

5-16-2011

# Optimization of culture conditions for equine bone marrow mesenchymal stem cells and their differentiation into osteoblasts

Elizabeth R. Ackell

University of Connecticut - Storrs, [elizabeth.ackell@gmail.com](mailto:elizabeth.ackell@gmail.com)

---

## Recommended Citation

Ackell, Elizabeth R., "Optimization of culture conditions for equine bone marrow mesenchymal stem cells and their differentiation into osteoblasts" (2011). *Master's Theses*. 119.  
[https://opencommons.uconn.edu/gs\\_theses/119](https://opencommons.uconn.edu/gs_theses/119)

This work is brought to you for free and open access by the University of Connecticut Graduate School at OpenCommons@UConn. It has been accepted for inclusion in Master's Theses by an authorized administrator of OpenCommons@UConn. For more information, please contact [opencommons@uconn.edu](mailto:opencommons@uconn.edu).

# **Optimization of culture conditions for equine bone marrow mesenchymal stem cells and their differentiation into osteoblasts**

Elizabeth Rose Ackell

B.S., University of Connecticut, 2008

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

at the

University of Connecticut

2011

**APPROVAL PAGE**

Master of Science Thesis

**Optimization of culture conditions for equine bone  
marrow mesenchymal stem cells and their  
differentiation into osteoblasts**

Presented by

Elizabeth Rose Ackell, B.S.

Major Advisor \_\_\_\_\_  
Kristen E. Govoni

Associate Advisor \_\_\_\_\_  
Steven A. Zinn

Associate Advisor \_\_\_\_\_  
Thomas A. Hoagland

University of Connecticut  
2011

## **Acknowledgment**

I offer my sincerest gratitude to my major advisor, Dr. Kristen Govoni, for supporting me throughout my graduate research. I attribute my successes to her patience and guidance, because without her time and encouragement this thesis would not have been possible. I am also very grateful for the members of my graduate committee, Dr. Steven Zinn and Dr. Thomas Hoagland. Their expertise and assistance were invaluable throughout my research.

I am extremely grateful to Dr. Alfredo Sanchez for taking the time to collaborate with us in the aspiration of bone marrow. His attention to detail and commitment to the health of our horses was essential for our success. In addition, many thanks go to Mr. John Bennett and Mrs. Kathy Pelletier. Their support for my research, from the background education they provided me as an undergraduate, to providing me with horses and stall space, was much appreciated.

My appreciation for my fellow lab mates, Nidhish Francis and Maria Procopio, is indescribable. The time and effort they provided to support my research was always above and beyond any that I could repay. I would also like to thank all of the undergraduate and graduate students, faculty, and staff of the Department of Animal Science for their assistance and continual support.

Finally, I thank my parents, Kenneth and Susan Ackell, and my fiancé, David Glynn, for their continual support throughout my education. Their encouragement touched my heart and has driven me to succeed.

Thank you all so much, your kindness will not be forgotten.

## Table of Contents

Approval Page.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
List of Tables.....	vi
List of Figures.....	vii
List of Abbreviations.....	viii
Review of Literature	
Introduction.....	1
Equine Fracture.....	2
Regulation of the differentiation of MSC into osteoblast cells.....	8
Mesenchymal Stem Cells.....	11
Rationale.....	18
Hypotheses.....	19
Objectives.....	19
Materials and Methods	
Animals.....	20
Serum Collection.....	20
Bone Marrow Aspiration.....	21
Post Procedural Horse Care.....	22
Culture of Bone Marrow Stromal Cells.....	23
Cell Passage.....	24
Cell Proliferation Assay.....	25
Cell Differentiation.....	26
Statistical Analysis.....	31
Results	
Population Rates of Different Cell Populations.....	32
Effect of FBS vs. HS on Cell Proliferation.....	32
Effect of hBMP-2 and DEX on Differentiation of eBMSC into Osteoblasts.....	33
Gene Expression During Differentiation of eBMSC into Osteoblasts.....	33
Figure Legends.....	35

Discussion.....	43
Appendices.....	49
References.....	63

## List of Tables

Table	Page
1. Cell Culture Medias.....	24
2. RT-PCR Primers.....	30

## List of Figures

Figure	Page
1. A similar cell number was observed between early and late adherence eBMSC in the absence and presence of serum.....	37
2. Similar BrdU incorporation was observed between early and late adherence eBMSC in the absence and presence of serum .....	37
3. Proliferation was greater in cells cultured with FBS compared with HS.....	38
4. Human BMP-2 treatment did not alter ALP enzyme activity.....	39
5. Dexamethasone treatment increased ALP enzyme activity.....	39
6. In the presence of DEX, no effect of hBMP-2 was observed.....	40
7. Confirmation of osteoblast differentiation by Alizarin Red Stain.....	41
8. Increased mRNA expression of ALP and OC during differentiation.....	42
9. Decreased expression of Tbx3 during differentiation.....	42



## List of Abbreviations

$\alpha$ -MEM	minimal essential medium $\alpha$
AB	amphotericin B
ALP	alkaline phosphatase
AMSC	adipose-derived mesenchymal stem cell
BG	$\beta$ -glycerol 2-phosphate
BMP	bone morphogenetic protein
BMSC	bone marrow mesenchymal stem cell
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
DEX	dexamethasone
DNA	deoxyribonucleic acid
e	equine
FBS	fetal bovine serum
h	human
HS	horse serum
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
OC	osteocalcin
Osx	osterix
PBS	phosphate buffer saline
PS	penicillin/streptomycin
r	rat
rfu	relative fluorescence units
rlu/s	relative light units per second
RT-PCR	reverse transcriptase polymerase chain reaction
Runx2	runt-related transcription factor 2
SE	standard error
Tbx3	T-box 3
TGF- $\beta$	transforming growth factor beta
VC	L-ascorbic acid-2-phosphate (vitamin C)

## **Literature Review**

### **Introduction**

The horse industry is a multibillion dollar industry where the health of the animals is of great focus for horse owners (Byers, 2011). Bone fractures are an aspect of equine health failure that is continually catastrophic and often fatal. In addition to discussing fracture, this review will outline the process by which bone tissue is regularly maintained and self-healed. Also, traditional methods of healing fractures that have limited use and success will be discussed. The lack of success that is common with the traditional methods has led to the need for additional research to identify more successful and economical ways of healing fractured bone. This review will identify advancements in research, including the use of adult stem cells, specifically, mesenchymal stem cells (MSC), in fracture healing. The main focus of previous research in this area has been in rodent and human models. The previous research has shown that MSC are an accessible and functional source for bone regeneration. Past work has increased understanding of the isolation, proliferation, and differentiation of MSC in rodent and human models. Recent studies suggest that equine (e) MSC may be used for the advancement of fracture healing, but more research is required in this species. This literature review will present data supporting the advancements of this novel method for use in healing equine fractures.

## **Equine Fracture**

In the horse industry, millions of dollars are spent on the training and health of horses that are used for all aspects of equine sports. Thoroughbred racing is a well known equine sport where maintaining animal health is one of the largest expenses to owners and trainers. An especially important aspect of animal health is structural soundness. A fracture, or break in structural soundness, can be devastating. It is estimated that annual losses due to bone fractures in the horse-racing industry exceed \$10 million (Lewis, 2008). It has been determined that 7% of all Thoroughbred racehorses incur musculoskeletal injuries, and almost one half of these injuries are catastrophic (Bathe, 1994; Estberg et al., 1998). Research shows that more than 300 actively racing Thoroughbreds die following a fracture injury each year, and that most of these deaths are related to injuries of the bones of the forelimb (Hill et al., 1986). To repair a fracture, surgery costs vary greatly between \$2,000 and \$10,000 per surgery (Beyer, 2006). This does not include the post-surgery care and rehabilitation which can cost anywhere between \$100 and \$2,000 per day for the first three to four weeks after the surgery (Sacks, 2006; Blanchette, 2008). The increased costs associated with care following fractures are often due to slow healing and lack of efficient treatments. This has driven researchers to find quicker, safer, and more effective methods of healing broken bones in horses. This review will go into detail about the mechanisms of fracture, the current methods of healing equine fractures, research to advance healing, and the possible use of MSC for fracture healing.

## ***Mechanism of Fracture***

To better understand how a fracture can be healed, this review will first discuss the biology of bone fracture. Perren (2008) states that a fracture is the result of an incongruence in structural stiffness that leads to a loss of support and locomotion. A fracture destroys the integrity of a bone and disables limb or joint function (Perren, 2008). Fractures can be divided into one of three categories (Riggs, 2002). The first is monotonic fracture, where in the case of a fall or crash, a bone is loaded with more force than it is strong enough to handle for a single moment. The second category is pathological fracture, where a bone gradually loses strength due to conditions such as osteoporosis, and is unable to withstand normal body weight. The third type of fracture is fatigue fracture, where a bone is continually over-loaded with pressure, such as an excess of high intensity workout, and is progressively eroded due to the great level of fatigue (Riggs, 2002). Some researchers have suggested that most racing fatalities (52.5 to 72.7 %) occur when a horse falls and in a single moment an otherwise healthy bone is broken with a monotonic fracture (McKee, 1995). However, Stover (2003) states that most skeletal injuries are due to repetitive overuse, when a bone that has incurred microdamage and subsequent repair over time, no longer has the strength to support the increased stress, and a fatigue fracture occurs. In both monotonic and fatigue fracture, the methods used to repair and heal the fracture, detailed later in this review, are the same. However, before understanding the methods to heal a fracture, it is important to understand how bone is naturally maintained and repaired.

## ***Bone Maintenance***

Bone tissue undergoes continuous remodeling that allows the tissue to maintain mineral homeostasis (Raggatt and Partridge, 2010). Bone remodeling occurs in four stages: 1) during the activation phase, an initial remodeling signal, such as direct mechanical strain or hormone action, stimulates osteoclast formation, 2) during the resorption phase, osteocytes recruit osteoclasts to dissolve and degrade the mineralized bone matrix, 3) during the formation phase, new bone tissue is formed directly at the sites of bone resorption as a response to mechanical and hormonal stimulation, and 4) during termination phase, mature osteocytes signal that a quantity equal to that of resorbed bone has been replaced (Raggatt and Partridge, 2010). The steps listed here are regularly active in healthy individuals for the maintenance of bone tissue.

Through the four steps outlined above, bone tissue is able to maintain homeostasis throughout the lifetime of an animal. In the early stages of life, the third and fourth steps are more active, to ensure the formation of new bone for growth (Raggatt and Partridge, 2010). To withstand mechanical strain, such as the force placed upon the legs of a horse when galloping at full speed, bone cycles through the four steps, to ensure that integrity of the bone is maintained (Raggatt and Partridge, 2010). Finally, in later stages of life, malnutrition or disease conditions, the first two stages of bone remodeling are more active, and bone is degraded without the corresponding reformation (Pagani et al., 2005). However, when a bone is fractured, these four steps alone are not sufficient to heal a fractured bone.

## ***Repair of Fractures***

Fracture healing is a process that occurs in four stages (Schindeler et al., 2008).

1) The inflammation stage occurs in response to the soft tissue and vascular damage that occurs with a fracture (Schindeler et al., 2008). This damage leads to the activation of wound healing pathways, such as the Jun N-terminal kinase pathway, that combat infection and advance clotting (Ip and Davis, 1998). 2) Soft callus formation begins when chondrocytes and fibroblasts dominate the area and provide mechanical support to the fracture. 3) A hard callus is formed in a stage also known as primary bone formation. In this stage increased osteoblast activity results in the formation of mineralized bone matrix. However, this hard callus is irregular, so in the final stage, secondary bone formation must occur, and 4) during secondary bone formation, osteoclast resorption activity is coupled with osteoblast formation (Schindeler et al., 2008).

The third and fourth stages of fracture healing are similar to the processes that bone undergoes for regular remodeling. However, the mechanisms that stimulate bone remodeling and fracture healing are different and healthy bone formation arises from two different areas. In bone remodeling, the bone formation occurs in the area where the bone is weakest, at the site where resorption occurred due to osteoclast activity (Raggatt and Partridge, 2010). In fracture healing, the bone formation arises directly from the periphery of the soft callus in areas of stability (Schindeler et al., 2008). The natural process of fracture healing is activated immediately following a break in bone, but it has been found that over 50% of all fractures incurred by horses are not successfully healed via this process (Kraus et al., 2005). Due to the inability to heal after a bone fracture and

the resulting loss of horse use, veterinarians have stepped in with various methods of treatment to ensure survival and future use.

### ***Basic Methods of Healing***

When veterinarians first worked to assist the natural fracture healing process, the goal was to simply achieve solid union of both sides of the fracture (Muller, 1963). The most basic and original methods to aid healing were mechanical. The original methods, such as external splinting with plaster, along with stall rest to reduce movement, are still used today. External splinting is successful in achieving solid union in a previously fractured area; however, it does not allow for full recovery of mobility (Perren, 2008). Often, full recovery is not achieved because basic methods, such as external splinting, do not aid healing and can cause additional damage to joints and soft tissue that are also injured when a bone is broken (Perren, 2008). The next advancement in the healing of fractures was surgical stabilization via internal fixation (Perren, 2008). Also still utilized today, surgical stabilization is done with the goal of creating a motionless neutral environment. Veterinarians surgically implant metal plates and screw the plates into the bone fragments at the site of the fracture (Perren, 2008). This method allows the force applied to the injured area to be absorbed by the plate, allowing the bone more freedom to heal (Perren, 2008). Surgical stabilization allows solid union and does not damage local joints and soft tissue, or prevent the natural healing of these tissues (Perren, 2008). However, due to the forced closure of the fracture gap, there is no way to monitor the healing process. Thus, to avoid re-fracture it is essential to leave the implant in for an extended period of time (Perren, 2008). The lack of healing to damaged joints and soft

tissues, along with the inability to monitor and confirm successful fracture healing, limit recovery following a fracture.

### ***Recent Advancements in Healing Fractures***

Recently, the goal in fracture treatment has been expanded to include a successful healing of the fracture, and also to allow the full recovery of the function of the injured limb and associated joint. The successful recovery will allow the horse to have full mobility and to serve a useful purpose, in the sport or industry where they were originally trained (Perren, 2008). The goal in fracture treatment has evolved over time as understanding of the anatomy of the horse has evolved. The focus of research and treatments is transitioning from a mechanical approach to a cell and environment based approach. It is now known that there are four elements that are critical for bone repair: 1) adequate blood supply to the region of growth, 2) osteoinductive signals provided by growth factors, 3) osteogenic cells capable of responding to these signals, and 4) an osteoconductive matrix to support these cells (Gamradt and Lieberman, 2004). Researchers are working to better understand these four elements with the goal of using novel techniques that incorporate a combination of these elements to enhance the efficiency and success of fracture healing.

Novel methods to provide each of these elements, blood supply, osteoinductive signals, osteogenic cells, and supporting matrix, have been identified. First, researchers have identified peripheral blood progenitor cells, as an ideal cell type to promote vascularity, to ensure an adequate blood supply, during bone repair (Lee et al., 2008). Second, to enhance osteoinductive signals, growth factors such as bone morphogenic



proteins (BMP) are being used to successfully treat fractured bones (Govender et al., 2002). Third, MSC have been identified as a cell population with osteogenic potential capable of responding to osteoinductive growth factors (Hanada et al., 1997). Last, to provide the osteoconductive matrix to support osteogenic cells, researchers have been experimenting with porous forms of mineralized calcium, known as hydroxyapatite (Okamoto et al., 2006). Whereas researchers understand that these four elements must be combined to have the greatest impact on fracture healing, it is necessary to understand how each element is individually regulated. By focusing on the cells that are active in fracture healing and the factors that are essential for their success, researchers will be able to enhance the naturally acting mechanisms to improve fracture healing. Recent research has identified that MSC are multipotent cells with the osteogenic potential to be used for therapeutic repair of fractures because they are involved in the last three of the four steps of fracture repair (Schindeler et al., 2008). By gaining a better understanding of MSC, researchers will be able to progress towards more advanced methods of fracture healing.

### **Regulation of the differentiation of MSC into osteoblast cells**

To use MSC for fracture healing it is important to understand the process of differentiation that they go through to become osteoblast cells. This process has been well-characterized in rodent models. Osteoblast differentiation occurs when a cells differentiate from MSC into osteocytes (Caplan, 2009). This process begins with the proliferation of MSC which increases the cell number to ensure an adequate population for new bone formation. Next, the MSC differentiate into osteochondro-progenitor cells.

Following this first differentiation a cell will undergo commitment to osteogenesis. Once a cell is committed to the osteoblast lineage it will differentiate into a preosteoblast cell. Next, the preosteoblast will fully differentiate into an osteoblast. Finally, to complete bone formation the osteoblast will mature to an osteocyte (Caplan, 2009). These steps do not occur spontaneously, they are regulated by a number of factors. To use these cells for fracture healing, it is not only important to understand the steps through which MSC differentiate into the osteoblast, it is also important to understand the complex regulation of this process.

