Characterization and comparison of cell frequency, growth, and multipotential differentiation of adult mesenchymal stromal cells derived from equine bone marrow and adipose tissue

Martin Andreas Vidal
Louisiana State University and Agricultural and Mechanical College, mvidal@vetmed.lsu.edu

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CHARACTERIZATION AND COMPARISON OF CELL FREQUENCY, GROWTH, AND MULTIPOTENTIAL DIFFERENTIATION OF ADULT MESENCHYMAL STROMAL CELLS DERIVED FROM EQUINE BONE MARROW AND ADIPOSE TISSUE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Veterinary Clinical Sciences

by

Martin A. Vidal
B.S., University of Wisconsin-Madison, 1992
M.S., University of Wisconsin-Madison, 1995
B.V.Sc., The University of Liverpool, 2000

May 2008
DEDICATION

With my deepest admiration for our most elegant, noble and athletic companion:

The horse

and to my most precious allies, my parents Professor Carl Rudolf and Jacqueline Vidal

and my siblings Silvia Natalia, Stefan Matthias and Rudolf Leonard Vidal.

My life’s journey and personal development and all my pride and love for the horse and
the profession of equine veterinary medicine and science are rooted within my family and

our love and support for each other.
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<th>Full Form</th>
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<tbody>
<tr>
<td>AGC</td>
<td>Aggrecan</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose tissue derived stromal cell</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>Cbfa-1</td>
<td>Core binding factor alpha</td>
</tr>
<tr>
<td>CD</td>
<td>Cell doubling number</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>Col2A1</td>
<td>Collagen alpha 1 type 2</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>DDFT</td>
<td>Deep digital flexor tendon</td>
</tr>
<tr>
<td>DJD</td>
<td>Degenerative joint disease</td>
</tr>
<tr>
<td>DT</td>
<td>Cell doubling time</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>hrIL-1 beta</td>
<td>Human recombinant interleukin-1 beta</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>IRAP</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Bone marrow-derived mesenchymal stromal cell</td>
</tr>
<tr>
<td>OC</td>
<td>Osteocalcein</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>Runx</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>SDFT</td>
<td>Superficial digital flexor tendon</td>
</tr>
<tr>
<td>SL</td>
<td>Suspensory ligament</td>
</tr>
<tr>
<td>Sox</td>
<td>Sry-type high mobility group box containing gene</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>UMB</td>
<td>Urinary bladder matrix</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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ABSTRACT

Equine bone marrow-derived mesenchymal stromal cells (MSCs) and adipose tissue-derived stromal cells (ASCs) were compared for frequency within their respective tissues, cell doubling characteristics and differentiation multipotential in culture based on histochemical staining and compositional analysis. Equine MSCs and ASCs from young adult horses were harvested and isolated from sternal bone marrow and supragluteal subcutaneous adipose tissue, respectively, and grown up to passage 10 (P10) to determine cell doubling characteristics. Limit dilution assays were performed on primary and passaged (P2, P4) MSCs and ASCs to determine the frequency of colony forming units with a fibroblastic phenotype (CFU-F), and the frequency of MSC differentiation into adipocytes (CFU-Ad) and osteoblasts (CFU-Ob). Pellet cultures of MSCs and ASCs at P2 were performed in chondrogenic media with or without transforming growth factor (TGF-β3) and bone morphogenic protein (BMP-6). Collagen type II expression, glycosaminoglycan and DNA content and pellet size were measured.

Primary MSCs doubled more slowly than subsequent MSC passages (DT = 4.9 ± 1.6 days compared to 1.4 ± 0.22 days). Doubling time of ASCs (2.1 ± 0.9 days) was significantly slower than that of MSCs. Primary MSC frequency was 1 in 4,224 ± 3,265 nucleated BM cells while the frequency of ASC was 1 in 2.3 ± 0.4 nucleated stromal vascular fraction cells. Primary and subcultured MSCs showed robust adipogenic and osteogenic differentiation potential. MSC pellet cultures developed collagen type II expression by Day 7 and hyaline matrix by Day 14. ASC pellets only exhibited mild matrix or collagen formation under electron microscopic examination but showed no immunohistochemical expression of collagen type II. MSC pellets supplemented with
growth factors were larger (p <0.0033) and showed significant increases in GAG concentration by Day 14 compared with all other pellets of both cell types (P<.0001).

The frequency, *in vitro* growth rate, and adipogenic and osteogenic differentiation potential of young adult horses are similar to those documented for MSCs of other species, whereas in vitro growth rate of ASCs differs from human ASCs. MSCs show earlier osteogenesis compared with ASCs and more robust chondrogenesis in the presence or absence of human recombinant TGFβ3 and BMP6.
CHAPTER 1.
A REVIEW OF FUNDAMENTAL CONCEPTS IN STEM CELL BIOLOGY AND MULTIPOTENTIAL CHARACTERISTICS AND MECHANISMS
1.1 Introduction to Stem Cell Biology: Definitions and Nomenclature

Stem cells are functional units responsible for generation of embryonic and adult tissue.\(^1\) The description of stem cells dates back to the 19th century, when Edmund Beecher Wilson described the term as synonymous to a mitotically quiescent primordial germ cell.\(^2\) However, the bulk of the research into the biology of mesenchymal stem cells started well over forty years ago when Friedenstein and his coworkers showed that bone marrow derived cells could differentiate into cells of mesenchymal origin, such as osteoblasts.\(^3\) During that time it was also shown that demineralized bone or bone extracts were capable of causing cartilage or bone formation when implanted either subcutaneously or intramuscularly.\(^4-6\) During the 1970s Caplan and his coworkers demonstrated that mesenchymal cells from embryonic stage 24 chick limb buds could be isolated and were able to differentiate in vitro into various mesenchymal tissues such as bone, cartilage and muscle. Later it was found that exposure of these limb bud cells to demineralized bone extracts would induce chondrogenic differentiation in a dose-dependent fashion, which lead to the isolation of a demineralized bone crude extract with known dose dependent “chondrogenic stimulating activity” (CSA). This protein was isolated, purified and characterized as a heterodimer of what are now known as bone morphogenic proteins.\(^5,7-10\)

These chick limb bud mesenchymal cells and the presence of CSA in demineralized bone extracts as well as the aforementioned work by Urist and coworkers lead to the suspicion that mesenchymal stromal cells also exist in adult tissues, which appeared consistent with the enhanced efficiency of fracture repair when fresh bone marrow was used in large bone defects.\(^5\) Further support of this theory was work by Friedenstein\(^11\) and Owen\(^12,13\) that showed that bone marrow-derived cells are capable of adhering to petri dishes and appeared to have osteogenic and
adipogenic potential. This work set the stage for exploring the potential of adult mesenchymal stromal cells (MSC) from varying tissue sources for the purpose of regenerative tissue repair.

1.1.1 Nomenclature of Mesenchymal Stromal Cells (MSCs)

For many years, the literature has published a confusing variety of different terminology in its description of stem cells from different tissue sources. The property of bone marrow-derived mesenchymal stromal cells to adhere to the plastic culture dish prompted the original descriptive name of ‘plastic-adherent progenitor cells’. The literature often refers to the bone marrow-derived stem cells with plastic adhering properties as mesenchymal stem cells, which became popular through Arnold Caplan’s work and descriptions. However, it has been shown that not all of these mesenchymal cells are in fact multipotent. Pittenger and his coworkers showed that merely 70% of the unfractioned nucleated cell pool of marrow-derived cells was capable of adipogenic, osteogenic and chondrogenic differentiation whereas the remainder of the cells did not undergo chondrogenic differentiation. Hence it was concluded during the Annual Meeting of the International Society for Cellular Therapy (ISCT) in 2000 that there is insufficient evidence that all unfractioned fibroblast-like adhering cells have stem-like activity.

It is generally accepted that unlike normal fibroblasts, such as those of dermal origin, those isolated from bone marrow by plastic adherence have a multipotential biological properties but are not a uniform population of cells. In fact, only a very small proportion of the marrow-derived nucleated cell pool is capable of forming a colony-forming unit of fibroblasts (CFU-F) demonstrating multipotential. Therefore, it has been proposed by Horwitz and coauthors to name these cells multipotent mesenchymal stromal cells and suggest that only cells that have met specified stem cell characteristics may be termed ‘mesenchymal stem cells’; this compromise ascribes the popular ‘MSC’ acronym to both cell populations and maintains continuity within the
literature. However, in the context of stromal cell descriptions from multiple tissue sources, the
term ‘mesenchymal stromal cell’ and its common acronym MSC, which merely refers to the
embryological origin of this cell type, can create confusion. If the cell is derived from bone
marrow it may be appropriately called BMSC, which is also a common acronym of these cells, in
order to distinguish the origin of this type of MSC from those of other origins.

Nomenclature from stromal cells of adipose tissue varies widely including descriptions
such as adipogenic reticular cell, mesenchymal cell, fibroblast, stromal cell and adipose-
derived adult stem cell (ADAS). A recent consensus reached at the Second Annual
International Fat Applied Technology Society meeting (IFATS, October 3-5, 2004, Pittsburgh, PA) was to use the acronym ‘ASC’ for adipose-derived stromal cells or adipose-derived stem
cells.

1.1.2 Stem Cell Definition and Self-renewal

Stem cells are clonogenic in nature capable of repeated cell division over many
generations without differentiation into a specific cell lineage. Genetic characteristics and
environmental cues such as cytokines, transcription factors, cell-to-cell or cell-to-matrix
interactions are believed to direct stem cells into a committed precursor cell lineage, which then
gives rise to mature progeny cells, which include nonrenewing progenitor cells and terminally
differentiated effector cells. Whether stem cells truly maintain absolute self-renewal potential
remains controversial. Marrow-derived MSCs have been shown to have a highly variable self-
renewal potential in culture. Friedenstein and his coworkers have shown that the cyclic
division known to occur in cell culture is not characteristic for cells in vivo. The cue for stem
cells to enter into the cell cycle appears to depend on serum growth factors and the addition of
increased concentration of growth factors such as the fibroblast growth factor-2 (FGF-2). This
can cause expansion rates of more than 50 population or cell doublings (CD) compared with control basal media cultures, which are usually bound by senescence to less than 50 CDs as postulated by the Hayflick limit, which is discussed later.28,29

Another factor, which appears to effect the expansion rate as well as maintenance of multipotentiality of human mesenchymal stromal cells, is lowering the seeding density of cells in culture.27,30 Colter and coworkers showed dramatic differences in expansion rates when cells were seeded at low compared with high densities.27 This work is supported by a recent report, which showed that human bone marrow-derived MSCs seeded at 10 cells/cm² expanded approximately 500-fold within 12 days with a maximum cell doubling time of approximately 10 hours.31 When the same cells were seeded at 1,000 cells/cm², they expanded only 30-fold, which merely resulted in a 5-fold greater final cell count relative to the low density plating. The effect of seeding density on the differentiation potential of cells was illustrated by Sekiya31 and his coworkers who showed that after adipogenic induction of passage 3 human MSCs caused significant reduction in absorbance levels for Oil Red O after isopropyl alcohol extraction when cells were seeded at 1,000 cells/cm² compared with those seeded at 50 cells/cm².

The growth characteristics of MSCs depend on the species and the tissue origin. Mesenchymal stromal cells derived from bone marrow show an initial lag phase of cell proliferation in primary culture, which accelerates through a log phase and eventually plateaus.26 Cell doubling times of MSCs derived from bone marrow vary slightly among species, reported as approximately every 48 hours for dogs32 and Rhesus Macaques33 whereas human postnatal MSCs require approximately 24 hours.34 Furthermore, age-related decreases in growth rates have been reported for intestinal stem cells using an irradiation model to simulate tissue aging in mice35. Also differences between the in vitro culture conditions, including the concentrations of
fetal bovine serum or the use of other serum substitutes such as horse serum, have to be an important consideration in the comparative assessment of cell growth characteristics as determined in separate publications.

1.1.3 **Stem Cell Replicative Control**

Exciting new research in recent years has begun to elucidate the complex regulatory control mechanisms that are involved in stem cell self-renewal. This process is believed to be directed by cell intrinsic factors, epigenetic control, small RNA regulators, and cell-extrinsic signals. Among the intrinsic transcription factors are orphan nuclear receptor TLX, polycomb transcriptional repressor Bmi1, high-mobility-group DNA binding protein Sox2, and basic helix-loop-helix Hes genes. Epigenetic control occurs through histone modifying enzymes and chromatin remodeling proteins whereby histone modification can involve acetylation, methylation, phosphorylation, ubiquitylation, sumolyation and ADP-ribosylation. Histone acetylation has been most thoroughly examined and is regulated by histone acetylases (HATs) and histone deactylases (HDACs). It has been shown that HDAC-mediated transcriptional repression occurs through deacetylation of histone lysine residues and thereby restricts access of transcription factors to their target genes. These processes are believed to be essential for the maintenance, replication, and differentiation of neuronal stem cells.

More recently the epigenetic control of self-renewal through histone methylation has gained attention. The mechanism occurs through chromatin modifications of target genes assuming a bivalent conformation facilitated by histone methylation, which is a dynamic process regulated by methyl transferases and demethylases. Transcriptional control of self-renewal in neuronal stem cells is closely related to the degree of lysine methylation leading to either mono-, di-, or tri-methylated histones. Gene-specific ‘active’ or ‘repressive’ methylation complexes are
then responsible for inducing replicative capacity through histone H3 lysine 4 (H3K4) methylation, as seen in embryonic and neuronal cells. In contrast, repressive neuronal stem cell genes carry histone H3 trimethyl K9 and histone H3 monomethyl K20 as well as histone H3K27, of which the latter has also been reported for embryonic stem cells. The methylation status is also linked to the differentiation capacity of stem cells whereby histone H3 trimethyl K20 has been found abundantly in differentiation neuronal stem cells. These methylation modifications are known to occur on target genes downstream to transcription factors Oct4, Nanog, c-Myc, KIf4 and Sox2, which have been shown to be able to reprogram fibroblasts to a pluripotent state and are therefore believed to be important upstream regulators of the self-renewal mechanism.

Cellular processes including development, proliferation, and differentiation are also controlled by small non-coding RNAs. A large family of the small non-coding RNAs recently identified, are the miRNAs (micro-RNA) which are single-stranded fragments transcribed by RNA polymerase II from miRNA genes (class II genes) and are known as negative regulators of eukaryotic gene expression. Primary transcripts of miRNA, called pri-miRNAs, are converted inside the nucleus to pre-miRNAs, which are hairpin precursors extruded into the cytoplasm where they cleaved into mature miRNAs. These miRNAs are then complexed to an RNA-induced silencing complex (RISC) and presented to target mRNAs leading to their cleavage and subsequent translatational repression. It is believed that about one-third of all animal genes are regulated by miRNAs and of all miRNAs thus far identified the majority (70%) appears to occur in the brain, suggesting their importance in neuronal function.

Cell-extrinsic signals occur through direct physical interactions between stem cells and their biological niches are are soluble factors, which include Wnt, Notch and Sonic hedgehog.
The Wnt/β-catenin signaling has been shown to be important in the proliferation pathway of neuronal stem cells through downstream targets such as cyclin D1. Also Wnt3a and Wnt5a have both been associated with stimulation of murine neuronal precursor cell proliferation and differentiation. Notch signaling occurs by direct binding of a Notch ligand of one cell to the Notch receptor of an adjacent cell, which facilitates the release of the intracellular Notch domain and its translocation into the nucleus where it causes downstream effector expression such as for genes of the Hes family. Notch is believed to maintain the self-renewable state of stem cells. Finally, the sonic hedgehog (Shh) gene also is considered an important neural progenitor cell mitogen in both the embryonic and adult brain maintaining cell proliferation through mediation by the zinc finger-containing transcription factor Gli1.

Finally, it is believed that receptor tyrosine kinase signaling regulates stem cell proliferation and self-renewal, controlled in part by extracellular ligands such as transforming growth factor-alpha (TGFα), epidermal growth factor (EGF), and fibroblast growth factor (FGF). It has been postulated that neuronal stem cells may be influenced by other extracellular signals such as the Wnt, Notch and Shh derived from neighboring cell types, such as astrocytes and neuroblasts, as well as ependymal and endothelial cells through interaction with intrinsic signaling pathways such as the Wnt/-catenin-cyclin D1, Notch-hes1/5 and Shh-Gli1.

1.1.4 Definition of Stem Cell Potency

Stem cells are categorized into classes of potency: (1) Totipotent cells such as those in a fertilized egg are capable of forming an entire organism and are therefore able to give rise to any embryonic and extra-embryonic cell type; (2) Pluripotent cells are capable of forming tissues of all embryological origins such as endodermal, mesodermal and ectodermal tissues; whereas (3)
multipotent cells, such as mesenchymal stromal cells of bone marrow\textsuperscript{17,57,58}, are believed to be restricted to the differentiation of one or more cell types within a single embryological germ layer, and therefore limited to formation of mesenchymal tissues such as blood, fat,\textsuperscript{17} bone,\textsuperscript{26,59} cartilage,\textsuperscript{57} and muscle.\textsuperscript{60} However, MSCs have also been reported to form tissues such as liver\textsuperscript{61} and lung,\textsuperscript{62} which are considered of endodermal origin or nerves,\textsuperscript{17,58,63-65} which are ectodermal tissue. Therefore, the mesenchymal cells may have greater pluripotentiality than originally thought.

1.1.5  **Stem Cell Plasticity**

The differentiation process was traditionally believed to be a progressive downstream commitment and irreversible in nature. However, more recently studies have challenged this concept suggesting that once stem cells have ‘committed’ themselves to a specific cell lineage, they may still be able to transdifferentiate into another cell lineage. This would support a more flexible system in which cells may quickly respond to changing regenerative cues in the microenvironment of healing tissues.\textsuperscript{66} Song and his coworkers reported that once progenitor cells have differentiated into adipogenic, osteogenic or chondrogenic cell lines, transdifferentiation of each committed cell line can occur if the cells are aposed to appropriate inductive culture conditions of the other two cell lines. Transdifferentiation into each of the other two cell lines was demonstrated through upregulation of mRNA levels of specific factors associated with adipogenesis, osteogenesis and chondrogenesis.\textsuperscript{67} Song and his coworkers\textsuperscript{67} have suggested that his phenomenon may be due to potential progenitor cell contamination from possibly multiple stem cells sources within one cell pool or due to cell-to-cell fusion between progenitor cells and a tissue-specific cells. Also, the potential ability of cells to reverse to a precursor cell stage through dedifferentiation and subsequent redifferentiation into a different
lineage has been speculated but requires further investigation. Research is ongoing to substantiate the validity of this suggested plasticity of stem cells, defined as “the conversion of the cell of one tissue lineage into a cell of an entirely distinct lineage, with the concomitant loss of the tissue-specific markers and function of the original cell type and acquisition of markers and function of the transdifferentiated cell type”.\textsuperscript{64,68}

1.1.6 \textbf{Embryonic versus Adult Stem Cells}

The potential application of stem cells in human tissue engineering has caused considerable controversy within the academic, public and political arenas. In the layperson’s eyes, stem cells are commonly associated with embryonic origin and thus associated with the destruction of human embryos. Therefore, human embryonic stem cell research, under the current political atmosphere, is restricted to only a few established cell lines. Embryonic stem cells (ES) are believed to have a much longer lifespan compared with the adult stem cell, which is directly related to their telomerase activity. Human ES cells have been expanded for up to 250 population doublings\textsuperscript{69} (PDs) maintaining their pluripotency and normal karyotype. Therefore, these cells are considered more versatile compared with the adult mesenchymal stem cell. However, in addition to the ethical issues associated with ES cells, reports that these cells can form teratomas in vivo have pushed the emphasis of research more toward postnatally derived stem cells.

Embryonic stem cells are derived\textsuperscript{70,71} from the inner cell mass (ICM) of the blastocyst consisting of an outer cell layer, the trophectoderm, which later develops into the placental corion and a fluid-filled blastocoele containing a cluster of cells, called the ICM. It is still not clear, however, whether all cells of the ICM are pluripotent and whether ES cells are derived from a single or a population of ICM cells within the blastocyst.\textsuperscript{64} Depending on the species,
blastocysts are approximately 1 week-old preimplantation embryos. The embryonic ICM contains pluripotent stem cells (epiblast), which develop into the species-specific organism consisting of the three embryonic layers, the endoderm, mesoderm, and ectoderm. Embryonic stem cells are defined as capable of differentiation into all tissue lineages and have much longer cell culture longevity compared with adult stem cells, which display replicative senescence similar to all other eukaryotic cell lines as stated in the Hayflick limit, a term that was coined by Burnette in recognition of Hayflick’s discovery. Interestingly, adult cells will conform to this predetermined maximum lifespan of approximately 50 cell doublings after recovery from a frozen state.

The replicative lifespan has been shown to be directly related to telomerase activity, which was first sequenced by Blackburn and her coworkers in ciliated protozoa, Tetrahymena thermophila. This discovery allowed length measurements of telomeres, which are the cellular replicometer and which are suspected to shorten in aging cells until a critical telomere length eventually determines cellular senescence. Greider and Blackburn discovered telomerase, an enzyme that adds telomere repeats to the chromosomes and thereby maintains replicative cell activity such as seen in embryonic stem cells and cancer cells. Telomerase is reduced in hemopoietic stem cells (HSC) and variable or even absent in somatic cells. Embryonic stem cells have been reported to survive for 1 to 2 years in culture with cell doubling times of 36 to 48 hours. Cell aging through serial propagation in culture is related to other degenerative changes, which include abnormal cytoplasmic structures, changes in metabolism, loss of methyl groups, reiterated DNA sequences, reduction of replicative efficiency and growth rate. Apoptosis and tumorigenicity are possibly associated with a certain genetic instability of these aging cells. The putative relationship to cancer has been proposed as signaling pathways, such as
those regulated through PTEN, notch, sonic hedgehog and wnt, recently reported for hemopoietic stem cells (HSCs)\textsuperscript{87-92}, are also related to oncogenesis.\textsuperscript{93} ES cells can replicate through extended population doublings beyond those manifested for eukaryotic cells. However, Deasy and coworkers showed that muscle-derived stem cells (MDSCs) can also be expanded over more than 300 PDs during 225 days in cell culture.\textsuperscript{93} These cells showed normal \textit{in vitro} phenotypic characteristics (ScaI+/CD34+/desmin\textsuperscript{low}) for up to 200 PDs, at which point CD34 was lost from the surface marker profile concomitant with a loss of muscle regeneration, myogenic activity and increased growth on soft agar. These results demonstrate that adult stem cells appear to have the ability to replicate long term but that cell aging is inevitable and may be related to cancer development.

1.1.7 \textbf{Mesenchymal Stromal Cell Frequency}

Pittenger and his coworkers reported MSC frequency in human bone marrow of between 1 per 10\textsuperscript{4} to 1 per 10\textsuperscript{5} nucleated cells.\textsuperscript{17} Donor age is a factor influencing human MSC frequency. Their numbers have been reported to decline in an age-related manner from 1 in 10,000 (newborns) to 1 in 2,000,000 (an 80 year-old person).\textsuperscript{94} Canine\textsuperscript{32} and feline\textsuperscript{95} bone marrow-derived MSC frequency has been reported to be 1 in 2.5 x 10\textsuperscript{4} and 1 in 3.8 x 10\textsuperscript{5}, respectively. Our data show an equine MSC frequency among the total nucleated BM cells of 1 in 4.2 x 10\textsuperscript{3} (based on CFU-F) in young horses ranging in age from 9 months to 5 years. However, MSC frequency in mice\textsuperscript{96} has shown considerable variation for primary cell frequencies (up to ten fold) between individual subjects. Murine bone marrow-derived MSC frequency determined from CFU-fibroblasts (F)/- adipocyte (Ad)/-osteocyte (Ob) studies ranged between 1 in 10.8 X 10\textsuperscript{3} to 1 in 3.45 X 10\textsuperscript{4}.\textsuperscript{97} One to 2 orders of magnitude differences in numbers of bone marrow-derived equine, human\textsuperscript{17}, canine\textsuperscript{32}, feline\textsuperscript{95} and murine\textsuperscript{96} MSC
frequency may be related to differences in the quantitative methods, age of the donor animals or some biological features unique to a particular species. Low plating density and large culture volume may influence cell survival relative to microtiter conditions, influencing the detected number of MSCs. Age-related correlations in MSC frequency or cell doubling times (2.1 days/cell doubling) could not be detected from non-hematopoietic bone marrow (nhBM) recovered from femoral heads of patients (27-81 years, n = 16) undergoing hip arthroplasty. The authors reported MSC frequencies as high as 1 in 13,000 nucleated cells from these patients.

