

Isolation and characterization of Porcine Monocyte-Derived Mesenchymal Cells

by

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B.S., Kansas State University, 2006

M.S., Kansas State University, 2008

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Abstract

Monocytes are leukocytes in peripheral blood that differentiate into macrophages in the context of the inflammatory response. Leukocytes are easy to isolate from a blood sample by inexpensive standardized methods, such as the Ficoll-based density gradient. We have found that monocytes isolated from peripheral blood of pigs and grown using simple procedures produce large numbers of mesenchymal cells that exhibit differentiation into mesodermal lineages *in vitro*.

Peripheral blood samples were obtained from 2, 4, and 6 months old male pigs. The cells were isolated by a Ficoll-based density gradient and cultured in 20% FBS in DMEM media, on uncoated tissue culture vessels. All isolates exhibited mesenchymal morphology and continued to expand at least to passage 7. The expansionary potential was greatest for the cells obtained from the 2 mo. old pigs. We isolated similar cells from porcine fetal livers (gestation day 60), at which time hematopoiesis is occurring in the liver. Therefore, these cells are present from at least mid-gestation through 6 months, the approximate age of puberty in pigs.

In regards to immune-phenotype, the cells are strongly positive for the leukocyte marker CD 14 and SLA-DR-II. Approximately 50% of the cells are positive for CD 45, and they are negative for CD 105, CD 31, and CD 90. The monocyte-derived cells express mRNAs for TLR-3,4,5, 7, and 9. They also express the pluripotency-associated gene Nanog but only weakly express Sox-2 and Oct-4. *In vitro* the cells are capable of differentiation into adipogenic, osteogenic, and chondrogenic lineages. They also exhibit phagocytosis as measured by *in vitro* assay. We tested their ability to support the porcine reproductive and respiratory virus *in vitro* but they were not supportive using standard techniques. Initial attempts have also failed to support myogenic differentiation.

The cells isolated in this study represent a novel subset of monocytes with characteristics overlapping those of mesenchymal stem cells. Swine are physiologically similar to humans and further work is needed to characterize these cells for regenerative medicine applications.

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Acknowledgements

I would like to acknowledge the support of my parents and my head advisor, Dr. Duane Davis in the completion of this work. My many thanks as well to the laboratory personnel who have helped me in the completion of this thesis and the development of this project.

Dedication

This thesis is dedicated to my parents, who have been my greatest source of support for my university studies in the USA.

Chapter 1 - Introduction

Monocytes are a heterogeneous population of leukocytes of clinical significance as these cells have a crucial role in the process of inflammation and thus have an important function in the clearance of foreign invaders. In addition, monocytes have a range of essential physiological properties, such as: phagocytosis, differentiation, and chemotaxis potential (Goldsby, 2002). Moreover and as described in this dissertation, monocytes are the only leukocytes in peripheral blood from which cells with multipotent characteristics have been derived.

Several groups report the isolation and characterization of properties of unique monocyte subsets obtained from human blood. Amongst these descriptions are: fibroblastic macrophages, (Zhao et. al., 2003), programmable cells of monocytic origin, (Ruhnke et. al., 2005), peripheral blood insulin producing cells, (Zhao et. al., 2007), monocyte derived multipotential cells/progenitors (Kuwana et. al., 2003), and CD 45 progenitors (Rogers et. al., 2007).

The common denominator amongst all of these subsets is the expression of the leukocyte marker CD 14. In addition, most monocyte cultures are grown on RPMI based media formulations with the addition of 10% FBS, and do not require the use of coated tissue culture vessels for expansion and culture. Monocyte derived multipotential cells/progenitors are an exception to this last characteristic in that they require fibronectin coated vessels for isolation (Kuwana et. Al., 2003).

The research in this dissertation describes another unique monocyte-derived cell the monocyte-derived mesenchymal cell (MDMC) and provides initial characterization of its immunophenotype, phagocytic activity, gene expression, and expansionary characteristics in vitro. These cells have characteristics overlapping traditional monocytes and mesenchymal stem cells their morphology and mesodermal differentiation potential.

Chapter 2 - Literature review

Origin of cells in the blood

To set the context for the work in this dissertation this section provides some current concepts about the origin and functions of monocytes. The cellular components of the blood are divided into two general categories: the erythrocytes, and the leukocytes. The following descriptions are adapted from Goldsby (2002) and Carlin et al (2013).

Erythrocytes are the red blood cells and their function is to deliver oxygen to all other tissues of the body. Leukocytes a heterogeneous population of cells. They are essential for the innate and adaptive immune response found throughout mammalian species (Goldsby, 2002).

Leukocytes are found primarily in the bone marrow or the spleen. All leukocytes share a common ancestor. The hematopoietic stem cells reside in the bone marrow of adult mammals and are the common ancestor of all leukocytes Hematopoietic stem cells give rise to copies of themselves and also to progeny that will eventually become other cell types. Hematopoietic stem cells have a polygonal morphology. They give rise to both erythrocytes and leukocytes.

Hematopoietic stem cells are considered to be pluripotent in that they differentiate to all the blood cell types, however ‘pluripotent’ as applied to blood stem cells has a different meaning than the common definition applied to totipotent cells, such as a fertilized egg. In the latter case pluripotency is the ability to differentiate to each of the cell types in the body.

Hematopoietic stem cells are found in different anatomical locations, depending on the stage of development. First, they are present in an extraembryonic membrane, the yolk sac. At the third month of gestation (in humans), these hematopoietic stem cells migrate from the yolk sac to the fetal liver and then to the spleen. Finally, and in the weeks prior to parturition,

hematopoietic stem cells move to the bone marrow, their almost exclusive location postnatal (Goldsby, 2002).

Hematopoietic stem cells are naturally scarce in numbers. The frequency of hematopoietic stem cells is estimated to be less than one per 50,000 cells in the bone marrow. (Goldsby, 2002). Hematopoietic stem cells give rise to two main subgroups of cells: the myeloid, and lymphoid progenitors. Myeloid progenitors give rise to phagocyte cells, erythrocytes, and megakaryocytes, which are the precursor of platelets. Lymphoid progenitors, on the other hand, give rise to T-lymphocytes, B-lymphocytes, and Natural Killer cells. In general terms, myeloid progenitors are characterized for their ability to carry out a cellular function known as phagocytosis. The most prominent characteristic of lymphocytes is their ability to act as coordinators of elements the immune system.

Amongst the most commonly known progeny of the myeloid progenitors are cells that are capable of professional antigen processing and presentation. Antigen processing depends on successful phagocytosis of a pathogen. The most well characterized 'professional' phagocytes are progeny of myeloid cells the: eosinophils, basophils, neutrophils, macrophages, and monocytes.

Among the myeloid progenitors, monocytes are the subject of renewed interest as these cells might very well be the only circulating stem cells present at all stages of development. Traditionally, monocytes are known to give rise to macrophages and dendritic cells upon exposure to soluble proteins naturally present in circulating blood. Recent research reports, however, hint to a much higher degree of functional variety for monocytes. In addition, novel functions and subsets continue to be discovered for these cell types. Similarly, monocytes might

have far reaching implications on the incidence, progression and possible treatment of hematological malignancies (Goldsby, 2002).

Monocytes

In vivo models of disease, such as genetically engineered mice, have yielded insight into different monocyte subsets. Mice engineered to be devoid of particular chemokines and integrins have helped identify the subsets of monocytes that rush to sites of inflammation (Carlin, et. al., 2013). Similarly, mice devoid of certain toll like receptors have yielded insight in the understanding of monocyte functions as discussed in Carlin et. al. (2013).

Inflammation is a process that entails many elements and different subsets of monocytes specialize in responding to different elements of the response. Upon breach of the innate barriers of the self, molecular signals, such as chemokines, lead to the recruitment of neutrophils into the area. About a day after this event, a subset of monocytes expressing the lymphocyte antigen 6c (Ly6c+) appear on site and serve to coordinate and orchestrate the cellular immune response. This subset of monocytes exits the bone marrow and extravasates to critical areas. Many chemokines are involved in this process, and one of the most prominent ones is chemokine receptor type 2 (CCR2). Once on site, these monocytes differentiate into subsets of macrophages and dendritic cells. These differentiated progeny, in turn have a pivotal role in phagocytizing and clearing foreign invaders. In addition, the differentiated phagocytes have a major role in the stimulation of T-helper lymphocytes (Carlin, et. al., 2013).

Another monocyte subset has almost opposite characteristics to the one just described. It expresses low levels of Major Histocompatibility Complex type II (MHC II) and LyC 6 antigen and is generally unable to interact with other leukocytes such as the T-lymphocytes. These monocytes have a high expression of CX3 C chemokine receptor 1 (CXCR1), and express high

levels of the transcription factor Nr4a1. They are capable of interaction with antibodies and with the B-lymphocytes that produce them. Differentiation into other phagocytes has not been found for these cells, and their primary function is thought to be to aid in the repair of muscular traumas (Carlin, et. al., 2013).

The primary function for the LyC 6 – monocytes is recruitment into endothelial tissue to mediate phagocytosis of compromised cells. These monocytes set up residence in the vicinity of compromised endothelial cells and recruit neutrophils for clearing compromised entities. LyC 6 – monocytes are brought into alert when double stranded DNA that is associated with viruses bind to their TLR-7 receptors (Carlin, et. al., 2013).

Culture of monocytes

A common method to isolate monocytes is based on density after centrifugation in a ficoll gradient. In this method whole blood is overlaid on a ficoll preparation and then centrifuged. Upon the creation of a density gradient from whole blood, the leukocytes form a distinct white band on the tube called the buffy coat. The buffy coat contains a heterogeneous mixture of leukocytes present in the blood sample. From this point the common method of separating monocytes is to allow for selective attachment of cells (Zhao et al., 2003). After plating the cells in the buffy coat and allowing a period of attachment on tissue culture vessels changing the medium is expected to remove other cell types. Standard tissue culture techniques can be applied to isolate and characterize monocyte subsets.

Several groups of researchers have reported growth and characterization of monocytes under different conditions. The different subsets of monocytes grown under various conditions have particular immune phenotypes and gene expression patterns. Amongst the universal parameters for the monocyte lineages are positive expression of clusters of differentiation (CD)

markers such as CD 14, CD 45, and CD 105 (Goldsby, 2002). CD 14 is a molecule at the surface of a cell that acts as a receptor for lipopolysaccharides (LPS), a common component of bacterial cell walls. When LPS is bound to CD 14, monocytes enter an alert state. CD 45 is also known as the leukocyte common antigen (LCA), and is expressed in all hematopoietic stem cells. CD 105 acts as a modulator of cellular responses to growth factors.

Monocytes are traditionally known as precursors to professional antigen presenting, the phagocytic macrophages. During the maturation process pro-monocytes enlarge and ultimately migrate to specific anatomical locations to differentiate. The maturation process in the blood usually takes 8 hours. Among the end differentiation states of monocytes in particular tissues are the phagocytes. Phagocytes are cells that have the ability to serve as antigen presenting and phagocytosis centers (Goldsby, 2002).

Phagocytes are essential cells of the immune system that aid in clearing and processing of pathogens. Among phagocytes are macrophages and dendritic cells. When monocytes differentiate into macrophages, the cells increase in volume, and the organelles increase and become more complex. As a result of these changes, macrophages have the capabilities and cellular machinery to digest and process antigens in a process known as phagocytosis. Phagocytosis is also seen in monocytes but the process is more active in differentiated progeny cells such as macrophages and dendritic cells (Goldsby, 2002).

First, an antigen on a pathogen adheres to the cell membrane of a phagocyte and adherence leads to the enlargement of proximal areas of the cell membrane. The elongated membrane is called pseudopodia and it ultimately surrounds and engulfs the pathogen whose antigen is bound to the cell membrane. The pathogen is thus engulfed in a membrane inside the

cell called a phagosome. The phagosome enters the endocytic pathway fuses with an acidic organelle called the lysosome. The resulting complex is a phagolysosome.

Lysosomes are organelles that contain acidic enzymes such as lysozyme and other acidifying agents that digest and decompose the antigen into its elementary constituents. The digested material is then eliminated in a process called exocytosis.

Professional antigen presenting cells have the ability to present the digested antigens. Digested antigens are processed after phagocytosis and then presented at the surface of the cell. The antigens are presented in the context of MHC I, for interaction with cytotoxic lymphocytes of MHC II for interaction with helper lymphocytes.

This leads to the effective mounting of a cellular adaptive response. Examples of professional antigen presenting cells are dendritic cells and macrophages. Monocytes are not traditionally considered to be professional antigen presenting cells (Goldsby, 2002).

Phagocytosis, and general characteristics of phagocytes

Phagocytosis is one of the earliest specializations to appear during development. In humans professional phagocytes can be found in the embryonic yolk sac at the fifth week of gestation (Rehakova et. al., 1998). The initial recognition of the antigen by the phagocytic cell is critical for phagocytosis to occur. The degree to which an antigen is successfully phagocytized is directly dependent on internalization by the pseudopodia. Since phagocytes have cell receptors sensitive to the Fc portion of antibodies, those antigens that are coated by antibodies are better internalized. The synergistic action of specific cell receptors for a particular antigen, and those for the Fc portion of antibodies results in enhanced phagocytosis.

These cell receptors are of particular importance, since MHC class II molecules present antigens to the T-helper Lymphocytes. The T-helper lymphocytes are the coordinators for the

adaptive immune responses against foreign invaders to the body. When a phagocytes is in an alert state, it immediately express more MHC class II molecules on its surface in order to alert the humoral and cell mediated responses into action.

Opsonization is the process by which pathogens are first covered with specific antibodies prior to interaction with a phagocytic cell. Opsonization greatly enhances the rate of phagocytosis of a pathogen. Normal plasma and high concentrations of monoclonal antibodies are commonly used to coat antigens prior to phagocytosis. In this context, normal plasma and monoclonal antibodies are known to act as opsonins.

The ultimate fate of successfully phagocytized pathogens is either exocytosis or antigen presentation. Exocytosis, the process by which the digested pathogen is expelled out of the cell is the most common of the two end stages. Nevertheless, antigen presentation occurs when digested material inside of the cell is coupled to MHC class II receptors and transported to the cell surface (Goldsby, 2003).

In the laboratory a practical convenient method for evaluation of phagocytosis is the use of fluorescent beads. The beads are shipped with a fluorophore inside and are washed prior to use to remove surfactants and antimicrobials that could interfere with the assay. For phagocytosis assays, beads with 2 micron diameter are traditionally employed, as smaller sizes are more likely to be endocytosed indiscriminately (Blanchette et.al., 2009), (Steinkamp et. al., 1982).

The ratio of opsonized beads to target cell is usually kept at 30 to 1. Nevertheless, some research groups have published studies with ratios as high as 100 to 1. The rate of phagocytosis is dependent on the chances of collision amongst the beads, and the target cells. The chances of collision, in turn depends the number of the much smaller beads to the much bigger cells (Berry, et. al, 1946).

Once in the reaction vessel, the mixture of cells and beads is kept at a temperature of 37°C, as most cellular processes are favored by that temperature. In addition, gentle rocking is exerted on the reaction vessels to avoid the settling down of beads and target cells due to gravity. For the assays to yield relevant results, the target cell population should be homogeneous. Most commonly, the cells must be present devoid of other cellular contaminants such as erythrocytes, that would interfere with the beads to target cells interactions (Savard et.al., 2000).

Under *in vitro* settings, phagocytosis is inhibited by low temperatures, as well as the use of saline devoid of ions. For most mammalian cells, cellular processes are slowed down at 4°C, and cellular pumps cannot carry out necessary functions when they are on media devoid of ions (Parod and Brain, 1983).

In addition, for the process to successfully occur, an extended period of time must be allowed. On average, the time allowed for incubation of opsonized beads to target cells is 90 min. Phagocytosis assays are typically measured via flow cytometry or fluorescence microscopy. The use of a flow cytometry based assay allows for a more quantitative approach to the number of phagocytic cells in a sample. In addition, a much higher number of cells can be analyzed, thus making the results more statistically significant. Typically, 20.000 events or more are analyzed per sample tube in a flow cytometer based phagocytosis assay (Fattorossi et. al., 1989).

In order to make this measurement, a sample dedicated to setting the baseline background fluorescence of the cells only is employed. The quantum emission of the fluorescent beads is much higher than that of the background level, thus easily allowing for the discrimination of internalized beads. The most common depiction of this phenomenon is the use of a graph with the number of events on the Y-axis, and the fluorescence intensity on the X-axis (Fattorossi et. al., 1988). For more accurate results debris, and other unwanted populations can be electronically

excluded by the use of specific gates (Talbot et.al., 1998; Zahn et.al., 1997). So long as the cells are centrifuged at speeds of at least 1000 x G, most of the free (not phagocytized) beads are washed away, leaving only internalized beads for analysis on the flow cytometer (Savard et.al., 2000).

For phagocytosis to occur the cytoskeleton of the cell must be rearranged. The physical engulfment of a pathogen involves deep elastic changes in the cytoskeleton of the cell. For this elastic change to happen, actin fibrils must be able to polymerize and depolymerize. Fungal toxins such as cytochalasin D have been used to block phagocytosis. Cytochalasin D has the ability to block actin polymerization at the molecular level. When monocytes in culture are exposed to concentrations ranging from 5 to 10 μ M Cytochalasin D, phagocytosis is inhibited. The compound is toxic to cells and exposure for 15 to 30 min. is enough to inhibit actin polymerization without affecting cell viability (Ribes, et. al., 2010; Sanguedolce et.al., 1992; Zahn et.al., 1997; Steinberg et.al., 2007).

Furthermore, stimulation of certain components of the innate immune system impact phagocytosis. Specifically, stimulation of toll like receptors (TLR's) with their respective ligands has a positive impact on the rate of phagocytosis. TLRs 2, 4, and 9 recognize molecular structures and pathways used by bacterial pathogens. When these TLRs are bound to their ligands, the phagocyte becomes alert and primed for enhanced phagocytosis (Ribes et. al., 2010).

Current knowledge of phagocytes leads us to believe that such cells are highly responsive to a variety of cytokines and become activated in specific and non-specific manners when exposed to pro- inflammatory cytokines. Ability to display phagocytosis has also been linked with ability of the phagocyte to serve in the maturation and regeneration of wounds. Upon

phagocytosis of apoptotic cells, debris, and foreign substances on the site of injury, macrophages are believed to take on a regenerative role (Ungeforen et. al., 2015).

Monocyte-derived cells with stem cell properties

Several reports identify cell populations that are separated from whole blood by density gradients, attachment to culture dishes, and display characteristics of stem cells. Most of these reports deal with monocyte-derived cells as indicated by their surface epitopes. However besides their monocyte origin the relationships of these cell populations to each other are unclear. The names and initial references for the monocyte-derived cells are fibroblastic macrophages (f-macrophages, Zhao et al., 2003), monocyte-derived multipotential cells/progenitors (MOMC/MOMP, Kuwana et al., 2003) and programmable cells of monocytic origin (PCMOs, Dreskke et al., 2006).

Another group identified peripheral blood stem cells that have hematopoietic markers but are not identifiable as monocytes. These are PB-IPCs (peripheral blood insulin producing cells, Zhao et al., 2007), that are positive for CD9, CD45, and CD117 but not the hematopoietic stem cell marker CD34 or markers for lymphocytes CD3 (T cells) or CD20 (B cells) or monocytes/macrophages CD14 and CD11b/Mac-1. Also of interest are the CD 45 progenitors identified by Rogers et. al. (2007). This is a rare population found in human umbilical cord blood. It is considered lineage negative and is prepared by negative selection for CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66, and cells expressing glycoprotein A and positive selection for CD45. Regarding the monocyte-derived stem cells, there are differences. These cells differ in their sources, initial treatments, and the culture conditions employed as well as the characteristics evaluated. These are discussed below.

Fibroblastic Macrophages (Zhao et. al., 2003). This procedure first differentiates monocytes to macrophages and then, in the presence of M-CSF and LIF two morphologies are found. One is round (called standard, s) and the other is fibroblast-like (f). The f cells proliferate and the s cells don't.