The differentiation of MSC into osteoblasts is a complex process that is regulated by a number of transcription factors that have been characterized in rodent models. Early differentiation is regulated by the transcription factors runt-related transcription factor 2 (Runx2), distal-less homeobox 5, Twist, trans-acting transcription factor 3, and  $\beta$ -catenin (Komori, 2006). The transcription factors osterix (Osx), activating transcription factor 4, fragile fiber 1, and early growth response 2 are active in the later stages of differentiation (Komori, 2006). Of these, the two master transcription factors that regulate osteoblast differentiation are Runx2 and Osx. Runt-related transcription factor 2 is the earliest marker of osteoblast differentiation as it is the factor that commits the osteo-chondro progenitor cells to the osteoblast lineage (Franceschi and Xiao, 2003). This transcription factor, Runx2, also maintains the cells in an immature, pre-osteoblast stage until other transcription factors become active (Franceschi and Xiao, 2003). Further down the osteoblast lineage this factor is not expressed in more mature osteoblasts (Franceschi and Xiao, 2003). Downstream of Runx2, Osx is essential for differentiation from the pre-osteoblast to the osteoblast. It has been shown in a knock-out mice model that deletion of

Osx results in a complete lack of osteoblasts and little to no mineralization in the embryo (Nakashima et al., 2002).

Another transcription factor that has recently been identified to play a role in osteoblast differentiation is T-box 3 (Tbx3; Govoni et al., 2009). T-box 3 is one of 18 genes in the T-box family that are essential for a wide variety of developmental processes (Wilson and Conlon, 2002). The T-box family is highly conserved among most species and is associated with a number of genetic disorders and cancers (Wilson and Conlon, 2002). The T-box family of genes was first identified as a region of homology on the DNA binding domain containing the *Brachyury* gene, which is associated with short-blunt ended tails in mice (Chapman et al., 1996). One human disorder linked to the T-box family of genes is ulnar-mammary syndrome. This disorder, caused by a mutation in the Tbx3 gene, is associated with posterior limb deficiency or duplication (Bamshad et al., 1997). It is known that Tbx3 is essential during bone development, in embryonic and postnatal stages, and that it is an important regulator of the cell cycle (Bamshad et al., 1997). T-box 3 stimulates proliferation by inhibiting senescence (Fan et al., 2004). With regard to murine osteoblasts, it is known that over-expression of Tbx3 inhibits osteoblast differentiation and the expression of Runx2 and Osx (Govoni et al., 2009). To date, the expression of Tbx3 has not been characterized in the equine model.

The transcription factors Runx2, Osx, and Tbx3, which regulate osteoblast differentiation, are not independently active, and require signals from a combination of other proteins. Research has identified that Runx2 and Osx are modified by a number of proteins involved in the Wnt and transforming growth factor beta (TGF- $\beta$ ) signaling

pathways, respectively (Gaur et al., 2005; Heino and Hentunen, 2008). There are many signaling pathways that regulate osteoblast differentiation; however, the focus of this thesis will be on these two main pathways as they are the master regulators of the two essential transcription factors. Wnt signaling directly stimulates Runx2, thus stimulating the differentiation of MSC down the osteoblast lineage (Gaur et al., 2005). Signaling from the TGF- $\beta$  superfamily, which includes bone morphogenetic proteins (BMP), stimulates osteoblast differentiation by regulating expression of Osx (Heino and Hentunen, 2008). One area of research that is lacking, is the understanding of the pathways that regulate Tbx3 action. There are few data available, in any species, regarding the upstream regulation of Tbx3 and its downstream effects on other transcription factors associated with osteoblast differentiation. By understanding how these transcription factors are regulated, it is possible to understand when they are expressed during the differentiation of MSC into osteoblasts. Understanding the expression of transcription factors that are active in rodent and human models will allow the characterization of the differentiation of eBMSC into osteoblasts. This characterization will improve understanding of the mechanisms that regulate the differentiation of eBMSC to osteoblasts. With improved understanding of transcription factors, veterinarians be able to use them as a tool in novel methods to improve fracture healing.

### **Mesenchymal Stem Cells**

Stem cells are classified as zygotic, embryonic, or adult, based on their origin (Herzog et al., 2003). All of these stem cells are capable of self renewal and

differentiation into at least one mature cell type (Herzog et al., 2003). Adult stem cells, such as hematopoietic stem cells and MSC, have pluripotent ability, but based on their origin, some are more pluripotent than others (Horwitz et al., 2002; Herzog et al., 2003). One origin of adult stem cells is bone marrow. Found in the interior of the long and flat bones of the mammalian body, bone marrow is made up of stroma and hematopoietic cells (Dominici et al., 2004). The stroma contains a variety of cells that have regenerative capabilities that contribute to many diverse tissues (Dominici et al., 2004). These regenerative cells, including MSC, are a collection of stem cells with restricted genetic programs (Dominici et al., 2004). Mesenchymal stem cells have the ability to differentiate into fat (adipocytes), bone (osteoblasts), muscle (myocytes), connective tissue (fibroblasts), and cartilage (chondrocytes) (Fortier et al., 1998). The ability of the MSC to differentiate into osteoblasts makes this cell population an ideal candidate for use as an advanced method of treatment for fractured bone. Before the MSC can be used in fracture healing they must be obtained from the adult animal.

### ***Sources of MSC***

Mesenchymal stem cells can be isolated from various tissues in the adult body, including bone marrow and adipose tissue (Gamradt and Lieberman, 2004). Bone marrow is the most commonly used, and adipose tissue is the second most commonly used source of MSC (Baksh et al., 2007). When comparing these two tissue sources, research has been determined that there is no significant difference in cell proliferation between cell lines isolated from adipose or bone marrow (Colleoni et al., 2009). In addition to the similarities in proliferation, adipose-derived MSC (AMSC) and bone

marrow MSC (BMSC) display similar morphological changes after exposure to osteogenic medium (Colleoni et al., 2009). However, more extensive research has determined that the isolation of MSC from bone marrow shows less variability in cell counts than MSC isolated from adipose tissue (Colleoni et al., 2009). Colleoni et al. (2009) propose that greater variability in MSC from adipose tissue is due to the tissue processing, specifically enzymatic digestion that is necessary to isolate the MSC. In addition to differences in cell count variability, the most important differences for MSC used in fracture healing are seen when comparing osteogenesis of human (h) BMSC with osteogenesis of hAMSC. Following culture in osteogenic medium hBMSC underwent a much greater increase in alkaline phosphatase (ALP) activity compared with hAMSC (Im et al., 2005). Also, greater mineralization was identified in hBMSC than in hAMSC, suggesting that bone marrow may be a better source of MSC than adipose tissue for differentiation into osteoblasts (Im et al., 2005). Through this review of literature it was determined that bone marrow is the ideal source of MSC for osteoblast differentiation.

### ***Identification of MSC***

Researchers have developed a basic understanding of MSC and have been able to expand that understanding to develop criteria for identifying the cells. Mesenchymal stem cells make up 1 out of 100,000 nucleated cells in bone marrow (Pittenger et al., 1999). Though the population is small, multiple therapeutic doses can be obtained from one small portion of bone marrow (Haynesworth et al., 1992). It has been identified that MSC selectively attach to tissue culture plastic and display spindle-shaped morphology (Bruder et al., 1997; Im et al., 2005). The MSC are the only cell population in the bone

marrow that have these two properties (Bruder et al., 1997). For this reason the adherence to plastic is used to isolate MSC from all other cells in the bone marrow and the morphology is used to visually confirm isolation.

In the human model, minimal defining criteria for MSC have been outlined (Dominici et al., 2006). MSC must be plastic-adherent, express CD73, CD90, and C105 surface markers, and have the ability to differentiate into osteoblasts, adipocytes, and chondroblasts (Dominici et al., 2006). To further define these cells, they should also show long-term survival *in vivo*, have self renewal capacities, and possess the ability for tissue repopulation with multilineage differentiation (Horwitz et al., 2005).

Unfortunately, much less defining criteria is available for eMSC (Schauwer et al., 2010). Limited availability of species-specific monoclonal antibodies results in the current limitation in immunophenotyping the eMSC (Paris and Stout, 2010). It has been recently suggested that in addition to plastic adherence and tri-lineage differentiation, eMSC should express CD29, CD44, and CD90 (Schauwer et al., 2010). This suggestion was supported by research by Guest et al. (2008) where the expression of these markers, CD29, CD44, and CD90, was confirmed in eBMSC.

### ***Characterization of eBMSC***

This section will discuss the characteristics of MSC that were first identified in hMSC. Although many of these characteristics have also been identified in the equine model, they are not as extensively examined. Researchers have found that hMSC have a finite lifespan of 15 to 50 doublings, resulting in the possibility for over a billion-fold

expansion (Gamradt and Lieberman, 2004; Grove et al., 2004). Importantly, through these doublings and following cryopreservation, MSC have the ability to retain their undifferentiated phenotype and pluripotency (Bruder et al., 1997; Gamradt and Lieberman, 2004). Cell recovery following thawing from cryopreservation regularly exceeded 95% (Bruder et al., 1997). Finally, for the use of MSC in fracture healing, there was no significant difference between basal ALP activity of cell populations that were differentiated before or after cryopreservation (Bruder et al., 1997).

Research has determined that characteristics such as adherence to tissue culture plastic and spindle shaped morphology in eMSC are consistent with characteristics of MSC from other species (Violini et al., 2009). These cells retain their undifferentiated phenotype and pluripotency through proliferation, maintaining the multilineage ability to undergo adipogenic, osteogenic, and chondrogenic differentiation (Vidal et al., 2006; Arnhold et al., 2007). Also consistent with reports on hMSC, eMSC are not changed when the cells undergo cryopreservation for 1 to 6 months (Arnhold et al., 2007). Research has found no significant difference between the osteogenic differentiation potential of eBMSC before and after cryopreservation (Arnhold et al., 2007).

Through more extensive research, it has been identified that while all hMSC are plastic adherent, there are two different time periods for adherence (Bruder et al., 1997). The first group of cells adheres quickly, in approximately 12 hours and gives rise to colonies soon after adherence (Bruder et al., 1997). The second type requires more time, approximately 2 days, until adherence and do not yield colonies until several days after adherence (Bruder et al., 1997). In the human model both cell types retain their ability to



differentiate along the osteogenic lineage and show no difference in their rates of cell division when exposed to osteogenic media (Bruder et al., 1997). To date, no work has been done in the equine model to identify differences in cells with different adherent times.

### ***Ideal Culture Conditions for MSC***

Research has been done to identify the optimal culture conditions for enhancing the number of MSC through the determination of cell population doubling and colony size (Eslaminejad et al., 2009). Serum type is one key factor that has been tested, comparing fetal bovine serum (FBS) to autologous serum or serum from the same animal. It has been shown, with no statistical difference in cell number doubling or changes in deoxyribonucleic acid (DNA) copy number, that hBMSC are no more responsive to culture in autologous serum than they are to culture in FBS (Dahl et al., 2008). However, researchers found that rat (r) BMSC grown in autologous serum reached confluence, in primary and sub cultures, faster than rBMSC grown in FBS (Eslaminejad et al., 2009). In addition, rBMSC grown in autologous serum showed significantly greater growth rates and larger colony sizes than rBMSC grown in FBS (Eslaminejad et al., 2009). Finally, rBMSC cultured in autologous serum or FBS express alkaline phosphatase (ALP) and osteopontin at similar concentrations, determined by reverse transcriptase polymerase chain reaction (RT-PCR; Eslaminejad et al., 2009). Improved understanding of the ideal methods for proliferation of eMSC will allow researchers to expand isolated samples for further experimentation and for use in therapeutic does.

### ***Differentiation of MSC into Osteoblasts***

For the use of MSC in fracture healing, further research is required to develop an understanding of the differentiation of MSC into osteoblasts. To drive hMSC differentiation into osteoblast cells, ideal culture conditions have been determined. In a human model, culture in medium containing dexamethasone (DEX; 25mg/mL), vitamin C phosphate (VC; 300 ug/mL), and  $\beta$ -glycerophosphate (BG; 10mM) resulted in osteogenic differentiation (Jaiswal et al., 1997). This was duplicated in a rat model, where similar concentrations of VC, BG, and DEX were essential for osteoblast differentiation (Ter Brugge and Jansen, 2002). Finally, in an equine model, osteogenic differentiation has been achieved using similar concentrations of VC and BG supplementation in the presence and absence of DEX, similar to findings in other species (Colleoni et al., 2009; Toupadakis et al., 2010). Although osteoblast differentiation is achieved with and without DEX treatment, cultures treated with DEX show significantly greater increases in osteonectin gene expression than cultures without DEX treatment, suggesting that DEX may enhance osteoblast differentiation (Carpenter et al., 2010).

A number of methods have been used to confirm the differentiation of MSC into osteoblasts. These methods are essential to further characterization of the differentiation in the equine model. Visual confirmation of differentiation is determined with the presence of calcium mineralization indicated by ALP or von Kossa staining (Henrichsen, 1956; Jaiswal et al., 1997). Other methods include determining ALP enzyme activity and real time PT-PCR analysis of the expression of genes such as Runx2 and Osx at different

stages of osteoblast differentiation (Jaiswal et al., 1997; Govoni et al., 2006; Carpenter et al., 2010).

The methods above allowed researchers to establish the basic culture conditions for osteoblast differentiation. Once these conditions were established, Hanada et al. (1997) and Murray et al. (2010) were focused on methods to enhance differentiation. In a rat model, treatment with BMP-2 increased bone nodule formation and calcium content of MSC (Hanada et al., 1997). Similarly, eBMSC treated with BMP-2 displayed an increase in ALP enzyme activity and gene expression, suggesting that treatment with BMP-2 increased eBMSC differentiation into osteoblasts (Murray et al., 2010). A better understanding of the factors and mechanisms that enhance the ability of MSC to differentiate into osteoblasts will allow researchers to continue work examining the novel use of MSC in fracture healing.

## **Rationale**

The studies described in this literature review indicate that eBMSC can be isolated, proliferated, and differentiated into osteoblast cells (Akahane et al., 2008). To date, the regulation of the differentiation of eBMSC into osteoblast cells has not been characterized. Identification of essential transcription factors will provide a better understanding of the process of differentiation. A better understanding of differentiation can be used to enhance the process through manipulation of growth and transcription factors. Understanding and enhancing eBMSC differentiation into osteoblast cells may aid in the therapeutic use of MCS in fracture healing. Based on the research utilizing

BMSC in rats and humans, along with the preliminary work in eBMSC, several hypotheses were developed.

### **Hypotheses**

- 1) Horse serum will induce greater proliferation of eBMSC than FBS
- 2) The addition of Dex and BMP-2 will increase differentiation of eBMSC into osteoblasts
- 3) Expression of Tbx3 will decrease during osteoblast differentiation.

### **Objectives**

To test these hypotheses the following objectives were developed. 1) To isolate eBMSC and optimize the cell culture conditions; 2) to optimize the differentiation of eBMSC into osteoblast cells; and 3) to determine expression of key transcription factors during eBMSC differentiation into osteoblast.

## **Materials and Methods**

### **Animals**

Morgan horses (n = 6; 3 males, 3 females) from the University of Connecticut herd were used for the collection of serum and BMSC. All animals were between the ages of 3 and 5 years old at the time of sample collection. Before all procedures, the veterinarian performed a complete physical examination. The physical examination included body temperature, heart rate, respiratory rate, capillary refill time, mucous membrane color, and abdominal and thoracic auscultation. For sample collections, animals were restrained in stocks wearing a halter in a closed room. All protocols used in this experiment were approved by the Institutional Animal Care and Use Committee at the University of Connecticut (Protocol: A09-021).

Bone marrow aspirates from the first three animals were used to optimize isolation of the MSC from the bone marrow. All experiments detailed in this section were performed on the BMSC from the final three animals. All experiments were repeated two to four times using cells from each of the three animals. Results were consistent across animals and replications. Due to the consistency of results and for ease of reporting, the data presented are the results from one replication from one animal.