1.1.8 Adult Stem Cell Sources

Adult mesenchymal stromal cells with multipotent differentiation potential can be found in many different tissues of the body. It is not clear whether mesenchymal cells are recruited in response to natural cell turnover and replenishment or injury from niches in close proximity to these sites or from bone marrow. Multipotent cells have been isolated from tissues such as the lung, liver, kidney, spleen, thymus, placenta, pancreas and even the brain and nerves as well as mesenchymal tissues such as cartilage, muscle and adipose tissue. In the horse, bone marrow and adipose tissue are the most useful and accessible sources for MSCs. The progenitor cells harvested from these sites in other species have the ability to replicate as undifferentiated cells and to differentiate into various mesenchymal phenotypes, including bone, cartilage, fat, muscle, tendon and marrow stroma.

1.1.9 Mesenchymal Stem Cell Niches

Under in vivo conditions mesenchymal stem cells are believed to multiply and differentiate under much more stringent control than their characteristic expansive growth seen in cell culture conditions. These multipotent cells are believed to reside in specific niches within their respective tissues, which are responsible to both restrain their regenerative capacity and to
prevent tumor formation as well as shield them from molecular signals, which could induce their cell cycle or differentiation. Hence, these niches provide a balance between the benefit of replenishing the demand for mature cell lineages and avoiding the detriment of uncontrolled proliferation of progenitor cells.104

The concept of stem cell niches and their prevailing cellular and molecular mechanisms have only very recently been targeted by research efforts. The most well-defined niches thus far are those of the hemopoietic stem cell (HSC), the intestinal stem cell (ISC), the hair follicle stem cell (HFSC)104 and the neural stem cell niche.105 Cancer development is believed to be associated with aberrations in the control of stem cell proliferation in their niches and therefore they provide excellent target for therapeutic intervention in cancer treatment.106,107

Osteoblastic cells provide a niche environment for precursor cells such as the HSC. Genetically altered mice produce osteoblast–specific PTH/PTHrP receptors (PPR) and high levels of the Notch ligand Jagged-1, which is a HSC growth factor87, which leads to increaseds in vivo Notch1 activation in HSCs as well as cultured HSC numbers.108 Parathyroid hormone (PTH) activation of PPR caused an increase in cultured osteoblast and HSC numbers, which was abolished by γ-secretase, a Notch inhibitor. Furthermore PTH injection increased HSC number and survival seen after bone marrow transplantation from the wild-type animals.108 It is believed that direct contact between HSCs and N-cadherin+CD45- osteoblastic (SNO) cells lining the endosteal bone surface mediate cell proliferation through N-cadherin and beta-catenin molecules and through BMP receptor type IA (BMPRIA) controlled signaling of bone morphogenetic proteins (BMP) in osteoblastic cells.109 Signaling pathways such as those mediated through Wnt, Notch and Hedgehog are also important in regulating HSC growth and proliferation and modulation of these transcription factors via the inhibition of glycogen synthase kinase-3 (GSK-
3) activity have been suggested\textsuperscript{110}, yet are not fully understood.\textsuperscript{104} Bone marrow contains multiple niches for HSCs such as the endosteal and a vascular niche and it has been suggested that due to the proximity of stromal stem cells in bone marrow, similar niches are also present for the adult mesenchymal stem cell.\textsuperscript{111}

1.1.10 Stem Cell Phenotyping

It is important to be able to recognize and identify stem cells based on specific phenotypic characteristics in order to define stem cell function and their mechanism of tissue repair. In vivo models employed for the study of mesenchymal or hemopoietic cell “stemness” allow the localization of these cells based on immunohistochemical detection of stem cell surface markers. However, attempts to isolate characteristic cell surface markers of a definitive stem cell phenotype from varying tissue sources have thus far been unsuccessful.\textsuperscript{58,68} Phenotyping these cells currently relies on the use of a combination of cell surface markers rather than specific surface antigens. There are distinctive differences between the different stem cell types with regard to the presence or absence of hematopoietic antigens, CD14, CD34, and CD45 on MSCs.\textsuperscript{17,112} A number of surface markers such as STRO-1, CD14, CD29, CD 44, CD49a, HOP-26 (CD63), CD71, CD73, CD90, CD106, CD120a, CD124, and SB-10 (CD166) antigens have been described for MSCs.\textsuperscript{14,17,112-114} Surface markers (CD13, CD29, CD44, CD73 and CD90) have also been used to isolate adipose tissue-derived stromal cells.\textsuperscript{14} In this study primary stromal vascular fraction cells expressed these markers to less than 54%, whereas the surface marker profile of subcultivated ASCs increased to > 90% of positive cells. These results show that the primary nucleated cell pool in stromal vascular fractions derived from adipose tissue and the nucleated cell population harvested from bone marrow stroma are heterogeneous in nature consisting of varying cell types and stem cells at different stages of differentiation, which may
variably restrict their multipotential. Approximately two-thirds of all marrow-derived stromal cells show tri-potential when tested under adipogenic, osteogenic and chondrogenic conditions. The remaining portion of the cells were bipotential, capable of adipogenesis and osteogenesis, suggesting that these two differentiation processes are more closely related in their upstream gene regulation than the control of chondrogenesis. Species-related differences with respect to stem cell marker expression and the putative plasticity of stem cells can further complicate the identification of stem or lineage committed cells.

1.1.11 Effects of Oxygen Tension on Stem Cell Activation

An important difference between stem cell niches in the body and traditional cell culture conditions is the difference in oxygen concentrations. The use of cultured cells in tissue engineered constructs or in cell-based approaches introduces a cell previously cultured at 21% O₂, to hypoxic conditions within the implanted tissue environment. Mesenchymal stromal cells undergo significant metabolic changes under such conditions. MSCs live in close proximity and metabolic relationship to hematopoietic stem cells suggesting that these cells live in similar bone marrow niches. This tissue is known to be hypoxic at 1 to 2% O₂. The proliferative effects of hypoxic conditions within bone marrow on HSCs are well known.

Oxygen levels are intricately related to self-renewal and differentiation of mesenchymal stromal cells. Culturing human MSCs under hypoxic conditions of 2% O₂ enhanced proliferation of in monolayer culture and within 3D scaffolds. Hypoxia at 2% also reduced differentiation processes such as chondrogenesis and osteogenesis whereas at slightly higher oxygen concentrations (5%) human adipose-derived stromal cells demonstrated the opposite phenomenon of reduced proliferation rates and increased adipogenesis compared with cells cultured under traditional 20% O₂ conditions. Grayson and coworkers recently showed that
Hypoxia resulted in approximately 30-fold differences in human MSC expansion rates and multilayer cell growth after confluence had been reached in monolayer cultures. Hypoxia also had a positive effect on mRNA levels of upstream regulators responsible for cell growth, such as Oct-4 and HIF-2 as well as extracellular matrix proteins such as collagen I, fibronectin and connexin-43, which is involved in gap junction formation. Hypoxia (1%) apparently did not affect important regulators of adipogenesis such as PPARγ and ADD1/SREBP1c, or downstream markers such as lipoprotein lipase and aP2. Yet adipogenesis clearly appeared to be induced and enhanced compared with cells grown under 2% and 21% normoxic conditions, as measured by lipid droplet and triglyceride accumulation.

Hypoxia inducible factors (HIFs) are proteins belonging to the family of bHLH-PAS proteins and act as transcription factors responsible for cellular changes in response to hypoxia. HIFs bind to hypoxia regulated elements (HRE) at the promoter region of well over 150 target genes involved in cell metabolism, survival, motility, basement membrane integrity, angiogenesis, hematopoiesis and a number of other functions. HIF proteins consist of an α (HIF-1α) and β (HIF-1β) subunit. Regulation of HIF activity is largely dependent on the stability of its α subunit, which increases under decreasing O2 concentrations. The DNA binding and transcription depends on the stability of the HIF-α protein, which needs to bind the constitutively expressed HIF-β as well as other coactivators such as CBP/p300 within the nucleus at the HRE sequence.

Differentiation mechanisms appear to be down-regulated by HIF as well as Notch. The intracellular domain of Notch is released from the plasma membrane and transported to the nucleus after proteolytic cleavage in response to Notch receptor binding with Jagged ligands. The recruitment of Notch and binding to a DNA complex with other coactivators (MAML, CSL
and p300) leading to downstream activation of target genes such as Hes-1 and Hey-2, requires binding of HIF-1.\textsuperscript{121,124}

The precise interaction between HIFs, Notch and Oct4, which is predominantly responsible for regulating ES cell differentiation and the potential interaction with other proteins such as BMP and TGFβ is still under investigation. TGF has been shown to induce stability of the HIF-1a through inhibition of PDH2, which is a proline hydroxyase targeting degradation of the HIF-a subunits under normoxic conditions.\textsuperscript{125} It appears that low oxygen levels as seen in intact tissues such as bone marrow will undermine differentiation processes keeping progenitor cells potentially stored in microenvironments and protected from oxidative stresses.

1.1.12 The Concept of “Stemness”

The true test of mesenchymal stromal cell “stemness” beyond their ability to differentiate \textit{in vitro} into specific lineages is the demonstration of tissue functionality in animal implantation studies and disease models to progress their application in regenerative tissue repair. The ability of MSCs to self-renew and expand in culture, to differentiate into different cell lines dependent on the appropriate stimuli, and to regenerate tissue only encompasses part of the MSC potential. The early experiments in which MSCs were injected into mice under experimental conditions resembling osteogenesis imperfecta, demonstrated that the procedure resulted in a small but significant increase in bone strength.\textsuperscript{126} As recently reviewed by Prockop\textsuperscript{127} similar functional improvements were noted in animal models for human diseases such as Parkinson’s disease, stroke, myocardial infarction and spinal cord injury. Surprisingly, the clinical effect, which was attributable to the cell-based approach, did not appear to be correlated to engraftment or differentiation of stem cells. Only few donor MSCs in experiments where human cells were implanted into immunodeficient mice after induction of coronary infarction through ligation of
the anterior descending coronary artery were found based on sensitive assays of tracing human chromosomal in the animal tissues.\textsuperscript{128} In clinical cases of osteogenesis imperfect, less than 1\% of donor cells were found within osseous and dermal as well as other tissues.\textsuperscript{126} These results showed that tissue formation alone could not account for the improvement of the clinical or experimental conditions, suggesting that MSCs affected the repair process in yet a different ways.

1.1.13 \textbf{Immunotolerance}

Immunosuppressive effects of MSCs in response to allogeneic transplantation in both clinical and experimental models suggest that these cells play an important role in this function. The precise mechanisms by which MSCs are tolerated are poorly understood. However, experiments show that MSCs can suppress mixed-lymphocyte reactions in culture suggesting that MSCs may allow tissue repair following allogeneic bone marrow engraftment by inciting immunesuppression.\textsuperscript{129} Experimental T cells-mediated autoimmune encephalomyelitis (EAE) in a murine multiple sclerosis model have shown that the systemic use of MSCs will result in drastic improvement of the associated symptoms.\textsuperscript{130} In a caprine femoropatellar inflammatory arthritis model the use of stem cells resulted in lowering the inflammatory response.\textsuperscript{131}

The mechanisms by which MSCs are believed to avoid allogeneic recognition are partly related to cell surface phenotype wherein MSCs are positive for MHC class I, but negative for MHC class II, CD40, CD80 and CD86 markers. While the MHC Class I molecules alone can incite a T cell response, other surface molecules are required for a secondary response by the T cells.\textsuperscript{132} Therefore, it may be feasible to use MSCs in allogeneic transplantations without immunosuppression in the recipient. Also, MSCS are believed to inhibit CD8+ and CD4+ cells such as natural killer cells and thereby evade an innate immune response. Therefore, gene-
modified MSCs have been proposed as a therapeutic instrument for targeting diseases and medical conditions in which MSCs can introduce healing factors or altered genetic sequences to replace the body’s own defective codes “under the radar” of the immune system’s vigilant guard. MSCs have been used as carriers for interferon-β delivery to tumors in mice.\textsuperscript{133} Also, gene therapy with genetic codes for vascular endothelial growth factor has been shown to improve myocardial function in infracted rats\textsuperscript{134} \textsuperscript{135}, bone formation through the delivery of BMPs\textsuperscript{136-138} and osteopenia via the delivery of merely a normal collagen type I gene into young humans with skeletal malformations due to osteogenesis imperfecta.\textsuperscript{126,139}

MSCs may be able to provide immune-suppressed microenvironments and therefore modulate T cell reactions through the synthesis and release of a multitude of soluble factors\textsuperscript{140,141}, prostaglandins and interleukins such as IL-10\textsuperscript{142} or through direct cell-to-cell contact.\textsuperscript{143}

1.1.14 **Mesenchymal Stem Cell Trophism**

MSCs used as feeder cells in co-culture with hematopoietic cells were found to produce copious amounts of cytokines and chemokines in culture.\textsuperscript{144} The patterns of these secreted factors changed once the cells were injected into new environments such as the brain, which suggested that MSCs promote repair of damaged cells. Hence adult marrow-derived Mesenchymal Stem Cells (MSCs) are now known to exert these trophic effects on their environment by secretion of a variety of cytokines and growth factors, which act in paracrine and autocrine fashion by suppressing the local immune system, inhibiting fibrosis (scar formation) and apoptosis, enhancing angiogenesis, and stimulating mitosis and differentiation of other stem cells intrinsic to the damaged tissues.\textsuperscript{145} These trophic effects represent a separate function of
stem cells apart from the direct differentiation into committed cell lines required to regenerate injured tissue.

Potapova and coworkers have shown that human MSCs (hMSC) cultured in monolayer or as three-dimensional aggregates in hanging drops (spheroids) secreted vascular endothelial growth factor (VEGF), basic fibroblast growth factor, angiogenin, procathepsin B, interleukin (IL)-11, and bone morphogenetic protein 2, which affected endothelial cell migration, extracellular matrix invasion, proliferation, and survival in vitro.\textsuperscript{146} The concentrations of these paracrine factors increased 5-20 times in medium conditioned by hMSC spheroid cultures. Differences in stimulation of in vitro endothelial cell survival and in vivo umbilical vein endothelial cell proliferation, migration, and basement membrane invasion were noticed between media conditioned by hMSC spheroids and those conditioned by monolayers of hMSCs.\textsuperscript{146} Examples of the indirect MSC effects on its environment through induction of trophic factors are the release of cytokine such as granulocyte colony-stimulating factor (G-CSF), stem cell factor, macrophage colony-stimulating factor (M-CSF) and interleukins such as IL-6, which are known supporters of hematopoiesis.\textsuperscript{147} The MSCs are attracted to injured tissue sites by chemoattractant factors such as stromal-cell-derived factor 1 (SDF-1), which mobilizes CD117-positive MSCs, or endothelial progenitor cells, from the circulatory system to the ischemic and injured tissue such as an infarcted myocardium.\textsuperscript{148} The release of G-CSF from MSCs or other inflammatory cells will also have a direct mobilizing effect on recruitment of additional MSCs.\textsuperscript{149} Hence, MSCs are closely involved with a multitude of processes, which support different aspects of tissue repair, further demonstrating the potential importance of these cells in the tissue repair process via systemic or direct delivery through properties other than cell differentiation and replacement of injured tissue.
1.1.15 Stem Cell Homing

One of the important attributes of stem cells, which is still poorly understood, is their ability to specifically home onto injured tissues. It is known that adhesion molecules, chemokine receptors and their ligands are important for tissue-specific homing of leukocytes as well as hemopoietic stem cells. Information is also slowly becoming available regarding the expression of these receptors and adhesion molecules in mesenchymal stem cells. Studies that show MSC localization predominantly in ischemic injured tissues, such as experimentally-induced myocardial infarctions in rats, suggest that MSCs deposited in the left ventricular chamber are attracted by and directed to specific receptors and ligands in these injured tissues.\textsuperscript{150} Interestingly, this particular study also showed that the infused MSC localization within the injured heart soon dissipated (within 4 hours).\textsuperscript{151} Cells infused intravenously appeared to get trapped within the lung parenchyma. This effect can be avoided using vasodilators such as sodium nitroprusside.\textsuperscript{152}

Understanding of MSC homing requires appreciation of MSC expression of chemokines (CCR1, CCR2, CCR3, CCR4, CCR7, CCR9, CCR10, CX3CR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6)\textsuperscript{153-158} and their receptors from the different chemokine families, integrin molecules such as α1, α2, α3, α4, α5, αv, β1, β3, β4 and other adhesion molecules such as VCAM-1, ICAM-1, ICAM-3, ALCAM and endoglin/CD105.\textsuperscript{151,159,160} These molecules may be of specific importance in certain tissues such as the integrin VLA-4 and adhesion molecule VCAM-1, which have been found to be crucial in human MSC attachment to the endothelium. Considerable variability within the literature regarding the chemokine expression has been attributed to the heterogeneity of MSCs and their potential to home toward different tissues.\textsuperscript{151}
1.1.16 Mitochondrial Transfer and Stem Cell Fusion

MSC support in tissue repair was found when MSCs were cocultured with modified endothelial cells (A549 $\rho^0$ cells) containing non-functional mitochondria. The coculture resulted in colonies of functional A549 cells with MSC mitochondrial DNA but no genomic MSC donor DNA. The process occurred through a direct exchange of mitochondria or mitochondrial DNA between A549 $\rho^0$ cells and MSCs without cell fusion. However, spontaneous fusion has been reported between ESCs and co-cultured somatic cells, resulting in a stem cell biomarker transfer. In the context of investigating stem cell plasticity, these studies appeared added confusion because in order to determine plasticity between differentiated cell lines robust techniques for detection of stem cell fusion are required. Reprogramming of somatic cells using fusion partners such as embryonic stem cells, embryonic germ cells, embryonal carcinoma cells and undifferentiated cells designed to reset gene expression to embryonic state without altering the DNA is an emerging tool based on epigenetic mechanisms designed for future cell-based therapies.

1.2 Multipotential and Differentiation Pathways

After cell culture experiments have established the self-renewal potential of mesenchymal stromal cells from varying tissue sources, it is necessary to demonstrate their multipotential characteristics by induction of stem cells into adipogenic, osteogenic and chondrogenic cell lineages.

Molecular mechanisms of differentiation have still not been completely elucidated. Baksh and her coauthors have recently proposed that adult stem cells undergo differentiation into separate compartments, the first of which is governed by transcriptional modification of the multipotent MSC without phenotypic change, followed by the second compartment, which is
characterized by phenotypic modification of the precursor cell into a specific cell line.\textsuperscript{58} Mesenchymal stromal cells reside within their respective niches and remain quiescent and arrested in the G0/G1 stage of the cell cycle until an environmental signal such as a growth factor stimulates proliferation and eventually differentiation.\textsuperscript{58} It is believed that the MSC cell division is asymmetric resulting in two daughter cells, one of which is an exact replica of the original cell and the second is a precursor cell that contains a different genetic transcription pattern compared with the mother cell. The precursor cell further divides to give rise to bipotent and tripotent precursor cells, which morphologically still resemble the multipotent stem cell but may then further differentiate into unipotent cells eventually giving rise to varying cellular phenotypes such as those osteoblasts, adipocytes, chondrocytes, myocytes or tenocytes. The regulation of transitions between the multipotent, partially committed precursor cells and the fully committed cells is not completely understood. However, it is known that there is a temporal relationship in which cytokine growth factors and extracellular matrix proteins interact with multipotent and committed precursor cells. In the search for common control genes between differentiation pathways such as osteogenesis, adipogenesis, and chondrogenesis, experiments have been conducted to examine expression profiles of 39,000 transcripts that showed at least a 1.5-fold increase in RNA levels after induction. The results showed that 914, 947 and 52 genes are activated during osteogenesis, adipogenesis and chondrogenesis, respectively, whereby approximately 25\% (235) of the genes upregulated during osteogenesis and adipogenesis are common to both differentiation processes as opposed to 10 and 3 between adipogenesis and chondrogenesis or chondrogenesis and osteogenesis, respectively.\textsuperscript{58} A total of 8 genes (period homolog1 [PER1], nebulette [NEBL], neuronal adhesion molecule [NRCAM] FK506 binding protein 5 [FKBP5], interleukin1 type II receptor [IL1R2] zinc finger protein 145 [ZNF145],
tissue inhibitor of MMP4 [TIMP4] and serum amyloid A2) were common to all three mesenchymal lineages differentiation processes, suggesting that these genes possibly represented a form of master control or were genes responsible for regulation of cellular processes common to all three lineages, such as cell adhesion, organization of the actin cytoskeleton and inflammatory pathways. The relative abundance of common genes during adipogenic and osteogenic differentiation suggests that these two processes are more similar and may therefore share common precursor cells, compared with the chondrogenic differentiation process.

1.2.1 Adipogenesis

Adipose tissue was traditionally considered a storage depot for energy up until the 1980s. Since then, however, molecular biological techniques have completely altered our understanding of the role of adipose tissue in the body. A variety of gene products were isolated from adipose tissue such as adipin, tumor necrosis factor (TNF)-alpha, plasminogen activator inhibitor-1 as well as adipokines such as leptin and adiponectin, and it was found that these factors were regulatory during energy homeostasis under feeding and fasting conditions. Additionally it has been shown that adipose tissue is related to insulin sensitivity and obesity, as well as in artherogenesis and inflammation. Contrary to suggestions of the older literature that individuals are born with a set number of adipocytes, it is now believed that based on evidence of $^3$H-thymidine incorporation into new adipocytes in rats fed high caloric diets, that adipogenesis occurs throughout the life time of an individual or animal and that therefore obesity is governed by adipocyte hypertrophy and hyperplasia.

Adipogenesis and associated tissue engineering applications are important considerations in human medicine due to injuries and diseases such as burns, avulsions, lipodystrophy, post-surgical defects after breast tumor removal and congenital defects such as Poland syndrome or
Romberg’s disease, all of which often cause tissue defects due to absence or loss of adipose tissue and therefore significant cosmetic dissatisfaction. Current approaches to correct such defects with dermal or free adipose tissue grafts, collagen injections and synthetic materials have major disadvantages such as immune reactions, shrinkage, absorption and fibrous tissue and oil cyst replacement as in the case of free adipose tissue grafts. The aim is to design soft tissue scaffolds which are large enough to provide cosmetic relief to the injured tissues and which can integrate into the recipient’s tissue bed thereby replacing the current use of artificial devices such as breast implants. The engineering is challenging because scaffold size and pore size limit to cell seeding and adipocyte growth and expansion.

Equine veterinary medicine, however, does not currently have much use for such cosmetic interventions and therefore it begs the question as to why adipogenesis is an important factor to consider in our efforts to study the multipotential of equine stem cells. As previously alluded to it is conventionally considered one of the three pillars of differentiation through which cells will demonstrate their multipotential capacity. However, it also known that adipogenesis and osteogenesis are intimately related to each other and with the more recent discoveries of adipokines and their important endocrine functions within the body, adipose tissue has gained recognition beyond its functions of energy storage and a physical protection of underlying tissues. Therefore, it is important to appreciate the intricacies of adipogenic and osteogenic control as it may provide an understanding for future therapeutic interventions in non-union or mal-union of bone healing, which is a recognized problem in equine veterinary medicine.

1.2.1.1 Molecular Control of Adipogenesis

Adipogenic induction is typically achieved with induction media containing ligands for nuclear hormone receptors involved in both adipogenesis and/or osteogenesis, such as
dexamethasone for the glucocorticoid receptors or indomethacin and thiazolidinediones for the peroxisome proliferator activated receptor (PPARγ). The following is a description of the molecular, transcriptional, cellular and hormonal controls known to regulate adipogenesis.

The very early molecular events determining a primitive mesenchymal precursor cell to differentiate into an adipocytic lineage remain unknown. Characteristic for the first stage of fat cell differentiation is an initial growth arrest which is then followed by several cell divisions, known as clonal expansion. During this process the transcription factors peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein (C/EBPα) are expressed, which in turn are associated with a second growth arrest followed by complete differentiation. It is believed that PPARγ and C/EBPα have antimitotic properties. The activation of PPARγ appears to correlate with the demise of the DNA binding activity of E2F/DP, which is an important regulator for many genes associated with cell growth and promoting cell division.

A number of different preadipocytic cell lines such as the 3T3-L1 and 3T3-F442A isolated from nonclonal Swiss 3T3 by Green and colleagues as well as TA1 and Ob have been used to conduct studies regarding adipogenesis. These cells are indistinguishable from fibroblasts but are already committed to an adipocytic lineage and therefore will differentiate readily into mature adipocytes within 4 to 6 days when exposed to agents such as cAMP, insulin or glucocorticoids. In 3T3-L1 cells, the transition from a growth-arrested preadipocyte to a fully differentiated fat cell has been reported in association with a PPARγ mediated induction of cyclin-dependent kinase inhibitors such as p18 (INK4c) and p21 (Waf1/Cip1). C/EBPα has also been associated with the expression of p21.