Methods of isolation and culture. Human monocytes are isolated from the buffy coats of 500 mL of peripheral blood, plated at $2-3 \times 10^7$ cells per 15-cm dish in RPMI medium 1640 with 10% FBS for 8-12 h in an 8%CO₂ atmosphere. The floating cells are removed and attached cells removed with forceful pipetting.

The resulting cells are 90-95% CD14 positive and are plated at 1×10^5 cells /ml in 8-well lab-tek chamber slides (0.4ml/well). Half of the medium is replaced every 5-7 d. Cells cannot be lifted with standard enzymatic methods so they are dispersed using 2% lidocaine in PBS. Treatment with M-CSF (50 ng/ml) yielded 35% f-macrophages. Adding LIF (1,000 units/ml) to M-CSF yielded approximately 50% f-macrophages and the remainder s-macrophages. The f-macrophages include dividing cells and by 14 d their number increases and the number of s-macrophages decreases.

Characteristics of the cells obtained. Fibroblastic macrophages display fibroblast morphology and exhibit monocyte markers (CD14, CD34, and CD45). Macrophage, T-lymphocyte, epithelial, neuronal, endothelial, and hepatic differentiation was induced.

Differences between the f-macrophages and the s-macrophages are that the former exhibited less IL-10, TNF-alpha, TNF3-RII, HLA-DR, and HLA-DQ. The f-macrophages exhibited less killing of human leukemia cells than the standard cells. However the f-cells stimulated more lymphocyte proliferation. Also the f-cells had fewer lipid droplets and expressed less leptin and PPAR gamma.

The f-cells had higher levels of the hematopoietic stem cell marker CD34. The authors postulated that the f-macrophages are the progenitors of s-macrophages. They estimated that the f-cells double in in culture about every 3 d.

Programmable cells of monocytic origin (PCMOs, rat cells: Dreskke et. al., 2006, human cells: Ruhnke et al., 2005). PCMOs are generated from circulating monocytes by a process that is considered de-differentiation. This is accomplished by exposure to M-CSF and IL-3 for 6 d and the process is patented by F. Faendrich (Kremer et al., 2009 cited in Zachos et al., 2014). Human PCMOs were differentiated to hepatocytes and tested in an immune-deficient mouse model and the rat cells were tested in an allogeneic heart infarct model. They can differentiate chondrogenically (Puffe et al., 2008). While they are not to be capable of osteogenesis they do promote osteogenic differentiation of MSCs in co-culture (Zachos et al., 2014).

When human monocytes were cultured in medium with M-CSF and IL-3 they proliferated and formed colonies. By 5 to 6 d they became confluent by proliferating and increasing in cell size. Both cell number increased and DNA synthesis was demonstrated. Some cells remained positive for CD14 but the percentage is reduced to about 46% by d 6. Toll-like receptors (2, 4, 7, and 9), and Klf4 are downregulated during dedifferentiation but Oct4 and Nanog are upregulated (mRNA for Sox2 was not detected) (Ungreforen et al., 2010).

Methods of isolation and culture (Dreskke et al., 2006). After density gradient centrifugation the buffy coat is isolated from a sample of whole blood from male Lewis rats. The buffy coat is then plated onto uncoated tissue culture vessels and incubated 2 h. The cells from the buffy coat are seeded at 1.3×10^7 cells/cm² and are cultured in 10% FBS in RPMI supplemented with 2 mM glutamine, 100 U/ mL penicillin, and 100 mg/mL streptomycin.

Upon completion of the initial incubation the nonadherent cells are removed by gentle washing with PBS. Adherent cells are cultured in 10% FBS in RPMI 1640, supplemented with 140 μ M β -mercaptoethanol, 5 ng/mL murine M-CSF, and 0.4 ng/mL murine IL-3. After 6 d the adherent cells are harvested non-enzymatically and considered to be PCMOs.

As determined by flow cytometry tests, the cells isolated in this fashion are CD 11b +, and CD 90 +, corresponding to monocytic and stem cell markers, respectively. On the basis of these results the murine cells isolated by means of this protocol are analogous to the PCMOs of human origin described previously (Ruhnke et al., 2005).

Characteristics of the cells obtained. PCMOs were given by intra-myocardial and intravenous injections in murine models of myocardial infarction. The SRY gene, indicating the cells as from the male donor PCMOs, was detected in the infarcted hearts. There was significant improvement in myocardial function in PCMO-transplanted animals as compared to controls. As compared to traditional monocytes (controls), PCMO-treated rats had significantly higher expression of VEGF-A and increased neovascularization near the infarction zone.

PCMOs are capable of expressing homing receptors to engraft in the area of infarction. Expression of the chemokine receptors CXCL3, and CXCR4 is significantly higher in PCMOs derived from infarcted and non-infarcted animals as compared to traditional monocytes. Interestingly, expression of the chemokine receptor CXCR3, and its ligands CXCL 9, CXCL10, and CXCL11, in addition to VEGF-A are significantly upregulated in PCMOs derived from infarcted animals as opposed to their counterparts in healthy animals.

The authors reported that small numbers of transplanted cells were found in the area of infarction. By both routes of administration the PCMOs were consistently identified near the

infarction area. Nevertheless, long term (+ 60 d) presence of the cells was low and only detected by PCR for SRY suggesting low levels of long term engraftment.

Peripheral blood insulin producing cells (PB-IPCs, Zhao et.al., 2007). These cells are obtained from adult human blood and have characteristics of both embryonic and hematopoietic lineages. Specifically, these cells express the transcription factors Oct-4 and Nanog, along with well recognized hematopoietic markers, such as: CD 9, CD 45, and CD 117. Nevertheless, these cells did not express CD 34, a hematopoietic stem cell marker, and had expression patterns for markers closely associated with monocytes and macrophages. A number of lines of evidence were reported to indicate the islet- β -cell progenitor characteristics of PB-IPCs. For instance, PB-IPCs expressed β -cell-specific transcription factors for the insulin gene. In addition, PB-IPCs were able to produce insulin and improve the status of diabetic in mice. In subsequent work PB-IPCs were differentiated to astrocyte-like cells (Li et al., 2015).

Methods of isolation and culture. Procedures used were the same as those used previously for isolating stem cells from human cord blood (Zhao et al., 2006). Buffy coats were isolated from human peripheral blood samples. The age range for the blood donors was 20 to 62 years. Density gradient separation was used to obtain the buffy coat. Peripheral blood samples were overlaid on top of Ficol-Hypaque 1.077 and then centrifuged to generate a density gradient.

RBCs were eliminated by means of a red blood cell lysis buffer. The cells were seeded into tissue culture vessels and cultured in 7% FBS in RPMI, and incubated at 37°C, 8% CO₂. Cells from the buffy coat were seeded at a rate of 1 x 10⁶ cells/mL with 25 mL in 150 x 15 mm Style Petri dishes (Beckton Dickinson Labware, Franklin Lakes, NJ) . PB-IPCs were injected into *in vivo* murine diabetes models at a rate of 5 x 10⁶ cells/mouse via an intra-peritoneal route.

Characteristics of the cells obtained. The authors detected expression of particular gene transcripts in peripheral blood mononuclear cells (PBMCs) that caused them to conclude there is a subset of PBMCs that express insulin mRNA. In addition, other transcripts associated with insulin, such as: basic leucine zipper MafA, and Nkx6.1 were found. Similarly, transcripts for somatostatin and ghrelin, which are common products of the pancreatic islets were also identified.

PB-IPCs were isolated by means of selective attachment to tissue culture vessels. The insulin transcription factors group: MafA, Nkx6.1, Pdx1, and NeuroD1 were strongly expressed in PB-IPCs as measured by western blots. In addition, enzymes the insulin convertases: PC1/3, and PC2 were expressed in PB-IPCs. ELISA assays showed that insulin and its by product C-peptide were secreted by PB-IPCs.

PB-IPCs expressed elevated levels of CD-9, tetraspanin, CD-45, leukocyte common antigen, and CD-117, stem cell factor receptor. However, PB-IPCs were negative for CD-34, a hematopoietic stem cell marker, CD-3, a T-cell marker, and CD-20, a B-cell marker. PB-IPCs were negative for CD-14, and CD 11b, markers commonly associated with the monocyte/macrophage lineage.

Moreover, PB-IPCs were negative for HLA-DR, CD-40, and CD-80, which are markers that are associated with monocytes and macrophages. Very few cells expressed HLA-DQ, and CD-86 (less than 10%). PB-IPCs strongly expressed HLA-ABC, a marker found in almost all nucleated adult cells.

In regards to embryonic stem cell markers, western blot analyses showed that the transcription factors Oct-4, and Nanog were expressed in PB-IPCs. Results from *in vivo*

experiments in which PB-IPCs were injected into diabetic mice showed *de novo* production of insulin.

Monocyte derived multipotential cells/progenitors, MOMCs (Kuwana et. al., 2003).

MOMCs have been shown to differentiate into endothelial cells and osteoclasts (Kuwana et. al., 2003). When the cells from the buffy coat were plated on fibronectin coated tissue culture vessels, a fraction of the cells attached immediately. Clusters of circular cells attached as early as 24 hrs. after initial plating and by d4 and 5 adherent cells with a fibroblast morphology were present (Kuwana et. al., 2003).

These adherent cells have proliferative potential and are referred to as monocyte derived multi-potential progenitors (MOMPs) by Kuwana et al.(2003) and monocyte-derived multi-potential cells (MOMCs) by Seta and Kuwana (2010).

When viewed with the transmission electron microscope, MOMCs had a characteristic spindle shape with pseudopodia, lysosomes, and endocytic vesicles that are commonly associated with macrophages (Kuwana et. al., 2003).

MOMCs appear to be a subset of CD 14 positive monocytes. Differentiation of MOMCs required fibronectin binding and exposure to factors derived from CD 14 negative peripheral blood cells. These cells were able to be cultured *in vitro* for up to 5 passages before they stopped proliferating. As measured by gene and protein expression associated with particular lineages, MOMCs are able to undergo multilineage differentiation (Kuwana et. al., 2003).

Methods of isolation and culture. The cells from the buffy coat were plated at a very high density, 2×10^6 / mL on tissue culture vessels coated with fibronectin (10 μ g/ mL)by incubating overnight at 4°C (Kuwana et. al., 2003). In later work Seta et al., (2012) concluded that producing MOMCs depends on their binding to the RGD domain of fibronectin. The media

used was 10 % FBS in DMEM, supplemented with 2 mM L-glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin.

Cells from the buffy coat were plated in the fibronectin-coated tissue culture vessels and allowed to incubate at 37°C, 5% CO₂. The medium was replaced every 3 d, and unattached cells were removed. The cells were cultured for a total of 4 weeks (Kuwana et. al., 2003).

After about a week in culture, the cells were lifted and used for assays or re-plated on coated plates and expanded up to passage 10. After two weeks in culture, these cells stopped dividing but remained viable for a maximum of 4 weeks (Kuwana et. al., 2003). As measured by immunological analyses, MOMCs expressed markers that are closely associated with hematopoietic and monocytic lineages. Nevertheless, MOMCs did not express those markers that are associated with dendritic cells (Kuwana et. al., 2003).

Characteristics of cells obtained. MOMCs showed morphological characteristics that share traits of monocytes, endothelial, and mesenchymal cells. They are CD 14, CD 34, CD 45, and collagen type I positive (Kuwana et. al., 2003). MOMCs were able to serve as stromal supporting cells for hematopoietic cells, at least *in vitro*.

As described by Kuwana et al. (2003) the immunophenotype of MOMCs sets them apart from monocytes and macrophages which are CD 14 positive, CD 45 positive, and CD 34 negative; endothelial progenitors which are CD 34 positive, and CD 45 negative; and mesenchymal progenitors which are negative for CD 14, CD 34, CD 45, and Type I collagen (Kuwana et. al., 2003). MOMCs also expressed CD 34, and CD 105. However, MOMPs did not express CD 117, and CD 133. MOMCs expressed markers associated with the endocytic lineage including CD 144, and VEGFR1. Similarly, MOMCs expressed the mesenchymal associated

markers associated markers Collagen type I, III, fibronectin, and vimentin (Kuwana et. al., 2003).

The number of MOMCs increased in culture up to passage 5, at which point MOMCs stopped proliferating. As the cells reach passage 5 in culture cell division significantly slowed and the number of dead cells increased (Kuwana et. al., 2003).

MOMCs are able to differentiate to macrophage, dendritic cell, osteogenic, myogenic, chondrogenic and adipogenic lineages. Initially after generating MOMCs 50-80% differentiated to adipocytes, 45-60% expressed SkM-actin when myogenic differentiated, almost all cells formed calcium deposits for osteogenic differentiation. Type II collagen was expressed in all adherent cells after chondrogenic differentiation. After 3 week culture the number of cells undergoing differentiation was lower. The estimated numbers of cells that differentiated to different lineages was reduced for osteogenic (8%-15%), myogenic (4%-17%), and adipogenic (18%-35%) but the authors stated that chondrogenic differentiation was increased after 3 weeks (Kuwana et. al., 2003).

MOMCs expressed Nanog and Oct-4 (Seta, and Kuwana, 2010). A comparative analysis of MOMCs with monocytes, macrophages, and dendritic cells revealed expression of stemness genes in MOMCs but not the other cells. Amongst the other genes that were differentially expressed in MOMCs were DLG3 and myosin X. DLG3 is a gene thought to have a negative impact on cell proliferation. Differential expression of this and related genes might account for the MOMCs resistance to immortalization with a lentiviral vector containing E6, and E7 human papilloma virus oncogenes. Myosin X is a gene with a pivotal role on filopodia formation, as well as potential enhancement of the cell's migration capabilities (Seta, and Kuwana, 2010).

Lastly, the AML-1 transcription factor is differentially expressed in MOMCs, as measured by semi quantitative PCR. AML-1 is commonly expressed in hematopoietic lineages. AML-1 serves as an activator or suppressor to many hematopoietic genes. AML-1 also has direct involvement in the differentiation and maturation of endothelial progenitor cells. The latter, are similar to MOMCs in that they have a similar level of CD 34 expression, as well as similar differentiation capabilities (Seta et. al., 2012).

CD 45 progenitors (Rogers et. al., 2007). A rare population of CD 45 progenitors obtained from umbilical cord blood displays great proliferative potential when cultured in FSFL medium. These cells represent a homogeneous population of mesenchymal cells that have great expansionary potential, as well as a range of differentiation capabilities. In addition, these cells express high levels of Oct-4 and Nanog, both of which are transcription factors strongly associated with pluripotency.

Methods of isolation and culture. Human umbilical cord blood was collected in ACD coated blood collection tubes to prevent coagulation. A density gradient was obtained by making a dilution of collected blood with Pentastarch at a rate of 1:5. This mixture was then centrifuged at 50 x G, for 10 min. at 10°C. All the fractions except for the sedimented red blood cells were collected and centrifuged at 400 x G for 10 min. at 10°C. Afterwards, the supernatant was aspirated and the pellet was resuspended in 10% serum in IMDM. The resuspended pellet was mixed with a matching volume of 20% DMSO in serum, used as freezing media. The cells were then stored in liquid nitrogen until needed.

Upon thawing a negative selection column was used to deplete CD-2, CD-3, CD-14, CD-16, CD-19, CD-24, CD-56, CD-66, and cells expressing glycoprotein-A. These cells are referred

to as being lineage negative or Lin^{minus}. Similarly, a positive selection column was used to isolate CD-45 positive cells.

Thawed cells that were filtered through the selection columns, were cultivated on FSFL medium for proliferation and prior to differentiation. FSFL medium is defined as StemSpan™ medium (Stem Cell Technologies, Vancouver, Canada) with Iscove's MDM (Invitrogen, CA, USA), 1% BSA, 10 µg/mL insulin, 200 µg/mL human transferrin, 10⁻⁴ M 2-mercaptoethanol, and 2 mM L-glutamine. This medium was further supplemented with 25 ng/mL stem cell factor, 25 ng/mL Flt-3 ligand, and 50 ng/mL fibroblast growth factor-4, 50 ng/mL heparin, and finally 10 µg/mL low density lipoprotein. The cells were incubated in this media at 37°C, 5% CO₂ for 8 d until confluence. Half of the medium was changed every 48 h.

Characteristics of the cells obtained. The CD-45positive Lin^{minus} cells proliferated readily on the FSFL media formulation, experiencing as much as a 10 fold increase in total cell numbers over 8 days of culture. These cells stained negative for the mesenchymal cell marker vimentin. However, when these cells were cultured for an additional 6 d in Mesencult medium (Stem Cell Technologies, Vancouver, Canada), the cells were positive for vimentin.

Several media formulations were tested for the initial growth and expansion of the umbilical cord blood cells that were filtered through the selection columns. The media formulations tested were FSFL medium, 20% FCS in DMEM, Mesencult medium alone, and a combination of FSFL media first and Mesencult medium later formulations. Only the cells that have been cultured in FSFL, and FSFL first were able to proliferate *in vitro*.

As demonstrated by an *in vivo* model, CD-45positive Lin^{minus} cells grown on FSFL media were able to graft on the stroma of NOD/SCID mice, but were unable to serve as hematopoietic stem cells. Under *in vitro* settings, CD-45positive Lin^{minus} cells, express Oct-4,

and Nanog, as measured by western blots. In addition, these cells were able to differentiate along endothelial, osteoclast, and neural lineages.

Supplemental reports of progenitor cells obtained from peripheral blood.

Nonhematopoietic mesenchymal stem cells (Kawada et.al., 2004). G-CSF induces a subset of cells from the bone marrow to enter circulation on a mouse model for myocardial infarction. These bone marrow derived cells are mesenchymal in nature and engraft into the damaged tissue to ultimately form cardiomyocytes in an in vivo mode. In addition, adherent cell cultures demonstrate the ability of these cells to differentiate into osteogenic and adipogenic lineages.

Methods of isolation and culture. Bone marrow cells are isolated by mechanical extraction from femurs of C57BL/6 mice and immediately resuspended in 2% Fetal Calf Serum in HBSS –Ca, -Mg, 10 mM HEPES, 100 U/ mL penicillin, and 100 μ / mL streptomycin. On the basis of cell sorting by means of the MoFlo instrument, a subset of cells with the following immunophenotype is obtained: CD 34⁻, c-kit⁺, Sca-1⁺, Gr-1⁻, Mac-1⁻, B220⁻, CD 3⁻, and Ter 119⁻.

Characteristics of cells obtained. Immunological and histological analysis of heart tissue from C57BL/6 mice that had suffered myocardial infarction revealed that when the nonhematopoietic mesenchymal stem cells were transplanted into these animals the myocardial lesion was populated by the transplanted cells. The transplanted cells engrafted to the site of injury and expressed: α -actinin, sarcomeric myosin, as well as cardiac troponin-I. The transplanted cells were not detected into other tissue samples, such as those from: liver, kidney, and skeletal muscle.

Endothelial progenitor cells (Hristov et. al., 2004). In bone marrow and peripheral blood there resides a subset of endothelial progenitor cells. These cells are first located in the bone marrow and have the phenotype: CD 133⁺, CD 34⁺, and VEGF-R2⁺. Later during development these cells migrate into peripheral blood and progressively diminish in the

expression of those markers. In peripheral blood these cells are: VE-cadherin⁺, endothelial nitric oxide synthase⁺, and vWF⁺. The frequency at which these cells are present in peripheral blood appears to be very low in adult individuals.

Methods of isolation and culture. A subpopulation of CD 34⁺, and CD 133⁺ cells is initially isolated from bone marrow or adherent peripheral blood samples on the basis of cell sorting. Upon initial isolation, the cells are cultured in fibronectin coated tissue culture vessels in Fetal Calf media- based cell culture media. Normally, the culture media is supplemented with VEGF, bFGF, and EGF. These supplements are intended to stimulate the proliferation of endothelial cells.