### **Serum Collection**

At least one week before bone marrow aspiration, blood was taken to provide serum for cell culture experiments. The side of the neck, in the area of a jugular vein, was clipped and prepared aseptically. Animals were sedated with detomidine

hydrochloride (Dormosedan; Pfizer) at a dose of 0.02 mg/kg body weight, administered intravenously to ensure a safe and efficient sample collection. Approximately 500 mL of blood was taken and stored in 50 mL tubes. Blood was incubated at room temperature for 2 to 4 hours to allow clotting. The blood was then refrigerated at 4° C for 10 to 12 hours. Next, the tubes were centrifuged at 2,016 rcf for 30 minutes at 4° C. In a sterile environment, the serum was transferred by gently pipetting (using a sterile transfer or serological pipette) into a new tube. The serum was filtered through 0.22 µm pores. Next, the serum was frozen at -20° C for 12 hours. The next day, the serum was thawed to room temperature and heat inactivated. To heat inactivate the serum, it was incubated at 56° C for 30 minutes. Following heat inactivation serum was filtered through 0.22 µm pores and stored at 4° C for immediate use or -20° C for future use in cell culture experiments (Toupadakis et al., 2010).

### **Bone Marrow Aspiration**

Before the bone marrow aspiration procedure, the neck of the horse was clipped and prepared aseptically, to allow access to the jugular vein. Next, approximately 8 mL of 2% lidocaine were used to infiltrate the subcutaneous area directly above a jugular vein. At this site a 14 gauge, 6 inch catheter was inserted. Once the catheter was secured, the horse was sedated with detomidine hydrochloride (Dormosedan) at a dose of 0.02 mg/kg body weight, and butorphanol tartrate (Torbugesic; Fort Dodge) at a dose of 0.1 mg/kg body weight, administered intravenously. Next, the area of the sternum was clipped and prepared aseptically. Approximately 8 mL of 2% lidocaine were used to infiltrate the subcutaneous tissue, muscle, and periosteum to provide local analgesia.

After local infiltration, the area was prepared surgically using sterile gloves. A #10 surgical blade was used to make a stab incision through the soft tissue area of the sternum. A Jamshidi bone marrow biopsy needle was introduced through the stab incision and advanced through the muscle layers until it made contact with the ventral surface of the sternebrae (Smith et al., 2003). Pressure was applied to advance the needle until it was seated in the bone to a depth of 1 to 2 cm. The stylet was then removed and a 20 mL syringe containing heparin was attached. Multiple quick and forceful aspirations were done to dislodge bone marrow particles with minimal peripheral blood contamination. A total of 5 to 15 mL of bone marrow were obtained from each horse. Once the sample was obtained, the stylet was replaced and the Jamshidi needle removed. The skin stab incision was closed with one to three skin staples. A sterile gauze with povidone iodine ointment (Betadine; Purdue Pharma) was held over the area for 5 minutes to facilitate hemostasis.

### **Post Procedure Horse Care**

Following bone marrow aspiration, all horses were housed in a 12 x 12 foot stall. Horses were provided with clean water ad libitum, and hay and grain twice daily at maintenance rations. Horses remained in the stall for 24 hours per day for three days following the procedure. For the fourth through fourteenth day following the procedure, animals were hand-walked daily, for increasing periods of time from 5 to 60 minutes. Physical examination was performed twice daily for the first 5 days, and then once daily for the next 7 days. This included taking temperature, heart rate, and respiratory rate, along with evaluation of intestinal motility and the biopsy site. Horses received flunixin

meglumine (Banamine; Schering-Plough) at 1.1 mg/kg body weight, twice daily for the first three days and then once daily for days four through six. The skin staples were removed from the biopsy site 14 days after collection of the bone marrow sample. Following the 14 day stall rest, staple removal, and passing veterinary examination, the horse was returned to the herd and resumed normal daily activity.

### **Culture of Bone Marrow Stromal Cells**

After bone marrow was aspirated, the sample was placed on ice and brought to the laboratory. The sample was transferred from the aspiration syringe into a 50 mL tube and centrifuged at 1,000 rcf for 3 minutes at room temperature. The supernatant was removed and the pellet re-suspended in 10 mL minimum essential medium  $\alpha$  ( $\alpha$ -MEM; Invitrogen) and 40 mL ammonium chloride (STEMCELL, cat. no. 07800) to lyse red blood cells. The sample was vortexed and placed on ice for 10 minutes. The sample was then centrifuged at 1,000 rcf for 3 minutes at room temperature. The supernatant was removed and the cells were resuspended in  $\alpha$ -MEM. The sample was vortexed and visually analyzed under the microscope to ensure red blood cell lysis. Ammonium chloride treatment was repeated if red blood cells were still present. When the red blood cells were lysed the sample was filtered through 70  $\mu$ m cell strainer. The sample was then reconstituted in maintenance media (Table 1) and plated in one 100 mm cell culture dish for every 2 mL original bone marrow. The cell culture dishes were incubated at 37° C, 5% CO<sub>2</sub> for 20 minutes. After a 20 minute incubation period, non-adherent cells were removed from the cell culture dish. The non-adherent cells were counted and replated at 6 or 12 million cells per plate in maintenance media (Table 1). The adherent cells that



attached within 20 minutes, on the original plates, were provided with 6 mL fresh maintenance media (Table 1). Cells were incubated at 37° C, 5% CO<sub>2</sub> for three days. After three days of incubation, 4 mL fresh maintenance media (Table 1) were added to the plates. After 6 days of incubation, media and non-adherent cells were removed and the plate was washed with phosphate buffered saline (PBS; Animal Cell Culture Facility, Storrs, CT) to remove residual cells and debris. After washing with PBS, 6 mL of maintenance media (Table 1) was added to the plates. To continue cell growth, maintenance media (Table 1) was replaced every three days until cells reached 70% confluence.

**Table 1: Cell Culture Medias**

Maintenance media	$\alpha$ -MEM + 10% FBS + 1% PS + 1% AB
Proliferation serum free media	$\alpha$ -MEM + 0.1% BSA+ 1% PS + 1% AB
ALP serum free media	$\alpha$ -MEM + 0.1% BSA + 1% PS + 1% AB + VC
Differentiation media	$\alpha$ -MEM + 10% FBS + 1% PS + 1% AB + VC + BG + DEX

**Abbreviations:**

AB = amphotericin B;  $\alpha$ -MEM = minimum essential medium  $\alpha$ ;  
 BG = glycerol 2-phosphate disodium salt hydrate; BMP-2 = bone morphogenic protein;  
 BSA = bovine serum albumin; DEX = dexamethasone; FBS = fetal bovine serum;  
 HS = horse serum; PS = penicillin/streptomycin concentrate;  
 VC = L-ascorbic acid 2-phosphate sesquimagnesium salt

**Cell Passage**

When the cells reached about 70% confluence they were passed (Appendix 1). First, cells were rinsed with PBS. Next, the cells were lifted from the plate after a three minute incubation with 0.25% Trypsin + 0.5 mM EDTA (Animal Cell Culture Facility, Storrs, CT) + 50% PBS and a cell scraper. Cells were removed from suspension by centrifugation at 1,000 rcf for 3 minutes at room temperature and reconstituted in

maintenance media (Table 1). To expand cells, they were re-plated in cell culture dishes at 200,000 cells per dish (Appendix 1).

### **Cell Proliferation**

To determine proliferation, two methods were utilized: alamarBlue assay and 5-bromo-2'-deoxyuridine (BrdU) assay. The alamarBlue assay is an indicator of cell number because it uses a water soluble dye that is an oxidation-reduction indicator (Nakayama et al., 1997). The indicator, resazurin, is reduced to resorufin and fluoresces in response to metabolic reactions in the cell. The BrdU assay indicates cell proliferation by substituting labeled BrdU for thymidine thus indicating which cells are actively replicating their DNA through the fluorescence in the BrdU (Malaval et al., 1999). For alamarBlue (Appendix 2) and BrdU (Appendix 3) assays, cells were passaged once and plated in a 96-well plate at a density of 3,000 cells in 100  $\mu$ L maintenance media, per well. Cells were allowed to adhere for 48 hours. Next, proliferation serum free media (Table 1) was used to rinse the cells. Following rinsing fresh proliferation serum free media (Table 1) was added. Twenty-four hours after serum deprivation, the cells were treated with or without 5% FBS, or 5% HS in proliferation serum free media (Table 1) for 48 hours. For the alamarBlue assay cells were rinsed twice with PBS before the addition of a 1:10 dilution of alamarBlue indicator (Invitrogen) to  $\alpha$ -MEM at 100  $\mu$ L per well. The plate was incubated for 4 hours at 37° C, 5% CO<sub>2</sub>. Following incubation, fluorescence was detected by the plate reader (Synergy 2, BioTek) at 540/35 x 600/40 nm with sensitivity set at 54 (Appendix 2). For the BrdU assay, the Cell Proliferation ELISA, BrdU (chemiluminescent) Kit (Roche Applied Science) was used, according to

the protocol provided by the manufacturer, after the 48 hour treatment. Specifically, the cells were incubated with BrdU labeling solution at a concentration of 10  $\mu$ L/well for 4 hrs at 37° C. After the incubation, the labeling solution was removed, cells were fixed, and DNA was denatured. This was followed by the addition of anti-BrdU-POD antibody (100uL/well) which binds to the BrdU incorporated in the newly synthesized DNA. The excess antibody was removed, and 100  $\mu$ L of the substrate solution was added to each well and incubated for 5 min at room temperature. Finally, the chemiluminescence was detected by the plate reader (Synergy 2, BioTek; Appendix 3).

## **Cell Differentiation**

### ***Alkaline Phosphatase Activity***

To determine osteoblast differentiation, ALP enzyme activity was determined (D'Ippolito et al., 1999). Alkaline phosphatase enzyme activity is only found in differentiated bone where calcium deposits are formed. For ALP assays (Appendix 4), cells were passaged once and plated in a 96-well plate at a concentration of 6,000 cells in 100  $\mu$ L maintenance media, per well. Forty-eight hours after plating, cells were rinsed twice and then re-plated in ALP serum free media (Table 1). After 24 hours, the cells were treated in the presence or absence of 30 or 60 ng/ml hBMP-2 (PeproTech), 25 mg/ml DEX (Sigma Aldrich), or both in ALP serum free media (Table 1; Murray et al., 2010). Seventy-two hours after treatment, cells were washed twice with PBS and permeabilized with 100  $\mu$ L of 0.1% triton, followed by a freeze and thaw. Of the total 100  $\mu$ L, 40  $\mu$ L and 20  $\mu$ L of the lysate were transferred to two new 96-well plates for determination the ALP activity and protein concentration, respectively. To determine

ALP activity, 200  $\mu$ L of the ALP substrate, p-Nitrophenyl phosphate (Sigma Aldrich), were added to each well. The absorbance was read immediately (0 hr) and kinetically every 10 minutes for approximately two hours after substrate addition at 405 nm using the plate reader (Synergy 2, BioTek). The protein concentration was determined by measuring the absorbance following treatment with Quick Start Bradford Protein (Bio-Rad). The protein concentration was standardized with serial dilutions of bovine serum albumin (BSA; Sigma Aldrich). The ALP activity was standardized to the cellular protein content (Appendix 4).

### ***Staining***

To confirm osteoblast differentiation, Alizarin Red Staining (Appendix 5) and Alkaline Phosphatase Staining (Appendix 6) were performed. Equine BMSC were plated in 6-well plates with 150,000 cells per plate in 2 mL of maintenance media (Table 1). When cells reached 90% confluence, media was changed to differentiation media (Table 1). Staining was performed on separate plates on Days 0 and 18. Alizarin Red Staining identifies osteoblast differentiation by identifying calcium deposits in cells with red dye. Briefly, media was removed from the culture plate, the plate was fixed with cold methanol, and then frozen at -20° C for 12 hours. After freezing, the methanol was removed, the plate was stained the Alizarin Red, and incubated on a rotator at room temperature for 10 minutes. After incubation, the stain was removed and the plate was air-dried (Appendix 5). Alkaline Phosphatase Staining identifies ALP enzyme products in osteoblast cells. Briefly, the media was removed and cells were fixed in cold 100% methanol for 20 minutes at room temperature. After fixation, methanol was removed,

and substrate-diazonium solution was added to each well. Next, cells were incubated for 30 minutes in 37°C, the stain was removed, and the plate was stored at 4°C wrapped in foil (Appendix 6).

### ***Messenger Ribonucleic Acid (mRNA) Gene Expression***

To determine gene expression during differentiation, cells were passaged once and plated in 6-well plates at 150,000 cells per well in maintenance media (Table 1). When cells reached 90% confluence, media was changed to differentiation media (Table 1) and this was considered Day 0. Media was changed every three days from Day 0 through Day 18. In addition to media being changed every three days, sample plates were also taken for RNA extraction every three days (Appendix 7). For RNA extraction, media was removed from the plate and each well was rinsed once with 2 mL of PBS. Following rinsing, 1 mL of TRI Reagent (Sigma Aldrich) was added to each well and wells were scraped with a pipette tip to lift cells. The TRI reagent mixture was then transferred to a micro-centrifuge tube and frozen at -80° C. When all desired samples were collected all micro-centrifuge tubes were thawed and vortexed. This was followed by the addition of one-fifth volume of > 99% Chloroform (Sigma Aldrich). The tube was then mixed and incubated for 2 to 3 minutes at room temperature. After the incubation, the tubes were centrifuged at 12,000 rcf for 15 minutes at room temperature. The aqueous, clear, supernatant phase was carefully transferred to a new micro-centrifuge tube. Next, RNA was extracted using Rneasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer (Appendix 7). Following extraction, RNA concentration was determined by Nano-Drop spectrophotometer and contaminating DNA was removed

(Appendix 8). DNA was removed by 30 minute digestion at 37°C using the DNase in the TURBO DNA-free kit (Ambion; Appendix 6). RNA concentration was once again determined by NanoDrop spectrophotometer. Next, RNA quality was determined using the Experion RNA StdSens Analysis Kit protocol (Bio-Rad). Once concentration and quality were determined, all RNA samples were made into 10 µL aliquots at a concentration of 30 ng/µL (total 300 ng) to be used for reverse transcription RT-PCR.

To reverse transcribe the RNA samples (Appendix 9), 1 µL of Oligo (dT) primer (Ambion) was incubated with 10 µL of the RNA sample at 70° C for 10 min in the S1000 Thermo Cycler (Bio-Rad). This was followed by the addition of the master mix containing 1 µL of dNTP, 2 µL of DTT, 5.5 µL of 5X Buffer and 0.5 µL of SuperScript II (Invitrogen). Next the samples were returned to the Thermo Cycler and the reaction conditions were 37°C for 3 min, 42°C for 60 min, 4°C for 3 min, and 90°C for 2.5 min. Once the reactions were complete, 80 µL of nuclease free water was added to the samples (Appendix 9).

Real-time RT-PCR analysis was used to determine the expression levels of the genes listed in Table 2. Real-time RT-PCR (Appendix 10) was performed using 7900HT Fast Real-Time PCR system (Applied Biosystems). Each reaction contained 10 µL of 2X Power SYBR Green Master Mix (Applied Biosystems), 1 µL each of 10 mM of forward and reverse primer (Table 2), 3 µL of nuclease free water, and 5 µL of cDNA. The total of 20 µL of the reaction was loaded onto a Fast Optical 96 well reaction plate (Applied Biosystems; Appendix 10). Once real-time RT-PCR was complete, the  $\Delta C_T$  values were determined by subtracting the  $C_T$  value for the control gene from the  $C_T$  value for the

gene of interest. Once the  $\Delta C_T$  was determined for the control and the treatment groups,  $\Delta\Delta C_T$  was calculated to express the relative quantification of gene expression (Govoni et al., 2006).

**Table 2: RT-PCR Primers**

<i>Primers</i>	
Gene	(5' – 3')
Tbx3 (Forward)	GCA TCC CTT TCT CGT CTC TG
Tbx3 (Reverse)	GAC CAT CTC GGT ACC CCT CT
Osx (Forward)	GCT CAC TAT GGC TCC AGT CC
Osx (Reverse)	AAG GTC ACT GCC CAC AGA GT
Runx2 (Forward)	CAG ACC AGC AGC ACT CCA TA
Runx2 (Reverse)	GCA GCA TTC TGG AAG GAG AC
ALP (Forward)	GAC ATG ACC TCC CAG GAA GA
ALP (Reverse)	GCA GTG AAG GGC TTC TTG TC
Type I Collagen (Forward)	TTG ACC CTA ACC AAG GAT GC
Type I Collagen (Reverse)	TTC TTG GCT GGG ATG TTT TC
OC (Forward)	GTG CAG AGT CTG GCA GAG GT
OC (Reverse)	CCA GCC AAT GAT CCA GGT AG
GAPDH (Forward)	ATC ACT GCC ACC CAG AAG AC
GAPDH (Reverse)	GTG AGC TTC CCA TTC AGC TC

**Abbreviations:**

ALP = alkaline phosphatase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; OC = osteocalcin; Osx = osterix; Runx2 = runt-related transcription factor 2; Tbx3 = T-box 3

***Primer design and validation***

The primers listed (Table 2) were used in real-time RT-PCR to amplify the associated equine genes. These primers were designed using FASTA sequences (from [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and Primer3 ([frodo.wi.mit.edu/primer3](http://frodo.wi.mit.edu/primer3)). To ensure that the primer only amplified the desired equine gene, Blast was used ([blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)). In the laboratory, PCR was performed using the primers across a temperature gradient to ensure gene expression and optimize to use at

60°C (Appendices 11 and 12). Next primer concentration was optimized to 10 µmol using real time RT-PCR efficiency curve. Real-time RT-PCR was performed using serial dilutions of control cDNA with serial dilutions of primer. Results of the real time RT-PCR with serial dilutions were analyzed with Applied Biosystems SDS Software version 2.4 Standard Curve option following Absolute Quantification (Alonso et al., 2004).

