Culture and Characterization of Endothelial Colony Forming Cells from Peripheral Blood, Bone Marrow, and Umbilical Cord Blood of Horses

by

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Abstract

Endothelial progenitors cells (EPCs), are a type of progenitor cell originating in the bone marrow and can be found circulating in peripheral blood, bone marrow, and umbilical cord blood. EPCs play an essential role in the formation of new blood vessels as well as maintaining vascular repair and homeostasis. These cells have become an intense area of interest in regenerative therapy due to the fact that they augment the formation of new blood vessels and promote endothelialization. Recent research has also shown that in humans with cardiovascular disease and metabolic disease there is a reduction in the number and function of EPCs. Since endothelial progenitor cells have yet to be investigated in horses and given the prevalence of diseases that can result in vasculature damage to horses, studying equine EPCs is an advantageous endeavor. Utilizing established protocols for human EPC isolation, we proposed that a specific subtype of EPCs, endothelial colony forming cells (ECFCs), could be isolated and cultured from peripheral, bone marrow, and umbilical cord blood samples from horses. Upon successful isolation and expansion, cells were characterized as true ECFCs using functional assays of acetylated low density lipoprotein (Ac-Di-LDL) uptake assay and vascular tube formation during culture in a Matrigel Basement Membrane Matrix. Cells from peripheral blood samples were analyzed for expression of specific cell markers including CD34, CD105, vascular endothelial growth factor 2 (VEGFR-2), and Von Willebrand factor (vWF). These markers were assessed via indirect immunofluorescence. Flow cytometry analysis was performed using the cell markers

CD14 and CD105. The number of cell colonies were recorded as well as cell performance in characterization assays including percent positive cells for the uptake of Di-Ac-LDL and vascular tube scoring of the Matrigel tubule formation assay. Cell performance and maximum number of passages before cell senescence were assessed on cells from peripheral blood samples through assessment of uptake of Ac-Di-LDL and vascular tube formation. Investigating equine endothelial progenitor cells and establishing culture and characterization methods is the initial step in gaining knowledge as to how these cells could be beneficial in equine regenerative medicine. Further investigation of the ECFCs will be carried out in future studies upon the establishment of a successful isolation protocol.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

EPC	Endothelial Progenitor Cell
ECFC	Endothelial Colony Forming Cell
Dil-Ac-LDL	Acetylated Low Density Lipoprotein
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
vWF	Von Willebrand Factor
ESC	Embryonic Stem Cells
iPSC	Induced Pluripotent Stem Cell
DNA	Deoxyribonucleic Acid
HSC	Hematopoietic Stem Cell
ECM	Extracellular Matrix
PEG	Poly (ethylene glycol)
PVA	Polyvinylalcohol
MSC	Mesenchymal Stem Cell
VEGF	Vascular Endothelial Growth Factor
EOC	Early Outgrowth Endothelial Cell
LOC	Late Outgrowth Endothelial Colony Forming Cell
CEPCs	Circulating Endothelial Progenitor Cells
BOECs	Bone Outgrowth Endothelial Cells
LOEPCs	Late Outgrowth Endothelial Progenitor Cells

CACs	Circulating Angiogenic Cells
EPDCs	Endothelial Progenitor-Derived Cells
ECs	Endothelial Cells
SDF-1	Stromal Cell-Derived Factor-1
MMP-9	Matrix Metalloproteinase 9
NO	Nitric Oxide
PGLS-1	P-selectin Glycoprotein Ligand-1
ICAM-1	Intercellular Adhesion Molecule 1
IGF2	Insulin-like Growth Factor 2
IGF2R	Insulin-like Growth Factor 2 Receptor
ATP	Adenosine Triphosphate
EHS	Englebreth-Holm-Swarm
TGF-β	Transforming Growth Factor Beta
Ac-LDL	Acetylated Low Density Lipoprotein
VPN	Vascular Permeability Factor
RNA	Ribonucleic Acid
WPBs	Weibel-Palade Bodies
Ang-2	Angiopoetin 2
CD	Cluster of Differentiation
LPS	Lipopolysaccharide
MPC	Mesenchymal Progenitor Cells
RGD	Tripeptide Arginine-Glycine-Aspartic Acid
EBM-2	Endothelial Basal Medium

PBS	Phosphate Buffered Saline
DAPI	4',6-diamidino-2-phenylindole
TRITC	Tetramethylrhodamine Isothiocyanate

Chapter 1: Literature Review

Stem Cells a Brief Overview

Late outgrowth endothelial colony forming cells are a type of endothelial progenitor cell which are classified as an adult stem cells. Stem cells are an undifferentiated cell type that can undergo efficient and accurate self-renewal through cell division even after periods of inactivity. Stem cells are able to differentiate into many cell types in the body and, in some cases, any type of cell in a multicellular organism.

There are two main types of stem cells, pluripotent and adult stem cells. Pluripotent stem cells, as there name suggests (pluri = several and potent = having great power), are able to differentiate and give rise to all three germ layers. Germ layers, the ectoderm, mesoderm, and endoderm, are the initial layers of cells that will form during embryogenesis. The ectoderm, which is the external germ layer, will give rise to cells such as skin, neuronal cells, and pigment cells. The mesoderm differentiates into cells such as cardiac, skeletal muscle, blood, and endothelial. Finally, the endoderm will give rise to lung, pancreatic, thyroid, and other internal organ cells. Traditionally, pluripotent stems cells are associated with originating from the inner cell mass of the blastocysts and are termed embryonic stem cells (ESC). The blastocyst is a spheroid formed about 5 days post fertilization consisting of the trophoblast (outer layer of cells), the blastocoel (cavity which is fluid filled), and the inner cell mass (collection of cells on the interior of the blastocyst). The inner cell mass is separated from the other two components of the

blastocyst and are plated on feeder cells and allowed to expand giving rise to embryonic stem cells.

The second type of stem cell is the adult stem cell. Adult stem cells can come from a variety of sources such as adipose tissue, bone marrow, peripheral blood, and umbilical cord blood. Adult stem cells have various levels of potency including multipotent and oligopotent progenitor cells. Multipotent progenitor cells have a finite proliferative ability, yield oligopotent progeny, and have the ability to self-renew; however this ability is limited when compared to pluripotent cells. Oligopotent progenitor cells will only differentiate into a limited array of cell types, have limited ability to proliferate, and typically cannot undergo self-renewal.

In search for alternative sources of pluripotent stem cells, a second type of pluripotent stem cell entered onto the stage in 2006 called induced pluripotent stem cell (iPSCs) [1]. This stem cell type is obtained by genetically reprogramming adult cells so they will take on pluripotent embryonic stem cell characteristics. Shinya Yamanaka's research team demonstrated four factors (Oct4, Sox2, cMyc and Klf4) used to genetically reprogram cells and produce iPSCs [1]. The discovery of iPSCs has given researchers the ability to bypass the use of embryos as a source of pluripotent stem cells. The ability to transform somatic or rather adult stem cells into a pluripotent state of being is another step forward in stem cell research. This process of using strong stimuli to produce iPSCs can avoid the use of genetically reprogrammed methods that rely on lentiviral or retroviral deoxyribonucleic acid (DNA) to reprogram the genes which are often unsuitable for use in human stem cell therapy. Induced pluripotent cells can increase the risk of tumor formation from multiple germlines. iPSCs reprogrammed without foreign

DNA and vectors allows the cells to better suit the needs for stem cell therapy and reduce the risk of adverse outcomes. The potential benefits of stem cell therapy and regenerative medicine are extensive even with the current scope of clinical applications being narrow; however, there is still an abundance of research left to explore.

Regenerative Medicine

The National Institute of Health defines regenerative medicine as the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage, or congenital defects. It is not uncommon for a lower level species such as a salamander that is lacking a limb to undergo total limb regeneration. However, only slight levels of regeneration are seen in higher mammals. An example would be the missing portion of a fingertip being regenerated. Regeneration in higher mammals is primarily seen at a cellular level such as epidermal cell turnover [2]. The appeal of studying how tissues are regenerated has increased and combined the efforts of various fields including stem cell biology, developmental biology, bioengineering, tissue engineering, and material engineering. Although re-growing an amputated limb would be a much celebrated accomplishment, regenerative medicine had to initiate from the beginning with understanding the biological mechanisms behind the process of regeneration in nature.

The study of stem cells has advanced greatly since the discovery of hematopoietic stem cells in mice in the 1960s. Hematopoietic stem cells (HSC) are the most highly studied type of stem cell to date [2-4]. Since their discovery the ability to isolate, characterize, culture, and utilize various stem cells for therapy has improved. Stem cell

therapy has been administered in therapeutic applications using stem cells alone, stem cells combined with a scaffold, and stem cells repopulating decellularised tissues. Stem cells can be harvested from a niche such as bone marrow, umbilical cord blood, or peripheral blood, expanded in culture, and then injected back to the site of injury. The avenue of stem cells being used as a regenerative therapy is appealing in that it is non-invasive and requires a fairly uncompleted procedure. Although experimental procedures using stem cells have shown promise in diseases such as cardiac disease, ligament regeneration, and stroke, the exact mechanism by which these cells are allowing for improvement of damaged areas is still unclear. It is thought that perhaps the cells are not just acting themselves to regenerate the injured tissue but have a paracrine effect as well. It is also known that following injection of a stem cell suspension, the percentage of the cells remaining at the site of injection and thus afflicted area is low. Studies injecting stem cells are continuing to move forward; however, to date, the only injectable cell therapy that is widely used clinically is injectable hematopoietic stem cells [5, 6]. The knowledge that stem cells do not stay localized to the injection site and the known benefit of adding growth factors to stem cell suspensions has led many researchers to study combining stem cells and supporting bioactive materials.

Bioengineered scaffolds can be produced from a wide range of natural and synthetic materials or a combination of the two. Bioengineered scaffolding is typically solid whereas a bioengineered matrix is typically not solid. They can be injectable or non-injectable and can have a range of complexity. Repair of tissues takes place in the extracellular matrix (ECM), environment and many bioengineered scaffolds can be and are often fashioned to mimic the ECM in hopes to generate the most appropriate scaffolds

for a given application. Scaffolds can be designed and fine-tuned for specific applications including the architecture of the material, bioactivity, and mechanical properties such as stiffness and elasticity [7]. One of the most important factors to consider with bioengineered scaffolds is if the scaffold is compatible with the tissue and that it will not have adverse effects. Scaffolds allow for cell adhesion and migration, maintenance of cell location at the site of injury to be treated, and delivery of nutrients and/or growth factors to enhance cell survival and function. Scaffolds can also be designed to be degradable or non-degradable depending on the application and can be designed to have the appropriate degradation rate allowing for optimal tissue regeneration process of the injured area.

These three-dimensional bioengineered structures can be generated in a few main approaches [7]. The first is the construction of a porous scaffold that is made prior to the seeding of cells. Natural materials used in the scaffold construction will typically allow for better biocompatibility, but have limitations in the ability to fine tune the mechanical and physical properties of the substance. The other option is to use synthetic materials (inorganic or organic) or a hybrid using both natural and synthetic materials. With the choice of synthetic materials, the gain in control over the physical and mechanical properties results in a loss of optimal biocompatibility. Another process is cell sheet development which allows cells to produce their own ECM and then they can be harvested as sheets without the use of enzymatic removal of the cells from the *in vitro* culture conditions. The use of cell sheets is advantageous for regenerative medicine to repair endothelium and epithelial areas that are damaged; however, they have a disadvantage due to the inability to produce thick layers of cell sheets. Another approach

is the use of a decellularised ECM, which allows for the natural environment to be most similar for the re-seeding of cells. All of the cell components other than the ECM of the tissues will be removed by specific decellularization techniques which can include the combination of chemical, physical, and enzymatic processes [7-9]. A final method to mention is a process where living cells are encapsulated in a hydrogel that is formed by crosslinking of the material through ionic or covalent interactions. Some of the most well-studied and widely used substances for cell encapsulation include naturally occurring algae polysaccharides, collagen, hyaluronic acid, chitosan, poly (ethylene glycol) (PEG), and polyvinylalcohol (PVA) [7].

The future of regenerative and stem cell therapies hold great promise. One of the most advanced areas of regenerative medicine and tissue engineering is the work that has been done for the treatment of individuals suffering from severe burns, diabetic ulcers, and other traumatic chronic wounds [10, 11]. The development of materials mimicking the natural epidermal and dermal layers of skin that will aid in healing of wounds have been and are continuing to be investigated. In addition to the material, the use of cells incorporated into these biomaterials has also emerged as a treatment option and shown to be of benefit to severe and chronic wound healing [11-14]. This use of biomaterials with and without cells is working to function as a support or scaffold for the regeneration of healthy tissue and to aid in the adhesion, differentiation, and growth of cells during the wound healing process [13, 14]. Some treatment options include skin grafting either though autograpfts, allografts, or xenografts, and tissue engineered constructs [15]. Various tissue engineered construct options are commercially available for people that include epidermal substitutes, dermal substitutes, and composite substitutes [11, 12, 16].

However, the biggest challenge to the creation of complex tissues of any type when using biological scaffolds is the vascularization of the engineered tissue [17]. Methods to augment vascularization are pore size of the hydrogel, use of growth factors, and encapsulation of cells such as endothelial progenitors that produce blood vessels. By using stem cells, and in some cases accompanied by engineered scaffolds, steps are being made to move towards regenerating tissues, engineering complex tissues, and possible organ replacement. The use of stem cells in cases of cardiac myocardial infarction has made advancements. The heart is no longer thought to be an organ incapable of regeneration but in fact an organ that has regenerative potential from cardiac and other progenitor cell types [18]. There is evidence that injecting a stem cell suspension into the damaged area of the heart can have potential benefits in reducing scar size and increasing heart function; however, more studies need to be done before actual clinical applications become more available [19]. The benefits and potential for applications of stem cells continues to grow and outstanding research will continue and clinical applications of regenerative therapies are going to become more common and widely accepted.

Regenerative medicine and regenerative therapies are not foreign concepts in the horse industry and it is continuing to be a rising area of interest. Studies have shown promising results using stem cell treatment as a method for tendon injuries treatment and is becoming a standard procedure in many places using stem cell types such as mesenchymal stem cells (MSC), being harvested from either the bone marrow or from adipose tissue [20, 21]. These samples are processed and then cultured, expanded, and injected back into the horse at the site of injury. The use of biomaterial scaffolds to optimize the function of stem cells is becoming a focus of interested in research for

injected stem cells that do not maintain the location at the injection site. Work in this area in the horse has begun with 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 therapy [22-25]. Again, with regenerative medicine in horses, vascularizing tissues will be a key issue to address and although engineering complex tissues may not be an immediate clinical goal in the horse, in order to continue to advance the field of equine regenerative medicine, the development of methods and understanding of vascularization of biological scaffolds is essential. In any case, where vascularization of a tissue is required, endothelial progenitor cells are an appealing cell type in that they can form blood vessels *de novo*.

The Vascular System

The circulatory system is vital to maintain life and consists of the heart and blood vessels. The heart acts as a pump to circulate blood through the body via the blood vessels. Blood is a fluid that mammals use as a delivery mechanism for nutrients and removal of metabolic waste products, both being essential for the healthy functioning of the body. Blood is comprised of red blood cells that shuttle oxygen throughout the body, white blood cells that are active in the immune system, platelets which work to prevent excessive bleeding, and plasma that holds all cells in suspension. Plasma is made up of mainly water with a small percentage of proteins (albumins, gloubulins, minerals, sugars, fats, hormones, vitamins, and anti-clotting factors). Monocytes and adult stem and progenitor cells such as endothelial progenitor cells are also found circulating in peripheral blood.

There are several terms used to describe the formation of blood vessels and each term is associated with a type of blood vessel formation. Angiogenesis is defined as the formation of new capillaries from preexisting vessels via sprouting or splitting [26]. Vasculogenesis is defined as the *de novo* formation of a primary vascular network from the assembly of precursor cells such as progenitor cells Arteriogenesis is the remodeling of existing blood vessels resulting from blood flow, sheer stress, and vascular dilation [26]. Capillogenesis is the universal formation of capillaries while the individual undergoes growth and development [26]. Finally, neovascularization is a general term that encompasses any new blood vessel formation of any size in the adult [26].

Angiogenesis occurs in response to specific signals causing an endothelial cell located on an existing vessel to send a single, long pseudopodia out initiating the formation of a new vessel. This extension will continue developing outward until it encounters another existing vessel where it will then attach and allow for blood flow to occur through the newly formed vessel. In embryonic development, transmembrane protein ephrin-B2 (in arteries) and the receptor Eph-B4 (on veins) mediate signals sent between cells when two endothelial cells are in contact with each other. This aids in the proper organization of vessels and regulation of the growth of blood vessels based on tissue need [27]. Usually tissues have a need for more blood flow to provide oxygen and this need results in the stimulation of angiogenesis. The lack of oxygen results in the creation of a hypoxic environment. This hypoxic environment increases hypoxia-inducible factor 1 which encourages the production of VEGF, which will act on endothelial cells to encourage angiogenesis. There are two cell phenotypes that work together in angiogenesis. They are called the leading cell which is migratory and

polarized, and stalk cells that will proliferate during the extension of the new vessel [28]. The Notch signaling pathway plays an important role in directing which cells will become leading cells and which cells will become stalk cells. The Notch signaling pathway is considered to be a highly conserved communication pathway between cells both in embryonic and adult life. Endothelial cells will respond to VEGF by selecting a leading cell and then producing proteases that will digest the basil lamina of the existing vessel allowing for the migration of the leading endothelial cell in the direction of the VEGF gradient. Leading cell selection is thought to be the result of the interaction of VEGF and notch/DLL4 signaling in a feedback loop mechanism, where once the leading cell is selected, all surrounding cells are inhibited from taking on the same leading cell phenotype [28]. Cell proliferation will take place by the stalk cells to form the new vascular tube structure with the production of a lumen and basement membrane [28, 29]. The increase in blood flow to the tissue that had previously been experiencing a hypoxic environment will have a negative feedback action causing the hypoxia-inducible factor 1 to become inactive subsequently degraded due to the increase in oxygen. It is important to note that there are many other growth factors that could play a role in angiogenesis, such as fibroblast growth factor and angiopoetin 1, which is thought to be a key partner for VEGF and helps with the stabilization of the newly formed vessels [30].