Prior to differentiation into mature fat cells the preadipocytic cell enters a second period of growth arrest following clonal expansion. Attempts to define the beginning and the end of the
adipogenesis process in culture are difficult, because the process is asynchronous in nature occurring progressively in cell clusters making mRNA level expressions patterns difficult to determine. However, it appears that adipogenesis is subject to a cascade of events. An early biomarkers of the differentiation process is lipoprotein lipase. The appearance of this protein is closely followed by a number of transcriptional factors, which result in the expression of the mature adipocyte phenotype and the production of a number of adipogenesis end products. These include glycerophosphate dehydrogenase, fatty acid synthase, the adipocyte-specific fatty acid binding protein (aP2), malic enzyme, the glucose transporter GLUT 4, the insulin receptor and considerable triglyceride accumulation.

1.2.1.2 Transcriptional Control of Adipogenesis

The key players involved in the transcriptional cascade of adipogenesis are members of the CCAAT/enhancer binding protein (C/EBP) family, especially C/EBPα, C/EBPβ, and C/EBPδ, the transcription factors peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ ligands, such as the thiazolidinedione (TZD) class of anti-diabetic drugs, bind with a range of affinities (Kd = 50 – 700 nM). Other ligands such as 15 deoxy-Δ12,14 prostaglandin J2 (15dPGJ2) and polyunsaturated fatty acids such as oleate and linoleate177,178 may also bind to PPARγ but with much lower affinity (Kd = 2 – 50 μM range).

The CCAAT/enhancer binding proteins belong to the family of basic leucine zipper class of transcription factors and they appear to play a role in both adipogenesis and osteoblast development.179,180 These proteins have a profound effect on the fat cell maturation of cultured preadipocytic cell lines under adipogenic induction. It is known that mRNA and protein levels of CEBPβ and C/EBPδ will rise early during adipogenesis followed by C/EBPα which is induced later in the differentiation process, before other end-product genes of fat cells.179 The role of
C/EBPs in adipogenesis has been studied with the help of knock out mice\textsuperscript{181}, antisense RNA experiments blocking the expression of factors like C/EBP\textgreek{a} and embryonic fibroblast models lacking C/EBP\textgreek{b} and/or in combination with C/EBP\textgreek{d}.\textsuperscript{182}

The peroxisome proliferator-activated receptor-\textgreek{g} (PPAR\textgreek{g}) is part of a heterodimer formed with retinoid X receptor (RXR).\textsuperscript{179} PPAR\textgreek{g} was discovered through cloning specific trans-acting factors which bound to the cis-elements (ARE6 and 7 and ARF6) found within the enhancer located within the 5′ flanking region of aP2. PPAR\textgreek{g} governs the function of most adipocyte-specific genes and is required for the fat-selective enhancers within the genes encoding aP2, lipoprotein lipase (LPL)\textsuperscript{183} and phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme of gluconeogenesis.\textsuperscript{184} In most models of diabetes, gene expression of PEPCK is enhanced in the liver and believed to contribute to the increased hepatic glucose output.\textsuperscript{185}

As stated earlier, transcriptional control of adipogenesis follows along a cascade of events whereby hormonal cues may initially activate CEBP\textgreek{b} and C/EBP\textgreek{d}, which then activate expression of the PPAR\textgreek{g}-RXR\textgreek{a} complex subsequently causing the activation of C/EBP\textgreek{a}. The factor ADD1/SREBP1 is also induced during adipogenesis and is regulated in response to variations in food intake.\textsuperscript{186,187} The exact role of is still not completely understood but its contribution to adipogenesis in regulating triglyceride and fatty acid metabolism has been established. It is considered a weaker stimulus for adipogenesis compared with PPAR\textgreek{g} or the C/EBP proteins\textsuperscript{179}, yet it is believed that ADD1/SREBP1-expressing cells in conditioned medium can activate PPAR\textgreek{g} directly or indirectly via promotion of an endogenous PPAR\textgreek{g} ligand.\textsuperscript{187} Adipocyte determination and differentiation-dependent factor 1 (ADD1) is a member of the basic helix-loop-helix leucine zipper (bHLH-LZ) family of transcription factors that binds
to two distinct DNA sequences and has been associated with cholesterol homeostasis, adipocyte development, and expression of fatty acid synthase (FAS) and lipoprotein lipase (LPL), two key regulators of fatty acid metabolism.\textsuperscript{186}

1.2.1.3 \textbf{Cellular and Hormonal Control of Adipogenesis}

Hormonal induction of adipogenesis involves activation of preadipocyte IGF-1 receptors (IGFR) by IGF-1 or insulin leading to activation of two separate signal transduction pathways, Akt and Ras, which activate adipogenesis via currently unknown effectors. Cyclic AMP (cAMP) is induced via methylisobutylxanthine (MIX) contributing to adipogenic differentiation by induction of C/EBP\(\beta\) either via the cAMP element binding protein or other unknown mechanisms. Two further adipogenic activation pathways are mediated either through glucocorticoids which bind to the glucocorticoid receptor (GR) and thereby resulting in C/EBP\(\delta\) activation and preadipocyte factor (Pref1) repression. Pref1, a preadipocyte cell surface glycoprotein, has also been implicated in mediating an antiadipogenic effect of growth hormone (GH) in rats.\textsuperscript{188} A variety of growth factors and cytokines act as inhibitors of adipogenesis affecting mitogen-activated protein kinase (MAPK) which phosphorylates PPAR\(\gamma\) and its heterodimerization partner RXR.

1.2.2 \textbf{Osteogenesis}

Work by Pittenger\textsuperscript{17}, Muraglia\textsuperscript{189} and their coworkers has shown that only about 30\% of human MSCs have tri-lineage (adipogenesis, osteogenesis, chondrogenesis) differentiation potential. The heterogeneity of the differentiation potential was further underlined by the fact that the remaining two thirds of the cells were either bipotential, capable of adipogenesis and osteogenesis, or unipotent in chondrogenesis.\textsuperscript{189} These data suggested that there may be a closer relationship with regard to the upstream regulatory control genes or even common precursors.
between adipocytes and osteoblasts compared with chondrocytes.\textsuperscript{58} With the help of DNA microarray technology the profiling of global gene expression has allowed to investigate patterns gene activation during the three differentiation processes. It has been shown that among 39,000 transcripts 914 responded to osteogenesis, 947 to adipogenesis and 52 during chondrogenesis at levels of 1.5 or higher compared with controls. A total of 235 genes were shared by osteogenic and adipogenic differentiation where as chondrogenesis only shared 3 and 10 genes with osteogenic and adipogenic differentiation processes, respectively. Eight genes were upregulated during all three differentiation events. These genes included period homolog1 (PER1), nebulette (NEBL), neuronal adhesion molecule (NRCAM), FK506 binding protein 5 (FKBP5) interleukin 1 type II receptor (IL1R2), zinc finger protein 145 (ZNF145) tissue inhibitor of metalloproteinase 4 (TIMP4) and serum amyloid A2.\textsuperscript{58} These genes are known to regulate a variety of basic cellular housekeeping processes including cell adhesion, protein folding and inflammation rather than representing master control genes.

The transcription factors regulating adipogenesis have an inverse relationship in the control of osteogenesis.\textsuperscript{167,190,191} Certain conditions that enhance adipogenesis, such as glucocorticoid treatment, ovariectomy, immobilization and aging leading to increasing numbers of adipocytes and osteopenia.\textsuperscript{192-195} It has become increasingly more evident during the 1990s that mesenchymal stromal cells were able to assume both osteoblastic and adipocytic phenotype suggesting possible transdifferentiation mechanisms, which may naturally be related to the proximity of marrow adipose tissue and bone.\textsuperscript{167,196,197} The close but reciprocal balance between osteoblastogenesis and adipogenesis has stimulated discussions of how this relationship may be used in the future for therapeutic interventions.
1.2.2.1 **Molecular Control of Osteogenesis**

PPAR\(\gamma\) is an important transcription factor involved in osteogenic regulation. The ligands for this nuclear hormone receptor are known to inhibit osteogenesis. The importance of the PPAR\(\gamma\) and its ligands involved in this process is evident from studies in which PPAR\(\gamma\)-deficient mice spontaneously demonstrated osteogenesis but failed in adipogenesis.\(^{198}\) High affinity ligands such as rosiglitazone were able to increase adipogenesis and concurrently reduce bone density, whereas osteogenesis seemed to be affected by the low affinity ligand troglitazone.\(^{199,200}\) Similar to other transcription factors such as Lef1 and Twist, PPAR\(\gamma\) binds to the runt homology DNA (RDH) binding domain and thereby inhibits the DNA binding site for the runt-related transcription factor Runx2/ Cbfa-1 (core binding factor alpha), which is a central regulatory protein for osteoblastogenesis.\(^{201}\)

Runx2 expression appears early during embryogenesis within the prechondrocytic mesenchyme in two isoforms, the ubiquitous MRIPV Runx2 and the osteoblast-related MASNS which later become involved in bone formation. Unlike most other transcription factors, Runx2 is also expressed in post-mitotic stages of mesenchymal cell division, which is believed to augment MSC commitment into and osteogenic phenotype. The differentiation process, requires an arrest of the cell cycle, similarly as described above during adipogenesis, and Runx2 is known to affect downstream target genes, the CDK inhibitors (CKIs) p21 and p27, which inhibit progression into the S-phase.

Thus Runx2 plays a critical role in determining the fate of cell differentiation during the development of bone. It functions as a molecular switch in determining processes of osteogenesis and chondrogenesis and is closely intertwined with other regulatory processes such as Wnt/b-catenin signaling pathway which activate Runx2 in MSCs via the Tcf1/Lef1 regulators to induce
osteoblastogenesis. Furthermore, Runx2 expression is controlled by down-regulation through the Nkx3.2 homeodomain after its induction by BMP2. Both Sox9 (Sry-type high mobility group box containing gene 9) and the Nkx3.2 are required for chondrogenesis during the endochondral bone formation process. The control of prechondrogenic expression of Runx2 by Nkzx3.2, as well as PTH/PTHrP, is considered a key point in determining chondrogenesis. It is necessary to undermine premature bone formation and to allow MSCs commitment into the chondrogenic lineage. In turn, Runx2-dependent activation of vascular endothelial growth factor (VEGF), matrix protein collagen type X and the matrix metalloproteinase MMP9 are required for endochondral ossification subsequent to the hypertrophic chondrocytes stage during which Nkzx3.2 is down-regulated. CEBP and Runx2 (Cbfa1) also act synergistically to enhance osteocalcin (OC) expression.

1.2.3 **Chondrogenesis**

The enormous economic implications and the loss of horses due to osteoarthritic and degenerative joint diseases in horses has made chondrogenesis and the cytokines and growth factors involved in the differentiation and maturation process of cartilage repair a important focus of equine orthopedic research. As discussed below in the section on cartilage repair in horses, it is now generally accepted that certain growth factors such as insulin-like growth factor-1 (IGF-1) and transforming growth factor-beta1 (TGFβ1) are important substances, which may benefit cartilage healing as an exogenous source during the early healing phase, or through genetic modification of chondrocytes or progenitor cells. As discussed earlier, stem cells affect tissue healing through cytokine release and immunemodulation in addition to tissue regeneration. Therefore, these cells are considered an essential for the repair of cartilage. However, there is now increasing evidence that mesenchymal stromal cells from varying equine tissue sources are
not all created equal. Koerner and coworkers compared bone marrow and peripheral blood progenitor cells and reported that only stromal cells derived from bone marrow were capable of chondrogenesis in a pellet culture model. Giovannini and coworkers, however, showed that equine blood-derived fibroblast-like cells do express proteoglycans in pellet cultures and concluded that these cells are capable of chondrogenesis yet are inferior to bone marrow-derived cells. Koch and his coworkers showed that pellet cultures derived from cord blood progenitor cells stain for glycosaminoglycans (saffarin O) and showed cartilage morphology with lacunae containing chondrocytes. Collagen type II expression, however, was not examined in this study. Most recently Kisiday and his coworkers have compared the chondrogenic potential of bone marrow and adipose tissue-derived mesenchymal stromal cells in agarose and peptide hydrogel cultures with or with out treatment of TGFβ1. The results again demonstrate the superiority of marrow stromal cells which produced more aggrecan-like proteoglycans under both hydrogel culture conditions compared with ASCs. Histological analysis of hydrogel cultures containing TGFβ1 for proteoglycan and collagen type II containing matrix as well as collagen type II expression evaluated with RT-PCR was only detectable in MSCs but not ASCs.

Other work has shown that human ASCs cultured with TGFb1, ascorbate and dexamethasone have similar biochemical marker profiles to those of articular chondrocytes. Awad and coworkers showed that human ASCs appear to demonstrate adequate chondrogenesis when cultured on agarose, alginate and gelatin scaffolds despite differences in protein and proteoglycan synthesis between the different scaffold cultures. Considering the biological (cell viability and morphology), biochemical (protein, proteoglycan, glycosaminoglycan and hydroxyproline synthesis) and mechanical properties (compressive modulus) the gelatin scaffold appeared to perform best overall followed by alginate and the agarose scaffolds. More recently
Hennig and coworkers compared the chondrogenesis between ASCs and MSCs under the effects of BMP6 and TGFβ1.\textsuperscript{218} It was found that ASCs showed reduced messenger level of BMP-2,-4, and -6 and failed to show significant mRNA levels of TGFβ-receptor-I. Accordingly, ASCs did not show significant chondrogenic response when just treated with TGFβ1, even at increased concentrations of 50ng/ml. The combination of TGFβ1 and BMP6, however, improved the chondrogenic capacity by similar levels of gene expression (aggrecan 1 (AGC), collagen alpha 1 type 2 (Col2A1), and COMP) as those seen for MSCs just under TGFβ stimulus. These results confirm earlier work by Im and coworkers who demonstrated inferior matrix production in ASC cultures compared with MSCs cultures and further supports the results of Kisiday’s work, which also showed nominal responses of ASCs to TGFβ1 compared with MSCs.

Hegewald\textsuperscript{219} and coworkers have provided evidence that the joint environment itself is an important chondrogenic stimulus to mesenchymal cells within the joint as the synovium is a rich source of mesenchymal stromal cells capable of chondrogenesis.\textsuperscript{220,221} Hegewald’s work has shown that hyaluronic acid or autologous joint fluid can induce \textit{in vitro} chondrogenesis in pellet cultures. Proteoglycan expression was increased due to addition of hyaluronic acid or varying concentrations of autologous joint fluid, but relatively lower compared with MSC cultures under chondrogenic induction with TGFβ1. Immunohistochemistry showed that the addition of 5, 10 or 50% synovial fluid concentrations to the chondrogenic protocol had significant effects on collagen type II expression.\textsuperscript{219}

The effect of synovial fluid is most likely related to growth factor release from soft tissues such as the synovium, the different types of synoviocytes and its progenitor cells. The effects of IGF-1\textsuperscript{222}, TGFβ1\textsuperscript{211,222,223}, fibroblast growth factor-2 (FGF2)\textsuperscript{224} on MSCs have been
studied by several groups and have all shown to enhance the chondrogenic potential of MSCs or even ASCs.

1.2.4 Current Knowledge about Equine Mesenchymal Stromal Cells

Equine stem cell biology includes mesenchymal cells from different tissues other than bone marrow and adipose tissue. Table 1 summarizes the main findings on all stem cell types thus far studied for the horse including adipose- and bone marrow-derived mesenchymal stromal cells, embryonic stem cells, and peripheral blood- and cord blood-derived progenitor cells. The research efforts on equine ESCs, however, are more comprehensive and therefore deserve a more discussion at this point.

1.2.5 Multipotentiality after Cryopreservation of Stem Cells

Cryopreservation of stem cells is an essential tool in tissue engineering and deserves a brief discussion. The application of cell-based therapies and gene alterations of stem cells to increase efficiency and efficacy relies on the complete functionality of these progenitor cells to undergo differentiation pathways, release cytokines and regenerate tissues after cryopreservation. Cryopreservation causes damage to the cell via extra- and intracellular ice formation, dehydration and due to the effects solutions in which the cells are suspended. The rapid rate at which cells are frozen is important in order to promote vitrification of the freezing solution rather than crystal formation within the cells. The process of freezing causes cellular dehydration through emigration of water from the cell and intracellular crystallization of water associated with mechanical damage due to crushing extracellular ice formation. Solute concentrations in non-frozen solutions such as high salt solutions are detrimental to the cell because during freezing these solutes are excluded from the crystal structure of the ice and thereby cause further damage to the cell. Conditions which cause an increase in viscosity, depress freezing
temperatures and cause rapid cooling are believed to promote vitrification, a process characterized by amorphous ice formation rather than crystalization. Prior to cryopreservation cells and other small biological tissues are readily penetrated by cryoprotectants such as ethylene and propylene glycol, glycerol, dimethyl sulfoxide (DMSO) and trehalose. Cells can easily be frozen in temperatures of liquid nitrogen of -196°C. Combinations of cryoprotectants are less toxic and therefore more effective than a single agent.

The expansion ability of stem cells after cryopreservation has been documented by Xiang and coworkers who cultured cryopreserved mesenchymal stromal cells obtained from bone marrow for 15 passages after resuscitation of the cells. These authors also found that these previously frozen MSCs were capable of pluripotent differentiation into mesodermal cell lines such as chondrocytes and adipocytes as well as ectodermal neurocytes under the appropriate conditions. The identification of the characteristic committed cell phenotype was supported by both histochemical analysis of safranin O heterochromia and procollagen type II expression in the case of chondrocytes, lipid droplet accumulation for adipocytes and nestin and neuron specific endolase RNA expression for neurocytes. Human MSCs undergo osteogenic differentiation based on evidence of increased alkaline phosphatase expression and nodule formation. This group also examined the growth kinetics of these cells and found that cryopreserved cells showed no significant differences in their self-renewal ability compared with fresh cells over the course of 10 passages. The post-cropreservation osteogenic potential of MSCs is further supported by Yoshikawa and coworkers who implanted cryopreserved cells after thawing into hydroxyapatite (HA) constructs and found through histochemical evaluation of in vitro culture experiments using plated cells and cells seeded onto HA-blocks as well as after
subcutaneous implantation experiments into syngenic rats, that these cells elicited extensive bone formation and elevated mRNA levels for alkaline phosphatase and osteocalcin activity.\textsuperscript{230}

The number of cryopreserved cells appears to have an effect on the post-thaw viability of adipose-derived mesenchymal stromal cells.\textsuperscript{231} It was found that the optimal cell number for cryopreservation purposes was approximately $0.5 \times 10^5$ cells/ml freezing medium consistent of 10% DMSO, 10% Ham’s F12 medium and 80% fetal bovine serum. The number of alkaline phosphatase expressing cells required increased three-fold after freezing ASCs compared with fresh cells. However, this phenomenon was not correlated to colony forming units undergoing osteogenesis. More work is yet required in the area of stem cell cryopreservation to optimize post-thaw cell viability, which is important to obtain the required cell numbers for cell-based therapies and implantation techniques in clinical medicine and surgery.

1.3 \textbf{Current Status of Stem Cell Biology in Equine Veterinary Medicine}

The treatment of orthopedic injuries and diseases has rapidly advanced in the past twenty years with the advent of improved surgical technology, instruments and techniques, such as through arthroscopy and new and improved implant materials and designs. However, the horse will always be disadvantaged in its ability to heal quickly and efficiently from significant injuries such as fractures and tendon and ligament injuries or diseases and developmental conditions such as a laminitis and subchondral bone cysts. The reason lies within its significant body weight, the resulting forces on soft and hard tissues, and the horse’s inability to psychologically cope with ambulatory failure because of its keen fright and flight response. The result of prolonged periods of significant lameness or non-weightbearing is all too often the laminitic breakdown within the contralateral foot capsule. Therefore, there is a real need to invent and develop new treatment techniques which are designed to improve dynamic stability across fracture sites and to increase

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the speed and efficiency of healing. These will help horses regain ambulatory control and use of their injured tissues to avoid further complications and decrease the significant wastage particularly common in the competitive equestrian industry.

Particularly painful and debilitating diseases commonly seen in athletic horses are flexor tendonitis and suspensory desmitis. These injuries are distinct from those observed in humans as they most often occur as core lesions within the soft tissue rather than complete ruptures such as Achilles tendon injuries in human athletes. Tendon injuries belong economically to the most important orthopedic injuries related to the loss of use of horses from all aspects of an enormous equine industry of 9.2 million horses. The equestrian industry contributes $39 billion dollars to the yearly US economy of which the racing industry alone is responsible for approximately 26%. The annual incidence of superficial digital flexor tendon injury seen just in Thoroughbred race horses ranges from 7 to 43%. Tendon and ligament injuries are characterized by a slow and inadequate healing process, owing to the low metabolic rate of tenocytes, mechanically inferior scar tissue formation, and the highly strenuous demands on these soft tissue structures during exercise and, especially, competition. The reinjury rate of SDFT injuries is also high ranging from 67% in Thoroughbred race horses due to the high-speed demands to between 20 to 40% in horses used for show jumping, dressage, polo and other competitive equestrian disciplines.

In recent years, stem cell-based therapeutic approaches have been used in equine veterinary medicine to treat the predominantly effected soft tissue structures such as the superficial digital flexor tendon (SDFT), deep digital flexor tendon (DDFT), or the suspensory ligament (SL) and its branches. Traditionally these injuries have been treated with a myriad of therapeutic interventions ranging from blistering and firing tendons to medical and surgical

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1 [http://horsecouncil.org/economics.html](http://horsecouncil.org/economics.html)
treatments. Medical treatments include intralesional or paralesional injections of anti-inflammatory drugs, tendon matrix constituents or lysyl oxidase inhibitors to prevent collagen type 3 cross-linking. Alternative treatments with laser, ultrasonographic, electromagnetic and extracorporeal shock wave therapies have also been reported. Surgical approaches such as the superior check ligament desmotomy are used in the hope of producing a favorable mechanical environment for tendon healing. However, none of these modalities have stood the test of time and have often produced questionable results and undesirable consequences. Controlled clinical studies are difficult to conduct due to the lack of client compliance, and experimental in vivo studies have not provided much more than a 60 – 70 % prognosis of recovery.

It is generally accepted that intrinsic tendinitis or desmitis, characterized by lesions within the core of the tendon or ligaments, particularly affecting the SDFT, is due to injury subsequent to repetitive loading and strain. The precise etiopathogenesis and associated molecular processes have not yet clearly been defined but are most likely explained by a combination of the current multiple theories related to mechanical, molecular, vascular and perhaps hyperthermic processes. Subclinical and often non-inflammatory events have been shown to result in either tenocyte overstimulation or understimulation and subsequent gene expression of catabolic factors causing further degeneration. Conversely, in clinical tendinitis overt inflammation within the lesion secondary to overloading and fibril disruption occur. The reason that the aforementioned treatment options do not or only partially address the actual problem of the disrupted tissue, is that most of them do very little to regenerate the actual structure of the tissue and to prevent the formation of non-elastic scar tissue incompatible with its natural function of shock absorption.
The notion that transferring bone marrow into injured tendons may be beneficial to the healing process within injured tendons was due to the introduction of growth factors and the potential of resident mesenchymal stromal cells. The original report described an unusually high success rate of 86% within a population of 100 horses.\textsuperscript{237} Whereas scientifically debatable, this report provided important stimulus to further investigate the merit and potential of equine stem cells and their combination with growth factors and bioabsorbable scaffolding materials, the three pillars of tissue engineering.