### **Statistical Analysis**

Data were analyzed by analysis of variance (ANOVA) using SAS 9.2 software (SAS Institute Inc.). Data are presented as mean ± standard error (SE) and significant difference was determined at  $P \leq 0.05$ .



## **Results**

### **Proliferation Rates of Different Cell Populations**

To determine if early and late adherence cell populations proliferate at different rates, cell number and BrdU incorporation were determined. When cells were cultured in the absence of serum, a significant difference in the number of cells between early and late adherence groups was not observed [ $285,831 \pm 7,462$  and  $272,720 \pm 7,462$  relative fluorescence units (rfu), respectively;  $P > 0.2$ ; Figure 1]. Similar to cell number, no difference was observed in the BrdU incorporation of early and late adherence cells cultured in the absence of serum [ $320,731 \pm 29,848$  and  $341,486 \pm 29,848$  relative light units per second (rlu/s), respectively;  $P > 0.6$ ; Figure 2]. In both early and late adherence cell populations, treatment with FBS increased cell number  $134 \pm 5\%$  and  $133 \pm 5\%$ , respectively ( $P < 0.0001$ ; Figure 1). Finally, for early and late adherence cells there were similar increases in BrdU incorporation after FBS treatment of early ( $173 \pm 16\%$ ) and late ( $160 \pm 16\%$ ) adherence cells ( $P < 0.0001$ ; Figure 2).

### **Effect of FBS vs. HS on Cell Proliferation**

To identify the optimal culture conditions for eBMSC the cells were cultured in the presence or absence of FBS or HS. Following a 48 hour treatment period, compared with control cells cultured in proliferation serum free media, cells treated with FBS had greater increase in cell numbers ( $133 \pm 5\%$ ) than cells treated with HS ( $116 \pm 5\%$ ;  $P < 0.02$ ; Figure 3A). In addition, when compared with control cells cultured in proliferation

serum free media, cells treated with FBS had greater BrdU incorporation ( $167 \pm 6\%$ ) than cells treated with HS ( $120 \pm 6\%$ ;  $P = 0.004$ ; Figure 3B).

### **Effect of hBMP-2 and DEX on Differentiation of eBMSC into Osteoblasts**

No effect of hBMP-2 was observed at 0, 30, and 60 ng/ml [ $140 \pm 87$ ,  $143 \pm 81$ , and  $159 \pm 81$  milliunits ALP per milligram protein (mU/mg), respectively], on ALP activity ( $P > 0.8$ ; Figure 4). Dexamethasone treatment increased ALP activity 6-fold ( $2,661 \pm 72$  and  $405 \pm 54$  mU/mg, control and DEX, respectively;  $P < 0.0001$ ; Figure 5).

To determine if the presence of DEX would improve the effect of hBMP-2 on differentiation, eBMSC were treated with the combination of DEX and hBMP-2. The addition of DEX to the culture did not improve the effect of hBMP-2 at 0, 30, or 60 ng/ml ( $2,661 \pm 57$ ,  $2,310 \pm 76$ , or  $2,621 \pm 76$  mU/mg, respectively;  $P > 0.05$ ; Figure 6).

### **Gene Expression During Differentiation of eBMSC into Osteoblasts**

To confirm that eBMSC successfully differentiated into osteoblasts by Day 18 of culture, cells were stained with Alizarin Red on Days 0 and 18 (Figure 7A and 7B). By Day 18 of culture a 4-fold increase in Alizarin Red stain was observed; demonstrating successful differentiation into osteoblasts ( $P < 0.05$ ; Figure 7C). Confirm of eBMSC differentiation into osteoblasts was also determined with a 50% increase in ALP staining, from Day 0 to Day 18 ( $P < 0.05$ ; data not shown). As expected during differentiation, ALP expression increased by Day 3 and remained increased through Day 18 of culture ( $P < 0.05$ ; Figure 8). Osteocalcin expression also significantly increased over 200-fold by Day 18 of culture ( $P < 0.05$ ; Figure 8). Expression of Runx2 increased 3-fold on Day 6

and remained increased through Day 18 of culture ( $P < 0.05$ ; Figure 9). Osterix expression increased as early as Day 3 and increased 9-fold by Day 18 ( $P < 0.05$ ; Figure 9). The changes observed in these osteoblast makers, ALP and OC, and transcription factors, Runx2 and Osx, demonstrate successful differentiation of eBMSC into osteoblasts. T-box 3 expression increased 2-fold at Day 3, but then decreased greater than 4-fold by Day 15 of culture ( $P < 0.05$ ; Figure 9).

## Figure Legends

Figure 1: A similar cell number was observed between early and late adherence eBMSC in the absence and presence of serum. Cells (early and late) were cultured in the presence (0.1% BSA + 10% FBS) or absence (0.1% BSA) of serum. Cell number was determined by alamarBlue assay and expressed as rfu. Data are presented as mean  $\pm$  SE. Bars with different letters (a or b) are significantly different at  $P < 0.05$ . BSA = bovine serum albumin; eBMSC = equine bone marrow stem cells; FBS = fetal bovine serum; rfu = relative fluorescence units; SE = standard error.

Figure 2: Similar BrdU incorporation was observed between early and late adherence eBMSC in the absence and presence of serum. Cells (early, with 20 minute adherence and late, with 24 hour adherence) were cultured in the presence (0.1% BSA + 5% FBS) or absence (0.1% BSA) of serum. Proliferation was determined by BrdU assay and expressed as rlu/s. Data are presented as mean  $\pm$  SE. Bars with different letters (a or b) are significantly different at  $P < 0.05$ . BrdU = 5-bromo-2'-deoxyuridine; BSA = bovine serum albumin; eBMSC = equine bone marrow stem cells; FBS = fetal bovine serum; rlu/s = relative light units/second; SE = standard error.

Figure 3: Proliferation was greater in cells cultured with FBS compared with HS. eBMSC were treated with 0.1% BSA (control), 0.1% BSA + 5% FBS, or 0.1% BSA + 5% HS and effects of treatment on proliferation were determined by cell number (A) and BrdU incorporation (B). Data are presented as mean  $\pm$  SE and expressed as a % of control. \* indicates a significant difference between the two treatments at  $P < 0.02$ . BrdU = 5-bromo-2'-deoxyuridine; BSA = bovine serum albumin; eBMSC = equine bone marrow stem cells; FBS = fetal bovine serum; HS = horse serum; SE = standard error.

Figure 4: Human BMP-2 treatment did not alter ALP enzyme activity. Cells were treated with 0, 30, or 60 ng/ml hBMP-2. Differentiation was determined by ALP enzyme activity. Data are presented as mean  $\pm$  SE. A significant difference was not observed between treatment groups at  $P \geq 0.8$ . ALP = alkaline phosphatase; hBMP-2 = human bone morphogenetic protein 2; SE = standard error.

Figure 5: Dexamethasone treatment increased ALP enzyme activity. eBMSC were cultured with (0.1% BSA + 25 mg/mL DEX) or without (0.1% BSA; Con) DEX. Differentiation was determined by ALP enzyme activity. Data are presented as mean  $\pm$  SE. \* indicates a significant difference between the two treatments at  $P < 0.0001$ . ALP = alkaline phosphatase; BSA = bovine serum albumin; Con = control; DEX = dexamethasone; eBMSC = equine bone marrow stem cells; SE = standard error.

Figure 6: In the presence of DEX, no effect of hBMP-2 was observed. eBMSC were treated with 0, 30, or 06 ng/ml hBMP-2 in the presence of DEX (25 mg/mL). Differentiation was determined by ALP enzyme activity. Data are presented as mean  $\pm$  SE. A significant increase was not observed with treatment at  $P > 0.6$ . ALP = alkaline phosphatase; BSA = bovine serum albumin; DEX = dexamethasone; eBMSC = equine bone marrow stem cells; hBMP-2 = human bone morphogenetic protein 2; SE = standard error.

Figure 7: Confirmation of osteoblast differentiation by Alizarin Red Stain. eBMSC were treated with differentiation media for 18 days. Cells were stained with Alizarin Red on Day 0 (A) and 18 (B) of culture. Images are a representative of 6 replicates. Quantification of mineralization (C) is presented as mean  $\pm$  SE. \* indicates a significant difference between Days 0 and 18 at  $P < 0.05$ . eBMSC = equine bone marrow stem cells; SE = standard error;

Figure 8: Increased mRNA expression of ALP and OC during differentiation. Cells were plated in differentiation media and mRNA expression was determined by real-time RT-PCR. RNA were collected for analysis every three days between Day 0 and Day 18 of culture, with  $n = 6/\text{day}$ . Data are presented as mean fold change from Day 0  $\pm$  SE. \* indicates significant difference from Day 0 at  $P < 0.05$ . mRNA = messenger ribonucleic acid; ALP = alkaline phosphatase; OC = osteocalcin; RT-PCR = reverse transcriptase polymerase chain reaction; SE = standard error.

Figure 9: Decreased expression of Tbx3 during differentiation. Cells were plated in differentiation media and mRNA expression were determined by real-time RT-PCR. RNA were collected for analysis every three days between Day 0 and 18, with  $n = 6/\text{day}$ . Data are presented as mean fold change from Day 0  $\pm$  SE. \* indicates that a significant change in mRNA expression from Day 0 was observed for all genes on all days, except for Tbx3 expression on Day 6. mRNA = messenger ribonucleic acid; Osx = osterix; RT-PCR = reverse transcriptase polymerase chain reaction; Runx2 = runt-related transcription factor 2; SE = standard error; Tbx3 = T-box 3.

Figure 1

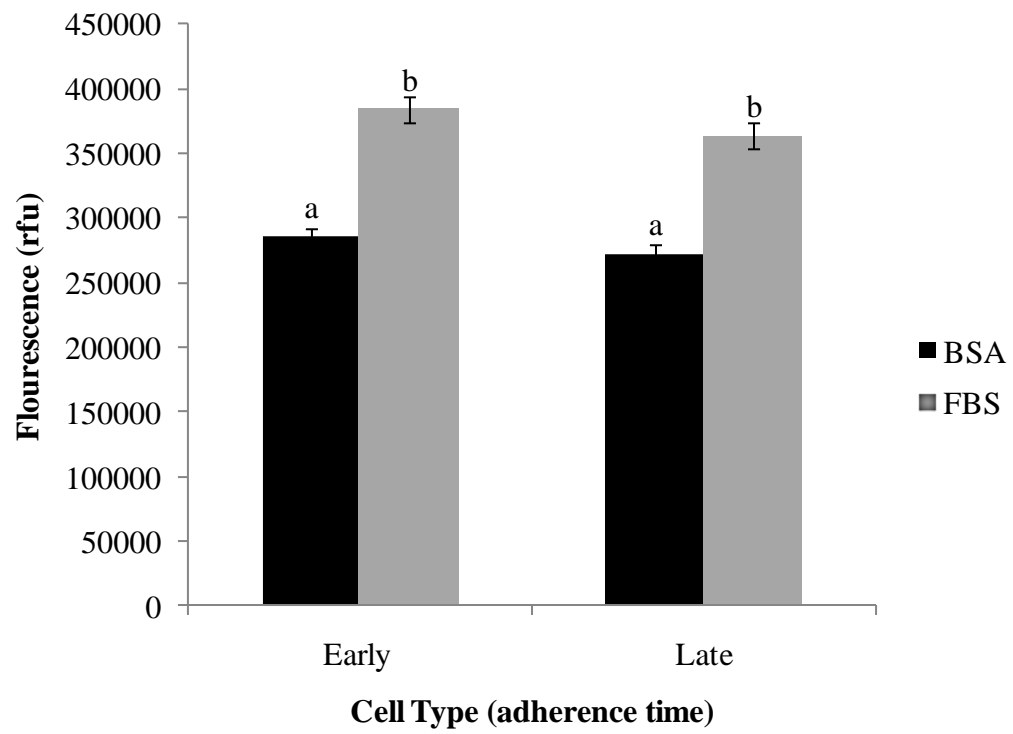


Figure 2

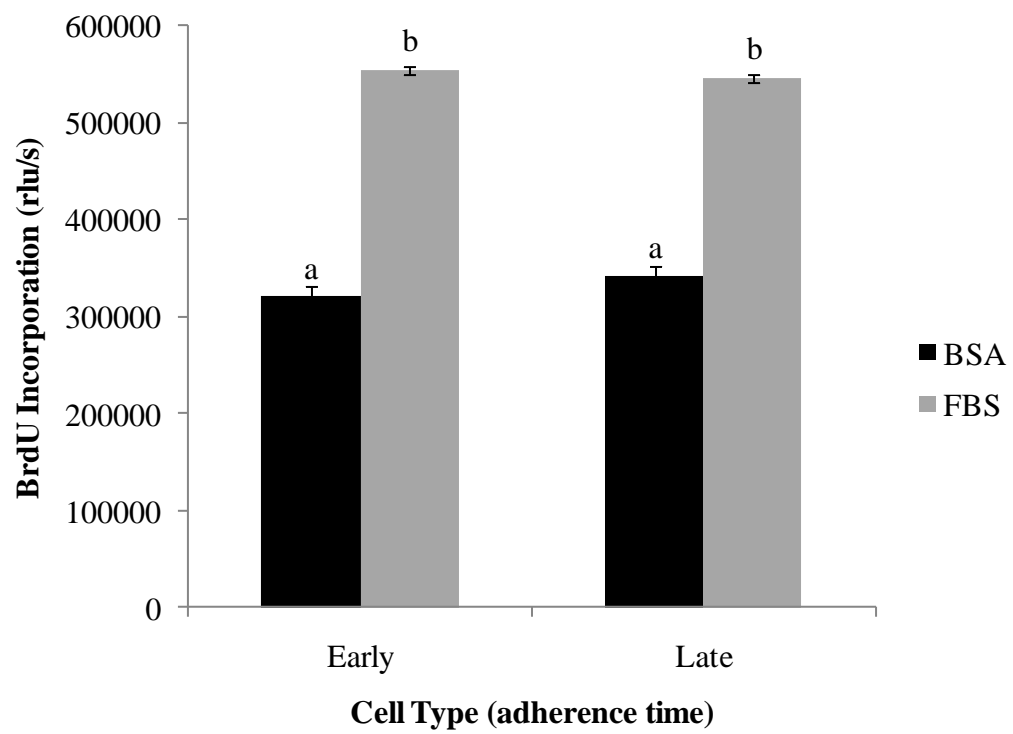
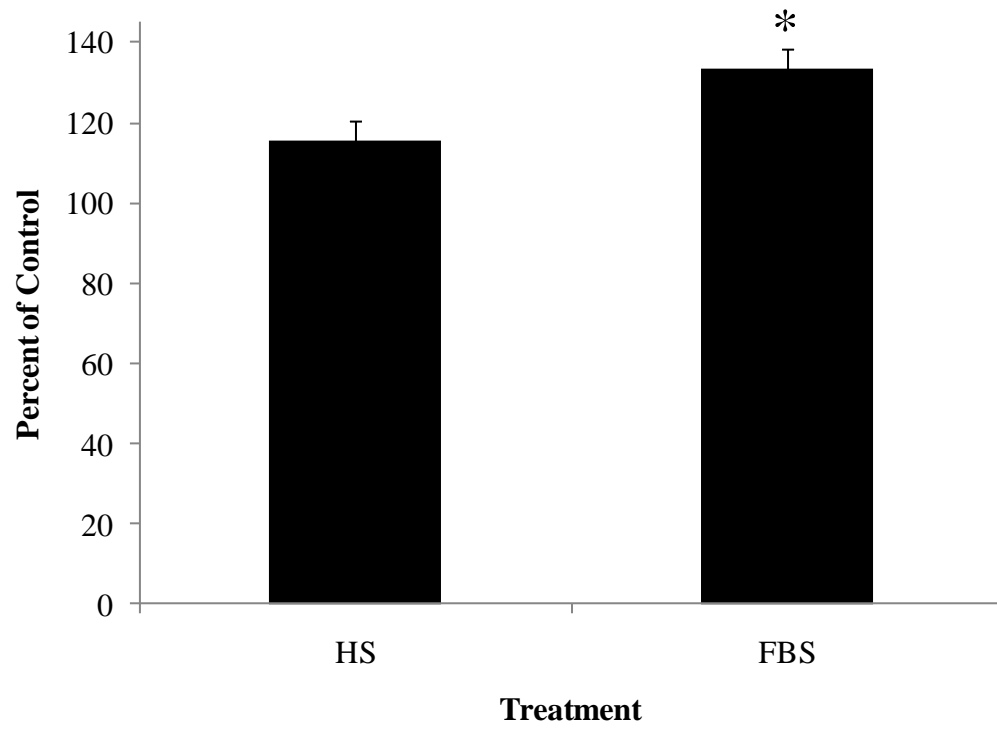


