ISOLATION, CHARACTERIZATION, AND *IN VITRO* PROLIFERATION OF CANINE BONE MARROW, ADIPOSE TISSUE, MUSCLE, AND PERIOSTEUM-DERIVED MESENCHYMAL STEM CELLS

BY

AGATHA H. KISIEL

A Thesis
Submitted to the Graduate Faculty
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Companion Animals
Faculty of Veterinary Medicine
Atlantic Veterinary College
University of Prince Edward Island

Your file Votre référence
ISBN: 978-0-494-82262-3
Our file Notre référence
ISBN: 978-0-494-82262-3

Conditions for the Use of the Thesis

The author has agreed that the Library, University of Prince Edward Island, may make this thesis freely available for inspection. Moreover, the author has agreed that permission for extensive copying of this thesis for scholarly purposes may be granted by the professor or professors who supervised the thesis work recorded herein or, in their absence, by the Chair of the Department or the Dean of the Faculty in which the thesis work was done. It is understood that due recognition will be given to the author of this thesis and to the University of Prince Edward Island in any use of the material in this thesis. Copying or publication or any other use of the thesis for financial gain without written approval by the University of Prince Edward Island and the author's written permission is prohibited.

Requests for permission to copy or to make any other use of material in this thesis in whole or in part should be addressed to:

Chair of the Department of Companion Animals

Faculty of Veterinary Medicine

University of Prince Edward Island

Charlottetown, P. E. I

Canada C1A 4P3

SIGNATURE PAGE

(iii) & (iv)

REMOVED

Abstract

Objective: To isolate and characterize mesenchymal stem cells (MSCs) from canine muscle and periosteum, and to compare the proliferative capacity of bone marrow, adipose tissue, muscle, and periosteum-derived MSCs (BMSCs, AMSCs, MMSCs, and PMSCs, respectively).

Sample Population: Seven canine cadavers.

Procedures: Characterization of MSCs was based on their plastic adherence and morphology, immunofluorescence of MSC-associated cell surface markers, and expression of pluripotency-associated transcription factors. Morphological and histochemical methods were used to evaluate differentiation of MSCs cultured in adipogenic, osteogenic, and chondrogenic media. Passage one MSCs, cultured in triplicate, were counted at 24, 48, 72, and 96 hours to determine tissue specific-MSC proliferative capacity. Mesenchymal stem cell yield/gram of tissue was calculated for confluent passage one MSCs.

Results: Successful isolation of BMSCs, AMSCs, MMSCs, and PMSCs was based on their plastic adherence and morphology, positive expression of CD44 and CD90, negative expression of CD34, CD45, and CD146, mRNA expression of SOX2, OCT4, and NANOG, and adipogenic and osteogenic differentiation. The proliferative capacity was not significantly different between BMSCs, AMSCs, MMSCs, and PMSCs over a four day culture period. However, periosteum provided a significantly higher MSC yield/gram of tissue once confluent in passage one (mean ± SD of 19,400,000 ±

12,800,000 of PMSCs/gram of periosteum obtained in a mean ± SD of 13 ± 1.64 days).

Conclusions and Clinical Relevance: Canine muscle and periosteum are sources of MSCs. Periosteum is a superior tissue source for MSC yield, and may be useful in allogenic applications.

Acknowledgements

I would like to acknowledge my supervisors, Dr. Trina Bailey and Dr. Caroline Runyon, for giving me this opportunity to complete a Masters project and a Small Animal Surgery Residency. I appreciate your support and mentorship.

I would also like to thank Dr. McDuffee for her guidance in this project and her expertise in the field of mesenchymal stem cells.

Thank you to Dr. Gelens, a member of my Masters Supervisory Committee, for your support and enthusiasm.

Thank you to Blanca Esparza Gonzalez and Rodolfo Nino-Fong for teaching me the laboratory techniques necessary for mesenchymal stem cell culture. Your expertise and guidance helped me complete this project.

Thank you to Elmabrok Masoud for your statistical support, to Dr. Andrea Bourque for your help with histopathology, and to Dr. David Sims for your guidance in imaging techniques. I really appreciate your willingness to help.

Thank you to the Atlantic Veterinary College Internal Research Grant Fund and the Atlantic Canada Opportunities Agency for financial assistance.

I would also like to thank the Atlantic Centre for Comparative Biomedical Research for providing the use of laboratory equipment.

To my mom, who has always believed in me and supported me in all my dreams.
I could not have done this without you. I love you.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

MSC	Mesenchymal stem cell
BMSC	Bone marrow-derived MSC
CFU-F	Colony forming unit fibroblast
OA	Osteoarthrits
AMSC	Adipose tissue-derived MSC
ES	Embryonic stem cells
FACS	Fluorescence activated cell sorting
αMEM	Alpha minimal essential medium
PDGF	Platelet derived growth factor
FGF	Fibroblast growth factor
FCS	Fetal calf serum
DMSO	Dimethylsulfoxide
G-CSF	Granulocyte colony stimulating factor
M-CSF	Macrophage colony stimulating factor
VEGF	Vascular endothelial growth factor
HGF	Hepatic growth factor
IGF-1	Insulin –like growth factor 1
bFGF	Basic FGF
PlGF	Placenta-derived growth factor
MCP-1	Monocyte chemoattractant protein 1
MIP-1	Macrophage inflammatory protein 1
IDO	Indoleamine 2,3 deoxygenase
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
MMSC	Muscle-derived MSC
PMSC	Periosteum-derived MSC
SD	Standard deviation
FITC	Fluoroscein isothiocyanate
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase PCR
DMEM	D minimal essential medium
ALP	Alkaline phosphatase
P0	Passage zero
P1	Passage one

Chapter 1: Introduction

1.1 Definition of a Mesenchymal Stem Cell

A mesenchymal stem cell (MSC) is an undifferentiated cell that can proliferate and self-renew as well as differentiate into various connective tissue cells such as osteoblasts, adipocytes, and chondrocytes.[1,2] A MSC is also defined as a multipotent cell that can differentiate into various, but limited number of cell types.[3,4]

Mesenchymal stem cells are defined based on a number of criteria that evaluate their morphologic, physical, phenotypic, and functional properties.[5] The International Society for Cellular Therapy has provided a list of criteria to define human MSCs. This includes: 1) ability to adhere to plastic, 2) be positive for cell surface markers CD73, CD90, and CD105, 3) be negative for cell surface markers CD 11b, CD14, CD19,CD29α, CD34, CD45, HLA-DE, and 4) ability to differentiate into osteoblasts, adipocytes, and chondroblasts.[6] Canine MSCs have been identified based on similar criteria.[7,8]

The proteome of human MSCs is being identified and used to define MSCs.[9] The proteome of MSCs, defined as the entire complement of proteins, is based on six functional groups of proteins: cell surface markers, responsiveness to growth factors, developmental signaling cascades, extracellular matrix interaction, regulation of transcription and translation, cell number regulation, and protection against cellular stress.[10] The individual proteins themselves are not specific to MSCs, but their combination can help identify MSCs as long as tissue sources and culture conditions are kept similar.

The definition of a MSC is controversial because no specific cellular marker exists to identify a MSC *in vitro* or *in vivo*.[5] Mesenchymal stem cells may have the ability to transdifferentiate into non-mesodermal cells like neurons and hepatocytes,[9] and consistent nomenclature is lacking.[12] In the literature, other names synonymous with bone marrow-derived MSCs (BMSCs) include bone marrow stromal cells,[13] mesenchymal stromal cells, marrow stromal cells, mesodermal progenitor cells, marrow isolated adult multilineage inducible cells, and colony forming unit-fibroblasts (CFU-F).[10] These numerous synonyms likely exist because bone marrow was originally isolated as the first source of MSCs and is still one of the most commonly used tissues in experimental studies.[14]

1.2 Sources of Mesenchymal Stem Cells

Mesenchymal stem cells have been isolated from humans, baboons, rabbits, pigs, rats, mice,[5] sheep,[15] horses,[16] cows,[17] dogs,[18] and cats.[13] Tissue sources include bone marrow, periosteum, adipose tissue, synovium, muscle,[19,20,21] peripheral blood, and the central nervous system.[8] There is belief that MSCs are associated with pericytes so that any vascularised tissue source could be a potential source of MSCs.[22] To the author's knowledge, bone marrow,[8] adipose tissue,[18] umbilical cord vein,[7] and umbilical cord blood[11] are the only canine tissue sources reported to provide MSCs.

1.3 Clinical Significance

Treatment of many canine orthopedic and neurologic diseases often does not result in the desired clinical outcome or patient's return to normal function, despite the advances in veterinary surgery and medicine. These diseases include but are not limited

to osteoarthritis, spinal cord injury, and abnormal bone healing. Mesenchymal stem cells have shown tremendous promise in experimental and clinical models of veterinary and human diseases.[3,19,23-27] Therapeutic applications, as reported in the canine literature, have contributed to our basic understanding of canine MSCs.

Osteoarthritis (OA) affects many canine joints and can be managed satisfactorily in some cases with weight control, activity modification, analgesics, chondro-protectants, physiotherapy, acupuncture, and surgery but can be debilitating to some patients despite these modalities.[28,29] Black, et al.[30] conducted a randomized, double-blinded, placebo controlled experiment assessing the effects of coxofemoral intra-articular injection of autologous adipose-derived MSCs (AMSCs) in dogs clinically affected with bilateral coxofemoral OA. The treatment group had significant improvement in lameness at a walk and trot, pain on manipulation, and pain free range of motion as assessed by a veterinarian using a numeric rating scale. This was in contrast to the control group (placebo) that failed to show improvement based on veterinary and owner assessments. In a similar study, a 30-40% improvement in functional disability and lameness in dogs with elbow OA was found up to 180 days after intra-articular injection of AMSCs.[31] Even though this was not a randomized, blinded and placebo controlled study with objective assessments, the results were promising. The authors suggested that the beneficial results seen may be due to MSC secretion of IL-1a, a molecule shown to improve OA in an equine model. It was also suggested that the MSCs had paracrine effects on the resident population of endogenous stem cells or that they underwent chondrogenic differentiation themselves.

Spinal cord trauma, intervertebral disc disease, and degenerative myelopathy are

examples of common neurological disorders that can have guarded to grave prognoses despite appropriate surgical and/or medical interventions. The potential benefit of BMSCs has been shown in a canine disc degeneration model.[32] Eighteen beagles were divided into three groups: control, nucleotomy with fluoroscopic guided injection of BMSCs (treatment group), and a nucleotomy group only. The treatment group showed a significantly higher disc height index, stronger disc signal intensity on T2 weighted MR images, and significantly higher proteoglycan content compared to the nucleotomy group. Macroscopically the treatment group looked similar to the control group and lacked the narrowed disc space and connective tissue invasion seen in the nucleotomy group. The results supported the notion that the injected MSCs decelerated the effects of disc degeneration.

Enhanced bone regeneration is especially important in cases of severely comminuted fractures, delayed unions, and non-unions. The use of MSC based therapy in bone healing may allow for successful union, hasten the recovery period and result in less morbidity to the patient. Bruder, et al.[33] evaluated the effect of cultured autologous BMSCs on the healing of a critical-sized defect in canine femurs. Significant new bone formation was present in the group treated with the carrier loaded with MSCs; atrophic non-union was present in all untreated femurs at 16 weeks. Because MSCs have shown to be involved in all four strategies of bone regeneration (osteogenesis, osteoinduction, osteoconduction, and osteopromotion) their role in bone tissue engineering is an area of clinical interest.[3]

Despite the positive results of clinical and experimental MSC studies in animals, there are many unknown factors associated with their use. For example, in the literature

on canine MSCs, the ideal cell transplantation number, cell yield per gram of canine donor tissue, and ideal tissue source is not definitively known. Conventionally, high numbers of MSCs have been used in veterinary and human cell based therapies.[23,34,35] Wagner et al[36] report that in human medicine, 1-5 million cells/kg are administered intravenously or directly into the tissue. Superior tissue sources have been identified for human and rat MSCs depending on their intended use, both *in vitro* and *in vivo*.[20,21,37,38]

It is clear that further basic knowledge surrounding canine MSCs is required and is justified based on their potential therapeutic applications in veterinary medicine. Identifying potential donor tissue sources of canine MSCs, characterizing their phenotype, as well as determining the cell yield per gram of donor tissue would contribute to our understanding of canine MSCs.

1.5 References

1. Fortier L. Stem cells: classifications, controversies, and clinical applications. *Vet Surg* 2005;34:415-423.

2. Barry F, Murphy J. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 2004;36:568-584.

3. Kraus K, Kirker-Head C. Mesenchymal stem cells and bone regeneration. *Vet Surg* 2006;35:232-242.

4. Kamashina H, Farese J, Storm J, et al. The frequency, growth kinetics, and osteogenic/adipogenic differentiation properties of canine bone marrow stromal cells. *In vitro Cell Dev Biol Anim* 2008;44:472-479.

5. Javazon E, Beggs K, Flake A. Mesenchymal stem cells: paradoxes of passaging. E*xp Hematol* 2004;32:414-425.

6. Dominici M, LeBlanc K, Meuller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 2006;8:315-317.

7. Zucconi E, Vieira NM, Bueno DF, et al. Mesenchymal stem cells derived from canine umbilical cord vein-a novel source for cell therapy studies. *Stem Cells Dev* 2010;19:395-402.

8. Csaki C, Matis U, Mobasheri A, et al. Chondrogenesis, osteogenesis, and adipogenesis of canine mesenchymal stem cells: a biochemical, morphological, and ultrastructural study. *Histochem Cell Biol* 2007;128:507-520.

9. Park HW, Shin J, Kim C. Proteome of mesenchymal stem cells. *Proteomics* 2007;7:2881-2894.

10. Maurer M. Proteomic definitions of mesenchymal stem cells. *Stem Cells Int* 2011;3:1-9.

11. Seo M, Jeong Y, Park J, et al. Isolation and characterization of canine umbilical cord blood derived mesenchymal stem cells. *J Vet Sci* 2009;10:181-187.

12. Beyer Nardi N, da Silva Meirelles L. Mesenchymal stem cells: isolation, *in vitro* expansion and characterization. *Handb ExpPharmacol* 2006;174:249-282.

13. Martin DR, Cox NR, Hathcock TL, et al. Isolation and characterization of multipotent mesenchymal stem cells from feline bone marrow. *Exp Hematol* 2002;30:879-886.

14. Pountos I, Corscadden D, Emery P, et al. Mesenchymal stem cell tissue engineering: techniques for isolation, expansion, and application. *Injury* 2007;38S4:S23-S33.

15. Mrugala D, Bony C, Neves N. Phenotypic and functional characterization of ovine mesenchymal stem cells: application to a cartilage defect model. *Ann Rheum Dis* 2008;67:288-295.

16. Koch T, Berg L, Betts D. Current and future regenerative principles, concepts, and therapeutic use of stem cell therapy and tissue engineering in equine medicine. *Can Vet J* 2009;50:155-165.

17. Bosnakovski D, Mizuno M, Kim G, et al. Isolation and differentiation of bovine bone marrow mesenchymal stem cells. *Cell Tissue Res* 2005; 319:243-253.

18. Neupane M, Chia-Cheng C, Kiupel M, et al. Isolation and characterization of canine adipose-derived mesenchymal stem cells. *Tissue Eng Part A* 2008;14:1007-1015.

19. Niemeyer P, Szalay K, Luginbuhl R, et al. Transplantation of human mesenchymal stem cells in a non-autogenous setting for bone regeneration in a rabbit critical-size defect model. *Acta Biomater* 2010;6:900-908.

20. Sakaguchi Y, Sekiya I, Yagishita K, et al. Comparison of human stem cells derived from various mesenchymal tissues. *Arthritis Rheum* 2005;52:2521-2529.

21. Yoshimura H, Muneta T, Nimura A, et al. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* 2007;327:449-462.

22. da Silva Meirelles L, Fontes A, Covas D, et al. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev* 2009;20:419-27.

23. Kanaya A, Deie M, Adachi N, et al. Intra-articular injection of mesenchymal stromal cells in partially torn anterior cruciate ligaments in a rat model. *Arthroscopy* 2007;23:610-617.

24. Centeno CJ, Busse A, Kisiday J, et al. Regeneration of meniscal cartilage in a knee treated with percutaneously implanted autologous mesenchymal stem cells. *Med Hypotheses* 2008;71:900-908.

25. Murphy JM, Fink DJ, Hunziker EB, et al. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum* 2003;48:3464-3474.

26. Nandoe Tewarie RS, Hurtado A, Bartels RH, et al. Stem cell based therapies for spinal cord injury. *J Spinal Cord Med* 2009;32:105-114.

27. Coutts M, Keirstead HS. Stem cells for the treatment of spinal cord injury. *Exp Neurol* 2008;209:368-377.

28. Rychel JK. Diagnosis and treatment of osteoarthritis. *Top Companion Anim Med* 2010;25:20-25.

29. Fritsch D, Allen TA, Dodd CE, et al. Dose titration effects of fish oil in osteoarthritic dogs. *J Vet Intern Med* 2010;24:1020-1026.

30. Black LL, Gaynor J, Gahring D, et al. Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. *Vet Ther* 2007;8:272-284.

31. Black LL, Gaynor J, Adams C, et al. Effect of intraarticular injection of autologous adipose-derived mesenchymal stem and regenerative cells on clinical signs of chronic osteoarthritis of the elbow joint in dogs. *Vet Ther* 2008;9:192-200.

32. Hiyama A, Mochida J, Iwashina T, et al. Transplantation of mesenchymal stem cells in a canine disc degeneration model. *J Orthop Res* 2008;26:589-600.

33. Bruder S, Kraus K, Goldberg V, et al. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* 1998;80:985-996.

34. Vidal MA, Kilroy GE, Johnson JR, et al. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet Surg 2006;35:601-610.

35. Pal R, Venkataramana NK, Jaan M, et al. Ex vivo-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study. *Cytotherapy* 2009;11:897-911.

36. Wagner J, Kean T, Young R et al. Optimizing mesenchymal stem cell-based therapeutics. *Curr Opin Biotechnol* 2009;20:531-536.

37. Hayashi O, Katsube Y, Hirose M, et al. Comparison of osteogenic ability of rat mesenchymal stem cells from bone marrow, periosteum, and adipose tissue. *Calcif Tissue Int* 2008;82:238-247.

38. De Ugarte DA, Morizono K, Elbarbary A, et al. Comparison of multilineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* 2003;173:101-109.