Vasculogenesis is the process by which endothelial cells differentiate and proliferate in an area that was previously avascular [30]. The development of vascular networks, once thought only to occur during embryonic development, is seen in postnatal life allowing for the construction of new vasculature and the maintenance, homeostasis, and repair of established vasculature. This ability of the body to undergo vasculogenesis

in adult life is now attributed to the action of EPCs. In the beginning of human development, the formation of blood vessels will appear on the egg yolk sac, which is located outside of the embryo. In the mesoderm collections of mesenchymal cells will form adjacent to the extraembryonic endoderm [31]. These collections of cells are called blood islands and located here are hemangioblasts that will go through initial differentiations. Cells located on the outside of the blood islands will differentiate into precursors for endothelial cells and the hemangioblasts located on the inside will differentiate into hematopoietic precursors. This vascularization of the yolk sac will begin about 7.5 days after conception in humans. The aggregates of cells will continue to proliferate and differentiate resulting in the establishment of the embryonic stems of the yolk sac, arteries and veins, the dorsal aortae, and the cardinal veins [31]. Vascular endothelial growth factor along with VEGF receptors (VEGFR) are the initial endothelial cell specific signaling pathways to be established [31, 32]. The establishment of the vasculature will continue as the peripheral cells of the blood island work to produce capillaries, arteries, and veins of the developing embryo.

Both endothelial cells and endothelial progenitor cells contribute to postnatal vasculogenesis. Vasculogenesis includes the establishment of new vessels either in the healthy body or in situations of tumor growth. The main regulator of postnatal vasculogenesis is VEGF. Other factors that could play a potential role are granulocyte macrophage colony stimulating factor, stem cell growth factor, and insulin [32]. By understanding postnatal vasculogenesis, there is the potential to better understand how to assist therapeutically in diseases that involve ischemia or in cases of severe wounds. It will also allow for engineered tissue constructs to be more successful and viable. There

are also potential benefits when treating tumors through blocking of signals and growth factors that would work to establish a vascularized tumor. The major role that endothelial cells and endothelial progenitor cells play in vascularization of tissues has led to their intensive investigation of their basic biology and what benefits we can gain from utilizing these cells in regenerative medicine and tissue engineering.

Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) are defined as an oligopotent stem cell. They can differentiate into only a single terminally differentiated cell type, endothelial cells, which line the heart and blood vessels of vascular system. EPCs were first described in 1997 by Asahara *et al*, and since their discovery they have been investigated to be used as biomarkers of diseases and used in regenerative medicine therapies [33-35]. EPCs can be found and harvested from bone marrow, peripheral blood and umbilical cord blood; however, they originate in the bone marrow [33]. Bone marrow is located in the interior of long bones of the body and is the location of red blood cell production and storage as well as the production and storage location of many types of adult stem cells. EPCs can be mobilized from the bone marrow by factors such as severe endothelial damage, stromal cell-derived factor-1, matrix metalloproteinase, and vascular endothelial growth factor (VEGF) [36-39]. Once EPCs are circulating in the bloodstream, they home to sites of injury, undergo proliferation, and differentiate into mature endothelial cells to reconstruct damaged areas of vasculature [36, 40].

To date, there has not been a specific marker acknowledged to be an accurate identifier of endothelial progenitor cells [41]. This lack of a single specific marker has

also been difficult to determine based on the fact that the nomenclature for EPCs has varied greatly over the years of investigation. The varied nomenclature is most likely the result of the fact that there are subtypes within the main term "EPC". There are two main subtypes of endothelial progenitor cells, early outgrowth endothelial colony forming cells (EOCs) and late outgrowth endothelial colony forming cells (LOCs or ECFCs), both with specific characteristics. Other terms that have been used include circulating endothelial progenitor cells (CEPCs), bone outgrowth endothelial cells (BOECs), late outgrowth endothelial progenitor cells (LOEPCs), circulating angiogenic cells (CACs), and endothelial progenitor-derived cells (EPDCs), to name a few [33]. These cell types have traditionally been characterized by using a combination of cell markers, functional properties, and phenotypic observations. EOCs are characterized by appearing in culture in less than 7 days and being positive for the endothelial markers of CD31, CD34, CD105, vascular endothelial growth factor receptor 2 (VEGFR2), von Willebrand Factor(vWF). The two distinguishing factors of EOCs from ECFCs is that EOCs are positive for hematopoietic markers of CD14, CD45, and CD133 and will not form vascular tubes *in vitro* [33, 35, 42, 43]. ECFCs, which are well characterized in humans, appear in culture after 7 days and show uptake of low density lipoprotein. ECFCs are positive for endothelial markers of CD31, CD34, CD105, vascular endothelial growth factor receptor 2 (VEGFR2), von Willebrand Factor (vWF), and are negative for hematopoietic markers of CD14, CD45, and CD133 [33, 35, 42, 43]. ECFCs also have the ability to form vascular tubes *in vitro* unlike EOCs which cannot. ECFCs are considered to fit the true definition of an endothelial progenitor cell in that the cells can undergo vasculogenesis and the terminally differentiated progeny are endothelial cells

(ECs) [43]. ECFCs are characterized by having a single layer of cell growth with cobblestone appearance to the cells. Table 1 outlines the differences between EOCs and ECFCs.

EPCs function in the process of the maintenance of vascular homeostasis and repair in postnatal life. However, the exact mechanisms and specific functions of EPCs in addition to how to characterize these cells are continually debated. Endothelial cells that line blood vessel walls need to be replaced over time due to various reasons such as denudation, natural endothelial cell turnover, and injury to the endothelium. Figure 1 shows the process of endothelial cells turnover based on the information presented by Richardson and Yoder [42]. The healthy endothelium undergoes natural turnover of cells when the endothelium is damaged or undergoes denudation. Platelets that are found circulating in peripheral blood will aggregate at the damaged area followed by the recruitment of proangiogenic hematopoietic cells. Finally, ECFCs will home to the site under repair, adhere, and undergo proliferation and eventually differentiate into mature endothelial cells. For the healing of a traumatic injury, hemostasis will occur allowing for the discontinuation and prevention of any further hemorrhage. Recruitment of ECFCs will shortly follow hemostasis. ECFCs adhere, proliferate, allow for lumen formation, and ultimately differentiate into mature endothelial cells resulting in healing of the wound. It is obvious that the maintenance of vascular homeostasis and repair of a traumatic injury requires not only ECFCs but other cells types as well; however, without ECFCs there would be an incomplete set of tools to undergo effective regeneration and repair.

Differences Between ECFCs and EOCs		
Marker/functional test	**Late Outgrowth Endothelial Colony Forming Cells (ECFCs)**	**Early outgrowth cells (EOCs)**
Cells appear in culture	Days > 7	Days < 7
Cell Expansion	Significant outgrowth	Little outgrowth
Endothelial markers	$CD31^+/CD34^+/CD105^+/CD146^+$ $VEGFR2^+/VE\text{-}Cadherin^+/vWF^+$	$CD31^+/CD34^+/CD105^+/CD146^+$ $VEGFR2^+/VE\text{-}Cadherin^+/vWF^+$
Hematopoietic markers	$CD14^-/CD45^-/CD133^-$	$CD14^+/CD45^+/CD133^+$
In vitro vascular tube formation	YES	NO
In vivo neovascularization	YES, more than EOC	YES

Table 1: Differences in characteristics between the two subtypes of endothelial progenitor cells (EPCs), late outgrowth endothelial colony forming cells (ECFCs) and early outgrowth cells (EOCs).

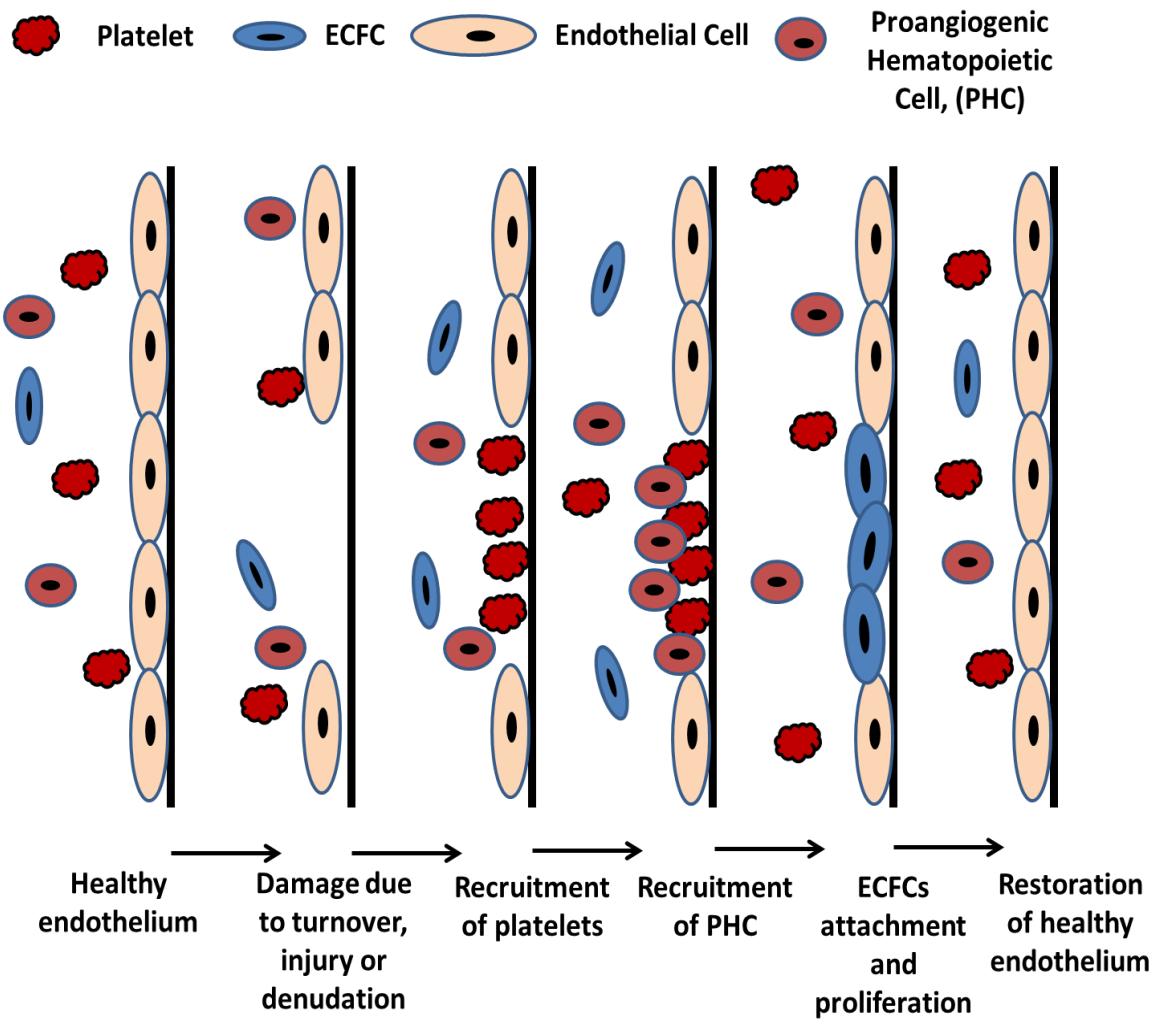

Figure 1: Maintenance of vascular homeostasis and repair by ECFCs. The healthy endothelium will have natural turnover of cells, have repair of damaged areas, or cell replacement when endothelial cell denudation occurs. Platelets will aggregate at the damaged area that are found circulating in peripheral blood followed by the recruitment of proangiogenic hematopoietic cells and finally ECFCs will home to the site of repair, adhere and undergo proliferation and eventually differentiate into mature endothelial cells. Modified from Richardson, M.R. and M.C. Yoder, *Endothelial progenitor cells: quo vadis?* J Mol Cell Cardiol, 2011. 50(2): p. 266-72.

EPCs have also been shown to be a contributor to complications when increased vascularization is unwanted. Tumor enlargement upon reaching a size of 1-2 mm^3 requires neovascularization of the tumor for further growth and nutrient support. Thus, the developing tumor will release mobilization factors that will cause the release and homing of EPCs to the site of the tumor [44]. The further development of a tumor is not considered a beneficial occurrence in that tumors themselves, even if non-malignant, pose health problems and are the result of an imbalance of normal homeostasis. However, because EPCs home to tumors, they may have the potential to aid in therapeutic treatment of tumors by allowing for the delivery of genes, proteins, and other methods of treatments to the tumor site. There are also potential ways to improve tumor imaging methods [44]. As with most biological systems the balance of EPCs is tightly regulated where unwanted outcomes would occur with either lack of or abundance of EPCs.

Mobilization and homing of EPCs is a complex process with more exact information regarding the homing and mobilization of EPCs still unknown. Mobilization and recruitment of EPCs is usually referring to the release from a stem cell niche, in this case, bone marrow. The niche allows for the storage of stem cells where they exist in an undifferentiated state and are an army of regenerative capacity waiting to be called upon and deployed. As stated previously, signals such as severe endothelial damage, stromal cell-derived factor-1 (SDF-1), matrix metalloproteinase 9 (MMP-9), and vascular endothelial growth factor (VEGF), cue the release of EPCs from the bone marrow niche [36-39]. VEGF is essential for EPC maintenance, proliferation, chemotaxis, and differentiation and is a mobilizer of EPCs and spurs angiogenesis [45]. Other factors

have been shown to play a role in the mobilization of EPCs such as the activation of the MMP-9 pathway. The MMP-9 pathway will allow for the conversion of the Kit ligand (KitL), which is membrane bound, into a soluble Kit ligand (sKitL) [36, 45]. The soluble KitL will allow for heightened movement of cells that are VEGFR-2^{+}, (EPCs), moving from bone marrow to the peripheral blood stream [45]. Another point to mention is that MMP-9 is thought to be dependent on nitric oxide (NO), which is concluded based on the fact that in endothelial NO knockout mice, there is a reduction in MMP-9 activity even when mice were stimulated with VEGF [45]. SDF-1 recruits and retains stem cells in areas that are experiencing ischemia [46]. SDF-1 is upregulated when inflammation is present as well as other situations of hypoxia, changes in mechanical forces, or changes in the extracellular matrix [45, 47]. SDF-1 alone is not able to initiate neovascularization, and VEGF may be the additional signal needed for successful neovascularization to occur. There are other studies that show exercise increases the number of circulating EPCs, and this increase could be the result of an increase in VEGF [48, 49]. Erythropoietin and statins have also been shown to increase circulating EPCs resulting from the release of the cells from the bone marrow; however, long term use of statins can have the inverse effect and reduce the number of circulating EPCs [45]. Erythropoietin is thought to increase the mobilization of EPCs from the bone marrow through upregulating VEGF [50]. The exact mechanism of how statins increase the number of circulating EPCs is not known. However, the involvement of nitric oxide related mechanisms, such as the stimulation of the Akt/eNOS pathway, are thought to be involved [51, 52]. Hristov *et al.* have illustrated the mobilization of EPCs from the bone marrow and is depicted in Figure 2.

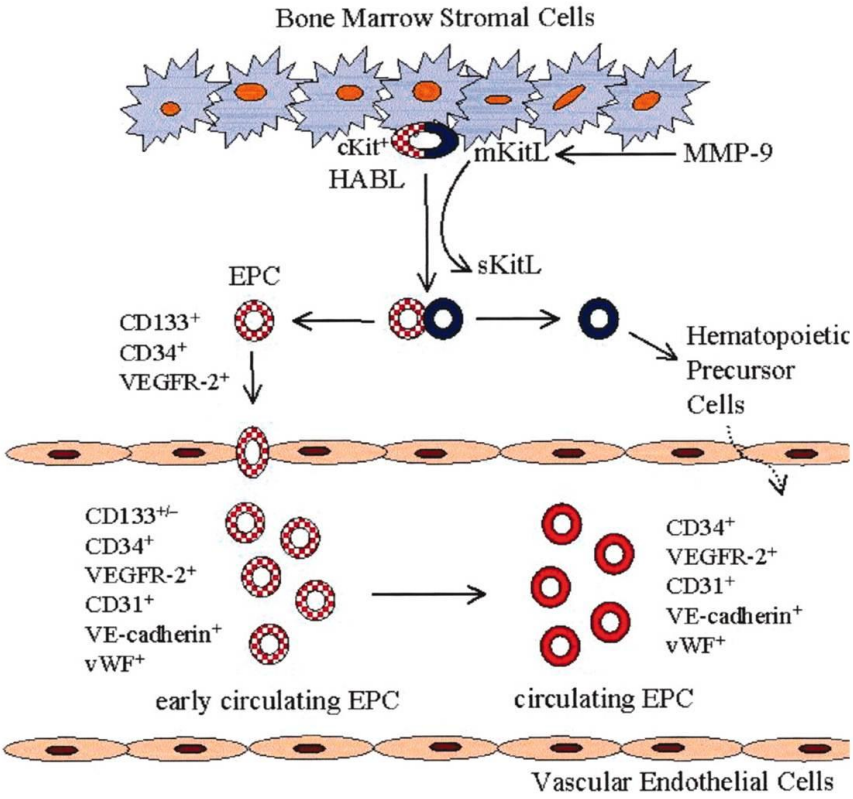

Figure 2: EPCs mobilization from the bone marrow. This process is the result of a variety of factors including the activation of matrix metalloproteinase-9 (MMP-9) acting to convert membrane-bound Kit ligand (mKitL) to Kit ligand which is soluble (sKitL) resulting in the mobilization of endothelial progenitor cells (EPCs) from the bone marrow into peripheral circulation. Also shown is the early circulating EPCs and the markers on which they are positive for (CD133, CD34, VEGFR-2, VE-cadherin and von Willebrand Factor (vWF) whereas once the EPCs have matured settled into circulation they are no longer positive for CD133. Permission to use image from Williams and Wilkins/Wolters Kluwer Health: Arteriosclerosis, Thrombosis, and Vascular Biology. Hristov, M., W. Erl, and P.C. Weber, *Endothelial Progenitor Cells: Mobilization, Differentiation, and Homing.*, 2003. 23(7): p. 1185-1189.