Figure 3

A



B

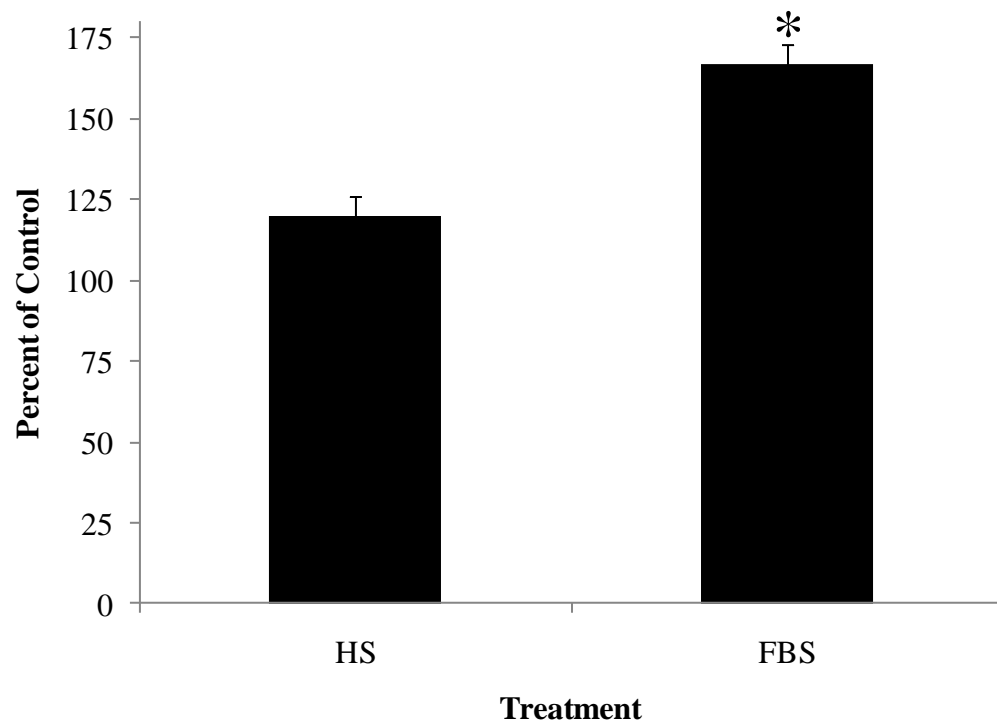


Figure 4

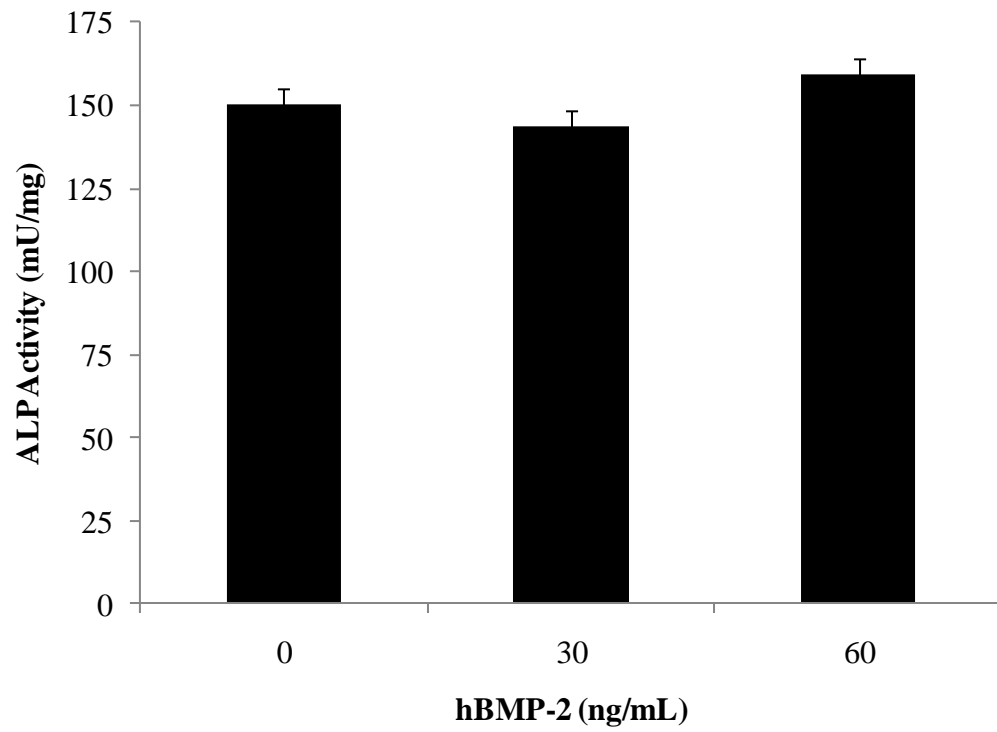


Figure 5

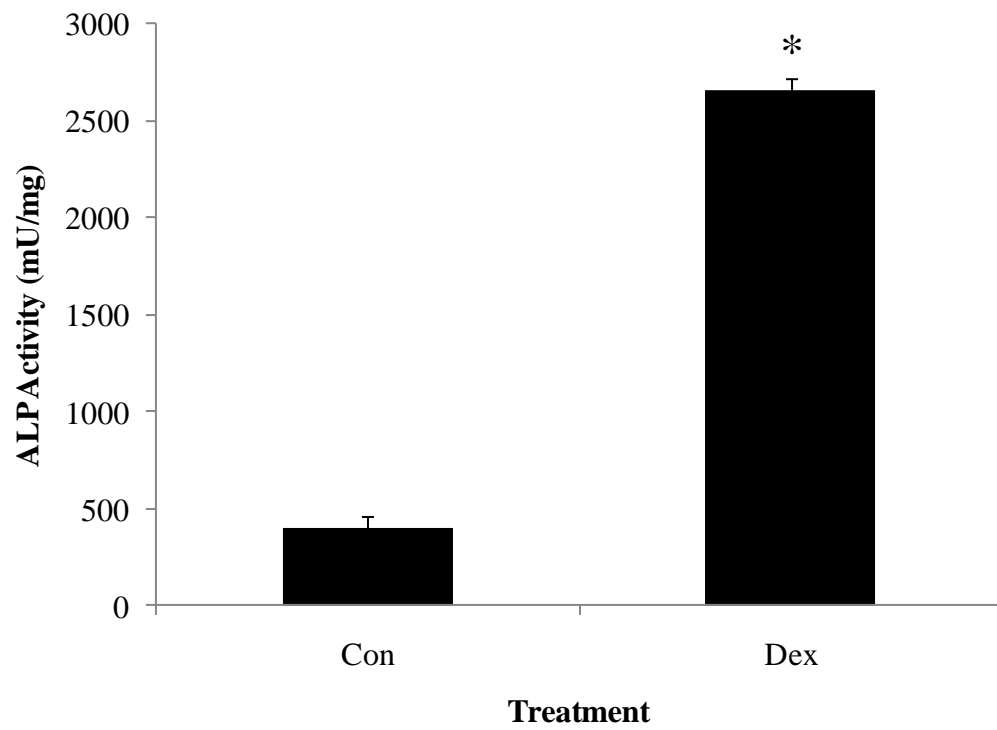




Figure 6

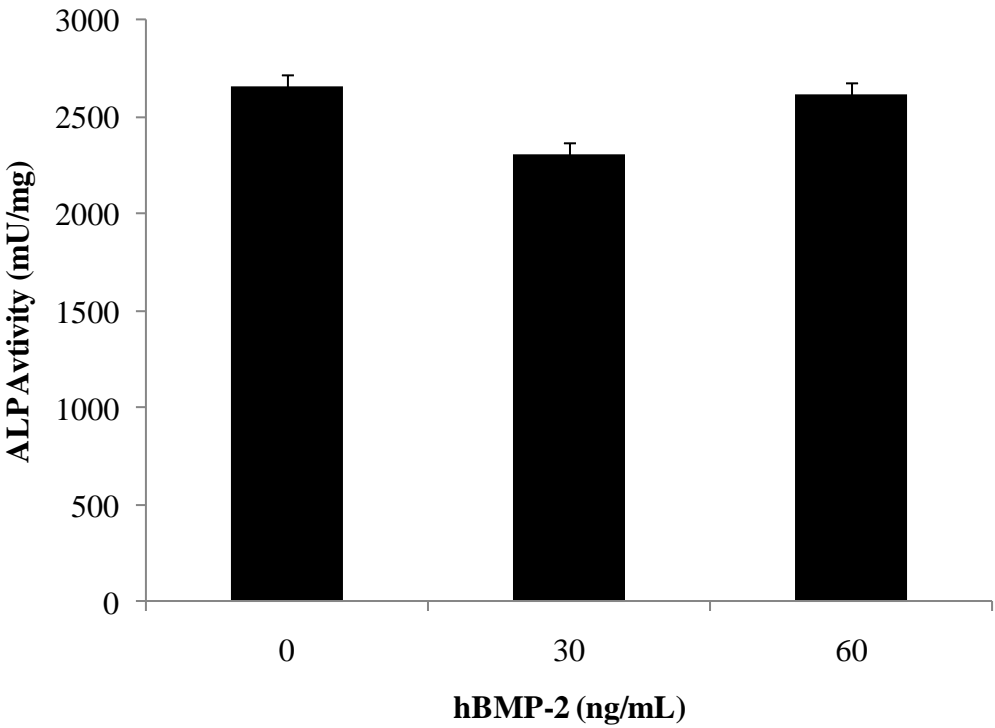


Figure 7

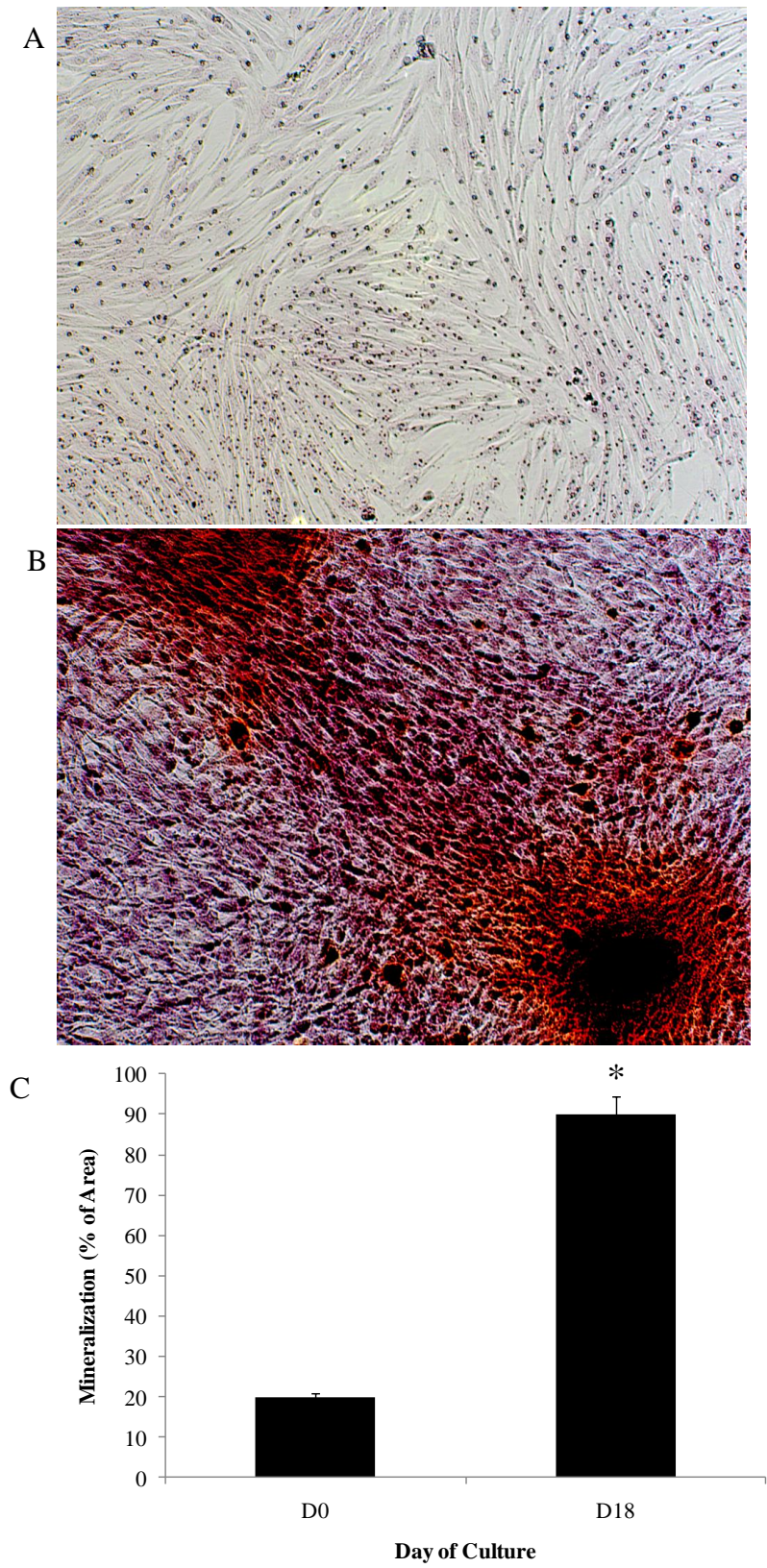


Figure 8

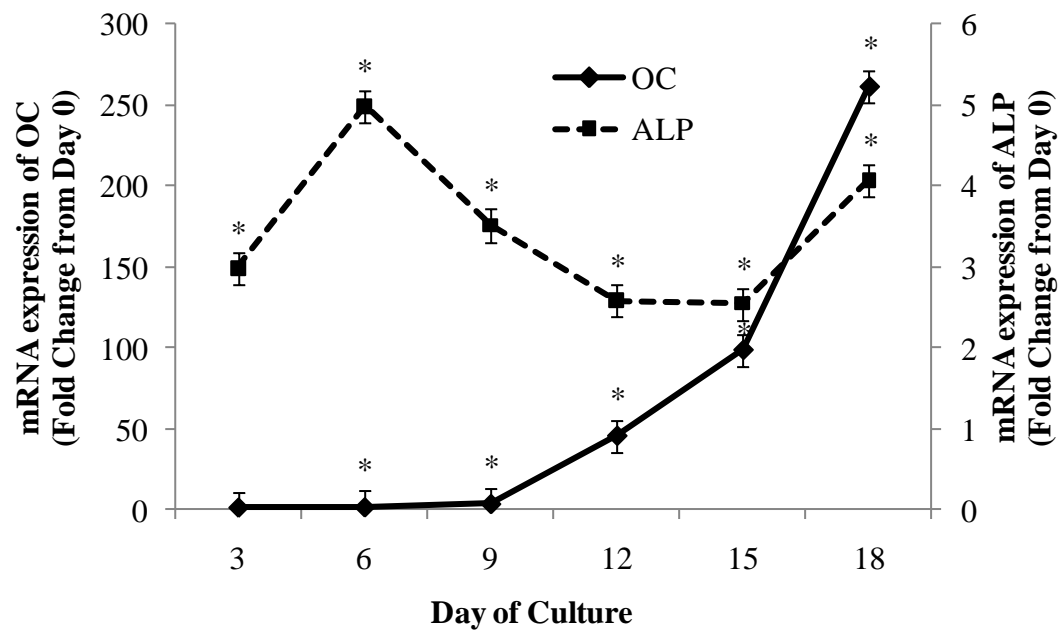
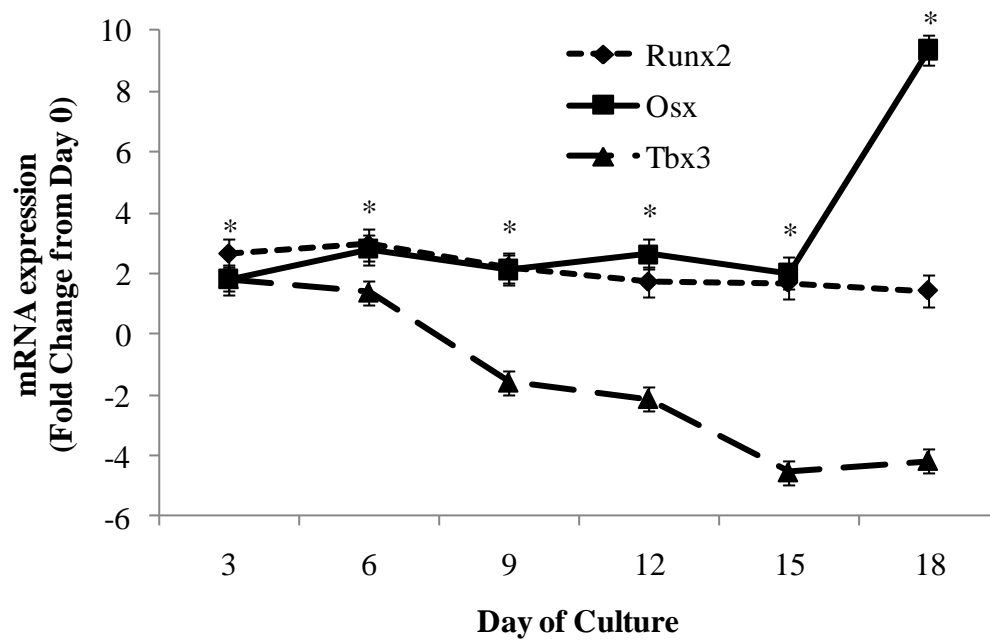