Chapter 2: Literature Review

2.1 Embryonic Stem Cells and Mesenchymal Stem Cells

Both embryonic stem cells (ES) and MSCs have shown promise in the field of tissue engineering and regenerative medicine. Embryonic stem cells have the unlimited ability to self-renew, and like MSCs, are undifferentiated cells.[1] Embryonic stem cells are obtained from the pre-implantation stage of an *embryo*, or more specifically the inner cell mass of a blastocyst.[2] In comparison to MSCs, ES have a longer life span attributed to their greater telomerase activity.[2] The derivation and maintenance of ES in culture is difficult, whereas MSCs are easily isolated and expanded.[3] This is only one of many reasons why scientists have looked for alternatives to ES. Major ethical concerns exist regarding the destruction of human *embryos* for scientific use; laws governing such science are varied across the world.[4] Embryonic stem cells are allogenic and carry the risk of immune rejection which is not seen with immunotolerant MSCs. Tumour development is also a risk with ES.[5,6] Identification of ES is similar to that of MSCs in that specific cellular markers are used to define them, but more definitive identification is provided with evidence of unlimited proliferation and pluripotency. Pluripotency is a cell characteristic that describes its ability to differentiate into a cell of all three embryonic germ layers (endoderm, ectoderm, and mesoderm).[4] Other techniques for definitive identification is observation of teratoma formation after subcutaneous injection of ES into severe combined immunodeficiency mice[1] or by evaluating germline transmission of the ES.[2] There are marked similarities and differences between ES and MSCs. Research of stem cell therapy in both these fields will likely continue in full force because of the therapeutic potential in a variety of diseases.[3,7]

2.2 Isolation and Expansion

Mesenchymal stem cells can be isolated from a wide array of tissues and from a number of species.[7] The location of the tissue source itself can vary and result in isolation of MSCs with different inherent properties. For example, canine subcutaneous fat is reported to provide a greater MSC yield in comparison to omental and inguinal adipose tissue.[8] Bone marrow can be collected from the humerus, femur, or ilium in dogs. Although many options exist for tissue collection, ideal sources include sites that can provide the optimum amount of tissue, with minimal invasiveness, morbidity, and cost. Studies are currently attempting to identify tissue sources that can provide the most ideally functional MSC depending on the intended application.[9]

Numerous protocols exist to isolate MSCs once tissue is harvested. Bone marrow-derived MSCs can be obtained with the classic protocol that is based on the adherence property of MSCs, with density centrifugation, or red blood cell lysis using cytotoxic materials. The classic method involves isolating the mononuclear cell portion of a centrifuged sample of bone marrow (which contains MSCs) and plating in a culture dish with growth media. The buffy coat is the source of the mononuclear cells; however, contamination with hematopoietic cells occurs with this method. Contamination can alter the micro-environment of MSCs and affect their phenotype as well as growth characteristics.[10,11]

The density centrifugation technique theoretically reduces the degree of MSC contamination by separating the erythrocytes and granulocytes into separate layers from the mononuclear portion. Harvested bone marrow is layered over a solution of Ficoll (synthetic neutral highly branched hydrophilic polysaccharide with a density of 1.077)

or Percoll (colloidal solution of silica particles with a density of 1.088).[12] After centrifugation, the mononuclear cells are separated from the erythrocytes, granulocytes, and platelets. The mononuclear cells are collected and plated in a culture dish with growth medium. Bourzac et al.[12] found that equine BMSC yield was six fold higher with the Percoll density centrifugation in comparison to the classic method; however no significant differences were seen between the Percoll and Ficoll density gradient solutions. The density centrifugation method is reported to be a time consuming and difficult technique. Frequent manual handling also increases the risk of bacterial contamination. The lack of standardization makes comparisons among studies challenging.[10]

The red blood cell lysis technique involves using a cytotoxic material like ammonium chloride to disrupt the erythrocyte cell membrane and eliminate it from culture. This method is faster and has very little toxic effects on other cells. The technique was shown to be effective in isolating human BMSCs and resulted in a higher number of colony forming units/ml of bone marrow with significantly larger colonies in comparison to Ficoll density centrifugation.[10]

Isolation of MSCs from solid tissues is most commonly performed with enzyme digestion or explant culture techniques. With enzyme digestion, the tissues are washed to remove debris, minced, and digested with a collagenase solution (type I collagenase for example). The collagenase disrupts the peptide bonds in the collagen molecules to release the resident cells[13] which are obtained and plated after filtration and centrifugation. Both techniques result in cellular contamination with fibroblasts, endothelial cells, pericytes, blood, and stromal cells,[13-15] but enzymatic digestion is more

expensive, time consuming for large samples, and can affect cell viability.[16] With explant culture, the tissues are cut into smaller pieces, dispersed in a culture dish and allowed to adhere for a few minutes before a culture medium is applied. Progenitor cells migrate from the tissue within a few days of culture and are harvested after trypsinization (a method used to release flask adhered cells).[17-19] Jing et al.[16] found that primary explant culture of murine adipose tissue provided a higher yield of stromal cells per tissue weight in comparison to enzyme digestion.

Regardless of the isolation technique used, MSCs present in culture flasks adhere to the surface of the flask and proliferate. A concentrated population is obtained with washing of the cells to remove contaminated, non-adherent cells, and lifting of MSCs to eliminate trypsin-insensitive cells.[20] It is interesting to note that with changes in media some MSCs are lost with the non-adherent population of cells, but can be re-cultured and increase the MSC yield by 36.6%.[13] Because all of the above isolation techniques result in suboptimal contamination, methods have been developed to select specifically for MSCs. These include immunodepletion and immunoselection techniques using fluorescent activated cell sorting (FACS) or magnetic bead sorting.[13,21,22] With FACS, the cellular solution can be labelled with specific monoclonal antibodies that are tagged with a fluorescent dye and will bind to the desired cell population (ie. hematopoietic cells for immunodepletion and MSCs for immunoselection). The cells are then separated based on whether or not they fluoresce. In immunoselection magnetic bead sorting, antibodies that would bind to the cell surface proteins of MSCs are coated with magnetic beads so when an external magnetic field is introduced, the MSCs labelled with antibody separate themselves from non-labelled contaminates. The disadvantage

of immunoselection and immunodepletion is the potential for altering the epigenetics and viability of MSCs as well as preventing further use of antigenic methods to isolate cells.[11]

The next step following isolation of the desired population of MSCs is expansion. Expansion can be affected by a number of factors that are related to donor or culture conditions.[23] Donor dependent factors include species,[3] age,[7] sex, presence of systemic disease, and injury.[13] The frequency of MSCs isolated from adult mice and female mice was lower than their counterparts (immature mice and male mice, respectively) in one study.[24] Nie, et al.[25] compared MSC properties between healthy patients and those affected with systemic lupus and found that the latter had different MSC morphology and slower proliferation rate with development of senescence after four passages.

Culture condition factors can include isolation technique,[15,16] cell seeding density,[26] number of passages, type of media and growth factors, and other environmental factors.[13,23] For instance, human BMSCs cultured in platelet rich plasma had a higher expansion rate, while still maintaining their multipotential characteristics, in comparison to those cultured in fetal calf serum (FCS).[27] The isolation and expansion rate of equine umbilical cord-derived MSCs was improved when they were cultured on fibronectin coated plates.[23] Fibronectin is an extracellular matrix protein that provides a substrate for adhesion, proliferation, and migration. Grayson, et al.[28] were able to show that human BMSCs cultured in hypoxic conditions (2%) had a thirty fold increase in expansion compared to BMSCs cultured in normoxic conditions (20%).

Culture medium commonly consists of a basal medium, like alpha minimal essential media (αMEM), FCS, and/or other growth factors like platelet derived growth factor (PDGF) and fibroblast growth factor (FGF).[13] The basal medium contains essential nutrients and electrolytes. Fetal calf serum is a source of growth factors and is one of the most commonly used additives to cell cultures. However, drawbacks exist with its use. It has been shown to demonstrate variable and inconsistent performance results, especially between lot numbers[29] and carries the risk of disease transmission and immune reactions.[13,30] As a result, alternatives are being investigated and include platelet rich plasma, platelet lysate, and serum-free media.[29-31]

Cells can be expanded in monolayer cultures made of glass, polystyrene, and plastic culture flasks, and Petri dishes or in three dimensional cultures of alginate, hyaluronic acid, collagen, fibrin, and chitosan.[13] Three dimensional scaffolds are typically utilized to induce chondrogenesis,[32,33] or provide a scaffold for implanted MSCs in chondrogenic[34,35] or osteogenic applications.[36] Few studies exist comparing monolayer cultures to three dimensional culture systems with respect to MSC expansion properties, but some findings have been contradictory. In one study, three dimensional culture with a hydroxyapatite/chitosan gelatin scaffold showed improved cell adhesion, expansion and osteogenic differentiation of human BMSCs.[37] Another revealed that MSCs failed to proliferate in an alginate culture.[38]

The numerous isolation and expansion protocols that exist make comparisons among studies difficult. However, researchers are trying to identify the optimal conditions for MSC expansion and differentiation. In addition, researchers are trying to investigate culture conditions that would closely mimic the *in vivo* situation. Ultimately

the goal is to understand MSCs *in vivo* and maximize the therapeutic application of MSCs grown *in vitro*.

2.3 Cryopreservation

Cryopreservation is a freezing method that halts the chemical, biochemical, and physical properties of cells when they are subjected to cryopreservation temperatures.[39] The ability to successfully cryopreserve cells and tissues is a monumental asset in the field of tissue engineering and medicine. It allows for maintenance of cells or tissues that cannot be used immediately, and provides a large source of viable cells that can be used at any time for research or clinical applications.[40] Studies specifically evaluating cryopreservation of MSCs have shown successful cryopreservation of canine AMSCs,[40] equine peripheral blood-derived MSCs,[41] and human-derived BMSCs[42] and AMSCs.[15]

Cells and tissues are placed in a cryoprotectant prior to freezing. The most common cryoprotectant used is dimethylsulfoxide (DMSO), but glycerol is another example.[43] Cryoprotectants are thought to reduce cell injury by preventing intra and extracellular ice formation,[44] altering the concentration of harmful electrolytes within the cells, and stabilizing cell proteins and plasma membranes.[39] Researchers are evaluating the addition of caspase inhibitors, as caspase has a role in cellular apoptosis.[44] Dimethylsulfoxide is superior to glycerol because it rapidly penetrates into most cells. However the disadvantage is its cell toxicity.[43] Toxicity can be decreased with using a lower concentration of DMSO, decreasing the time to exposure pre-freeze and post-thaw, and using a lower freezing temperature.[39]

Toxicity has also been documented in patients receiving cryopreserved stem cells. In one study, 2.2% of the transplant patients had DMSO-related side effects.

These included cardiovascular, respiratory, neurological, and renal disorders.[30,44] Other concerns include microbiological contamination which has been reported in 0-4.5% of human cases in one study.[44] Although clinical application of cryopreserved cells has not been described in the canine literature, the potential risks associated with their use warrants caution and investigation in veterinary applications.

Long term preservation of cells requires storage in a mechanical freezer with a temperature of at least -80°C.[39] The temperature of vapour phase nitrogen is -156°C and of liquid nitrogen is -196°C.[44] An estimated shelf life of 1000 years is reported for cells stored in liquid nitrogen tanks.[39] Storage in liquid nitrogen tanks is the recommended technique.[43] However, Berz et al.[44] recommend storage of DMSO samples in the vapour phase of nitrogen because of the potential spread of infectious agents (aspergillosis and viruses) through the liquid phase. Many cryopreservation techniques, including freezing temperature, freezing and thawing rate, cryopreservatives, length of cryopreservation, and thawing temperatures are still controversial.[44]

Various studies have been conducted to show that cryopreserved MSCs are viable. Martinello et al.[40] evaluated canine AMSC morphology, vitality, telomerase activity, surface marker phenotype, and trilineage differentiation capability after 10-12 months of cryopreservation. The cryopreserved MSCs showed similar morphology and surface marker expression in comparison to fresh MSCs, and were successfully differentiated down the adipogenic, osteogenic, and myogenic lineages. However, the proliferation capability was decreased in the early passages which was supported by concurrent telomerase concentrations. This was in contrast to human BMSCs that did not show a difference in proliferation capacity, although they had been cryopreserved

for only one week. The morphology, recovery rate, and cell activity has been reported to be sustained in MSCs even after bone marrow, from which they have been obtained, has been cryopreserved for 21-25 years.[45]

Human BMSCs were shown to have a cell viability of greater than 90%, and maintain their expansion and differentiation properties after 5-16 days of cryopreservation.[42] Successful therapeutic potential was seen when these cells were injected into the myocardium of patients with ischemic cardiomyopathy. The clinical improvements were significant and resulted in resolution of symptoms. Greater than 90% viability was seen in human BMSCs cryopreserved for 0.3-37 months in one report,[46] and greater than 70% after cryopreservation for 30 months in another.[30]

Contradictory reports regarding the successful cryopreservation of the mononuclear cell fraction of bone marrow also exist. Successful cryopreservation of the mononuclear cell fraction of bone marrow would negate the need for specialized equipment and support staff at the collection facility; immediate cell culture techniques would not be required. Casado-Diaz, et al.[47] found that mononuclear cells cryopreserved for 4-8 weeks resulted in an MSC population with a high division rate, typical MSC surface marker phenotype, and with osteogenic and adipogenic differentiation capabilities. This is in contrast to Samuelsson, et al.[30] who did not recommend cryopreservation of bone marrow mononuclear cells as a source of MSCs because a sufficient number of MSCs was not generated and their immunosuppressive capabilities were affected.

Cryopreservation of MSCs is possible, relatively safe, and effective. Although many techniques are still debated, MSCs can be cryopreserved with current methods for

a number of years and still maintain a high degree of viability. The ability to cryopreserve MSCs further enhances their use in cell based therapies.

2.4 Paracrine Effects

Mesenchymal stem cells function by differentiating into specific cellular lineages and secreting growth factors and cytokines that promote cellular regeneration and tissue repair.[48] Mesenchymal stem cells were once thought to be therapeutic by differentiating into a specific cell lineage, like an osteoblast; but many of the therapeutic effects are now considered to be a result of their paracrine properties.[49] This understanding paves the way for potential cell-free based therapies. According to da Silva Mierrelles,[49] MSC paracrine effects can be broken down into four categories: trophic, anti-scarring, chemo-attractant, and immunomodulatory. The immunomodulatory property of MSCs will be discussed further in chapter 2.5.

Mesenchymal stem cells are considered to be trophic because they stimulate progenitor cells to proliferate and differentiate. They are anti-apoptotic and angiogenic. For example, MSCs support hematopoeisis by producing granulocyte colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), IL-6, and stem cell factor, and not by differentiating into hematopoietic cells themselves.[48] They nourish the hematopoietic environment.

The anti-apoptotic function of MSCs can serve to limit the degree of tissue injury.[50] Vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF-1) are a few cytokines involved in this process.[49] Shabbir, et al.[51] conducted a study where 4 million porcine-derived MSCs were injected intramuscularly into the hamstring muscles of hamsters with cardiomyopathy. A 60%

decrease in cardiac myocyte apoptosis was found on histopathology and the myocyte density was 80% higher in the treatment group compared to the control group. They concluded that the lower levels of cTnI, a marker of tissue injury, and myocyte regeneration were a result of the anti-apoptotic effect of MSCs.

In the same study, angiogenesis was demonstrated by a 30% higher cardiac capillary density in the treatment group. Kinnaird, et al.[52] demonstrated MSC related angiogenesis in a murine hindlimb ischemia model. An increased return to blood flow and greater hindlimb arterial cross section was seen in mice that received BMSC injections. These authors also documented expression of a number of cytokines with angiogenic properties. These include basic fibroblast growth factor (bFGF), VEGF, placental growth factor (PlGF), and monocyte chemoattractant protein 1 (MCP-1).[49]

The mechanism of anti-scarring is not completely understood, but anti-fibrotic effects have been seen with MSC secretion of HGF and adrenomedullin.[49] In a murine model of chronic kidney disease, the interstitial volume and collagen, and smooth muscle actin areas were decreased and associated with reduced interstitial fibrosis in cases that received MSCs intravenously.[53] Basal expression of VEGF and BMP-7 was present in MSCs, and the renal tissue of MSC treated animals had increased expression of these factors in comparison to the control group. Ortiz, et al.[21] found that the degree of pulmonary fibrosis, as a function of pulmonary collagen, was decreased in mice with bleomycin induced pulmonary injury after immediate treatment with systemic BMSCs. The authors hypothesized that the anti-fibrotic effects could be mediated by MSC differentiation into alveolar epithelial cells or pulmonary stem cells. They also speculated that the MSCs secreted cytokines that antagonized pro-inflammatory

mediators like TNF- α and/or removed hyaluronan and osteopontin, known contributors to fibrosis.

Numerous molecules have been identified to have a role in MSC's ability to chemoattract other cells. These are molecules like MCP-1 and 3, macrophage inflammatory protein (MIP-1), Rotoxin-3, SDF-1, and IL-8.[49] These molecules can function in an indirect fashion to target progenitor cells, white blood cells, memory and naïve T cells, B cells, and NK cells and promote secretion of other factors that would modify the microenvironment.[48,49]

Mesenchymal stem cells cultured in hypoxic conditions have demonstrated increased expression of multiple cytokines, like VEGF, that have trophic, anti-fibrotic, and chemo-attractant properties.[54] This is interesting because hypoxia is present in acutely injured tissues and mediates tissue necrosis. The ability to upregulate these protective mediators supports evidence for the role of MSCs in tissue regeneration and repair. Mesenchymal stem cells can also be genetically modified to upregulate gene expression of these growth factors.[55] Species and gender differences have been shown to impact the therapeutic effect of MSCs with respect to their paracrine function[55] and synergistic behavior among these cytokines has been reported.[52]

Functional improvements have been seen in cases that have had little MSC engraftment.[56] Studies have also shown that cell free MSC conditioned medium can result in functional improvements similar to MSC treated groups and in contrast to control groups.[57] In Shabbir, et al.'s[51] hamster heart failure model, animals injected with MSC conditioned medium showed similar improvements in ventricular function, tissue regeneration, and reduced cell death and scarring in comparison to the MSC

treatment group. This study provides further evidence that many positive therapeutic outcomes of MSC treatments are a result of their paracrine effects and not necessarily MSC differentiation.

In summary, MSC paracrine effects include attenuation of tissue injury, angiogenesis, scar tissue prevention, and chemo-attraction, including the recruitment and stimulation of progenitor cells.

2.5 Mesenchymal Stem Cells and the Immune System

Mesenchymal stem cells have shown both inhibitory and stimulatory effects on the immune system.[49,58] They are not rejected by the immune system, regardless of their source (autologous, allogenic, and even xenogenic) and are therefore characterised as immunoprivileged.[3,59] This property supports the idea of banking stem cells and providing an off-the-shelf source for allogenic applications. Transplants can be performed among individuals without having concerns about incompatible immunophenotypes and the option for treating diseases like graft versus host disease, Crohn's disease, and organ transplant rejection exist.[58] The incidence of graft versus host disease was shown to be significantly reduced in those patients who received MSCs concurrently with their bone marrow transplant.[60] The applications and mechanisms for MSC immunotolerance is an area of active research.[60-62]

Mesenchymal stem cells have been shown to evade immunosurveillance based on the expression of the following immunophenotype: major histocompatibility complex I positive (MHC I^+), MHC II^-, $CD40^-$, $CD80^-$, $CD86^-$, $B7\text{-}1^-$, and $B7\text{-}2^-$.[61,63] In order to stimulate an immune reaction, cells require at least MHC II and/or MHC I and the appropriate co-stimulatory molecules.[64] Mesenchymal stem cells typically express MHC

I which allows for initial activation of T lymphocytes, but the signalling cascade fails to progress because of the absence of required co-stimulatory molecules.[63] Glennie, et al.[65] confirmed that antigen stimulated T cell activation was not affected by MSCs in culture because the upregulation of CD25 and CD69, expected to be seen with T cell activation, was not altered by their presence. Expression of MHC on MSCs can be mediated by pro-inflammatory cytokines like IFN-γ.[58] In the presence of lower levels of this cytokine, MSCs have upregulated expression of MCH II and act as antigen presenting cells. In this situation they are immunostimulatory.

The proposed mechanism governing the relationship between MSCs and lymphocytes has varied. Di Nichola, et al.[66] concluded that soluble factors like HGF and TGF-β1, produced by MSCs, were responsible for the suppression of lymphocytes rather than direct cell to cell contact as has been hypothesized by others.[60] This group was able to demonstrate reduced suppression of lymphocyte proliferation when monoclonal antibodies against these factors (HGF and TGF-β1) were added to cell cultures with MSCs. They were also able to demonstrate similar T cell inhibition with the addition of these factors in MSC free culture; the effects of these soluble factors were additive. This is in contrast to another group that confirmed reduced immunoreactivity because of direct cell to cell contact between MSCs and lymphocytes.[60] Suppressed lymphocyte reactivity was not seen in cultures made with MSC supernatant; MSCs needed to be present in culture and the degree of immunosuppression was dose dependent. Glennie, et al.[65] speculate that the mechanism of action includes both cell to cell contact and the cytokine environment.

