp, Switzerland.
b. HyClone Laboratories Inc, Logan, Utah.
c. Ti Eclipse, Nikon Corp, Melville, NY.
d. Lonza, Visp, Switzerland.
e. BD Biosciences, Bedford, Mass.
f. Poietics Human Endothelial Colony Forming Cells, Lonza, Visp, Switzerland.
g. NH 373 cells, American Type Culture Collection, Manassas, Va.
h. BD Matrigel Basement Membrane Matrix, BD Biosciences, Bedford, Mass.
i. Dil-Ac-LDL, Biomedical Technologies Inc, Stoughton, Mass.
j. DAPI, Life Technologies, Grand Island, NY.
l. Worthington Biochemical Corp, Lakewood, NJ.
m. Cytomation MoFlo High Speed Cell Sorter, Beckman-Coulter Inc, Brea, Calif.

References