Characteristics of cells obtained. Over time, these cells gradually lose the expression of CD 133, CD 34, and VEGF-R2, and instead become VE-cadherin⁺, endothelial nitric oxide synthase⁺, and vWF⁺. However, after 4 days in culture the expression of those markers also begins to diminish and the cells become strongly positive for CD 14, CD 11b, and CD 11c. At this stage, these cells have very limited proliferative potential and are able to secrete G-CSF. These cells are able to differentiate into endothelial lineages.

Fibrocytes (Bellini and Mattoli, 2007). Fibrocytes are a population of mesenchymal cells that have properties overlapping those of hematopoietic stem cells, monocytes and fibroblasts. When primed with pro-fibrotic cytokines and growth factors, fibrocytes produce large amounts of collagen and differentiate into myofibroblasts.

Methods of isolation and culture. Fibrocytes were identified in biopsy samples from injured tissues. Under laboratory conditions, fibrocytes are obtained on the basis of adherent cell cultures from peripheral blood incubated under standard tissue culture conditions for 10-14 days. The culture media utilized for fibrocyte isolation typically has a high level of serum.

Characteristics of cells obtained. Fibrocytes are mesenchymal cells in nature and have a natural role in a variety of reparative processes as part of the body's efforts to repair damaged tissues. In regards to immunophenotype, fibrocytes are characterized amongst many other parameters as being: CD 45⁺⁺, CD 90⁻, CD 11a⁺⁺, CD 11b⁺⁺, CD 14^{+/-}, MHC-I⁺⁺, MHC-II⁺⁺, CD 40⁺, CD80⁺, CD 34⁺⁺, CD 105⁺⁺, CD 4⁻, CD 19⁻, and CCR1⁺. In addition, fibrocytes secrete high levels of collagen I, fibronectin, and even higher levels of vimentin. Fibrocytes lack expression of vWF.

Multipotent mesenchymal stromal cells in blood (He, et. al., 2007). These cells are found in low frequency amongst all the other leukocytes in blood. Multipotent mesenchymal stromal cells in peripheral blood are consistently found in many different instances and provide evidence for a systemic migration of a group of cells from the bone marrow to other sites of the body even in adults.

Methods of isolation and culture. Multipotent mesenchymal stromal cells are isolated on the basis of primary cultures of adherent cells from peripheral blood samples.

Characteristics of cells obtained. Multipotent mesenchymal stromal cells are: CD 34⁻, HLA-DR⁻, c-Kit⁻, CD 133⁺, CD 14⁻, CD 45⁻, CD 31⁻. These cells are able to differentiate along: osteogenic, adipogenic, and myogenic lineages. In addition, these cells secrete collagen type I, II, III, and VI, as well as fibronectin. In addition, these cells do exhibit a significant expression of the transcription factor Oct-4, which is commonly associated with pluripotency.

Culture modified mononuclear cells (Gulati, et. al., 2003). Upon vascular injury, a population of cells that are endothelial progenitors in nature rush to the site of mechanical injury and play a pivotal role in the process of vascular repair. These progenitor cells are naturally present in peripheral blood and are readily expanded under standard laboratory conditions. These cells are thought to be actually a heterogeneous mixture of leukocytes, mostly composed of CD 14⁺ cells and a minority of CD 133⁺ subpopulations. A synergistic relationship amongst the cells in this heterogeneous mixture is thought to be responsible for the engraftment and repair into damaged tissue.

Methods of isolation and culture. A volume of 25 mL of whole blood from rabbits was separated by means of the density gradient centrifugation method using Histopaque 1083 and centrifuging at 1800 rpm for 25 min. The buffy coat was then collected and the cells were counted and seeded into 6-well-plates. The cells were seeded at a rate of 5×10^6 c/mL and the wells were coated with fibronectin at a rate of $1 \mu\text{g}/\text{cm}^2$. The cell culture media used was endothelial growth media-2 (EGM-2, Clonotech). Adherent cells from the seeded buffy coat multiplied readily and were passaged on day 3 after initial plating and then cultured in fibronectin coated plates and with the same media as described above.

Characteristics of cells obtained. Adherent cells that had been in culture for a week revealed a heterogeneous morphology. In days 9-12 of culture, the cells developed a cobblestone morphology and were characterized by a marked increase in proliferative capacity while maintaining contact inhibition. At day 7 in culture, the cells were CD 14⁺ and were able to incorporate acetylated LDL, a feature characteristic of endothelial lineages. The cells were also RAM-11⁺, a marker commonly associated with macrophages. At day 12 in culture, the cells

exhibited a marked decrease in expression of CD 14, and RAM-11, while being strongly positive for CD 31, a marker commonly associated with mesenchymal stem cells.

Clinically significant and functional improvements were observed in allogeneic models of vascular disease as a direct consequence of the administration of the culture modified mononuclear cells.

CD 14⁺, CD 34^{low} cells as the source of circulating endothelial progenitors

(Romagnani, et. al., 2005). CD 14⁺ peripheral blood leukocytes are a highly diverse population of cells with a variety of functions, some of them not fully characterized to date. Of all these cells, the CD 14⁺, CD 34^{low} represent a minute fraction, ranging from only 0.6% to 8.5%. In their natural state, these cells express high levels of the embryonic stem cell markers: Nanog, and Oct-4. However, when differentiation of such cells was induced into endothelial lineages, the stem cell markers are downregulated. These cells are highly plastic in that they are able to differentiate into endothelial, osteoblast, adipocyte, and even neural lineages. Many reports corroborate that when endothelial progenitor cells are infused into a damaged host, neovascularization occurs. Typically, these endothelial progenitor cells are derived from adherent peripheral blood mononuclear cells that have been stimulated with VEGF and express lectin.

Methods of isolation and culture. Whole human blood was collected from healthy donors, and incubated in 0.5% FCS in phosphate buffer pH 7.2, supplemented with 20 mM EDTA for 20 min., at 4°C. CD 14⁺ cells were then sorted out of the blood by means of a magnetic column. The cells were then plated into 6-well-plates at a rate of 8 x 10⁶ cells/ well. The wells were coated with fibronectin, and the media used was 20% FCS in endothelial basal medium (EBM-MV), supplemented with EGM-MV SingleQuotes, and 100 ng/mL human recombinant VEGF.

Characteristics of cells obtained. Cells from peripheral blood that were cultured under the conditions mentioned for 5 days at passage 1, were adherent and stained highly positive for acLDL, Ulex-lectin. In addition, these cells at passage 1, and just 5 days into culture were CD 14⁺, CD 11c⁺, CD 16⁺, CD 31⁺, CD 86⁺, CD 105⁺, and HLA-DR⁺, while lacking expression of CD 34, and CD 133. At the transcriptional level, these findings were confirmed as the transcripts for all the markers that were expressed at the protein level were found to be upregulated. Interestingly, transcripts for CD 133 were not found to be upregulated while those for CD 34 were, despite the lack of protein expression for CD 34. More sensitive protein expression analyses found CD 34 to be expressed, albeit below the detection threshold of flow cytometry. During the 5 days of initial culture, CD 14⁺ CD 34^{low} cells expressed a high level of the pluripotent stem cell markers: Nanog and Oct-4. However, this expression became virtually negligible by day 11 of culture under the conditions mentioned. Lastly, the cells were able to differentiate into osteoblasts, as demonstrated by positive staining of: alkaline phosphatase, and alizarin red. At the transcriptional level, differentiated osteocytes did upregulate the expression of Osterix, and Runx2 both of which transcription factors strongly associated with osteocytes. Adipocyte generation was assessed by the acquisition of characteristic morphology and staining with Oil Red O. At the transcriptional level, differentiated adipocytes did upregulate the expression of AP-2, PPAR γ both of which transcription factors strongly associated with adipocytes. Finally, cells differentiated into neural lineages as they did upregulate GFAP, neurofilament 200, NSE, as measured by RT-PCR.

Equine peripheral blood progenitors (Koerner et. al., 2006). Cells with fibroblastic morphology have the capability to differentiate into chondrogenic, adipogenic, and osteogenic

lineages. These are cells that are obtained on the basis of ficcoll-gradient centrifugation of whole blood. Prior to blood extraction, the animals are stimulated with G-CSF in an effort to mobilize the cells of interest into the bloodstream. Upon isolation, the cells are cultured in fibronectin or collagen type I coated tissue culture vessels. The cells isolated in this manner were CD 34⁻, CD 105⁺, SH2⁺, SH3⁺.

Methods of isolation and culture. Blood samples were obtained in heparinized blood collection tubes from the jugular vein of horses of an average age of 9.3 years. Blood samples obtained in this manner were processed in a 1 hr. window of time from the collection point. The volume used for each blood sample was 36 mL. First, the samples were allowed to stay at room temperature for 20 min. in order to allow RBC sedimentation. The opaque supernatant, devoid of RBC contamination was carefully removed and placed into 15 mL Ficcoll for the generation of a density gradient. The centrifugation parameters were 1.600 x G, for 20 min. at 10°C. The buffy coat was placed into a new conical tube and washed twice with PBS, after which the cells were counted. Afterwards, the cells were washed again in PBS.

Cells were then resuspended in 20% FCS in DMEM-F12, supplemented with 100 IU/mL Penicillin, and 100 µg/mL Streptomycin. The cells were seeded in tissue culture vessels at a rate of 1.6×10^5 c/cm², and incubated under standard tissue culture conditions for up to 2 weeks without media changes. Upon confluency, the cells were seeded at a new rate of 2.7×10^4 c/cm².

Characteristics of cells obtained. In all, blood samples from 33 different equine donors were obtained and processed as mentioned in methods of isolation and culture. Of the 33 animals, cells with mesenchymal morphology were isolated from only 12 animals. After 2 weeks in culture only 1-5 cell colonies appeared in the T-75 tissue culture vessels the cells were in. As the cells proliferated, the cells adopted a more elongated shape as opposed to the original

mesenchymal morphology. The cells had a very short lifespan in culture, as evidenced by a progressive decrease in proliferation rates as passaging advanced. The cells were able to proliferate in culture for a maximum of 6 passages. In addition, the cells were affected adversely by the use of trypsin, as a cell dissociation agent during passaging. The cells were able to differentiate into chondrogenic lineages, as evidenced by positive staining for safranin O, Alcian Blue, and Collagen type II. Osteogenic differentiation was evidenced by positive staining with Alkaline-Phosphatase and the acquisition of cubical shapes. Adipogenic differentiation was demonstrated by positive staining with Oil Red O.

Human monocyte subsets (Shantsila et.al., 2011). Human monocytes are in fact a heterogeneous subset of leukocytes represented in 3 major groups. Altogether, these cells have a variety of functions, ranging from immunological to atherogenesis, and neovascularization processes. The simplest manner to segregate each subset of monocyte is by the expression profile of CD 14, CD 16, and CCR2. The first monocyte subset is defined as being: CD 14⁺, CD 16⁻, and CCR2⁺. The second monocyte subset is defined as being: CD 14⁺, CD 16⁺, and CCR2⁺. The third monocyte subset is defined as being: CD 14^{low}, CD 16⁺, and CCR2⁻. All of the subsets are represented in the bone marrow. Those monocytes on the second subset present characteristics and proliferation as that seen in mesenchymal progenitors.

Methods of isolation and culture. Sample volumes of 30 mL of whole blood were allowed to coagulate and the buffy coat was isolated on the basis of Ficcoll centrifugation. Out of the buffy coat, the three monocyte subsets were isolated on the basis of magnetic cell sorting. Each subset was then suspended in RPMI-1640, supplemented with 2mM L-glutamine, 100 µg/mL gentamicin, 100 µg/mL penicillin, and 1 µg/mL LPS endotoxin, at a rate of 1×10^6 c/mL.

The cell suspensions were then incubated in polystyrene tubes with gentle shaking, and under standard tissue culture conditions. The cells were harvested at 24 hrs.

Characteristics of cells obtained. The first population of monocytes was found at a rate of 84.6 ± 5.77 % of all monocytes. The second population of monocytes was found at a rate of 5.90 ± 3.40 % of all monocytes. The third population of monocytes was found at a rate of 9.51 ± 3.74 % of all monocytes. The second population of monocytes had the largest size as measured by the forward scatter, as compared to the other 2 populations. In addition, the second population of monocytes had the highest density of TLR-4, integrin receptor $\beta 2/CD-18$, CXCR4, Tie2, CD-163, KDR, VEGF receptor 1, and CD-115. In regards to the functional characterization of the subsets, the activity of the NF κ B was assessed by means of intracellular IKK β level assessment.

In this regard, the first population of monocytes had a significantly higher level of IKK β , as compared to the other subsets. The first and second monocyte populations had a significantly higher level of phagocytosis as compared to the third monocyte subset. In response to LPS stimulation, the first and second monocyte subsets did produce TNF- α . In response to LPS stimulation, monocytes in the first subset had a significant upregulation of IL-1 β , IL-6, MCP-1, and IKK β . Conversely, monocytes in the second subset had a significant upregulation of IL-10.

Monocyte derived endothelial progenitors (Shantsila et. al., 2012). Monocytes give rise to CD 34⁺, KDR⁺ endothelial progenitor cells. These cells are believed to have a therapeutic role in heart failure and their progressive decrease is strongly correlated with a negative outcome in cardiovascular diseases.

Methods of isolation and culture. Blood samples were collected from donors in EDTA-blood collection tubes. Blood samples were processed within 1 hr. of collection. A volume of 100 μ L of blood was incubated with labeled antibodies for CD 14, and CD 16 for 15 min. in the

dark. Afterwards, red blood cells were lysed by means of BD lysing solution, incubated for 10 min, and then washed in PBS and finally analyzed in the cell sorter so as to separate the 3 monocyte subsets. Once the subsets had been separated, the cells were similarly stained against CD 34, and KDR.

Characteristics of cells obtained. Based on the samples obtained from whole blood of patients with and without heart failure, CD 34⁺, KDR⁺ endothelial progenitor cells, derived from classic and intermediate monocytes (subsets 1 and subset 2) were significantly reduced in those patients that had heart failure. This pattern was not observed for those CD 34⁺, KDR⁺ endothelial progenitor cells, derived from the third monocyte subset. Higher levels of the endothelial progenitors as derived from the monocyte subsets in question, are associated with enhanced angiogenesis and tissue repair.

Cord blood derived multipotent stem cells (Zhao, et. al., 2012). These cells have a remarkable ability to modulate T lymphocytes to the degree that they might be clinically useful to treat autoimmune diseases, such as type I diabetes.

Methods of isolation and culture. Human cord blood were obtained from healthy donors and plated in serum-free medium by Lonza (Walkerville, MD) and incubated under standard tissue culture conditions. When the cells reached confluency, they were lifted and seeded in the stem cell educator device. The stem cell educator device consisted of a chamber device made out of medical grade plastic with 9 disc surfaces in which the cells grow. At the top of the chamber device, there is an opening that allows the infusion of leukocytes from the patient, and at the bottom there is a collection port for the retrieval of the leukocytes that have been co-cultured with the allogeneic stem cells. The leukocytes are then returned to the patient. The leukocytes are

obtained from the patient's blood and are initially separated by means of density gradient centrifugation prior to being exposed to the cord cells in the chamber device.

Characteristics of cells obtained. Cells isolated from cord blood stained positive for embryonic cell markers, Oct-4, Nanog, SSEA-3, and SSEA-4, as well as the common leukocyte marker CD 45. Up to 12 weeks after administration of the patient's own leukocytes through the stem cell chamber device, no significant alterations were noted on the overall leukocyte count on the patient, as compared to the normal level. Furthermore, no fever was induced as a result of the treatment at any time point. Stem cells, as isolated from the cord blood and placed in the chambered device, were adherent and did not detach from the chambers and into the patient's blood. This was evaluated by staining the outflow of the leukocytes from the device for Oct-4, Nanog, SSEA-3, and SSEA-4, by means of flow cytometry. Furthermore, because of the low immunogenicity of the cord blood stem cells, no HLA-matching was required and no clinical signs of immunorejection were seen in the individuals with type I diabetes. Individuals with type I diabetes who had their leukocytes circulate through the chambered device and then injected back onto them, had an improvement in β -cell function, as demonstrated by an increase in C-level peptide production and a decreased need for insulin therapy.

Interestingly, the T-reg lymphocytes, defined as being CD 4⁺, CD 25⁺, and Foxp3⁺ was significantly increased 4 weeks after the treatment of the patient's own blood through the chambered device with the cord blood stem cells. At that same time point, the plasma levels of TGF- β 1 showed a significant increase. Similarly, expression of IL-4, as well as IL-12 were significantly increased, whereas IL-5, and IL-13 was decreased. Taken together, these characteristics provide evidence for the regulation of the autoimmune response in response to contact with allogeneic cord blood stem cells.

Chapter 3 - Monocyte-Derived Mesenchymal Cells (MDMCs):

Isolation, in vitro expansion, and initial characterization.

Introduction.

Monocytes are a type of leukocytes that serve as precursors for professional antigen presenting cells such as macrophages and dendritic cells. As part of the inflammation process, monocytes migrate to the site of pathogen entry and secrete inflammatory cytokines. In addition, monocytes differentiate into macrophages that play an essential role in clearing invading organisms.

Monocytes originate in the bone marrow of mammals and are present at relatively low levels in peripheral blood. As precursors of professional antigen presenting cells, monocytes are capable of phagocytosis, have a particular immuno-phenotype, and express genes expression associated with professional antigen presenting cells.

Recently several monocyte derived cells with stem cell properties have been described. These include (f-macrophages, Zhao et al., 2003), monocyte-derived multipotential cells/progenitors (MOMC/MOMP, Kuwana et al., 2003) and programmable cells of monocytic origin (PCMOs, Dreskke et al., 2006). These each represent unique populations of cells derived from the buffy coat of density gradient separations.

The procedures to obtain each of these cell types involve specialized culture conditions and in general initially harvest small numbers of cells that must be greatly expanded *in vitro*. We have identified methods for producing another unique monocyte derived mesenchymal cell (MDMC) that can be expanded much more quickly than the previously described cells

originating from monocytes and here we report the isolation of MDMCs from peripheral pig blood using standard media and uncoated plastic dishes.

From the buffy coat of a 7 mL sample of whole blood we routinely obtain in excess of 5×10^6 cells by 10 d after initial plating and the cells can routinely be expanded through 6 or more passages producing more than 6 population doublings. Here we describe some characteristics of these cells including their immune-phenotype, expression of some TLR and stem cell genes, differentiation into some mesodermal lineages, and the numbers produced by pigs of different ages. We also report the isolation of similar cells from mid gestation fetal livers.

Materials and methods.

Postnatal porcine monocytes.

All animal blood samples were collected in accordance with the approved Institutional Animal Care and Use Committee protocol no. 3354. All samples were obtained from the pigs housed at the swine teaching and research center, Kansas State University. Peripheral blood monocytes were isolated by density gradient separation. A blood sample was collected by puncture of the external jugular vein on Heparin coated blood collection tubes from healthy donor pigs of two, four, and six months of age. Blood (7 mL) was diluted with 21 mL of PBS (pH 7.2, -Ca, -Mg, 1 X, Gibco, Grand Island, NY, USA).

Warmed Histopaque® 1083 (10 mL, Sigma Life Science, St. Louis, MO, USA) was layered over the diluted sample in a 50 mL conical tube and the gradient centrifuged (800 x G, for 45 min.) with the brake off. The plasma fraction was aspirated and saved to use for opsonization (-20°C). The opaque interphase (buffy coat), containing the leukocytes was transferred to a separate 15 mL conical tube and brought to 10 mL with PBS (-Ca, -Mg). The cells were precipitated (800 x G, for 15 min) and the supernatant was removed. The pellet was resuspended in 10 mL of PBS (-Ca, -Mg) and the cells precipitated (400 x G, for 5 min). All centrifugations were at 7°C. The cells isolated from the buffy coat were not mixed with cells from other isolations. Therefore each culture corresponded to a single blood sample (7 mL).