Stimulated T lymphocytes are preferentially bound by MSCs in comparison to quiescent T lymphocytes and B lymphocytes.[64] Mujamdar, et al.[64] were able to demonstrate that MSCs bind to both $CD4^+$ (T helper cell) and $CD8^+$ (cytotoxic T cell). Mesenchymal stem cells could act as antigen presenting cells to T lymphocytes when they were cultured in tetanus toxoid and IFN-γ (to induce expression of MHC II). The authors speculated that the cell to cell interaction seen between MSCs and lymphocytes and the resultant production of cytokines seen in this study could potentially occur *in vivo* under the appropriate conditions.

Inhibition of lymphocyte suppression has been reported to be both reversible and irreversible. Di Nichola, et al.[66] showed that suppressed murine T lymphocytes could be stimulated with allogenic dendritic cells, phytohemagglutinin, and IL-2 once removed from culture with MSCs. The proliferation rate was similar to that of control cultures. Krampera, et al.[60] reported that the naive and memory T cells were both suppressed while in culture with MSCs, but when the MSCs were removed, naive cells were no longer suppressed. In contrast to this, Glennie, et al.[65] reported that MSC mediated suppression of $CD4^+$, $CD8^+$, and B cell proliferation was irreversible despite culture with IL-2 and antigen.

Antigen presenting cells are not required to mediate MSC immunomodulation.[58] However, the maturation of antigen presenting cells, affected by soluble factors like prostaglandin E2 and IL-6 produced by MSCs, has been reported to affect T cell suppression.[58,67] Lymphocyte immunosuppression has been reduced in studies that decreased PGE2 production with PGE2 inhibitors.[68,69] Nitric oxide, HGF, and TGF-β1,

and indoleamine 2,3 deoxygenase (IDO), an enzyme upregulated in MSCs exposed to pro-inflammatory molecules, have also been shown to suppress T cell proliferation.[22,58]

Mesenchymal stem cell immunomodulation is complex with many likely mechanisms involved. Much of what is known stems from *in vitro* experiments with manipulation of culture conditions that may or may not represent the *in vivo* environment. It is interesting that not only do MSCs fail to elicit an immune reaction in allogenic and xenogenic applications, but they show suppressive and permissive effects on the immune system depending on the local environment.

Chapter 2.6 Homing Mechanism

Homing is the ability of cells to migrate to and engraft into tissues depending on the environment to which they are exposed.[3,56,58] Karp, et al.[70] defines homing as "the arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium". Mesenchymal stem cells home to inflamed, ischemic, malignant, and bone marrow tissue as well as to other normal tissues.[70] Once their destination is reached, MSCs exert their function in a number of ways: cellular differentiation, production of cytokines, growth factors, and hormones, stimulation of other progenitor cells, cell fusion or transfer of mitochondria.[71] The aim of this chapter is to illustrate examples of clinical applications, proposed mechanisms of action, and the challenges and solutions associated with MSC engrafting.

Successful MSC homing has been reported. Kopen, et al.[72] showed that mouse BMSCs injected into the cerebrospinal fluid of neonatal mice migrated throughout the forebrain and cerebellum, along the normal route of post natal development, and underwent neuronal differentiation in response to the microenvironment. These cells

followed the same expected path of resident neuroprogenitor cells. In another study, systemically administered MSCs homed to the site of injury and improved functional outcome in a model of rat cerebral ischemia.[73] Type II diabetes was induced in mice to evaluate the effect of systemically administered human MSCs. Preferential migration of MSCs was seen in the pancreas and kidneys of affected rats. Human fibroblasts were used as a control and could not be identified in any of the organs examined.[71] In a swine myocardial infarction model, BMSCs directly injected into the infarcted tissue showed engraftment in the necrotic tissue up to six months post-treatment and resulted in improved cardiac function in comparison to the control group.[74]

The ability of MSCs to home is related to their individual expression of chemokine receptors and adhesion molecules as well as that of their target tissues.[22] Leukocytes have a very well developed homing mechanism and that knowledge has contributed to the understanding of MSC homing. Leukocyte homing is defined by the adhesion cascade: tethering, rolling, firm adhesion, and transmigration.[58] This is similar to MSCs in that cytokine expression can be modified by inflammation, chemokine signals allow for cell adhesion to endothelial cells, and MSCs transmigrate into tissues, followed by navigation through the extracellular matrix.[75]

Mesenchymal stem cells migrate towards growth factors in a dose dependent fashion,[49] preferentially migrate to injured tissues and tumours,[3,78] and can also cross the blood brain barrier.[73] Proteins reported to be involved in MSC migration in culture include MCP-1,[76] MIP-1α, SDF-1 and IL-8.[77] Toll-like receptors[58] and lower concentrations of bFGF are also reported to support migration of MSCs.[77]

CD44 is a MSC surface protein that allows MSCs to bind to endothelial cells through E-selectin.[49] Selectins are a family of adhesion molecules that participate in the rolling adhesion and migration of MSCs. This concept was demonstrated when MSCs showed reduced rolling in mice with deficient endothelial P selectin.[79] Integrins are another class of adhesion molecules. Specific integrins $\alpha 1$-$\alpha 5$, $\beta 1$, $\beta 3$, and $\beta 4$ have been shown to be expressed on MSCs.[67]

VCAM-1 is an adhesion molecule expressed by both endothelial cells and MSCs. It is expressed at sites of tissue injury and is a receptor for VLA-4, a ligand expressed in 50% of MSCs.[67] Studies have clarified the role of VCAM-1 as an important molecule involved in MSC homing by showing that administration of anti-VCAM-1 antibodies results in reduced adhesion between rat MSCs and vascular endothelium.[58]

Mesenchymal stem cells express receptors for ligands of the chemokine family. The most common receptor reported is CXCR4 and is the only receptor that binds to SDF-1, a potent chemokine upregulated by injured tissues.[80] CXCR4 is not specific to MSCs.[81] Potapova, et al.[82] report that 24 hours after isolating MSCs, the expression of CXCR4 diminished from 30%. This is in agreement with other studies that suggest that CXCR4 concentration on the cell surface of MSCs is typically low (<1%) but can be upregulated with the addition of cytokines in culture or through retroviral transduction.[77,80] CX3CR1, a receptor present on MSCs, binds with fractalkine, another chemokine upregulated in injured tissues as shown in a rat model of hypoglossal nerve injury.[77] Other chemokine receptors reported to be expressed by MSCs, and likely

involved in their homing capability include: CCR1, CCR4, CCR7, CXCR5, CCR10,[67] CCR2, and CCR5.[77]

Endothelial transmigration is considered a result of MSC expression of matrix metalloproteinases like MMP-2, and MTI-MMP, and TIMP-1.[75] In addition to MMPs, gelatinases have been shown to disrupt the endothelial basement membrane and extracellular matrix in order to allow MSCs to migrate across a chemokine gradient. Matrix metalloproteinase inhibitors result in poor migration of MSCs across endothelium and have been seen in overly confluent cultures.[70]

Mesenchymal stem cells have been reported to have low engraftment rates.[70] Suboptimal engraftment rates are considered a potential cause for the transient effects seen with MSC therapy.[80] Factors affecting engraftment include: duration of cell culture, route of administration, MSC size and number, timing of administration, animal irradiation, and genetic engineering. Rombouts, et al.[22] revealed that 55-65% of freshly administered MSCs could be recovered from the bone marrow of sublethally irradiated mice and resulted in complete renewal of the lymphohematopoietic system within four weeks. In contrast, administration of MSCs cultured for 48 hours resulted in complete absence of MSCs within lymphohematopoietic organs. Therefore, cultured MSCs demonstrated reduced engraftment in comparison to fresh MSCs. Intravenous administration of MSCs was inferior to that of intraventricular injection for engraftment of MSCs in a model of heart disease.[67] Intra-arterial injection close to the targeted organ is reported to provide higher engraftment rates, potentially because of bypassing filter organs like the spleen, liver, and lung.[70] Mesenchymal stem cells can lodge in pulmonary vasculature because of their relatively large size and result in fewer cells

having the opportunity to engraft into targeted tissues. Sodium nitroprusside was shown to decrease entrapment in the pulmonary vasculature and therefore may improve the number of MSCs available for homing to other tissues.[83] Karp, et al.[70] report improved engraftment with administration of higher cell numbers and earlier in the course of disease. Higher engraftment was seen in the bone marrow of mice exposed to total body irradiation than those without.[84] Lastly, human AMSCs that were retrovirally transduced to over express CXCR4 showed better homing in comparison to their control.[80] Therefore, the engraftment ability of MSCs is low, but techniques are being established to improve this characteristic.

Tracking of MSCs *in vivo* is another important factor in the understanding of MSC engraftment. Researchers are investigating methods that allow for minimally invasive, repeatable, and quantitative approaches to identify MSCs *in vivo*.[81] Cells can be labelled directly with radionuclides like ^{111}In oxine or nanoparticles like superparamagnetic superoxide and visualized with gamma scintigraphic imaging or magnetic resonance imaging, respectively.[85,86] The disadvantages of these techniques include the inability to detect cells with cell division or radio-decay, and the non-specific detection from dead cells.[81] Cells can be indirectly labelled with reporter genes. For instance, MSCs can be transduced with firefly luciferase labelling and visualized with bioluminescent imaging.[86] The advantage of using reporter genes lies in the expectation that MSCs can be observed for longer periods.[81] Histopathological techniques exist as well but are more useful to evaluate the fate of MSCs.[87]

In summary, MSCs demonstrate some ability to migrate and dock to various tissues. Preferential migration to inflamed and cancerous tissue has been demonstrated.

A plethora of chemokines and their respective receptors are involved in their migration, adhesion, and transmigration. Current investigations continue to define the role of these proteins as well as evaluate the manipulation and identification of engrafted MSCs in order to improve potential therapeutic applications.

2.7 Therapeutic Applications

Mesenchymal stem cells have shown tremendous promise in the treatment of a variety of human and veterinary diseases. Exploitation of properties such as simple isolation and expansion, tolerance of cryopreservation, paracrine and immunomodulatory effects, as well as MSC homing capability has advanced their therapeutic potential. Applications have been demonstrated in, but not limited to, orthopaedic, neurologic, endocrine, renal, and oncologic diseases.

Abundant reports exist in the literature regarding the application of MSCs in orthopaedics. These include bone healing, osteochondral defects, osteoarthritis, and tendon disorders.[3,20,88] Cases with poor bone stock (as a result of comminution or tumour excision), delayed healing and non-unions, or systemic disease can be challenging and benefit from the use of MSCs. Niemeyer, et al.[89] compared the regenerative potential of autologous and xenogenic BMSCs, both in a calcium deficient hydroxyapatite scaffold, in a critical size radial bone defect model in immunocompetent New Zealand white rabbits. The regenerative potential was evaluated with radiography, micro-computed tomography, histology, and biomechanically by testing the stiffness in four point bending. The autologous MSC group showed significantly improved healing of the defect (22.7% of defect filled with bone) when compared to control (9.08% with

the carrier and 2.15% without the carrier). The xenogenic MSC group displayed inferior results demonstrated by all parameters evaluated (only 6.63% of the defect was filled with bone). Researchers have been able to demonstrate improved bone healing in an unchallenged stabilized tibial fracture model in mice treated systemically with BMSCs.[90] The fracture callus volume was greater, contained more cartilage and bone content, and had increased toughness in mice treated with MSCs in comparison to the control group. Mesenchymal stem cells were observed to migrate to the fracture site in a time and dose dependent manner.

Osteogenesis imperfecta is a genetic disorder of collagen deficiency, most commonly reported in people that results in poor bone quality, structural deformities, and great risk of fracture.[91] Unaltered bone marrow was administered intravenously to human patients with *osteogenesis imperfecta* and resulted in a 44-77% increase in bone mineral content, improved growth, reduced frequency of fractures and histopathological evidence of dense new bone formation in patients three months after treatment. One case had 37 fractures thirteen months prior to treatment, whereas only 3 occurred within 6 months after. These authors believed improvements were a result of MSC bone engraftment and osteoblast stimulation. The transplant was tolerated well except for one patient that developed sepsis, pulmonary insufficiency, and neurological signs. In another study, significant improvements in bone strength and growth, and reduction in fracture incidence were documented in affected mice that received *in utero* transplantation of culture expanded fetal blood MSCs.[93] It was interesting to note that the transplanted cells engrafted to various organs, but was greatest to bones and sites of fracture healing. Toxicities associated with MSC transplants were not reported.

The use of MSCs in the treatment of tendon disorders is a common practice in equine medicine.[14] Schnabel, et al.[94] evaluated the effect of locally injected BMSCs and IGF enhanced BMSCs in an equine model of bilateral tendinitis. Mechanical testing revealed that injected superficial digital flexor tendons were stiffer than controls, but significance was not reached. Ultrasound examinations also did not differ between groups. However, histological scores were better in the tendons that were injected with MSCs. Genetic enhancement of the MSCs with growth factor did not reveal any benefit, despite reports of improved tendon healing with local injections of IGF-1 alone. Controlled and randomized clinical trials of MSC treated tendon disorders in the equine do not exist in the literature. However, comparisons among studies have been made evaluating injury recurrence rates, an important outcome measure, and have found decreased incidence of recurrence in horses treated with MSCs.[95,96] In addition, horses that receive MSC treatment sustain their reinjury later in comparison to those who are managed conservatively (ie. 83 days versus 44 days, respectively).[96]

Mesenchymal stem cells have shown to be an attractive option in the treatment of osteoarthritis. Murphy, et al.[97] excised the anterior cruciate ligament and medial meniscus of goats to create a model of osteoarthritis. Six weeks after the initial surgery, autologous BMSCs in combination with hyaluronan were injected intra-articularly. The most interesting finding was that the medial meniscus had shown regeneration, and in those cases only, was the degree of osteoarthritis decreased in comparison to the control group that received a hyaluronan injection only. Reduced articular cartilage degeneration, osteophyte remodelling, and subchondral bone sclerosis were seen in the treatment group. A significant increase in meniscus volume was also seen in a human

patient treated with intra-articular BMSCs.[98] However, cartilage repair was not seen in dogs with induced stifle osteochondral defects treated with collagen implants loaded with MSCs.[99] The only difference in this group compared to the control group was significantly greater subchondral bone production consistent with more of an osteogenic contribution.

The consequences of spinal cord and brain injury can be grave; it is therefore no surprise that researchers are trying to evaluate the utility of MSCs in their treatment. Li, et al.[100] evaluated the effect of umbilical cord-derived MSCs in the treatment of severe acute spinal cord injury in dogs. The treatment group included dogs with local delivery of MSCs and the control groups consisted of those without any treatment and dogs who only received saline injection. Spinal cord regeneration was not evident based on histopathology and MRI, but the dogs had significantly improved functional recovery based on the Olby score and somatosensory evoked potentials. A randomized and controlled phase I/II human clinical trial by Bang, et al.[101] evaluated the effect of BMSCs in the treatment of severely debilitated ischemic stroke patients. Patients that received autologous, culture expanded BMSCs systemically showed significant improvement in neurological recovery based on functional assessments.

Mesenchymal stem cells have been applied to the treatment of experimentally induced type II diabetes in mice.[71] Mice developed severe hyperglycemia and were given systemically administered human BMSCs as a treatment and human fibroblasts as a control. Only the MSCs homed specifically to the pancreas and kidneys of affected mice. Results revealed significant reduction in blood glucose levels and increased levels

of circulating insulin in the treatment group. Therapeutic effects were also evident in the renal parameters evaluated.

Ischemia reperfusion induced acute renal failure has been treated experimentally in rats using MSCs. Renoprotection was evident in rats that received autologous BMSCs via intracarotid injection immediately and twenty-fours after renal ischemia.[102] This was not seen in control rats administered fibroblasts. Significant improvements were seen in renal function of MSC treated rats based on serum creatinine and blood urea nitrogen. Lower injury scores and reduced apoptosis were seen on renal histopathology. The improvements in renal parameters were likely a function of MSC's paracrine effects; as very few labelled MSCs were seen in the kidneys, differentiation into tubular or endothelial phenotypes was not present, and expression of anti-inflammatory cytokines was increased.

Lastly, MSCs are currently being used in the fight against cancer. They home to tumours and can be modified to deliver anti-tumour therapies.[103] Mesenchymal stem cells have been shown to engraft in brain tumours, and after modification, release factors like IL-12, tumour necrosis factor related apoptosis inducing ligand (TRAIL), and IFN-β, resulting in reduced tumour growth and improved survival times.[104] Loebinger, et al.[105] demonstrated apoptosis of lung, breast, squamous, and cervical cancer when co-cultured with TRAIL expressing human BMSCs. *In vivo* evaluation of TRAIL MSCs was performed in xenograft models of subcutaneous and metastatic pulmonary tumours; significant reductions in tumour burden were seen. Studeny, et al.[106] demonstrated that unaltered human MSCs co-injected with melanoma cells in a xenografic mouse model stimulated tumour development. However, administration of

MSCs modified to express IFN-β inhibited tumour growth. Therefore, MSCs have the potential to be used as an anti-tumour modality.

In conclusion, MSCs have shown positive results in the treatment of oncologic, renal, endocrine, neurologic, and orthopaedic applications. Benefits have been seen with both local and systemic administration. The majority of treatments have been shown to be safe, as reports of tumours, adverse immune responses, and infections are rare.[10,107] Large scale, randomized and controlled studies are the required next step to prove the role of MSCs in tissue engineering and regenerative medicine.

2.8 References

1. Rippon HJ, Bishop AE. Embryonic stem cells. *Cell Prolif* 2004;37:23-34.

2. Fortier L. Stem cells: classifications, controversies, and clinical applications. *Vet Surg* 2005;34:415-423.

3. Barry F, Murphy J. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 2004;36:568-584.

4. Alison MR, Poulsom R, Forbes S, et al. An introduction to stem cells. *J Pathol* 2002;197:419-423.

5. Hayes B, Fagerlie SR, Ramakrishnan A, et al. Derivation, characterization, and *in vitro* differentiation of canine embryonic stem cells. *Stem Cells* 2008;26:465-473.

6. Van Orman JR, Weihrauch D, Warltier DC, et al. Myocardial interstitial fluid inhibits proliferation and cardiomyocyte differentiation in pluripotent embryonic stem cells. *Am J Physiol Heart Circ Physiol* 2009;4:H1369-H1376.

7. Malgieri A, Kantzoni E, Patrizi MP, et al. Bone marrow and umbilical cord blood human mesenchymal stem cells: state of the art. *Int J Clin Exp Med* 2010;3:248-269.

8. Neupane M, Chia-Cheng C, Kiupel M, et al. Isolation and characterization of canine adipose-derived mesenchymal stem cells. *Tissue Eng Part A* 2008;14:1007-1015.

9. Harichandan A, Buhring H. Prospective isolation of human MSC. *Best Pract Res Clin Haematol* 2011;24:25-36.

10. Horn P, Bork S, Diehlmann A, et al. Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. *Cytotherapy* 2008;10:676-685.

11. Hsiao F, Cheng C, Peng S, et al. Isolation of therapeutically functional mouse bone marrow mesenchymal stem cells within 3 h by an effective single-step plastic-adherent method. *Cell Prolif* 2010;43:235-248.

12. Bourzac C, Smith L, Vincent P, et al. Isolation of equine bone marrow-derived mesenchymal stem cells: a comparison between three protocols. *Equine Vet J* 2010;42:519-527.

13. Pountos I, Corscadden D, Emery P, et al. Mesenchymal stem cell tissue engineering: techniques for isolation, expansion, and application. *Injury* 2007;38S4:S23-S33.

14. Black LL, Gaynor J, Gahring D, et al. Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. *Vet Ther* 2007;8:272-284.

15. Baptista L, do Amaral R, Carias R, et al. An alternative method for the isolation of mesenchymal stromal cells derived from lipoaspirate samples. *Cytotherapy* 2009;11:706-715.

16. Jing W, Jingang X, Zhonghua X, et al. Explant culture: an efficient method to isolate adipose-derived stromal cells for tissue engineering. *Artif Organs* 2010;33:105-112.