Figure 9



## Discussion

The use of eBMSC as a therapeutic aid in fracture healing requires their isolation, *in vitro* expansion, and reintroduction into the fracture site. This study indicated that isolation, proliferation, and *in vitro* osteoblast differentiation of eBMSC is possible. Following isolation, the culture conditions for both proliferation and differentiation of eBMSC into osteoblasts were optimized. FBS was identified as the ideal serum for eBMSC proliferation, and it was determined that DEX is essential for optimal differentiation of eBMSC into osteoblasts. This research also allowed us to identify and characterize the expression of a number of genes associated with eBMSC differentiation into osteoblasts. The expression of osteoblast makers ALP and OC, and transcription factors, Runx2 and Osx was characterized. Based on the similar expression of these genes in the equine cells, compared with the human and murine models, it was determined that the regulation of eBMSC differentiation into osteoblasts is similar to that of other species. It was also determined that the transcription factor, Tbx3, is expressed in the eBMSC, and that the expression of Tbx3 is reduced when osteoblast differentiation occurs. Although eBMSC were successfully cultured and mechanisms that may regulate the differentiation of eBMSC into osteoblasts were identified, further work is needed. This work should identify key factors that may be used to improve eBMSC differentiation into osteoblast cells, as well as perform clinical trials to determine the *in vivo* response to this therapeutic method.

The isolation of eBMSC from six horses was successful. This cell culture system resulted in similar cell growth characteristics as observed in the system used by Vidal et al. (2006). Similar cell numbers and times between plating and confluence were

observed (Vidal et al., 2006). Previous groups have successfully used  $\alpha$ -MEM for cell culture and observed cell viability at  $83 \pm 11\%$  following cryopreservation (Arnhold et al., 2007). This cell culture system has similar success with  $\alpha$ -MEM and cryopreservation, thus demonstrating that these methods allow for optimal culture and experiments with eBMSC.

Using alamarBlue and BrdU experimental assays, no differences in proliferative abilities between the early and late adherence cell populations were observed. These two different cell populations were first identified in a human model (Bruder et al., 1997). These researchers identified hBMSC that selectively attached to tissue culture plastic, but did not have uniform adherence times or colony growth (Bruder et al., 1997). Although the adherence times were different, Bruder et al. (1997) determined that both populations of cells retained their capacity to differentiate along the osteogenic lineage (Bruder et al., 1997). Based on these data, it was concluded that both early and late adherence cell populations are adequate for additional experiments with eBMSC. The population of cells with the late adherence time was used for these experiments because a greater number of cells was available from this population.

Also using alamarBlue and BrdU experimental assays, a greater increase in the number of eBMSC and BrdU incorporation was observed in cells treated with FBS compared with cells treated with HS. This led to the conclusion that FBS was a more successful stimulator of proliferation than HS in this model. This contradicts data from a rat model (Eslaminejad et al., 2009), but supports recent research in an equine model (Toupadakis et al., 2010). In a rat model, it was found that rBMSC cultured in

autologous serum reached confluence by Day  $7 \pm 1$  while cells cultured in FBS reached confluence by Day  $12 \pm 1.7$  (Eslaminejad et al., 2009). Similar to the findings in this thesis, a recent publication reports that eBMSC cultured in FBS had significantly greater cell numbers than eBMSC cultured in HS ( $1.87 \times 10^6 \pm 0.44 \times 10^6$ , and  $1.05 \times 10^6 \pm 0.26 \times 10^6$ , respectively; Toupadakis et al., 2010). These researchers suggested that a greater concentration of HS may be required in culture to achieve the proliferation rates achieved by culture in FBS (Toupadakis et al., 2010). The differences observed between HS and FBS may be due to the following reasons. First, autologous serum, HS, is produced from an animal much older than the animal from which FBS is produced, and thus the autologous serum has a lower nutrient content. Second, the commercially produced FBS may have a greater level of purity than the HS that is produced in the laboratory. Based on these results, for future research with this cell culture protocol, all eBMSC were cultured in media containing FBS. The use of FBS is advantageous because FBS is a commercially available product of good quality and its use will provide more consistency in research performed in different laboratories.

After identifying the optimal cells and culture conditions for proliferation, the evaluation of the differentiation of eBMSC into osteoblast cells was possible. Differentiation was confirmed by increased ALP enzyme activity, and Alizarin Red and ALP Staining. It was also determined that during osteoblast differentiation gene expression of osteoblast markers were consistent with previous reports in other species models (Pagani et al., 2005). These findings were consistent with reports in hBMSC, where ALP expression increased early in differentiation, while OC expression had greatest increase on the final days of culture (Pagani et al., 2005). These results were

also consistent with reports from research done with rBMSC on the expression of key transcriptional activators of osteoblast differentiation (Heino and Hentunen, 2008). In a rat model and this eBMSC model, expression of Runx2 was greatest during the first six days of differentiation culture and Osx expression was greatest in the final days of culture (Heino and Hentunen, 2008). These results are also consistent with findings in MSC from multiple tissue sources, such as adipose and peripheral blood from human and rodent species, where increases in Runx2 and Osx are observed during differentiation of MSC into osteoblast cells (Heino and Hentunen, 2008). These results, with equine gene expression at comparable time points with known expression in rodent and human models, confirmed that this technique for osteoblast differentiation was successful. These findings also established that equine osteoblast differentiation is regulated similarly to osteoblast differentiation in other species.

To determine if growth factors BMP-2 and DEX would improve differentiation of eBMSC down the osteoblast lineage in this model, ALP activity was determined following treatment. BMP-2 and DEX are known to improve differentiation of MSC into osteoblasts in other models, and it was hypothesized that similar effects would be observed in this equine model. First, the addition of hBMP-2, in both the absence and presence of DEX, had no effect on ALP enzyme activity, and thus did not improve the differentiation of the eBMSC into osteoblasts. These findings were in contrast to reports from human and equine BMSC where treatment with similar doses of BMP-2 increased ALP enzyme activity and gene expression 14-fold (Carpenter et al., 2010). It is possible that other BMP or growth factors, such as BMP-7 or fibroblastic growth factor, may be more effective for the improvement of differentiation. Second, similar to previous work

in eBMSC, the addition of DEX to culture significantly increased ALP enzyme activity (Ter Brugge and Jansen, 2002). This demonstrated that the addition of DEX is critical to ensuring optimal osteoblast differentiation

To date, there has been no research on the novel transcription factor Tbx3, in the equine species. This is the first evidence that Tbx3 is expressed in eBMSC. In addition, it was determined that as eBMSC differentiate into osteoblasts there is a decrease in expression of Tbx3. These findings are consistent with previous research demonstrating that the over-expression of Tbx3 represses osteoblast differentiation in mouse BMSC (Govoni et al., 2009). Therefore, it is speculated that to allow eBMSC to differentiate into osteoblast cells, expression of Tbx3 must be inhibited. Further research is needed to determine if the inhibition of Tbx3 is indeed required for osteoblast differentiation in the equine model, and if Tbx3 interacts with other key transcription factors such as Runx2 and Osx.

To fully understand the differentiation of eBMSC into osteoblasts, further work is needed to identify other novel transcription factors that regulate differentiation. It will be important to determine the effects of key regulators, such as BMP and fibroblastic growth factor, on eBMSC differentiation into osteoblasts. It is speculated that if the addition of these factors to cell culture can increase eBMSC differentiation into osteoblasts *in vitro*, then the addition of these factors to cell culture prior to re-introduction into a wound site may improve the therapeutic properties of the eBMSC. Research has indicated that in a rat model, BMSC cultured with the addition of fibroblastic growth factor resulted in increased OC gene expression and bone nodule formation (Hanada et al., 1997).



In addition to understanding differentiation of eBMSC into osteoblasts, further work is needed on the introduction of these cells into fracture sites. Limited work has been done in the equine model, but work in a human model determined that the transplantation of plastic adherent BMSC resulted in successful engraftment along with significant acceleration of growth (Horwitz et al., 2002). Also, it has been reported in a rat model that MSC combined with porous hydroxyapatite ceramics result in bone formation, four weeks after ectopic implantation (Okamoto et al., 2006). This bone formation was confirmed with significant increases in ALP and OC content in tissue taken from the location of implantation (Okamoto et al., 2006). Further work was done to determine the effect of subcutaneous injection of osteogenically treated sheets of MSC, compared with non-osteogenically treated sheets. Through dissection, this group determined 100% new bone formation when osteogenically treated sheets of MSC were injected subcutaneously, compared with implantation of control cell sheets (Akahane et al., 2010). These studies indicate that the implantation of MSC is possible and successful in regenerating bone.

Controlled trials in the equine model will be necessary to identify the key mechanisms for reintroduction of the eBMSC into a wound for fracture healing. These clinical trials will help determine the cell numbers and growth factor concentrations required for successful healing. With this research, veterinarians will eventually be able to use these techniques and methods to improve fracture healing in horses.

## Appendices

### Appendix 1 - Cell Culture

1. Warm the bottles of maintenance media ( $\alpha$ -MEM (Invitrogen, cat. no. 12571) + 10% FBS (HyClone Laboratories, cat.no. SH30910.03) + 1% PS (Animal Cell Culture Facility, Storrs, CT) + 1% AB (Fisher Scientific, cat. no. BP264550)), PBS, and 50% Trypsin-EDTA + 50% PBS in water bath at 37°C. Note: Do not place the bottle with Trypsin-EDTA directly in water.
2. Wipe down the working area in the hood with a 70% ethanol soaked gauze sponge. Spray or wipe the pipette gun and a 50 mL tube with 70% ethanol and place them in the hood.
3. Wipe the bottles from the water bath with 70% ethanol gauze and place them into the hood.
4. Once all above steps are done remove the cell culture dishes from the incubator and place them into the hood .
5. Tilt the plate of cells. Using a glass Pasteur pipette and the vacuum pump, remove the media from the plate without touching the bottom of plate with the pipette tip.
6. Tilt the plate and use a transfer pipette to gently add PBS to the plate, from the side wall. Then swirl the PBS over the plate to rinse the cells.
7. Tilt the plate and use a glass Pasteur pipette and the vacuum pump to remove the PBS from the plate.
8. Add 50% trypsin-EDTA + 50% PBS directly onto the cells.
9. Incubate the plate for 3 min at 37°C.
10. During this time add maintenance media (3 mL per culture dish) to the 50 mL tube.
11. Remove the plate from incubator and place it in the hood.
12. Tilt the plate and with a transfer pipette draw up some of the 50% trypsin-EDTA + 50% PBS. Using the liquid in the pipette rinse the bottom of plate.
13. Use a cell scraper to clear the entire plate of cells.
14. When the cells are detached, use a transfer pipette to transfer the 50% trypsin-EDTA + 50% PBS and cells from the plate to a 50ml tube.
15. Centrifuge the 50mL tube at 2,000 rpm for 3 minutes at room temperature (22°C to 26°C). Note: remember to balance the centrifuge with a water blank.
16. Once the centrifuge is complete return the tube to the hood. Use a glass Pasteur pipette and the vacuum pump to remove the supernatant from cell pellet that remains at the bottom of the tube.
17. Add 1,000  $\mu$ L of maintenance media, to the cell pellet. Pipette up and down to re-suspend the cells.
18. Add at least 9 mL of maintenance media to the 50 mL tube (Note: amount of media to use to re-suspend the pellet varies according to the number of plates available and type of experiment). Add the media slowly, and then pipette up and down until the pellet is broken up.
19. Using the hemocytometer count the cells and determine the total number of cells.
20. Plate cells at the necessary density in the required plates for further use (proliferation or differentiation).

21. Swirl the plates to ensure that the cells are evenly distributed.
22. Label each plate with cell type and origin, passage number, initials, date, and plate number.
23. Check plates under the microscope to ensure cells are evenly distributed in an appropriate confluency.
24. Incubate the plates, clean up, and wipe down the hood with 70% ethanol.

## **Appendix 2 - alamarBlue assay**

### **Day 0 (Plating cells in 10% fetal bovine serum)**

1. The cells are passed once (as per the cell passage protocol) before plating.
2. A 96 well plate is plated at a concentration of 3,000 cells/well in 100  $\mu$ L/well of maintenance media.

### **Day 2 (Cells in serum free environment)**

1. Proliferation Serum Free Media ( $\alpha$ -MEM + 0.1% BSA (Sigma cat. no. A7888) + 1% PS + 1% AB) is thawed in water bath at 37°C.
2. Dump off the media in 96-well plate on to a paper towel placed in fume hood.
3. Load 50  $\mu$ L of media to all the wells, mix gently, and dump it off.
4. Load 100  $\mu$ L of media to all the wells and mix.
5. Place the plate in a 5% CO<sub>2</sub> incubator at 37°C.

### **Day 3 (Treatment of cells)**

1. Layout a design for the control and different treatment wells (stock media with 0.1% BSA-control, 5.0% FBS – treatment wells).
2. Prepare treatment groups with different concentrations of calf serum and stock media in 15 mL tubes. (Be sure to make double the volume required for each treatment group).
3. Dump off the media in 96 well plate on to a paper towel placed in fume hood.
4. Load 100  $\mu$ L of control and treatment media to the designated wells using a multipipette.
5. Mix gently and kept in CO<sub>2</sub> incubator.

### **Day 5 (alamarBlue assay)**

1. Dump off the media (control and treatment) in 96 well plates.
2. Rinse all the wells with 100  $\mu$ L of PBS.
3. Dump off the PBS, and re-wash with another 100  $\mu$ L of PBS.
4. Add alamarBlue indicator (Invitrogen, cat. no. DAL1025) to plain  $\alpha$ -MEM medium in 1:10 dilution (9 mL of  $\alpha$ -MEM + 1mL of alamarBlue for 96 wells) NOTE : Take care not to expose alamarBlue to sunlight. Add alamarBlue indicator just before wells are loaded, keeping it in fumehood for a long time greatly reduces activity.
5. Dump off the PBS from the 96 well plates.
6. Load 100  $\mu$ L of solution ( $\alpha$ - MEM + alamarBlue) to all wells and mix gently.
7. Wrap the plate with aluminum foil and placed in CO<sub>2</sub> incubator.
8. Set Synergy 2 to read plate to allow for warm up.
9. After 4 hours, fluorescence is detected by Synergy 2, Biotek plate reader 540/35 x 600/40 nm, Sensitivity 54.

### **Appendix 3 - BrdU assay**

#### **Day 0 (Plating cells in 10% fetal bovine serum)**

1. The cells are passed once (as per the cell passage protocol) before plating.
2. A 96 well plate is plated at a concentration of 3,000 cells/well in 100  $\mu$ L/well of maintenance media.

#### **Day 2 (Cells in serum free environment)**

1. Proliferation Serum Free Media is thawed in water bath at 37°C.
2. Dump off the media in 96-well plate on to a paper towel placed in fume hood.
3. Load 50  $\mu$ L of media to all the wells, mix gently, and dump it off.
4. Load 100  $\mu$ L of media to all the wells and mix.
5. Place the plate in a 5% CO<sub>2</sub> incubator at 37°C.