The supernatant was removed and the pellet resuspended in 1 mL of standard media, consisting of 20% FBS (Gibco, Grand Island, NY, USA), in DMEM + GlutaMAX™-I (1 X, Gibco, Grand Island, NY, USA) supplemented with antibiotic/antimycotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone® ,Gibco, Grand Island, NY, USA), Normocin™ (50 mg/mL, InVivoGen, San Diego, CA, USA), β-mercaptoethanol (55

μM), and Gentamicin (50 mg/ mL, Gibco, Grand Island, NY, USA). Aliquots (80 μL) of suspended cells were pipetted into each of the wells of two, six well plates and 2 mL of standard medium added. The isolates were incubated (38.5°C, 5% CO_2) with media was changed every 3 to 4 d. Unattached cells were discarded with spent media. After 10 d the cells were lifted with 0.5% Trypsin-EDTA (5 mg/ mL, Life Technologies, Carlsbad, CA, USA) and the harvested cells were seeded at a density of 10,000 cells/ cm^2 in tissue culture grade plastic vessels in standard medium. When the cells achieved 90 % confluence (4-5 d) they were passaged into new dishes at the same seeding density and in the same medium as for the initial plating. Cell isolates were kept in culture up to 13 passage if they continued to expand.

Fetal porcine monocytes.

Pregnant gilts were slaughtered at d 60 of gestation and the uterus removed. Fetal livers were removed and a sample (3 g) placed in a 50 mL conical tube containing 1% Bovine Serum Albumin (BSA) in PBS (-Ca, -Mg) and transported to the clean bench. The sample was transferred to a 100 μm cell strainer over a 50 mL conical tube and the tissue was gently forced through the filter with a plastic syringe plunger. The filtrate was allowed to flow (after gentle mixing) for 20 min. The filtrate volume was adjusted to 7 mL with 1% BSA in PBS (-Ca, -Mg).

Afterwards, the monocytes were isolated on a Histopaque® 1083 density gradient as described above. Viability of isolated cells was determined using a micro-capillary flow cytometer (Guava EasyCyte Plus, Millipore, Billerica, MA, USA) and Guava Via Count reagent. The cells were plated on standard media as described for peripheral blood monocytes at a density of 20,000 cells/ cm^2 on uncoated T-75 tissue culture vessels.

At 24 hrs. after initial plating as described, all of the media was replaced for half of the flasks in culture. For the other half of the cells in culture, all of the media was replaced 4 d after

initial plating. From this point on, the cells were incubated and treated under the same standard conditions as described for the cells isolated from peripheral blood.

Immunohistochemistry.

Flow cytometry was performed on fixed and blocked cells in suspension. The epitopes of interest were: porcine CD 14 (R & D Systems®, cat # MAB4597), porcine CD 45 (AbD Serotec, cat # MCA1222F), and porcine CD 105 (Abcam), as markers for the leukocyte lineages. The following markers were also evaluated as they are commonly used to assess mesenchymal stem cells: porcine CD 31 (AbD Serotec, cat # MCA1746PET), porcine SLA Class II-DR (AbD Serotec, cat # MCA2314F), and porcine CD 90 (Abcam). Analysis for leukocyte markers was carried out at passages 3 and 7, whereas analysis for mesenchymal stem cell markers was carried out at passage 8. Upon detaching and counting the cells in culture, the cells were fixed with 4% Paraformaldehyde for 10 min. at room temperature. Before staining, the cells were washed 3 times in 1 mL of PBS (-Ca, -Mg), then resuspended in 1 mL of 5% Normal Goat Serum in PBS (-Ca, -Mg) to block for non-specific antigens. The samples were unstained, isotype control, and antibody reacted samples were evaluated.

The samples were then centrifuged at 1,000 x G for 5 min, and then resuspended on 100 μ L of PBS (-Ca, -Mg) and 10 μ L of PBS (-Ca, -Mg), isotype control was added to the cells in the isotype control, or primary labeled antibody was added to the cells in the stained sample. Samples were mixed by gentle pipetting and incubated at (4°C, protected from light for 40 min). After staining cells were washed twice in 1 mL of PBS (-Ca, -Mg) and analyzed using the Guava Express Pro acquisition software. The Isotype control was used to establish the baseline fluorescence emission settings for the samples for each of the epitopes.

Phagocytosis assays.

Preparation of opsonized beads working stock.

Fluoresbrite™ Yellow/Green fluorescent beads (2µm, 5.68×10^6 beads/µL, Polysciences, Inc., Warrington, PA, USA) were dispensed into a sterile 1.7 mL microcentrifuge tube. From this point forward all were done on a non-sterile basis. The beads were washed in HBSS (+Ca, +Mg, Gibco, Grand Island, NY, USA).

A 100 µL volume of beads was mixed with 1 mL of HBSS (+Ca, +Mg), centrifuged (5,000 x G for 5 min, 3 times). Between each centrifugation the pellet was resuspended in 1 mL of HBSS (+Ca, +Mg). In preparation for opsonization, the pellet was resuspended on 100 µL of 0.45 µm filtered porcine non-autologous plasma.

From this resuspended pellet, 16 µL was further diluted with 984 µL of 0.45 µm filtered porcine non-autologous plasma. For opsonization, beads were incubated (37°C, protected from light, for 1 hr.) with gentle vortexing at 30 min. The working stock was used within 3 days of processing.

The opsonized bead working stock contained 9×10^6 beads in a volume of 100 µL of non-autologous filtered plasma. The bead stock was kept protected from light at 4°C, and warmed to 37°C prior to use. Each opsonized bead stock was used within 3 d after preparation.

Phagocytosis:

Adherent cells (passage 6) were detached with 0.5 % trypsin-EDTA. An aliquot of 3×10^5 viable cells was allocated to each of 3 microcentrifuge tubes: cells only, cells and beads at 4°C, and cells and beads at 37°C.

Initially the sample tubes were centrifuged (800 x G, for 10 min. at 22°C) and pellets resuspended in 100 µL of 5% FBS in HBSS (+Ca, +Mg) and 100 µL of filtered (0.45 µm)

porcine non-autologous plasma was added to a separate microcentrifuge tube and labeled “plasma only”. Similarly, 100 μ L of opsonized bead stock was added to 2 separate microcentrifuge tubes. These 2 microcentrifuge tubes were labelled “bead stock”.

The microcentrifuge tubes labelled “cells only, and cells and beads at 37°C” were pre-incubated in the 37°C orbital shaker at 200 rpm (Lab Line Instruments Inc., Melrose Park, IL, USA), protected from light. The microcentrifuge tubes labeled “plasma only”, and one of the microcentrifuge tubes of “bead stock”, were also incubated alongside these samples, and under the same conditions, for 1 hr.

Similarly, the microcentrifuge tube labeled “cells and beads at 4°C” was incubated at 4°C on a Nutating mixer at 200 rpm (GyroMini, Labnet International Inc., Edison, NJ, USA), protected from light. The microcentrifuge tube labeled “bead stock” was incubated in the same conditions.

All the incubations were for 1 h. Afterwards, the volume of the microcentrifuge “plasma only” was added to the cells only microcentrifuge sample. The volumes of the microcentrifuges “bead stocks” were added to the cells and beads at 4°C, and cells and beads at 37°C samples.

The microcentrifuge tubes labelled “cells only, and cells and beads at 37°C” were incubated again in the 37°C orbital shaker (200 rpm). Likewise, the microcentrifuge tube labeled “cells and beads at 4°C” was incubated at 4°C on a Nutating mixer at 200 rpm. All the incubations were for 1.5 hr., and were protected from light.

Finally, all tubes were centrifuged (1,000 x G, for 5 min.) and pellets resuspended in 500 μ L of ice cold HBSS (-Ca, -Mg, Gibco, Grand Island, NY, USA) 3 times. The samples were

analyzed using the Guava Easy Cyte Plus (Millipore, Hayward, CA, USA) bench top flow cytometer.

Cells in plasma without fluorescent beads served as a negative control. An elliptical gate was set on the side scatter/ forward scatter dot plot to select the cell population and this gate was applied to the histogram for green fluorescence.

RNA extraction.

RNA was extracted from cultured cells at passage 7 using Qiagen Rneasy® Micro kit according to the manufacturer's instructions. Cells (8×10^5 - 1×10^6) were lysed in Buffer RLT containing β -mercaptoethanol and stored frozen (-80°C) until completion of the extraction procedure. An on-column DNase digest (RNase-free DNase Set, Qiagen) was performed to remove genomic DNA. Columns were eluted twice with 20 μl 50°C nuclease-free water for a final volume of 16 μl . Quantity of RNA was determined with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific), and RNA quality checked using the Agilent 2100 Bioanalyzer with the Agilent 6000 Nano kit at Kansas State University's Center of Biomedical Research Excellence (COBRE).

Quantitative Real Time PCR

RNA underwent reverse transcription (10 ng/ μl of RNA) using Applied Biosystems TaqMan RT reagents in a total reaction volume of 30 μl . cDNA was stored at -20°C until analysis. The cDNA was subjected to quantitative RT-PCR using TLR-3, TLR-4, TLR-5, TLR-7, TLR-9 (components of the innate immune system), Oct-4, Sox-2, Nanog (pluripotency genes) and 18S (housekeeping gene) ribosomal subunit primers. All primers for the genes of interest were selected on the basis of previously published sequences that are specific to swine. Primer

sequences (Invitrogen) were as follows: TLR3 FWD: 5'- GACCTCCCGGCAAATATAAC-3', TLR3 REV: 5'- GGGAGACTTTGGCACAATTC-3', TLR4 FWD: 5'- TGTGCGTGTGAACACCAGAC-3', TLR4 REV: 5' AGGTGGCGTTCCTGAAACTC-3', TLR5 FWD: 5'- CCAGTCCTGCACTTGGAATG-3', TLR5 REV: 5'- GATTGGGCAGGTTCTGAAG-3', TLR7 FWD: 5'- CGGTGTTTGTGATGACAGAC-3' , TLR7 REV: 5'- AACTCCCACAGAGCCTCTTC-3', TLR9 FWD: 5'- GGCCTTCAGCTTCACCTTGG-3', TLR9 REV: 5'- GGTCAGCGGCACAAACTGAG-3', Oct4 FWD: 5'- AGAAGGATGTGGTCCGCGT-3'. Oct4 REV: 5'- ACTGCTTGATCGTTTGCCC-3'. Sox2 FWD: 5'- TTCCATGGGCTCAGTGGTCAA-3'. Sox2 REV: 5'-TGGAGTGGGAAGAAGAGGTAAC-3', Nanog FWD: CCCGGGCTTCTATTCCTACCA, Nanog REV: TACCCACACGGGCAGGTT, 18s FWD: 5'- GAGGTTCGAAGACGATCAGA-3'. 18s REV: 5'- TCGCTCCACCAACTAAGAAC-3'.

The reactions were assembled using 10 ng of cDNA, 100 μ M (stock) each of FWD and REV primers, 10 μ l of power SYBR green master mix (Applied Biosystems, Rostercity, CA), diluted with nuclease-free water to make a 20 μ l reaction per well in a 96 well plate. All samples were analyzed with an Eppendorf Realplex.

Each plate contained neonatal swine testes cDNA to evaluate assay variation. Threshold values were averaged across plates for each gene in order to allow comparisons. Relative expressions of the genes of interest were normalized to the 18s mRNA endogenous control and calculated by means of the $\Delta\Delta$ Ct method.

Statistical analysis.

Effects of donor age on the number of cells at each passage was evaluated using the mixed procedure of SAS and means compared using the Tukey method for multiple comparisons

(Cary, NC). Donor pigs was considered to be a random variable and passage number was considered to be a fixed effect.

The number of doublings was calculated using the formula: $3.32 \times (\text{Log}(\text{Viable cells at passaging}) - \text{Log}(\text{Viable cells originally seeded})) + \text{starting doublings}$. A total of 2 subsamples were cultured and counted up to passage 7 and passage 4 for each of the isolates used in the peripheral blood and fetal liver studies respectively. For the calculation of non-cumulative population doublings at each passage, the difference was taken in between the corresponding previous cumulative population doubling and the corresponding population doubling at that passage so as to obtain the population doubling without the cumulative term.

Polarization of cells and differentiation attempt into dendritic cells prior to viral infectivity assay.

Cells were cultured in standard media described as above up to passage 3. At passage 3, the cells were cultured in 10% FBS in DMEM supplemented with basic fibroblast growth factor (bFGF) at a rate of 10 ng/mL in order to induce myogenic differentiation. Similarly, the cells were also exposed to 10% FBS in DMEM supplemented with Lipopolysaccharide (LPS), at a rate of 1 $\mu\text{g}/\text{mL}$ so as to induce macrophage differentiation. The cells were plated at a density of 2,500 c/cm^2 prior to exposure to the corresponding differentiation media formulation. Cells were cultured for a total of 3 days under standard tissue culture conditions described as above. At the end of the 3 days incubation period, no morphological changes were observed as a result of exposure to differentiation media, and the cells were discarded.

Prior to exposure to stocks of fluorescently labeled Porcine Reproductive Respiratory Virus (PRRV), cells were cultured in standard media up to passage 6. At passage 7, the cells were plated at a density of 10,000 c/cm^2 , and cultured in 5% FBS in DMEM with A/a, as

described in the standard media formulation. The cells were allowed to reach 50-70% confluency prior to exposure the virus stock. Prior to virus exposure, the cells were exposed to 5% FBS in DMEM with A/a, supplemented with 20 ng/mL of: IL-4, IL-10, and LPS for a total of 48 hrs. in order to polarize the cells for the viral infectivity assays. Differentiation into dendritic cells was also attempted by exposure of the cells to IL-4, and GM-CSF at 2 ng/mL, and 5 ng/mL respectively for a 7 days under standard tissue culture incubation conditions.

For the last virology trial, the cells were cultured at a plating density of 2,500 c/cm². The cells were cultured in polarization media as described above. The cells were exposed to the dendritic cell differentiation media described with the concentration for IL-4, and GM-CSF increased to 20 ng/mL, and 50 ng/mL respectively.

Neither of the polarization, and dendritic cell differentiation made the cells susceptible to viral infection. Furthermore, the morphological characteristics of the cells in culture were virtually unchanged without regard of the culture conditions tested.

Exposure of cells cultured in standard media to virus stock did not lead to virus infection. Because the receptor for the PRRV is CD-168, it can be noted that the cells in culture lack this cell receptor. Nevertheless, cells in culture proved to be susceptible to infection with lentivirus, as shown by preliminary trials.

Mesodermal differentiation potential of MDMC's

Early passage (P4-5) cells from 2 month, 4 month and 6 month old pigs were used to assess the adipogenic, chondrogenic, and osteogenic potential of MDMC's. To test adipogenic potential, cells (1x10⁴ viable cells/cm²) were plated in tissue culture treated plates and cultured with complete growth medium at 38.5°C in a humidified atmosphere of 5% CO₂. When cells reached 80-90% confluence, complete growth medium was replaced with StemPro®

Adipogenesis Differentiation Medium (Gibco). MDMC's were cultured continuously in the differentiation medium for 21 days by replacing the medium every 3 d. After 21 d, MDMC's were washed 3 times with PBS and fixed with 4% paraformaldehyde solution for 15 min at room temperature, then washed twice with PBS to remove residual paraformaldehyde. Fixed cells were stained with Oil Red O (Sigma Aldrich) and Harris Hematoxylin. When visualized under light microscopy, lipid droplets appeared red and nuclei blue.

For chondrogenic differentiation, MDMC's were cultured in chondrogenesis differentiation medium (StemPro® Chondrogenesis Differentiation Kit; Gibco®) according to the kit instructions. MDMC's suspended in complete growth medium at 2.0×10^7 viable cells/ml were seeded in 20 μ L droplets in the center of wells of a multi-well plate to generate micromass cultures. After cultivating micromass cultures for 6 h at 38.5°C in a humidified atmosphere of 5% CO₂, 1 mL of warmed chondrogenesis differentiation medium was added per well and the plate was incubated for 21 d with the medium replaced every 3 d. After 21 d the differentiation medium was removed and the cells were washed 3 times with PBS. Then, cells were fixed with 4% paraformaldehyde solution for 15 minutes at room temperature, rinsed twice with PBS and stained (30 min with Alcian Blue ((1% w/v; MP Biomedicals, LLC, Solon, OH) solution prepared in 0.1 N HCl). Stained cells were rinsed 3 times with 0.1N HCl and distilled water added to neutralize the acidity. Blue color-stained cells indicated the synthesis of proteoglycans by chondrocytes.

Osteogenic differentiation of MDMC's was investigated using the StemPro® Osteogenesis Differentiation Kit (Gibco). MDMC's were seeded in culture wells at 5×10^3 viable cells/cm² and cultured in complete growth medium at 38.5°C until they reached 80-90% confluence. The medium was replaced with pre-warmed osteogenesis differentiation medium

prepared according to the kit instructions. MDMC's were cultured continuously in the differentiation medium for 21 d with medium replacement every 3 d. After 21 d, the MDMC's were washed 3 times with PBS and fixed (4% paraformaldehyde for 15 min at room temperature). The fixed cells were rinsed twice with PBS. Alizarin Red S (Sigma Aldrich) solution (2% w/v, pH 4.2, prepared in distilled water) was added and incubated 3 min. The stained cells were rinsed 3 times with distilled water and visualized under a light microscope. Cultured cells undergoing osteogenic differentiation showed increased mineral deposition and were stained red by Alizarin Red S dye.

MDMC's cultured in complete growth medium were stained along with each of the differentiation treatments to serve as negative controls.

Chapter 4 - Results.

Establishment and expansion of monocyte-derived mesenchymal cells (MDMCs) obtained from peripheral blood of 2, 4, and 6 months old pigs.

Independent of the age of the donor, cells with a mesenchymal morphology (Fig. 4.1) became established and proliferated during the 10 d initial incubation period. The average number of viable cells obtained at passage 1 was a little over 5×10^6 . This resulted from the initial cells isolated from a 7 mL sample of porcine peripheral blood from the jugular vein.

Cultures of MDMCs were established for 2- to 6-mo-old castrated male pigs. At each blood collection, two 7 mL blood samples were collected from each pig. Each sample was subjected to a separate density gradient and the cells grown in separate flasks to gain information on the repeatability of the isolation and culture procedures.

All attempts to establish cultures of MDMCs were successful and the cultures were allowed to grow to passage 10 if they continued to expand. At least one cell isolate from each age group was able to expand to passage 10 or greater. All cultures established from 2 month old donors continued to expand to passage 10. Cells from 4 mo.-old pigs continued to passage 10 for 2 of 3 pigs. For cultures from 6 mo.-old pigs, the cells obtained from 1 animal did not continue to expand past passage 7, cells from another animal expanded through passage 8, and cultures from the final pig expanded to passage 10.

Cell numbers harvested at passages 1 to 7 are presented in Table 4.1. There was a difference ($P < 0.05$) in the number of viable cells attributed to passage up to passage 7 and there was an interaction ($P < 0.05$) between donor age and passage number. The interaction occurred because the number of cells harvested from 6-mo old donors declined in later passages.

Similar to viable cells recovered at each passage there was an interaction ($P < 0.05$) of donor age by passage for the cell doublings at each passage (Table 4.2). This interaction resulted because the number of doublings for the 6-mo donors declined in later passages. The cumulative potential for expansion of cell numbers is presented in Fig. 4.2. In the graph, it can be noted that there is an upward trend for the cumulative population doublings for each of the age groups. Nevertheless, the cumulative doublings for the 6 mo.-old pigs flattened and failed to increase after passage 6. This difference is significant ($P < 0.05$) at passage 7 compared to cells from the other age groups.

Graphs of the cumulative doublings for each donor for ages 2-, 4-, and 6-mo are in figures 4.3, 4.4, and 4.5 respectively. The data show that the growth of cells isolated from each blood sample from the same pig provided quite similar results, the only exception being pig 4-1 (4 mo.) after passage 5 and pig 27-9 (6 mo.) after passage 6. Further the results within age group are quite consistent, except the decline in expansion after passage 5 in some pigs noted above.