17. Resau J, Sakamoto K, Cotrell J, et al. Explant organ culture: a review. *Cytotechnology* 1991;7:137-149.

18. Tuli R, Tuli S, Nandi S, et al. Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone. *Stem Cells* 2003;21:681-693.

19. Yameen Z, Leavesky D, Upton Z, et al. Multilineage differentiation potential of bone and cartilage cells derived from explants culture. *Open Stem Cell J* 2009;1:10-19.

20. Kraus K, Kirker-Head C. Mesenchymal stem cells and bone regeneration. *Vet Surg* 2006;35:232-242.

21. Ortiz L, Gambelli F, Mcbride C, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;100:8407-8411.

22. Rombouts W, Ploemacher R. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* 2003;17:160-170.

23. Schuh W, Friedman M, Carrade D. Identification of variables that optimize isolation and culture of multipotent mesenchymal stem cells from equine umbilical cord. *Am J Vet Res* 2009;70:1526-1535.

24. Katsara O, Mahaira LG, Iliopoulou EG, et al. Effects of donor age, gender, and *in vitro* cellular aging on the phenotypic, functional, and molecular characteristics of mouse bone marrow-derived mesenchymal stem cells. *Stem Cells Dev.* 2011 Feb 15.

25. Nie Y, Lau C, Lie A. Defective phenotype of mesenchymal stem cells in patients with systemic lupus erythematosus. *Lupus* 2010;19:850-859.

26. Kamashina H, Farese J, Storm J, et al. The frequency, growth kinetics, and osteogenic/adipogenic differentiation properties of canine bone marrow stromal cells. *In vitro Cell Dev Biol Anim* 2008;44:472-479.

27. Vogel JP, Szalay K, Geiger F, et al. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and *in vivo* bone formation in calcium phosphate ceramics. *Platelets* 2006;17:462-469.

28. Grayson W, Zhao F, Bunnel B, et al. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2007;358:948-953.

29. Chase L, Lakshmipathy U, Solchaga L, et al. A novel serum-free medium for the expansion of human mesenchymal stem cells. *Stem Cell Res Ther* 2010;1:8-19.

30. Samuelsson H, Ringden O, Lonnies H, et al. Optimizing *in vitro* conditions for immunomodulation and expression of mesenchymal stromal cells. *Cytotherapy* 2009;11:129-136.

31. Goedecke A, Wobus M, Krech M, et al. Differential effect of platelet-rich plasma and fetal calf serum on bone marrow-derived human mesenchymal stromal cells expanded *in vitro*. *J Tissue Eng Regen Med.* 2011 Jan 14.

32. Hsu SH, Huang TB, Cheng SJ, et al. Chondrogenesis from human placenta-derived mesenchymal stem cells in three-dimensional scaffolds for cartilage tissue engineering. *Tissue Eng Part A.* 2011 Mar 7.

33. Kim H, Lee J, Im G. Chondrogenesis using mesenchymal stem cells and PCL scaffolds. *J Biomed Mater Res A* 2010;92:659-666.

34. Wang Y, Kim U, Blasioli D, et al. *In vitro* cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. *Biomaterials* 2005;26:7082-7094.

35. Bosnakovski D, Mizuno M, Kim G, et al. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. *Biotechnol Bioeng* 2006;93:1152-1163.

36. Zhao F, Grayson W, Ma T, et al. Effects of hydroxyapatite in 3-D chitosan-gelatin polymer network on human mesenchymal stem cell construct development. *Biomaterials* 2006;27:1859-1867.

37. Zhang L, Su P, Xu C, et al. Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. *Biotechnol Lett* 2010;32:1339-1346.

38. Karlsen T, Mirtaheri P, Shahdadfar A, et al. Effect of three-dimensional culture and incubator gas concentration on phenotype and differentiation capability of human mesenchymal stem cells. *J Cell Biochem* 2011;112:684-693.

39. Karlsson J, Toner M. Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials* 1996;17:243-256.

40. Martinello T, Bronzini I, Maccatrozzo L. Canine adipose-derived-mesenchymal stem cells do not lose stem cell features after a long term cryopreservation. *Res Vet Sci* 2010; doi:10.1016/j.rvsi.2010.07.024.

41. Martinello T, Bronzini I, Maccatrozzo L, et al. Cryopreservation does not affect stem characteristics of multipotent cells isolated from equine peripheral blood. *Tiss Eng Part C* 2010;16:771-781.

42. Chin S, Poey A, Wong C, et al. Cryopreserved mesenchymal stromal cell treatment is safe and feasible for severe dilated ischemic cardiomyopathy. *Cytotherapy* 2010;12:31-37.

43. Meryman H. Cryopreservation of living cells: principles and practice. *Transfusion* 2007;47:935-945.

44. Berz D, McCormack E, Winer E, et al. Cryopreservation of hematopoietic stem cells. *Am J Hematol* 2007;82:463-472.

45. Huang YZ, Shen JL, Gong LZ, et al. *In vitro* activity of human bone marrow cells after cryopreservation in liquid nitrogen for 21 - 25 years. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2010;18:224-229.

46. Kotobuki N, Hirose M, Machida H, et al. Viability and osteogenic potential of cryopreserved human bone marrow-derived mesenchymal cells. *Tissue Eng* 2005;11:663-673.

47. Casado-Diaz A, Santiago-Mora R, Jimenez R, et al. Cryopreserved human bone marro mononuclear cells as a source of mesenchymal stromal cells: application in osteoporosis research. *Cytotherapy* 2008;10:460-468.

48. Caplan A, Dennis J. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 2006;98:1076-1084.

49. da Silva Meirelles L, Fontes A, Covas D, et al. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev* 2009;20:419-27.

50. Caplan A. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009;217:38-324.

51. Arsalan Shabbir, David Zisa, Gen Suzuki, et al. Heart failure therapy mediated by the trophic activities of bone marrow mesenchymal stem cells: a noninvasive therapeutic regimen. *Am J Physiol Heart Circ Physiol* 2009;296:1888–1897.

52. Kinnaird T, Stabile E, Burnett M, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circ Res* 2004;94:678-685.

53. Ninichuck V, Gross O, Segerer S, et al. Multipotent mesenchymal stem cells reduce interstitial fibrosis but do not delay progression of chronic kidney disease in collagen4A3-deficient mice. *Kidney Int* 2006;70:121-129.

54. Gnecchi M, Zhang Z, Ni A, et al. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* 2008;21:1204-1219.

55. Zisa D, Shabbir A, Suzuki G, et al. Vascular endothelial growth factor (VEGF) as a key therapeutic trophic factor in bone marrow mesenchymal stem cell-mediated cardiac repair. *Biochem Biophys Res Commun* 2009;390:834-838.

56. Prockop D. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). *Clin Pharmacol Ther* 2007;82:241-243.

57. Shimada I, Spees J. Stem and progenitor cells for neurological repair: minor issues, major hurdles, and exciting opportunities for paracrine-based therapeutics. *J Cell Biochem* 2011;112:374-380.

58. Yagi H, Soto-Gutierrez A, Parekkadan B, et al. Mesenchymal stem cells: mechanisms of immunomodulation and homing. *Cell Transplant* 2010;19:667-679.

59. Bruder S, Kraus K, Goldberg V, et al. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* 1998;80:985-996.

60. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naïve and memory antigen-specific T cells to their cognate peptide. *Blood* 2003;101:3722-3729.

61. LeBlanc K, Tammik C, Rosendahl K, et al. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003;31:890-896.

62. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002;30:42-48.

63. Javazon E, Beggs K, Flake A. Mesenchymal stem cells: paradoxes of passaging. E*xp Hematol* 2004;32:414-425.

64. Majumdar M, Keane-Moore M, Buyaner D. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci* 2003;10:228-241.

65. Glennie S, Soeiro I, Dyson P, et al. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005;105:2821-2827.

66. Di Nichola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;99:3838-3843.

67. Chamberlain G, Fox J, Ashton B, et al. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007;25:2739-2749.

68. Aggarwal S, Pittenger M. Human mesenchymal stem cells modulate allogeneic immune cell response. *Blood* 2005;105:1815-1822.

69. Jarvinen L, Badri L, Wettlaufer S, et al. Lung resident mesenchymal stem cells isolated from human lung allografts inhibit T cell proliferation via a soluble mediator. *J Immunol* 2008;181:4389-4396.

70. Karp J, Sock Leng Teo G. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 2009;4:206-216.

71. Lee R, Seo M, Reger R, et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci U S A* 2006;103:17438-174343.

72. Kopen G, Prockop D, Phinney D. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A* 1999;96:10711-10716.

73. Chen J, Li Y, Katakowski M, et al. Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *J Neurosci Res* 2003;15:778-786.

74. Shake J, Gruber P, Baumgartner W, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* 2002;73:1919-1926.

75. Van Linthout S, Stamm C, Schultheiss H, et al. Mesenchymal stem cells and inflammatory cardiomyopathy: cardiac homing and beyond. *Cardiol Res Pract* 2011;6:1-8.

76. Wang L, Li Y, Chen J, et al. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. *Hematol* 2002;7:113-117.

77. Ji J, He B, Dheen T, et al. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* 2004;22:415-427.

78. Stagg J. Mesenchymal stem cells in cancer. *Stem Cell Rev* 2008;4:119-124.

79. Ruster B, Gottig S, Ludwig R, et al. Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006;108:3938-3944.

80. Bobis-Wozowicz S, Miekus K, Wybieralska E, et al. Genetically modified adipose tissue-derived mesenchymal stem cells overexpressing CXCR4 display increased motility, invasiveness, and homing to bone marrow of NOD/SCID mice. *Exp Hematol* 2011. Mar 21.

81. Lee Z, Dennis J, Gerson S. Imaging stem cell implant for cellular-based therapies. *Exp Biol Med* 2008;233:930-940.

82. Potapova I, Cohen I, Doronin S. Apoptotic endothelial cells demonstrate increased adhesiveness for human mesenchymal stem cells. *J Cell Biochem* 2009;219:23-30.

83. Gao J, Dennis JE, Muzic RF et al. The dynamic *in vivo* distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs* 2001;169:12–20.

84. Francois S, Bensidhoum M, Mouiseddine M, et al. Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. *Stem Cells* 2006;24:1020-1029.

85. Kraitchman D, Tatsumi M, Gilson W, et al. Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. *Circulation* 2005;112:1451-1461.

86. Arbab A, Janic B, Haller J, et al. *In vivo* cellular imaging for translational medical research. *Curr Med Imaging Rev* 2009;5:19-38.

87. Shan L. FluidMAG iron nanoparticle-labeled mesenchymal stem cells for tracking cell homing to tumors. Molecular Imaging and Contrast Agent Database (MICAD) [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2004-2010. 2009 Dec 23 [updated 2010 Feb 16].

88. Cancedda R, Dozin B, Giannoni P, et al. Tissue engineering and cell therapy of cartilage and bone. *Matrix Biol* 2003;22:81-91.

89. Niemeyer P, Szalay K, Luginbuhl R, et al. Transplantation of human mesenchymal stem cells in a non-autogenous setting for bone regeneration in a rabbit critical-size defect model. *Acta Biomater* 2010; 6: 900-908.

90. Granero-Molto F, Weis J, Miga M, et al. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells* 2009;27:1887-1898.

91. Chanda D, Kumar S, Ponnazhagan S. Therapeutic potential of adult bone marrow-derived mesenchymal stem cells in diseases of the skeleton. *J Cell Biochem* 2010;111:249-257.

92. Horwitz E,, Prockop D, Fitzpatrick L, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999;5:309-313.

93. Guillot P, Abass O, Bassett D, et al. Intauterine transplantation of human fetal mesenchymal stem cells from first-trimester blood repairs bone and reduces fractures in osteogenesis imperfect mice. *Blood* 2008;111:1717-1725.

94. Schnabel L, Lynch M, van der Meulen M, et al. Mesenchymal stem cells and insulin-like growth factor-1 gene-enhanced mesenchymal stem cells improve structural

aspects of healing in equine flexor digitorum superficialis tendons. *J Orthop Res* 2009;27:1392-1398.

95. Frisbie D, Smith R. Clinical update on the use of mesenchymal stem cells in equine orthopaedics. *Equine Vet J* 2010;42:86-89.

96. Smith, R. Mesenchymal stem cell therapy for equine tendinopathy. *Disabil Rehabil* 2008;30:1752-1758.

97. Murphy JM, Fink DJ, Hunziker EB, et al. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum* 2003;48:3464-3474.

98. Centeno CJ, Busse A, Kisiday J, et al. Regeneration of meniscal cartilage in a knee treated with percutaneously implanted autologous mesenchymal stem cells. *Med Hypotheses* 2008;71:900-908.

99. Yamazoe K, Mishima H, Torigoe K, et al. Effects of atelocollagen gel containing bone marrow-derived stromal cells repair of osteochondral defect in a dog. *J Vet Med Sci* 2007;69:835-839.

100. Li J, Byeon Y, Ryu H, et al. Transplantation of canine umbilical cord blood-derived mesenchymal stem cells in experimentally induced spinal cord injured dogs. *J Vet Sci* 2007;8:275-282.

101. Bang O, Lee J, Lee P, et al. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol* 2005;57:874-882.

102. Togel F, Hu Z, Weiss K, et al. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol* 2005;289:F31-F42.

103. Loebinger M, Kyrtatos P, Turmaine M, et al. Magnetic resonance imaging of mesenchymal stem cells homing to pulmonary metastases using biocompatible magnetic nanoparticles. *Cancer Res* 2009;69:8862-8867.

104. Nakamizo A, Marini F, Amano T, et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;65:3307-3318.

105. Loebinger M, Eddaoudi A, Davies D, et al. Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer. *Cancer Res* 2009;69:4134-4142.

106. Studeny M, Marini F, Champlin R, et al. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-β delivery into tumors. *Cancer Res* 2002;62:3603-3608.

107. Wakitani S, Okabe T, Horibe S, et al. Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. *J Tissue Eng Regen Med* 2011;5:146-150.

Chapter 3: Hypotheses and Objectives

Hypothesis

1. Canine bone marrow, adipose tissue, muscle, and periosteum are sources of MSCs.

This will be based on:

a) All four donor tissue-derived MSCs being adherent to plastic and having the typical fibroblastic morphology.

b) The isolated MSCs expressing cell surface markers CD44, CD90, and CD146 based on immunohistochemistry. They will not express the hematopoietic cell surface markers D34 and CD45.

c) The isolated MSCs expressing pluripotency associated transcription factors OCT4, SOX2, and NANOG using RT-PCR and gel electrophoresis.

d) The isolated MSCs being differentiated down the adipogenic, chondrogenic, and osteogenic lineages. With adipogenic differentiation, lipid vacuoles will form and stain positively with Oil Red O. In a chondrogenic pellet culture system, a chondrocyte matrix will form and stain positively with Alcian blue and Safranin O. With osteogenic differentiation, bone nodules will be apparent and stain positively with Von Kossa and Alizarin Red stain.

2. Canine muscle and periosteum-derived MSCs will have an equivalent, if not superior, proliferation potential compared to bone marrow and adipose tissue-derived MSCs.

Objectives

1. To confirm the ability to isolate post natal MSCs from canine bone marrow and adipose tissue.

2. To isolate post natal MSCs from canine skeletal muscle (MMSCs) and periosteum (PMSCs).

3. To determine which of the four donor tissues has the greatest proliferation potential.

4. To determine the MSC yield/gram of donor tissue after the cells are grown to confluency in two passages (also known as P1), a clinically relevant time frame.

Chapter 4: Sample Selection and Preparation

4.1 Dog Selection

Bone marrow, adipose tissue, muscle, and periosteum were collected from seven randomly selected dogs that were euthanized as part of a population control program at local animal shelters (this was not a deliberate randomization process; tissues were collected as dogs became available). All experimental protocols were reviewed and approved by the Institutional Animal Care Committee following guidelines of the Canadian Council on Animal Care. The dogs were young adult, mixed breeds weighing between 20- 35 kg. Four dogs were intact males, one was a neutered male and two were females of unknown ovariohysterectomy status. Tissues were collected immediately after euthanasia, when a lack of a heartbeat was confirmed, in an aseptic manner.

4.2 Isolation and Culture of Mesenchymal Stem Cells from Bone Marrow

Bone marrow was harvested from the proximal aspect of both humeri in six dogs and from one humerus in one dog using a 15 gauge Illinois bone marrow biopsy needle (CareFusion, San Diego, CA) with 2500 IU heparin (Leo Pharma Inc., Thornhill, ON) per 12 mL collecting syringe. The maximal amount of bone marrow that could be harvested using this technique (5-27.5 mL) was collected and suspended in α minimal essential medium (αMEM) (Invitrogen, Toronto, ON). Bone marrow samples were kept on ice for immediate transport to the onsite laboratory. The samples were divided in half and placed in 50 mL centrifuge tubes (BD Falcon, Franklin Lakes, NJ) and centrifuged at 1500 x *g* for 10 minutes. The buffy coat was collected and placed in a T75 flask (Corning Incorporated, Corning, NY) with 10 mL of standard medium (SM)

which was composed of αMEM supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories Inc., Etobicoke, ON), 2 mM L-glutamine (Invitrogen, Toronto, ON), 10000 IU penicillin and 10 mg streptomycin/mL (Sigma, Oakville, ON), and 250 μg/mL amphotericin B (Invitrogen, Toronto, ON). Cell cultures were maintained in a humidified 5% carbon dioxide and 95% air atmosphere incubator at 37°C. Unattached cells were removed after 48 hours by washing with sterile phosphate buffered saline (PBS) (Invitrogen, Toronto,ON). The medium was renewed three times per week. The passage 0 cells were cultured until they reached > 75% confluency or were present in culture for 11 days. At that time the cells were detached, counted, and subcultured, or cryopreserved for further studies using cryopreservation medium (90% FBS and 10% dimethyl sulfoxide (Sigma, Oakville, ON)).

4.3 Collection and Cryopreservation of Adipose Tissue, Muscle, Periosteum, and Bone

Adipose tissue was collected from the dorsum of the sacrococcygeal region. A 3 cm skin incision was made proximal to the tail base. The underlying subcutaneous tissue was excised and collected from each dog and placed in αMEM solution and kept on ice. The muscle and periosteum were collected from the hindlimb; the tissues were collected from both hindlimbs in the first four cases. A standard craniomedial approach was made to the stifle and extended distally over the tibia. The cranial tibial muscle (15.30-42.26 g) was excised, cut into smaller pieces, and placed in chilled αMEM solution on ice. The periosteum was incised on the medial aspect of the proximal tibia and elevated using a periosteal elevator. Approximately 0.49-1.60 g of periosteum was collected from each dog and placed in chilled αMEM solution. Cortical bone (0.9 g)

was collected from the proximal tibia of one dog using Lempert rongeurs and placed in αMEM solution similar to the other tissues. The bone was collected for potential use in further studies of MSC osteogenic differentiation.

Tissues were processed within 24 hours; tissues that were not processed immediately were kept on ice and refrigerated at 4°C. Cold, sterile PBS was placed in Petri dishes to keep a moist environment for the tissues while being cut into 1 cm segments. Tissue segments were placed into 2 mL cryovials (Corning Incorporated, Corning, NY) and submerged in freezing media composed of 92.5% PBS and 7.5% dimethyl sulfoxide. The cryovials remained at room temperature for 30 minutes to allow for freezing media to penetrate the tissue. The samples were then placed in styrofoam containers and put in a -80°C freezer for a minimum of 24 hours. All the samples were placed into a liquid nitrogen tank within 72 hours of processing.