#### **Day 3 (Treatment of cells)**

1. Layout a design for the control and different treatment wells (stock media with 0.1% BSA-control, 5.0% FBS – treatment wells).
2. Prepare treatment groups with different concentrations of calf serum and stock media in 15 mL tubes. (Be sure to make double the volume required for each treatment group).
3. Dump off the media in 96 well plate on to a paper towel placed in fume hood.
4. Load 100  $\mu$ L of control and treatment media to the designated wells using a multipette.
5. Mix gently and kept in CO<sub>2</sub> incubator.

#### **Day 5 (BrdU assay, utilizing Cell Proliferation ELISA, BrdU (chemiluminescent) Kit and Protocol (Roche Applied Science, cat. no. 11669915001))**

1. Add 10  $\mu$ L/well BrdU labeling solutions to the plate and incubate for 4 hrs at 37°C.
2. The labeling solution is removed.
3. Add 200  $\mu$ L/well FixDenat to the cells and incubate for 30 minutes at room temperature.
4. Remove the FixDenat solution.
5. Add 100  $\mu$ L/well Anti-BrdU-POD working solution and incubate for 90 minutes at room temperature.
6. Excess antibodies are removed by dumping off the solution and rinse the wells three times with 200  $\mu$ L/well of washing solution.
7. The washing solution is dumped off.
8. The bottom of the plate is sealed with a black adhesive film.
9. Add 100  $\mu$ L/well of the substrate solution to each well.
10. The plate is incubated for 5 minutes at room temperature.
11. The luminescence is read at BioTek plate reader with auto sensitivity.

## **Appendix 4 - ALP assay**

### **Day 0: (Plate cells)**

1. Plate cells at 6,000 cells/well in 96 well plate in maintenance media at 100  $\mu$ L/well.
2. Incubate for at 37°C.

### **Day 2: (Change to serum free media)**

1. Rinse with 100  $\mu$ L of proliferation serum free media two times.
2. Make a stock media of ALP serum free media ( $\alpha$ -MEM + 0.1% BSA + 1% PS + 1% AB + 300  $\mu$ g/mL VC (Sigma Aldrich, cat. no. A8960) + 10 mM BG (Sigma, cat. no. G9891)).
3. Add 100  $\mu$ L/well of the above solution and incubate for 24 hours.

### **Day 3: (Add Appropriate Growth Factors)**

1. Make a stock of ALP serum free media.
2. Used the above media and h-BMP 2 (PeproTech, cat. no. 120-02) and 25 mg/mL DEX (Sigma Aldrich, cat. no. D2915) to make your treatments.
3. Remove old media from wells without rinsing.
4. Add 100  $\mu$ L/well for control and treatments and incubate for 72 hours.

### **Day 6: (Permealize the cells and perform ALP assay)**

1. The procedures for this day can be performed under non-sterile conditions.
2. Dump off the media from the cells.
3. Rinse two times with warm PBS using 100  $\mu$ L/well.
4. Add 100  $\mu$ L of 0.1% triton to each well.
5. Shake at medium speed at room temperature for 15 to 20 minutes.
6. Wrap in a parafilm.
7. Freeze at -80°C for at least 15 minutes (optional – after shaking the cells can be stored at -80°C until future use).
8. Obtain two 96 well plates.
9. Thaw the ALP substrate by placing the tube in the water bath and also thaw out the 96-well plate at room temperature.
10. Scrape the cells using the tips on a multipipettor and pipette mix the cell lysates. Make sure that no bubbles are contained in the pipette tip when the lysate is transferred to the new 96-well plate: Set multipipettor to 40  $\mu$ L, hold the button on the pipettor down then begin scrapping the wells; once well is sufficiently scrapped release the button and remove the lysate from the well.
11. Transfer 40  $\mu$ L of the cell lysate (for ALP) into one 96-well plate.
12. For protein analysis add 80  $\mu$ L Distilled H<sub>2</sub>O then add 20  $\mu$ L of the cell lysate (for protein concentration) into one 96-well plate.

**ALP activity**

1. Prepare two Blank wells with 40  $\mu\text{L}$  of 0.1% Titron/well.
2. Add 200  $\mu\text{L}$  of the ALP Substrate (pNPP; Sigma Aldrich, cat. no. P7998-100) to all of the wells (including blank).
3. Immediately read (0 hour) absorbance at 405 nm, and then incubate at 37°C and read after 5 hours of incubation.

**Protein estimation**

BLANK- in the next available column, make two blank wells. First well: 20  $\mu\text{L}$  of 0.1% Triton and 80  $\mu\text{L}$  of Distilled  $\text{H}_2\text{O}$ . Second well: 100  $\mu\text{L}$  of Distilled  $\text{H}_2\text{O}$

SAMPLE- 20  $\mu\text{L}$  lysate and 80  $\mu\text{L}$  Distilled  $\text{H}_2\text{O}$ . ( $\text{H}_2\text{O}$  added first)

DYE- 2 mL Bio-Rad Protein Assay Reagent (Bio-Rad, cat. no. 500-0205) and 8 mL  $\text{H}_2\text{O}$  (1:5 dilution)

STANDARDS- utilizing BSA (Sigma Aldrich, cat no. A7888-100G)

Stock: 100  $\mu\text{g}$  BSA/ 1 mL  $\text{dH}_2\text{O}$  = for example: 30 mg BSA/300mL  $\text{dH}_2\text{O}$

1. Of the 300mL stock take 150  $\mu\text{L}$  and add 150  $\mu\text{L}$   $\text{dH}_2\text{O}$   
{final concentration is 50  $\mu\text{g}/\text{mL}$ }
  2. Of the resulting solution take 150  $\mu\text{L}$  and add 150  $\mu\text{L}$   $\text{dH}_2\text{O}$   
{final concentration is 25  $\mu\text{g}/\text{mL}$ }
  3. Of the resulting solution take 150  $\mu\text{L}$  and add 150  $\mu\text{L}$   $\text{dH}_2\text{O}$   
{final concentration is 12.5  $\mu\text{g}/\text{mL}$ }
  4. Of the resulting solution take 150  $\mu\text{L}$  and add 150  $\mu\text{L}$   $\text{dH}_2\text{O}$   
{final concentration is 6.25  $\mu\text{g}/\text{mL}$ }
  5. Of the resulting solution take 150  $\mu\text{L}$  and add 150  $\mu\text{L}$   $\text{dH}_2\text{O}$   
{final concentration is 3.125  $\mu\text{g}/\text{mL}$ }
1. Into the next available column add 100  $\mu\text{L}$  of each standard/well (7 wells total; include 100  $\mu\text{g}/\text{mL}$ (from stock) and 0  $\mu\text{g}/\text{mL}$ (distilled  $\text{H}_2\text{O}$ ))
  2. Add 200  $\mu\text{L}$  of the dye to all wells on the plate.
  3. Incubate the plate for 5 minutes and read the plate absorbance at 595 nm.

## **Appendix 5 - Alizarin Red Staining**

1. Remove media from culture plate
2. Wash with PBS
3. Fix with ice cold 100% methanol, in freezer (-20°C) over night (12 hours)
4. Remove methanol
5. Rinse once with DI H<sub>2</sub>O
6. Stain with 1:2 Alizarin Red:media mixture
7. Incubate at room temperature while rotating
8. Remove Alizarin Red stain
9. Rinse with DI H<sub>2</sub>O until background is clear
10. Wash with PBS and rotate with PBS at room temperature for 15 minutes
11. Remove PBS and air-dry the plate

### Alizarin Red Stain

200mL DI H<sub>2</sub>O

3.42g Alizarin Red powder (Sigma Aldrich cat. no. A5533)

Adjust to pH 4.2

Add DI H<sub>2</sub>O to make final volume of 250mL

Filter (0.22µm pores)



## Appendix 6 - ALP Staining

1. Gently remove the media from the plates and rinse once with warm PBS.
2. Remove PBS and fix the cells in cold 100% methanol (-70°C) using 2 mL in each well for 20 minutes at room temperature (no rotation).
3. During fixation, make the substrate and the diazonium salt solutions (These are light sensitive so cover tubes with foil and shut off lights). Do not combine substrate-diazonium solution (ALP staining solution) until you are ready to add since this solution is unstable at assay pH.
4. Remove methanol and rinse 1-2X's with PBS.
5. Remove the PBS and add 2 mL of the substrate-diazonium solution (ALP staining solution) to each well and incubate for 30 minutes in 37°C incubator, every 10 min swirling the cells.
6. Remove the staining solution and add PBS. Check under the microscope to identify ALP positive cells (red).
7. Store at 4°C and wrap in foil.

### ALP Buffer (pH 8.6)

6.055 g Tris

5.84 g NaCl

0.147 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

0.372 g KCl

0.203 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

Make in 1 liter of  $\text{H}_2\text{O}$  (distilled water)

Adjust to pH 8.6

### Substrate (Falcon tube 50 mL)

8 mg naphthol AS-TR phosphate

9 mL ALP Buffer

### Diazonium salt (Falcon tube 50 mL)

6 mg fast red violet LB

3 mL ALP Buffer

### Substrate-diazonium solution (ALP Staining Solution)

1 mL Diazonium salt

9 mL Substrate

## **Appendix 7 - RNA Extraction – Rneasy Mini Kit (Qiagen, cat.no. 74104)**

1. Cells are plated in 6-well plates (150,000 cells/well, 2 mL maintenance media).
2. Cells are allowed to reach 90% confluence and are then changed to differentiation media ( $\alpha$ -MEM + 10% FBS + 1% PS + 1% AB + VC + BG + DEX). RNA is then extracted every three days from day 0 to day 18.
3. Tilt the plate of cells. Using a glass Pasteur pipette and the vacuum pump, remove the media from the plate without touching the bottom of plate with the pipette tip.
4. Tilt the plate and use a transfer pipette to gently add PBS to the plate, from the side wall. Then swirl the PBS over the plate to rinse the cells.
5. Tilt the plate and using a glass Pasteur pipette and the vacuum pump remove the PBS from the plate.
6. Add 1 ml TRI Reagent (Sigma Aldrich, cat. no. T9424) to each well and scrape with pipet tip.
7. Transfer it to a 1.5 mL microcentrifuge tube, vortex, and homogenize.
8. Incubate for 5 minutes at room temperature.
9. At this point microcentrifuge tubes may be frozen (-80°C) and the protocol may be continued at a later date (after thawing at room temperature and vortexing)
10. Add 200  $\mu$ L chloroform (Sigma Aldrich, cat. no. C2432-500) to each tube and mix by inverting 5 to 7 times.
11. Incubate for 2 to 3 minutes at room temperature.
12. Centrifuge at 12,000 x g for 15 minutes.
13. Transfer the aqueous phase (transparent, clear top layer) into a new micro centrifuge tube (approximately 500  $\mu$ L).
14. Add one volume (500  $\mu$ L) of 70% EtOH to each tube and vortex the tubes.
15. Transfer 700  $\mu$ L of the sample into the RNA easy spin column.
16. Centrifuge at 8000 x g for 30 seconds and discard the flow through.
17. Add 700  $\mu$ L Buffer RWI and centrifuge at 8000 x g for 30 seconds.
18. Discard the flow through and the collection tube, and place the filter into a new collection tube.
19. Add 500  $\mu$ L of Buffer RPE and centrifuge at 8000 x g for 30 seconds.
20. Discard the flow through and add 500  $\mu$ L of RPE buffer.
21. Centrifuge at 8000 x g for 2 minutes and discard the flow through.
22. Centrifuge at 8000 x g for 1 minute.
23. Discard the flow through and the collection tube, and put the filter into a new capped collection tube.
24. Add 50  $\mu$ L of Rnase free water and centrifuge at 8000 x g for 1 minute.
25. Take 35 to 40  $\mu$ L from the collection tube and put back on the filter. Centrifuge at 8000 x g for 1 minute.
26. Collect the eluted RNA and spec it in the Nanodrop spectrophotometer.
27. Aliquot and store in the -80°C freezer.

## **Appendix 8 - TURBO DNA-Free Protocol (Ambion, cat. no. AM1907)**

1. Thaw the reagents (10x Turbo DNase Buffer, Turbo DNase, DNase inactivation reagent ) on ice.
2. Add 0.1 volume of 10x Turbo Dnase Buffer ( i.e. 5  $\mu$ L for 50  $\mu$ L of RNA sample) and 1  $\mu$ L Turbo DNase to the RNA sample and mix gently ( This is for routine DNase treatment with RNA con. of <10  $\mu$ g/50  $\mu$ L of sample. If it is more than this con., dilute your sample to have 10  $\mu$ g/50  $\mu$ L using  $C1V1=C2V2$ )
3. Incubate at 37°C for 20 to 30min
4. Add 0.1 volume of resuspended DNase Inactivation reagent (5  $\mu$ L for 50  $\mu$ L of RNA sample) and mix well. NOTE: The DNase Inactivation reagent should be vortexed and mixed properly before use since it is a turbid white solution.
5. Incubate for 5 min at room temperature (22 to 25°C) while mixing it occasionally by flicking or tapping
6. Centrifuge at 10000 Xg for 1.5 min
7. Transfer the supernatant to a fresh tube. NOTE: Avoid disturbing the pellet as much as you can, since transfer of a small portion of the pellet could interfere in your further procedures especially reverse transcription. Usually about 46 to 48  $\mu$ L can be obtained.
8. The final DNA-free RNA con. is determined by Nano drop1000.

## Appendix 9 - Reverse Transcriptase PCR

1. Thaw the reagents on ice (OligoDT (dT) primer (Ambion, cat.no. 5730G), Superscript II DTT, 5X buffer and reverse transcriptase (Invitrogen, cat. no. 18064-014))
2. Add 1  $\mu\text{L}$  of OligoDT to each 10  $\mu\text{L}$  aliquot of RNA sample (30 ng/ $\mu\text{L}$ ) in 8 tube strips. This makes it a total of 11  $\mu\text{L}$ .
3. Set the PCR machine to run the following program at 20  $\mu\text{L}$ :
  - 70°C for 10 min
  - 4°C for 20 min
  - 37°C for 3 min
  - 42°C for 1 hr
  - 4 °C for 3 min
  - 90°C for 2.30 min
  - 10°C forever
4. Place the tube strips into PCR machine and start step1, 70°C for 10 min
5. During the 10 minute step 1, make the master mix.

### Master Mix

5X Buffer-	5.5 $\mu\text{L}$
dNTP-	1 $\mu\text{L}$
DTT-	2 $\mu\text{L}$
SuperscriptII	0.5 $\mu\text{L}$
Total Sample:	9 $\mu\text{L}$

NOTE: Add SS II last just before it is time to add master mix to samples

The above calculations are per sample, multiply it by the number of samples +1 for pipetting errors.

6. Hit pause on the PCR machine when the temperature has dropped to 4°C for the next step 2.
7. Take the samples with RNA and Oligo (dT) out of the machine and keep it on ice
8. Add SSII to the master mix, mix gently
9. Add 9  $\mu\text{L}$  of master mix to each sample , making it a total of 20  $\mu\text{L}$  .( Samples are kept on ice while adding the master mix)
10. Place the samples back into the machine, hit proceed, and hit proceed again to move to step 3, 37°C for 3 min. This means the step 2 is skipped.
11. When Reverse Transcription is complete add 80uL of Nuclease free water to samples and store at -20°C.

## Appendix 10 - Real-time RT-PCR

1. Thaw the Reagents on ice.(Syber green (Applied Biosystems, cat. no. 4367659) wrapped in foil, F and R primers, and cDNA)
2. Turn the lights off before you begin the experiment.
3. Make Master Mix (amounts are per reaction) for each gene you will be amplifying:

2X Syber Green	10 $\mu$ L
Forward Primer	1 $\mu$ L
Reverse primer	1 $\mu$ L
Nuclease Free water	3 $\mu$ L
Total:	15 $\mu$ L
4. Add 5  $\mu$ L of cDNA (diluted 1:5, 20  $\mu$ L of cDNA + 80  $\mu$ L of Nuclease free water) each well in 96 PCR optical plate. Make sure to pipette to the bottom of the well.
5. Add 15  $\mu$ L of master mix to each well in Fast Optical 96 well reaction plate (Applied Biosystems, cat. no. 4346906)
6. Seal it with a PCR adhesive film tightly to prevent evaporation
7. Centrifuge the plate at 1000 RPM for 1-2 minutes.
8. Run it in Q-PCR Machine with this program:

50°C for 2 min	
95°C for 10 min	
95°C for 15 s	X 40 cycles
60°C for 1 min	
95°C for 15 s	
60°C for 15 s	
95°C for 15 s	

## Appendix 11 - Polymerase Chain Reaction (PCR)

1. Thaw reagents on ice ( Promega Green Master mix, forward and reverse primers, and cDNA)
2. Make Master mix of 20  $\mu\text{L}$  for one reaction
  - Nuclease free water      5.5  $\mu\text{L}$
  - Green master mix   12.5  $\mu\text{L}$
  - Forward Primer    1.0  $\mu\text{L}$
  - Reverse Primer    1.0  $\mu\text{L}$

NOTE: This is for one sample, multiply it by the # of samples+2 for pipetting errors.
3. Transfer 20  $\mu\text{L}$  of the master mix to into each tube of the 8 tube strips
4. Add 5  $\mu\text{L}$  of the cDNA to 8 tube strips, making it a total of 25  $\mu\text{L}$ /tube. This should be done just before it is ready to got the PCR machine.
5. Place the tubes into PCR machine and run the program:
  - 94°C for 3 min
  - 94°C for 30 s
  - 60°C for 1 min
  - 72°C for 1 min
  - 72°C for 5 min
  - 10°C forever

X 40 cycles
6. Once the PCR is complete ( about 2 1/2 hr), it is stored at 4°C.