Current cell-based approaches to treating tendon and ligament injuries currently used in equine veterinary medicine employ the use of supraphysiological numbers of mesenchymal stromal cells either just isolated from adipose tissue (ASC) or isolated and expanded from bone marrow (MSC). It is important to recognize the difference between these two approaches, as the cells isolated from adipose tissue are a nucleated cell pool referred to as the stromal vascular fraction, that contains a relatively high number of mesenchymal cells (~200,000 ASCs/gram of fat), but which have not been expanded. MSCs occur in much lower numbers within bone marrow and therefore have to be expanded in culture before implantation. Cell culture of MSCs for 10 to 14 days, however, will result in much greater cell numbers than the primary ASC isolates from the stromal vascular fraction. Hence there are vast differences and variations in the applied cell numbers between these two treatment options, which may affect tissue healing. It is currently assumed that these high numbers of MSCs and ASCs will regenerate normal tissue more effectively compared with the body’s natural ability. Initial reports from clinical data in the United Kingdom have shown that the use of bone marrow-derived cells appeared to reduce the re-injury rate in both racehorses and sport horses by approximately 20 to 30%, whereby it seemed to be important that this therapeutic modality was used early in the course of injury.\textsuperscript{238,239}
1.4 **Gene Delivery in Mesenchymal Stromal Cells**

Gene therapy involves the insertion of genetic sequences via vectors such as viruses into somatic cells, which will change the recipients’ genome but, unlike germline gene therapy, will not cause inheritance of the genetic alteration to the next generation. Mesenchymal stromal cells are a potential way to introduce genes for the augmentation of cell differentiation and the neoformation of tissues. Biologically suitable cell scaffolding and a combination of stem cells and gene-enhanced cell differentiation are currently considered the most effective manner of tissue engineering. Genetically modified cells have been investigated in a variety of tissues such as bone\(^{240,241}\), cartilage\(^{242,243}\), tendons\(^{244}\), brain\(^{245}\) and other nervous tissues\(^{246}\) and the heart\(^{247}\) and in diseases such as diabetes\(^{248}\) and cancer control\(^{249}\) through the use of hemopoietic stem cells. In equine veterinary medicine, the predominant application of recombinant proteins and gene therapy has focused on musculoskeletal diseases such as cartilage resurfacing, fracture healing, tendon/ligament repair and wound management. Growth factors of potential clinical relevance are those of the bone morphogenic protein family (BMPs), the transforming growth factor-\(\beta\) family (TGF\(\beta\)), insulin like growth factors (IGFs), platelet derived growth factor (PDGF), fibroblast growth factors (FGFs), epidermal growth factors (EGFs) and activins and inhibins.\(^{250}\) Stewart and co-workers showed that FGF-2 treatment of monolayer marrow-derived MSCs resulted in increased mRNA level of collagen type II and aggrecan as well as total glycoaminoglaycan and DNA content from pellet cultures under chondrogenic conditions.\(^{224}\) *In vitro* experiments with equine chondrocytes demonstrate that IGF-I appears to have both a protective function within the joint as well as a role in supporting extracellular matrix growth. The combination of IGF-I and corticosteroids have demonstrated a protective effect on proteoglycan degeneration in response to an interleukin (IL)-1 induced inflammatory
response. The anabolic effect of IGF-1 appears to be dose-dependent and related to the matrix synthesis by chondrocytes grown in fibrin discs. Proteoglycan content is augmented by pretreatment of bone marrow derived MSCs with TGF-β. Equine in vivo models have shown that treatment of full-thickness cartilage lesions in the femoropatellar joint with human recombinant IGF-I enhanced fibrin clots resulted in improved collagen type II deposition within 6 months after treatment. Using scaffolds composed of fibrin and chondrocytes treated with IGF-I resulted in superior collagen type II deposition compared with controls not receiving IGF-1. The exact molecular mechanisms of this anabolic effect remain to be established.

In the equine athlete, cartilage damage is a sequel to the inflammatory events that occur in osteoarthritic joints. In osteoarthritis, stem cells are depleted locally, and their proliferation and differentiation capacity appears to be reduced. This suggest the potential value of stem cell delivery into osteoarthritic joints in the hope of reducing the progressive destruction of the joint and possibly facilitating repair. Gene enhancement of MSCs in the joint with cartilage protective growth factors such as IGF-1 and possibly factors which will reduce the inflammatory process within osteoarthritic joints may well further our investigations toward a successful treatment for osteoarthritis in the horse and other species. It has been shown in the rabbit model that the severe inflammatory and proteoglycan destructive effects of intra-articular injections of human recombinant interleukin-1 beta (hrIL-1 beta) was inhibited by the IL-1 receptor antagonist (IL-1ra) also referred to as IRAP. The use of an adenoviral vector for gene delivery was first successful in the laboratory animal model. Equine synoviocytes cotransduced with IGF-1 and/or IL-1ra coding sequences were able to partially or fully reverse the detrimental effects of
IL-1 exposure to cartilage explants via AdIGF-I-transduced cultures or AdIGF-I/AdIL-1Ra-transduced synovial cocultures, respectively. The combination of IGF-1 and IL-1ra has recently been reported in an elegant study using the same vector model in the horse to treat microfractured chondral defects. Iatrogenic defects had filled in after 16 weeks of a controlled rehabilitation regime with levels of proteoglycan and collagen type II significantly above those of the sham treated controls. Therefore, IL-1ra appears to play a role as a component in the treatment of arthritis. Gene therapy via cell transduction with this mediator appears to be a suitable method of delivery. Recombinant IL-1ra proteins are known to have a short half-life of 4 to 6 hours after intravenous administration in humans, requiring molar IL-1ra/Il-1 ratios of 10 to 100 to demonstrate efficacy. The use of viral particles as reported in the previously described study is not suitable for clinical application in animals or humans. The combined application of gene encoded IGF-I and IL-1ra may further benefit from transduction into mesenchymal stromal cells. Beyond their obvious function as cell and tissue replacement, other known advantages such as their cytokine release and anti-inflammatory effects are important. A recent study in sheep using joint destabilization via medial meniscectomy as a model of osteoarthritis showed that MSC-based therapy had a significant effect on articular cartilage structure maintenance and the reduction of subchondral sclerosis.

Most of the research efforts in regenerative tissue repair for musculoskeletal disease in horses thus far have concentrated on cartilage repair and resurfacing due to osteoarthritis and trauma induced arthritis, leading to the substantial yearly losses that occur in the competitive equestrian industries. In contrast to this body of literature, research into stem cell, scaffolding and growth factor or gene-mediated approaches to fracture and subchondral bone cyst repair, arthrodesis, and conditions such as malunion or non-union, which are less well described in
horses compared with humans, dogs and other species has been limited. Fracture repair in the
horse is fraught with many possible complications related to the body weight of these animals
and the susceptibility of laminar tissues in contralateral feet to increased forces during fracture
healing. Bone regeneration has so far been limited to cancellous bone grafts used during the
surgical fracture repair or arthrodesis procedures. MSC implantation into mechanically suitable
scaffolding materials has been investigated in other species for some time. However, due to the
tremendous body weight of the horse, the majority of the literature on fracture repair has focused
mostly on internal or external fixation techniques to allow immediate post-operative recovery
and ambulation on the affected limb. Most scaffolding materials required to fill large fracture
defects are not yet sufficiently strong or have not been tested under the conditions a horse would
impose. However, recent studies in sheep using autologous MSCs seeded onto porous
hydroxyapatite ceramic have been encouraging but will require validation of mechanical
suitability before this material is used in the equine model.264

A recent study using equine MSCs has shown that adenoviral vector transduction of
BMP-2 and BMP-6 resulted in significant upregulation of these two osteogenic factors under
appropriate osteogenic induction.241 The use of a gene-mediated approach showed accelerated
differentiation and ossification of monolayer cultured cells as well as transduced MSCs seeded in
three-dimensional alginate culture systems for both cytokines. BMP-2 transduced cells showed a
very sharp increase (2514-fold) in BMP2 expression during the first 48 hours and subsequently
leveled off after 12 days (20-fold), whereas RNA levels of BMP-6 rose sharply by 2 days (6236-
fold) after induction but only reduced 10-fold over the course of three weeks, suggesting that
there is a time-dependent role for both of these genes in osteogenesis. Equine-specific microarray
analysis showed that a number of other genes, such as those related to inflammation (IL8,
CXCL2), osteogenesis (cartilage oligomeric matrix protein [COMP], procollagen α 1, BMP6 precursor, SMAD6, procollagen α-1 type III precursor (COL3A1), parathyroid hormone-related peptide) and cell differentiation (inhibin β A subunit, keratinocyte growth factor [fgf-7], fibulin-1 [FBLN1], and retinoid acid receptor responder [RARRES1]) were upregulated in response to BMP induction. Genes commonly related to tissue repair, which did not appear to be affected by BMP-2 or -6 were aggrecan core protein, biglycan, decorin, dermantan sulfate proteoglycan, fibronectin, insulin-like growth factor II, SOX-9, collagen type II.241

A number of other growth factors may soon be used for genetic enhancement of mesenchymal stromal cells in the attempt to optimize regenerative tissue repair. Some of the growth factors which have gained increasing attention for their application for equine musculoskeletal diseases are the platelet derived growth factor (PDGF) and TGF-β. These growth factors can be derived from autologous platelet concentrates deposited in injured tissues. These are presumed to benefit the healing process through degranulation and release of PDFG and vascular endothelial growth factor. These factors appear to augment neovascularization, through stimulation of endothelial cells and TGF-β, which is known to stimulate mitosis and differentiation in osteoblastic progenitor cells and subsequently osteoid production.250 Increased concentrations of PDGF and TGF-β have recently been reported in a study, which showed that PRP has anabolic effects on explants of the equine superficial digital flexor tendon (SDFT). This was determined by quantitative RT-PCR of collagen type I (COL1A1), and III (COL3A1) and COMP mRNA levels, and it was also found that PRP does not activate catabolic factors such as MMP-3 and MMP-13.265 Interestingly, acellular bone marrow appears to have even greater effects on COMP and total protein synthesis of equine suspensory fibroblast cultures compared with PRP and equine serum.266 Bone marrow supernatant has mostly anabolic effects but factors
such as age may change the profile of the protein composition in acellular bone marrow as a thus far unidentified protein in bone marrow supernatant has been reported to have inhibitory effects on osteoblast proliferation in aging rats. The promotive effect of platelet-rich plasma derived growth factors on differentiation and tissue regeneration in wound healing involves thrombin activation, platelet degranulation and growth factor release leading to improved epithelial differentiation and improved collagen bundle organization. Platelet-rich plasma enhances bone healing in defects around canine dental implants.

1.4.1 Cartilage Repair

Cartilage degeneration associated with osteoarthritis is one of the major orthopedic concerns in both human and veterinary medicine. Hence, cartilage repair has been investigated for many years with promising advances in scaffold material development as well as growth factor and cell integration. A cartilage repair protocol as reviewed recently by Caplan consists of expanding MSCs collected from bone marrow in monolayer culture before seeding upon fibronectin-coated hydroxyapatite (HA) sponges. The high-molecular weight HA has been found to be chondroinductive and anti-angiogenic. Studies using MSCs in implanted vascular-excluded porous CaP ceramic vehicles have shown that an avascular microenvironment encourages MSCs to form cartilage. Furthermore the oligomers of the degraded HA scaffold trigger the neovascularization of hypertrophic cartilage followed by replacement with vascularized bone and the integration of the neocartilage with the host cartilage.

The use of growth factors such as insulin like growth factor-1 (IGF-I) integrated into a fibrin base to recruit stem cells from within the cartilage and the deeper layers of an equine femoral trochlear ridge cartilage defect model has been examined showing that. Also, chondrocyte and MSC integration into a fibrin base has been investigated and the results have
shown that the fibrin glue itself improved collagen type II concentration (expressed as a percentage of collagen type I) in the repair tissue from 27% to 43% when compared with defects that were not filled with fibrin. However, adding IGF-I to the fibrin clot further improved collagen type II expression to 47%, which was found to be significantly different from just using the fibrin scaffold without the growth factor. However, adding autologous chondrocytes into to the fibrin base yielded the best results showing a collagen type II expression of 62%.276 Adenovirus transduction of IGF-1 into chondrocytes has shown to improve healing during the first 1 to 2 months after injury whereas less benefits were seen after long term-follow-up of the injury.277 Similar results of an early response in cartilage were also seen when MSCs implanted into experimental cartilage defects in equine femoropatellar joints.278 These results suggest that IGF-1 and genetic modification of chondrocytes with IGF-1 as well as MSC implantation have positive effects on cartilage healing. Further work using other combinations of other scaffolds such PRP (platelet rich plasma) and stem cells programmed by growth factors and gene modulations to differentiate into chondrocytes is on the way and has recently been introduced at the annual ACVS Symposium.

1.4.2 Tendon/Ligament Repair

Equine tendon and ligament injuries are the second most important orthopedic condition in equine athletes and hence revenue in the competitive equestrian industry every year. The use of stem cells for tendon repair in horses, however, has been limited to cell replacement therapy since to date stem cells or bone marrow aspirates have merely been injected without an additional matrix. Growth factors such as IGF-I, have been used along with stem cells to enhance collagen type I expression. The use of this growth factor in collagenase-induced lesions has shown that tendons treated with IGF-I expressed increase Cell proliferation and collagen content
and there was overall less soft tissue swelling in the treated tendons as compared with saline treated tendon controls. A separate study examining the temporal expression of IGF-I and transforming growth factor-β1 (TGF-β1), and extracellular matrix proteins collagen type I and III found that TGF-beta1 expression occurred within 1 week of the wound healing process and therefore much earlier than IGF-I, which peaked 4 weeks after the injury and remained elevated until week 8. Messenger RNA levels of both collagen types remained high for 8 weeks after reaching their peak within the first 7 days. The conclusion of these studies was that IGF-I is an important factor in the tendon healing process and that early exogenous IGF-1 administration may benefit tendon healing by elevating low endogenous levels of the growth factor during the first four weeks after the injury.

So far no adequate scaffolds have been added into core lesions of tendons along with stem cells. The only exception is a product with the somewhat misleading name of ACell, Inc. (www.acell.com), which is an injectable product and does not contain any stem cells. It is a lyophilized acellular powder, which is derived from extracellular urinary bladder matrix (UMB) of specific pathogen free (SPF) pigs. ACell has had mixed anecdotal success and after a period of legal disputes about proprietary rights their products are once again commercially available on the market again. The only peer-reviewed paper reports that 84.4% of 77 horses with proximal/body suspensory ligament lesions, 86.7% of 15 horses with suspensory branch lesions and 92.9% of 14 animals with superficial digital flexor tendon were found to be sound and returned to work within 12 months or more after treatment. Ultrasonographic examination of the lesions revealed considerable fluid infiltration at 5 and 30 days post injection but had improved fiber pattern and minimal edema formation by Day 60.
The use of stem cells for equine tendon and ligament strain injuries was established during the mid 1990s in the form of bone marrow transfers from the sternum directly into the tendon lesion. A retrospective study by Herthel\textsuperscript{237} reported an 86\% success rate of horses returning to work after 6 months. This report suffered from a lack of classification of tendon lesions, breed heterogeneity and use of the treated equine population as well as a lack of appropriate follow-up data. The results, however, appeared to provide an impetus in the equine research community and commercial market. Stem cells are now commercially available for horses from two different companies. Some companies (VetStem, Inc., Poway, CA, USA; Evostem Finland Ltd, Kangasala, Finland) isolate autologous stem cells from adipose tissues and VetStem Inc. has more recently also advertised their services for canine tendon injuries commonly observed in the dog racing industry. Other companies (VetCell BioScience Limited, London, United Kingdom) provide autologous stem cell isolates from bone marrow. Since 2002, the American and British stem cell companies report that between 400 to 600 horses have been treated and claim approximately 70\% prognosis for racehorses to return to racing.\textsuperscript{281} However, no controlled studies have so far been undertaken to show the efficacy of these cells to improve tendon healing. A common measure of a successful tendon repair is if a horse is able to race three or more times without reinjury and such data are still lacking.

Surgical implants based on collagen-suture scaffolds seeded with mesenchymal cells have not yet been employed for tendon lacerations exhibiting partial or complete transsection in horses. However, in rodent models these scaffolds have been successfully used. The constructs are designed by aligning a suture material under tension within a collagen scaffold and then seeding the collagen with mesenchymal cells. The transplanted cells will orientate themselves along the suture material and begin to contract the collagen. Such constructs were used in a
rabbit Achilles tendon repair model by Young and coworkers which resulted in significant improvement in morphological, structural, material and mechanical properties compared with simply reapposing the transected tendon with suture material. The inserted constructs improved cellular alignment and elongation as well as improved fiber bundle thickness and crimp parameters of the repaired tendon segment.

Synthesis of the first in vitro engineered tendon based on rat Achilles tendon cells which were first grown to confluence in monolayer culture and later allowed to self-assembly within a cylinder between two anchor points. The resultant scaffold free tissue was composed of aligned small diameter collagen fibrils and a large number of cells as well as an excess of non-collagenous extracellular matrix and showed structural, morphological and also mechanical characteristics similar to that of chick embryo tendons.

The search for a mechanically optimal scaffold or engineered tendon implants is ongoing. Further research is still required to elucidate the tenocyctic potential of MSCs and ASCs, the role of mechanical forces on mesenchymal stem cell differentiation, and the impact of growth factors and other mediators as well as the cell to matrix interaction to optimize the results of scaffold development is required.

1.4.3 Bone Repair

Bone marrow transplants provide an osteoinductive and osteoconductive environment for resident stem cells and have been used in horses to augment repair of bone fractures and joint arthrodesis procedures. While the use of MSCs in bone repair has been extensively investigated in other species, applications of MSCs for the repair of larger equine fractures have been hampered by the lack of suitable scaffolding materials. Such materials require a number of other parameters, the most important of which is that the material is mechanically sensitive to the
site providing sufficient compressive strength to withstand the rigorous biomechanical demands of an adult horse. Furthermore, the material has to be porous in nature to allow or encourage cell attachment and access to bioreactive materials aimed to encourage cellular proliferation and differentiation. Since most scaffolding materials will have different mechanical properties than the host tissue, they will either need to be incorporated into the tissue or facilitate bioabsorption.

Such scaffolding materials does not yet exist which will continue to limit effective bone repair in equine orthopedic surgery. Stem cells and appropriately sized scaffolding materials would be most beneficial in the repair of large bone defects in fractured equine long bones. Fracture repair in horses is subject to tremendous mechanical challenges and possible complications, unlike those that occur in any other species in which osteosynthesis and fracture repair is commonly performed. The horse’s post-operative survival is largely dependent on its ability to bear weight on the repaired leg during and immediately after recovery from surgical correction of a fracture. Therefore, fractures with large bone defects are very unstable and despite internal fixation and coaptive cast stabilization will bear a guarded to poor prognosis.

Bone marrow biopsies harvested intra-operatively from the tuber coxae and less frequently from the sternum or the proximal medial tibia have been used to augment osteogenesis, osteoinduction, osteoconduction and osteopromotion for orthopedic procedures such as fracture repair and arthrodesis techniques. Bone marrow biopsy tissue lacks mechanical stability and has a relatively low number of progenitor cells per unit weight of tissue, which limits the use and versatility of this technique. Their use in cystic lesions such as the common medial femoral subchondral bone cyst in young horses has been met with limited success due to bone marrow necrosis after implantation into the lesion. Therefore, the rate-limiting step in the
tissue engineering for equine fracture repair will be the design of new implant materials with the appropriate mechanical properties.

1.5 **Other Potential Applications of Stem Cells in Equine Veterinary Medicine**

Other potential applications for stem cells in equine veterinary medicine including traumatic and degenerative skeletal condition such as arthrodesis, mal- and non-union during fracture healing, degenerative joint disease (DJD), dermal injuries, wound management and vascular diseases such as equine laminitis and ischemia reperfusion injuries after intestinal insults. While the treatment of intestinal distension-related ischemia reperfusion injuries with stem cells is still only conceptual, another very significant vascular disease of the horse, laminitis, has recently received increasing attention.

1.5.1 **Equine Laminitis and the Equine Metabolic Syndrome**

Acute laminitis is a debilitating, painful, and potentially career-ending and life-threatening disease of the soft tissues (laminae) of the equine digit. At the pathological level, laminitis reflects an induced vascular dysfunction afflicting the vasculature of the hoof, which leads to laminar edema formation, tissue ischemia, activation of additional systemic or local inflammatory mediators, and enzymatic abnormalities. The ultimate result of these processes is laminar tissue injury/necrosis, lameness and eventually mechanical failure with rotation or sinking of the distal phalanx within the hoof capsule. In other words, laminitis destroys the tissues responsible for supporting the horse by suspension of the distal phalanx within the hoof capsule.

Laminitis causes high morbidity and mortality for the horse and leads to economic and emotional costs for the owners. Approximately 15% of horses in the US are afflicted with laminitis in their life, and 75% of these develop lameness and debilitation necessitating
euthanasia. Monetary losses associated with equine laminitis are estimated at $13 million per year associated with diagnosis, treatment and animal loss due to complications. This, combined with an incomplete understanding of the disease, helps explain why 3,500 equine veterinarians responding to the American Association of Equine Practitioners (AAEP)-sponsored survey considered it the most important equine disease and the number one area for future research (http://www.aaep.org/).

The pathogenesis of laminitis is not fully understood; however, there are a number of pathological and experimental observations that link this equine disease to human conditions such as diabetes and Raynaud’s syndrome.\textsuperscript{285-287} Inactive horses often develop visceral obesity and subsequently exhibit a “metabolic syndrome” characterized by insulin resistance, glucose intolerance, hyperlipidemia, and hypertension, a spectrum resembling human type 2 diabetes mellitus. The onset of acute laminitis routinely precedes the diagnosis of the equine metabolic syndrome since the metabolic syndrome often goes unnoticed.\textsuperscript{287} Horses afflicted with laminitis frequently display a peripheral Cushing-like disorder characterized by elevated levels of serum glucocorticoid and increased steroid converting enzyme (11\textbeta-hydroxysteroid dehydrogenase-1) activity within the laminar tissues.\textsuperscript{288-290} In man, clinical investigators have postulated that localized conversion of glucocorticoids by 11\textbeta-hydroxysteroid dehydrogenase-1 in adipose tissue promotes the onset of metabolic syndrome and type 2 diabetes mellitus.\textsuperscript{291,292} Overexpression of this enzyme in adipose tissue of transgenic mice produces all of the physiologic changes expected in metabolic syndrome.\textsuperscript{291} \textit{In vitro} studies have determined that the pathological tissue changes occurring in laminitis result from glucose deprivation and associated matrix metalloproteinase activation.\textsuperscript{293} Likewise, \textit{in vitro} analyses have demonstrated that equine endothelial cell function is inhibited by homocysteine, a risk factor in man for
atherosclerosis and small vessel disease. In summary, equine laminitis represents a small vessel dysfunction directly associated with a human-like “metabolic syndrome”.

Some of the benefits of adult stem cell application is apparent from studies which have shown that MSCs from humans and rodents may readily differentiate into endothelial cells and are capable of expressing potent angiogenic growth factors. Arterial infusion of adult bone marrow-derived stem cell populations has been shown to protect humans and rodents from the necrotic consequences of prolonged vascular ischemia/reperfusion of the hind limb. These findings support the potential value of adult bone marrow stem cells for the treatment of horses suffering from acute ischemic injury associated with laminitis.

A number of animal species are used as surrogate models for small vessel disease in man, including the mouse, rat, dog, and pig. Each of these animals offers certain advantages. Rodents provide inbred, genetically defined animals available in large numbers at relatively low cost. The dog and pig, with their larger body mass, provide a vasculature more closely approximating that of humans. However, large animals such as the horse are rarely used as a preclinical model due to its cost and the lack of suitable research facilities accommodating its use in most major medical centers. Nevertheless, horses suffer from diseases relevant to the human condition and thereby offer a unique opportunity as an experimental model. With its greater size and weight-bearing loads, the demands placed on small vessels in the equine model are expected to meet or exceed those required in man. Thus, this offers the unique possibility to test the safety and efficacy of an adult stem cell therapeutic in a clinical veterinary setting. The outcome of in vivo experimental trials will have direct commercial application in veterinary medicine and will provide a large animal pre-clinical test for future human applications.
1.6 **Conclusions**

Equine stem cell biology is just now beginning to blossom and benefits from all the work that has already been performed in other species. The horse is a unique model as it is the most athletic domestic animal of all that are likely to receive the benefit of technical advances in regenerative tissue repair. Most of the diseases currently under investigation in the horse are of an orthopedic nature, for which cell-based therapy may prove to be useful in augmenting osteosynthesis for equine fractures, arthrodesis procedures and subchondral bone cysts, tendon and ligament repair and cartilage resurfacing. However, the horse’s bodyweight and its unparalleled impressive athletic ability pose the most challenging constraints for tissue engineering and it is postulated that advances in this species will inevitably translate to successful therapeutic results in other species such the human and the dog. Therefore, it is important to strike the delicate balance between the four different constituents of successfully engineering or supporting the repair of new tissues in the injured equine athlete. Current efforts are directed toward establishing the basic understanding of the frequency and growth characteristics of stem cells from varying tissues in culture in order to amplify their numbers required for tissue engineering. Furthermore it is important to demonstrate the in vitro multipotential differentiation capacity of equine stem cells before these cells are then further tested for functionality in the tissues of interest.