4.4 Isolation and Culture of Mesenchymal Stem Cells from Adipose Tissue, Muscle, and Periosteum

Cells were isolated from tissues using an enzyme digestion technique. Cryopreserved adipose and muscle tissues were warmed in a water bath of 37°C for approximately 5 minutes until the liquid was defrosted. Tissue handling was performed with sterile technique in a biosafety cabinet. Each tissue was removed from the cryovials and placed in a 50 mL centrifuge tube containing 25 mL of sterile PBS. The tissue was rinsed with PBS, weighed and minced. Minced tissue was placed in centrifuge tubes containing 10 mL of 2000 units/mL collagenase Type I (Invitrogen, Toronto, ON), vortexed and placed in a 37°C incubator. The tubes were vortexed every 20 minutes and incubated for a total of 60 minutes. Once the tissue was digested, 10

mLs of SM was added to the mixture to inhibit further enzyme digestion. The cell suspension was filtered through a 100 μm filter (BD Falcon, Franklin Lakes, NJ) followed by a 70 μm filter (BD Falcon, Franklin Lakes, NJ) and centrifuged at 377 x *g* for 10 minutes. The supernatant was removed and the cell pellet was resuspended in SM. Viable cell numbers, based on 0.4% Trypan blue (Sigma, Oakville, ON) dye exclusion, were counted with a hemocytometer. Adipose tissue cells were plated at a mean ± standard deviation (SD) cell density of $2.2x10^4 \pm 0.84x10^4$ cells/cm^2 and muscle cells were plated at a mean ± SD cell density of $3.6x10^4 \pm 1.2x10^4$ cells/cm^2 with SM.

Isolation of cells from the periosteum was similar to that of adipose tissue and muscle except that the minced tissue was pre-treated with 2000 units/mL type I collagenase for 10 minutes. The partially digested tissue was rinsed and treated with type I collagenase for an additional 160 minutes. Viable periosteal cells were plated at a mean ± SD cell density of $3.2x10^4 \pm 2.2x10^4$ cells/cm^2 with SM.

Adipose tissue, muscle, and periosteum-derived cell cultures were maintained, as passage 0 cells, in a humidified 5% carbon dioxide and 95% air atmosphere incubator at 37°C. The medium was renewed three times per week. The cells were grown to 75-100% confluency, at which time they were detached, counted, and subcultured or cryopreserved for further studies.

4.5 Remarks

Bone marrow collection *post mortem* in the majority of these cases proved to be difficult as compared to clinical experience collecting bone marrow in sedated or anesthetized dogs. The Institutional Animal Care Committee required that bone marrow samples be collected from these dogs *post mortem*. The volume of bone marrow

collected in this study was much less compared to the amount easily obtained in clinical cases. For example, in five out of seven cases, only 5 mL of bone marrow could be aspirated even when using both proximal humeri. This is in comparison to over 20 mL that can be collected from one humerus in a clinical case. Twenty seven and a half milliliters were easily and unexpectedly obtained from the last case. A possible explanation for this includes a coagulopathy; however this could not be determined at the time of tissue collection and was not clinically apparent (no obvious signs of petechiae or scleral or submucosal hemorrhage). Blood clotting *post mortem* is the most likely reason for difficulty in collecting samples.

Technical error on behalf of the collector could not be ruled out but was thought unlikely because of previous and future successes in collecting bone marrow in this fashion. A medicine clinician who is experienced in bone marrow collection tried to help and found the same difficulty; therefore, making technical error less likely.

The proximal humerus is not a commonly reported location for collection of bone marrow in canine MSC experimental studies, but it yields adequate volumes of high-quality marrow in clinical veterinary bone marrow aspiration.In reported *ante mortem* research cases, the bone marrow was collected from the femur during a total hip replacement; the volume of collection was not reported but the cells would become confluent within 7 days.[1,2] In reported *post mortem* research cases, bone marrow was collected from the iliac crest,[3] or from the femur through flushing.[4] It is interesting to note that only 5 mL was obtained from the iliac crest and 3-10 mL was obtained from the femur in those studies. Neither of those studies reported the cell yield or time to confluency; however, Volk, et al.[2] did report limited cell numbers of the first passage

resulting in prioritizing what experiments were to be done. The significance of low volume bone marrow collection on MSC isolation and proliferation is not reported. The total mononucleated cell count increases with increasing volume of bone marrow, but the frequency of BMSCs (which is related to the total mononucleated cell count) can be variable.[3] The frequency of BMSCs can also be affected by the donor age, culture conditions, and blood contamination. Cell seeding density is known to affect proliferation rates, and may be a factor in ultimate cell yield.[3] Comparisons among studies are difficult because of variable conditions and absence of information in the reports.

Collection of 5 mL of bone marrow, in case number two, resulted in a zero yield of MSCs, even with culturing and subculturing media for up to two weeks. It was the author's observation that the cells in the first passage of a majority of these cases were slower to grow and become confluent compared to the cells obtained from clinical cases. However, when evaluating the MSC yield/mL of bone marrow collected in dogs of this study and those of clinical cases, this may not necessarily be true. For example, with collection of approximately 5 mL of bone marrow from study dogs, we could obtain approximately 1.5 x 10^6 BMSCs/mL of bone marrow in a mean of 16 days. In clinical cases, 10-15 mL of bone marrow was collected and resulted in an approximate yield of 3 x 10^5 to 6 x 10^5 BMSCs/mL of bone marrow in P0 and 1 x 10^6 to 2.5 x 10^6 BMSCs in a period of two to three weeks. Further investigation would be required to evaluate the effect of *ante* and *post mortem* bone marrow collection on MSC yield.

4.6 References

1. Csaki C, Matis U, Mobasheri A, et al. Chondrogenesis, osteogenesis, and

adipogenesis of canine mesenchymal stem cells: a biochemical, morphological, and ultrastructural study. *Histochem Cell Biol* 2007;128:507-520.

2. Volk SW, Diefenderfer DL, Christopher SA, et al. Effects of osteogenic inducers on cultures of canine mesenchymal stem cells. *Am J Vet Res* 2005;66:1729-1737.

3. Kamishina H, Deng J, Oji T, et al. Expression of neural markers on bone marrow-derived canine mesenchymal stem cells. *Am J Vet Res* 2006;67:1921-1928.

4. Kamashina H, Farese J, Storm J, et al. The frequency, growth kinetics, and osteogenic/adipogenic differentiation properties of canine bone marrow stromal cells. *In vitro Cell Dev Biol Anim* 2008;44:472-479.

Chapter 5: Mesenchymal Stem Cell Determination

5.1 Plastic Adherence

5.1.1 Introduction

A defining characteristic of MSCs is their ability to adhere to plastic.[1-4] Isolation of MSCs based on this method alone is recognized as the classic technique.[5] Friedenstein and colleagues first demonstrated this property in the 1970's; this group identified an adherent population of fibroblast-like cells after delayed rinsing of culture dishes plated with whole bone marrow.[6] They termed these cells "fibroblast colony forming units" and documented their proliferative capacity and osteogenic differentiation.[7] These cells are now known as MSCs.

Mesenchymal stem cells are not the only cell type to initially adhere to plastic. Contamination can occur with macrophages, lymphocytes, fibroblasts, endothelial, hematopoietic, and smooth muscle cells.[8,9] However, contaminating cells can be eliminated with deprivational media (ie. serum only media),[9] and repeated washing of cells.[10] Adherence to plastic and stromal cells by hematopoietic stem cells is reported to occur through adhesion molecules, cytokine receptors, and extracellular matrix proteins, and is likely a similar mechanism seen with MSCs.[11]

Species differences do exist. Isolation of BMSCs is most challenging in murine species because they have a higher proportion of adherent contaminating hematopoeitic cells.[12] Hematopoeitic contamination in human BMSC samples is less than 30%.[6]

To resolve the issue of contamination, various experiments have been conducted to optimize the isolation of MSCs. Cell adhesion has been improved by coating plates with extracellular matrix proteins like fibronectin.[13] Contamination can be reduced by

using density centrifugation of bone marrow samples.[5] Immunoselection and immunodepletion techniques can be performed on fresh tissue samples prior to initial culture to isolate the desired MSC population.[14] Jarocha, et al.[14] found that positively selected CD105 and CD271 MSCs had a greater proliferative capacity and reached confluency faster than BMSCs isolated with Ficoll gradient centrifugation. Also, fibrin microbeads were successfully used to isolate MSCs from human peripheral blood whereas the plastic adherence method did not.[15]

In summary, MSCs can be isolated based on their adherence to plastic. Contamination with other cell populations affects the efficiency of this method and drives the search for novel techniques. The first gold standard step in proving that the cells isolated in this study are MSCs, is to document their adherence to plastic.[16]

5.1.2 Study Findings

Plastic adherent cells with the typical fibroblast phenotype were isolated and expanded from all four donor tissues from all seven dogs similar to studies reported in the canine literature.[12, 16-19] The only difference was that density gradient separation was not used to isolate BMSCs. A phase contrast inverting microscope was used to visualize cells in culture. Initially they were non-adherent and appeared as floating circular bodies. But within 48 hours, fibroblast like cells appeared and were adherent to the culture flask; they were present as individual cells or in very small colonies. At this time BMSC flasks were washed, and media was changed in the other tissue-derived MSC flasks to remove non-adherent cells. Adherent fibroblast-like cells continued to proliferate and became the dominant phenotype. With each media change, the non-adherent circular cells were seen with less frequency. Kamashina, et al.[12] report these

cells to be leukocytes and hematopoietic progenitor cells. The adherent cells were also successfully detached using trypsin. These cells were further utilized for MSC determination and proliferation studies. The adherence property of the cells isolated supports the notion that they are MSCs.

5.2 Morphology

5.2.1 Introduction

Mesenchymal stem cells have a characteristic fibroblast-like morphology when initially adherent to culture flasks.[20] This is true for MSCs derived from different tissue sources and different species.[12,21] Mesenchymal stem cells isolated from canine bone marrow and adipose tissue have been described in this fashion.[16,19,22] However, the morphology of canine MMSCs and PMSCs has not been described because literature characterizing their *in vitro* characteristics is lacking. Kamashina, et al.[12] described initial canine BMSC cultures to be heterogenous in nature. The culture flasks contained a population of large flattened cells, and short and long spindle shaped cells. As MSCs became confluent, the long spindle shaped cells predominated. Mesenchymal stem cell morphology does not change with cryopreservation[18] but does with toxicity associated with culture media reagents,[19] cell differentiation[16] and cell senescence.[18]

5.2.2 Study Findings

Mesenchymal stem cells derived from all four donor tissues, and from all seven dogs, had the typical fibroblast morphology as described in the literature. Adherent fibroblast-like cells were seen as early as 48 hours after initial plating. Similar to Kamashina, et al.,[12] the predominant cell type was the long spindle shaped cell, but short

spindle shaped and flattened cells were also seen (Figures 1 and 2). Cells would initially be seen dispersed in very small colonies and would expand in colony size and number until tightly packed at confluency. The majority of the colonies (derived from bone marrow, adipose tissue, and periosteum) became 80-100% confluent within 6 to 8 days of initial seeding of T75 flasks. This finding was similar to BMSCs in another study.[16] However, MMSCs consistently took longer, and only achieved 45-75% confluency during this time frame. Mesenchymal stem cell morphology was similar regardless of tissue source.

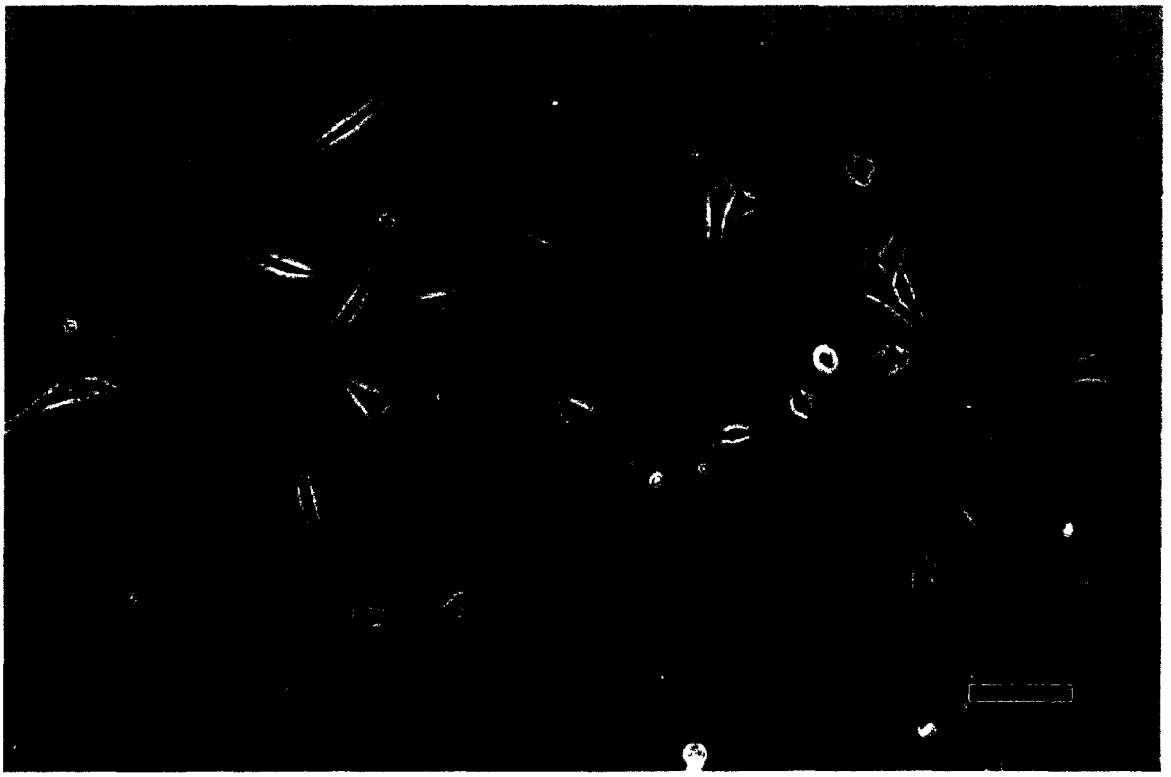

Figure 1. Light microscopic image of MMSCs that were plated 24 hours previously. A heterogenous population of MSCs can be seen; in particular long spindle shaped cells, as well as more flattened cells and cigar shaped cells.
Magnification is 10 x. Scale bar = 100 μm

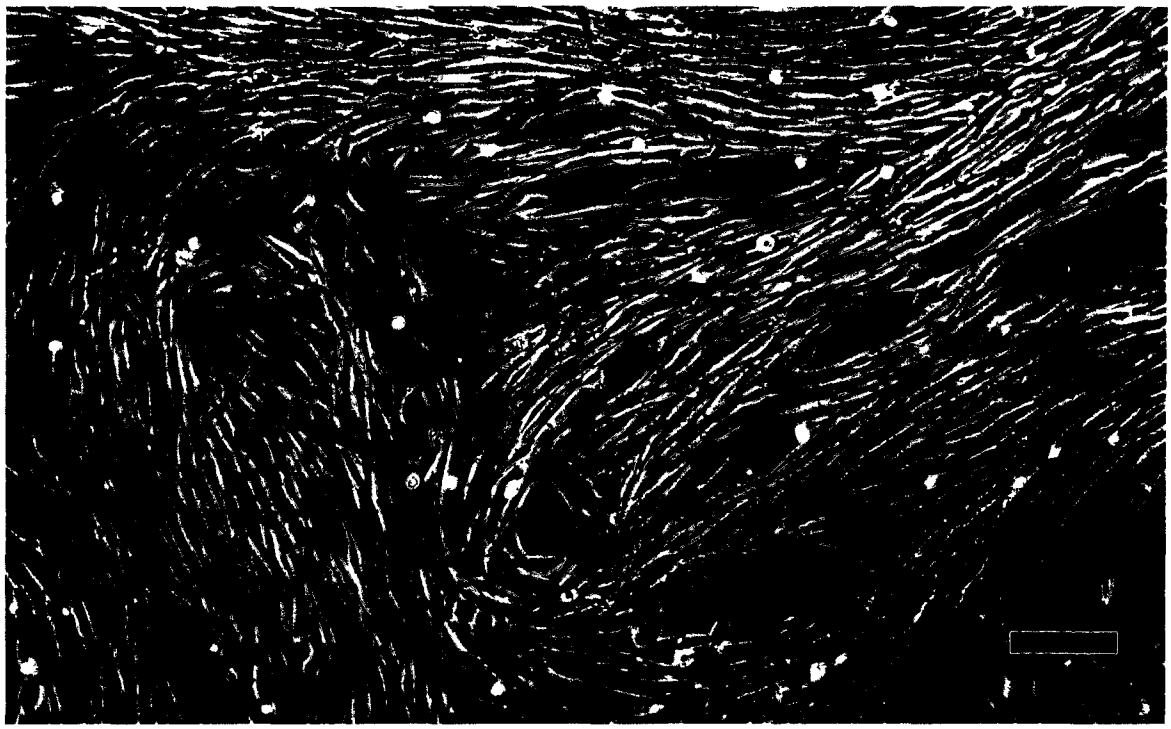

Figure 2. Light microscopic image of MMSCs that are nearly confluent after being plated six days previously. The long spindle shaped morphology characteristic of MSCs can be clearly seen. Magnification 10 x. Scale bar = 100 μm

5.3 Cell Surface Markers

5.3.1 Introduction

Mesenchymal stem cells are also characterized by the cell surface proteins they express. However, no single definitive marker defines an MSC *in vitro* or *in vivo*.[35,37,42] Mesenchymal stem cell identification is based on the presence of a number of specific cell surface markers and the absence of others. According to the International Society for Cellular Therapy, human MSCs positively express cell surface markers CD73, CD90, and CD105 and negatively express cell surface markers CD 11b, CD14, CD19, CD29α, CD34, CD45, and HLA-DE.[1] Although defined guidelines have not been proposed in the canine literature, canine MSCs are commonly reported to positively

express CD90, CD29, and CD44.[12,16,20,22] Other less frequently reported positively expressed surface markers include CD105, CD33, and CD184.[20]

Identifying MSCs solely on cell surface markers is difficult for a number of reasons. First of all, MSCs share similar surface markers with other cells like endothelial, epithelial, and muscle cells.[23] Secondly, species differences exist in MSC phenotype. For instance, murine MSCs typically express CD34, a hematopoietic marker, whereas rat and human MSCs do not.[9,24] Lastly, necessary monoclonal antibodies against surface antigens may not exist for the species of interest nor cross react with antibodies available from other species. This potential limitation seen with immunocytochemistry can be overcome with PCR techniques.[25] Inconsistencies in cell surface marker expression can also exist because of differences in isolation methods, culture conditions, and species and tissue origin.[9,26]

The CD cell surface markers evaluated in this study include: CD34, CD44, CD45, CD90, and CD146. Cell surface antigens CD34 and CD45 are common markers of hematopoietic cells. Therefore they should not be expressed by MSCs. CD90 is a Thy-1 glycoprotein, and CD44 is a hyaluronate receptor-adhesion molecule.[23] CD146 is a trans-membrane glycoprotein that was conventionally considered a marker for endothelial cells. However, pericytes have been shown to express CD146 and are considered to be precursors of MSCs. In addition, this surface antigen is not detected on hematopoetic cells.[24,27] Therefore, we would expect canine MSCs to positively express cell surface antigens CD44, CD90, and CD146.