EPC homing has been thought to be similar to the process of the inflammatory response [45]. The adhesion molecules P-selectin and E-selectin that are present on endothelial cells play a role in the initial actions of homing EPCs. E-selectin has also been shown to mediate interactions between EPCs themselves [45, 53, 54]. P-selectin glycoprotein ligand-1 (PGLS-1), when expressed, has been shown to facilitate EPCs recruitment and potentially enhance their proangiogenic ability [53]. Other adhesion integrins such as β2-integrins allow for strong attachment of EPCs and migration to areas of damaged endothelium and was shown to aid in the homing of EPCs in a study looking at hind limb ischemia in mice [55]. One study looked at the glycoprotein intercellular adhesion molecule 1 (ICAM-1), and when it was upregulated, there was improved homing of EPCs to ischemic areas [56]. Another adhesion molecule to note is α4 that has been shown to assist EPCs to areas that are undergoing remodeling [56]. Recently, it has been investigated that insulin-like growth factor 2 (IGF-2) and its receptor (IGFR-2), stimulated various steps for homing of EPCs through the induction of hypoxic environments [40]. Homing of EPCs is a multi-variant process that is not the result of one signal. Other signals contributing to the homing of EPCs are SDF-1 and MMP-9 as well as lymphocyte function associated antigen 1 activation, stem cell factor, and cytoskeleton rearrangement [46]. It is a possibility that depending on location, injury, or disease, different homing signals or a combination of signals will be involved in the recruitment of EPCs for specific regenerative functions.

The number and function of circulating EPCs have been shown to be decreased and impaired in diseases that cause endothelial damage such as cardiovascular disease and diabetes [57-63]. Some studies have suggested that circulating levels of EPCs

correlate to the Framingham risk factor for cardiovascular risk where a decrease in cell viability *in vitro* was also seen in patients with a high risk cardiovascular disease [64]. Discussed numbers of EPCs could also lead to a lack of endothelial regeneration thus causing endothelial dysfunction and cardiovascular damage [65]. Endothelial progenitor cells have also been investigated in patients with diabetes, and studies have shown that there are a reduced number and function of EPCs, which correlates to the issues of vascular dysfunction and ischemia seen in diabetic patients [66, 67]. Gestational diabetes may also have a negative effect and alter the function of EPCs leading to the offspring having a higher risk of cardiovascular disease and diabetes [68]. One side of the investigation of EPCs includes the study of the biology and function of EPCs in states of disease versus non-diseased states. On the other end of the investigation of EPCs includes how we can utilize EPCs in regenerative therapy applications. There are encouraging studies that show EPCs have been used therapeutically with promising results for non-healing wounds, limb ischemia, and cardiovascular ischemia, and are essential for vascularization of engineered tissues [69-71].

Characterization of EPCs:

EPCs have been isolated and investigated in people, dogs, mice, pigs, sheep, and chickens but have yet to be isolated and characterized in horses [72-78]. Isolation methods that have been investigated vary from whole blood isolation, density gradient centrifugation, filtration devices, magnetic bead cell sorting, and high speed cell sorting based on fluorescently labeled cell markers. EPC cell types can be harvested from peripheral blood, bone marrow, and umbilical cord blood. The isolation method used

when Asahara first identified EPCs was by using magnetic beads coated with CD34 or VEGFR-2 in hopes to bind $CD34^+$ or $VEGFR\text{-}2^+$ cells which were then sorted through fluorescently labeled high speed cell sorting. The cells were plated for culture, expanded, and analyzed [35]. Density gradient centrifugation is often used to isolate the mononucleate portion of whole blood which is then plated with supplemented media optimized for the support of endothelial progenitor and endothelial cells and allowed to incubate for the development of cell colonies [79, 80]. Density gradient centrifugation allows for the erythrocytes to aggregate due to a specific agent and move through the separating solution and sediment at the bottom of a tube. The mononucleate layer of cells is less dense and will be located above the layer of density gradient material and beneath the layer consisting of plasma from the whole blood sample. There are devices developed that allow for the capture of mononucleate cells while running blood though a filter. The mononucleate cells are captured on the filter paper then flushed from the paper and placed into culture [81]. EPCs can be isolated from whole blood by allowing for the EPCs present in blood to adhere to the bottom of a polystyrene cell culture flask while mixed with supplemented media. After adherence of cells, the blood-media mixture is removed and the attached cells are allowed to incubate and proliferate forming cell colonies to then be used for expansion and analysis [82]. As stated, EPCs have not been previously investigated in horses and in this study isolation methods included whole blood isolation and density gradient centrifugation. Magnetic bead or filtration isolation methods could be investigated in future studies.

As mentioned already, due to the fact that there is no single marker to distinguish EPCs and with the different subtypes of EPCs, a combination of characterization methods

are used to identify these cells. The collection of methods usually includes the phenotypic appearance of the cells, time till appearance in culture, functional assays, and analysis of specific cell markers. The methods to characterize EPCs chosen to be used in the characterization of ECFCs from healthy adult horses included vascular tube formation *in vitro*, uptake of acetylated low density lipoprotein, and analysis of VEGFR-2, vWF, CD14, CD34, and CD105 specific cell markers. Background on these methods will be discussed in more detail.

Vascular Tube Formation:

The ability of endothelial cells and ECFCs to produce vascular tube structures *in vitro* is an essential characterization tool to evaluate not only cell identity, but functional ability throughout the lifespan of the cell. One way to assess vascular tube formation is the use of Matrigel Basement Membrane Matrix (BD Biosciences). Matrigel is produced from the extraction of the basement membrane of Englebreth-Holm-Swarm (EHS), mouse sarcoma. Matrigel is rich in extracellular matrix proteins and has the following constituents: laminin, collagen IV, heparan sulfate proteoglycans, and entactin/nidogen [83, 84]. Other components of Matrigel that work to support a variety of cell types have been shown to be TGF-β, insulin-like growth factor, fibroblast growth factor, epidermal growth factor, and tissue plasminogen activator [85, 86]. The matrigel membrane allows for cell attachment and differentiation and in the case of ECs and ECFCs, a way to evaluate vasculogenesis *in vitro* [87, 88]. Other cell types often supported by matrigel are epithelial cells, neurons, cardiac cells and hepatocytes. For more than two decades, it has been known that endothelial cells will form blood vessel like structures when plated

on a basement membrane with the cells organizing themselves into tubes containing lumens and then ceasing cell proliferation once the tube structures are formed [89]. The laminin in the matrigel works to promote cell adhesion, proliferation, and differentiation of the ECs or ECFCs seeded on top of the matrix, and the growth factors present will aid in the support and promotion of vascular tube formation. This two dimensional assay itself is considered to be straight forward; however, it is important to note cell seeding density because too few cells will result in incomplete tubule formation and too many cells will produce monolayers rather than tubes. The optimal range of cell seeding densities has been shown to be around 15,000 cells per well of a 96-well plate (4,800 cells/cm^2) [89]. Vascular tubule formation assays are essential to determine healthy functioning endothelial cells, evaluate ECs vasculogenesis, and are highly used when studying ECs and ECFCs in culture.

Uptake of Acetylated Low Density Lipoprotein

A characteristic of endothelial cells is their ability to uptake acetylated low density lipoprotein (Ac-LDL). This is accomplished by the "scavenger cell pathway" where the Ac-LDL binds to the LDL receptor located on the membrane of the endothelial cell and is taken up by endocytosis into the cytoplasm [90, 91]. Once inside the cell the Ac-LDL is hydrolyzed by lysosomal activity. This process is advantageous as a way to characterize and isolate endothelial cells. The Ac-LDL is tagged with the florescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL), which allows the cells to be imaged by florescent microscopy, flow cytometry, and high speed cell sorting devices. Another benefit to this method of characterization and

isolation of ECs is that the Dil-Ac-LDL has no adverse effect on the cells and cells can be isolated, sorted, and then expanded in culture without effecting cell growth rate [91, 92]. This characteristic of ECs is also found in their precursor cells endothelial progenitor cells and is often used as a characterization method when isolating and analyzing EPCs function [35, 58, 73, 75, 79, 81, 93, 94]. The uptake of Dil-Ac-LDL is an easy and common assay to establish the identity of endothelial cell types, but since other cell types (such as macrophages) also have the ability to uptake Ac-LDL, this functional assay should not be the sole characterization method.

Vascular Endothelial Growth Factor:

Vascular endothelial growth factor (VEGF), a signaling protein, functions as an essential regulator of angiogenesis. The discovery of VEGF was the result of various groups of researchers identifying a factor that induced vascular leakage (vascular permeability factor, VPN), as a mitogen of the endothelial cell that bound heparin, and then finally the determination of VEGF itself by identifying an amino acid sequence of a protein that only promoted growth of endothelial cells [95]. VEGF falls into the category of growth factors called the cysteine-knot superfamily which is characterized by all of its members containing a cysteine knot design located at the end of a beta-sheet with four strands [96, 97]. The VEGF gene itself is arranged containing eight exons and seven introns which leads to the formation of several isoforms based on variations in spliceosome activity. The six isoforms include the major isoforms $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189,}$ and $VEGF_{206}$, and the less frequently seen isoforms $VEGF_{145}$ and $VEGF_{183}$. $VEGF_{165}$ is the most commonly seen isoform, and a slice variant of this isoform identified

(labeled as $VEGF_{165}b$) and it is speculated to be a negative regulator of receptor activity [98]. Once the VEGF molecules were identified and studied the investigation of receptors for VEGF began. The initial two receptors identified are VEGFR-1 (Flt-1) and VEGFR-2 (KDR or Flk-1) with VEGFR-2 having a higher binding affinity [95]. Other VEGF receptors include VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGF acts as a mitogen to endothelial cells in both micro and macrovasculature signaling for the cells to undergo cell division. VEGF has been shown to stimulate angiogenesis and has been shown in studies to aid in the formation capillary like structures from endothelial cells in 3-D gels [99, 100]. VEGF, as previously mentioned, is a stimulator for EPC mobilization from the bone marrow. VEGF has also been seen to increase serine proteases urokinase-type, tissue-type plasminogen activators, and metalloproteinase interstitial collagenase. This is important to note based on their ability to aid the sprouting and migration of endothelial cells as the ECM is degraded making room for angiogenesis to occur [100]. VEGF also functions in vascular permeability which could be an essential step in angiogenesis [101, 102]. Glucose transport has also been shown to be activated by VEGF correlating to the increased energy needs when angiogenesis is occurring [100]. Vasodilation is also an effect that VEGF can exhibit and has been looked at in previous studies looking at vasodilation and heart contractility. VEGF has been of interest for the potential as a therapeutic agent. In tumor growth, there is unwanted cell growth and subsequent angiogenesis of the tumor. By utilizing VEGF inhibitors, there could be the potential to reduce the angiogenic activity at the tumor location inhibiting the tumors ability to continue to grow and receive nutrients. In 2004, the FDA approved a VEGF inhibitor, bevacizumab. This inhibitor was used with cytotoxic chemotherapy to treat

carcinomas. Other drugs have also entered the market for the treatment of individuals with cancer and individuals with macular degeneration [103]. It is clear that both allowing for the action of VEGF to promote angiogenesis as well as learning how to inhibit the action of VEGF has beneficial properties in regenerative and therapeutic medicine.

Vascular endothelial growth factor receptor 2 (VEGFR-2), is a cellular signaling receptor for VEGF. Unlike VEGFR-1 which promotes the development of hematopoietic cells, VEGFR-2 is indispensable in the development of endothelial cells and is the main communication avenue for signals from VEGF to the vascular endothelium [104]. VEGFR-2 is a type III transmembrane kinase receptor with seven immunoglobin domain extracellur regions, a transmembrane domain, and an intracellular domain with a tyrosine kinase. When VEGF binds to VEGFR-2, intracellular signals that effectively promote the survival, proliferation, permeability, and migration of EPCs and ECs will occur. Proliferation of ECs and EPCs happens through the phosphorylation of the extracellular signal-regulated kinase (Erk) pathway which comes about through the PKC-dependent pathway calling upon the activation of Phosphoinositide phospholipase C gamma (PLC-γ) [104]. PLC-γ is crucial for angiogenesis based on the fact that PLC-γ deficient mice will die while still in utero with an underdeveloped vascular system [105]. Migration of ECs and EPCs is crucial while angiogenesis is underway. These cells will move through the ECM as enzymes degrade the ECM allowing for a path to be made for the cells. Shb protein is an adapter protein and is silenced by small interfering ribonucleic acid (RNA), that will result in the reorganization of the cytoskeleton and cause cell migration. The phosphorylation of VEGFR-2 can lead to alteration of actin due to the action of VEGF.

Finally, when PI3K is activated it will regulate cellular migration via multiple growth factors. ECs and EPCs survival is encouraged by VEGF and signals transmitted through VEGFR-2 that phosphorylates apoptotic and anti-apoptotic proteins as well as by integrin activity. Permeability of the vasculature as a result of VEGF and the initiation of endothelial nitric oxide synthase (eNOS) facilitates the production of NO [104]. VEGFR-2 is essential for proper functioning of ECs and EPCs and is commonly used as a characterization marker when studying both ECs and EPCs.

Von Willebrand Factor:

Endothelial cells have the ability to synthesize, store, and secrete the adhesive glycoprotein Von Willebrand factor (vWF) [106]. vWF is critical to hemostasis and when deficient, the resulting disease, von Willebrand disease, is the most common congenital bleeding disorder [107]. vWF is a mediator of the adhesion of platelets between endothelial surfaces and the subendothelial matrix. The second important function of vWF is that it is a carrier and stabilizer for factor 8 (FVIII). FVIII is a critical blood clotting factor, and a deficiency or inability to code for this gene results in the bleeding disorder of hemophilia A, which results in an inefficiency to properly and swiftly form blood clots. vWF is a large gene and consists of 52 exons with a total of twelve domains (three A, three B, two C and four D) [108]. When vWF is synthesized in the EC it begins as a precursor polypeptide followed by cleavage resulting in the dimerization of the peptide to pro-vWF from non-covalent associations and covalent polymerization. vWF can be released from ECs or stored [109]. Storage of vWF is in Weibel-Palade bodies (WPBs) and the formation of WPBs is initiated by the presence of

vWF [107]. In hemostasis, vWF will bind to fibrillar collagen type I and III where it then begins its facilitative role in adhesion of platelets in the peripheral blood system [108]. vWF has two specific receptors for platelets which are glycoprotein GPIbα, located in the GPIb-IX-V complex, as well as the αIIbβ3, located in the GPIIb-IIIa complex. Even at shear stress levels of >5,000 s^{-1}, when GPIbα binds to domain A1, vWF can maintain platelets on the surface of the vasculature. This binding will eventually allow for platelet aggregation [107]. vWF may also work in the regulation of angiogenesis and pathways that could be involved are integrin αvβ3, VEGFR-2 signaling and angiopoetin 2 (Ang-2). Interestingly, high levels of vWF are actually an inhibitory agent in angiogenesis [107]. The staining for the intracellular vWF protein for the identification of EPCs and ECs is advantageous in the fact that it not only identifies the cells but confirms that they are functioning cells and have the capability of angiogenesis and hemostasis.

CD14, CD34, and CD105

CD stands for cluster of differentiation and is an identification protocol for the numerous "CD" cell markers of cells within the body [110]. Of the common markers used for the identification of ECs and EPCs (CD31, CD34, CD105, CD14, CD45, CD133) three were chosen to be used as identifiers for the study of equine endothelial colony forming cells. The markers chosen were CD14, CD34, and CD105. This was based on having both endothelial (CD34 and 105) and hematopoietic markers (CD14), as well as markers where antibodies that cross reacted with the horse species were available.

The receptor CD14 is specific to the lipopolysaccharide (LPS) complex and the LPS binding protein is important for the immune system to discern LPS. The

discernment of LPS is crucial for the immune system to identify bacteria which contain LPS that is specifically located on the outer membrane of gram negative bacteria [111]. CD14 will associate with Toll-like receptor 4 (TLR4) which aids in the activation of the immune system. When LPS or LPS-binding protein interacts and binds to CD14, a signal transduction event will result where CD14 "shuttles" the LPS to TLR4, which activates down-stream signals leading to synthesis and subsequent release of inflammatory cytokines (Tumor necrosis factor-alpha, Interleukin-6 and Interleukin-1 beta) will occur [112, 113]. When macrophages interact with LPS complexes adhesive actions are initiated as well as the induction of macrophages to begin synthesis of tumor necrosis factor [114]. CD14 has traditionally been considered to be present on monocytes, macrophages, dendritic cells, and cells of myeloid lineage [112]. One study investigated the number of CD14 receptors were present on monocytes and the showed that through fluorometric assays 110,000 CD14 molecules were seen on human monocytes [114]. In addition to CD14 being found as a membrane bound receptor, CD14 can be found in a soluble form in human plasma [115]. CD14 has also been studied in horses with monoclonal antibodies produced to better investigate cells marker expression as well as to investigate the immune response in horses [112]. CD14 has been commonly used as a marker for distinguishing hematopoietic stem cells and to characterize EPCs [33, 42]. When using CD14 in characterizing EPCs it is important to make note that EOCs will express the hematopoietic marker of CD14 whereas ECFCs will not express CD14 [33, 42]. The analysis of CD14 expression of EPCs will allow for a better determination of what type of EPC is being isolated.

CD34, a transmembrane phosphoglycoprotein, is found on hematopoietic progenitor cells, endothelial cells, endothelial progenitor cells and embryonic fibroblasts [116]. There is growing evidence that MSCs are positive for CD34 as well as cell types such as interstitial dendritic cells and epithelial progenitor cells [117]. The structure of CD34 has been studied over the years and it has revealed that the phosphoglycoprotein has an extracellular domain, a single transmembrane helix, and then a cytoplasmic tail that contains phosphorylation sites. The extracellular domain is comprised of serine, threonine, and proline profoundly that is O-glycosylated and sialyated. Other features of the extracellular domain include sites for N-linked glycosylation, a cysteine-bonded globular domain, and juxtamembrane stalk [118]. For almost four decades CD34 has been primarily used to identify hematopoietic stem cells as well as progenitor stem cells [118]. Although the use of CD34 as an identifying marker is quite common, the actual function of CD34 remains to be determined. Some functions thought to be carried out by CD34 are cell adhesion, homing of hematopoietic stem cells, enhancing proliferation which in turn blocks differentiation, and the adhesion of lymphocytes that is mediated by L-selectin specifically associated with the vascular endothelium [117, 118]. The ability of the CD34 protein to assist in the adhesion of lymphocytes is based on the interaction between the L-selectin present on lymphocytes and the glycosylated CD34 protein of high endothelial venules [118]. This process allows for the lymphocytes to be recruited to secondary lymphoid organs.