Establishment and expansion of MDMC-like cells obtained from fetal livers on d 60 of gestation.

To gain some information on the developmental timing of the MDMCs we evaluated cells isolated from fetal livers. The cells were harvested from fetuses removed after the slaughter of pregnant gilts. At d 60 the liver is the site of hematopoiesis and thus the cells present represent an early stage of development.

Regardless of the timing of the initial media change, cells with a mesenchymal morphology became established and proliferated more slowly than peripheral blood MDMCs, and required approximately 30 d to reach confluency. The average number of cells obtained at passage 1 was a little over 16 million viable cells from multiple samples. This corresponds to cells isolated from a 7 mL sample extracted from 4 g of fetal liver. However, only a few isolates continued to expand.

Cell numbers harvested at passages 1 to 4 are presented in Table 4.3. In this experiment we also evaluated the effect of timing of the first media change (24 h vs 3 to 4 days). Because the sample size was not adequate, no multiple comparisons were made.

Cultures of MDMCs were established for normal and 24 h media change timings for cell lines of fetal liver monocytes using the procedures developed for deriving peripheral blood MDMCs. For each fetal liver, two 7 mL samples were collected. Each sample was subjected to a separate density gradient and the cells grown in separate flasks in duplicates to gain information on the repeatability of the isolation and culture procedures.

These cultures were allowed to grow to passage 4 if they persisted. In total, eight separate cell isolates from eight different liver samples were started for each media change group. However, only three cell isolates from each media change treatment persisted to passage 4.

The potential for expansion of cell numbers is presented in Fig. 4.6 and 4.7. These cultures were much less successful in generating expanded cell numbers and there was considerably more variation in the successful expansion of these cultures as compared to results with peripheral blood from postnatal pigs.

Immunophenotype of MDMCs from peripheral blood.

Cells at passages 3 and 7 were evaluated. Percentage of MDMCs expressing CD 14, CD 45, and CD 105 remained relatively unchanged over passage and for cells from the different age pigs. Overall, the percentage of cells expressing CD 14 is high (70 to 90%), whereas approximately half of cells express CD 45 on their surface (Table 4.4). We found essentially no evidence of CD 105 positive cells. The mesenchymal stem cell marker CD 31, and the co-stimulatory molecule CD 90 also are not expressed on MDMCs. Similarly, fixed cells from a 6 month old animal at passage 5 revealed that SLA-DR-II is expressed at levels comparable to that of CD 14 (Table 4.5).

Immunophenotype of fetal liver cells.

Percentage of cells positive for CD 14 and CD 45 was high (65 to 99%) regardless of media change treatment but CD 105 positive cells were not detected (Table 4.6). The mesenchymal stem cell marker CD 31, and the co-stimulatory molecule CD 90 are not expressed. However, the macrophage marker SLA-DR-II, is expressed (Table 4.7).

Phagocytosis.

The internalization of fluorescent beads was temperature sensitive and markedly reduced at 4°C as compared to 37°C for all of the MDMCs from either peripheral blood or fetal liver origin. MDMCs are capable of phagocytosis, and the cells from the 4 mo. old donors phagocytosed more beads than other samples (Table 4.8). The phagocytic capabilities were similar for cells from peripheral blood and fetal livers.

Gene expression.

To gain more insight into the nature of the MDMCs we evaluated expression of three genes associated with embryonic stem cells (Oct 4, Nanog, and Sox2) that are also expressed by pig Wharton's jelly cells, a type of MSC. Because monocytes are part of the innate immune system we also evaluated several toll-like receptor genes. Each of these genes appear to be expressed at the RNA level by MDMCs, although some are expressed at low levels (Table 4.9). A buffy coat isolate harvested 24 h after initial plating and MSCs from a porcine Wharton's Jelly (WJ) isolate were included for comparison. For the WJ cells relatively higher expression of Nanog, and somewhat higher expression of Sox 2 than for Oct 4 are consistent with earlier observations (Carlin et al., 2006). Based on these two comparisons MDMCs express Oct4, Sox2, and Nanog at low levels and less than WJ MSCs.

Results for differentiation procedures.

After 21 d in differentiation media, cells from all 3 MDMC isolates demonstrated osteogenic and chondrogenic differentiation as assessed by appropriate staining. MDMC isolates from both the 4- and 6-mo old pigs also stained positive for adipogenic differentiation, but MDMC's isolated from the 2 month old pig were negative for adipogenesis.

Evidence of adipogenic differentiation included a change in morphology from fibroblastic to spherical and the formation of intracellular lipid droplets. The accumulated lipid vacuoles were visualized by Oil Red O stain (Figure 4.8). MDMC's cultured in complete growth medium showed no evidence of lipid droplet development and did not stain with Oil Red O.

Osteogenic differentiation conditions for 21 days resulted in matrix calcification as identified by Alizarin Red staining. The dye-calcium complexes with extracellular calcium deposits exhibited a bright orange-red color (Figure 4.9). MDMC's from the 6 month old pig also exhibited the formation of lipid droplets; in order to visualize this, one well was stained with both Alizarin Red, Oil Red O, and Hematoxylin (Figure 4.10). MDMC's cultured in complete growth medium did not stain with Alizarin Red.

Micromass cultures of MDMC's in chondrogenesis differentiation medium began to produce cell pellets within 5 days; pellets increased in size and number for the duration of the 21 day differentiation procedure. Pellets stained positive with Alcian blue dye (Figure 4.11), indicating the presence of sulfated proteoglycan, a matrix component of mature chondrocytes. MDMC's cultured in complete growth medium did not stain with Alcian blue.

Figures and tables

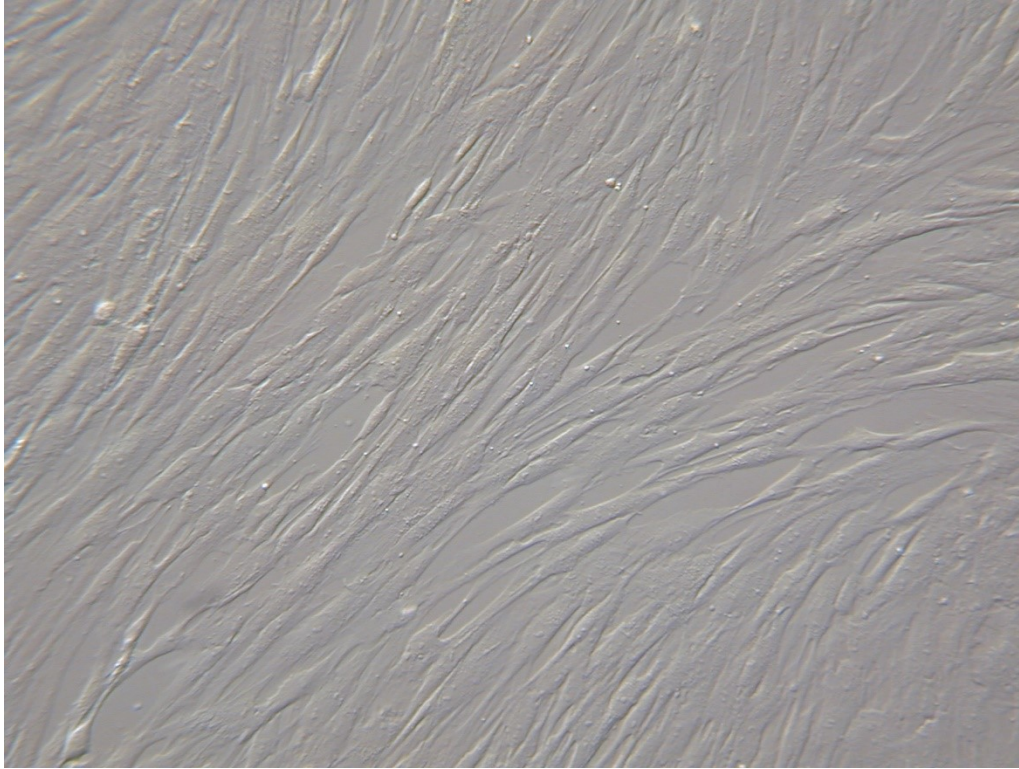


Figure 4.1 MDMCs obtained from a 4 mo. old male donor (4-1). The cells shown are at passage 5.

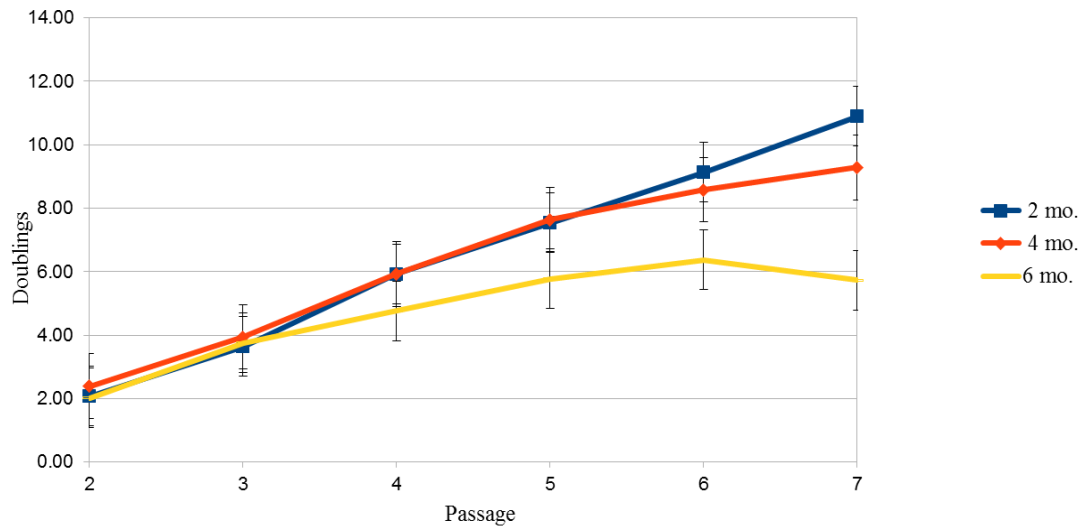


Figure 4.2 Cumulative cell doublings by MDMCs during passage 2 to 7. The non-cumulative doublings are shown in table 4.2.

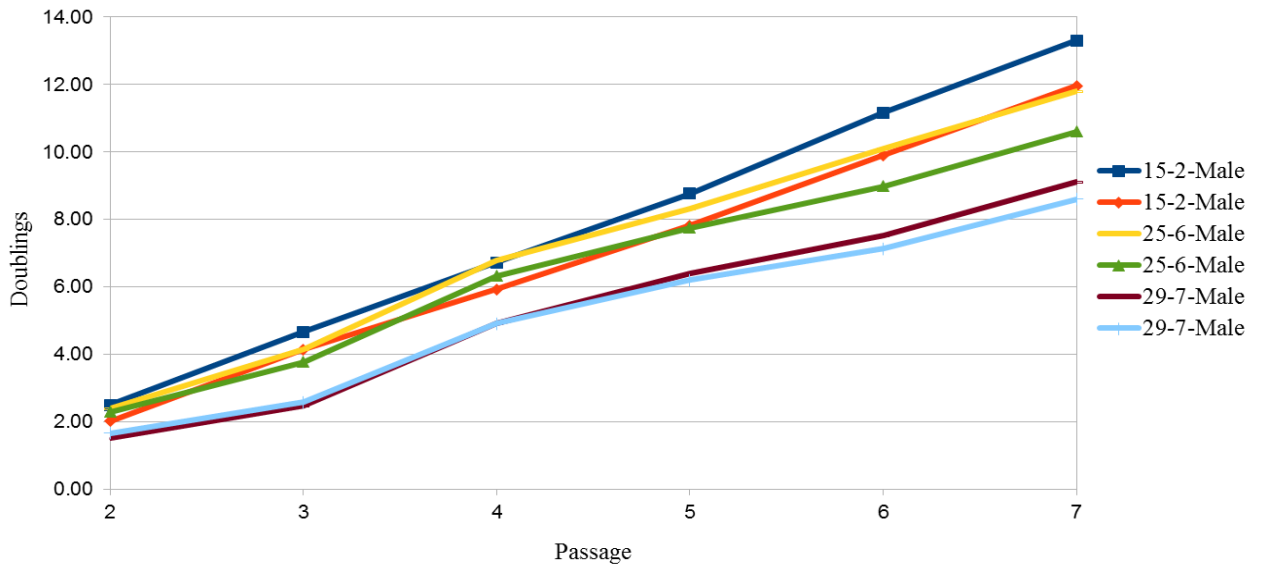


Figure 4.3 Cumulative cell population doublings for cells harvested from 2 month old pigs. Numbers of viable cells grown from individual buffy coats (2/pig) are shown.

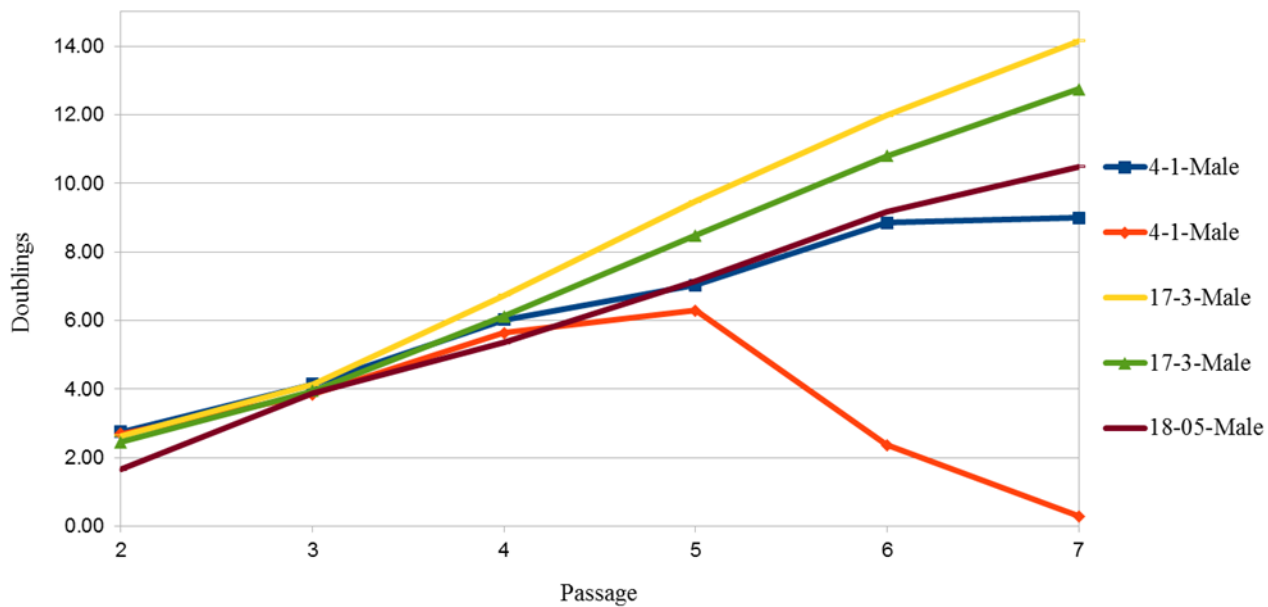


Figure 4.4 Cumulative cell population doublings for cells harvested from 4 month old pigs. Numbers of viable cells grown from individual buffy coats (2/pig) are shown.

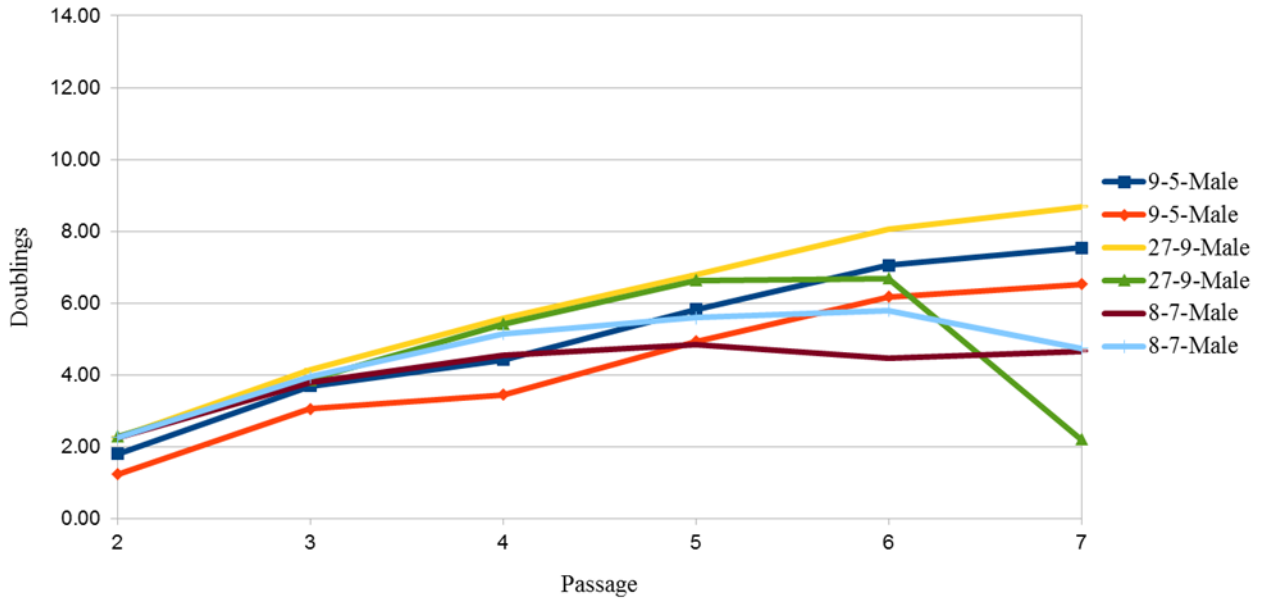


Figure 4.5 Cumulative cell population doublings for cells harvested from 6 month old pigs. Numbers of viable cells grown from individual buffy coats (2/pig) are shown.

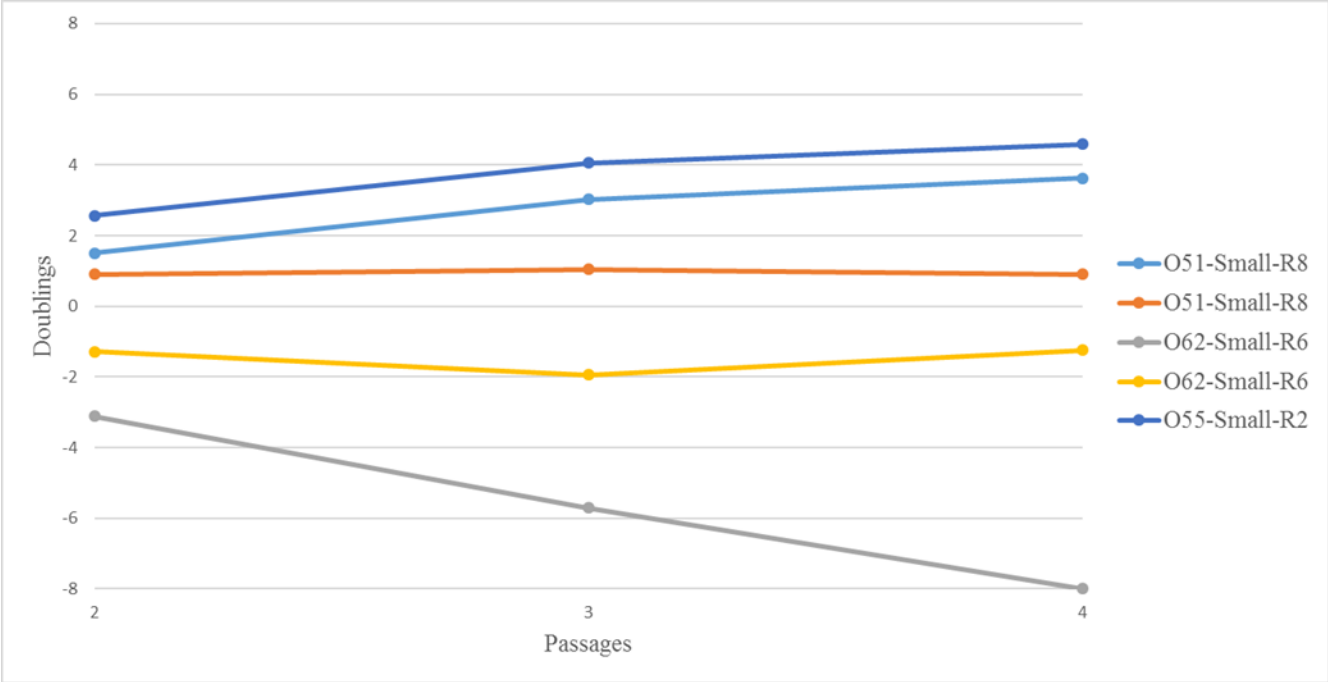


Figure 4.6 Cumulative cell population doublings for cells harvested from fetal liver and with initial medium changes 3 to 4 days.