5.3.2 Immunocytochemistry and Immunofluorescence

Immunohistochemistry is a qualitative method that detects specific antigens within tissues through antigen-antibody reactions. More specifically, immunohistochemistry is the localization of antigens in tissues and immunocytochemistry is the localization of antigens in cells.[28] Successful immunohistochemistry techniques rely on appropriate antigen preservation, specific staining, a well characterized antibody, and use of a label that is easily visible.[29] Light microscopes, fluorescent microscopes, and electron microscopes can be used to visualize antigen-antibody reactions when they are tagged with dyes or enzymes, fluorochromes, and ferritin or colloidal gold, respectively. The reactions can also be tagged with radioactive isotopes and visualized with autoradiography.[29]

Ideally the tissue should be prepared in such a fashion that the antigen of interest maintains its reactivity and accessibility with the antibody.[30] The fixation method prevents loss of tissue and soluble antigens, allows localization of antigen-antibody reactions, and can remove lipids that may interfere with their binding. Tissue samples may include impression smears, cryostat sections, freeze-dried samples, and paraffin sections. Cultured cells can also be processed.[30,31] Many fixation methods exist but the choice depends on the expected antigen-antibody reaction and its location.[29] Examples include ethanol, acetone, carbon tetrachloride, formalin, and sodium thiosulfate.[30]

Although fixation of tissues is desired, it can affect the avidity between the antigen and antibody by altering the epitope conformation. Therefore antigen retrieval methods have been developed to unmask the epitope. These techniques involve heating (microwave, autoclave, steam heating, or water bath) or using enzymes like

protease.[29,32] Many antigen retrieval techniques have optimal fluid pH and molarity that needs to be taken into account. The theory is that antigen retrieval methods either improve the penetration of antibodies through fixed tissues, or they reverse the conformational changes that occurred to the antigens during the fixation process.[32]

Immunoglobulins can attach nonspecifically to tissue components by hydrophobic and electric forces, resulting in unreliable results. The addition of albumin or non-immune serum from the species donating the secondary antibody will occupy the majority of the non-specific binding sites and minimize nonspecific staining.[29]

Immunohistochemistry can be performed via the direct or indirect method. The direct method involves using only one antibody that is labeled. In the indirect method, the first antibody is unlabelled and reacts with the epitope of interest (primary antibody). A secondary antibody, which is labeled, is used to recognize the primary antibody; it is typically developed against the gammaglobulin of the species that donated the primary antibody.[29] The direct method maybe be advantageous because it is quicker and simpler, but it is less sensitive than the indirect method.[30]

The type of antibody that can be used, monoclonal or polyclonal, also varies. Monoclonal antibodies are developed to recognize single epitopes. Although they are expensive, difficult, and time consuming to generate, they are desired because they are much more specific and have less background staining compared to polyclonal antibodies, designed to recognize many epitopes. Polyclonal antibodies, on the other hand, have a greater affinity, wider reactivity, are less sensitive to fixation, and are stable over greater ranges of pH and salt concentrations.[32,33]

Immunofluorescent staining utilizes antibodies labeled with a fluorochrome. These are "substances which emit light of longer wavelengths than that of the excitation absorption."[30] The most commonly used fluorochrome is fluoroscein isothiocyanate (FITC) which emits a green light, with a wavelength of 490 nm, when activated. Rhodamine isothiocyanate is another and produces fluorescence of a red color.[29] Other examples of fluorochromes include: peridinin chlorophyll protein, green fluorescent proteins, Texas red, Cy3 and Cy5.[31] Counterstaining to identify nuclei of non-immunoreactive tissue can be done with agents like methyl green.[29] Fluorochromes are typically easily conjugated to antibodies at a ratio of 1:2 and don't alter the reactivity of the antibody.[30] They do require visualization with a fluorescent microscope. Mercury vapour or xenon arc lamps are used, and provide light with the appropriate wavelength to visualize the fluorescence of antigen-antibody reactions.[29] However, samples do lose fluorescence with microscopic viewing and with time.[28,30] Fading can be slowed by keeping samples in the dark or adding compounds like 1,4-diazobicyclo-(2-2-2)-octane, phenylenediamine, or n-propyl gallate.[29,31]

Positive and negative controls are very important in immunohistochemistry. A tissue sample with the expected and desired antigen-antibody reaction should be included as a positive control. Negative controls can be obtained by using a heterologous antibody,[30] omitting the primary antibody or using an inappropriate secondary antibody with the indirect method, or by using tissue samples without the targeted antigen.[29] Positive and negative controls should be handled in the exact manner as the tissue of interest.

Nonspecific staining can occur with nonspecific binding of antibodies and reagents, cross reaction with endogenous antibodies, reaction with similar epitopes on different antigens,[32] inadequate removal of unconjugated fluorochromes, excessive fluorochrome:antibody ratio, reaction with non-immunogenic contaminants like dead cells, and with autofluorescence.[30]

Factors that can affect reaction detection threshold include: sample collection, fixation, and processing, section thickness, antigen retrieval methods, and the type, dilution, and incubation time and temperature of the antibody.[33] Many variables affect the potential outcome of immunohistochemistry experiments. Therefore, the use of controls and pilot runs is important in defining the best conditions for optimal results.

5.3.3 Technique

Antibodies

- Mouse monoclonal to canine CD34; labeled with FITC-IgG prepared by affinity chromatography on Protein G from tissue culture supernatant.
  (AbD serotec, Raleigh, NC)
- Rat monoclonal to human, cat, cow, dog, horse, and pig CD44; labeled with FITC-protein G purified (Abcam Inc., Cambridge, MA)
- Rat monoclonal to canine CD45; labeled with FITC- IgG prepared by affinity chromatography on Protein G from tissue culture supernatant.
  (AbD serotec, Raleigh, NC)
- Mouse monoclonal to canine CD90
  (Accurate Chemical and Scientific Corporation, Westbury, NY)

- Goat anti-mouse IgM antibody; labeled with FITC-IgM prepared by affinity chromatography

  (Cedarlane Laboratories Limited, Burlington, ON)
- Mouse monoclonal to human CD146; species reactivity includes mouse, rabbit, and canine; labeled with ALEXA FLUOR® 488

  (Millipore, Billerica, MA)

Cells derived from bone marrow, adipose tissue, muscle, and periosteum from three dogs were expanded in SM to 80-100% confluency in passage one for use in evaluation of cell surface CD protein markers with fluorescent labeled specific antibodies. Isolated canine MSCs were evaluated by immunofluorescence microscopy for MSC specific markers CD90 and CD44, and hematopoetic stem cell markers CD45 and CD34, and the pericyte specific marker CD146. Cells were seeded at a cell density of 1500 cells/cm^2 in a 24 well plate (Corning Incorporated, Corning, NY) for 48 hours. Cells were fixed with 4% paraformaldehyde (Fisher Scientific, Nepean, ON) for 15 minutes at room temperature. Non-specific binding was blocked with 1% bovine serum albumin (Fisher Scientific, Nepean, ON) for one hour at room temperature followed by the addition of diluted primary antibodies in a dark environment. Cells were incubated with mouse anti-dog CD34:FITC (1:1000), rat monoclonal to CD44:FITC (1:1000), rat anti-dog CD45:FITC (1:1000), primary mouse monoclonal to CD90 (1:1000), and mouse anti-human CD 146:ALEXA FLUOR®488 (2:1000) overnight at 4°C. Controls included wells that did not have any added antibody. A secondary, goat anti-mouse IgM antibody, tagged with FITC, was used to detect the CD90 antibody (1:1000). After the addition of the secondary antibody, cells were incubated for one hour at room

temperature. The cells were washed and counterstained with Hoechst 33258 (Invitrogen, Toronto, ON) (pentahydrate bis-benzamide dye solution, 1µl in 10mL distilled water) for five minutes at room temperature in a dark environment. Pictures were taken with a fluorescent microscope.

5.3.4 Results

Cells isolated from all four donor tissues and cultured under standard conditions in passage one strongly expressed the cell surface antigen CD90 and weakly expressed C44, based on positive staining with green immunofluorescence. Bone marrow-derived MSCs from two out of three dogs showed very weak positive expression of CD45; the other three tissue-derived MSCs were negatively labeled for this cell surface marker. None of the isolated cells stained positively for CD34 and CD146 (Figures 3 and 4). Control wells did not demonstrate immunofluorescence.

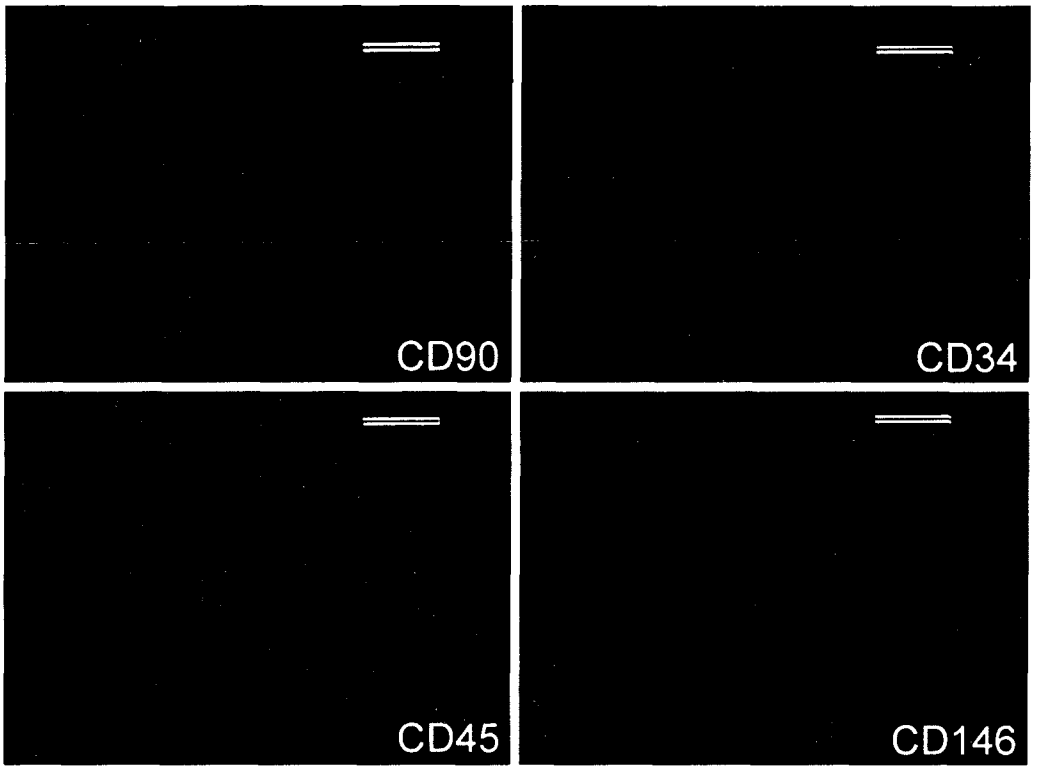

Figure 3. Immunofluorescence labelling of PMSCs. The isolated MSCs showed strong positive staining for stem cell surface specific marker CD90. They were negatively labelled for hematopoetic stem cell surface markers CD34 and CD45 and for CD146. Magnification is 20 x. Scale bar = 100 μm

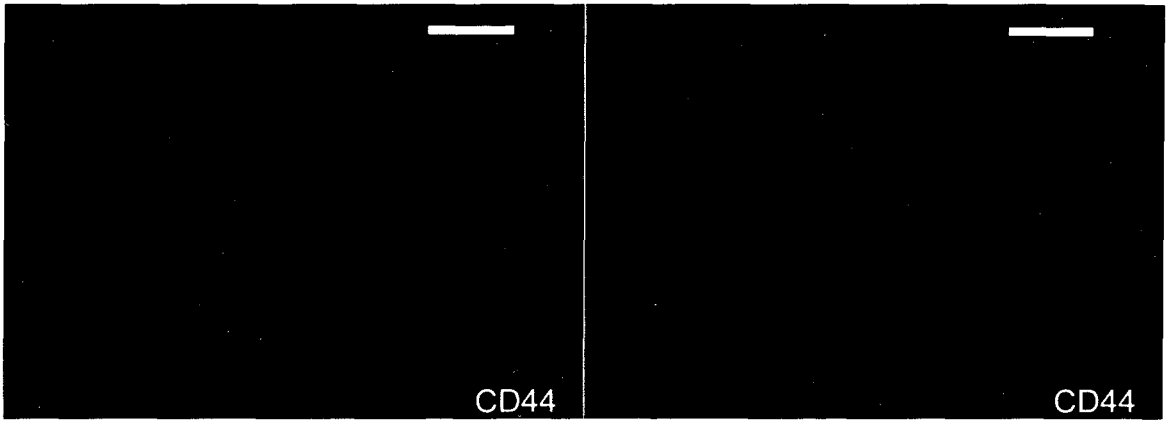

Figure 4. Immunofluorescence labeling of BMSCs for cell surface marker CD44. In the image on the right, very weak positive immunofluorescence can be seen on the cell surface of BMSCs which is consistent with positive expression of this cell surface marker. The image on the left represents the positive staining of the nuclei of these cells. The images were not overlapped as in Figure 3 because of further loss in image quality. Magnification is 20 x. Scale bar = 100 μm

5.3.5 Discussion

Mesenchymal stem cells isolated from canine bone marrow, adipose tissue, muscle, and periosteum positively expressed cell surface antigens CD44 and CD90, and were negatively labeled for CD34 and CD146. Expression of cell surface antigen CD45 was not seen in any tissues, except for bone marrow.

It is understood that the presence or absence of specific cell surface antigens, like those evaluated in our study, define the phenotype of human MSCs. But the literature indicates that species differences exist.[24,34] It is therefore important to evaluate the MSC phenotype of individual species. In our study, MSCs derived from all four donor tissue sources had positive expression of surface markers CD90 and CD44. These results are in agreement with those that evaluated MSCs from canine bone marrow[19,24,] and adipose tissue,[35,36] as well as equine[25,37] and human-derived MSCs.[21]

The majority of the MSCs were negatively labeled for the hematopoetic receptors CD34 and CD45. Bone marrow-derived MSCs from two dogs stained very weakly for CD45 using immunofluorescence. This finding could potentially be due to non-specific staining as was postulated by Braun, et al.[25] who found equine AMSCs to have a weak signal for CD45 using flow cytometry but negative genetic expression of the surface marker using RT-PCR. Non-specific staining may have been a result of conjugation with non-immunogenic contaminants, or binding to a similar epitope on another antigen. The positive expression of CD45 could also be a reflection of hematopoietic contaminants that can be present in the early passages of MSC cultures.[24]

All four donor tissue-derived MSCs were negatively labeled for CD146. Evaluation of this cell surface antigen was of interest because it is a marker of pericytes,

which are now thought by many, to be the source of MSCs. Reng and colleagues[38] identified CD146 as a surface marker of canine vascular endothelial cells and pericytes. However, this marker has not been commonly evaluated in MSCs of animal species. Sorrentino, et al.[39] isolated human BMSCs that were positive for this marker, in addition to the more commonly expressed cell surface antigens, CD90 and CD105.

A few possibilities exist for the lack of CD146 immunoreactivity in our study. It could be that canine BMSCs, AMSCs, MMSCs, and PMSCs do not express this cell surface antigen, as a species difference to human MSCs, despite their MSC identity. Another possibility is that pericytes are not the source of MSCs. But a more likely explanation is that variable cell surface antigen expression is seen as a consequence of differences in isolation methods, culture conditions, and tissue origin.[9,40] It is possible that because we did not have a positive control for this marker the detection threshold for immunofluorescence was not reached. We could have purchased human BMSCs or canine pericytes as a positive control to evaluate this further.

Although immunofluorescence microscopy was a satisfactory technique in qualitative evaluation of cell surface marker expression, and its use is reported by others,[16,37] additional assessments using flow cytometry and RT-PCR could have provided quantitative measurements, as described in current literature. These techniques may have provided more definitive answers regarding the expression of CD45 and CD146 in our samples. The name flow cytometry is used interchangeably with fluorescence activated cell sorting. It can analyze single cells and separate them based on surface antigens, DNA content, light scatter, calcium flux, or intracellular pH.[31] Unlike immunofluorescence, it is rapid, sensitive, and provides a quantifiable and

objective measurement.[30] RT-PCR is quantitative and can detect expression of low density expression markers as well as use fewer cells to characterize phenotype in comparison to immunocytochemistry.[40]

5.4 Expression of Pluripotency Associated Transcription Factors

5.4.1 Introduction

The molecular basis governing MSC's self renewal and ability to differentiate along several cell lineages is currently an area of great research interest. The proliferation and multipotency properties of MSCs have been defined, but the identity of transcription factors involved and their role is rather unclear. In comparison, transcription factors that regulate embryonic stem cell characteristics are better understood and are now being evaluated in MSCs.[41]

Three transcription factors that have been identified to have pivotal roles in ES pluripotency and self renewal are OCT4, NANOG, and SOX2.[42,43] Studies have shown that pluripotency and self-renewal are lost when these factors are downregulated.[44] Other genes involved in maintaining pluripotency and self-renewal capacity are regulated by these three "master" transcription factors.[42,45,46] These include NrOb1, Sall4, C-Myc, and K1E4 to name a few.[47]

OCT4, also known as OCT 3[45] or POU5F1,[48] is a transcription factor expressed in undifferentiated ES and during mouse embryogenesis.[49] It prevents expression of genes involved in cell differentiation. Knock out of OCT4 was lethal in embryonic mice,[45] and thus demonstrates its importance for embryological development. SOX2 null embryos also die soon after implantation; in mice, SOX2 was shown to be required for trophectoderm formation and uterine implantation.[50] Genes of the SOX family are

responsible for organogenesis and early embryonic development.[51] NANOG is a more recently described transcription factor that has similar roles in ES. Overexpression of NANOG has been shown to inhibit ES differentiation, whereas reduced expression correlates with increased cell differentiation.[42,52] Cells have also been virally induced with OCT4, SOX2, and NANOG in combination, into pluripotent cells with similar characteristics to ES.[43]

OCT4, NANOG, and SOX2 mRNA and protein expression have been documented in MSCs derived from a variety of tissues and species. In one study, all three transcription factors were seen in human MSCs derived from adipose tissue, heart and dermis, and OCT4 and NANOG were seen in MSCs derived from bone marrow.[53] Embryonic stem cell markers have also been identified in MSCs derived from dental pulp, peripheral blood, liver, bone marrow, and adipose tissue of other studies.[53] Canine AMSCs[17] and equine bone marrow and umbilical cord-derived MSCs[54] also demonstrated their expression. However, others have failed to demonstrate the presence of these transcription factors in MSCs. For example, Pierantozzi, et al.[41] demonstrated the absence of OCT4 and SOX2 in human BMSCs, AMSCs, and heart derived MSCs.

Debate exists regarding the role and function of OCT4, SOX2, and NANOG in MSCs. These transcription factors are of interest because of their role in ES pluripotency and proliferation. Techniques used to evaluate pluripotency associated transcription factors are similar among studies and utilize immunocytochemistry and polymerase chain reaction (PCR) methods.

5.4.2 Reverse Transcriptase Polymerase Chain Reaction

Polymerase chain reaction is one of the most commonly practiced molecular techniques used to amplify specific segments of DNA. Semiquantative analysis of genetic variation and presence of gene expression can be evaluated using PCR.[55] In routine PCR, DNA is used as the starting material, whereas with reverse transcriptase PCR (RT-PCR), mRNA is used as the starting block to synthesize cDNA.[56] mRNA expression can also be quantitatively measured using real time RT-PCR.