## **Appendix 12 - DNA – Agarose gel**

### **Preparation of a 2% agarose gel (mini gel)**

1. Weigh out 1 g of agarose
2. Add it to a conical flask containing 50 mL of TAE buffer
3. To dissolve, heat it in a microwave for about 1.30 minutes (solution starts boiling by this time). NOTE: take out the flask and swirl it in between to avoid spillage and for proper dissolution
4. Allow it to cool to about 50-60°C (i.e. cool to touch)
5. Add 3 µL of ethidium bromide stock solution (10 mg/mL) to the liquid gel. Gently swirl to mix. NOTE: WEAR GLOVES! Ethidium bromide is TOXIC MUTAGEN. Proper care is to be taken in disposal.
6. Pour the liquid gel into the gel forming cassette.
7. Place the combs at one end to form the wells, allow it to solidify (It take about 20 minutes)

### **Loading the wells**

1. Fill the mangle apparatus with the loading Buffer TAE
2. Load ladder to the first well (1 µL of loading Dye + 5 µL of DNA ladder (100 bp ladder, Promega). This mixing can be done over a small piece of parafilm.
3. Rest of the wells are loaded with 10 µL of the sample (PCR products). There is no need to add the loading dye to it, since the PCR product by itself contains the dye(green master mix)
4. Close the lid, making sure that the electric field is generated as desired (Run to Red).
5. Connect the electrodes to the battery and turn it on. Look for bubbles rising up on either side of the electrodes.
6. Run it at 115 volts for about 45 minutes ( i.e., until the dye front reaches almost bottom of the gel)
7. Visualize the gel.

## References

- Akahane, M., A. Nakamura, H. Ohgushi, H. Shigematsu, Y. Dohi, and Y. Takakura. 2008. Osteogenic matrix sheet-cell transplantation using osteoblastic cell sheet resulted in bone formation without scaffold at an ectopic site. *J. Tissue Eng. Regen. Med.* 2(4): 196-201.
- Akahane, M., H. Shigematsu, M. Tadokoro, T. Ueha, T. Matsumoto, Y. Tohma et al. 2010. Scaffold-free cell sheet injection results in bone formation. *J. Tissue Eng. Regen. Med.* 4(5): 404-411.
- Alonso, A., P. Martín, C. Albarrán, P. García, O. García, L. F. de Simón et al. 2004. Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Sci. Int.* 139(2-3): 141-149.
- Arnhold, S. J., I. Goletz, H. Klein, G. Stumpf, L. A. Beluche, C. Rohde et al. 2007. Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. *Am. J. Vet. Res.* 68(10): 1095-1105.
- Baksh, D., P. W. Zandstra, and J. E. Davies. 2007. A non-contact suspension culture approach to the culture of osteogenic cells derived from a CD49<sup>low</sup> subpopulation of human bone marrow-derived cells. *Biotechnol. Bioeng.* 98(6): 1195-1208.
- Bamshad, M., R. C. Lin, D. J. Law, W. C. Watkins, P. A. Krakowiak, M. E. Moore et al. 1997. Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. *Nat. Genet.* 16(3): 311-315.
- Bathe, A. P. 1994. 245 fractures in thoroughbred racehorses: Results of a 2-year prospective study in newmarket.
- Beyer, A. 2006. Life, savings contribute to difficult calculation. *The Washington Post*.
- Blanchette, A. 2008. A second chance for abby: A horse with a broken leg was one day away from being euthanized when a group of boarders at horseplay ranch in corcoran pulled together to raise money for her expensive life-saving surgery. *McClatchy - Tribune Business News*. : n/a. from the ABI/INFORM Complete database.
- Bramlage, L. R. 1983. Long bone fractures. *Vet. Clin. North Am. Large Anim. Pract.* 5(2): 285-310.
- Bruder, S. P., N. Jaiswal, and S. E. Haynesworth. 1997. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J. Cell. Biochem.* 64(2): 278-294.



- Byers, D. 2011. Funding cannot keep pace. *Thoroughbred Times*.
- Caplan, A. I. 2009. Why are MSCs therapeutic? new data: New insight. *J. Pathol.* 217(2): 318-324.
- Carpenter, R. S., L. R. Goodrich, D. D. Frisbie, J. D. Kisiday, B. Carbone, C. W. McIlwraith et al. 2010. Osteoblastic differentiation of human and equine adult bone marrow-derived mesenchymal stem cells when BMP-2 or BMP-7 homodimer genetic modification is compared to BMP-2/7 heterodimer genetic modification in the presence and absence of dexamethasone. *J. Orthop. Res.* 28(10): 1330-1337.
- Chapman, D. L., N. Garvey, S. Hancock, M. Alexiou, S. I. Agulnik, J. J. Gibson-Brown et al. 1996. Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev. Dyn.* 206(4): 379-390.
- Colleoni, S., E. Bottani, I. Tessaro, G. Mari, B. Merlo, N. Romagnoli et al. 2009. Isolation, growth and differentiation of equine mesenchymal stem cells: Effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor. *Vet. Res. Commun.*
- Dahl, J. A., S. Duggal, N. Coulston, D. Millar, J. Melki, A. Shahdadfar et al. 2008. Genetic and epigenetic instability of human bone marrow mesenchymal stem cells expanded in autologous serum or fetal bovine serum. *Int. J. Dev. Biol.* 52(8): 1033-1042.
- D'Ippolito, G., P. C. Schiller, C. Ricordi, B. A. Roos, and G. A. Howard. 1999. Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *Journal of Bone and Mineral Research.* 14(7): 1115-1122.
- Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause et al. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. *Cytotherapy.* 8(4): 315-317.
- Dominici, M., C. Pritchard, J. E. Garlits, T. J. Hofmann, D. A. Persons, and E. M. Horwitz. 2004. Hematopoietic cells and osteoblasts are derived from a common marrow progenitor after bone marrow transplantation. *Proc. Natl. Acad. Sci. U. S. A.* 101(32): 11761-11766.
- Eslaminejad, M. B., L. Rouhi, M. Arabnajafi, and H. Baharvand. 2009. Rat marrow-derived mesenchymal stem cells developed in a medium supplemented with the autologous versus bovine serum. *Cell Biol. Int.* 33(5): 607-616.
- Estberg, L., S. M. Stover, I. A. Gardner, B. J. Johnson, R. A. Jack, J. T. Case et al. 1998. Relationship between race start characteristics and risk of catastrophic injury in thoroughbreds: 78 cases (1992). *J. Am. Vet. Med. Assoc.* 212(4): 544-549.

- Fan, W., X. Huang, C. Chen, J. Gray, and T. Huang. 2004. TBX3 and its isoform TBX3+2a are functionally distinctive in inhibition of senescence and are overexpressed in a subset of breast cancer cell lines. *Cancer Res.* 64(15): 5132-5139.
- Fortier, L. A., A. J. Nixon, J. Williams, and C. S. Cable. 1998. Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *Am. J. Vet. Res.* 59(9): 1182-1187.
- Franceschi, R. T., and G. Xiao. 2003. Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways. *J. Cell. Biochem.* 88(3): 446-454.
- Gamradt, S. C., and J. R. Lieberman. 2004. Genetic modification of stem cells to enhance bone repair. *Ann. Biomed. Eng.* 32(1): 136-147.
- Gaur, T., C. J. Lengner, H. Hovhannisyanyan, R. A. Bhat, P. V. Bodine, B. S. Komm et al. 2005. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J. Biol. Chem.* 280(39): 33132-33140.
- Govender, S., C. Csimma, H. K. Genant, A. Valentin-Opran, Y. Amit, R. Arbel et al. 2002. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: A prospective, controlled, randomized study of four hundred and fifty patients. *J. Bone Joint Surg. Am.* 84(12): 2123-2134.
- Govoni, K. E., S. K. Lee, R. B. Chadwick, H. Yu, Y. Kasukawa, D. J. Baylink et al. 2006. Whole genome microarray analysis of growth hormone-induced gene expression in bone: T-box3, a novel transcription factor, regulates osteoblast proliferation. *Am. J. Physiol. Endocrinol. Metab.* 291(1): E128-36.
- Govoni, K. E., G. R. Linares, S. T. Chen, S. Pourteymoor, and S. Mohan. 2009. T-box 3 negatively regulates osteoblast differentiation by inhibiting expression of osterix and runx2. *J. Cell. Biochem.* 106(3): 482-490.
- Grove, J. E., E. Bruscia, and D. S. Krause. 2004. Plasticity of bone marrow-derived stem cells. *Stem Cells.* 22(4): 487-500.
- Hanada, K., J. E. Dennis, and A. I. Caplan. 1997. Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. *J. Bone Miner. Res.* 12(10): 1606-1614.
- Haynesworth, S. E., J. Goshima, V. M. Goldberg, and A. I. Caplan. 1992. Characterization of cells with osteogenic potential from human marrow. *Bone.* 13(1): 81-88.

- Heino, T. J., and T. A. Hentunen. 2008. Differentiation of osteoblasts and osteocytes from mesenchymal stem cells. *Curr. Stem Cell. Res. Ther.* 3(2): 131-145.
- Henrichsen, E. 1956. Alkaline phosphatase in osteoblasts and fibroblasts cultivated in vitro. *Exp. Cell Res.* 11(1): 115-127.
- Herzog, E. L., L. Chai, and D. S. Krause. 2003. Plasticity of marrow-derived stem cells. *Blood.* 102(10): 3483-3493.
- Hill, T., D. Carmichael, G. Maylin, and L. Krook. 1986. Track condition and racing injuries in thoroughbred horses. *Cornell Vet.* 76(4): 361-379.
- Horwitz, E. M., P. L. Gordon, W. K. Koo, J. C. Marx, M. D. Neel, R. Y. McNall et al. 2002. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc. Natl. Acad. Sci. U. S. A.* 99(13): 8932-8937.
- Horwitz, E. M., K. Le Blanc, M. Dominici, I. Mueller, I. Slaper-Cortenbach, F. C. Marini et al. 2005. Clarification of the nomenclature for MSC: The international society for cellular therapy position statement. *Cytotherapy.* 7(5): 393-395.
- Horwitz, E. M., D. J. Prockop, L. A. Fitzpatrick, W. W. Koo, P. L. Gordon, M. Neel et al. 1999. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat. Med.* 5(3): 309-313.
- Im, G. I., Y. W. Shin, and K. B. Lee. 2005. Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? *Osteoarthritis Cartilage.* 13(10): 845-853.
- Ip, Y. T., and R. J. Davis. 1998. Signal transduction by the c-jun N-terminal kinase (JNK) — from inflammation to development. *Curr. Opin. Cell Biol.* 10(2): 205-219.
- Jaiswal, N., S. E. Haynesworth, A. I. Caplan, and S. P. Bruder. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J. Cell. Biochem.* 64(2): 295-312.
- Komori, T. 2006. Regulation of osteoblast differentiation by transcription factors. *J. Cell. Biochem.* 99(5): 1233-1239.
- Kraus, B. M., M. W. Ross, and R. C. Boston. 2005. Surgical and nonsurgical management of sagittal slab fractures of the third carpal bone in racehorses: 32 cases (1991-2001). *J. Am. Vet. Med. Assoc.* 226(6): 945-950.

- Lee, D. Y., T. J. Cho, J. A. Kim, H. R. Lee, W. J. Yoo, C. Y. Chung et al. 2008. Mobilization of endothelial progenitor cells in fracture healing and distraction osteogenesis. *Bone*. 42(5): 932-941.
- Lewis, J. M. 2008. New technology could help reduce bone fractures in horses. 2010
- Malaval, L., F. Liu, P. Roche, and J. E. Aubin. 1999. Kinetics of osteoprogenitor proliferation and osteoblast differentiation in vitro. *J. Cell. Biochem*. 74(4): 616-627.
- McKee, S. L. 1995. An update on racing fatalities in the UK. *Equine Veterinary Education*. 7(4): 202-204.
- Muller, M. E. 1963. Internal fixation for fresh fractures and for non-union. *Proc. R. Soc. Med*. 56: 455-460.
- Murray, S. J., K. S. Santangelo, and A. L. Bertone. 2010. Evaluation of early cellular influences of bone morphogenetic proteins 12 and 2 on equine superficial digital flexor tenocytes and bone marrow-derived mesenchymal stem cells in vitro. *Am. J. Vet. Res*. 71(1): 103-114.
- Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J. M. Deng, R. R. Behringer et al. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*. 108(1): 17-29.
- Nakayama, G. R., M. C. Caton, M. P. Nova, and Z. Parandoosh. 1997. Assessment of the alamar blue assay for cellular growth and viability in vitro. *J. Immunol. Methods*. 204(2): 205-208.
- Okamoto, M., Y. Dohi, H. Ohgushi, H. Shimaoka, M. Ikeuchi, A. Matsushima et al. 2006. Influence of the porosity of hydroxyapatite ceramics on in vitro and in vivo bone formation by cultured rat bone marrow stromal cells. *J. Mater. Sci. Mater. Med*. 17(4): 327-336.
- Pagani, F., C. M. Francucci, and L. Moro. 2005. Markers of bone turnover: Biochemical and clinical perspectives. *J. Endocrinol. Invest*. 28(10 Suppl): 8-13.
- Paris, D. B. B. P., and T. A. E. Stout. 2010. Equine embryos and embryonic stem cells: Defining reliable markers of pluripotency. *Theriogenology*. 74(4): 516-524.
- Perren, S. M. 2008. Fracture healing. the evolution of our understanding. *Acta Chir. Orthop. Traumatol. Cech*. 75(4): 241-246.
- Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca et al. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science*. 284(5411): 143-147.

- Raggatt, L. J., and N. C. Partridge. 2010. Cellular and molecular mechanisms of bone remodeling. *J. Biol. Chem.* 285(33): 25103-25108.
- Riggs, C. M. 2002. Fractures--a preventable hazard of racing thoroughbreds? *Vet. J.* 163(1): 19-29.
- Sacks, P. H. 2006. Barbaro is 'doing well' ; success rate 50-50, local vet says: [ALL edition]. *Telegram & Gazette.* : A.1. from the ABI/INFORM Complete database.
- Schauwer, C. D., E. Meyer, G. R. Walle, and A. V. Soom. 2010. Markers of stemness in equine mesenchymal stem cells: A plea for uniformity. *Theriogenology.*
- Schindeler, A., M. M. McDonald, P. Bokko, and D. G. Little. 2008. Bone remodeling during fracture repair: The cellular picture. *Semin. Cell Dev. Biol.* 19(5): 459-466.
- Seeherman, H., R. Li, M. Bouxsein, H. Kim, X. J. Li, E. Smith-Adaline et al. 2006. rhBMP-2/calcium phosphate matrix accelerates osteotomy-site healing in a nonhuman primate model at multiple treatment times and concentrations. *Journal of Bone and Joint Surgery American Volume.* 88(1): 144-160.
- Smith, R. K., M. Korda, G. W. Blunn, and A. E. Goodship. 2003. Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. *Equine Vet. J.* 35(1): 99-102.
- Ter Brugge, P. J., and J. A. Jansen. 2002. In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone. *Tissue Eng.* 8(2): 321-331.
- Toupadakis, C. A., A. Wong, D. C. Genetos, W. K. Cheung, D. L. Borjesson, G. L. Ferraro et al. 2010. Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. *Am. J. Vet. Res.* 71(10): 1237-1245.
- Vidal, M. A., G. E. Kilroy, J. R. Johnson, M. J. Lopez, R. M. Moore, and J. M. Gimble. 2006. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: Adipogenic and osteogenic capacity. *Vet. Surg.* 35(7): 601-610.
- Violini, S., P. Ramelli, L. F. Pisani, C. Gorni, and P. Mariani. 2009. Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by in vitro exposure to BMP-12. *BMC Cell Biol.* 10: 29.
- Wilson, V., and F. L. Conlon. 2002. The T-box family. *Genome Biol.* 3(6): REVIEWS3008.