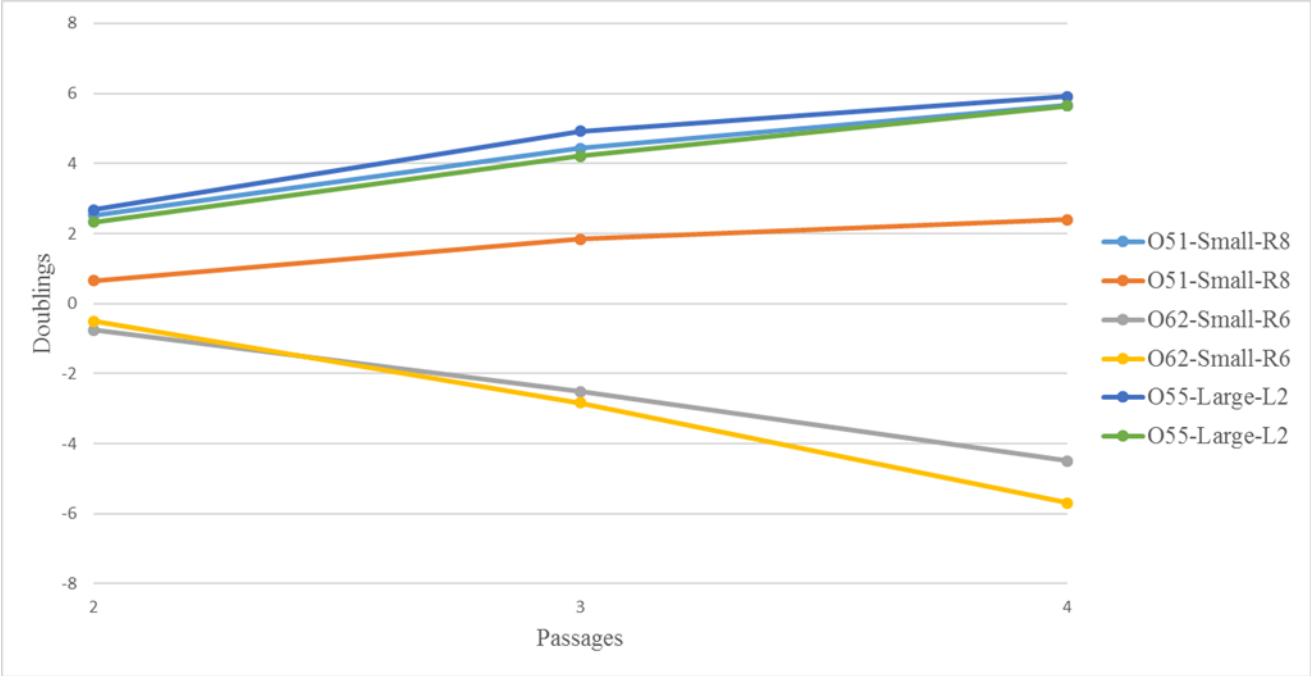


Figure 4.7 Cumulative cell population doublings for cells harvested from fetal liver and with initial medium changes at 24 hr. of initial culture and from that point on each 3 to 4 days.

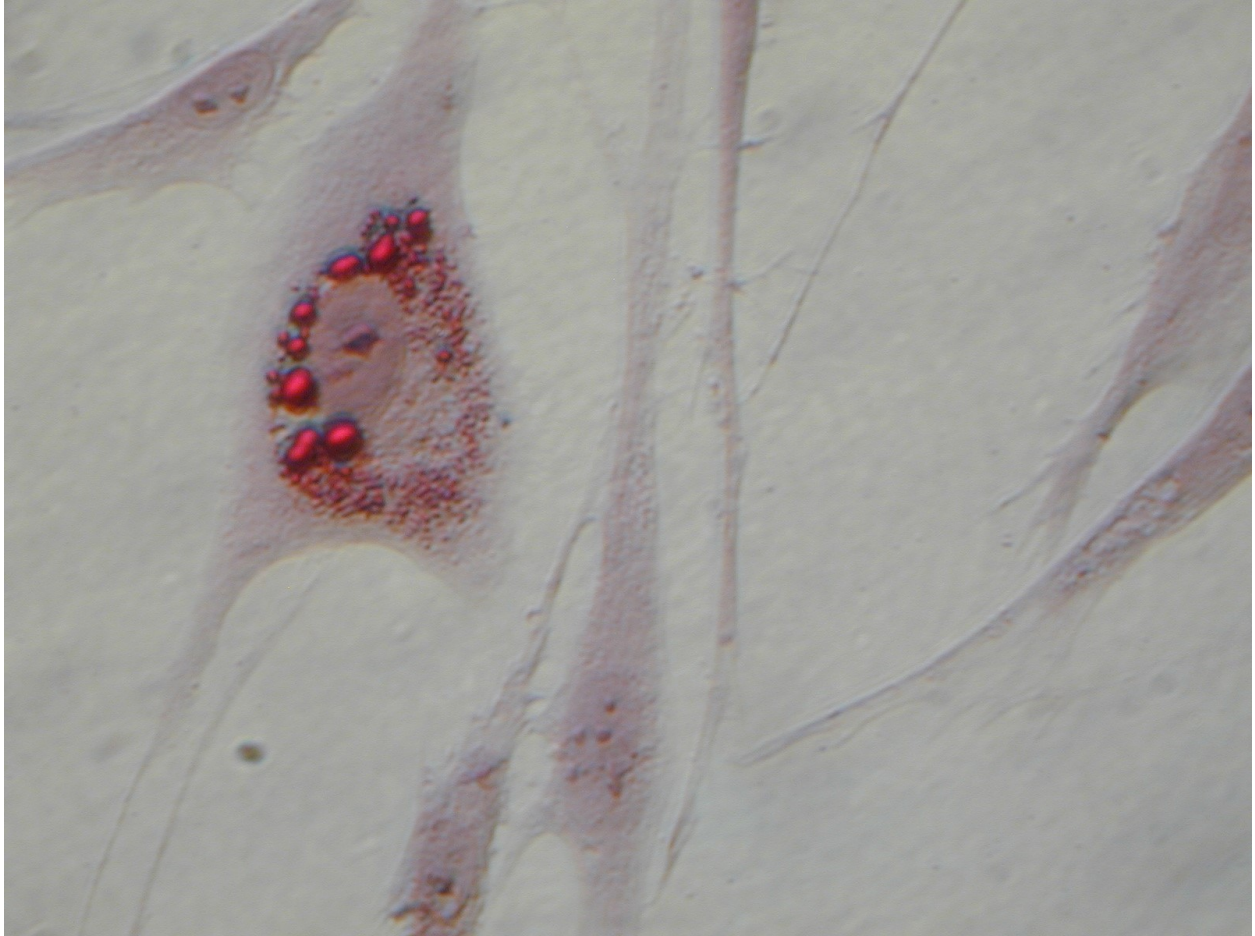


Figure 4.8 Adipogenesis differentiation.

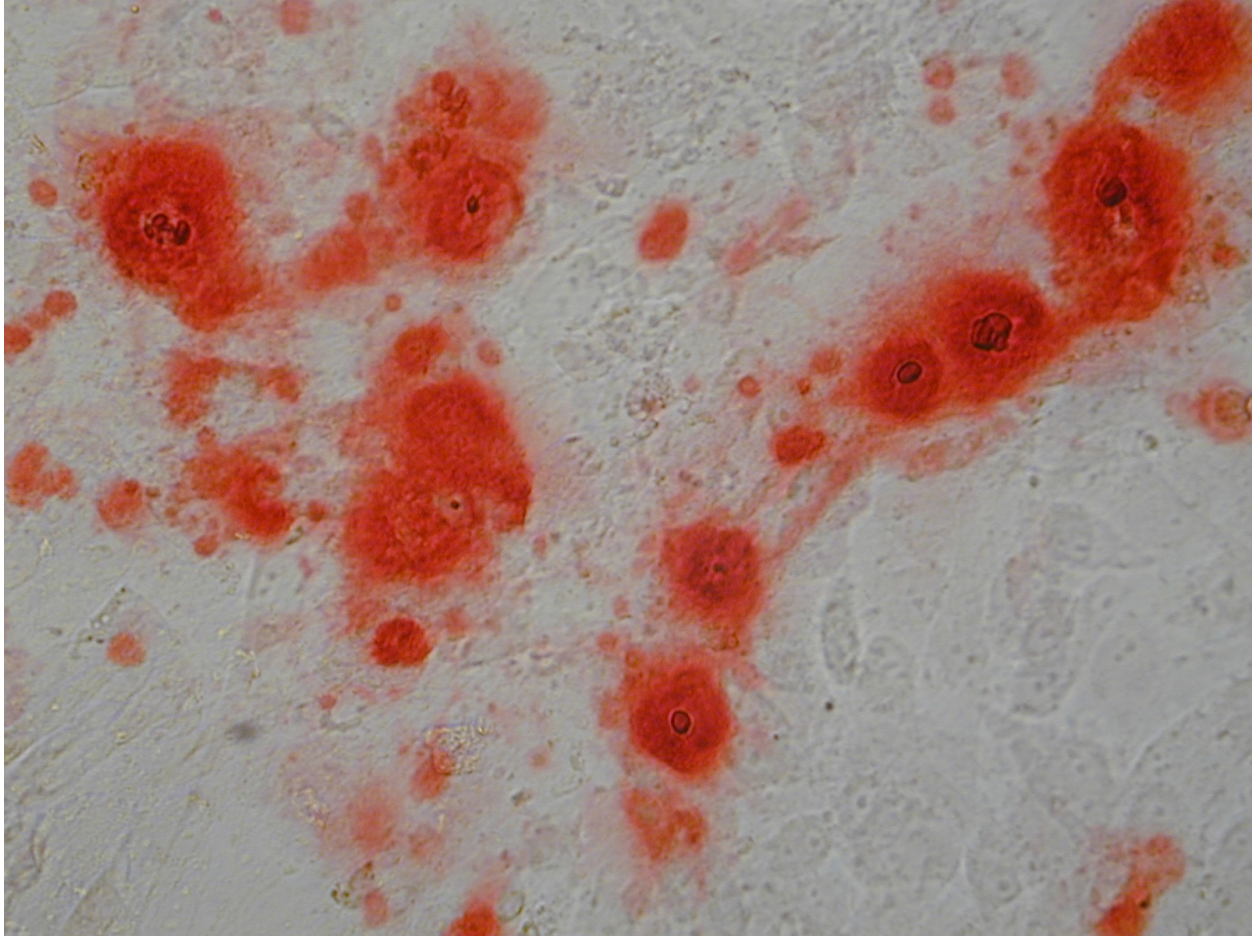


Figure 4.9 Osteogenic differentiation.

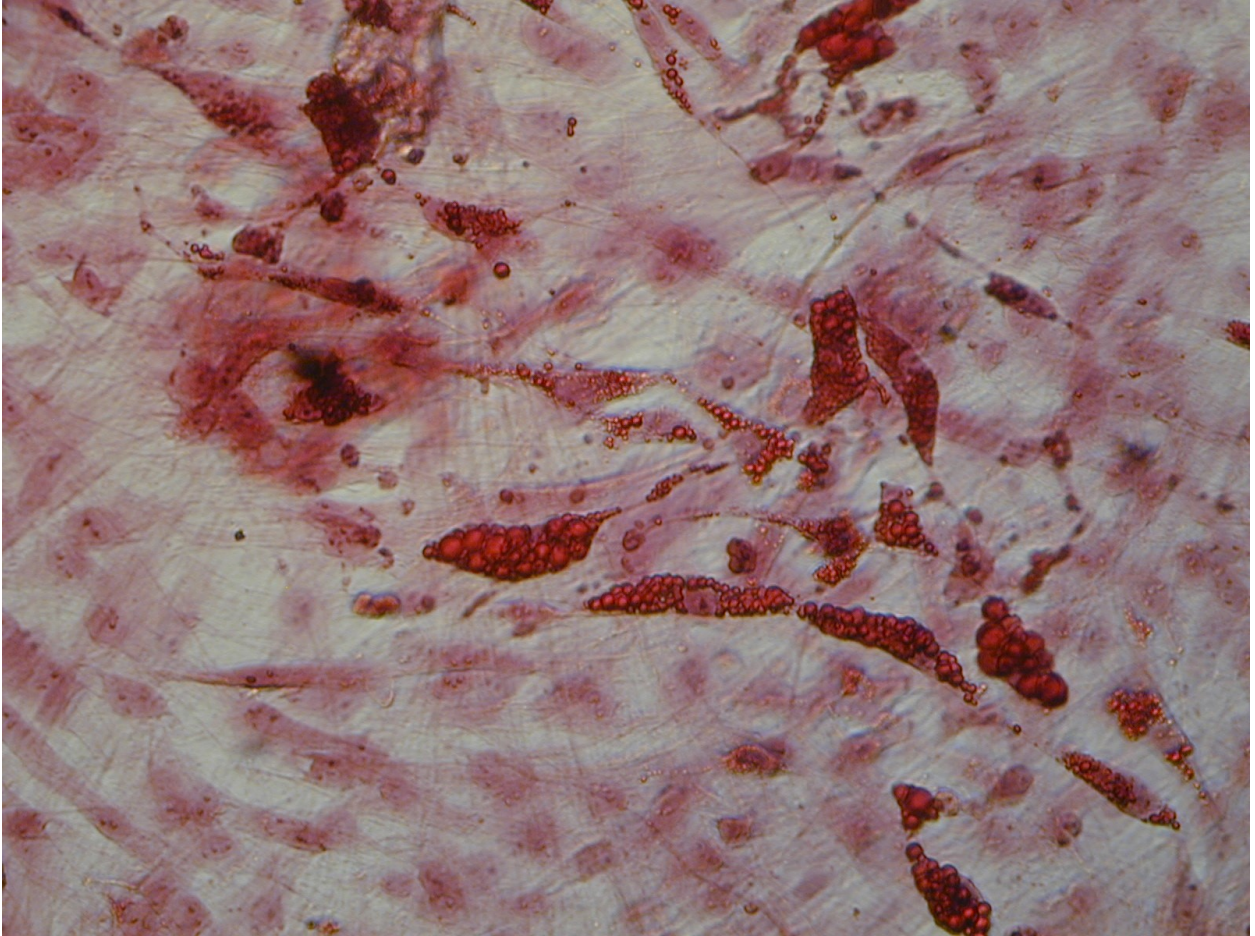


Figure 4.10 Osteogenic differentiation.

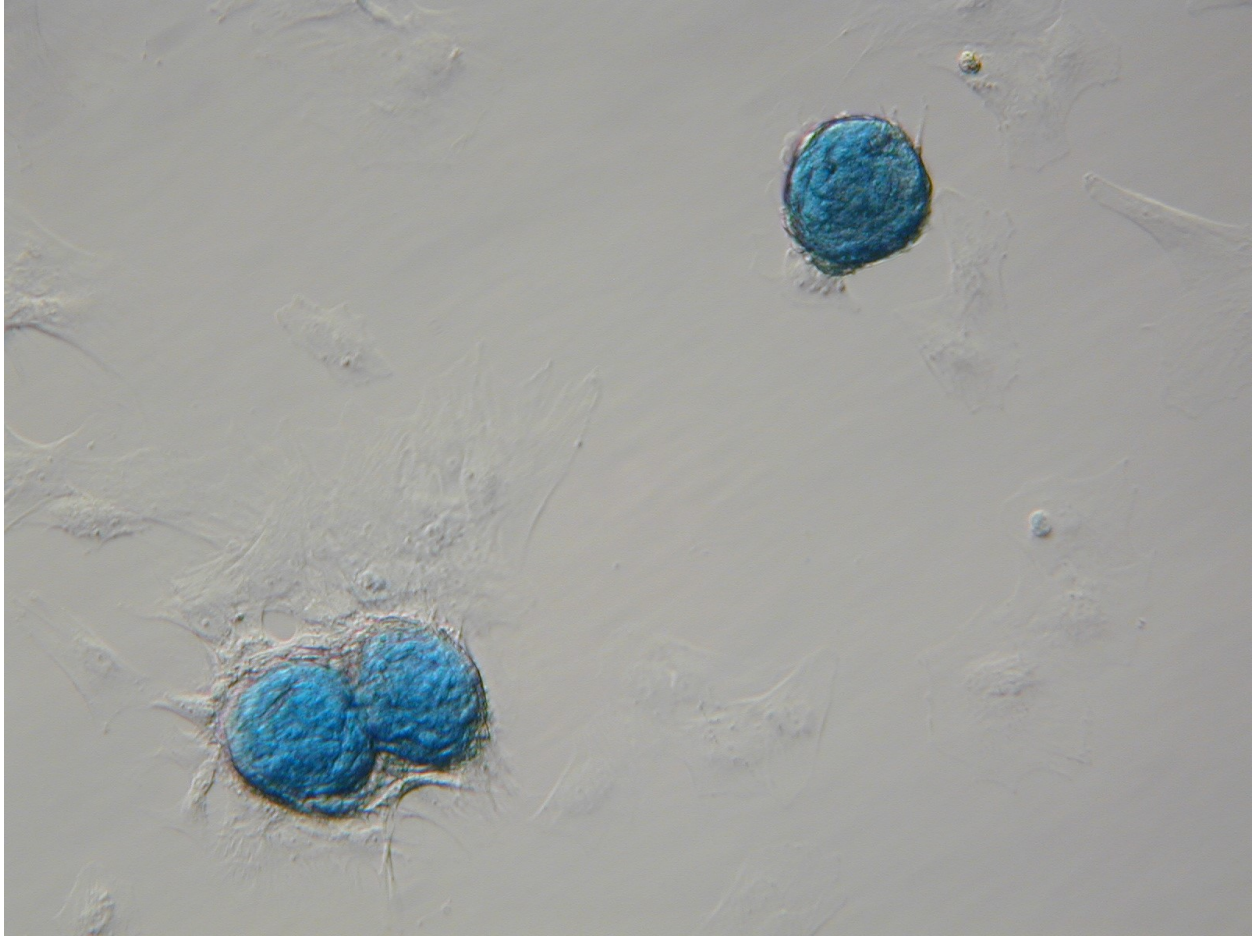


Figure 4.11 Chondrogenic differentiation.

Table 4.1 Number of MDMCs expanded from buffy coats of 2- to 6-mo old pigs.