CD34 proteins have been investigated in their role with proliferation and differentiation. By encouraging cell proliferation this in turn blocks cell differentiation. A few reasons favoring CD34 as functioning in this process is the fact that as a cell

matures there is a down regulation of CD34, and when fewer CD34 positive cells are present in embryonic and adult tissues in mice there seems to be a proliferation defect [118]. Enhancing cell adhesion in the case of lymphocytes has been seen with CD34 being shown to play a potential role in blocking cell adhesion. A specific member of CD34 family called podocalyxin and it is part of the glycocalyx of podocytes. It has been shown that low levels of podocalyxin can enhance integrin activity and adhesion, but inhibition of podocalyxin results in a reduced expression and a decreased adhesive activity [118]. Inhibiting CD34 does not allow for the adhesion action of CD34 to be carried out rather than CD34 having a blocking of adhesion itself. Homing assistance to hematopoietic $CD34^{+}$ stem cells has been studied in mice and when cells are $CD34^{-/-}$ there is a decrease in migratory ability of the cells [118]. As stated already, CD34 is a standard marker used in characterizing hematopoietic stem cells. When EPCs were first identified, CD34 was the selecting marker to isolate EPCs from human peripheral blood [35, 36]. Although more information is needed to accurately determine all functions of the CD34 protein and all cells containing CD34 proteins, it is in fact a standard characterization method and is used in this study to aid in the characterization of equine endothelial colony forming cells.

CD105 (endoglobin), a cell surface marker protein, is a homodimeric transmembrane protein. CD105 is largely found on endothelial cells and is involved in proliferation and hypoxia-inducible properties. It is also a co-receptor for transforming growth factor (TGF) beta one and beta three. CD105 has a large extracellular domain and a short intracellular domain with a transmembrane region that is hydrophobic. The extracellular domain will bind to the TGF-β1 and -β3 isoforms [119]. The amino acid

sequence of CD105 has the tripeptide arginine-glycine-aspartic acid (RGD) which is exposed on the extracellular domain and is a recognition structure seen on ECM proteins. Such proteins as fibronectin, vWF, vitronectin, fibrinogen, and collagen type 1 will be recognized by the RGD sequence. This recognition and adhesion will be important to processes of hemostasis, thrombosis, and inflammation. The primary function of CD105 is affiliated with TGF-β signaling which works to control proliferation, differentiation, homeostasis, and apoptosis [120]. TGF-β, when activated, can release angiogenic factors that originate from inflammatory cells, induce inflammation, as well as potentially suppress tumor progression. CD105 has also been looked at in playing a part in the endothelial nitric oxide synthase pathway. The endothelial nitric oxide synthase pathway works to produce NO within blood vessels and works as a regulatory process for tone of the vasculature system. CD105 is considered to be a cyclooxygenase-2 modulator within the nitric oxide synthase pathway, and is also thought to modulate the differentiation of endothelial progenitor cells to functioning mature endothelial cells. CD105 is important for angiogenesis which is shown by studies looking at either up-regulation or down-regulation of CD105 in tumor tissues (up-regulation) and lack of angiogenesis with *in vitro* studies (down-regulation) [120]. Also, CD105 knockout mice resulted in defects to the cardiac and vascular system that ended up in death of the mice during the early stages of embryonic development [121-123]. CD105 is important and often used as an identifying factor for endothelial cells. It has been shown that without CD105, proper function of endothelial cells, and thus angiogenesis, will not occur.

Although promise is being shown with EPCs, there are still studies and further research needed before the use of EPCs will have more clinical applications in human

regenerative medicine. The potential avenues to use EPCs in humans are encouraging and potential applications of EPCs in horses includes similar conditions seen in humans such as cases of decreased vascularization, complicated wounds, non-union fractures, and tendon and ligament repair. Veterinary regenerative medicine is a growing field and the use of MSCs in equine regenerative medicine is becoming more widely seen. Stem cell therapy has already been proven to be beneficial in the treatment of horses with ophthalmological pathologies [124]. This leads to the contemplation that EPCs could have the same or better beneficial outcome can benefit horses with ophthalmological pathologies. Although mesenchymal stem cells have had variable success in clinical application with injuries such as tendonitis, there is the potential that alternative types of stem cells used instead of or in addition to previously investigated cells could improve the outcome [125]. Using EPCs as a regenerative therapy in horses with equine metabolic syndrome or laminitis is also an apparent potential application. With the prevalence equine metabolic syndrome and chronic laminitis in horses, it is imperative to explore new and alternative avenues to aid in the treatment of these diseases. Intestinal ischemia in horses could also benefit from the therapeutic use of stem cells such as EPCs or MSCs to regenerate areas of tissue damaged from ischemia. Previous studies have shown that MSCs have been beneficial to reverse intestinal ischemia in rats [126]. The use of EPCs, with their neovascularization properties, could be beneficial or even more beneficial to treat intestinal ischemia.

With the vast potential clinical applications of EPCs in the horse field, the ability to isolate and correctly characterize these cells is imperative to allow for further research and therapeutic use of EPCs in horses. Continuing research on the biology of EPCs is

highly warranted due to their potential in regenerative medicine. Further understanding concerning the exact function of EPCs, homing mechanisms, and roles in disease states will go a long way to being able to take EPCs to more wide spread clinical applications across many species, not only horses.

Chapter Two: Materials and Methods

Peripheral Blood Isolation Method

All procedures involving animals were approved by the Auburn University animal care and use committee. Ten mL of peripheral blood from the jugular vein were collected into lithium heparin tubes from 24 adult horses. These horses were from the Auburn University College of Veterinary Medicine teaching and reproduction herd, and from the Auburn University Equestrian Team. The blood samples were transported on ice to the lab for processing. An isolation protocol for human ECFCs was used for the isolation of equine ECFCs [82]. Five mL of whole blood were placed onto uncoated T75 cm^2 cell culture flasks containing 15 mL of pre-warmed endothelial basal medium (EBM-2) containing growth factors (Lonza) and 10% horse serum (Hyclone). An additional 5 mL of supplemented media was used to rinse the pipette of any leftover blood and added to the flask for a total of 25 mL of blood-media mixture. The flask was incubated at standard cell culture conditions (37°C, 5% CO_2 and 95% humidity), for 24 hours to allow for cell adherence to the bottom of the polystyrene flask. After 24 hours, the blood-media mixture was gently removed as to not disturb attached cells. The flask was rinsed with 30 mL of phosphate buffered saline (PBS) followed with the addition of 15 mL of fresh supplemented media. Adherent cells were maintained at standard cell culture conditions and observed daily for colony formation with 30% of the media changed twice a week.

Once colonies developed, day of appearance and colony number were noted, and colony morphology was recorded via light microscopy, (Ti Eclipse, Nikon). Colonies

were harvested using trypsin/EDTA (0.25 mg/mL) and re-seeded onto a collagen coated T75 cell culture flask for cell expansion (rat-tail type I collagen at 50 µg/mL, BD Biosciences, Bedford MA). Cell expansion and morphology were recorded using light microscopy. Once cells reached 80%-90% confluence, they were harvested and used for characterization assays or frozen in liquid nitrogen for future experiments.

Bone Marrow Isolation Method

Bone marrow aspirates were collected from 3 horses belonging to the Auburn University College of Veterinary Medicine teaching herd while under sedation. The sternum was palpated to identify between the forelimbs to find the caudal most aspect of the sternum and was aseptically prepared for bone marrow aspirate collection. A stab incision was made with a #15 scalpel blade to the point of the sternum. A Jamshidi® bone marrow biopsy needle was inserted until coming into contact with the sternum. The needle was rotated with steady force applied until about 3 cm of the needle was into the sternal bone. A total of 30 mL (3, 10 mL syringes preloaded with heparin) were collected. After collection, the bone marrow aspirates were processed through density gradient centrifugation. Bone marrow was centrifuged to separate out the plasma, which was discarded. The remaining portion was diluted in a one to one ratio with EBM-2 supplemented media and mixed by gently pipetting the solution. The blood-media mixture was placed over 15 mL of Ficoll Paque Plus® (GE, Healthcare) and centrifuged for 30 minutes with no brake. The hazy mononucleate cell layer was harvested, rinsed with three times as much PBS, and centrifuged for collection of the cell pellet. The cell pellet was re-suspended and placed into a collagen coated cell culture flask with

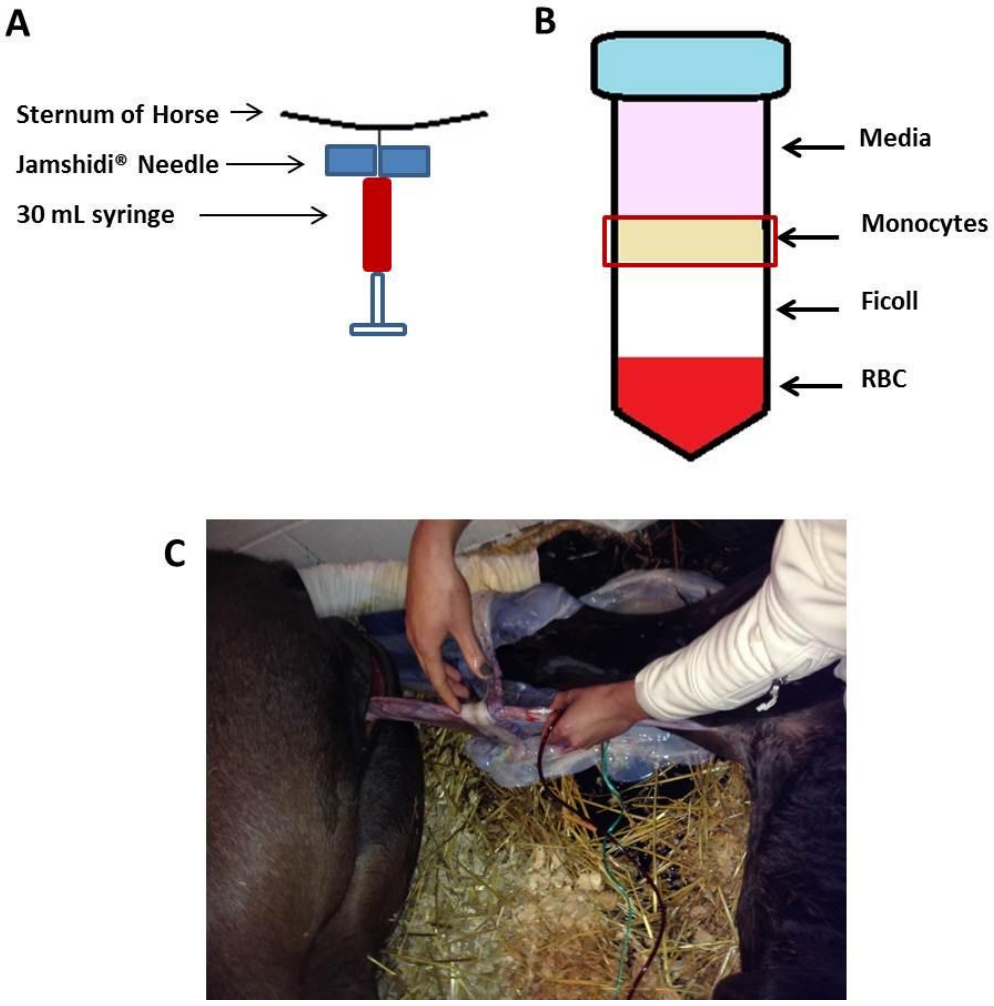

Figure 3. A) Standard collection procedure for bone marrow from the sternal bone of a horse using a Jamshidi® bone marrow biopsy needle B) Density gradient centrifugation of bone marrow and umbilical cord blood with the arrow showing the layer of monocytes harvested to isolate cells. C) Collection of umbilical cord blood.

EBM-2 media. Figure 3A and B depict the collection of bone marrow and the blood processed through density gradient centrifugation. Media was changed completely once sufficient cell attachment was achieved (2-3 days) followed with 30% media change two times a week while observing daily for cell colony formation. Once cells appeared, they were harvested as previously explained with the peripheral blood cell isolation and expanded, used for characterization assays, and cryopreserved.

Umbilical Cord Blood Isolation Method

Umbilical cord blood (UCB) was collected from the umbilical cord vein immediately after birth of the foal prior to the breakage of the umbilical cord itself. The umbilical cord was clamped 8 inches from the foal's umbilicus to reduce blood loss before collection. A 16 gauge needle was attached to a 30 mL syringe prefilled with heparin to collect 10 mL of umbilical cord blood. Figure 3C depicts the collection of umbilical cord blood immediately following foaling. UCB was processed by density gradient centrifugation. UCB was centrifuged and the plasma portion was discarded. The remaining portion was diluted in a one to one ratio with EBM-2 supplemented media and mixed by gently pipetting the mixture. The blood-media mixture was placed over Ficoll Paque Plus® and centrifuged for 30 minutes with no brake. The hazy mononucleate cell layer was harvested, rinsed with three times as much PBS, and centrifuged. The cell pellet was re-suspended and placed into a collagen coated cell culture flask with EBM-2 media. The media was changed completely once sufficient cell attachment was achieved (2-3 days) followed with 30% media change two times a week while observing daily for cell colony formation. Once cells appeared, they were

harvested as previously explained with the peripheral blood cell isolation and expanded, used for characterization assays and cryopreserved.

Matrigel Tubule Formation Assay

Equine ECFCs were harvested with trypsin/EDTA and seeded onto a 96-well cell culture dish containing 75 µl of BD Matrigel Basement Membrane Matrix (BD Biosciences), which had been incubated for 30 minutes prior to cell seeding. Multiple seeding densities were evaluated (7500-21,000 cell/well) to optimize tube formation, and 10,000 cells/well was determined to be optimal. All cells were used at a passage of 2 or 3 for initial Matrigel tubule formation. Human endothelial colony forming cells (LONZA, Switzerland) served as the positive control while mouse 3t3 fibroblast cells served as the negative control, and both cell types were seeded at the same cell density as the equine ECFCs. All cells were allowed to incubate at standard cell culture conditions for a period of 48 hours. Vascular tube formation was imaged at 0, 5, 24 and 48 hour time points at 4, 10, and 20x magnifications. The time to tube appearance and tube quality score (1-4 score) were recorded for each image and cell type. The scoring system for vascular tube formation was 1) no tube formation, 2) projecting tubes from cells but no connections between any cells, 3) vascular tube formation with connecting tubes in 50% or less of the field, and 4) high level of vascular tube formation with connecting tubes greater than 50% of the field. Three fields of view at 10x magnification per time point were used for scoring. Figure 4 shows examples of each tube score.

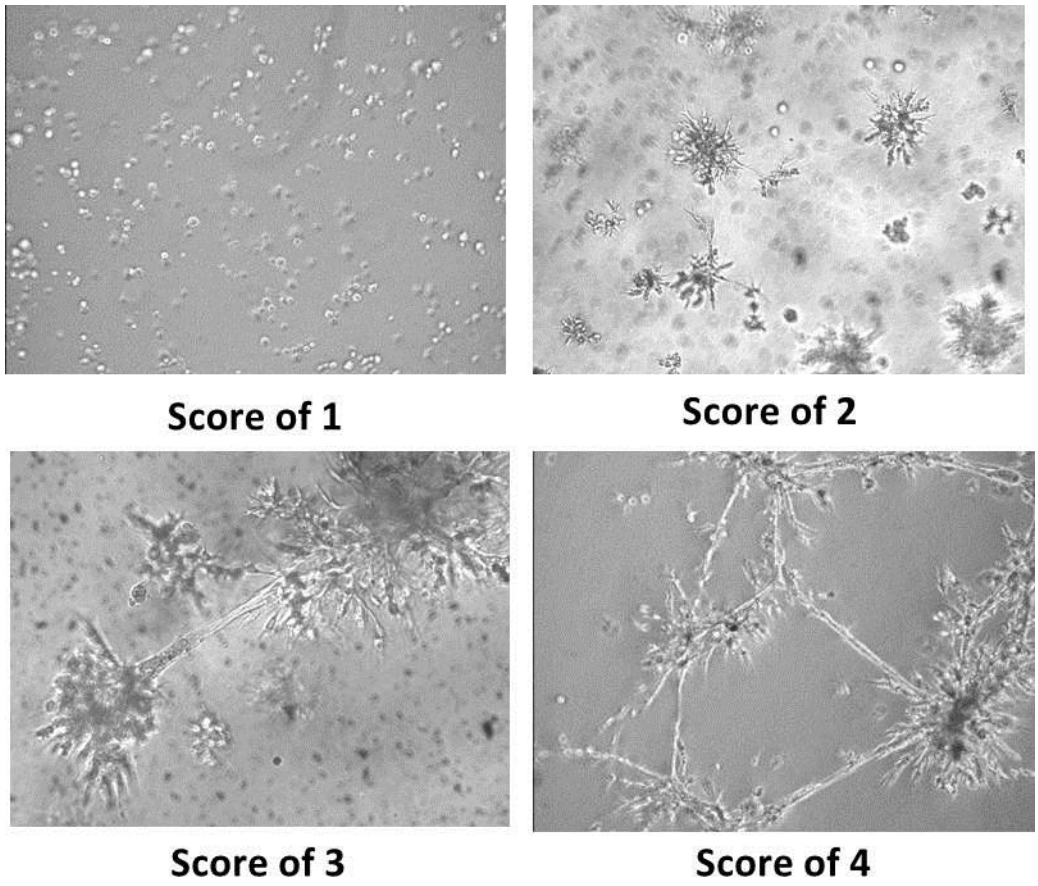

Figure 4. Scoring system used to evaluate vascular tube formation on Matrigel basement membrane matrix. Score were assigned based on a score of 1) no tube formation, 2) projecting tubes from cells but no connections between any cells, 3) vascular tube formation with connecting tubes in 50% or less of the field, and 4) high level of vascular tube formation with connecting tubes greater than 50% of the field. Three fields of view at 10x magnification per time point were used for scoring.