The natural environment of stem cells provides a mixture of cell-to-cell and cell-to-matrix cues and these progenitor cells require anabolic factors, an appropriate inductive and conductive matrix as well as mechanical stimulation to facilitate tissue alignment and maturation during the healing phase. However, unlike bone, some other specific tissue such as tendons and ligaments are not capable of providing a sufficient balance of these required factors resulting in
production of inferior scar tissue predisposing to reinjury in 58% of injured racehorses. Isolation of progenitor cell populations from adult horses has so far been unsuccessful. Mesenchymal stromal cells isolated from fetal tissues and young horses are believed to reside in the endotenon adjacent to the vasculature and in-between collagen fascicles, have demonstrated inferior multipotential compared with that recognized in bone marrow-derived MSCs. Therefore, it is likely that tendons and ligaments may obtain their requirement for stem cells after injury from a peripheral blood-derived or adjacent marrow-derived pool of progenitor cells. Current cell-based techniques have been aimed at using supraphysiological numbers of MSCs or ASCs. Prospective studies using MSCs report lower tendon reinjury rates and ASCs also appear to benefit the tissue organization after collagenase induced injury.

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CHAPTER 2.
CELL GROWTH CHARACTERISTICS AND DIFFERENTIATION FREQUENCY OF ADHERENT EQUINE BONE MARROW–DERIVED MESENCHYMAL STROMAL CELLS: ADIPOGENIC AND OSTEOGENIC CAPACITY

With permission from the *American College of Veterinary Surgery*
2.1 Introduction

The postnatal bone marrow contains marrow stromal cells, also known as mesenchymal stromal\textsuperscript{1} or stem cells (MSCs).\textsuperscript{2,3} These cells have the ability to differentiate along multiple lineage pathways \textit{in vitro} as summarized in recent reviews on stem cell characterization and clinical application in human\textsuperscript{2-7} and veterinary medicine\textsuperscript{8}. Since MSC-based therapeutic approaches have been investigated for some time in human medicine and surgery, veterinarians are now considering equine bone marrow-derived MSCs as potential therapeutic agents for musculoskeletal problems such as tendonitis, desmitis\textsuperscript{9}, as well as bone and joint\textsuperscript{10}-related conditions in the equine athlete. Recent \textit{in vivo} studies using a rabbit model have shown that MSCs can contribute to improved biomechanical characteristics following tendon repair.\textsuperscript{11,12} Multiple studies document the need for sufficient numbers of MSCs in order to optimize musculoskeletal tissue repair.\textsuperscript{3,5,8,13,14} It has been calculated that 70 million progenitor cells are required to produce a cubic centimeter of bone.\textsuperscript{15} Cell-based approaches to tendon and ligament injury repair have employed supra-physiologic numbers of MSCs.\textsuperscript{9,16} Awad and coworkers, using a rabbit model, found that greater MSC seeding densities affected cellular morphology and contraction kinetics in collagen scaffolds.\textsuperscript{17} Therefore, in order to generate the requisite number of cells required for tendon and ligament repair, it is necessary to characterize the growth characteristics of equine MSCs.

The majority of the published work documenting MSC differentiation potential, however, has been performed in species\textsuperscript{18} other than the horse, with the exception of the ability of equine MSCs to undergo chondrogenesis.\textsuperscript{19} Likewise, cell quantification and expansion data of bone marrow-derived MSCs have been reported for humans\textsuperscript{20,21}, dogs\textsuperscript{22}, sheep\textsuperscript{23}, rats\textsuperscript{24}, cats\textsuperscript{25} and Rhesus Macaques\textsuperscript{26} but not in the horse.
Therefore, the objectives of this study were to document and quantify the growth and
differentiation characteristics of equine bone marrow derived MSCs using limit dilution assays
for adipocytes, alkaline phosphatase positive cells, fibroblasts, and osteoblasts. This data will
assist future efforts to standardize the isolation, expansion, and transplantation of equine MSCs
in clinical practice.

2.2 Materials and Methods

2.2.1 Materials

All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or
Fisher Scientific International Inc. (Hampton, NH, USA) unless otherwise noted.

2.2.2 Animals and Signalment

Bone marrow (BM) was collected from the sternebrae of 5 horses ranging in age from 9
months to 5 years (mean ± SD: 2.4 ± 1.7 years) and 3 pony foals ranging in age from 17 to 51
days of age. All horses were Thoroughbreds, except for one 5-year-old Quarter horse. The bone
marrow collection procedure was approved by the Institutional Animal Care and Use Committee.

2.2.3 Bone Marrow Aspiration and MSC Isolation

The methods used for bone marrow aspiration27 and MSC isolation16,19 are similar to
other reports. Briefly, young horses were selected from the research herd and after sedation with
detomidine HCl (Domosedan® 0.04 mg/kg IV). The sternebrae were aseptically prepared and
local anesthetic (2% Lidocaine, 3 ml) was infiltrated into the subcutaneous tissue. A 40-ml
volume of bone marrow aspirate was collected using a 10-gauge, 3-inch bone marrow biopsy
needles and heparinized syringes (1,000 IU/ 10ml BM aspirate). Bone marrow was also
harvested from foals by sterile curettage of the sternebrae from fresh cadavers. The marrow
trabecular bone was then crushed and gently stirred before marrow stromal cell (MSC) isolation.
and expansion, which was performed immediately after the bone marrow collection for all animals.

The bone marrow aspirate was diluted in a 1:3 ratio with stromal medium consisting of DMEM-Ham’s F12 medium (vol/vol, 1:1) (HyClone, Logan, UT, USA), supplemented with a 1% antibiotic/antimycotic solution (MP Biomedicals, Irvine, CA, USA) and 10% characterized fetal bovine serum (FBS, HyClone) and layered over Ficoll-Paque® PLUS (Stem Cell Technologies, Vancouver, BC, Canada). The nucleated cells of the BM aspirate were fractionated over a Ficoll density gradient by centrifugation at 1400 rpm for 30 minutes at 4°C. The MSC-enriched cell population above the Ficoll layer was then aspirated and washed in calcium and magnesium-free Dulbecco’s balanced salt solution (CMF-DBSS) by further centrifugation at 1,200 rpm for 5 minutes at 4°C. The washed pellet was resuspended in 10 ml of stromal culture medium. Subsequently, a small aliquot of the cell suspension was centrifuged, the supernatant removed and the pellet was resuspended in an equal volume of a red cell lysis buffer for approximately 20 minutes (Sigma-Aldrich Co., St Louis, MO, USA). This extended exposure period resulted in lysis of both erythrocytes and nucleated cells. Subsequent trypan blue exclusion and hemocytometer counting was employed to determine the total number of nucleated cells per unit volume of bone marrow aspirate.

2.2.4 **Cell Doubling Method**

Primary cells from the subjects were plated at a density of $10^5$ nucleated cells per cm$^2$ in T25 culture flasks (Corning®, NY, USA) using the above described stromal culture medium. The cells were incubated for 6 days before the first medium change, allowing MSC adherence and facilitating removal of the nonadherent hemopoietic cell fraction. Thereafter, the medium was changed every 3–4 days until the adherent cell population reached approximately 80%
confluence. At this point, the adherent primary MSCs were passaged by digestion with 0.05% trypsin, counted with a hemocytometer, and a portion of the cells were re-seeded as “Passage 1” (P1) at 5 x 10^3 MSCs/cm². For the subsequent passages (P1 to P10) cells were inoculated in T25 flasks at 5 x 10^3 MSCs/cm² and allowed to multiply for 3 to 4 days to approximately 70 to 80% confluence before trypsinization and successive passage. Cell doubling times (DT) and numbers (CD) were calculated from hemocytometer counts and cell culture time (CT) for each passage according to the following two formulae:\(^2\):

\[
(1) \quad CD = \frac{\ln(N_f / N_i)}{\ln(2)} \\
(2) \quad DT = \frac{CT}{CD}
\]

(\(DT = \) cell doubling time; \(CT = \) cell culture time; \(CD = \) cell doubling number; \(N_f = \) final number of cells; \(N_i = \) initial number of cells)

All primary cells of the three foals were trypsinized and cryopreserved after they had reached approximately 80% confluence at P0. The cryopreservation medium contained 80% fetal calf serum, 10% DMEM, and 10% dimethyl sulfoxide (DMSO). Cryovials containing the MSCs were placed in a 5100 Cryo 1°C Freezing Container (Wessington Cryogenics, UK) for 24 hours at -80°C before transfer to liquid nitrogen. The MSCs from the three foals, used only for cell doubling experiments, were thawed at room temperature, counted with a hemocytometer to assess viability, and subsequently centrifuged at 1,200 rpm for 5 minutes before resuspension in stromal medium. The cells were then seeded for P1 at 5 x 10^3 cells/cm².

The MSCs isolated from the five adult horses were used only as freshly harvested cells that had not been cryopreserved. Cell cultures from each of the five horses were grown in duplicate for each passage, whereas those of the three foals were only grown as single cultures.
2.2.5 Colony Forming Unit (CFU) Assays

The CFU limit dilution assays were performed according to previously published methods. A portion of the original isolated bone marrow nucleated cell pool from the five adult horses was reserved for limit dilution assays to quantify colony forming units for fibroblasts (CFU-F), and cells capable of differentiation into adipocytes (CFU-Ad) and osteoblasts (CFU-Ob). Further CFU assays were performed at passage 2 (P2) and 4 (P4). Medium exchanges were performed every two to three days. Digital images were taken with an inverted phase contrast microscope at each passage and evaluated for cell morphology, distribution and confluence.

Primary cells were seeded and serially diluted (2-fold) across the 12 columns of four 96-well plates resulting in one plate for each CFU assay (CFU-F, CFU-Ad, CFU-Ob). Due to the anticipated low frequency of primary MSCs in P0, the nucleated bone marrow-derived cell pool was plated at greater per well densities relative to P2 and P4. Hence primary cells densities plated in 100 μL of stromal medium per well in each column were 1 x 10^5, 5 x 10^4, 2.5 x 10^4, 1.25 x 10^4, 6.25 x 10^3, 3.12 x 10^3, 1.56 x 10^3, 7.8 x 10^2, 3.9 x 10^2, 1.95 x 10^2, 9.76 x 10^1 and 4.88 x 10^1, respectively. The cells were fed with an additional 100 μL of stromal medium after a 2 to 3 day growth period and the medium was then changed every third day. The plates were incubated at 37 °C in a 5% CO₂ humidified incubator. Cells were cultured for a total of 9 days to establish colonies, at which time two plates were fixed as outlined below for CFU-F assays while the cells in the two remaining plates were maintained in culture with adipocyte differentiation media or osteogenic media for CFU-Ad or CFU-Ob assays, respectively, as described below. At P2 and P4, the cell seeding densities for the limit dilution assays (in 96-well plates) at these two passages were as follows: 5.0 x 10^3, 2.5 x 10^3, 1.25 x 10^3, 6.25 x 10^2, 3.13 x 10^2, 1.56 x 10^2, 7.8
x 10^1, 3.9 x 10^1, 1.9 x 10^1, 0.98 x 10^1, 0.49 x 10^1, 0.24 x 10^1. Otherwise, the limit dilution assays were conducted as described for the primary cells.

At the conclusion of the studies, the number of wells under each cell density that were negative for the specific marker (based on histochemical staining) was determined. Negative staining was defined as a well that failed to contain at least 20 positive cells. These values were then used to compute the frequency of the CFU progenitors according to the formula \( F = e^{-x} \), where \( F \) is the fraction of colony negative wells, \( e \) is the natural logarithm constant 2.71, and \( x \) is the number of colony forming units per well. Based on a Poisson distribution of a clonal cell lineage, the value of \( F_0 = 0.37 \) occurs when the number of total bone marrow-derived cells plated in a well contains a single colony forming unit.\(^{31}\)

### 2.2.6 CFU-Fibroblast (F) Assay

For CFU-F assays, cells were maintained in stromal medium for a total of 9 days. Following incubation, the cells were rinsed twice with prewarmed PBS. The cells were then fixed for 20 minutes in 10% formalin at room temperature and stored at 4°C until staining. The cells were stained in 0.1% toluidine blue in 1% paraformaldehyde in PBS for 1 hour and subsequently gently rinsed with tap water. Cells were counted using a phase contrast microscope and the MetaVue™ Imaging System (Universal Imaging Corporation, Downingtown, PA, USA) and aggregates of >20 toluidine blue staining cells per well were regarded as a positive CFU-F.

### 2.2.7 CFU-Adipogenesis (Ad) Assay

For CFU-Ad assays, cells were first cultured for 9 days in stromal medium to establish colonies. Thereafter, the cells were exposed to an adipogenic induction medium containing DMEM-Ham’s F12, 3% FBS, 1% antibiotic/antimycotic solution, biotin (33 \( \mu \text{mol/L} \)), pantothenate (17 \( \mu \text{mol/L} \)), 100nM insulin (1 \( \mu \text{mol/L} \)), dexamethasone (1 \( \mu \text{mol/L} \)),
isobutylinmethylxanthine (IBMX) (0.5 mmol/L), rosiglitazone (5 μmol/L) (Avandia™, Glaxo Smith Kline) and 5% rabbit serum (Invitrogen Corporation, Carlsbad, CA, USA) for 3 days. Thereafter, the same medium without the IBMX and the Avandia™ was used to maintain the adipocyte cell culture until day 6 when the cells were fixed for 20 minutes in 10% formalin at room temperature and later stained for neutral lipid accumulation with Oil Red O. The cells were stained for 20 minutes followed by 3 rinses with distilled water. With the use of a phase contrast microscope, cell aggregates were counted and quantified as CFU-Ad positive if the colonies contained >20 Oil Red O positive cells.

2.2.8 CFU-Osteogenesis (Ob) Assay

For CFU-Ob assays, cells were first cultured for 9 days in stromal medium to establish colonies. On day 9, cells were exposed to osteogenic induction medium (DMEM-Ham’s F12, 10% FBS, 1% antibiotic/antimycotic solution, β-glycerophosphate [10 mmol/L], dexamethasone [20 nmol/L] and sodium 2-phosphate ascorbate [50 μg/ml]). Culture in the osteogenic medium was maintained for 5 to 10 days until nodules were detected under phase contrast microscopy. The cells were fed 3 times per week. Upon completion, the cells were rinsed 3 times with 150 mM NaCl and then fixed in 70% ethanol and stored at 4°C. A 2% Alizarin Red solution was prepared in distilled water (pH 4.2) and the cells were stained for 10 minutes at room temperature and thereafter rinsed 5 times with distilled water. Cell aggregates were assessed under phase contrast microscopy for Alizarin Red staining mineralization and nodule formation.

2.2.9 Time Line of Differentiation

Primary cells of all donors were plated in 12-well-plates at $10^5$ cells/cm² and were allowed to incubate for 12 days for time line differentiation experiments and observation assessing osteogenesis and adipogenesis. To assess lineage specific differentiation, digital
images were obtained daily from each of 6 wells containing induced cells (in adipogenic or osteogenic media) and 6 wells containing control cells (in stromal medium). From two donors (an 18-month-old Thoroughbred and a 5-year-old Quarter Horse), cells were induced with adipogenic medium and cultured in four 12-well plates to day 2, 4, 6 and 10 and subsequently fixed and stained with Oil Red O. Cells form the same two donors were also induced with osteogenic medium and cultured in four 12-well plates. Based on our experience with human adipose tissue-derived stem cells (ASCs)\textsuperscript{30}, which require up to 3 weeks for mineralization to occur, it was decided to examine cultures with Alizarin Red staining at day 5, 10, 15 and 20. During the cell culture period, daily digital images were taken to monitor adipogenic and osteogenic changes of the cells. After the cells were stained, the 12-well plates were digitally scanned (Hewlett Packard scanner, USA) and images of the stained cells were taken from each plate.

2.2.10 **Statistics**

The values are reported as the mean and standard deviation. All statistical analyses were performed using the SAS 9.1.2 software package and the Type I error was maintained at 0.05 for all comparisons. The cell doubling data for each passage were compared using Proc GLM and the Tukey’s Studentized Range Test for pairwise multiple comparisons of the means. The same mean comparisons between passages were performed for the limit dilution assay results (CFU-F, CFU-Ad, CFU-Ob) after log transformation of the data. The modeling behavior of the MSC doubling number from passage 0 to 10 was determined by regression analysis using Proc Reg.

2.3 **Results**

2.3.1 **Cell Doubling Data**

Ficoll separation of bone marrow aspirates collected from the adult horses resulted in the
harvest of $6.4 \pm 3.4 \times 10^6$ nucleated cells/ml of aspirate. The cell viability after thawing the liquid nitrogen-stored primary foal MSCs was $64 \pm 6\%$. The cell doubling time for primary cells was calculated at $5 \pm 1.6$ days/cell doubling and is thereby significantly ($P < 0.0001$) longer than the cell doubling time of cells in the subsequent passages (Figure 1-A). There were no significant differences between cell doubling times of all subsequent passages, and an average cell doubling time of $1.4 \pm 0.26$ days/cell doubling was calculated for passages P1-P10. There was also no significant difference between the cell doubling data of each passage between the foals and the population of young adult horses used for this study. By passage 10, the cells had undergone $30 \pm 2.4$ cell doublings (Figure 1-B).

2.3.2 Cell Morphology Observations

The cellular morphology of primary cells included both large, widespread, and occasional multi-nucleated cells as well as spindle-shaped, mononuclear cells. This degree of heterogeneity decreased with subsequent passages as the smaller spindle-shaped fibroblastic cells appeared to predominate (data not shown). Cells grown to confluence grew in multiple layers indicating a lack of contact inhibition. In subsequent passages, the spindle-shaped fibroblastic cells detached more readily during trypsinization, leading to a morphologically homogenous cell population.

2.3.3 Cell and Time Line Differentiation

Cell differentiation of MSCs into adipocytes and osteoblasts (Figure 2-B and 2-C) was observed in all cultures under the appropriate conditions. A pilot study on adipogenic induction and differentiation of equine MSCs showed that the addition of 5% rabbit serum was required for consistent adipogenic results. After adipogenic induction, the cell morphology changed within 24 hours from the elongated confluent fibroblastic cells (Figure 3-A) to more oval-shaped cells, which exhibit a distinct ring of dark coarse granules around the cell periphery (Figure 3-F).
The data were compiled from duplicate cell cultures from young adult horses (n=5) and single cultures from foals (n=3). All values reflect the mean ± standard deviation. Data from passage 0-5 represent n=8 whereas data from passage 6-10 represent n=6. The DT (4.9 ± 1.6) of primary cells was significantly (P= 0.0001) greater than the average DT (1.4 ± 0.22 days/CD) of the subsequent passages. By passage 3 and 10, the equine MSCs had undergone 10.7 ± 1.5 and 30.4 ± 2.4 cell doublings, respectively. * indicates a statistically significant difference (p < 0.0001) in DT (A) between primary cells and that of subsequent passages. The MSC doubling number, after log transformation, behaved in a linear fashion (y = 1.27 + 0.255x, R² = 0.84).
Figure 2 – Colony Forming Unit (CFU) Assays and Staining.

The panels display representative photomicrographs for the following colony forming assays: (A) CFU-fibroblasts (F) detected by Toluidine Blue staining (20 X magnification); (B) CFU-Adipocyte (Ad) detected by Oil Red O staining (40 X magnification); and (C) CFU-Osteoblast (Ob) detected by Alizarin Red staining (10 X magnification). Bars represent a scale of 100 microns.
These granules appeared to develop into fat globules by day 2 (Figure 3-B). It was also noted that within 6 to 10 days after induction of adipogenic differentiation these cells were more likely to detach.

The adipogenesis time line differentiation experiment showed that lipid vacuoles became more numerous and larger with time (Figure 3-C and 3-D). The most notably visible differences occurred between day 0, equivalent to the cellular appearance of the control cells, and day 2 following induction.

During the limit dilution assays, osteogenesis of the equine MSCs typically occurred within 3 to 4 days after induction. Experiments were terminated when nodules had formed at the lowest cell dilutions within the 96-well plates, which on average occurred after 7.4 ± 2.6 days. The osteogenesis time line experiment showed rapid mineralization and nodule formation in all treatment wells and all plates by day 5 (Figure 4-B) for both donors’ cells. While the control wells showed some level of Alizarin Red staining (Figure 4-A), this was minor relative to the wells incubated under osteogenic conditions (Figure 4-B).

2.3.1 Limit Dilution Assays

Limit dilution CFU-F assays (Figure 3-A) determined that an adherent fibroblastic phenotype occurred at a frequency of 1 per 4,224 primary nucleated BM cells (Table 1). With subsequent passage, this population was significantly (P < 0.001) enriched; by passage 2 and 4 approximately 1 per 2.3-2.5 cells displayed an adherent fibroblastic phenotype (Table 1). CFU-Ad and CFU-Ob assays yielded frequencies of 1 per 7,114 adipocytes and 1 per 5,605 osteoblasts per primary nucleated BM cells, respectively. Subsequent passage significantly (P < 0.001) enriched for progenitors of both lineages. At passages 2 and 4, both lineages were detected at frequencies of 1 per 3.6 cells or greater.
Figure 3 – Adipogenesis (Time Line) and Oil Red O Staining.

Equine MSCs were maintained in stromal medium for 9 days until confluent. At this time (day 0), cells were induced with Adipogenic Differentiation Medium and maintained over a 10-day period. Representative photomicrographs (20 X magnification) of Oil Red O stained (top) and unstained (bottom) are displayed for days 0, 2, 4, 6, and 10 following adipogenic induction. Bars represent a scale of 100 microns.
Figure 4 – Osteogenesis and Alizarin Red Staining.

Equine MSCs maintained in Stromal Medium for 9 days were treated with Control (Stromal Medium) (A, C) or Osteogenic Inductive Medium (B, D) for an additional five days. Alizarin Red stained (A, B) and unstained (B, D) photomicrographs at 20X magnification are shown. Insets below the photomicrographs display images of the entire Alizarin Red stained plate under Control (left) and Osteogenic (right) conditions. Bars represent a scale of 100 microns.
Table 1 – MSC Limit Dilution Assays

<table>
<thead>
<tr>
<th>Passage</th>
<th>CFU-F</th>
<th>CFU-Ad</th>
<th>CFU-Ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>1:4224 ± 3265*</td>
<td>1:7114 ± 6657*</td>
<td>1:5605 ± 5266*</td>
</tr>
<tr>
<td>P2</td>
<td>1:2.28 ± 0.35</td>
<td>1:2.45 ± 0.01</td>
<td>1:2.45 ± 0.01</td>
</tr>
<tr>
<td>P4</td>
<td>1:2.54 ± 0.2</td>
<td>1: &lt; 2.44</td>
<td>1:3.63 ± 2.37</td>
</tr>
</tbody>
</table>

Table 1 summarizes the frequencies (original data) of colony forming units with fibroblastic phenotype (CFU-F), adipogenic capacity (CFU-Ad), and osteogenic capacity (CFU-Ob) within the pool of nucleated BM cells at three different passages (P0 [n=5], P2 [n = 5] and P4 [n= 4]). Values are expressed as the mean ± standard deviation. The number of donor animals is indicated in parentheses. * indicates statistically significant (P < 0.001) differences from other passages of the same CFU assay after log transformation of the data (transformed data not shown). The CFU frequencies (> 41%) for adipocytes at passage 4 could not be accurately calculated because of insufficient dilutions.
2.4 Discussion

Bone marrow stromal cells are easily separated from the nonadherent hemopoietic fraction of cells by culture and adherence to plastic dishes as early studies on human MSCs by Friedenstein\textsuperscript{34} and Owen\textsuperscript{35} and their coworkers have shown. The colonies established in monolayer culture are derived from a single bone marrow stromal precursor cell, the CFU-F.\textsuperscript{7,36} The current study evaluated the growth characteristics and colony forming unit frequencies of equine MSCs. The results of this study document that plastic adherent equine bone marrow-derived MSCs have a cell doubling rate of 1.4 ± 0.22 days. MSCs show an initial lag phase of cell proliferation in primary culture, which increases in subsequent passages similar to that documented in other mammalian species. A cell doubling time of approximately 48 hours has been reported for dogs\textsuperscript{22} and Rhesus Macaques\textsuperscript{26}. In humans, the cell doubling time of postnatal MSCs has been reported to be approximately 24 hours.\textsuperscript{21} Surprisingly, there was no significant difference in the cell doubling rate between foals and young horses. It is possible that a greater number of animals in each age group would have increased the likelihood of observing a difference in the cell doubling time between foals and young adult horses; however, it is also plausible that there is no difference in this parameter between donors in these age categories. Alternatively, the in vitro culture conditions employed may optimize cell growth independent of donor age and mask any inherent differences in doubling time. Further work will be necessary to address these questions.