Other techniques used to measure mRNA include northern blotting, ribonuclease protection assays, and *in situ* hybridization.[57] The advantage of RT-PCR, however, is that it only requires a small amount of starting sample and can detect rare gene expression.[58] RNA is obtained from the cytoplasm using detergents to lyse cells, phenol to extract proteins, and ethanol and centrifugation to precipitate out mRNA. Guanidinium/cesium chloride ultracentrifugation and the Chomczynski-Sacchi method are other techniques used to obtain whole cell RNA (from the cytoplasm and nucleus).[56] Once mRNA is harvested it can be synthesized into double stranded cDNA for PCR. mRNA is reverse transcribed using the enzyme reverse transcriptase.[28] Sources of reverse transcriptases include avian myeloblastosis virus and Moloney murine leukemia virus.[56] The concentration and quality of cDNA is measured using spectrophotometry. Optical density readings of the sample are taken at 260 and 280 nm. If the ratio is less than 1.75 the sample is of suboptimal quality because of contamination with proteins and should be modified.[56]

The following components are required for PCR: template, primers, *Taq* polymerase, deoxynucleotides, and a buffer.[55]

- Template: consists of the sample DNA with the desired DNA sequence.
- Primers: are short segments of DNA that are designed to recognize a portion of the target DNA. Both forward and reverse primers are used in PCR reactions; the forward primer is complementary to the 3' end of the antisense strand and the reverse primer is complementary to the 3' end of the sense strand. These primers used together flank the region of the desired DNA segment.[56] They should be specific, have high efficiency, be unlikely to form primer-dimers, and should ideally be on separate exons to avoid amplification of contaminating DNA.[57]
- *Taq* polymerase: is a heat resistant enzyme that is required to synthesize DNA by extending the primer-target sequence in a 5' to 3' direction.[28] It can do so at a rate of >60 nucleotides/second when at 70°C.[55] The source is *Thermus aquaticus*, a thermophilic eubacterial microorganism. DNA polymerase can also be obtained from *Thermus thermophilus, Bacillus stereothermophilus,* and *Thermococcus litoralis*.[58] 0.5 to 25μL per reaction are recommended when using *Taq* polymerase, but the recommendation differs when using DNA polymerase from other sources. Excess DNA polymerase is also discouraged because it can result in the synthesis of non-specific DNA products.[59]
- Deoxynucleotides: are composed of a cyclic five carbon sugar (deoxyribose), a purine or pyrimidine, and a phosphate group. These macromolecules are the building blocks used to synthesize DNA.[60] Excessive amounts of deoxynucleotides are required to allow for exponential amplification of DNA,[58]

but equal concentrations of each (dATP, dCTP, dGTP, dTTP) should be used to prevent mis-incorporation errors.[59]

- Buffers: are composed of salts like KCl and $MgCl_2$ and TrisHCl. They effect the specificity and amplification yield in PCR.[55]

Polymerase chain reaction is composed of two phases: the screening phase and the amplification phase.[59] The screening phase occurs in the early cycles when primers localize the selected DNA segment. The amplification phase occurs during cycles of exponential DNA production. Each cycle consists of three steps:

1) Denaturation: the reaction is subjected to temperatures of 92-97°C and disrupts the bonds between DNA strands resulting in their separation.[55]

2) Annealing: the temperature is lowered to 50-65°C and allows for primers to anneal to the template.[28]

3) Extension: DNA polymerase attaches to the primer-template complexes and extends the primer strand with deoxynucleotides.[59]

Reactions are typically run in a thermocycler for 30 cycles but cycles can be repeated until the desired amount of DNA is produced or until the substrates expire.[55] Twenty cycles of PCR can result in 1 million copies of DNA.[56] The PCR product can be visualized using gel electrophoresis, or can be quantified if using real time RT-PCR.

Gel electrophoresis allows for visualization of the gene products synthesized in PCR. Samples of DNA, which are stained with ethidium bromide, are placed in wells of agarose gel and stimulated to move through the gel by an electric current. DNA and RNA are both negatively charged and will migrate towards the positive charge based on

their molecular weight. Samples with a smaller molecular weight travel farther down the gel. The DNA products are visualized once exposed to ultraviolet light because they fluoresce.[28,60] Gel electrophoresis helps confirm that a single product was synthesized and that its size was consistent with the desired product.[58]

If gene expression is measured quantitatively, like with real time RT-PCR, a standard or reference gene should also be measured to normalize the amount of RNA added to the initial reaction.[61] Therefore if twice as much mRNA is added to a reaction, its quantity will be accounted for instead of interpreting the data as the sample truly having a two fold increase in gene expression. These reference genes are also termed housekeeping genes. These genes should be present in all cell types and should ideally be stable regardless of any experimental treatment. However, not all gene expression from control genes is resistant to imposed treatments; therefore each housekeeping gene should be validated. Examples of reference genes include glyceraldehyde-3-phosphate dehydrogenase mRNA, Beta actin mRNA, MHC 1 mRNA, and a variety of ribosomal mRNAs.[61]

The use of controls is especially important in PCR studies. Contamination can occur easily and result in the synthesis of non-specific DNA products.[59] Negative (no cDNA) and no reverse transcriptase controls are important to include for evaluation of contamination. Positive control samples are important to ensure that the reagents and primers work appropriately.[57]

5.4.3 Technique

Cells derived from bone marrow, adipose tissue, muscle, and periosteum from three dogs were expanded in SM to 80-100% confluency in passage one for evaluation of pluripotency gene marker expression using PCR.

Cells were seeded at a cell density of 4,200 cells/cm^2 in a 6 well plate (Corning Incorporated, Corning, NY) and cultured in SM until confluent. Total RNA was extracted from cells using Aurum^{TM} Total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) following manufacturer instructions. The RNA was treated with DNase to remove contaminating DNA. The cDNAs were synthesized from 1 μg total RNA using iScript^{TM} cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Primers derived from the coding regions of SOX2, NANOG, OCT4, the genes associated with pluripotency, and β2 Microglobulin, a housekeeping gene, are listed in Table 1.[17] Twenty five microlitres of PCR reactions were prepared with 1.6 μg cDNA, 0.1 μM of each primer, 12.5 μL of iQ^{TM} SYBR^{R} Green Supermix (Bio-Rad Laboratories, Hercules, CA) and sterile deionized water. PCR was run using the Rotorgene-6 RG 3000 (Corbett Research, Montreal, QC). Cycling conditions were as follows: 95°C for 5 minutes; 45 cycles at 95°C for 20 seconds, optimal annealing temperature (Table 1) for 20 seconds, and 72°C for 30 seconds. No template controls (nuclease free water instead of cDNA) were used as a negative control. The PCR products were stained with Syber Safe DNA gel stain (Invitrogen, Toronto, ON) and separated on 1% agarose gel (Biorad Laboratories Inc., Hercules, CA) by electrophoresis, and visualized under ultraviolet light. Digital images were captured with VisionWorks LS and UVP gel doc, Biospectrum AC Imaging.

Table 1. Primers used in RT-PCR

Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Annealing Temperature (°C)
OCT4	Forward GAGTGAGAGGCAACCTGGAG Reverse GTGAAGTGAGGGCTCCCATA	274	60
NANOG	Forward GAATAACCCGAATTGGAGCAG Reverse AGCGATTCCTCTTCACAGTTG	141	60
SOX2	Forward AGTCTCCAAGCGACGAAAAA Reverse GCAAGAAGCCTCTCCTTGAA	142	58
β2 Microglobulin	Forward TCTACATTGGGCACTGTGTCAC Reverse AAGAGTTCAGGTCTGACCAAG	136	60

5.4.4 Results

Bone marrow, adipose tissue, muscle, and periosteum-derived MSCs positively expressed the pluripotency-associated transcription factors SOX2, OCT4, and NANOG as determined by RT-PCR analysis and gel electrophoresis (Figure 5).

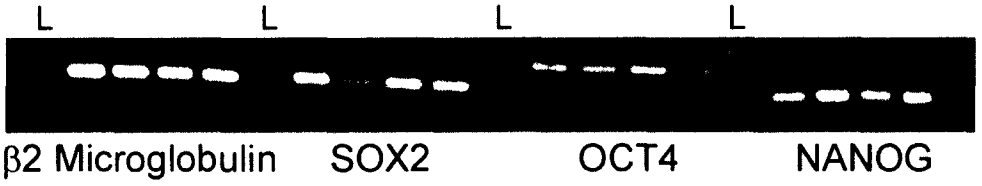

Figure 5. Positive mRNA expression of pluripotency associated transcription factors SOX2, OCT4, and NANOG and the housekeeping gene β2 MICROGLOBULIN in each of the four donor tissue-derived MSCs. The four bands above each transcription factor correspond with MSCs derived from bone marrow, adipose tissue, muscle, and periosteum, respectively. The DNA ladders are labelled L and each band corresponds to 100 bp with the lowest band representing 100 bp.

5.4.5 Discussion

OCT4, SOX2, and NANOG are pluripotency-associated markers expressed in embryonic stem cells.[62] Expression of these stem cell related transcription factors has

been reported in canine AMSCs,[17] equine BMSCs and umbilical cord MSCs,[54] and rhesus monkey BMSCs.[63] Our study found positive expression of OCT4, SOX2, and NANOG in canine BMSCs, AMSCs, MMSCs, and PMSCs using RT-PCR. This is the first report describing the positive expression of these transcription factors in canine BMSCs, MMSCs, and PMSCs.

OCT4, SOX2, and NANOG are transcription factors that work together in the maintenance of ES pluripotency and self-renewal.[46] Researchers are evaluating the presence and function of these factors in MSCs because of the similar differentiation and proliferation properties seen in ES. Other progenitor cells, like progenitor of mesodermal lineage cells and multipotent adult progenitor cells, that have characteristics similar to ES, also demonstrate elevated levels of ES pluripotency associated transcription factors.[64,65] An understanding regarding the molecular mechanisms behind MSC's plasticity is actively being sought. Once that is achieved, cell based therapies can be optimized.

Various studies have demonstrated the positive expression of embryonic stemness transcription factors in MSCs. Karaoz, et al.[66] isolated human BMSCs that expressed OCT4, NANOG, SOX2 as well as Rex-1, and FoxD-3 mRNA (all ES transcription factors). Isolated MSCs had the typical cell surface antigen phenotype and were differentiated down the adipogenic, osteogenic, and neurogenic lineages. Human BMSCs expressed OCT4, SOX2, and NANOG in another study.[67] In addition, OCT4 in BMSCs showed similar function to ES based on loss of function studies and chromatin immunoprecipitation DNA selection and ligation studies. Mouse AMSCs showed positive OCT4 expression with RT-PCR when isolated fresh as well as after two days in

culture.[68] Interestingly, expression of this transcription factor was no longer evident after three passages. Seo, et al.[69] documented the role of OCT4 expression in human umbilical cord blood MSCs by inhibition studies. Down regulated expression correlated with reduced cell proliferation and adipogenic differentiation capability, and affected expression of other pluripotency associated transcription factors.

Results of other studies contradict these findings. Mouse AMSCs and MMSCs, and human AMSCs did not have OCT4 expression.[68] Pierantozzi, et al.[41] reported that human BMSCS, AMSCs, and heart-derived MSCs did not express OCT4 and SOX2 using RT-PCR and immunocytochemistry. NANOG was expressed at a reduced level in comparison to ES. Level of NANOG expression did not appear to impact MSC proliferation and differentiation capability, thus questioning its role in this function. The other interesting finding in this study was that NANOG positive cells were a separate entity from CD146 positive cells. As mentioned earlier, CD146 has been proposed as a stem cell surface marker for human MSCs based on their relationship with pericytes. The authors question the results of studies reporting positive expression of OCT4, SOX2, and NANOG in MSCs because of their respective pseudogenes. Pseudogenes are expressed at high levels and have the potential to compromise PCR and immunocytochemistry results because of their high sequence homology.[70] Pierantozzi's group[41] claim to have avoided pseudogene detection through careful selection of primers. Pseudogenes have been identified for OCT4 and NANOG and not SOX2. Nonetheless, there is a possibility that our study targeted pseudogenes for these transcription factors.

Lack of controls is a major limitation in this study. We did not have either a positive or negative control; only a control to evaluate contamination was included. In one study, ES were used as a positive control and a sarcoma osteogenic cell line was used as a negative control.[41] In another study, OCT4 and SOX2 were expressed in porcine embryonic disc, porcine umbilical cord matrix cells, and porcine fibroblasts.[44] However, different levels of expression were observed with quantitative PCR; the former had the greatest level of expression and the latter had the least. In contrast to another study, OCT4 expression was not seen in human fibroblasts.[69] Therefore, the use of fibroblasts as a negative control is questionable, especially if using PCR in a qualitative fashion.

It is understood that MSCs are a population of heterogeneous cells. Perhaps some of the differences in study findings can be explained by the fact that MSCs are a mixture of progenitor cells with different cell fates.[41] Others believe that cell culture conditions play a major role in the expression pattern of MSCs[53] and result in an altered phenotype that doesn't represent the *in vivo* MSC. Although inconsistencies exist in the literature regarding MSC expression of pluripotency associated transcription factors, our study demonstrates that canine BMSCs, AMSCs, MMSCs, and PMSCs express OCT4, SOX2, and NANOG. This finding is similar to ES and other MSCs and lends support to the molecular basis of MSCs' pluripotency and proliferation properties.

5.5 Trilineage Differentiation

5.5.1 Introduction

One of the most important and convincing criteria in defining MSCs is based on their function. Mesenchymal stem cells should differentiate down the adipogenic,

chondrogenic, and osteogenic lineages *in vitro*.[9] This is important because the ability of MSCs to differentiate into different cell types distinguishes it from cells that may have similar morphology and cell surface markers, but truly are not MSCs. For instance, fibroblasts were shown to be plastic adherent, have a similar morphology, and expressed CD44, CD73, and CD105 similar to MSCs.[71] However, the fibroblasts had no colony forming capacity and could not be induced down the adipogenic, chondrogenic, and osteogenic cell lineages like MSCs.

Mesenchymal stem cells have been differentiated into adipocytes, chondrocytes, osteoblasts, myocytes,[72,73] and tenocytes[74] under appropriate biochemical, hormonal, and mechanical stimuli.[16,75] This has been described for both *in vitro* and *in vivo* conditions. Some claim MSCs can be differentiated into cardiomyocytes, and hepatic, neural and epithelial cells.[4,73,76,77] However, the differentiation potential of MSCs into non-mesodermal lineages is not agreed upon by everyone, with some saying results are not reproducible.[3,9] To confirm successful differentiation down specific cell lineages, a combination of morphological, immunophenotypic, and functional criteria are used.[24]

Mesenchymal stem cells can be differentiated down the adipogenic lineage when cultured with isobutylmethylxanthine,[78] dexamethasone, indomethacin, and insulin.[10,79] Neupane, et al.[17] reported improved adipogenic differentiation of canine AMSCs when rosiglitazone and rabbit serum were added to the induction medium. Successful differentiation occurs when lipid vacuoles form within cells. Oil red O and Sudan black are stains that can be used to visualize lipid vacuoles.[10,68] Nile red also stains intracellular lipid and is visualized with a fluorescent microscope.[80] Transcription

factors evaluated in determining the success of adipogenic differentiation include adiponectin, PPAR-γ,[16] fatty acid synthetase,[78] lipoprotein lipase,[10] and leptin.[17]

Chondrogenesis is best induced when MSCs are placed in three dimensional culture with the presence of TGF-β.[78] Bovine BMSCs cultured in collagen type II 3-D hydrogels demonstrated the best chondrogenesis based on morphology and mRNA expression of chondrogenic markers when compared to monolayer or alginate cultures.[76] Addition of collagen type II to a scaffold in another study also enhanced chondrogenic differentiation.[81] In chondrogenic assays, cells will aggregate and synthesize an extracellular matrix composed of glycosaminoglycans that can be visualized with Alcian blue, Toluidine blue, and Safranin O.[10,82] Cell numbers are estimated with measuring DNA content.[79] Glycosaminoglycan content can also be quantified using the 1,9-dimethylmethylene blue assay with spectrophotometry.[81] Immunohistochemistry techniques can be used to show expression of collagen type II, the most abundant collagen within hyaline cartilage.[76,83] One can also evaluate chondrogenic genetic markers that include aggrecan (expressed later in chondrocytes), fibromodulin (an early marker), decorin, type II collagen,[78] and SOX9.[22,76,81]

Mesenchymal stem cells are said to undergo osteogenesis as the default differentiation pathway.[83] Whether this is the natural fate of a MSC or the result of *in vitro* culture manipulations is unknown. Studies typically carry out experiments seeding cells in monolayer culture and inducing osteogenesis with media composed of β-glycerophosphate, ascorbic acid, dexamethasone, and FCS.[78,79,84] Although BMPs significantly improve osteogenesis in canine MSCs, dexamethasone is more important for osteoinduction of human MSCs.[85] The addition of 1,25-dihydroxyvitamin D3

resulted in increased mineralized extracellular matrix deposition in osteoinduced canine AMSCs.[17] Cells transform to a cuboidal shape and form nodules consistent with an osteoblast morphology and deposition of mineralized matrix, respectively.[10] Mineralized extracellular matrix can be visualized with Alizarin red or Von Kossa stain.[10,78] Alkaline phosphatase (ALP) activity[72] and calcium accumulation can also be quantified.[10] Collagen type I is a marker of bone formation as it is the most abundant collagen found in bone. It can be evaluated using immunohistochemistry techniques or RT-PCR.[16] RUNX2 and OSTEOCALCIN are other measurable osteogenic transcription factors.[16]

Myogenic differentiation has been documented by inducing MSCs with 5-azacytidine and amphotericin B.[24,78] Cellular markers for myocytes include myoD (a regulator in skeletal muscle development), and myosin (a marker of mature skeletal muscle).[3] To induce cardiomyocyte differentiation, AMSCs were co-cultured with ventricular cardiomyocytes.[68]

Differentiation down the neurogenic lineage has been documented in MSCs cultured with isobutylmethylxanthine and dibutyryl cAMP;[19,24] nerve growth factor, BDNF, and neurtophin-3; and DMSO/butylated hydroxyanisole, bFGF, and PDGF.[78] Researchers have confirmed their neurogenic lineage by demonstrating positive expression of anti-map 2,[68] NeuN, Gap43, Nestin, and neurofilament.[3] Both βIII-tubulin (a neuronal marker) and GFAP (a marker of mature astrocytes) were positively expressed in un-induced canine BMSCs, but GFAP expression was significantly increased in MSCs induced down the neurogenic lineage.[19] Fibroblasts were used as a control in this study and failed to express either of these two neural markers.

Most studies on MSCs *in vitro* do not mimic the *in vivo* situation. Since, ultimately the goal is to utilize MSCs in clinical applications, researchers are trying to develop *in vitro* experiments that simulate the *in vivo* environment. More recent reports in the literature are evaluating the effect of hypoxia on cell proliferation as well as differentiation capacity because hypoxic conditions are seen in traumatized tissues and fractures. Chung, et al.[75] report that human AMSCs and BMSCs have robust osteogenic potential under standard room air oxygen, but at 1% and 5%, AMSCs showed reduced osteogenic differentiation. Karlsen, et al.[81] showed no adverse effect of 6% O_2 and 7.5% CO_2 on the differentiation of human BMSCs. Currently, there is no consensus regarding which source of MSCs, AMSCs or BMSCs, has the best osteogenic potential under standard or hypoxic conditions.[75]

Others are evaluating the effect of mechanical forces on MSCs.[3] Again, this is to mimic the *in vivo* environment in which we hope to successfully utilize MSCs. Lower tensile strain (3%) results in upregulation of osteogenic related genes and MSC differentiation down the osteogenic lineage. In contrast, MSC exposure to greater tensile strains (10%) result in their differentiation into ligament and tendon cells.[86]

Demonstrating MSC trilineage differentiation is one of the gold standard steps in supporting MSC identity. Mesenchymal stem cells have been differentiated down numerous cell lineages, but adipogenic, chondrogenic, and osteogenic differentiation is most commonly reported. Numerous protocols exist for inducing differentiation; the same is true for evaluation of their cell lineage. Ultimately, one of the main attractions for the use of MSCs in clinical applications is their multipotentiality.

5.5.2 Technique

Cells in passage one, derived from bone marrow, adipose tissue, muscle, and periosteum from three dogs were used for adipogenic, chondrogenic, and osteogenic differentiation assays. All media changes were performed three times per week.

Adipogenesis

Cells were plated at a seeding density of 40,000 cells/cm^2 in 12 well plates (Corning Incorporated, Corning, NY) and supplemented with an adipogenic medium [isobutyl-methylxanthine (0.5 mmol/L) (Sigma, Oakville, ON), rosiglitazone (5 μmol/L) (Toronto Research Chemicals, Toronto, ON), dexamethasone (1 μmol/L) (Sigma, Oakville, ON), biotin (33 μmol/L) (Sigma, Oakville, ON), insulin (1μmol/L) (Sigma, Oakville, ON), pantothenate (17μmol/L) (Sigma, Oakville, ON), 10000 U penicillin and 10 mg streptomycin/mL, amphotericin B (250 μg/mL), L-glutamine (2mM), in D-minimal essential medium (DMEM) (Sigma, Oakville, ON), with 3% FBS and 5% rabbit serum (Invitrogen, Toronto, ON)] for 21 days. Equal numbers of cells were plated as controls and cultured in SM containing 5% FBS instead of 10%. Oil Red O staining was used in histochemical and morphological evaluation.