Uptake of Acetylated Low density lipoprotein

Equine ECFCs, human ECFCs, and 3t3 fibroblast cells were seeded at a cell density of 8,000 cells per cm^2 using 24-well cell culture plates. Wells containing equine and human ECFCs were coated with collagen at a concentration of 50µg/mL. All cells were incubated for 24 hours at standard cell culture conditions. After 24 hours Dil-labeled acetylated low density lipoprotein (Dil-Ac-LDL) (Biomedical Technologies Inc.) was added at a concentration of 50 µg/mL diluted in pre-warmed supplemented media appropriate for each cell type. Cells were incubated with the Dil-Ac-LDL for 4 hours at standard cell culture conditions. After incubation, the cells were washed with probe free media, fixed with 4% paraformaldehyde for 20 minutes, and then counter stained with 4',6-diamindino-2-phenylindole (DAPI) for 25 minutes. The cells were preserved with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA) and imaged with a fluorescent microscope at 10 and 20x magnifications. The uptake of Ac-Dil-LDL was visible with the tetramethylrhodamine isothiocyanate filter (TRITC, 528nm) and DAPI was visible with the 340 nm filter. Images were analyzed for percent positive cells with two separate overlaid images at different locations in the well at 10x magnification with all cells present in each field of view positively stained with DAPI counted as a cell. Cells stained with DAPI and showing uptake of Dil-Ac-LDL counting as a positive cell.

Passage to cell senescence and cell growth peripheral blood ECFCs

Cells were continuously passaged with the matrigel tubule formation assay and uptake of Dil-Ac-LDL performed at each passage as previously described. A seeding density of 10,000 cells/well was used for each passage. Tube formation in matrigel was

evaluated through the scale as previously described. The percentage of cells positive for uptake of Dil-Ac-LDL was recorded at each passage and compared between passages. The number of cell doublings (CD) per 24 hour period and population doubling time (PDT) per hour were determined. Cell seeding density at each passage, cell count at harvest, and time (hours) between passages were recorded to be used in the following equations for determining CD and PDT:

$CD = Log_2(C_H/C_s)$/number of days

PDT = CD/total number of hours

CD was the number cell doublings, C_H was the number of cells at harvest, and C_S was the number of cells at seeding. PDT was the total time in hours for the population to double.

Isolation of equine carotid endothelial cells

For a positive control cell for flow cytometry and indirect immunofluorescence, equine carotid endothelial cells were harvested from an adult horse at necropsy according to previously published protocols [127]. Briefly, isolated carotid arteries were digested with Type II collagenase (Worthington) and plated in DMEM with added HEPES, 10% fetal bovine serum (FBS), and 10% calf serum. Cells were passaged when they reached 80% confluence and re-seeded onto collagen coated flasks with supplemented endothelial growth media. When passaged cells reached 70% confluence, they were incubated with Dil-AC-LDL using the same protocol described above. The Dil-Ac-LDL fluorescent signal was detected with a 585/45 BP filter, and the brightest 22% of cells were gated and

sorted at 65 lb/in^2 into medium. The labeling and sorting of the cells was used to purify the ECs from any contaminating smooth muscle cells.

Immunofluorescent Staining

Equine ECFCs and ECs were evaluated for expression of endothelial cell markers vWF, VEGFR-2, CD34, and CD105 and the hematopoietic marker CD14 using indirect immunofluorescence (IF) at passages of 3, 4 and 5. All IF staining procedures were repeated with positive results 3 separate times. Negative controls for all specific cell marker antibodies consisted of staining the cells with the secondary antibody only. This verified that any positive staining seen was not due to background staining from the secondary antibody or non-specific binding of the secondary antibody. Equine ECFCs or ECs were seeded onto collagen coated glass coverslips at a cell density of 30,000 cells per coverslip and allowed to expand until reaching 80 to 90% confluence. All cells were fixed with 4% paraformaldehyde, washed, and blocked with 3% fetal bovine serum for 30 minutes. For the intracellular protein vWF, cells were permeabilized with 0.1% Triton x100 for 30 minutes prior to blocking. Coverslips were then incubated with the primary antibodies followed by the appropriate secondary antibodies. The antibodies were used in the following concentrations: vWF (A0082, DAKO), 1:200 for 3 hrs.; VEGFR-2 (sc-6251, Santa Cruz Biotechnology), 1:200 for 3 hrs.; CD34 (Clone RAM 34, eBioscience), 1:100 for 3 hrs.; CD105 (Clone SN6 AbD Serotec), 1:100 for 3 hrs.; CD14 (Clone 105, Wagner, Cornell University) 1:100 for 1 hour. Secondary antibodies (Alexa Fluor®488 anti-goat and anti-rabbit, Life Technologies; Streptavidin Dylight™ 550, Thermo Scientific) were all used at a concentration of 1:400 incubated overnight at 4°C for an

average of 18 hours. Cover slips containing stained cells were mounted on slides with Fluoromount-G and imaged using a fluorescent microscope. All antibodies are listed in Table 2.

Flow Cytometry

Harvested equine ECFCs were characterized with the endothelial cell surface marker of CD105, (AbD Serotec) and the hematopoietic surface marker of CD14 (Clone 105, Wagner, Cornell University) through flow cytometry. All cells were analyzed at passages of 3, 4, or 5 and staining and analysis was repeated twice for each cell marker. Cultured equine carotid endothelial cells served as the positive control for CD105 and monocytes from equine whole blood served as the positive control for CD14. Cultured cells were harvested and re-suspended in supplemented media and allowed to rest for cell surface marker regeneration after exposure to trypsin. Cells were prepared to have total of 5×10^5 cells for the staining process. Equine ECFCs and ECs were blocked with 10% horse serum for 30 minutes. Whole equine blood was collected in lithium-heparin tubes and aliquots of 100 µl were used for staining and analysis. Serum present in the whole blood provided natural blocking of the monocytes prior to the addition of the primary antibody. After incubation with the primary antibody, the red blood cells were lysed with 3 mL of lysis buffer before being stained with the secondary antibody. The primary antibody was added at a concentration of 2 ug per test for CD105 and 1 ug per test for CD14 for cultured cells and whole blood samples, and allowed to incubate at room temperature for 1 hour (CD105) or 30 minutes (CD14). All cells were washed and then stained with the appropriate secondary antibody (AlexaFluor 488, goat anti-mouse).

Cell Marker	Primary Antibody	Secondary Antibody	Primary Antibody Concentrations	Secondary Antibody Concentrations	Perm-eabilization	Blocking Buffer
vWF	Polyclonal rabbit, anti-human, Dako, Code A0082	Streptavidin, DyLight 550 Conjugated, Thermo Scientific, Rockford, IL (TRITC)	1:200 concentration 3 hr. incubation room temperature	1:400 concentration 24 hr. incubation 4°C	0.1% Triton x100 30 min.	3 % FBS/PBS 30 min.
VEGFR-2, (Flk-1)	Monoclonal mouse Anti human Flk-1 (A-3): sc-6251, Santa Cruz Bio-tecnhology,	Alexa Fluor® 488 Dye, Goat Anti Mouse Life Technologies (FITC)	1:200 concentration 3 hr. incubation room temperature	1:400 concentration 24 hr. incubation 4°C	None	3 % FBS/PBS 30 min.
CD 34	Monoclonal anti-mouse CD34 Biotin, Clone: RAM34 eBioscience 13-0341	Streptavidin, DyLight™ 550 Conjugated Thermo Scientific (TRITC)	1:100 concentration 3 hr. incubation room temperature	1:400 concentration 24 hr. incubation 4°C	None	3 % FBS/PBS 30 min.
CD 105	Monoclonal mouse Anti Human CD105, Clone: SN6 AbD Serotec	Alexa Fluor® 488 Dye, Goat Anti Mouse Life Technologies (FITC)	1:100 concentration 3 hr. incubation room temperature	1:400 concentration 24 hr. incubation 4°C	None	3 % FBS/PBS 30 min.
CD 14	Monoclonal mouse Anti-Horse Clone 105 B. Wagner Cornell University	Alexa Fluor® 488 Dye, Goat Anti Mouse Life Technologies (FITC)	1:100 concentration 1 hr. incubation room temperature	1:400 concentration 24 hr. incubation 4°C	None	3 % FBS/PBS 30 min.

Table 2: Antibody information including primary antibodies, secondary antibodies,concentrations, cell permeabilization, and cell blocking information for direct immunofluorescent staining of equine endothelial colony forming cells. Filter used included tetramethylrhodamine isothiocyanate filter (TRITC, excitation: 562nm, emission 576nm) and Fluorescein Isothiocyanate filter (FITC, excitation: 498nm, emission 520nm).

All cells were filtered and prepared for flow analysis with a BD Accuri C6 flow cytometer. A total of 10,000 events were collected for all samples recording events in a forward scatter versus side scatter plot with gates labeled as R1 including only positively stained cells to be used in analysis. The percentage of cells positive for the antibodies staining of the cells was recorded for all results.

Analysis

All continuous data are presented as mean ± standard deviation. Ordinal score data is presented as median and interquartile range (IQR). The percentage of LDL positive cells, cell doubling, and population doubling time over passages were analyzed using a one-way analysis of variance with Tukey's post-hoc comparison. The Matrigel tubule formation assay scores over each passage were analyzed with a Kruskal-Wallis test and Dunn's post hoc comparison and grouped early (P1-P4) and late (P5-P10) passages were compared with a two-tailed Mann-Whitney U test. All analyses were performed with a commercial statistics package (GraphPad Prism), and $P<0.05$ was considered significant.

Chapter Three: Results

Equine ECFC isolation and cell morphology

A total of 24 horses were sampled, including 15 warmbloods, 8 quarter horses, 1 paint, and 1 Arabian. Age range of horses sampled was 4 to 23 years of age, with a mean of 10.86 ± 4.42 years of age. There were 20 geldings and 4 mares. Three of 24 horses successfully produced colonies at 12 ± 2.45 days with 2.8 ± 1.45 colonies per mL of blood, which is similar to other studies with humans [74, 82]. Equine ECFCs exhibited single layer cobblestone morphology and showed significant outgrowth upon expansion from colony harvesting which is characteristic of endothelial colony forming cells. Figure 5 shows an ECFCs colony and the phenotypic qualities of the cells upon expansion.

A total of 3 horses were used for collection of bone marrow aspirates, 2 quarter horses (mares) and 1 warmblood (gelding). Two of the 3 horses produced colonies seen on day 6 ± 1.41 with 1.27 ± 0.47 colonies per mL. Equine bone marrow ECFCs had cobblestone morphology, significant outgrowth, and cells did not exhibit perfect cobblestone morphology as seen with the peripheral blood samples, but cells did show single layer of cell growth similar to what was seen with the peripheral blood sample ECFCs. A total of 3 pregnant mares (2 quarter horses and 1 warmblood) were available for UCB collections. Of the 3 mares that were sampled, 2 produced colonies. A total of 0.10 ± 0.09 colonies per mL were seen on an average of 14 ± 11.31 days. The sample from foal 1 produced 1 colony seen on day 22 post isolated and the sample from foal 2 produced 5 colonies 6 days post isolation. UCB cells showed cobblestone morphology,

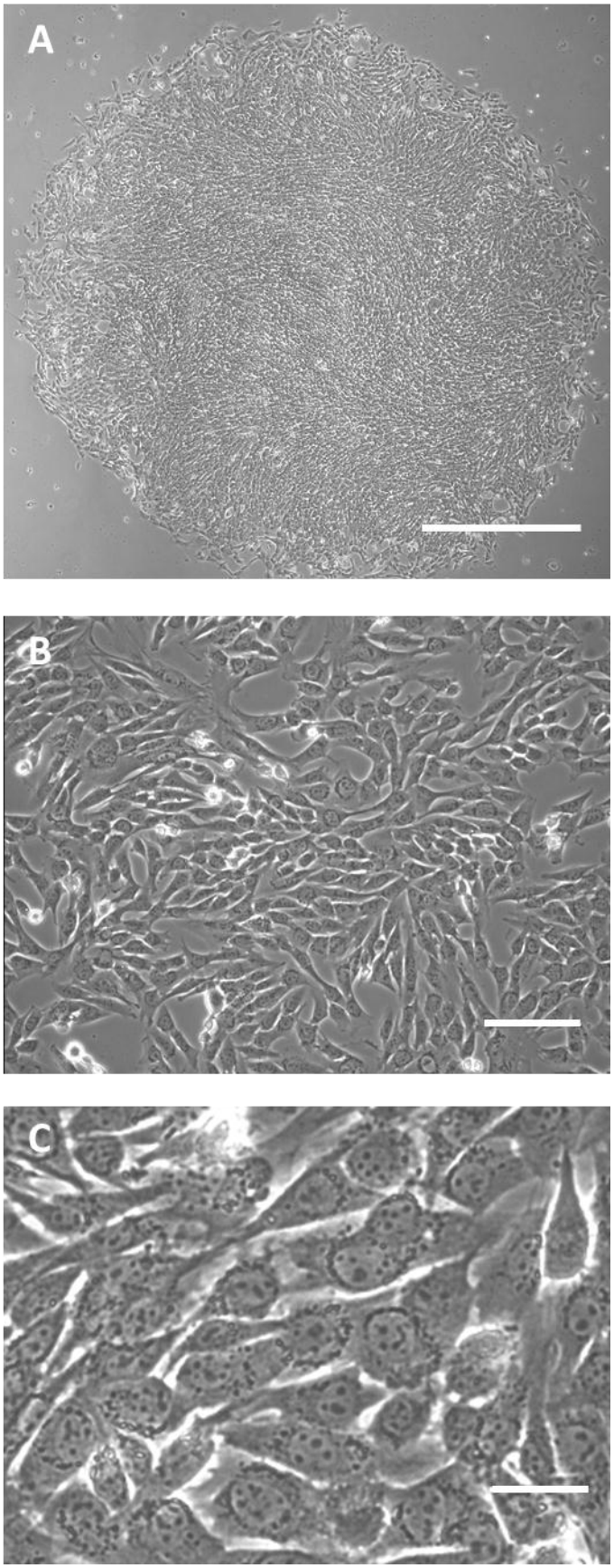

Figure 5. A) Confluent cell colony seen on day 12 after isolation. Scale bar represents 500 µm (4x magnification). B) Cells showed significant outgrowth on expansion and single layer cell growth. Scale bar represents 300 µm (10x magnification). C) Cobblestone morphology of the cells. Scale bar represents 50 µm (40x magnification).

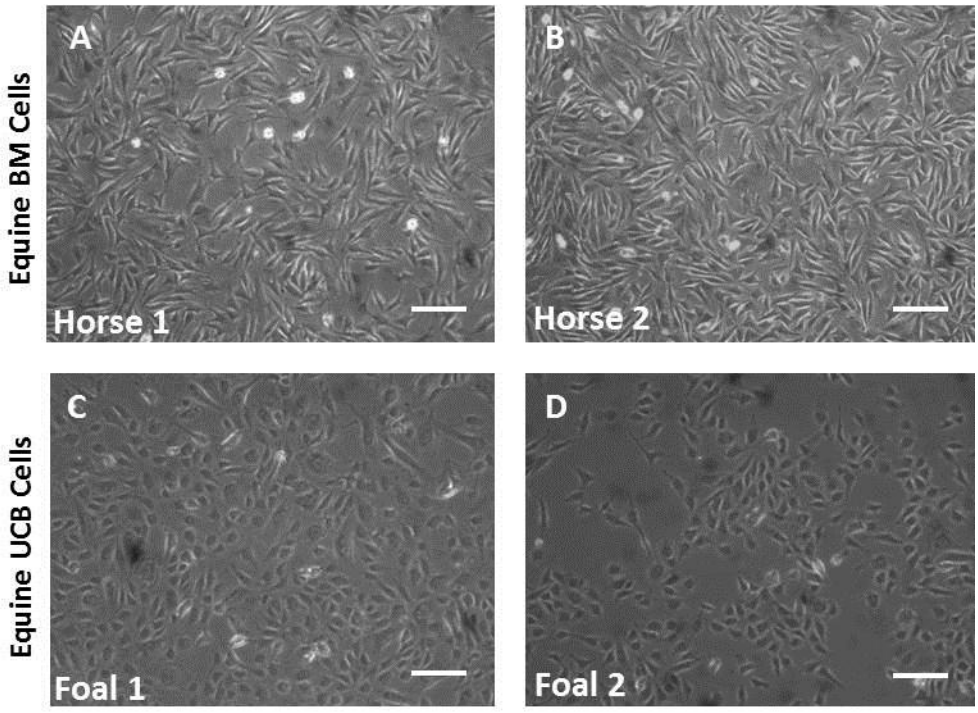

Figure 6. A) Bone marrow (BM) cells from horse A upon cell expansion and B) bone marrow cells from horse B upon cell expansion. BM cells exhibited less cobblestone morphology upon expansion. C) Umbilical cord blood (UCB) cells from foal 1 upon cell expansion and D) umbilical cord blood cells from foal 2. Scale bars represent 100 μm with images taken at 10x magnification.

but showed less robust outgrowth upon expansion. UCB cells also decreased in cell proliferation and increased in spindle and irregular shaped cells as passage increased from P1 to P8 based on qualitative observations. BM and UCB cells are shown in Figure 6.

Matrigel Tubule Formation Assay

Equine ECFCs formed vascular tubes at 24 hours post cell seeding onto matrigel basement membrane and maintained tube structure until 48 hours post seeding. Human ECFCs showed vascular tube formation at 5 hours post cell seeding and maintained tube structures until 48 hours post cell seeding. Fibroblast cells failed to form vascular tubes at any time point. Figure 7 shows the results for the matrigel tubule formation assay for the equine ECFCs, human ECFCs, and 3t3 cells at 0, 5 and 24 hours. Images of Matrigel tubule formation at 4x magnification were recorded allowing for the visualization of the branching networks that formed at 24 hours post seeding throughout the entire well resulting in a tubule formation score of 4 (Figure 7B). Bone marrow ECFCs formed vascular tubes *in vitro* with a score of 4 at 24 hours whereas the umbilical cord blood ECFCs did not form vascular tubes *in vitro* at any time point (Figure 8).