Pittenger and his coworkers reported that MSC frequency in human bone marrow of between 1 per 10\textsuperscript{4} to 1 per 10\textsuperscript{5} nucleated cells.\textsuperscript{18} Donor age is a factor influencing human MSC frequency. Their numbers have been reported to decline in an age-related manner from 1 in 10,000 (newborns) to 1 in 2,000,000 (an 80 year-old person).\textsuperscript{37} All of the equine donors used in the
current study were young animals for which a higher MSC frequency would be expected. Canine\textsuperscript{22} and feline\textsuperscript{25} bone marrow-derived MSC frequency has been reported to be 1 in 2.5 x 10\textsuperscript{4} and 1 in 3.8 x 10\textsuperscript{5}, respectively. Our data show an equine MSC frequency among the total nucleated BM cells of 1 in 4.2 x 10\textsuperscript{3} (based on CFU-F). However, similar to a report on MSC frequency in mice\textsuperscript{38}, considerable variation for primary cell frequencies (up to ten fold) between individual subjects was observed in our study. The current study has employed microtiter limit dilution assays to assess CFU frequency in the bone marrow. The values obtained were within a factor of 2 of those determined by this technique in murine bone marrow, where CFU-F/Ad/Ob frequencies ranged between 1 in 10.8 X 10\textsuperscript{3} to 1 in 3.45 X 10\textsuperscript{4}.\textsuperscript{29} The 1 to 2 orders of magnitude discrepancy in the literature reports of bone marrow-derived equine, human\textsuperscript{18}, canine\textsuperscript{22}, feline\textsuperscript{25} and murine\textsuperscript{38} MSC frequency may be related to differences in the quantitative methods, in addition to the age of the donor animals or some biological features unique to a particular species. Other studies have calculated CFU frequencies by plating at densities between 10\textsuperscript{3} to 10\textsuperscript{5} nucleated cells per cm\textsuperscript{2} and monitoring colony formations by visual inspection after set periods of time.\textsuperscript{25} The combination of low plating density and large culture volume may influence cell survival relative to microtiter conditions.

Following plating and the initial plastic adherence, the low frequency of the primary CFU populations was greatly enriched which was the reason for the reduced seeding density in the P2 and P4 analyses relative to the initial aspirate. These techniques were designed based on our observations using adipose-derived stem cells from human fat in which subcultivation has been found to enrich the presence of stromal cell surface markers such as CD13, CD29, CD44, CD73 and CD90 to > 90\% on ASCs populations compared with < 54\% of the initial primary stromal vascular fraction cells.\textsuperscript{39}
In addition to MSC frequency, the expansion characteristics of MSCs documented as cell doubling time and number during the early passages provide information relevant to tissue engineering applications that will require large cell numbers, especially where scaffold seeding is anticipated. The cell doubling time of 1.4 days/cell doubling approximates that of other species such as humans, dogs\textsuperscript{22} and Rhesus Macaques\textsuperscript{26}. However, it has been documented that the proliferation rate and maintenance of multipotentiality of human MSCs is dependent on the cell plating density in culture.\textsuperscript{40,41} Thus, it is important to standardize the isolation and expansion of MSCs. In our laboratory, a routine plating density of $5 \times 10^3$ cells/cm\textsuperscript{2} was employed based on previous studies using human adipose-derived stem cells.\textsuperscript{39} A recent report\textsuperscript{42} showed that human bone marrow-derived MSCs (passage 3) seeded at 10 cells/cm\textsuperscript{2} expanded approximately 500-fold within 12 days with a maximum cell doubling time of approximately 10 hours. When the same cells were seeded at 1,000 cells/cm\textsuperscript{2}, they expanded only 30-fold, which merely resulted in a 5-fold greater final cell count relative to the low density plating.

Sekiya\textsuperscript{42} and his coworkers also showed significantly reduced absorbance levels for Oil Red O after isopropyl alcohol extraction for passage 3 human MSCs that were seeded at 1,000 cell/cm\textsuperscript{2} compared with those seeded at 50 cells/cm\textsuperscript{2}. Furthermore, the length of pre-incubation (7 vs. 12 days) prior to adipogenic induction also influenced the degree of differentiation\textsuperscript{42}. This illustrates that further work will be required to optimize the seeding density and culture period to maximize stem cell yield.

Established adipogenic differentiation protocols\textsuperscript{43} have lately been further optimized for human bone marrow- and adipose tissue-derived MSCs. These protocols typically contain a mixture of insulin, dexamethazone, 1-methyl-3-isobutylxanthine and indomethacin.\textsuperscript{18,43,44} More recently, this protocol has been modified in our laboratory\textsuperscript{39} replacing indomethacin with rosiglitazone (5
μM), which is a member of the hypoglycemic drugs, thiazolidinediones or glitazones. These agents are used in humans for Type 2 diabetes due to their selective agonistic action on PPAR gamma nuclear receptors and thereby increasing insulin sensitivity. The same substitution was also adopted for the equine MSC cultures. Initial work using the same protocol as commonly used in our laboratory for human adipose-derived stem cells resulted in limited adipogenesis of the equine MSCs. Previous reports found that rabbit serum enhanced adipogenesis in vitro for human, rat and mouse MSCs. Janderova et al showed that by using 15% rabbit serum instead of fetal bovine serum approximately 90% of all human MSCs were Oil Red O positive within 6 days after adipogenic induction. Our studies demonstrated that the addition of 5% rabbit serum significantly improves adipogenesis in the equine MSCs in a comparable manner.

In conclusion, the current work documents the frequency and growth characteristics of multipotent equine bone marrow-derived MSCs from foals and young adult horses. Based on the CFU-F frequency of 1 in 4,224 (0.026%) within the nucleated cell pool, and an average yield of 64 million nucleated cells per 10 ml of bone marrow aspirate, it is feasible to obtain 1.664 X 10⁴ primary MSCs. Over a 23-day culture period equivalent to 14 cell doublings, a 16,384-fold (2¹⁴) cell expansion would yield approximately 250 million progenitor cells per 10 ml of bone marrow aspirate. Limited data are currently available concerning the number of cells required for the repair of tendon defects and strain injuries. However, efforts to repair skeletal defects in canine models have shown that 15 million cells/ml of implant volume were sufficient to obtain significantly improved bone production and regeneration. These calculations are supported by a study by Muschler and Midura who introduced a mathematical model, which calculated that 70 million osteoblasts would be required to produce a cubic centimeter of bone. Further work will be required in the equine model to reduce these basic science discoveries to clinical practice.
2.5 References


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CHAPTER 3.
CHARACTERIZATION OF EQUINE ADIPOSE TISSUE–DERIVED STROMAL CELLS: ADIPOGENIC AND OSTEOGENIC CAPACITY AND COMPARISON TO BONE MARROW-_DERIVED MESENCHYMAL STROMAL CELLS

With permission from the American College of Veterinary Surgery
3.1 **Introduction**

Adult adipose tissue is a source of fibroblast-like precursor cells\(^{334-337}\) capable of multipotential differentiation in a number of species. In the human literature these cells have gained importance because they are a readily accessible in large quantities. Recent investigations have focused on their application in the emerging field of regenerative medicine and surgery. Whereas the nomenclature of these cells varies widely,\(^{14}\) the consensus reached at the Second Annual International Fat Applied Technology Society meeting (October 3-5, 2004, Pittsburgh, PA) was to use the acronym ‘ASC’ for adipose derived stromal cells\(^{23}\) or adipose derived stem cells.\(^{24}\)

The academic and commercial veterinary communities have paid increased attention to stromal cells, harvested from adipose tissue (ASC) or bone marrow (MSC), as efficacious treatment options for the debilitating and career-ending musculoskeletal injuries and diseases of the equine athlete. The human and laboratory animal literature spanning 3 decades provides substantial characterization of these cells and their potential application to human diseases. In equine veterinary medicine, however, knowledge of equine stromal cells is limited and they are currently used with the implicit assumption that their basic characteristics, such as cell doubling time and differentiation potential, are similar to those of other species. We have recently reported on the frequency and cell doubling characteristics for equine bone marrow-derived MSCs.\(^{338}\) Similar to other species, equine MSCs rapidly expanded in monolayer culture to quantities sufficient for tissue engineering purposes and they exhibited an initial lag phase in culture.\(^{338}\) Expansion rates of primary cultured murine adipose tissue-derived ASCs have been reported to be up to 5-fold faster within the first 5 - 7 days compared with primary murine MSCs.\(^{339}\)
MSCs have stabilized in culture, their cell doubling time appears to be shorter than that of adipose tissue-derived ASCs suggesting that differences exist between these 2 cell types.

Autologous ASCs are relatively quickly isolated from adipose tissue by collagenase digestion. The resulting stromal vascular cell fraction (SVF) contains a greater proportion of stromal/stem cells per unit volume relative to bone marrow. Cell yields from adipose tissue may be influenced by age and the harvested tissue site. Because lesion size in injured tissues, such as tendon or bone, may require large amounts of cells for therapeutic efficacy, it is advantageous to know the cell doubling times for equine ASCs to optimize therapeutic cell harvest and expansion. Whereas the multipotentiality and proliferative rates of equine mesenchymal stromal cells derived from equine bone marrow and peripheral blood is documented, there is limited literature regarding these characteristics for equine adipose-derived mesenchymal cells. Our goal was to document the cell doubling characteristics of equine ASCs and their adipogenic and osteogenic differentiation potential.

3.2  Materials and Methods

Subcutaneous adipose tissue was collected from the region above the dorsal gluteal muscles of 5 horses (aged, 9 months to 5 years; mean ± SD, 2.4 ± 1.7 years). There were 4 Thoroughbred geldings (one 9- and two 18-months-olds and one 3-year-old) and a 5-year-old Quarter horse gelding. All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific International Inc (Hampton, NH) unless otherwise noted.

3.2.1  Adipose Tissue Harvest

Horses were sedated with detomidine HCl (0.04 mg/kg intravenously [IV]) and butorphanol (0.01 mg/kg IV), the area over the dorsal gluteal muscles was aseptically prepared, and skin and subcutaneous tissues were desensitized by local infiltration of 2% lidocaine using
an inverted L-block. A 10–15 cm incision was made parallel and ~15 cm abaxial to the vertebral column. Approximately 15 mL of adipose tissue were harvested over the superficial gluteal fascia for immediate ASC isolation and the skin incision apposed with nylon suture material.

3.2.2 ASC Isolation Method

The ASC isolation procedure was based on the technique previously reported for human ASC isolation. Adipose tissue was minced with a surgical blade, washed and briefly agitated with an equal volume of phosphate buffered saline (PBS) solution to promote separation into 2 phases. The upper phase consisted of the minced and washed adipose tissue and the liquid infranatant containing hemopoietic cells suspended in PBS was removed. The tissue was then digested in an equal volume of a filtered (0.2 μm) PBS solution containing 1% bovine serum albumin (BSA Type V; Sigma-Aldrich) and 0.1% of collagenase (Type I; Worthington Biochemical, Lakewood, NJ) with continuously shaking at 37°C for ~50 minutes. Subsequently, the sample was centrifuged at 260 g for 5 minutes. To complete stromal cell separation from primary adipocytes the sample was briefly and vigorously agitated again and then centrifuged at 260 g for 5 minutes resulting in a stromal-vascular fraction (SFV) pellet, which contains the nucleated cell portion of the adipose tissue harvest and therefore the ASCs.

A supernatant composed of oil, primary adipocytes and collagenase solution was removed after centrifugation and the SVF was resuspended in 10 mL stromal medium. The SVF is the remaining nucleated cell fraction of adipose tissue which contains the mesenchymal stromal cells. A small aliquot of the cell suspension was centrifuged and the pellet was resuspended in an equal volume of a red cell lysing buffer (Sigma-Aldrich) for ~20 minutes. This extended exposure period resulted in the lysis of both erythrocytes and nucleated cells.
facilitating nuclear trypan blue staining and therefore hemocytometer assessment of total nucleated cell numbers per unit volume of adipose tissue.

3.2.3 **Cell Doubling Method**

The cell doubling method and calculations described previously were used. Fresh primary nucleated cells from the SVF from each of the 5 horses were cultured in duplicate for each passage. Cells were plated in stromal culture medium at a density of $5 \times 10^3$ nucleated cells/cm$^2$ in T25 culture flasks (Corning®, Corning, NY). The adherent cell layer was rinsed 2 days later with warm PBS to remove any contaminating blood cells and the stromal medium was changed every 3 days until the adherent cell population reached ~80% confluence. Adherent primary ASCs were then passaged by digestion with 0.05% trypsin, and counted. Subsequent passages (P1 - P10) cells were inoculated in T25 flasks at $5 \times 10^3$ ASCs/cm$^2$ and allowed to multiply for 4 - 5 days to ~70-80% confluence before trypsinization and successive passage. Cell doubling times (DT) and numbers (CD) were calculated from hemocytometer counts and cell culture time (CT) for each passage according to the following 2 formulae:

\[
\begin{align*}
(1) \quad CD &= \ln(N_f / N_i) / \ln(2) \\
(2) \quad DT &= CT / CD
\end{align*}
\]

(DT = cell doubling time; CT = cell culture time; CD = cell doubling number; $N_f$ = final number of cells; $N_i$ = initial number of cells)

3.2.4 **Colony Forming Unit (CFU) Assays**

CFU limit dilution assays were performed according to previous methods. A portion of the original isolated SVF cell pool from the 5 horses was reserved for limit dilution assays to quantify colony forming units for fibroblasts (CFU-F), alkaline phosphatase expressing cells (CFU-ALP), and cells capable of adipogenic (CFU-Ad) and osteogenic (CFU-Ob)
differentiation. The same CFU assays were performed at passage 2 (P2) and 4 (P4). Medium exchanges were performed every 2-3 days. Digital images were taken with an inverted phase contrast microscope (Nikon TS100F, Nikon Instruments Inc., Lewisville TX) and CoolSpot CCD Camera (Nikon Instruments Inc.) at each passage and subjectively evaluated for cell morphology, distribution, and confluence.

Primary cells of the SVF, also referred to as passage 0 (P0), as well as cells of P2 and P4 were plated at 2-fold serial dilutions (5.0 x 10^3, 2.5 x 10^3, 1.25 x 10^3, 6.25 x 10^2, 3.13 x 10^2, 1.56 x 10^2, 7.8 x 10^1, 3.9 x 10^1, 1.9 x 10^1, 0.98 x 10^1, 0.49 x10^1, 0.24 x 10^1) across the 12 columns of 96-well plates suspended in 100 μL of stromal medium/well. In total 1 plate for each CFU assay (CFU-F, CFU-ALP, CFU-Ad, CFU-Ob) per passage (P0, P2, P4) per animal were seeded with cells. The plates were incubated at 37°C in a 5% CO₂ humidified incubator and the medium was changed every 2-3 days. Cells were cultured for 9 days to establish colonies, at which time the plates were fixed as outlined below for CFU-F and CFU-ALP assays, while the cells in the 2 remaining plates were maintained in culture and induced with adipocyte differentiation media or osteogenic media for CFU-Ad or CFU-Ob assays, respectively.

At the conclusion of the studies, the number of wells under each cell density that were negative for the specific marker (based on histochemical staining) was determined. Negative staining was defined as a well that failed to contain at least 20 positive cells (CFU-F, CFU-Ad) or one bone nodule (CFU-Ob). These values were then used to compute the frequency of the CFU progenitors according to the formula F = e^{-x}, where F is the fraction of colony negative wells, e is the natural logarithm constant 2.71, and x is the number of colony forming units per well. Based on a Poisson distribution of a clonal cell lineage, the value of F_0 = 0.37 occurs when
the number of total adipose tissue-derived cells plated in a well contains a single colony forming unit.\textsuperscript{321}

All cells used for the CFU assays were initially cultured in stromal medium for a total of 9 days. The cells assigned to the CFU-F and CFU-ALP assays were fixed and stained on day 9 and those in CFU-Ad and CFU-Ob cultures were induced at that time into the intended cell line as described below.

3.2.5 **CFU-Fibroblast (F) Assay**

Cells were rinsed twice with prewarmed PBS, fixed for 20 minutes in 10% formalin at room temperature and stored at 4°C until staining. The cells were stained in 0.1% toluidine blue in 1% paraformaldehyde in PBS for 1 hour and subsequently gently rinsed with tap water. Cells were counted using a phase contrast microscope and the MetaVue™ Imaging System (Universal Imaging Corporation, Downingtown, PA) and aggregates of >20 toluidine blue staining cells per well were considered a positive CFU-F.

3.2.6 **CFU-Alkaline Phosphatase (ALP) Assay**

Cells were rinsed twice with prewarmed PBS, fixed in 100% ethanol and stored at 4°C until staining. Thereafter, the cells were incubated for 1 hour in the presence of a solution containing 5-Bromo-4-chloro-3-indolyl phosphate dipotassium/nitrotetrazolium blue chloride (BCIP/NBT), which stains ALP positive cells purple. The plates were rinsed with water and cell aggregates were quantified with phase contrast microscopy and regarded as CFU-ALP positive if >20 cells stained for ALP.

3.2.7 **CFU-Adipogenesis (Ad) Assay**

Cells were exposed to an adipogenic induction medium containing DMEM-Ham’s F12, 3% FBS, 1% antibiotic/antimycotic solution, biotin (33 \(\mu\)mol/L), pantothenate (17 \(\mu\)mol/L),
100nM insulin (1 μmol/L), dexamethasone (1 μmol/L), isobutylmethylxanthine (IBMX) (0.5 mmol/L), rosiglitazone (5 μmol/L) (Avandia™, Glaxo Smith Kline, Cidra, Puerto Rico) and 5% rabbit serum (Invitrogen Corporation, Carlsbad, CA) for 3 days. Thereafter, the same medium without the IBMX and the Avandia™ was used to maintain the adipocyte cell culture until day 6 post-induction when the cells were fixed for 20 minutes in 10% formalin at room temperature and later stained for neutral lipid accumulation with Oil Red O. The cells were stained for 20 minutes followed by 3 rinses with distilled water. Phase contrast microscopy was used to count cell aggregates quantified as CFU-Ad positive if the colonies contained >20 Oil Red O positive cells.

3.2.8 **CFU-Osteogenesis (Ob) Assay**

Cells were exposed to osteogenic induction medium (DMEM-Ham’s F12, 10% FBS, 1% antibiotic/antimycotic solution, β-glycerophosphate [10 mmol/L], dexamethasone [20 nmol/L] and sodium 2-phosphate ascorbate [50 μg/ml]). Culture in the osteogenic medium was maintained for 5-10 days until nodules were detected with phase contrast microscopy and the culture medium was changed every 3 days. Upon completion, the cells were rinsed 3 times with 150 mM NaCl and then fixed in 70% ethanol and stored at 4°C. A 2% Alizarin Red solution was prepared in distilled water (pH 4.2) and the cells were stained for 10 minutes at room temperature and thereafter rinsed 5 times with distilled water. Cell aggregates were assessed with phase contrast microscopy for Alizarin Red staining mineralization and nodule formation.

3.2.9 **Time Line of Differentiation**

Primary cells of all donors were plated in 12-well-plates at 10⁵ cells/cm² and were allowed to incubate for 12 days for time line differentiation experiments and observation assessing osteogenesis and adipogenesis. To assess lineage specific differentiation, digital
images were obtained daily from each of 6 wells containing induced cells (in adipogenic or osteogenic media) and 6 control wells containing cells, which were maintained in stromal medium in the absence of inductive factors. For the adipogenic timeline differentiation experiment cells from 2 of 5 horses (18-month-old Thoroughbred, 5-year-old Quarter Horse) were plated in 12-well plates, induced with adipogenic medium and cultured to day 2, 4, 6 or 10 and subsequently fixed and stained with Oil Red O. Similarly, cells from the same two donors were prepared for osteogenesis timeline experiments and induced and cultured in osteogenic medium. Based on our experience with human adipose tissue-derived stem cells\textsuperscript{14}, which require up to 3 weeks for mineralization to occur, it was decided to examine the osteogenic cultures with Alizarin Red staining at day 5, 10, 15 and 20. During the cell culture period, daily digital images were taken to monitor adipogenic and osteogenic changes of the cells. After the cells were stained, the 12-well plates were digitally scanned (CanonScan8400F, Canon, Lake Success, NY) and images of the stained cells were taken from each plate.

3.2.10 Statistical Analysis

Cell culture data for ASCs were compared where appropriate to those recently reported on equine MSCs\textsuperscript{338}, which were harvested at the same time from the same horses. Values are reported as mean ± SD. All statistical analyses were performed using the SAS 9.1.2 software package (SAS Institute Inc., Cary, NC) and the Type I error was maintained at .05 for all comparisons.

Cell doubling data for each passage were compared using PROC GLM and the Scheffe’s test for pairwise multiple comparisons of the means. The modeling behavior of the MSC doubling number from passage 0 - 10 was determined by regression analysis using the PROC REG procedure. PROC GLM and Tukey’s Studentized Range Test was used for mean
comparisons of the limit dilution assay results (CFU-F, CFU-ALP, CFU-Ad, CFU-Ob) after log transformation of the data.

3.3 **Results**

The stromal vascular fraction provided a pool of primary nucleated cells (Fig 5A). The adherent cells presented in a mononuclear spindle-shaped form and appeared numerous and loosely distributed across the plates within 24 hours after plating.

3.3.1 **Cell Doubling Data**

The adipose tissue harvest yielded $3.0 \pm 1.4 \times 10^5$ nucleated cells/ml of tissue ($n = 5$). The cell doubling times across 10 passages (Fig 6A) had no significant differences between all passages and the overall mean DT across 10 passages was $2.1 \pm 0.9$ days/CD. By P10 the cells had undergone $28 \pm 2$ CDs (Fig 6B).

3.3.2 **Adipogenic and Osteogenic Differentiation and Time Line Data**

During 10 days in culture, equine ASCs had robust cell differentiation into adipocytes (Fig 7A to D) compared with non-induced cells (Fig 7E to H) in the control wells. Oil-Red-O staining of the control cells was negative indicating that there was no visible spontaneous adipogenesis. Successful adipogenesis required the addition of 5% rabbit serum as previously reported for equine MSCs. The cell morphology of the induced ASCs changed within 24 hours, displaying a distinct ring of dark coarse granules around the cell periphery, which developed into fat globules by day 2 after induction (Fig 5 Image C). Equine ASCs also had consistent mineralization and alizarin red positive calcium phosphate nodule formation, which occurred on average within $12 \pm 3.8$ days after induction of the ASCs.

3.3.3 **Alkaline Phosphatase Expression**

Alkaline phosphatase staining and expression in ASCs was compared with that in MSCs.
Figure 5 – Primary ASCs and Early Adipogenesis

Image A shows a population of primary ASCs (x 20 magnification) after 3 days in culture at ~70% confluence. Image B shows a confluent control cell population at Day 10 compared with an equally long cultured population of primary ASCs (Image C) 24 hours after induction of adipogenesis. Note the rapid changes in cellular morphology and arrangement of dark granules around the periphery of the preadipocytes, which were found to be oil droplets staining consistently with Oil-Red-O after 48 hours as seen in Panel III.
Figure 6 – Cell Doubling Time and Number of Primary and Passaged ASCs

The data were compiled from duplicate cell cultures from young horses (n=5). All values reflect the arithmetic mean ± standard deviation. Data from passage 0 – 7 represent n =5 whereas data from passage 8 – 10 represent n = 3. The overall data distribution was best described with a quadratic model (y = 1.73 + 0.369*X - 0.046*X²). The overall DT of primary and subcultured cells was 2.1 ± 0.9 days/cell doubling. No significant differences in DT were observed between individual passages. By passage 10 the equine ASCs had undergone 28 ± 2 cell doublings. The original CD data behaved in a linear fashion (y = 2.24 + 2.4*X, R² = 0.95).
Equine mesenchymal stem cells (ASCs) were maintained in stromal medium for 9 days until confluent. At this time (day 0), cells were induced with adipogenic differentiation medium and maintained over a 10-day period. Representative photomicrographs (x 20 magnification) of Oil Red O stained (top row) and control cells (bottom row) are displayed for days 2, 4, 6, and 10 after adipogenic induction. Bars in all photomicrographs represent a scale of 100 μ.
ALP expression was remarkably greater in undifferentiated cells of all primary MSC cultures and those of all subsequent passages (P2 and P4) compared with ASC cultures (Fig 8). ALP staining was visible with the naked eye in all CFU-ALP assays on MSCs across all passages compared with those conducted for ASCs where staining was present but detectable only with microscopy.