Age,mo	Passage							
	1 ^a	2 ^b	3	4	5	6	7	All
2	4.58 ± 0.54 ^{cx}	3.14 ± 0.54 ^x	2.22 ± 0.54 ^{xy}	3.71 ± 0.54 ^x	2.29 ± 0.54 ^{xy}	2.26 ± 0.54 ^{xy}	2.55 ± 0.54 ^{xy}	2.86 ± 0.28
4	5.00 ± 0.59 ^x	4.57 ± 0.59 ^x	2.46 ± 0.59 ^{xy}	3.33 ± 0.59 ^{xy}	2.76 ± 0.59 ^{xy}	1.62 ± 0.59 ^{xy}	0.76 ± 0.59 ^{xy}	2.52 ± 0.31
6	4.99 ± 0.54 ^x	3.04 ± 0.54 ^x	2.50 ± 0.54 ^x	1.53 ± 0.54 ^{xy}	1.65 ± 0.54 ^x	1.07 ± 0.54 ^{xy}	0.43 ± 0.54 ^y	1.72 ± 0.28
All	4.85 ± 0.32	3.52 ± 0.32	2.39 ± 0.32	2.66 ± 0.32	2.19 ± 0.32	1.58 ± 0.32	0.94 ± 0.32	2.69 ± 0.56

^aViable cells harvested approximately 10 d after plating the buffy coat.

^bViable cells obtained for passages 2 to 7, after plating 0.75 x 10⁶ viable cells.

^cLeast squares means ± standard error x 10⁶.

^{xy}Means with different superscripts differ (P < 0.05).

Table 4.2 Non-cumulative population doublings MDMCs grown from peripheral blood samples

Age,mo	Doubling						
	2 ^{ab}	3	4	5	6	7	All
2	2.07 ± 0.44 ^{cx}	1.57 ± 0.44 ^x	2.30 ± 0.44 ^x	1.61 ± 0.44 ^x	1.59 ± 0.44 ^x	1.77 ± 0.44 ^x	1.82 ± 0.32
4	2.41 ± 0.47 ^x	1.52 ± 0.47 ^x	1.95 ± 0.47 ^x	1.69 ± 0.47 ^x	0.91 ± 0.47 ^{xy}	0.67 ± 0.47 ^{xy}	1.53 ± 0.34
6	2.02 ± 0.44 ^x	1.73 ± 0.44 ^x	1.01 ± 0.44 ^{xy}	1.01 ± 0.44 ^{xy}	0.60 ± 0.44 ^{xy}	-0.64 ± 0.44 ^y	0.95 ± 0.32
All	2.17 ± 0.30	1.61 ± 0.30	1.75 ± 0.30	1.44 ± 0.30	1.03 ± 0.30	0.60 ± 0.30	1.43 ± 0.45

^aPopulation doublings at the end of passage 1. For all passages, cells took 4-5 days to confluency.

^bDoublings corresponding to passages 2 to 7, 0.75 x 10⁶ viable cells were plated initially for each passage.

^c Least squares means ± standard error .

^{xy}Means with different superscripts differ (P< 0.05).

Table 4.3 Fetal livers from all media change timings. Number of viable cells harvested through the first four passages resulting from plating the buffy coat extracted from 4 grams of fetal liver tissue. (n=3, for each media change timing).

Timing of initial media change	Passage				
	1 ^a	2 ^b	3	4	All
3-4 d	0.96 ± 0.78 ^c	1.96 ± 0.78	1.37 ± 0.78	0.94 ± 0.78	1.31 ± 0.72
24 hrs	2.01 ± 1.43	3.29 ± 1.43	2.89 ± 1.43	2.15 ± 1.43	2.58 ± 1.40
All	1.48 ± 1.00	2.62 ± 1.00	2.13 ± 1.00	1.55 ± 1.00	1.95 ± 1.11

^a First passage after plating the buffy coat, 1.5×10^6 cells were plated initially at passage 0.

^bFor passages 1 to 4, 0.75×10^6 viable cells were plated.

^c Least squares means ± standard error $\times 10^6$.

Table 4.4 Percent cells harvested from peripheral blood that expressed CD 14, CD 45, and CD 105 at passage 3 and passage 7.

Age, mo.	2						4		6						Means	
	15-2-Male		25-6-Male		29-7-Male		4-1-Male	17-3-Male	9-5-Male		27-9-Male		8-7-Male			
Pig	3	7	3	7	3	7	3	3	3	7	3	7	3	3	7	
CD-14	81	95	63	90	98	92	99	78	31	78	89	88	60	74.9	88.7	
CD-45	22	39	48	43	100	59	37	84	45	50	34	61	98	58.5	50.3	
CD-105	0.00	0.12	0.08	0.00	4.43	0.03	0.00	9.53	0.10	0.12	0.67	0.00	34.77	6.20	0.05	

Table 4.5 Immunohistochemistry at 24 h in culture after plating buffy coat for peripheral blood. Macrophage marker expression for peripheral blood sample.

Age in mo.	6- Cultured at 24 hrs. initial media change	6
Sample ID	6-1-Male	9-5-Male
Passage	1, 24 h after plating buffy coat	5
CD-14	80.44	78.44
CD-45	72.20	70.23
CD-105	0.63	0.04
CD-31	ND ^a	0.02
CD-90	ND ^a	0.04
SLA-DR-II	ND ^a	77.87

^aNot done

Table 4.6 Immunohistochemistry, CD 14, CD 45, CD 105. Fetal Liver samples.

Timing of media change for fetal liver sample	24 hr. upon initial plating of buffy coat, then every 3 to 4 days					Normal, every 3 to 4 days in culture		Means		
Sample ID	O62- Large- R1	O51- Small- R8	O51- Small-R8, flask B.	O55- Large- L2	O55- Large-L2, flask B.	O51- Small-R8	O55- Small- R2	24 hr.	Normal	All
Passage	3	3	3	3	3	3	3	3	3	3
CD-14	80.4	90.16	90.06	94.43	93.57	58.8	98.46	89.72	78.63	86.55
CD-45	88.53	99.04	80.85	88.7	92.13	80.97	65.92	89.85	73.45	85.16
CD-105	0.13	0.07	0.00	0.51	0.02	0.00	0.03	0.15	0.02	0.11

Table 4.7 Immunohistochemistry, CD 14, CD 45, CD 105, CD 31, CD 90, SLA-DR-II, for Fetal Livers.

Sample ID	O51- Large	O62- Small	O62- Large	Means
Passage	8	8	6	8
CD-14	93.3	94.23	ND ^a	93.77
CD-45	68.77	80.32	88.94	74.55
CD-105	0.50	0.00	ND ^a	0.25
CD-31	0.06	0.21	0.00	0.14
CD-90	0.88	14.30	0.00	7.59
SLA-DR-II	86.36	34.8	94.28	60.58

^a Not done.

Table 4.8 Phagocytosis assay. MDMCs from peripheral blood samples and fetal livers, all at passage 7.

Tissue	Peripheral blood						Fetal liver					Means		
Age in mo.	2		4		6		day 60 gestation							
Sample ID	15-2-Male	25-6-Male	17-3-Male	11-1-Male	9-5-Male	27-9-Male	O51 Small	O62 Small	O62 Large	O51 Large	O55 Large	Blood	Liver	All
Passage	7													
Temp. (4°C)	7.26	8.95	33.33	36.6	19.36	4.61	44.38	18.14	22.8	20.36	36.31	18.35	28.44	21.71
Temp. (37°C)	34.75	30.3	66.63	58.1	36.61	23.74	48.99	57.48	31.14	39.42	50.57	41.69	45.87	43.08

Table 4.9 RNA expression of some TLRs and pluripotency genes.

Sample	Age	Gene								
		n	TLR-3	TLR-4	TLR-5	TLR-7	TLR-9	Oct-4	Sox-2	Nanog
Porcine neonatal testis, Δ Ct (calibrator)	7d	1	17.063	15.853	17.027	18.027	17.854	20.921	20.766	15.759
Peripheral blood cells + 24 h ^a (Δ Ct)	6 mo.	1	0.418 (18.32)	8.954 (12.69)	0.010 (23.69)	14.188 (14.2)	20.874 (13.47)	0.043 (25.45)	0.004 (28.89)	0.004 (23.6)
pWJ MSCs ^b (Δ Ct)	birth	1	0.860 (17.28)	0.155 (18.54)	0.016 (23.01)	0.014 (24.23)	0.000 (28.92)	0.056 (25.09)	0.327 (22.38)	0.611 (16.47)
Peripheral blood, mean (range)	6 mo.	2	0.660 ^c (17.12-18.53) ^d	0.020 (20.40-24.06)	0.005 (24.18-25.77)	0.015 (23.91-25.04)	0.005 (25.39-27.55)	0.035 (25.37-26.46)	0.015 (26.62-27.49)	0.240 (16.86-22.11)
	4 mo.	2	0.600 (17.69-17.92)	0.000 (27.43-24.37)	0.005 (24.65-27.23)	0.005 (25.59-26.22)	0.000 (26.76-28.91)	0.015 (25.85-29.93)	0.010 (27.95-28.21)	0.030 (20.68-20.92)
	2 mo.	2	0.785 (17.33-17.50)	0.020 (21.06-23.10)	0.015 (22.92-24.30)	0.010 (23.88-27.27)	0.000 (26.94-29.37)	0.005 (28.02-29.41)	0.010 (26.91-28.06)	0.040 (19.98-20.82)
Fetal livers	Gest. d 60 ^e	5	0.678 \pm 0.12 (17.15-19.17)	0.022 \pm 0.01 (20.34-25.73)	0.024 \pm 0.01 (20.92-25.48)	0.026 \pm 0.01 (22.52-25.35)	0.008 \pm 0.00 (25.12-29.11)	0.034 \pm 0.01 (24.92-28.24)	0.044 \pm 0.02 (24.37-28.01)	0.030 \pm 0.01 (19.60-23.10)

^aPeripheral blood cells lysed 24 h after plating the buffy coat.

^bPorcine Wharton's jelly mesenchymal stem cells. Relative expression calculated using neonatal testis RNA as calibrator.

^cCells (8×10^5) were lysed at passage 7, and stored (-80°C) prior to RNA extraction. Mean \pm standard error.

^dRange for Δ Ct (CT for target/ CT for 18S).

^eLivers were harvested on d 60 of pregnancy.

Chapter 5 - Discussion

Differences between MDMCs, other monocyte-derived stem cells, and MSCs.

Recently there have been reports of monocyte-derived cells with stem-cell properties. These reports reveal a surprising plasticity for the newly described cells originating from monocytes in specific culture conditions. The properties for the different monocyte-derived cells differ from each other in important ways as do their apparent application potentials. Among the different cells derived from monocytes, MDMCs are unique for their rapid establishment in culture and proliferation that quickly (within one month) reaches cell numbers adequate for transplantation. As discussed by Ungefroren et al. (2016), the low expansion rate *in vitro* for the other monocyte-derived cells is a major handicap for their clinical application.

As commonly observed for primary cultures, the rate of expansion for MDMCs slows with advanced passages but not before producing millions of cells. Primary cell cultures are often preferred to cell lines in that they are less likely to undergo genetic reprogramming and transformation resulting in tumors and other unintended effects in a recipient host.

The growth potential of MDMCs is apparent particularly after passage 6. By this time the cells have undergone 6 to 10 doublings and 250 million to more than 4 billion cells have been produced from a 7 mL sample of whole blood. If more cells are needed the volume of blood collected could be increased substantially. The repeatable generation of these numbers was demonstrated for each donor. Therefore, repeated collection from a donor over time should be successful if that is important for generating even larger numbers of MDMCs.

Further differentiating MDMCs from some of the other monocyte-origin cells is that they can be grown without special dish coatings or growth factors beyond those in FBS. Specifically, they differ from MOMCs in that they do not express CD105 and CD31 and do not require

exposure to fibronectin during isolation or expansion (Kuwana et al., 2003). They also differ from f-macrophages, which require differentiation to macrophages and then culture with M-CSF and LIF (Zhao et al., 2003). Another monocyte-derived cell type, the PCMOs, require exposure to M-CSF and IL-3 (Dreskko et al., 2006).

Peripheral blood insulin producing cells (PB-IPCs) were described by Zhao et al. (2007). PB-IPCs are negative for CD14. Another rare stem cell population was reported by Rogers et al. (2007). They describe CD45 progenitors that require an elaborate in vitro environment and have only been obtained from umbilical cord blood. They are prepared by depleting the sample of several CD positive cells including CD14, are considered to be 'lineage negative' and are grown in a medium supplemented with transferrin, insulin, stem cell factor, Flt-3 ligand, fibroblast growth factor.

MDMCs have a morphology and mesodermal differentiation potential similar to mesenchymal stem/stromal cells (MSCs). MSCs attach to tissue culture vessels relatively quickly and differentiate into osteogenic, chondrogenic, and adipogenic lineages. However, the immunophenotype of MSCs and MDMCs is distinctly different. The immunophenotype of MDMCs is consistent with their monocyte origin in that they are positive for CD 14 and CD 45 but not markers associated with mesenchymal stem cells (CD105, CD31 and CD90; Dominici et al., 2006). We have also recently examined a few isolates for SLA-DR-II and found it to be present on both peripheral blood and liver origin cells. SLA-DR-II, the swine counterpart HLA-DR-II, is not present on MSCs (Dominici et al., 2006) but is on monocytes, further differentiating MDMCs from MSCs.

MDMCs start out as small populations of attached, spherical colonies in the tissue culture vessels. The abundance of these attached cells relative to all cells from the buffy coat is initially

very low. Over the course of a few days, these attached cells grow and expand from the initially attached colonies and acquire a more elongated shape. In a few more days the cells acquire the characteristic mesenchymal shape and exhibit a highly expansionary phase of growth. MDMCs are morphologically highly homogeneous population of cells even shortly after initial isolation as noted in forward scatter and side scatter plots throughout the culture process.

Immunophenotypic and gene expression characteristics of MDMCs.

Other characteristics examined, including phagocytosis and expression of TLR genes are consistent with the monocyte origin of MDMCs. However, these are also observed in other cell types including MSCs so they do not differentiate MDMCs from other cell types.

The following TLRs were evaluated as they are widely accepted as being expressed in mammalian monocytes: TLR 3 (ligand: ds RNA, typically found in viruses), TLR 4 (ligand: lipopolysaccharide (LPS), typically found in gram negative bacteria), TLR 5 (ligand: flagellin, typically found in many species of bacteria), TLR 7 (ligand: single stranded RNA, typically found in viruses), and TLR 9 (ligand: CpG DNA fragments, typically found in DNA viruses). Expression was detected for these TLR genes in MDMCs and expression of TLR3 was more similar to peripheral blood cells in the buffy coat than the other TLRs, which demonstrated considerably less expression in MDMCs than in the blood cells. The possible impact of this on MDMC functions will require further research. The genes that are strongly associated with pluripotency, Oct-4, Nanog, and Sox-2 are expressed at considerably lower levels in MDMCs than in neonatal testis. Similarly, the following CD markers were considered in the study as they are widely accepted as being expressed by monocytes: CD 14, LPS as its ligand, widely considered to be the crucial marker for monocytes, CD 45, commonly known as Leukocyte Common Antigen (LCA), and acts as an important regulator of T-cell antigen receptor signaling. CD 105, endoglin and its believed that it plays an important role in hematopoiesis.

MDMCs, as well as fetal liver monocytes expressed high levels of CD 14, intermediate levels of CD 45, and negligible levels of CD 34, CD 90, and CD 105. In addition, MDMCs as well as fetal liver monocytes expressed high levels of SLA-DR-II (The swine counterpart of HLA-DR-II). The resulting expression profile suggests that MDMCs are a type of leukocyte,

most probably a novel monocyte subpopulation. Interestingly, MDMCs were also capable of phagocytosis as measured by our *in vitro* assay, a characteristic that is the hallmark of phagocytes such as monocytes.

At passages 3, and passages 7, there was not a significant variability in regards to immunophenotype of CD 14, and CD 45 of MDMCs from donors of 2,4, and 6 months old, prompting to assume that such cells do not experience significant changes under the *in vitro* cell culture conditions employed. This may have the significance that whatever the function of such cells in the living organism might still be preserved even after initial extraction and subsequent expansion. In regards to the immunophenotype of MDMCs obtained from fetal livers, both CD 14 and CD 45 expression were high at passage 3 and passage 8.

MDMCs isolated from fetal livers.

Cells with the general characteristics observed for postnatal MDMCs were also isolated from fetal liver at mid gestation. However, these fetal cells were not easily expanded and generally grew more slowly than those isolated from peripheral blood. This might be related to the method of extraction of the cells or they may have different needs that are not met by the culture conditions that are effective for cells collected postnatally.

Potential applications for MDMCs.

Stem cells from embryonic and non-embryonic origin are the subject of intense study for potential clinical applications. Stem cells of embryonic origin pose major ethical as well as technical challenges, thereby leading to interest in stem cells from non-embryonic sources for potential clinical applications (Wang et. al., 2012). In virtually all reported instances, when stem cells are obtained from non-embryonic sources, they acquire a mesenchymal phenotype and are thus named MSCs. MDMCs are able to differentiate into mesodermal lineages making them similar to MSCs and this is important when considering how they might be used. Their osteogenic and chondrogenic differentiation is similar to MSCs and therefore their potential to aid in the regeneration of damaged or diseased tissues in the body is promising.

A large body of *in vivo* animal trials for many disease models reveals that for many diseases MSCs exert a positive clinical outcome. Nevertheless, the number of MSCs engrafting to the target organ is very small. Most of the *in vivo* studies focus on MSCs that had been administered in high numbers and intravenously. These observations suggest that the improvements observed are not only due to the desired differentiation of the MSCs *in situ*, but also due to the secretion of a particular cytokine profile by the cells.

Readily available, expandable, and autologous stem cells are a valuable resource for biological research and potentially have applications in biomedicine. Key applications, such as drug development and transplants are just two of the areas in which stem cells are making significant contributions. Stem cells and their progeny can help shed light into the specific processes that functional differentiation entails (Zhao, et al., 2003). Therefore, the prospect of obtaining stem cells from a donor's blood, differentiating them to a cell type affected by the disease, and then investigating treatments *in vitro* may be worth pursuing (Zhao., et al., 2003).

The ease of harvest and expansion of MDMCs could generate data in a reasonable time period for such applications.

One of the most intuitive uses of MDMCs is in the aid of tissue repair in various organs as a consequence of physical trauma/ disease. MSCs are being vigorously studied in areas of clinical interest for which there is insufficient or inadequate current therapy. For instance, MSCs are being tested in applications such as: acute myocardial ischemia, stroke, liver cirrhosis, amyotrophic lateral sclerosis, and even graft versus host disease (Wang, et. al., 2012).

Osteogenesis imperfecta (OI) is a bone disease that could be treatable with MSCs (Couzin-Frankel, 2016). OI is illustrative of the potential advantages MDMCs might have as a therapy. OI is a congenital disease that leads to extremely fragile bones. At its core, the disease is brought by significantly lower levels of type I collagen being produced by osteoblasts, the cells responsible for bone formation and remodeling. OI leads to increased number of bone fractures, abnormally low stature, and a shortened life span (Undale et al., 2009). MSCs have been shown to help ameliorate OI in children. Administration of allogeneic bone marrow MSCs into children afflicted with OI led to improvements. The percentage of engrafted cells remained very low, 2% at the most. However, these engrafted cells differentiated *in situ* into osteoblasts and as such lessen the overall impact of OI. The improvements caused dramatic enhancements in decreased number of fractures and a speeding of development (Undale et al., 2009).

There is reason to start stem cell therapy for OI in the fetus, and hope for this approach is buoyed by recent findings of the tolerance by the fetus of even mismatched cells as long as they come from the mother (Couzin-Frankel, 2016). The osteogenic potential of MDMCs harvested from maternal blood could be investigated as cell source to provide osteogenesis in affected

fetuses. It is likely that supplementary cell transplants would be needed after birth and those also could be provided by MDMCs expanded from the mother's blood samples.

Intrauterine administration of murine bone marrow cells into a murine fetus model of OI led to an increase of up to 20% more of type I collagen. Additionally, the intrauterine administration of allogeneic bone marrow cells into the diseased fetuses prevented lethality of OI in the animals in study. Furthermore, intrauterine delivery of bone marrow derived MSCs to a murine fetus model of OI, revealed that the transplanted cells engrafted into many tissues. Some of these engrafted cells differentiated *in situ* to trabecular and cortical bone cells (Undale et al., 2009).