Uptake of Acetylated Low Density Lipoprotein

Isolated equine ECFCs were positive for uptake of Dil-Ac-LDL. The ECFCs from the 3 horses had 74.9% ± 14.67% positive uptake of Dil-Ac-LDL. The human ECFCs were 100% positive for LDL uptake, whereas the 3t3 fibroblasts had no uptake.

Di-Ac-LDL images for equine and human ECFCs as well as 3t3 cells are shown in Figure 9A. Bone Marrow ECFCs showed robust uptake of Dil-Ac-LDL (Figure 9B) with a mean percent uptake of at 95.50 ± 2.50 at P4, 91.50 ± 1.50 at P6 and 93.00 ± 2.00 at P8 with no significant difference between passages seen (P=0.47). Umbilical cord blood cells showed an uptake of Dil-Ac-LDL (Figure 8C) at a mean percent of 96.67 ± 1.52 at P4, 85.67 ± 5.13 at P6 and 91.66 ± 6.51 at P8 with no significant difference between the passages (P=0.08).

Passage to cell senescence and cell growth of peripheral blood cells

The quality of vascular tube formation in equine ECFCs decreased as cell passage increased. The median score of tube quality was significantly different between passage 1 versus passages 4, 7, and 10 (Figure 10A). Overall, early passages 1-4 (median 2; IQR 2-3) had significantly higher tubule formation scores (P=0.0004) than later passages 5-10 (median 2; IQR 1-2) (Figure 10D). No ECFCs from any horse at any passage completely lost the ability to uptake LDL; however, a significant (P<0.0001) decrease in the percentage of cells positive for LDL uptake was identified in higher passages (Figure 10B and E). As passage number increased, cell doubling rate trended down; whereas population doubling time trended up (Figure 10). CD rates and PDT from passages 3 to 9 are shown (Figure 10F). Additionally, cells lost their characteristic cobblestone morphology and became more spindle-shaped as cell passage number increased, and an increase in the presence of vacuoles in the cytoplasm of the cell was seen (Figure 10C). Bone marrow cell never lost the ability to form vascular tubes when evaluated at passage 4, 6, and 8, whereas umbilical cord blood cells did not form tubes at

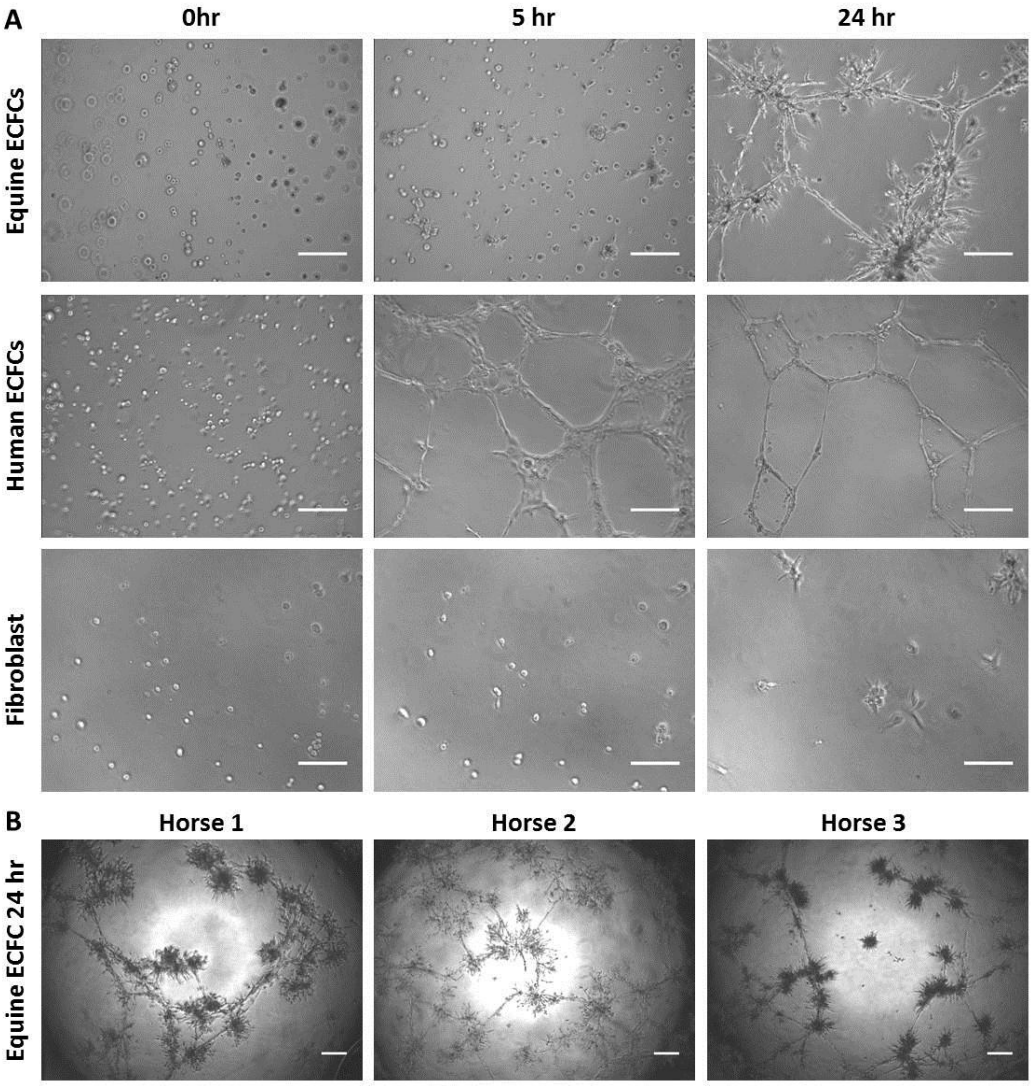

Figure 7. Matrigel tubule formation assay. A) Equine and human late outgrowth endothelial colony forming cells and 3t3 cells at 0, 5 and 24 hours respectively. Tube formation was at 24 hours which persisted for the equine and human ECFCs up to 48 hours. Seeding density was 10,000 cell/well of a 96-well plate for all cells. Any variations in appearance of cell densities in the images are from the differences in focus planes. 3t3 cells did not form tubes as expected. Scale bars represent 100 µm with images taken at 10x magnification. Cells were used at passage 2 and 3. B) Horse 1, 2 and 3, respectively showing vascular tube networks seen at 24 hours post seeding at 4x magnification with a tubule formation score of 4. Seeding density was 21,000 cells/well of a 96-well plate. Scale bar represents 1000 µm. Cell were used at passage 3 and 4.

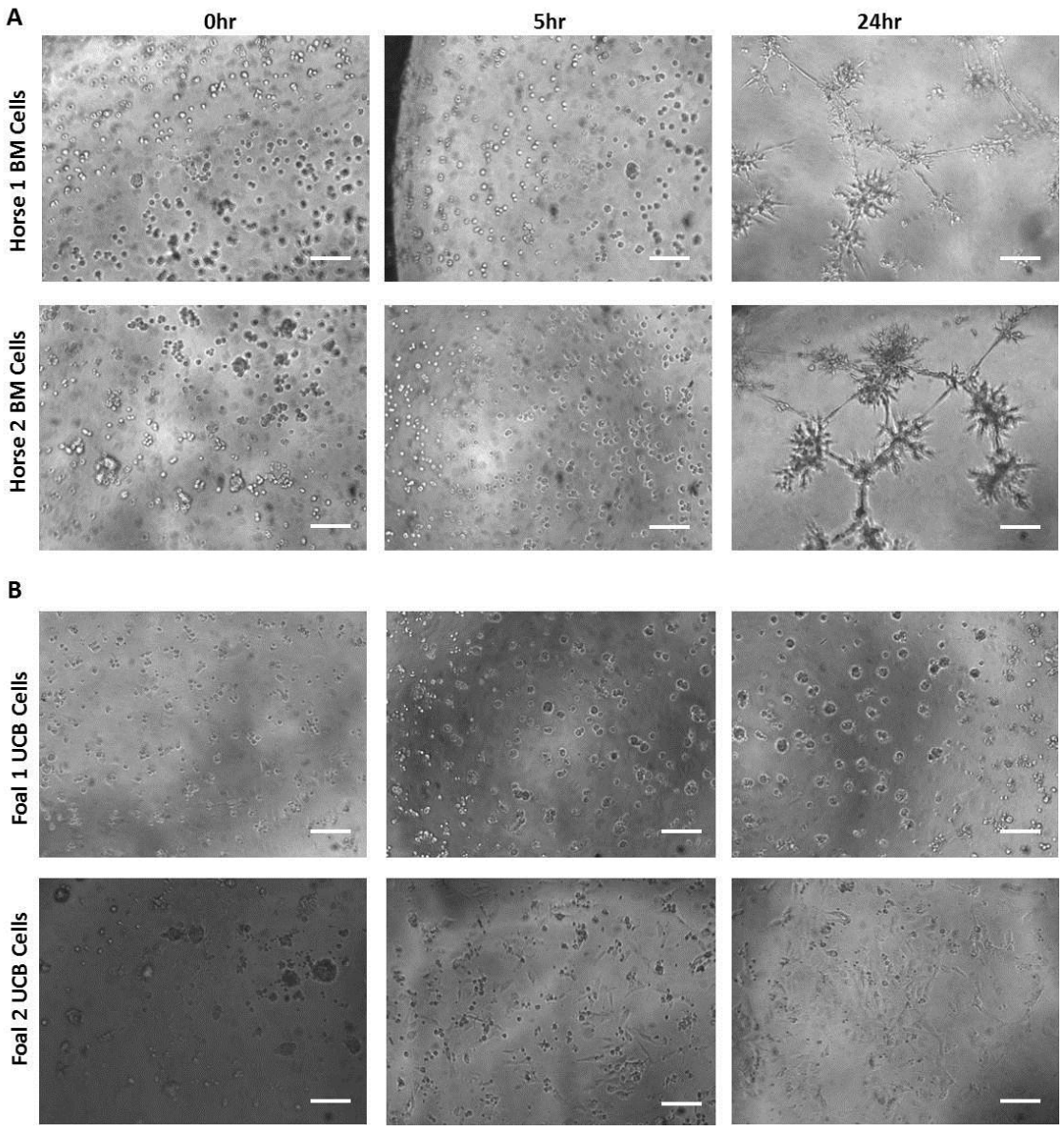

Figure 8. A) Bone marrow (BM) cells from horse A and horse B showed vascular tube formation on Matrigel at 24 hours post seeding at a score of 4. Cells were seeded at a density of 10,000 cells/well. Any variations in appearance of cell densities in the images are from the differences in focus plains. B) Umbilical cord blood (UCB) cells from foal 1 and foal 2 did not show vascular tube formation on Matrigel at any time point. Cells were seeded at a density of 10,000 cells/well. All scale bars represent 100 μm. All cells in the images are from passage 8.

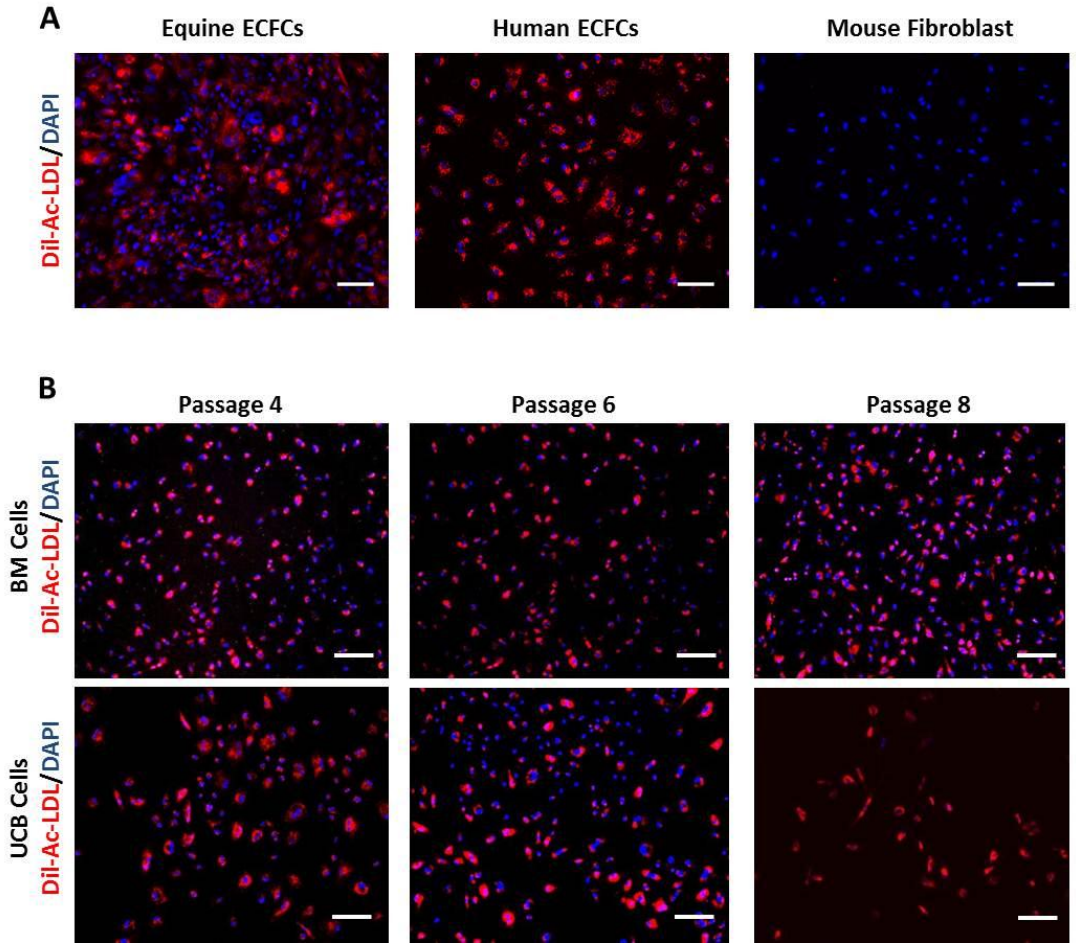

Figure 9. Uptake of acetylated low density lipoprotein (Dil-Ac-LDL) imaged with fluorescent microscopy (tetramethylrhodamine isothiocyanate filter [TRITC] 528nm). A) Equine ECFCs (passage 2) showed an 85% uptake of Dil-Ac-LDL visible by the red coloration of the cytoplasm of the cell, human ECFCs showed a 100% uptake of Dil-Ac-LDL, and 3t3 fibroblast cells as the negative control did not uptake any Dil-Ac-LDL. B) Bone marrow (BM) equine ECFCs at passages 4, 6, and 8 showed uptake of Dil-Ac-LDL. Umbilical cord blood (UCB) cells did show uptake of Di-Ac-LDL at passages 4, 6, and 8; however, cell proliferation dramatically decreased by passage 8. Bone marrow and umbilical cord blood cells showed no significant difference in uptake of Dil-Ac-LDL, (BM, P=0.47 and UCB, P=0.08, respectably). Nuclei are shown in blue being stained with 4',6-diamidino-2phenylindole (DAPI filter 340nm). Images were taken at 10x magnification and all scale bars represent 100 µm.

any passage, (Figure 11). Bone marrow and umbilical cord blood cells showed no significant difference in uptake of Dil-Ac-LDL, (BM, P=0.47 and UCB, P=0.08, respectively). It is important to note that upon qualitative observations the cell doubling rate of the umbilical cord blood cells decreased dramatically from passage 4 to passage 8, (Figure 9).

Isolation of equine carotid artery endothelial cells

Equine endothelial cells from the carotid arteries of both horses were successfully isolated with multiple small colonies of cobblestone morphology appearing by day 2 of culture after isolation. ECs were qualitatively analyzed for EC characteristics of vascular tube formation in matrigel with a score of 4 and uptake of Dil-Ac-LDL before moving forward with high speed cell sorting based on the uptake of Dil-Ac-LDL. ECs were successfully isolated through high speed cell sorting and placed back into culture with no adverse effects on cell phenotype or function. All assays and images of ECs are show in Figure 12.

Analysis of cell marker expression through immunofluorescent staining and flow cytometry

Equine ECs and all three equine ECFC samples stained positively for endothelial markers of vWF, VEGFR-2, CD34, and CD105 (Figure13). Immunofluorescent staining for CD14 was equivocal using an antibody that was optimized for flow cytometry.