3.3.5 Limit Dilution Assays

The adherent fibroblastic cell frequency in the stromal vascular fraction was 1 in 2.3 ± 0.4 primary nucleated cells as determined by the limit dilution CFU-F assays (Fig 9A). At P2 and P4, the frequency of cells with the fibroblastic phenotype did not change significantly. CFU-Ad and CFU-Ob assays for ASCs (Table 2) determined frequencies within the primary nucleated stromal vascular fraction cell population of 1 adipocyte progenitor per 4.9 ± 5.4 nucleated cells and 1 osteoblast progenitor in <2.5 ± 0.01 nucleated cells, respectively. Even the lowest dilution of primary nucleated cells seeded at 2.4 cells/well was not sufficient to reach the limit dilution necessary to calculate the CFU-Ob frequency at P0, as cell expansion and osteogenic induction still resulted in nodule formation. Subsequent passaging did not significantly enrich for progenitors of either the adipogenic or osteogenic lineage. In the stromal vascular fraction, CFU-ALP assays revealed a frequency of 1 in 3.64 ± 1.29 primary nucleated cells, whereas subsequent passages had significantly lower frequencies (P2: 1 in 97.3 ± 74.95; P4: 1 in 114.86 ± 132.61; Table 2). CFU-ALP and CFU-Ob did show significant differences at P2 and P4. ALP expression in bone marrow MSC cultures had frequencies for P0 of 1 in 15,035 ± 11,981, which was significantly lower when compared with P2 (1 in 4.53 ± 4.91) and P4 (1 in < 2.44), consistent with the frequency of CFU-F at the corresponding passages.338
Figure 8 – Alkaline Phosphatase Expression

The photomicrographs (x 10 magnification) show undifferentiated equine MSCs (Image A) and ASCs (Image B), which were maintained in stromal medium for 9 days before staining for alkaline phosphatase with BCIP/NBT. MSCs showed consistently more substantial and visible ALP staining in all limit dilution assays compared with ASCs where ALP staining could only be appreciated under microscopy.
Figure 9 – Colony Forming Units and Assays

The panel displays representative photomicrographs for the following colony-forming assays: (A) CFU-fibroblasts (F) detected by toluidine blue staining (x 20 magnification); (B) CFU-Adipocyte (Ad) detected by Oil-Red-O staining (x 20 magnification); and (C) osteoblastic [CFU-Osteoblast (Ob)] differentiation and mineralization of the extracellular matrix detected by Alizarin Red staining (x 10 magnification).
Table 2 – ASC Limit Dilution Assays

<table>
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<th>Passage</th>
<th>CFU-F</th>
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<th>CFU-Ob</th>
<th>CFU-ALP</th>
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<td>1:2.3 ± 0.4</td>
<td>1:4.9 ± 5.4</td>
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<td>1:3.64* ± 1.29</td>
<td>1:15,035† ± 11,981</td>
</tr>
<tr>
<td>P2</td>
<td>1:6.36 ± 3.76</td>
<td>1:6.52 ± 5.2</td>
<td>1:3.73 ± 1.48</td>
<td>1:97.3§ ± 74.95</td>
<td>1:4.53 ± 4.91</td>
</tr>
<tr>
<td>P4</td>
<td>1:2.54 ± 0.2</td>
<td>1: 4.32 ± 2.46</td>
<td>1:4.83 ± 5.34</td>
<td>1:114.86§ ± 132.61</td>
<td>1: &lt;2.44</td>
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</table>

*Indicates statistically significant \( P < .001 \) differences from other passages of the same CFU assay after log transformation of the data (transformed data not shown). §Statistically significant differences \( P < .001 \) were found for ASC cultures between CFU-ALP and the other assays within the same passage. CFU-ALP data for MSC cultures, which have been seeded and cultured as previously described\(^338\), are reported in this table. †Statistically significant differences \( P < .001 \) for MSC CFU-ALP assays were detected between P0 and the subsequent passages (P2 and P4). Insufficient dilutions prevented CFU frequencies calculations for osteoblastic lineage derived from ASCs at P0 and ALP expression of undifferentiated MSC at P4.

\( ^a \)Adipose tissue-derived stromal cells (ASC), \(^b\)Bone marrow-derived stromal cells (MSC)
3.4 Discussion

We designed this study to determine cell doubling times of equine adipose-derived ASCs and to establish that these cells retain their bipotentiality with subsequent passaging. This dataset was directly compared with equine MSCs because the 2 cell types were harvested and isolated in parallel studies from the same donor horses and cultured under the same conditions.

The cell morphology of SVF cells in primary cultures appeared heterogeneous in nature, yet compared with primary nucleated cell populations isolated from bone marrow SVF cells did not form distinct colonies but displayed a more uniform distribution upon adhering to the culture dish. Considerably fewer cells with a non-fibroblastic morphology were evident in the primary ASC cultures as compared with MSC primary cultures. The fibroblastic phenotype of ASCs was very similar to that of MSCs. ASCs did not display a lag period in their initial expansion rates as had been observed with MSCs.

Cell quantification of primary SVF nucleated cells in a population of middle-aged humans with mean body mass indexes of ~24-26 are reported to vary from ~300,000 - 404,000 cells/mL of lipoaspirate, values comparable to the present equine results. Consistent tissue collection and laboratory techniques are pivotal for maximizing cell yields as the number of adherent human SVF cell was found to vary significantly depending on the collagenase digestion times, sampling size, and sampling location. Our equine data showed variability in cell numbers ranging from 140,000 - 538,000 cells/mL of adipose tissue, which may also reflect donor-dependent differences. The reason for such individual variability of ASCs is yet unknown but it is possible that age or body mass may affect stromal cell numbers in adipose tissue. Age has been reported to inversely correlate with human marrow-derived MSC yields, yet there are conflicting reports in the literature concerning human ASC yields and age.
One study failed to demonstrate a correlation between human ASCs yields and age\textsuperscript{342}. In contrast, others observed that human stromal vascular cells showed a very significant negative correlation between glycerol-3-phosphate dehydrogenase expression and age,\textsuperscript{344} consistent with the conclusion that aging may diminish the number of cells capable of adipogenic differentiation. The small population size of the horses we studied was insufficient to provide insight into the role of age and weight as correlative variables in determining equine nucleated SVF cell yields. Cell doubling time of ~2 days for equine ASCs was more rapid than the ~4 days required by human ASCs\textsuperscript{14} cultured under similar conditions in our laboratory but was comparable to that of a recently established adult murine preadipocyte cell line (2 - 2.5 days).\textsuperscript{345} This suggests that there is some variability in ASC DT among species. This interspecies variation may be the result of different culture conditions and medium composition.

The harvest and expansion of both ASC and MSC cells have advantages and disadvantages. The harvest of MSCs is cosmetically more acceptable and the initial isolation process is less involved compared with that for ASCs. However, the sternal bone marrow collection technique commonly used with standing, sedated horses exposes them to potential complications like pneumothorax and pneumopericardium.\textsuperscript{346,347} In addition, it places the investigator in a precarious and vulnerable position. Whereas ASCs expand at a slightly slower rate compared with MSCs, the significantly greater number of available primary cells makes the ASC an interesting prospect for achieving the large cell numbers deemed necessary for tissue engineering applications.\textsuperscript{314} Our data imply that for a 10 mL adipose tissue sample with a yield of 300,000 nucleated SVF cells/mL of adipose tissue, the immediate available ASC number at a frequency of 43\% would be 1.3 x 10^6/mL. After 21 days in culture, the total ASC number would be 1.8 x 10^9 cells, which is significantly greater than comparable calculations for MSCs.\textsuperscript{338}
ASC frequency in our study (43%) is ~10-fold higher than reported for humans. It has been demonstrated that cell seeding density affects cell doubling rates in human MSCs and it is therefore likely that this adipose-derived stromal cell type will behave similarly under different seeding density conditions. Consequently, there is a need for optimization of seeding density and culture period to maximize equine ASC yield.

Cell seeding density, culture media, and additives such as thiazolidinediones and the rabbit serum are likely to influence the rate of adipose conversion in equine ASCs. In addition, there may be individual-dependent variation in the degree of adipogenic differentiation as it has been previously reported for human ASCs from different donors. Similar to our previous report on MSCs, the addition of rabbit serum was required to achieve any reliable adipogenesis and fat droplet accumulation. Under the same conditions in the CFU assays, adipogenesis was consistently more robust in ASC cultures compared with MSC cultures.

Differences between these 2 cell types were also noted in the time required for osteogenic differentiation. Time from osteogenic induction based on alizarin red positive nodule formation seen in the most diluted wells of the limit dilution assays was significantly longer for equine ASCs (12 ± 3.8 days) compared with their MSC relatives (7.4 ± 2.6 days); however, equine ASCs still appeared to produce bone nodules more rapidly than human ASCs, which required ~3 weeks. These results suggest that equine MSCs appear to have a greater osteogenic potential which would be consistent with the differences seen in ALP expression between undifferentiated ASCs and MSCs (Fig 1 Panel II). In contrast to our data, De Ugarte and coworkers could not find any significant differences in osteogenic differentiation capacity between human mesenchymal cells derived from adipose or marrow tissue. However, more recently differences in the osteogenic potential of human MSCs and ASCs have been reported by Im et al who
showed that ASCs expressed significantly less ALP and matrix mineralization during osteogenesis overall as compared with MSCs. It is interesting that even undifferentiated mesenchymal cells from marrow and adipose tissue show such profound differences in ALP expression. More detailed molecular work will be required to investigate the apparent reduction in ALP expression of undifferentiated ASCs during subsequent passaging and how that may affect their osteogenic potential. The CFU-Ob cultures of ACSs appeared to show the same frequencies of nodule formation, although matrix mineralization required more time. Future in vivo studies testing osteogenic applications will be required to determine the comparative efficacy of these 2 cell types, as ASCs may be less robust for such applications or require growth factor supplements such as bone morphogenic proteins to achieve the MSC potential, as has recently been described for the chondrogenic capacity of human ASCs.

Further quantitative analysis will be required to demonstrate differences in osteogenic and adipogenic marker expression between ASCs and MSCs. Studies in the human and murine literature suggest that there is an inverse relationship between osteoblastogenesis and adipogenesis. Transcriptional processes such as those mediated by the peroxisome proliferator activated receptor gamma have been implicated. Ligands of this receptor are known to activate adipogenesis and promote inhibition of osteogenesis by down-regulation of the osteoblast transcriptional regulatory protein Cbfa-1. Future investigations may lead to a more intricate knowledge of osteogenic disturbances related to delayed or non-union complications in equine bone healing and possibly to pharmacological and/or cell-based therapeutic interventions for such skeletal healing defects.

We concluded that equine adipose tissue-derived ASCs are capable of adipogenic and osteogenic differentiation and display expansion characteristics similar to those reported in the
murine literature. Equally important are the differences noted between the 2 equine stromal cell types, the ASC and MSC, for their adipogenic and osteogenic capacity and their pre-differentiation ALP expression. These findings have potential relevance to future equine veterinary tissue engineering and regenerative medical therapies.

3.5 References


31. Im GI, Shin YW, Lee KB: Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis Cartilage 13:845-853, 2005


CHAPTER 4.
COMPARISON OF CHONDROGENIC POTENTIAL IN EQUINE MESENCHYMAL
STROMAL CELLS DERIVED FROM ADIPOSE TISSUE AND BONE MARROW
4.1 Introduction

The two most common types of adult equine stem cells currently used for regenerative tissue repair are those derived from bone marrow (mesenchymal stem cells, MSC) and adipose tissue (adipose-derived stem cells, ASC). Both cell types are readily accessible in the horse for isolation, enrichment, and expansion. The multipotentiality of both cell types with respect to adipogenesis and osteogenesis has been documented.1-5 While in vitro chondrogenic potential has been described for equine MSCs, little attention has thus far been directed toward equine ASCs. Our recent work on the osteogenic and adipogenic potential of MSCs4 and ASCs5 has shown differences in cell frequency and in vitro growth characteristics, as well as the ability to express alkaline phosphatase and to produce bone nodules during osteogenesis. These results suggest inherent differences between MSCs and ASCs during adipogenesis and osteogenesis. Studies by Im and coworkers6 in rodent models showed that ALP staining and the amount of mineralized matrix deposition during osteogenesis was greater for MSCs compared with ASCs. Furthermore, they reported that ASCs appeared to have reduced chondrogenic potential relative to MSCs, based on the quantity of matrix production and cell morphology. Bone morphogenic proteins are a subgroup of the transforming growth factor superfamily and act as signaling factors which regulate cartilage and bone formation. Human ASCs showed reduced expression of the bone morphogenic proteins BMP-2, -4, and -6 relative to MSCs and did not express TGFβ receptor-1 mRNA.7 Consequently, greater concentrations of TGFβ did not enhance chondrogenesis of ASCs and only in combination with BMP-6 did ASCs express gene profiles similar to differentiated MSCs. It has also been reported that BMP6 enhances in vitro chondrogenesis of MSCs.8,9 Transforming growth factor beta-1 (TGF-β1) used in combination
with insulin like growth factor-1 (IGF-1) appears to enhance chondrogenesis in equine MSC cultures based on proteoglycan formation and procollagen Type II mRNA synthesis.\textsuperscript{10} Based on human and rodent models, it is important to evaluate potential differences between equine MSCs and ASCs and to establish which cells may be optimal for specific regenerative tissue applications. The aim of this study was to compare the chondrogenic potential of equine ASCs and MSCs pellet cultures in the presence or absence of a robust growth factor stimulus and to establish potential differences in their extracellular matrix composition.

4.2 Materials and Methods

4.2.1 Materials

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific International Inc (Hampton, NH) unless otherwise noted.

4.2.2 Animals and Signalment

Subcutaneous adipose tissue was harvested from the region above the dorsal gluteal muscles from 6 young Thoroughbred geldings (mean ± SD, 3.5 ± 1.1 years) and sternal bone marrow was collected from a different group of 5 Thoroughbred geldings (mean ± SD, 4 ± 1.4 years). The procedures were approved by the Institutional Animal Care and Use Committee prior to initiation of this study.

4.2.3 Cell Culture Studies

4.2.3.1 Bone Marrow Aspiration

The methods used for bone marrow aspiration\textsuperscript{11} and MSC isolation\textsuperscript{12,13} have been reported. Briefly, young horses were selected from the research herd and after sedation with detomidine HCl (Domosedan\textsuperscript{®} 0.04 mg/kg IV) the sternum was aseptically prepared and local anesthetic (2% Lidocaine, 3 ml) was infiltrated into the subcutaneous tissue. A 10-ml volume of
bone marrow aspirate was collected into heparinized syringes (300 IU/10ml BM aspirate) using a 10-gauge, 3-inch Silverman bone marrow biopsy needle. Marrow stromal cell (MSC) isolation and expansion was performed immediately after tissue harvest.

4.2.3.2 MSC Isolation Method

Bone marrow aspirates were diluted 1:3 with stromal medium consisting of DMEM-Ham’s F12 medium (vol/vol, 1:1) (HyClone, Logan, UT, USA), supplemented with a 1% antibiotic/antimycotic solution (MP Biomedicals, Irvine, CA, USA) and 10% characterized fetal bovine serum (FBS, HyClone) and layered over Ficoll-Paque® PLUS (Stem Cell Technologies, Vancouver, BC, Canada). Nucleated cells in the BM aspirate were fractionated over a Ficoll density gradient by centrifugation at 1,400 rpm for 30 minutes at 4°C. The MSC-enriched cell population above the Ficoll layer was then aspirated and washed in calcium and magnesium-free Dulbecco’s balanced salt solution (CMF-DBSS) by further centrifugation at 260 x g for 5 minutes at 4°C. The washed pellet was then resuspended in stromal culture medium. MSCs were expanded in primary culture (P0) to 80% confluence and then stored after cryopreservation as described below.

4.2.3.3 Adipose Tissue Harvest

Horses were sedated with detomidine HCl (0.04 mg/kg intravenously [IV]) and butorphanol (0.01 mg/kg IV), the area over the dorsal gluteal muscles was aseptically prepared, and skin and subcutaneous tissues were desensitized by local infiltration of 2% lidocaine using an inverted L-block. A 10–15 cm incision was made parallel and ~15 cm abaxial to the vertebral column. Approximately 15 mL of adipose tissue were harvested over the superficial gluteal fascia for ASC isolation. The skin incision was apposed with nylon suture material.
4.2.3.4 **ASC Isolation Method**

The ASC isolation procedure was based on a technique previously reported for human ASC isolation. Adipose tissue was minced with a surgical blade, washed and briefly agitated with an equal volume of phosphate buffered saline (PBS) solution to promote separation into 2 phases. The upper phase consisted of the minced and washed adipose tissue. The liquid infranatant containing hemopoietic cells suspended in PBS was discarded. The adipose tissue was then digested in an equal volume of a filtered PBS solution containing 1% bovine serum albumin (BSA Type V; Sigma-Aldrich) and 0.1% of collagenase (Type I; Worthington Biochemical, Lakewood, NJ) with continuously shaking at 37°C for ~50 minutes. The sample was centrifuged at 260 g for 5 minutes. To complete stromal cell separation from primary adipocytes, the sample was briefly and vigorously agitated and then centrifuged at 260 g for 5 minutes. After discarding the supernatant containing oil, primary adipocytes and collagenase solution, the stromal-vascular fraction (SFV) pellet containing the nucleated cell portion of the adipose tissue harvest including the adipose-derived mesenchymal stromal cells (ASCs) was then cultured in stromal medium. ASCs were expanded in primary culture (P0) and then stored after cryopreservation as described below.

4.2.3.5 **Cryopreservation of Cells**

Post-thaw cell viability is reported to depend on storage concentration and hence all cells were frozen at 0.5 million cells/ml after expansion in P0. The cryopreservation medium contained 80% fetal calf serum, 10% DMEM, and 10% dimethyl sulfoxide (DMSO). Cells were placed into a 5100 Cryo 11C Freezing Container (Wessington Cryogenics, Tyne and Wear, UK) for 24 hours at -80°C before transfer to liquid nitrogen.
4.2.3.6  **Chondrogenesis and Pellet Cultures**

For chondrogenesis experiments, primary cells (P0) were thawed and expanded (P1) to obtain approximately 12 million cells for subsequent P2 pellet cultures (n = 30) (Table 1). Cells were trypsinized and aliquots of 0.25 x 10^6 cells (P2) were placed into racked microtubes (ISC BioExpress, Kaysville, UT) tubes and centrifuged for 5 minutes at 240 g. The resulting pellets were then cultured and induced into chondrogenesis using DMEM/High glucose (10%), 1% antibiotic/antifungal solution, dexamethasone (100 nM), ascorbic acid 2-phosphate (50g/ml) and ITS+ (culture supplement containing bovine insulin, transferrin, selenous acid, linoleic acid and bovine serum albumin [BSA]; BD Biosciences, Bedford, MA) with or without transforming growth factor (TGFβ3) (10 ng/ml) and bone morphogenic factor (BMP6) (10 ng/ml). The 3 treatment groups were as follows: stromal medium (C), chondrogenesis medium (CH), and chondrogenesis medium plus TGFβ3 and BMP6 (CHGF). The medium was changed every second day in all cultures. Pellet cultures were terminated at Day 3, 7, 14 or 21 and then prepared for compositional studies. The experimental design for the chondrogenesis study is shown in Table 1.

4.2.4  **Compositional Analysis**

Pellets were either papain digested for quantification of total DNA and glycosaminoglycans (GAG) or fixed in 10% formalin and subsequently embedded in paraffin for staining and evaluation with light microscopy.

4.2.4.1  **Histology**

Multiple sections of all pellets were stained with alcian blue to evaluate proteoglycan deposition and selected pellets sections were stained with hematoxylin and eosin to evaluate
pellet morphology. Representative sections of the ASC and MSC pellet cultures and their
treatment groups were read and evaluated by a pathologist (DBP).

4.2.4.2 Immunohistochemistry

Immunostaining was performed using an automated immunostainer (Dako Corp., Carpinteria, CA). Paraffin-embedded sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with $\text{H}_2\text{O}_2$ (3.0%). Slides were pretreated with proteinase K (Dako Corp.) for antigen retrieval, blocked with horse serum (30 min) and incubated with the primary antibody for 30 min at 25°C. Mouse monoclonal antibody (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA), reported to cross-react against equine collagen Type II$^{16}$, was diluted 1:5. After rinsing, slides were incubated with horseradish peroxidase-labeled polymer (avidin- and biotin-free) conjugated to goat anti-mouse IgG (EnVision, Dako Corp.). Peroxidase activity was detected using the NovaRED substrate kit (Vector Laboratories, Inc., Burlingame, CA), and tissues were counterstained with Mayer’s hematoxylin. For each tissue, a conditioned medium negative control, that did not contain primary antibody, was evaluated and no staining was detected. Paraffin-embedded cartilage from the proximal third tarsus (T3) of a 3-year-old Thoroughbred gelding was used as a positive control.

4.2.4.3 Transmission Electron Microscopy

For transmission electron microscopic (TEM) tissue examination, representative samples taken from 3 donors of Day 21 MSC and ASC pellet cultures (C, CH, and CHGF) were used. Paraffin-embedded pellets were deparaffinized, rehydrated with water and then fixed, processed and embedded in an epon-araldite resin as previously reported.$^{17}$ Thin sections were prepared with an MT XL microtome (RMC Products, Tucson, Arizona), stained with uranyl acetate and lead citrate, and observed with an electron optical microscope (JEM-1011, JEOL Ltd.,
<table>
<thead>
<tr>
<th>Time Course</th>
<th>Analyses</th>
<th>C</th>
<th>CH</th>
<th>CH-GF</th>
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<th>MSC donors</th>
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1^Immunohistochemistry was performed on tissue from three donors for both MSC and ASC pellet cultures.  
2^Paraffin-embedded pellets used for histology were recovered and processed for TEM.

Cell lines from a total of 5 donors were used for MSC pellet cultures and from 6 donors for ASC pellet cultures. A total of 30 pellets per donor per cell type (MSC or ACS) were grown. Pellets were analyzed individually for histological examination and pooled by day (2 pellets per donor) for the biochemical analysis. Two alcian blue-stained sections per pellet for all donors were used to measure cross-sectional pellet size. The pellets were divided into 3 treatment groups as follows: stromal medium (C), chondrogenesis medium (CH), and chondrogenesis medium plus TGF\(\beta\)3 and BMP6 (CH-GF) and the medium was changed every third day. Pellets were harvested at Day 3, 7, 14 and 21 in culture and then prepared for the compositional studies.
Lewisville, TX). Paraffin-embedded articular T3 cartilage was processed in the same manner and used as a positive control.

4.2.4.4 Total DNA Quantification

Total DNA within the pellets was assessed with the Hoechst assay as previously published. Briefly, papain digested pellet DNA concentration was assessed against a calf thymus DNA standard curve after preparation in an assay solution (2M NaCl, 50mM sodium monobasic phosphate, pH 7.4) containing the Hoechst 33258 dye. Pellet total DNA was measured with a spectrophotometer (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT) and used to normalize GAG concentrations.

4.2.4.5 Glycosaminoglycans Quantification

Pellets were digested in 100 μg/ml of papain in 0.4 mM sodium acetate (pH 6.8), 10 mM EDTA, 200 mM l-cysteine at 60°C for approximately 24 hours. Glycosaminoglycan (GAG) synthesis within the pellets was measured by dimethylmethylene blue (DMMB) assay as previously described, using chondroitin sulfate C from shark cartilage as a standard.

Cross-sectional Pellet Size – The areas of 2 different cross-sections of all alcian blue-stained pellets from all donors were measured using the Image Pro Software (Cybernetics, Inc, Bethesda, MD) after calibration with a micrometer.

4.2.5 Statistical Analysis

The study design employed a repeated measures analysis of variance in a split plot arrangement of treatments. The main plot was arranged as a 2 by 3 factorial including 2 cell types (MSC, ASC), three treatments (C, CH, CHGF) and the cell type by treatment interactions. The error term used for the main plot was donor within cell type by treatment. The subplot factors included day, day by cell, day by treatment and day by cell by treatment interactions.
Tukey’s test was employed for post-hoc main effect comparisons and the T test was used for pair-wise comparisons of the least square means to examine the interaction effects. All statistical analyses were performed using the Proc GLM procedure of the SAS 9.1.2 software package (SAS Institute Inc., Cary, NC) and the Type I error was maintained at \( \alpha = .05 \) for all comparisons. Data are presented as the arithmetic mean ± standard error in all figures.

4.3 Results

4.3.1 H&E and Alcian Blue Histology

Histological examination of pellets stained with H&E showed that those cultured from MSCs produced a hyaline cartilage-like morphology by Day 14 (Fig. 10) based on characteristic lacunae formation containing round chondrocytes typically associated with maintenance of chondrogenesis.\(^{20}\) Pellets grown from ASCs maintained an immature fibroblastic tissue appearance for the duration of the study. MSC pellets showed consistently more intense proteoglycan staining than ASC pellets (Fig. 11). Mild alcian blue staining was notable as early as Day 3 in MSC pellets treated with growth factors, but was not apparent in ASCs until Day 14. Under chondrogenic conditions without added growth factors, proteoglycan staining was less intense for both cell types but MSC differentiated pellets showed proteoglycan staining by Day 7, which was a week earlier than for ASCs.