The mechanism by which the allogeneic, bone marrow MSCs led to an increase in the quality of life of the mice fetuses affected with OI is unknown, although it is largely believed that the effect is brought by a combination of site-specific differentiation into osteoblasts and the production of a cytokine profile that is favorable to the mice overall health. A leading belief is that the transplanted bone marrow MSCs lead to the reduction of stress, and apoptosis rates of already existing MSCs in the fetal mice, thereby creating a better niche for their normal functioning (Panaroni et al., 2009).

The significance of these findings in relation to MDMCs is that there are many compelling reasons to believe that MDMCs would have the same if not better effects into similar scenarios given the phenotypic characteristics that overlap with traditional MSCs, such as those in the bone marrow commonly found in mammalian species. In addition, MDMCs could easily be obtained from the same organism, facilitating autologous transplantation applications and presumably higher engraftment rates, and potentially clinical benefits similar to those observed with bone marrow MSCs.

MSCs with chondrogenic potential.

MSCs that have chondrogenic potential have been isolated from a variety of tissues such as bone marrow, synovium, perichondrium, and cartilage. The properties of these MSCs are thought to result from the anatomical niches where they are found. Nevertheless, clinical trials involving the use of MSCs are currently not as innovative as they were once due to some inconsistencies in results and heterogeneity of the extracted MSCs populations (Barry et al., 2011).

Bone marrow derived MSCs have also been explored in the context of cartilage repair with encouraging results. Given that MSCs are poorly immunogenic, engraft to several tissues in the body, and not just the target site, these cells have been administered in the context of helping either by themselves or by intermediate effects to the maintenance of cartilage that so readily depletes with aging. The number of chondro-progenitors increases in the *in vivo* animal models used, leading to at least some degree of cartilage repair (Gupta et al., 2012).

Potential advantages of using MDMCs in clinical applications.

Because the MDMCs acquire a mesenchymal phenotype during culture we hypothesized they might differentiate into cells of the mesodermal lineage and therefore we examined their potential for adipogenic, chondrogenic, and osteogenic differentiation using procedures used to differentiate MSCs into these lineages. These experiments were successful. The differentiation potential of MDMCs may be particularly important if further work supports these capabilities in models for regenerative medicine. In this regard it seems most likely they would be useful in autologous applications.

The simple isolation and expansion of MDMCs make them a good candidate for further study. Of particular interest is that based on the immunophenotype as well as expression of TLRs

and mesenchymal markers of pluripotency, MDMCs appear to be an intermediate population in between MSCs and monocytes.

Currently we have not attempted to isolate MDMCs from any other species. It will also be important to determine whether they can be found in older animals. Our studies provide an initial characterization of MDMCs but many questions remain to be answered about their characteristics, efficacy and safety in clinical applications. Their ability to grow to relatively large numbers without addition of special growth factors and their *in vitro* differentiation to three mesenchymal lineages indicate they may be useful for autologous biomedical applications. In addition, the ability of MDMCs to display phagocytosis is also indicative of a possible role in the process of wound maturation or regeneration, as current knowledge points to the fact that phagocytes also play a role in the maturation of wounds.

Furthermore, the implications of research into novel monocyte populations are potentially very favorable for the treatment of many diseases and monocytes may prove to be an autologous source of multipotent stem cells. To date, the overwhelming majority of research into characterization of monocyte subsets has been carried out for human blood, under conditions other than the ones described in this study. The study of porcine monocytes provides a research avenue in a species with physiological and immunological characteristics similar humans, while offering cost and safety advantages.

Bibliography.

- Barry, FP, Coleman, CM, D'Arcy, S, Murphy, JM, O'Sullivan, J. Mesenchymal chondroprogenitor cell origin and therapeutic potential. *Stem Cell Res & Ther.* 2 (2011). Doi: 10.1186/scrt49.
- Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. *Lab Invest.* 87(2007):858-870.
- Berry, JL, Leyendecker, RM, Spies, TD. "Comparative Studies of Phagocytosis in Normal and in Anemic Blood." *Blood.* 2(1947): 98-107.
- Blanchette CD, Woo YH, Thomas C, Shen N, Sulchek TA, Hiddessen AL. Decoupling internalization, acidification and phagosomal-endosomal/lysosomal fusion during phagocytosis of InLA coated beads in epithelial cells. *PLoS One.* 26(2009):e6056. doi: 10.1371.
- Carlin, R, Davis, D, Schultz, B, Troyer, D, Weiss, M. Expression of early transcription factors Oct-4, Sox-2, and Nanog by porcine umbilical cord (PUC) matrix cells. *Rep. Biol. Endoc.* 8 (2006): doi:10.1186/1477-7827-4-8.
- Carlin, LM, Stamatiades, EG, Auffray, C, Hanna, RN, Glover, L, Vizcay-Barrena, G, Hedrick, CC, Terence Cook, H, Diebold, S, Geissmann, F. "Nr4a1-Dependent Ly6Clow Monocytes Monitor Endothelial Cells and Orchestrate their Disposal." *Cell* 153(2013): 362-375.
- Couzin-Frankel, J. The savior cells? *Science.* 15 (2016):352:284-287.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D et al. Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. *Cytotherapy.* 8 (2006):315-317.
- Dresske, B, El Mokhtari, NE, Fandrich, F, Janssen, D, Plate, V, Reineke, A, Ruhnke, M, Siebert, R, Simon, R, Ungefroren, H. Multipotent Cells of Monocytic Origin Improve Damaged Heart Function. *Amer. J. Trans.* 6 (2006): 947-958.
- Fattorossi, A, Nisini, R, Pizzolo, JG, D'Amelio, R. New, Simple Flow Cytometry Technique to Discriminate between Internalized and Membrane-Bound Particles in Phagocytosis. *Cytometry* 10 (1989): 320-325.
- Goldsby, J, Goldsby, RA, Kindt, TJ, Osborne, BA, ed. *Immunology.* 5th ed. W. H. Freeman, 2002.
- Gulati, R, Jevremovic, D, Kleppe, LS, Lerman, A, Mueske, CS, Peterson, TE, Simari, RD, Vile, RG, Witt, TA. Autologous culture-modified mononuclear cells confer vascular protection after arterial injury. *Circulation.* 108 (2003):1520-1526.

- He, Q, Li, G, Wan, C. Concise review: multipotent mesenchymal stromal cells in blood. *Stem Cells*. 25(2007): 69-77.
- Hristov M, Weber C. Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *J Cell Mol Med*. 8(2004):498-508.
- Kawada H, Fujita J, Kinjo K, Matsuzaki Y, Tsuma M, Miyatake H, Muguruma Y, Tsuboi K, Itabashi Y, Ikeda Y, Ogawa S, Okano H, Hotta T, Ando K, Fukuda K. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. *Blood*. 104(2004):3581-3587.
- Koerner J, Nesic D, Romero JD, Brehm W, Mainil-Varlet P, Grogan SP. Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. *Stem Cells*. 24(2006):1613-1619.
- Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, Kawakami Y, Ikeda Y. Human circulating CD14⁺ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. *J Leuk Biol*. 74(2003):833-845.
- Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, Kawakami Y, Ikeda Y. Human Circulating CD 14⁺ Monocytes as a Source of Progenitors that Exhibit Mesenchymal Cell Differentiation. *J of Leuk Biol*. 74 (2003): 833-845.
- Kuwana, M, Seta, N. Derivation of Multipotent Progenitors from Human Circulating CD 14⁺ Monocytes. *Exp Hem*. 38 (2010): 557-563.
- Li H, Li J, Sheng W, Sun J, Ma X, Chen X, Bi J, Zhao Y, Li X. Astrocyte-like cells differentiated from a novel population of CD45-positive cells in adult human peripheral blood. *Cell Biol Int*. 39(2015):84-93.
- Panaroni C, Gioia R, Lupi A, Besio R, Goldstein SA, Kreider J, Leikin S, Vera JC, Mertz EL, Perilli E, Baruffaldi F, Villa I, Farina A, Casasco M, Cetta G, Rossi A, Frattini A, Marini JC, Vezzoni P, Forlino A. In utero transplantation of adult bone marrow decreases perinatal lethality and rescues the bone phenotype in the knockin murine model for classical, dominant osteogenesis imperfecta. *Blood*. 114(2009):459-468.
- Parod, RJ, Brain, JD. Uptake of Latex Particles by Macrophages: Characterization using Flow Cytometry. *Am Phys Soc*. 245(1983):C220-226.
- Pufe T, Petersen W, Fändrich F, Varoga D, Wruck CJ, Mentlein R, Helfenstein A, Hoseas D, Dressel S, Tillmann B, Ruhnke M. Programmable cells of monocytic origin (PCMO): a source of peripheral blood stem cells that generate collagen type II-producing chondrocytes. *J Orthop Res*. 26 (2008):304–313.

- Ribes S, Ebert S, Regen T, Agarwal A, Tauber SC, Czesnik D, Spreer A, Bunkowski S, Eiffert H, Hanisch UK, Hammerschmidt S, Nau R. Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated *Streptococcus pneumoniae* by murine microglia. *Infect Immun.* 78(2010):865-871.
- Rogers I, Yamanaka N, Bielecki R, Wong CJ, Chua S, Yuen S, Casper RF. Identification and analysis of in vitro cultured CD45-positive cells capable of multi-lineage differentiation. *Exp Cell Res.* 15 (2007):313:1839-1852.
- Romagnani P, Annunziato F, Liotta F, Lazzeri E, Mazzinghi B, Frosali F, Cosmi L, Maggi L, Lasagni L, Scheffold A, Kruger M, Dimmeler S, Marra F, Gensini G, Maggi E, Romagnani S. CD14+CD34^{low} cells with stem cell phenotypic and functional features are the major source of circulating endothelial progenitors. *Circ Res.* 97(2005):314-322.
- Ruhnke M, Ungefroren H, Nussler A, Martin F, Brulport M, Schormann W, Hengstler JG, Klapper W, Ulrichs K, Hutchinson JA, Soria B, Parwaresch RM, Heeckt P, Kremer B, Fändrich F. Differentiation of in vitro-modified human peripheral blood monocytes into hepatocyte-like and pancreatic islet-like cells. *Gastroenterology.* 128 (2005):1774-1786.
- Sanguedolce MV, Capo C, Bongrand P, Mege JL. Zymosan-Stimulated Tumor Necrosis Factor- α Production by Human Monocytes. *J of Imm.* 148 (1992): 2229-2236.
- Savard M, Bélanger C, Tardif M, Gourde P, Flamand L, Gosselin J. Infection of Primary Human Monocytes by Epstein-Barr Virus. *J of Vir* 74 (2000):2612-2619.
- Seta N, Okazaki Y, Izumi K, Miyazaki H, Kato T, Kuwana M. Fibronectin binding is required for acquisition of mesenchymal/endothelial differentiation potential in human circulating monocytes. *Clin Dev Immunol.* 2012 (2012):820827.
- Seta, N, Kuwana, M. Derivation of Multipotent Progenitors from Human Circulating CD 14+ Monocytes. *Experimental Hematology* 38 (2010): 557-563.
- Steinberg, BE, Scott, CC, Grinstein, S. High-Throughput Assays of Phagocytosis, Phagosome Maturation, and Bacterial Invasion. *Amer. Physiol. Soc.* 292 (2007): C945-952.
- Shantsila E, Wrigley B, Tapp L, Apostolakis S, Montoro-Garcia S, Drayson MT, Shantsila E, Wrigley BJ, Shantsila A, Tapp LD, Gill PS, Lip GY. Monocyte-derived and CD34+/KDR+ endothelial progenitor cells in heart failure. *J Thromb Haemost.* 10(2012):1252-1261.
- Steinkamp JA, Wilson JS, Saunders GC, Stewart CC. Phagocytosis: Flow Cytometric Quantitation with Fluorescent Microspheres. *Science* 215 (1982): 64-66.

- Talbot, NC, Paape, M, Worku, M. Selective Expansion and Continuous Culture of Macrophages from Adult Pig Blood. *Vet Immun Immunopath* 64(1998):173-190.
- Undale AH, Westendorf JJ, Yaszemski MJ, Khosla S. Mesenchymal stem cells for bone repair and metabolic bone diseases. *Mayo Clin Proc.* 84(2009):893-902.
- Ungefroren H, Hyder, A, Schulze, M, Fawzy El-Sayed, KM, Grage-Griebenow, E, Nussler, AK, Fändrich, F. Peripheral Blood Monocytes as Adult Stem Cells: Molecular Characterization and Improvements in Culture Conditions to Enhance Stem Cell Features and Proliferative Potential. *Stem Cells Int.* 2016 (2016):7132751. doi: 10.1155/2016/7132751.
- von Zahn J, Möller T, Kettenmann H, Nolte C. Microglial Phagocytosis is Modulated by Pro- and Anti-Inflammatory Cytokines. *Neuroimmunology* 8 (1997): 3851-3856.
- Wang S, Qu X, Zhao RC. Clinical applications of mesenchymal stem cells. *J Hematol Oncol.* 5(2012):19. doi: 10.1186/1756-8722-5-19.
- Zachos C, Steubesand N, Seekamp A, Fuchs S, Lippross S. Co-cultures of programmable cells of monocytic origin and mesenchymal stem cells do increase osteogenic differentiation. *J Orthop Res.* 32(2014):1264-1270.
- Zhao, Y, Glesne, D, Huberman, E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proc Natl Acad Sci U. S. A.* 100 (2003): 2426–2431.
- Zhao Y, Huang Z, Lazzarini P, Wang Y, Di A, Chen M. A unique human blood-derived cell population displays high potential for producing insulin. *Biochem Biophys Res Commun.* 360(2007):205-211.
- Zhao Y, Jiang Z, Zhao T, Ye M, Hu C, Yin Z, Li H, Zhang Y, Diao Y, Li Y, Chen Y, Sun X, Fisk MB, Skidgel R, Holterman M, Prabhakar B, Mazzone T. Reversal of type 1 diabetes via islet β cell regeneration following immune modulation by cord blood-derived multipotent stem cells. *BMC Med.* 10:(2012) 3. doi: 10.1186/1741-7015-10-3.

Appendix A - Preliminary experiments.

Preliminary experiments.

As preliminary experiments the cells were isolated as above, and viability was determined using a micro capillary flow cytometer (Guava EasyCyte Plus, Millipore, Billerica, MA, USA) and Guava Via Count reagent. The cells were plated at a seeding density of 10,000 cells/ cm² in tissue culture grade vessels. The cells were cultured under the same conditions as described above except for the culture medium.

In the preliminary experiments, the culture medium used was 10% FBS (Gibco, Grand Island, NY, USA), in RPMI (1 X, Gibco, Grand Island, NY, USA) supplemented with: Antibiotic/antimycotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone® , Gibco, Grand Island, NY, USA), Normocin™ (50 mg/mL, InVivoGen, San Diego, CA, USA), β-mercaptoethanol (55 µM), and Gentamicin (50 mg/ mL, Gibco, Grand Island, NY, USA).

For the media comparisons experiments, cells were plated under the same standard conditions described into one of the following media: 20% FBS in DMEM, 20% FBS in DMEM with fibronectin coating, 20% FBS in RPMI, and 20% FBS in RPMI with fibronectin coating, all media formulations had the same additional components as listed for the standard media formulation. Fibronectin was reconstituted in HBSS (+Ca, +Mg, Gibco, Grand Island, NY, USA) as per the manufacturer's instructions and allowed to coat the surface of tissue culture vessels 1 hr. in the clean bench prior to plating cells. Fibronectin from bovine plasma (Sigma Chemical Co., St. Louis, MO, USA) coating of selected tissue culture vessels was done at a concentration of 10 µg/cm².

In addition, suspension cultures were kept in tissue cultured grade vessels of all the corresponding supernatants obtained in the media comparison experiments. Cells in suspension cultures were kept under the same standard conditions as described above into separate wells of a 6 well plate.

Results of preliminary experiments.

Initially several media formulations and culture conditions were evaluated. Cell growth in RPMI with 10% FBS, a medium conventionally used for monocytes, consistently produced very little, if any, expansion of adherent cell numbers but viable cells could be maintained through several passages. Non-adherent cells grown in suspension from the buffy coat and cultured in RPMI with 10% FBS, proved unsuccessful for expanding cell numbers.

Increasing the FBS to 20% and using high glucose DMEM resulted in much better results in regards to expansionary potential. Another key was allowing the cells to remain in their initial dish until approximately d 10 before passaging. Fibronectin coating ($10 \mu\text{g}/\text{cm}^2$) did not improve results (Table. A.1), (Table. A.2). Therefore, the experiments reported on the materials and methods section were all conducted using cells that were grown in high glucose DMEM supplemented with β -mercaptoethanol and 20% FBS.

Regardless of the media formulation tested, the cells from the buffy coat attach to the surface of the tissue culture dish as early as 24 h and have a spherical shape that later turns into the characteristic polygonal shape of mesenchymal cells in culture. MDMCs proliferate in attached colonies over culture dish at first, and then proceed to grow to confluency of the surface in the 10 d during initial culture.

During initial testing we evaluated the potential for the MDMCs to support the porcine reproductive and respiratory (PRRS) virus. This was done with the help of Dr. Yongming Sang

in the Dept. of Anatomy and Physiology but the cells did not support virus growth. We also tested the potential for standard methods to differentiate the MDMCs to macrophages and dendritic cells but this was also not successful.

Appendix B - Tables for appendix

Table A.1 Cell growth using different media^a (donor: male 11-1, age: 4 mo.).

Culture medium	20% FBS in DMEM No coating		20% FBS in DMEM Coated with fibronectin (10 µg/cm ²)		20% FBS in RPMI Coated with fibronectin (10 µg/cm ²)		All media	
	Viable cells	Doubling	Viable cells	Doubling	Viable cells	Doubling	Mean no. of cells	Mean no. of doublings
1	.363 ^b	NA ^c	0.119	NA ^c	0.416	NA ^c	0.299	NA ^c
2	1.14	1.62	0.365	1.62	1.85	2.15	1.12	1.80
3	10.40	4.71	0.454	1.94	0.997	1.26	3.96	2.64
4	4.38	7.25	2.20	4.22	2.22	2.42	2.93	4.63
5	4.24	9.75	0.848	4.72	2.48	4.46	2.52	6.31
Total doublings/medium	NA ^c	23.33	NA ^c	12.50	NA ^c	10.29	NA ^c	15.37

^a No cell growth occurred for the 20% FBS in RPMI media formulation without fibronectin. For this preliminary experiment, plating density was not kept constant.

^b x 10⁶.

^cNot Applicable.

Table A.2 Cell growth using different media (donor: male 11-25, age: 4 mo.).

Passage	20% FBS in DMEM No coating		20% FBS in DMEM Coated with fibronectin (10 $\mu\text{g}/\text{cm}^2$)		20% FBS in RPMI No coating		20% FBS in RPMI Coated with fibronectin (10 $\mu\text{g}/\text{cm}^2$)		All media	
	Viable cells	Doubling	Viable cells	Doubling	Viable cells	Doubling	Viable cells	Doubling	Mean no. of viable cells	Mean no. of doublings
1	2.84 ^b	NA ^d	3.07	NA ^d	1.54	NA ^d	1.63	NA ^d	2.27	NA ^d
2	1.16	0.95	1.22	1.02	0.78	0.37	1.11	0.89	1.07	0.81
3	- ^c	- ^c	1.94	2.72	1.68	1.85	1.86	2.52	1.83	2.36
4	0.849	1.06	1.01	3.47	0.61	1.87	0.98	3.23	0.86	2.41
Total doublings	NA ^d	2.01	NA ^d	7.20	NA ^d	4.09	NA ^d	6.64	NA ^d	5.57

^a Plating density was 10,000 cells/cm². No cell growth occurred for the 20% FBS in RPMI media formulation without fibronectin.

^b x 10⁶.

^cSample volume partially lost.

^dNot Applicable.