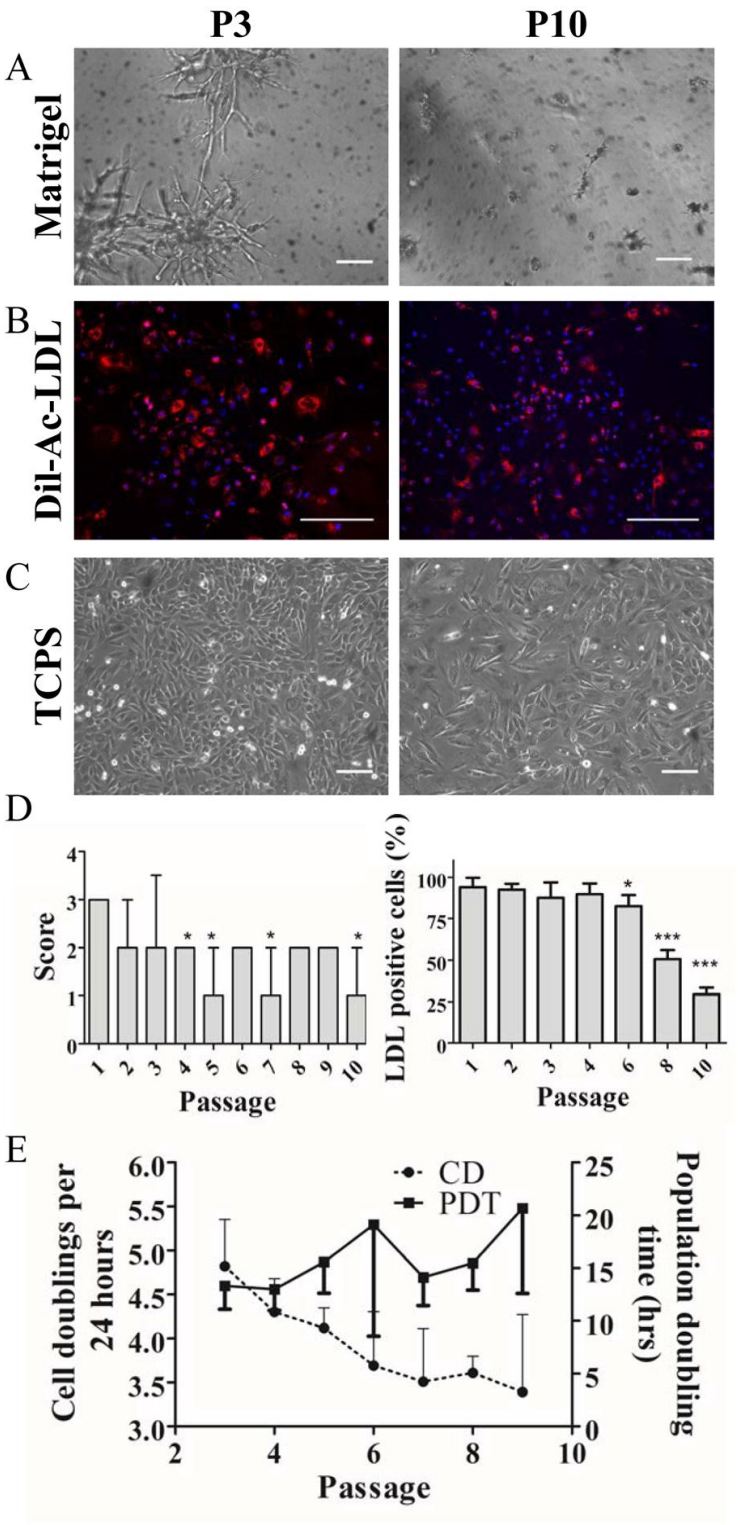

Figure 10. Evaluation of cell function at increasing passage number. A) Equine late outgrowth endothelial cells at passage 3 and 10, showing a decrease in tubule formation at higher passage. Panel A represents a score of 3 and panel B represents a score of 1. B) Uptake of Dil-Ac-LDL at passage 3 and 10 with a decrease in uptake (red coloration of cytoplasm) at higher passages. DAPI (blue) shows the staining of the nuclei. C) Early and late passages showing the loss of cobblestone morphology and increase in vaculated, spindle shaped cells in the later passage. D) The qualitative score of tubule formation decreased as passage increased. Overall, early passages 1-4 (median 2; IQR 2-3) had significantly higher tubule formation scores (P=0.0004) than later passages 5-10 (median 2; IQR 1-2). E) Dil-Ac-LDL uptake decreased significantly form passage 6 to passage 10 from earlier passages, (P<0.0001). F) Cell doublings (CD) showed a trend down as passage increased while population doubling time (PDT) trended up. Scale bars represent 100 µm for both the fluorescent and phase contrast microscope scales.

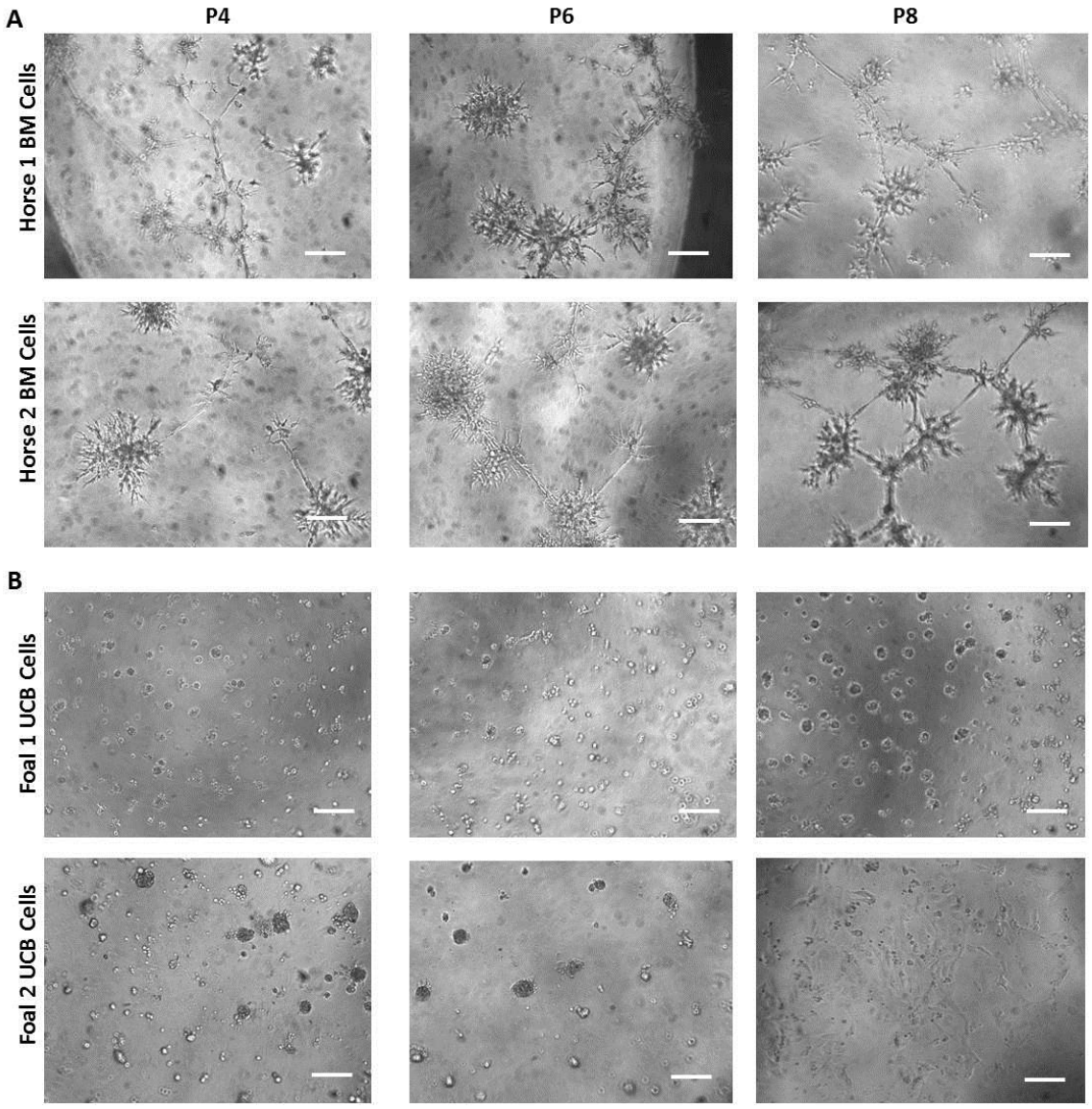

Figure 11. Evaluation of tubule formation as passage increased on bone marrow and umbilical cord blood cells. A) Bone marrow cell never lost the ability to form vascular tubes when evaluated at passage 4, 6, and 8. B) Whereas umbilical cord blood cells did not form tubes at any passage. Bone marrow and umbilical cord blood cells showed no significant difference in uptake of Dil-Ac-LDL, (BM, P=0.47 and UCB, P=0.08). It is important to note that upon qualitative observations the cell doubling rate of the umbilical cord blood cells decreased dramatically from passage 4 to passage 8, (Figure 9). Scale bars represent 100 µm.

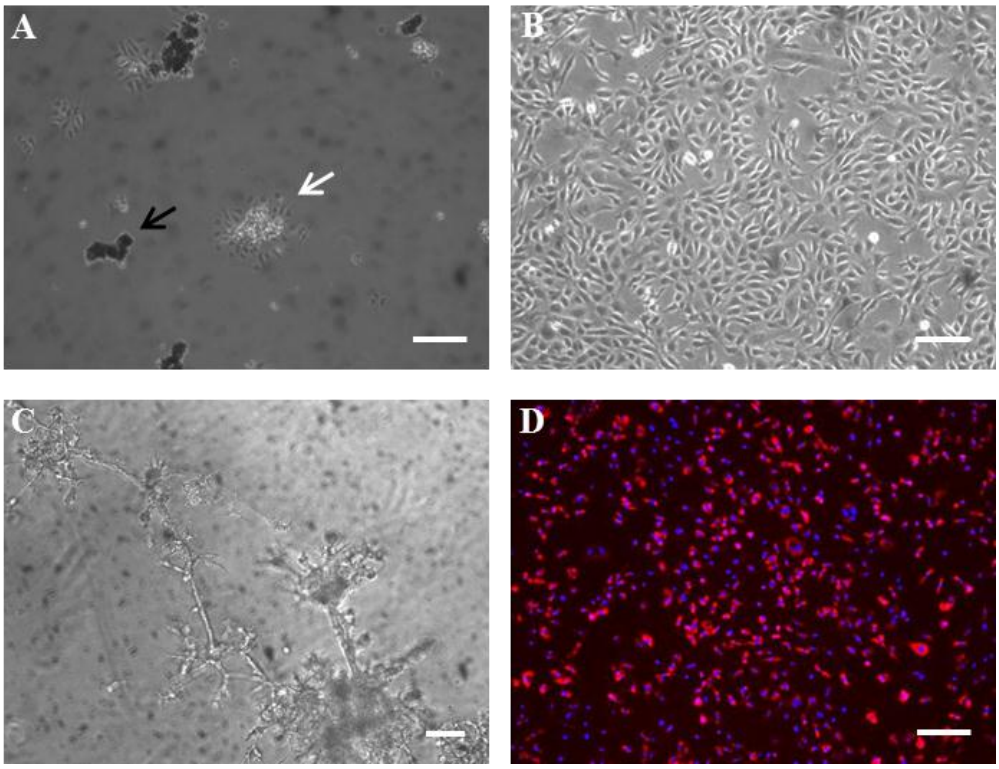

Figure 12. Isolation and characterization of equine endothelial cells (ECs) from the carotid artery. A) Several small colonies formed on day 2 of isolation. White arrow points to an ECs colony and the black arrow points to tissue debris from the digestion of the artery. B) ECs upon expansion after harvesting colonies. Cells showed significant outgrowth and cobblestone morphology. C) Matrigel assay showing ECs producing tube structures on Matrigel. D) ECs showed uptake of acetylated low density lipoprotein (Dil-Ac-LDL) by the red coloration of the cytoplasm and the blue depicts the DAPI stain of the nuclei. Scale bars represent 100 μm at 10x magnification (A, B, and D). Scale bar represents 200 μm at 20x magnification (C).

Flow cytometry was performed using the endothelial marker of CD105 and the hematopoietic marker of CD14. These two cell markers were chosen based on the fact that antibodies for both markers are specific to flow cytometry and had been previously shown to interact with equine cells. The other antibodies (CD34, vWF, and VEGFR-2) were unsuccessful on the ECFCs or the positive control ECs when tested for flow cytometry. All three samples of equine ECFCs isolated for this study stained positively for CD105 with 85.1 ± 13.6% positive cells. Horse number 2 showed the lowest percent positive staining (67.4%), whereas horse 1 and 2 were 90.7 and 91.2% positive, respectively. Equine endothelial cells were 99.6% percent positive. Scatter plots for flow cytometry analysis of CD105 are shown in Figure 14. ECFCs were positive for CD14 (81.1% ± 8.77). Horse 3 had the highest percent positive staining for CD14 of 91.2%, and horses 1 and 2 having similar percent positive staining of 76.7% and 75.4% respectively. Equine whole blood stained positive for CD14 (24.6%), and ECs were negative for CD14 (0.0%). Scatter plots for flow cytometry analysis of CD14 are shown in Figure 15.

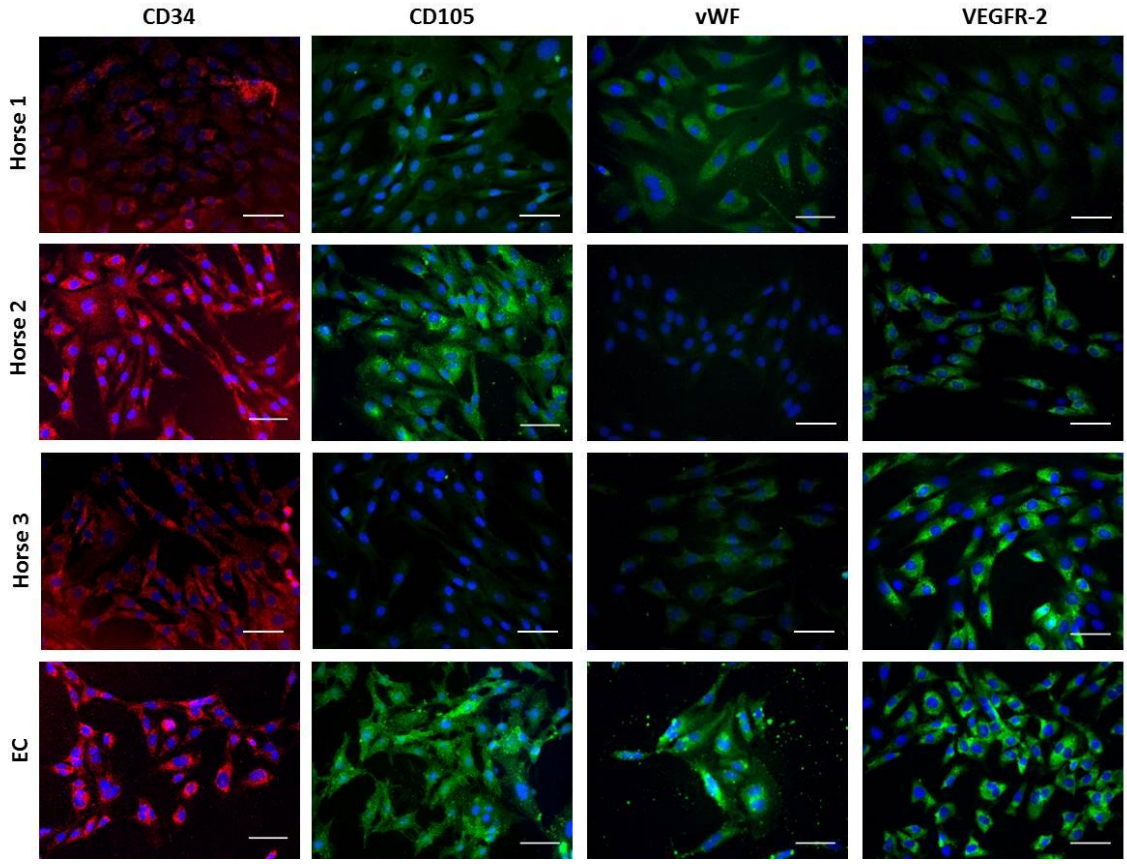

Figure 13. Immunofluorescent staining of endothelial markers. Equine late outgrowth colony forming cells (ECFCs) and equine carotid endothelial cells (ECs) were positive for CD34 (red) and CD105 (green), vWF (green), and VEGFR-2 (green). Nuclei are stained with 4',6-diamidino-2phenylindole visualized in blue (DAPI filter, 340nm). Scale bars represent 50 µm and all images were taken at 40x magnification. Visualization of red fluorescence was tetramethylrhodamine isothiocyanate (TRITC, excitation: 562nm, emission 576nm) and fluorescein isothiocyanate (FITC, excitation: 498nm, emission 520nm) for green visualization.

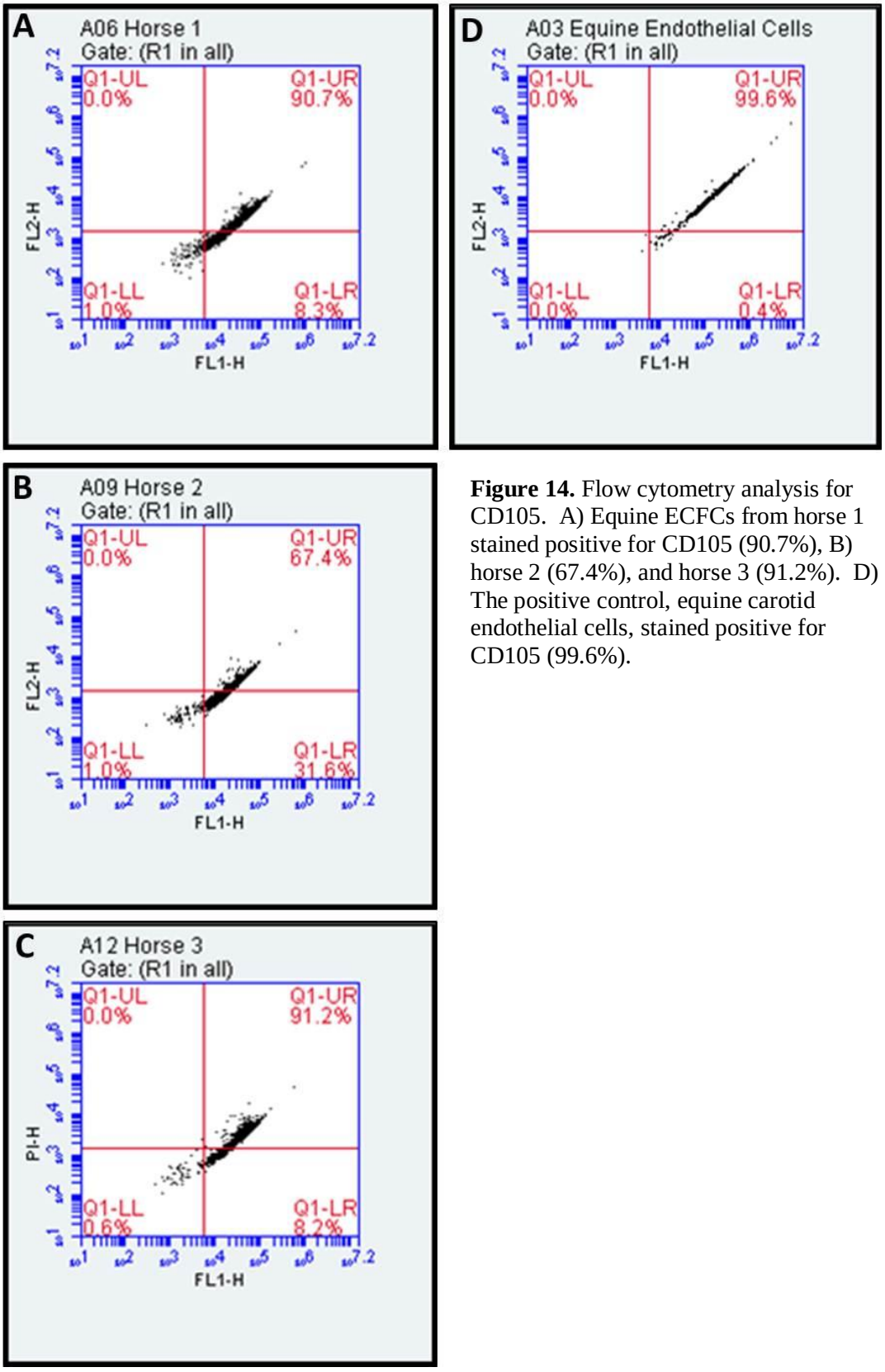

Figure 14. Flow cytometry analysis for CD105. A) Equine ECFCs from horse 1 stained positive for CD105 (90.7%), B) horse 2 (67.4%), and horse 3 (91.2%). D) The positive control, equine carotid endothelial cells, stained positive for CD105 (99.6%).