ASCs under chondrogenic conditions without growth factors pellets showed fibrillar matrix by Day 7. The outer pellet layers were often composed of cohesive and spindeloid cells arranged parallel to the surface varying in thickness from 3 to 10 cells. Inner layers tended to palisade and were variably arranged perpendicular to the surface. Under growth factor treatment the matrix density of the outer cell layer was increased at Day 14. Pellets showed a broad external zone of spindeloid cells arranged parallel to the surface approximately 150 to 200 \( \mu \)
Figure 10: Hematoxylin and Eosin (H&E) Staining of ASC and MSC Pellets

The figure shows that pellets cultured from both cell types showed an initial peripheral layer formation and maturation by Day 14 in form of a deeper palasading and perpendicular arrangement of cells. However, only MSC-grown pellets produced hyaline cartilage by Day 14. The appearance of these pellets showed the characteristic lacunae formation containing chondrocytes. Pellets grown from ASCs never showed evidence of hyaline cartilage formation but rather maintained the immature fibroblastic tissue appearance.
Figure 11: Alcian Blue Staining of ASC and MSC Pellet Cultures

Pellets cultured from MSCs showed consistently more intense staining than those cultured from ASCs. Mild proteoglycan staining was notable as early as Day 3 in MSC pellets treated with BMP6 and TGFβ3 but was not notable in ASCs until Day 14 under the same conditions. Chondrogenic conditions without added growth factors led to less intense proteoglycan staining for both cell types but MSC differentiated pellets showed proteoglycan staining by Day 7, which was a week earlier than was seen for ASCs. The ASC-CH and ASC-CHGF pellets of Day 14 pellet show evidence of an edge artifact.
in thickness. By Day 21 only one of the ASC pellets showed moderate amounts of dense hyaline extracellular matrix, which was not birefringent. Moderate alcian blue staining of extracellular matrix by Day 14 suggests proteoglycan expression in the stroma.

MSC pellets without growth factor treatment developed several layers of spindle cells arranged parallel to the surface by Day 3. Palisading spindle cells were arranged perpendicular to the surface. No extracellular matrix was seen, yet mild alcian blue staining was noted in the peripheral zone in CHGF MSC pellets. Within 4 days the outer zone showed spindle cells layers vary from 3 to 10 cells in thickness arranged parallel to the surface surrounded by moderate fibrillar extracellular matrix. There was no alcian blue staining of the outer zone of Day 7 MSCs pellets but moderate staining of the matrix in the deeper layers. Partial chondroid differentiation was noted, characterized by abundant extracellular matrix with round to stellate cells, often located within the lacunae and organized into rows perpendicular to the surface. Intense alcian blue staining was seen in the deeper cartilaginous appearing zone by Day 14 and the bulk of the pellet showed prominent chondroid differentiation. The matrix was eosinophilic near the surface but the thick chondroid differentiated zone appeared hyaline-like, typical of cartilaginous matrix and was only very mildly birefringent. Cells were round to oval and positioned within lacunae. Day 21 MSC pellets had a similar appearance but much of the matrix was birefringent under polarized light suggesting some collagen type I deposition as hyaline cartilage is usually not birefringent. There was evidence of variable central necrosis in most pellets of both cell types by Day 14.

4.3.2 Collagen Type II Immunohistochemistry

Collagen type II expression was noted by Day 7 in MSC pellets cultured with BMP6 and TGFβ3 (Fig. 12). No evidence of collagen staining was observed in ASCs under the same conditions.
The figure shows convincing Collagen Type II expression in MSC pellets when cultured with BMP6 and TGFβ3. ASC pellets did not show any evidence of collagen staining under the same conditions and are therefore not shown. Pellet cultures under chondrogenic conditions without growth factors showed mild staining by Day 14 whereas ASC pellets again did not express antigen for the collagen Type II antibody. All pellets were exposed to conditioned medium serving as negative control for unspecific staining and mature cartilage from the proximal surface of the third tarsus (T3) was used as positive control (data not shown).
MSC pellet cultures under chondrogenic conditions without growth factors showed mild staining by Day 14 whereas ASC pellets again did not express collagen type II. The positive controls of paraffin-embedded cartilage sections stained less intensely for collagen Type II than the pellets, presumably because the cartilage sections had previously been decalcified, a process that is thought to have affected the cartilage epitopes and therefore affecting antibody binding affinity.

4.3.3 **Transmission Electron Microscopy**

ASC pellets (CH and CHGF treated) from only one of three donors developed any appreciable amounts of collagen fibrils, whereas all examined MSC pellets (CH and CHGF; n = 3 donors) showed abundant collagen fibrils. MSC pellet cultures formed more fibrils and had more cohesive areas of extracellular matrix compared to ASC pellets. Collagen fibrils were virtually undetectable in the control (C) cultures. It was notable that pellets showed a random arrangement of fibrils similar to the transitional cartilage zone of articular cartilage (Fig. 13-E). Areas of linear fibril arrangement were seen in pellet cultures of both cell types but it was not possible to determine from which pellet layers they originated. MSC pellets (Fig. 13-D) appeared to have thinner collagen fibrils compared with articular cartilage (Fig 13-E) as well as less interfibrillar extracellular matrix. However, compared with ASC pellets (Fig. 13-B) MSC fibrils seemed thicker and more mature.

No compositional differences were discernable under TEM evaluation between CH and CHGF treatments.

4.3.4 **DNA Concentration**

DNA concentrations (Fig. 14) were significantly lower in control pellet cultures compared to those treated chondrogenic media with and without BMP6 and TGFβ3 for both
Figure 13: Transmission Electron Microscopy

Sections of ASC cultures (CHGF) shown in panel A and B clearly show collagen fibrils at 40k and 80k magnification, respectively. Panel C and D represent MSC pellets (CHGF) which had a similarly random fibril arrangement as the image of the transitional zone of equine articular cartilage (panel E, 40K magnification) but showing less interfibrillar extracellular matrix. Panel D (MSC pellet at 80K magnification) shows more mature and thicker fibril structure compared to that of ASC pellets (panel B, 80K magnification) but MSC fibrils still appear to have a smaller diameter compared with articular cartilage fibrils (Panel F, 80K magnification).
ASCs and MSCs (P < .0001) on Day 7. Significantly lower DNA concentrations were also seen for control cultures (P < 0.009) compared to chondrogenic cultures with and without growth factors on Day 14 (P < .0001) and 21 (P < .0001) compared to Day 7. The only other significant difference found was for control and CH ASC cultures on Day 21 (P = .02).

4.3.5 **GAG Concentration**

GAG concentrations (Fig. 15) normalized to total DNA concentrations were not significantly different for any cultures at Day 7. On Day 14, MSCs treated with growth factors showed significantly greater GAG concentrations than all other treatments for both cell types (P < .0001). On Day 21 MSCs cultured under chondrogenic conditions with and without growth factors expressed higher GAG concentrations compared with ASCs grown under the same conditions (ASC-CH vs. MSC-CH, P = .0044; ASC-CHGF vs. MSC-CH, P = .0076; ASC-CHGF vs. MSC-CHGF, P < .0001).

4.3.6 **Pellet Cross-sectional Measurements**

Cross-sectional pellet size of ASC-derived pellets (Fig. 16) did not vary in any of the treatments, with the exception of CH between Day 3 and 21 (P = .0062). Pellet sizes grown from CHGF-treated MSCs were significantly larger than CH-treated pellets on Days 14 (P = .0032) and Day 21 (P < .0001). Significant differences in CHGF treatments were found between Days 3 and 7 (P=.05), approaching significance between Days 3 and 14 (P = .058) and Day 21 was significantly different from Day 3 (P < .0001), 7 (P = .005) and 14 (P = .0043). CH-treated MSC pellets were larger than ASC pellets grown under the same conditions on Day 7 (P=.013). However, MSC pellets were larger compared to ASC pellets on Days 7 (P = .0078), 14 (P < .0001) and 21 (P < .0001) after culture under CHGF conditions. After Day 7 MSC pellet size of
Figure 14: Total DNA Concentrations

ASC pellet cultures of days 7, 14 and 21 and MSC Pellets cultures of days 7 and 14 grown under chondrogenic conditions (± growth factors) had significantly greater total DNA concentrations compared to their control cultures (P < .0001). The data are represented as the arithmetic mean ± SE (ASC cultures, n=6; MSC cultures, n=5).
Relative DNA corrected GAG concentrations were not significantly different for any cultures on Day 7. By Day 14 significant differences were seen between MSCs treated with growth factors and all other treatments for both cell types (P < .0001). A week later MSCs cultured under chondrogenic conditions with and without growth factors showed significant differences to ASCs grown under the same conditions (ASC-CH vs. MSC-CH, P = .0044; ASC-CHGF vs. MSC-CH, P = .0076; ASC-CHGF vs. MSC-CHGF, P < .0001). The data are represented as the arithmetic mean ± SE (ASC cultures, n=6; MSC cultures, n=5).
ASC-derived pellet cultures grown with or without growth factors showed no differences in cross-sectional pellet areas. Pellet sizes grown from MSCs were significantly different between CH and CHGF treatments on Days 14 (P = .0032) and Day 21 (P < .0001). On Day 7 significant differences were seen in CHGF treated ASC and MSC pellets (P < .0001). Day 21 MSC pellets treated with growth factor (CHGF) were significantly different from those of Days 3 (P < .0001), 7 (P = .005) and 14 (P = .0043). The comparison of CH treatments between MSC and ASC grown pellets showed significant changes only on Day 7 (P=.013). However, comparing CHGF treatments between the two cell types showed differences on Days 7 (P = .0078), 14 (P < .0001) and 21 (P < .0001). Cross-sectional areas of pellets grown in stromal medium (C) were not measured due to the lack of characteristic pellet structure. The data are represented as the arithmetic mean ± SE (ASC cultures, n=6; MSC cultures, n=5).

Figure 16: Pellet Cross-sectional Measurements
CH treated cultures decreased significantly (P = .016). A trend in decreasing pellet size was also noted for ASCs but was not significant. Pellet sizes were not assessed for control cultures because the lack of structural integrity caused a progressive loss of cell material in time despite careful media changes. The correlation between GAG corrected for DNA and pellet cross-sectional area approached significance at a p value of 0.076.

4.4 Discussion

The use of stem cells in equine veterinary medicine is beginning to expand to applications beyond tendon and ligament repair. MSCs injected directly into joints has significantly contributed to the healing process of experimentally induced meniscal lesions in sheep, rats and dogs.21-25 The application of stem cells in the traumatized and osteoarthritic equine joint was debated at the recent 2007 Symposium of the American College of Veterinary Surgeons debated. Results of a clinical study presented at this meeting showed that 10 out 15 horses suffering from a variety of joint related pathologies with poor prognosis recovered and returned to work after cell-based therapy with MSCs.26 It was suggested that MSCs are beneficial to articular cartilage recovery as long as the joint surface was not denuded of cartilage with evidence of subchondral bone exposure. Furthermore, it was postulated that gene-modified MSCs may provide more significant results.26

The in vitro chondrogenic potential of equine MSCs has previously been documented in monolayer cultures10,13,27, three-dimensional fibrin disks10, and pellet cultures.2,3,28,29 In a recent study, Kisiday and his coworkers compared chondrogenesis in equine adipose tissue and bone marrow-derived progenitor cells.16 The mesenchymal stromal cells were cultured in agarose and self-assembling peptide hydrogels with or without the transforming growth factor TGFβ1.16 The results of this study were consistent with our data, demonstrating the superiority of bone marrow
MSCs in their chondrogenic potential. Similar to our study, equine ASCs demonstrated the ability to synthesize extracellular matrix in the form of proteoglycans and glycosaminoglycans, yet unlike bone marrow MSCs, the adipose tissue–derived progenitor cells did not demonstrate any collagen Type II expression during the 3 week culture period. However, ASCs responded to TGFβ₁ treatment with elevated levels of hydroxyproline and glycosaminoglycan production in peptide hydrogel cultures.¹⁶ These results suggest that equine ASCs express receptors to TGFβ₁ or at least may respond to the growth factor through alternate pathways.

The differences in the chondrogenic potential between the two equine cell types are consistent with studies on human and rodent adipose and marrow-derived mesenchymal progenitor cells.⁶, ³⁰⁻³² However, Hennig³² and coworkers recently showed that human ASCs do not express significant levels of TGFβ receptor-I mRNA, suggesting that human ASCs do not have receptors for TGFβ₁. Human ASCs did not respond significantly to culture supplementation of TGFβ₁ at 10ng/ml or higher concentrations, levels comparable to those used in both in Kisiday’s and our current study. However, endoglin (CD105), a component of the TGFβ type I and II receptor complex, was expressed on both human ASCs³⁴ and MSCs³⁵ as part of their surface marker profile. Reports have suggested that dexamethasone³⁶ in chondrogenic protocols may suppress any stimulatory effects of TGFβ₁ and this may contribute to the observed tissue-dependent differences between MSCs.

The growth factor TGFβ₃ enhances chondrogenesis due to its putative role in the regulation of cell adhesion molecules, cytokines, and cytokine receptor synthesis.³⁷⁻³⁹ Estes and his coworkers have shown that BMP6 and TGFβ₃ controlled chondrogenic induction in human ASC-derived alginate bead cultures and increased collagen Type II (COL2A1) by 38-fold and 42-fold, individually, and by 56-fold when used in combination.⁴⁰ Also TGFβ₃ had significant
effects on collagen type X (COL10A1), suggesting a role in hypertrophic collagen synthesis. Recent work\textsuperscript{41} on MSCs derived from equine umbilical cord blood (UCB), reported that TGF\(\beta_3\) stimulated chondrogenesis; however, necessary controls were not presented to allow assessment of the relative influence of this growth factor on the chondrogenic potential.

Consistent with Estes et al\textsuperscript{40} but in contrast to our own findings in the equine model, Hennig\textsuperscript{32} and coworkers found that the addition of BMP6 resulted in similar chondrogenic induction of human ASCs and MSCs. If the work by Awad\textsuperscript{36} and coworkers on human ASCs is extrapolated to the equine ASC model, then the relatively higher concentration of dexamethasone in our study (100nM) compared to Hennig’s study (20nM) may have inhibited chondrogenic differentiation and accounted for the diminished ASC response compared to bone marrow MSCs. Regardless, our results indicate that there are inherent differences between these two equine cell types. Receptor profiles of ASCs may differ between these progenitor cells of different species. Further investigations will be necessary to determine whether the cytokine receptor profiles of equine ASCs and bone marrow MSCs differ and if this could account for their differential chondrogenic responses.

The immunohistochemical staining of collagen type II in MSC pellets demonstrated a pattern whereby signal was apparent within the zone below the superficial spindeloid cells layers and faded toward the center of the pellet. This pattern may be related to oxygen diffusion and may reflect the location of an oxygen tension gradient particularly suitable for chondrocytic synthesis of collagen type II fibrils. It is known that articular cartilage in vivo is bathed in joint fluid with a relatively low oxygen concentration (~ 5\% ).\textsuperscript{42} Proliferation of human adipose tissue-derived stem cells is inhibited at oxygen tension of 5\% but the rate of protein synthesis increases by 2-fold and that of collagen synthesis by 3-fold and glycosaminoglycan synthesis and lactate
production are increased as well. Also, both cartilage thickness and cell density affect oxygen tension of in vitro cartilage sections and oxygen tension has been associated with the regulation of chondrogenic induction and a concomitant decrease in cell proliferation. Thus, oxygen tension may play a role in the current study and merits further investigation.

The pellet morphology of equine ASC and MSC cultures showed considerable differences. Of note was the significant reduction of MSC pellet sizes under chondrogenic (CH) conditions during the second week in culture. Human chondrocytes and mesenchymal stem cells express the contractile protein, α-smooth muscle actin (SMA), which is believed to cause in vitro pellet formation after chondrogenic induction and is essential to cell-mediated contraction leading to tissue remodeling and collagen synthesis. Scaffold designs with variable resistance to cell-mediated contraction have been used to show that chondrocytic cell phenotype and collagen type II synthesis was superior in scaffolds permissive to contraction. Tissue remodeling, contraction and cartilage synthesis is enhanced by treating canine chondrocytes seeded onto collagen type II-GAG scaffolds with fibroblast growth-factor-2 (FGF-2). FGF-2 has also recently been shown to enhance chondrogenesis in equine mesenchymal stem cells due to an anabolic effect on glycosaminoglycan content and collagen type II expression. Consistent with these reports, the smaller pellet size seen in MSC chondrogenic cultures on Day 14 in this study coincided with the appearance of hyaline-like cartilaginous tissue shown in Figure 1. ASC pellets, however, showed a similar but non-significant trend in size reduction suggesting some degree of tissue remodeling consistent with the glycosaminoglycan deposition and the traces of collagen type II synthesis observed using TEM. In contrast the increased size of MSC pellets stimulated by BMP6 and TGFβ3 was quite striking, which appeared to obscure the contractile effect on pellet matrix remodeling during chondrogenesis and warrants further investigation.
The lack of the characteristic structure of pellets grown in stromal medium (C) prevented the measurement of their cross-sectional area. Failure to form a sufficiently robust extracellular matrix rendered the control pellets susceptible to cell loss during media changes. Only a few cultures in the control medium (C) formed pellets that remained intact for the duration the study; of these, most were derived from MSCs. The cellular loss in the control cultures due to media change is believed to account for the reduction in DNA content seen on Day 7 in control pellets. The ~three-fold reduction in total DNA content evident in CH and CHGF cultures after Day 7 was attributed to the progressive central necrosis in pellets of both cell types. The lack of differences among the normalized GAG concentrations between controls and the treated pellet cultures it thought to relate to relative differences in DNA and measured GAG concentrations between control cultures and treated cultures. Additional variation in the data may be due to the fact that MSCs and ASCs were isolated from two different populations of horses.

In summary, our data show that equine bone marrow derived MSCs exhibited superior chondrogenic potential compared to ASCs in the presence of stimulatory growth factors. Nevertheless, the easy access to ASCs in the horse makes these cells an attractive choice for tissue engineering purposes. Further investigations of receptor profiles of equine ASCs and MSCs are needed in order to optimize cell selection for the purpose of regenerative tissue repair and cartilage resurfacing in the horse.

4.5 References


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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS
The significance of these studies is that stem cells from equine bone marrow and adipose tissue can be reliably harvested, isolated and expanded to large numbers in relatively short periods. *In vitro* cell growth characteristics and differentiation multipotential provides a basis for the study of these cells in association with scaffold materials and cell-based therapeutic techniques. The data demonstrate that there are differences between the two cell types with respect to cell growth, cell marker expression and their differentiation potential, which will require further investigation to optimize culture conditions and determine receptor profiles to maximize their use for tissue engineering purposes.

Our studies have shown that MSCs and ASCs occur more frequently in bone marrow and adipose tissue compared with other species. However, the frequency data is quite variable and the population size was limited making it difficult to predict cell frequencies accurately. Both cell types exhibited robust adipogenesis and osteogenesis; however, osteogenesis occurred more rapidly in MSCs compared to ASCs. Undifferentiated MSCs expressed more ALP than ASCs, which would support the notion that MSCs are better equipped for osteogenesis compared with ASCs. Likewise, adipogenesis appeared more robust in ASC cultures compared with MSC cultures. These observations will require further molecular assessment but are consistent with the natural environment of these two cell types. Similar to other species both MSCs and ASCs maintained differentiation potential during later passages.

MSCs showed superior chondrogenic potential based on histological morphology, collagen type II immunohistochemistry and proteoglycan expression. Pellet morphology of MSCs was much more reminiscent of hyaline cartilage than the matrix organization of pellets grown from ASCs. Proteoglycan expression in ASC pellets compared with the controls suggests that these cells do respond to chondrogenic culture media and that BMP6 and TGFβ3 have an
additional positive affect on ASC differentiation and pellet maturation. However, the lack of collagen Type II expression and the morphological immaturity of ASC pellets suggest that ASCs may require different culture conditions and/or additional stimuli to perform similarly as MSCs. Under the present conditions the bone marrow derived stromal cells clearly show superior chondrogenesis as early as Day 7. Receptor expression profiles will need to be studied in the future to elucidate culture requirements for these two cell types to optimize the use of ASCs and MSC in tissue engineering. The high cell numbers that could easily be harvested in horses makes these cells a potential source of cell for regenerative tissue repair purposes. Future studies will be directed at optimization of their use in tissue engineering.

Our research efforts thus far, using equine stem cells have mostly concentrated on characterizing the in vitro growth and differentiation properties of these cells and we have also employed these cells clinically in studies on equine tendonitis. Because we can reliably generate large numbers of cells we have established the ground work for additional studies on optimizing culture conditions and on the effect of appropriate bioabsorbable scaffolds, cellular gene-enhancement and mechanoinduction prior to implantation, which may all play an important role in enhancing the potential of equine bone marrow and adipose tissue derived stem cells for their use in regenerative tissue repair.


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APPENDIX A: GRANTS AND PUBLICATIONS DURING DOCTORAL WORK

Grants (Funded)


Grants (Accepted But Not Funded)

Blackmer JM, Vidal MA, Gimble JM, Lopez MJ, Truax RE, Moore RM: "Tissue engineering: Growth of equine mesenchymal stromal cells and characterization of extracellular matrix in low-shear three-dimensional culture for use in tendon healing." USA Equestrian, December 2003, Role: PI. Amount: $9,630. (withdrawn due to acceptance of ACVS funding for same proposal)


Grants (Not Funded)


**Publications & Presentations**

**Peer-Reviewed Manuscripts**


Vidal MA, Robinson SO, Lopez MJ, Paulsen DB, Borkhsenious O, Johnson JR, Moore RM; Gimble JM: “Comparison of Chondrogenic Potential in Equine Mesenchymal Stromal Cells Derived from Adipose Tissue and Bone Marrow” (submitted to *Veterinary Surgery* December 2007)


Non-Peer-Reviewed Manuscripts


Research Abstracts And Proceedings


Scientific Poster Presentations


Sickler ER, **Vidal MA**, Robinson SO, Gimble JM, Lopez MJ, Stokes AM: (2006)“Adipogenic Differentiation and Characterization of Equine Bone Marrow-Derived Mesenchymal Stromal Cells” Howard Hughes Medical Institute Summer Undergraduate Research Poster Presentation, August 3rd, 2006, Louisiana State University, Baton Rouge, LA.


APPENDIX B: LETTERS OF PERMISSION

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Louisiana State University
Phone: (225) 578-9418
Fax: (225) 578-9559

“If we knew what we doing, it wouldn’t be called research” [A. Einstein]
VITA

Martin Andreas Vidal was born in May 1967, in Boulder, Colorado, where he spent the first five years of his life. He then grew up in Munich, Germany, and completed his secondary education at the Maximiliansgymnasium in Munich in 1987. After graduation Martin returned to the United States to study veterinary medicine. After a year of improving his English language skills while working partly as an animal caretaker at the School of Veterinary Medicine in Madison, Wisconsin, and as a trainer and exercise rider at a racehorse training facility near Rhinelander, Wisconsin, he then attended the University of Wisconsin in Madison, earning the degree of Bachelor of Science in Meat and Animal Science in 1992. Intrigued by ongoing research in nutrition, bone biology and fracture biomechanics at the Department of Meat and Animal Science, he decided to extend his education in these areas of interest, successfully completing his thesis and earning the degree Master of Science in Meat and Animal Science in 1995. Thereafter Martin was accepted at the University of Liverpool in England where he eventually studied veterinary medicine to earn his Bachelor of Veterinary Science in 2000 and he became a member of the Royal College of Veterinary Surgeons in the same year.

After graduation Dr. Vidal took a locum position as an associate equine veterinarian at the Minster Equine Veterinary Clinic in York located in Yorkshire, England. Nine months later, the outbreak of the foot and mouth disease in the Spring of 2002 drastically reduced the case load at the Minster Equine Veterinary Clinic and prompted Dr. Vidal to relocate to Newcastle upon Tyne in Northern England, where he took a position as a Temporary Veterinary Inspector for the British Department of Environment, Food and Rural Affairs (DEFRA) at the Kenton Bar, Newcastle Disease Emergency Control Center.
In August 2001, he then moved to Australia where he had accepted a one-year rotating internship in equine medicine, surgery and reproduction at the Goulburn Valley Equine Hospital in Congupna, Victoria. Thereafter Dr. Vidal was invited to stay for a further year as an associate veterinarian at the Goulburn Valley Equine Hospital before he started a combined Equine Surgery Residency/PhD program at the Louisiana State University School of Veterinary Medicine in Baton Rouge, Louisiana. Dr. Vidal successfully completed the surgical residency in July 2006 and is anticipating graduation as a Doctor of Philosophy in veterinary clinical science in May of 2008. Dr. Vidal is expected to take his specialty Board examinations with the American College of Veterinary Surgeons in February 2008 before embarking on his new tenure track faculty position for equine surgery at the University of California, Davis, in April 2008.