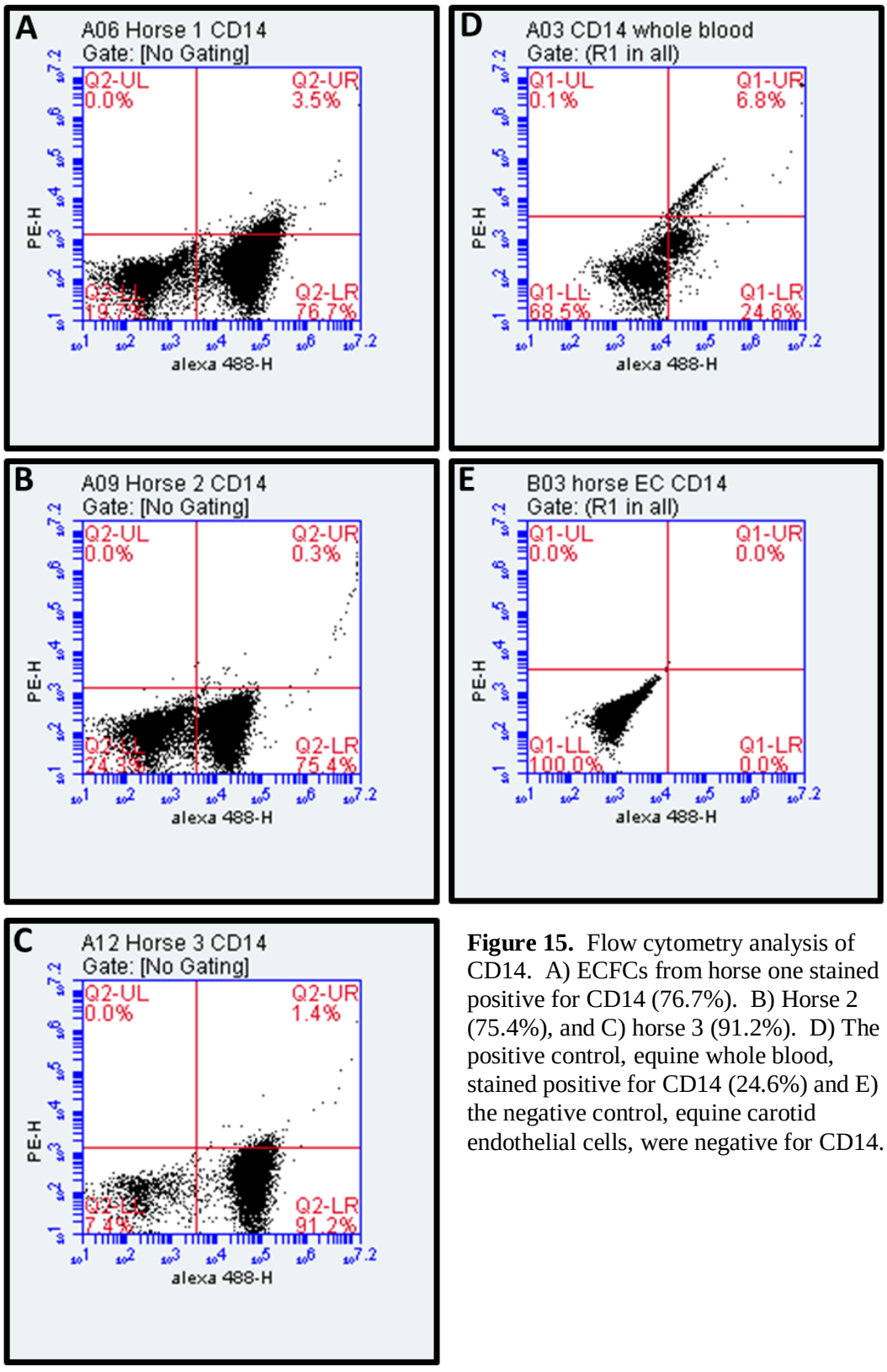

Figure 15. Flow cytometry analysis of CD14. A) ECFCs from horse one stained positive for CD14 (76.7%). B) Horse 2 (75.4%), and C) horse 3 (91.2%). D) The positive control, equine whole blood, stained positive for CD14 (24.6%) and E) the negative control, equine carotid endothelial cells, were negative for CD14.

Chapter Four: Discussion

The potential of EPCs in the future of regenerative medicine is exciting. This study demonstrates that the most important subtype of EPCs, ECFCs, can be isolated non-invasively from peripheral blood of healthy horses. ECFCs can also be isolated from bone marrow samples of healthy horses. Although collection of bone marrow samples is more invasive than collection of peripheral blood samples, bone marrow collection from horses is extremely common in the field of equine regenerative medicine. The ability to isolate and correctly characterize these cells will allow for further research and future therapeutic uses of EPCs in equine regenerative medicine.

As previously stated, engineering of complex tissues may not be an immediate clinical goal in the horse. However, showing that EPCs and ECFCs can be successfully isolated from peripheral blood and bone marrow samples of adult horses will aid in the advancement of equine regenerative medicine. The use of biomaterial scaffolds to optimize the function of stem cells is becoming more and more common and the biggest challenge to the creation of complex tissues using biological scaffolds is vascularization of the engineered tissues [17]. Further studies could be investigated for possible therapeutic uses EPCs for horses suffering from complicated wounds, non-union fractures, tendon and ligament repair, corneal disease, chronic laminitis, and intestinal ischemia. Looking at combining equine EPCs and bioengineered materials, given the vascularization properties of EPCs, could advance current and future regenerative medicine application for those various injuries or diseases. Investigating levels of circulating EPCs when comparing the normal horse to horses in a diseased state could also have interesting findings when studying diseases with vascular damage or disease.

Although ECFCs were successfully isolated, only 3 of 24 horses sampled produced cell colonies. The protocol used for the isolation of the equine ECFCs was optimized for the isolation of human ECFCs and only called for 5 mL of blood to be used for cell isolation. The large size of the horse and the size of the jugular vein (which affects the circulation of endothelial cells due to shear forces) could account for the low yield of equine ECFC seen in this study. In addition, growth factors and serum supplementation were optimized for the human. EPCs are known to be rare in circulation and studies with other species report various cell yields ranging from 4 to 6 colonies per mL being seen [74, 82]. Utilizing a larger blood sample volume and concentrating mononuclear cells with density gradient centrifugation are modifications of the isolation protocol currently under investigation in our laboratory. Bone marrow sampling, although more invasive, is standard protocol for collection of mesenchymal stem cells and bone marrow derived endothelial progenitor cells may be more concentrated in the bone marrow. By using density gradient centrifugation to isolate the mononucleate cell portion of the bone marrow aspirate, an increase in the number of colonies per mL was seen in this study (2.8 ± 1.45 colonies/mL from peripheral blood samples compared to 6 ± 1.41 colonies/mL from bone marrow aspirates). A higher success rate was seen for ECFC colony formation from the bone marrow samples (2 of 3 horses sampled) versus bone marrow, and the one bone marrow sample failing to produce colonies could have been from the result of processing issues with the separation of the cells through the density gradient medium. Umbilical cord blood is commonly known as a rich source of stem cells and it has been shown that umbilical cord blood is also a source for isolating EPCs [81, 128-130]. Human umbilical cord blood provides a good source of

mesenchymal stem cells to be banked and used in regenerative therapies [131-133]. The isolation of mesenchymal stem cells from horses is common and has shown good success rates in cell isolation and potential uses in clinical applications where regenerative medicine is warranted [134-137]. Cell colonies were produced by 2 of the 3 mares where umbilical cord blood samples were collected. Although the cells grew successfully in the EBM-2 supplemented medium and had monolayer cell growth with cobblestone characteristics to the cells appearance, these cells did not show significant outgrowth upon cell expansion, showed less proliferation, and reached cell senescence sooner than the peripheral and bone marrow ECFCs upon qualitative analysis. When looking at cell functional characteristics the umbilical cord blood cells did uptake Dil-Ac-LDL with similar percent positive cells when compared to peripheral and bone marrow blood ECFCs, but they failed to produce tubes during the *in vitro* vasculogenesis analysis. Given their inability to produce vascular tubes *in vitro*, their lack of robust outgrowth, low rates of proliferation, and their colony appearance being in close range of < 7 days in culture these cells had more characteristics that fell into the category of early outgrowth endothelial colony forming cells (EOCs) rather than ECFCs. There is ongoing analysis of cell from equine bone marrow and umbilical cord blood to gain more knowledge and aid in the determination of what the cells true characteristics are and thus identity. From this point forward in the discussion the focus will be only on peripheral blood sample ECFCs.

The isolated equine ECFCs from peripheral blood showed the characteristic cobblestone cell morphology with a single layer of growth which is a key method of distinguishing ECFCs from other cell types. The equine ECFCs formed in colonies after

7 days in culture and had significant outgrowth upon colony harvest and cell expansion. These observations and characteristics of colony appearance, cell morphology, and expansion further aided in the determination that the cells being isolated were ECFCs versus early outgrowth colony forming cells (EOCs), circulating endothelial cells, or other types of adult stem cells found in peripheral blood. All three equine ECFC samples formed tubes in the matrigel tubule formation assay. This characteristic confirms the cells ability to aid in the vascularization of tissues or used as a way to evaluate cell function when EPCs are being used as a biomarker in the equine species. The equine ECFCs formed tubes at 24 hours post cell seeding whereas human ECFCs showed tube formation at 5 hours post cells seeding. This suggests the components of the BD Matrigel Basement Membrane Matrix may be more suitable for the promotion of tubes from human ECFCs versus equine ECFCs. The appearance of the equine tubules was quite similar to tube formations observed in other species [138, 139]. Preliminary work by our collaborating laboratory research team has also shown that equine ECFCs form extensive vascular networks when cultured in a 3-dimensional hydrogel matrix whereas equine bone marrow derived MSCs did not (Seeto and Lipke, unpublished data). The uptake of acetylated low density lipoprotein is a common method used to characterize endothelial progenitor cells in that uptake of low density lipoprotein is a trait of endothelial cells [35, 43, 75, 91]. All three equine ECFCs samples in the study reliably up took Dil-Ac-LDL with 85% positive cells.

Characterizing stem and progenitor cells with cell surface markers is a standard approach, but presents specific challenges in horses because of lack of available antibodies. The antibodies that were chosen for this study either had evidence from the

literature of potential cross reactivity or were an anti-equine protein. Results of the immunofluorescent staining showed equine ECFCs were positive for the endothelial markers of vWF, VEGFR-2, CD34, and CD105. To strengthen the results of the staining, all cells were stained with the secondary antibody only to account for any background staining. Positive control cell (ECs) were included during the staining process lending more to the conclusion that the equine ECFCs in fact are staining positive for endothelial markers which characterizes them as endothelial colony forming cells. vWF is a glycoprotein functioning in hemostasis and endothelial cell adhesion [140]. The presence of the vWF protein on EPCs and ECs not only allows for proper cell function and adhesion when the vasculature is undergoing repair, but also allows for *in vitro* characterization of EPCs and ECs in that other cells types such as smooth muscle cells will lack vWF [141]. Vascular endothelial growth factor 2 is critical for endothelial cell development and is the key receptor that transmits VEGF signals to the endothelium [104]. VEGFR-2 intracellular signaling will effectively promote the survival, proliferation, permeability, and migration of EPCs and ECs [104]. For equine ECFCs to be successfully isolated and sustained in cell culture conditions they must have VEGFR-2 receptors that will bind to the VEGFR-2 in the growth medium. CD34 is a glycophosphoprotein found on lymphohematopoetic and early stem/progenitor cells, endothelial cells, and embryonic fibroblast cells [116]. The presence of $CD34^+$ EPCs is commonly seen as a way to distinguish EPCs from non-EPC and stem progenitors due to early studies showing $CD34^+$ (and VEGFR-2^+) cells isolated from umbilical cord blood, bone marrow, and peripheral blood differentiated into ECs when cultured *in vitro* [33]. CD34 is thought to play a role in the homing and adhesion of leukocytes when expressed

on EPCs and on EPC localization and adhesion. $CD34^+$ equine ECFCs indicate not only that they have endothelial cell characteristics, but that they possess the stem/progenitor phenotype that will allow for the cells to be used in regenerative therapy applications. CD105 is a homodimeric transmembrane protein that is associated with proliferation and hypoxia through transforming growth factor beta (TGF-β) signaling and is predominately found on endothelial cells [120]. TGF-β is a protein complex that governs both proliferation and cellular differentiation.

One unexpected result, but not entirely surprising, is that the isolated equine ECFCs were positive for CD14. CD14 is a receptor for the binding of lipopolysaccharide (LPS) complex and allows for the initiation of the immune response to bacteria and is used as a marker for macrophages and hematopoietic cells [112, 142]. ECFCs have been extensively characterized in humans and much controversy has existed in nomenclature and marker expression. There is not a single marker or group of markers that can distingush ECFCs from terminally differentiated endothelial cells which makes specific identification rely on both markers and other characteristics such as growth and function [33, 42]. One defining difference in EOCs versus ECFCs in humans is that EOCs express the hematopoietic markers CD14 and CD45 and ECFCs do not. This suggests hematopoietic origin of the EOCs. We do not believe that the cells isolated from the peripheral blood samples of horses in this study are EOCs due to the fact that they were positive for the endothelial markers, showed positive uptake of Dil-Ac-LDL, and formed vascular tubes *in vitro*. It is unlikely that cross-reactivity of the CD14 antibody caused a false positive since the equine ECs were negative for CD14 and the peripheral blood stained positive in a similar manner to previous work on equine monocytes. A probable

answer as to why these isolated equine ECFCs are positive for CD14 could lie in a species difference. Work in equine MSCs has shown that they are positive for CD14 suggesting that equine MSCs derive from CD14 positive cell [143]. CD14 was traditionally thought to be lacking on ECs, EPCs and MSCs; however, there is an increasing volume of literature that is contradicting the traditionally considered CD14 positive cell types showing that ECs, EPCs, and MSCs showing expression of CD14 [93, 142-146]. Traditionally, endothelial cells are sensitive to low levels of LPS, so it is not surprising that ECs would express CD14. When ECs are blocked with an anti-CD14 antibody they are not activated by LPS [142]. A study by Jersmann et al., demonstrated that human ECs do in fact express the cell surface marker CD14; however, the loss of CD14 expression as passage number increased was observed to be significant [142]. As the authors stated, assay protocols and cell culture conditions could be having an effect on the expression of CD14. Mesenchymal stem cells or mesenchymal progenitor cells are traditionally labeled as lacking the CD14 marker, but this has been contradicted in equine mesenchymal progenitor cells (MPC). Cells that were sorted as being positive for CD14 produced MPC colonies at a higher percentage than cells that were either unsorted or sorted as positive for CD14 [143]. When entering the study of isolating and characterizing endothelial colony forming cells from horses it was anticipated that the cells would be negative for CD14; however, by the equine ECFCs in this study expressing CD14 is not contradictory to recent observations from other studies [142, 143, 145, 146]. Interestingly, the CD14 in the equine MSC study was trypsin labile which was not the case in our study. More work on cell surface marker characterization over time and with various stimuli would be useful in further characterization of equine ECFCs.

Other studies have shown EPCs from peripheral blood to be positive for CD14 [147]. This leads back to the knowledge that no single marker is capable of identifying EPCs and EPCs subcategories and that a combination of characterization methods is needed to characterize EPCs.

In cell culture conditions, cells are not in their native environment, they are exposed to trypsinization and grown on surfaces not necessarily enhanced for cell performance. They are also are lacking the stimulation of shear forces that are seen in their natural environment of circulation. When investigating cells for use in therapeutic applications it is imperative to evaluate how long cells can remain in culture before losing their functional properties. The quality of equine ECFC tubules was diminished by passage 4, and ECFCs had a significant decrease in uptake of Dil-Ac-LDL between passage 6 and 8. It is the recommendation based on the results of this study that equine ECFCs be used for research and therapeutic application at a passage 5 or lower. This lifespan of the cells' performance is similar to other studies with human ECFCs in that cells can undergo about 100 cell doublings before reaching cell senescence. By having the ability to maintain equine ECFCs to passage 5 allows for significant cell expansion to allow for characterization assays and enough cells for therapeutic use of equine endothelial colony forming cells.

In final conclusion, based on the results of this study, ECFCs can be successfully isolated from peripheral blood samples and bone marrow samples of healthy adult horses. EOCs lack the vasculogenic abilities that ECFCs exhibit leading to the thought process that the umbilical cord blood cells seen in this study may be EOCs. More samples and further analysis is needed to determine the type and availability of EPCs found in equine

umbilical cord blood. When using equine ECFCs in regenerative medicine applications it is advised to use cells at a passage of 5 or less to obtain maximal functional properties of the cells. By furthering the understanding of the basic biology of these equine endothelial colony forming cells, we move closer to advancing the opportunities and applications of equine regenerative medicine. Given the importance of EPCs in human tissue engineering and regenerative medicine applications and the advances that have been made in equine regenerative medicine, a source of equine EPCs will be invaluable.

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