Oil Red O is a histochemistry technique utilized to demonstrate general lipids in a sample. With this method, lipids stain positively when red or yellow red, depending on the concentration.[87] Staining occurs through the process of adsorption.[88] Positive staining occurs because the dye is more soluble in lipid rather than the carrier it is soluble in.[89] Chayen et al.[87] describe Oil Red O as a strong colourant of fats.

Oil Red O Staining Protocol

1. Remove media from cells and wash with 2 mL of PBS.
2. Remove PBS.
3. Add 2 mL of 10% formalin and incubate for 15 minutes at room temperature.
4. Remove formalin.
5. Wash cells with 2 mL of distilled water.
6. Add 2 mL of 60% isopropanol to dish for 5 minutes at room temperature.
7. Remove isopropanol and let dish dry completely at room temperature. A hair dryer can be used to quicken the process.
8. Add 1 mL of Oil Red O working solution and incubate at room temperature for 60 minutes.
9. Remove Oil Red O solution and rinse dish with distilled water.
10. Wash the cells four times with distilled water.

Recipe for Oil Red O Stock Solution FW 408.5
1. Add 0.35 g Oil Red O and add to 100 mL of isopropanol.
2. Stir overnight.
3. Filter with 0.2 μm filter and store at room temperature.

Recipe for Oil Red O Working Solution (stable for 2 hours)
1. Add 6 mL of Oil Red O stock solution to 4 mL of distilled water.
2. Let mixture sit at room temperature and in a closed container for 20 minutes.
3. Filter with a 0.2 μm filter

Chondrogenesis

For chondrogenic differentiation, a pellet culture technique was used.[22] Five hundred thousand cells were placed in a 15 mL polypropylene tube (BD Falcon, Franklin Lakes, NJ) and centrifuged at 377 x *g* for five minutes to achieve a micromass pellet. The pellet was cultured for 28 days in chondrogenic media [DMEM supplemented with dexamethasone (10^{-7} M), ITS+1 (Sigma, Oakville, ON) (culture supplement containing bovine insulin, transferrin, selenous acid, linoleic acid, and bovine serum albumin), ascorbic acid 2-phosphate (50 μg/mL) (Sigma, Oakville, ON), L-glutamine (2 mM), 10000 U penicillin and 10 mg streptomycin/mL , and amphotericin B (250 μg/mL)] with and without human transforming growth factor β-1 (hTGF-β1)(10 ng/mL) (Millipore, Billerica, MA) in two dogs. The same pellet culture

technique was used in the third dog except the pellets were only maintained in culture for 21 days. Pellets were not cultured in a SM as a control equivalent because of the experience our laboratory has had with cell loss during the culture period. Vidal, et al.[35] report a similar experience and hypothesized that cells cultured using a micromass pellet technique in standard media lacked a compact structure resulting in cell loss with media changes. For microscopic evaluation, the pellets were embedded in paraffin, cut into 5 μm thick sections and stained with Hematoxylin and Eosin (H&E), Alcian blue and Safranin O.

Hematoxylin and eosin is one of the most commonly used stain in histopathology.[88] Hematoxylin is a natural dye that forms haematin when oxidized.[90] In the presence of metals, hematin will bind with lysine residues of nuclear histones; thus staining nuclei blue.[88,90] Eosin is an acid dye that binds to basic residues in cytoplasm, resulting in stains that are shades of pink.[88] It is speculated that the dye binds via electrostatic forces, but the mechanism of action is not fully known.[90]

Alcian blue at pH of 2.5 is the best for staining acidic mucosubstances a turquoise blue colour.[88] Glycosaminoglycans are also called acid mucopolysaccharides and include substances like hyaluronic acid and chondroitin sulfate.[87,88] Alcian blue is a positively charged dye that binds mucopolysaccharides because of their negative charge.[87] The critical electrolyte concentration method can be used to distinguish between different mucopolysaccharides.

Safranin O is used to stain cartilage, mucin, and mast cell granules; these substances are stained orange, whereas nuclei are stained black.[91] The intensity of

Safranin O staining has been shown to be directly proportional to the proteoglycan content in normal cartilage.[92]

Hematoxylin and Eosin Staining Protocol

De-parafinize and hydrate to distilled water includes steps 1-3
1. Place slides in xylene for 10 minutes.
2. Place slides into absolute alcohol for 2 minutes, followed by 95% alcohol and 70% alcohol for 2 minutes each.
3. Place slides in distilled water for 2 minutes.
4. Place slides in Harris' Hematoxylin for 6 minutes.
5. Rinse with tap water for 2 minutes.
6. Place in acid alcohol for 30 seconds.
7. Rinse with tap water for 1 minute.
8. Dip in ammonia water working solution for 10 seconds.
9. Rinse with tap water for 10 minutes.
10. Place in Eosin working solution for 6 minutes.

Dehydrate in alcohol and clear refers to steps 11 and 12.
11. Place in 95% alcohol, followed by absolute alcohol for 2 minutes and 4 minutes, respectively.
12. Place in xylene for 2 minutes.

Harris' Hematoxylin (6 week shelf life)
1. Mix together 15 g of hematoxylin crystals, 150 mL absolute ethanol, 300 g aluminum ammonium sulfate, 3 L of distilled water, 7.5 g of mercuric oxide, and 100 mL of acetic acid.

Acid Alcohol (1 week shelf life)
1. Make a 100:1 solution with 95% ethanol and concentrated HCl (Example: 100 mL of ethanol and 1 mL of HCl)

Ammonia Water Stock Solution (1 week shelf life)
1. Add 12 mL of ammonium hydroxide to 1 L tap water.

Ammonia Water Working Solution
1. Add 125 mL of stock ammonia water to 625 mL of tap water.

Eosin Stock Solution
1. Mix 30 g or Eosin Y (water soluble) with 600 mL of distilled water.
2. Add 2.4 L of 95% ethanol.

Eosin Working Solution (4 week shelf life)

1. Mix together 175 mL of eosin stock solution with 525 mL of 80% ethanol and 3.5 mL of acetic acid.

Alcian Blue Staining Protocol

1. De-parafinize and hydrate to distilled water.
2. Place in 3% acetic acid for 3 minutes.
3. Place in Alcian blue for 30 minutes.
4. Wash in running tap water for 10 minutes.
5. Rinse in distilled water.
6. Counterstain in nuclear fast red for 5 minutes.
7. Wash in running tap water for 1 minute.
8. Dehydrate slides and clear.

Acetic Acid Solution
1. Add 30 mL of acetic acid to 970 mL of distilled water.

Alcian blue Solution
1. Add 30 mL of Alcian blue (8GX) to 100 mL of 3% acetic acid.
2. Adjust the pH to 2.5

Safranin O Staining Protocol

With this stain, cartilage and mucins stain orange.

1. De-parafinize and hydrate slides to distilled water.
2. Stain slides with Weigert's iron hematoxylin in working solution for 10 minutes.
3. Wash slides in running tap water for 10 minutes.
4. Place slides in 0.001% fast green solution for 5 minutes.
5. Rinse slides quickly with 1% acetic acid for no more than 10-15 seconds.
6. Place slides in 0.1% Safranin O solution for 5 minutes.
7. Dehydrate and clear.

Weigert's Iron Hematoxylin Solution
1. Stock solution A is made of 1 g Hematoxylin and 100 mL of 95% ethanol.
2. Stock solution B is made of 4 mL 29% ferric chloride in water, 95 mL of distilled water, and 1 mL of hydrochloric acid.

Weigert's Iron Hematoxylin Working Solution
1. Mix equal parts Stock solution A and B.

0.001% Fast Green Solution
1. Mix 0.01 g of Fast green (FCF, CI 42053) with 1 L distilled water.

1% Acetic Acid Solution
1. Mix 1 mL of acetic acid with 99 mL of distilled water.

0.1% Safranin O Solution
1. Mix 0.1 g of Safranin O (CI 50240) with 100 mL of distilled water.

Osteogenesis

Cells were plated at a seeding density of 10,000 cells/cm^2 in 12 well plates and supplemented with osteogenic medium (αMEM supplemented with 5% FBS, dexamethasone (10^{-8} M), Beta-glycerophosphate (10mM) (Sigma, Oakville, ON), ascorbic acid-2 phosphate (50 μg/mL), L-glutamine (2mM), 10000 U penicillin and 10 mg streptomycin/mL , and amphotericin B (250 μg/mL)) for 8 weeks. Equal numbers of cells were plated as controls and cultured in SM containing 5% FBS instead of 10%. Von Kossa and Alizarin red staining were used for histochemical and morphological evaluation of osteogenic differentiation.

Von Kossa is a technique that stains calcium phosphates and carbonate groups black.[88] The stain reacts with phosphate in the presence of an acidic material.[93] The silver is deposited by replacing the calcium salt.[94] However, Von Kossa is not a specific stain for calcium. Calcium phosphate is deposited by osteoblasts and will stain with Von Kossa, but Von Kossa will stain phosphates that aren't necessarily part of a mineralized matrix.[93] Although this is a common technique to demonstrate osteogenesis in MSC cultures, others recommend utilizing additional stains like Alizarin red and/or osteogenic markers.[93]

Alizarin red stains calcium salts red or orange to yellow red.[88] It can, however, also stain for manganese, magnesium, barium, strontium, and iron.[95]

Von Kossa Staining Protocol

1. Remove media from dishes and rinse with PBS.

2. Fix in 10% formalin for 15 minutes.
3. Remove formalin and rinse dishes with distilled water. Leave in distilled water for 15 minutes.
4. Stain dishes with 2.5% silver nitrate solution for 30 minutes. Keep dish in bright light.
5. Remove silver nitrate and rinse dishes with distilled water three times.
6. Leave in distilled water for 15 minutes.
7. Add sodium carbonate formaldehyde for 5 minutes.
8. Remove sodium carbonate formaldehyde and rinse dishes with distilled water three ties. Leave in distilled water for 1 hour. Rinse again.

2.5% Silver Nitrate Solution
1. Add 2.5 g of silver nitrate to 100 mL of distilled water.

Sodium Carbonate Formaldehyde
1. Add 5 g of Na_2CO_3 to 25 mL of formalin and 100 mL of distilled water

10% Neutral Formalin Buffer
1. Add 16 g of Na_2HPO_4 and 4 g of NaH_2PO_4 to 100 mL of formalin and finally with distilled water up to 1 L total.

Alizarin Red Staining Protocol

1. Stain slides with Alizarin Red solution for 5 minutes.
2. Remove solution.

Alizarin Red Solution
1. Add 2.0 g of Alizarin Red to 100 mL of distilled water. Mix well.
2. Adjust the pH to 4.1 to 4.3% with 10% ammonium hydroxide.

5.5.3 Results

Bone marrow, adipose tissue, muscle, and periosteum-derived MSCs were successfully differentiated down the adipogenic and osteogenic cell lineages. Chondrogenic differentiation of MSCs derived from these four canine donor tissue sources was not demonstrated.

The cells cultured in adipogenic differentiation medium for 21 days demonstrated positive Oil Red O staining of lipid droplets (Figure 6). The number of

lipid droplets and intensity of staining was most impressive with MMSCs. Cells cultured in standard media did not develop lipid droplets nor had positive staining with Oil Red O.

Mesenchymal stem cells derived from bone marrow, adipose tissue, muscle, and periosteum from three dogs were set up in a pellet culture system in an attempt to induce chondrogenic differentiation. The suspended cells formed a visible white opaque pellet within two days. Cellular morphology and staining characteristics of each of the recovered pellets were all similar regardless of tissue source, time in culture, or whether the culture was supplemented with hTGF-β1 (Figure 7). Routine H&E staining of each of the pellets revealed a large central zone composed of eosinophilic amorphous necrotic material admixed with sparse karyhrrhectic debris (consistent with areas of tissue necrosis). The latter areas were surrounded by a thin peripheral layer of approximately one to ten cell layers thick comprised of bland, uniform, streaming, spindloid mesenchymal cells. These cells had fusiform nuclei, dense, fine chromatin, unapparent to small nucleoli and small amounts of poorly defined pinkish blue cytoplasm. Spindloid cells were separated by small amounts of pale, eosinophilic matrix which appears blue when stained with Alcian blue but which did not stain appreciably with Safranin O. There was no evidence of chondrocytes, lacuna, or a chondroid matrix to support chondrogenic differentiation in any of the examined BMSC, AMSC, MMSC, and PMSC samples.

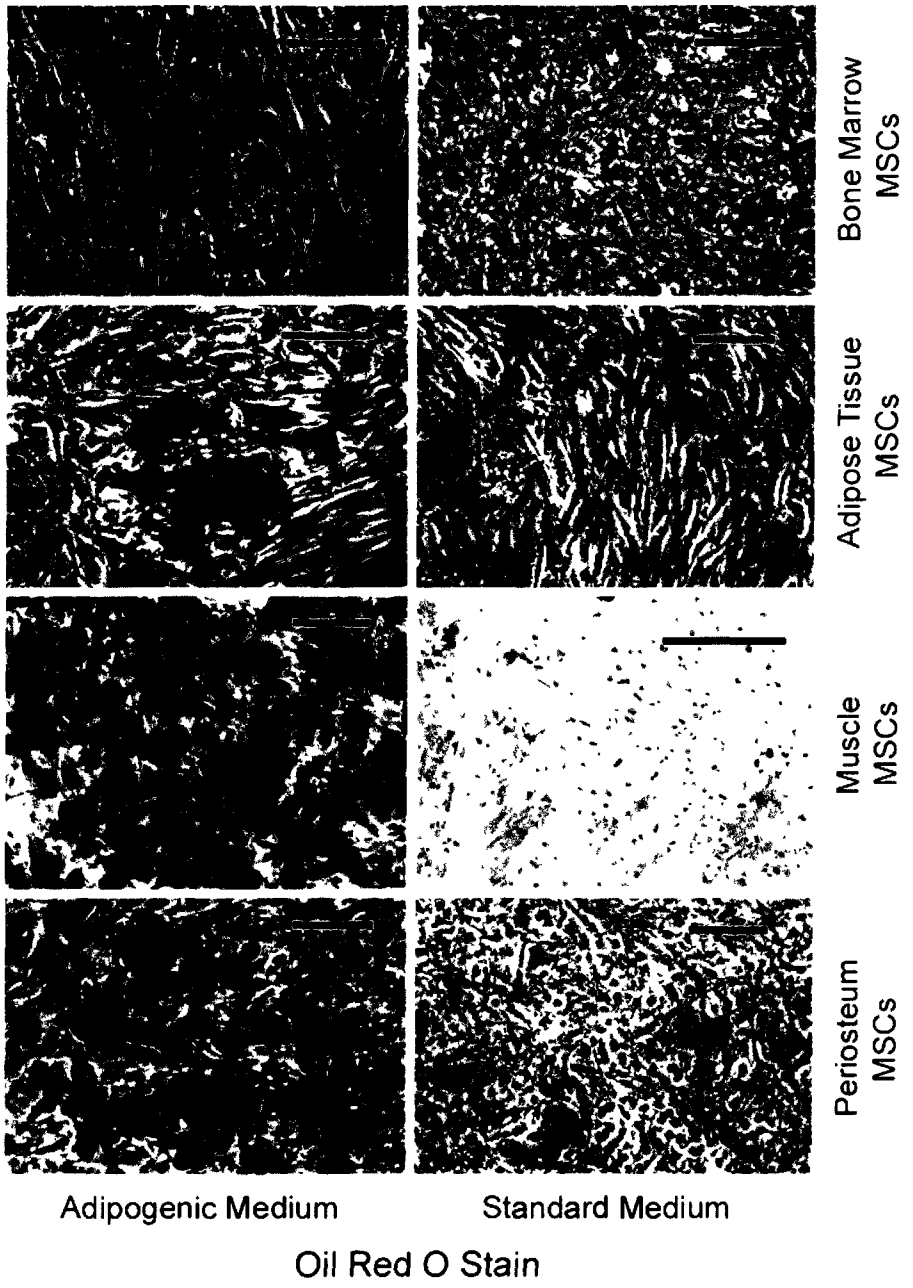

Figure 6. Light microscopic demonstration of adipogenic differentiation of BMSCs, AMSCs, MMSCs, and PMSCs. Isolated cells, cultured in adipogenic medium for 21 days, showed positive staining of lipids with Oil Red O. Control cells cultured in standard medium, as seen on the right, do not have evidence of lipid vacuole formation. Magnification is 20 x. Scale bar = 40 μm

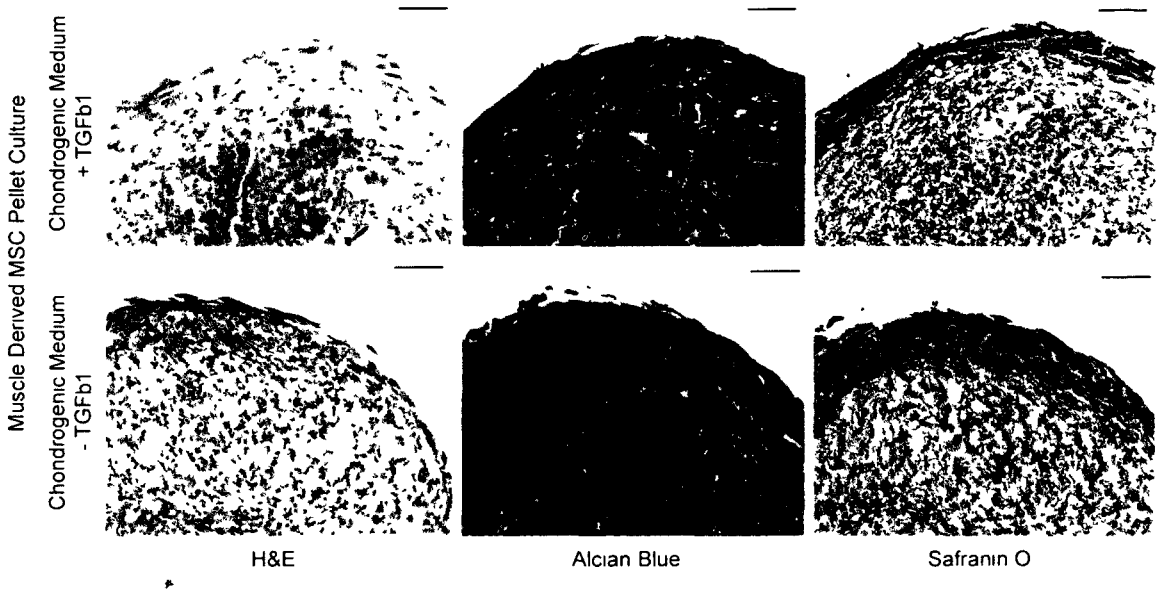

Figure 7. Photomicrograph of MMSCs cultured in a chondrogenic medium with and without hTGF-β1 for 28 days. The sections were stained with H&E, Alcian blue, and Safranin O. There was no appreciable difference in the cellular morphology or staining characteristics of the pellets subjected to the above culture conditions. The images show a large central area of tissue necrosis with a mesenchymal population in the periphery; chondrogenic differentiation is not supported by these histological sections. Magnification is 40 x. Scale bar = 100 μm

Osteogenic differentiation was demonstrated in all four donor tissue-derived MSCs in three dogs after induction with an osteogenic medium for eight weeks (Figures 8 and 9). The morphology of the MSCs progressed from a fibroblast- like appearance to a polygonal shape followed by the formation of nodular aggregates. These nodular aggregates stained positive with Von Kossa and Alizarin red, demonstrating the presence of a phosphate mineral composition and the presence of calcium salts, respectively. The positive stain uptake of the nodules using both techniques helps confirm the presence of mineralization and differentiation of the MSCs down the

osteogenic cell lineage. MSCs cultured in standard media did not form nodules and did not stain positive with Von Kossa or Alizarin red.

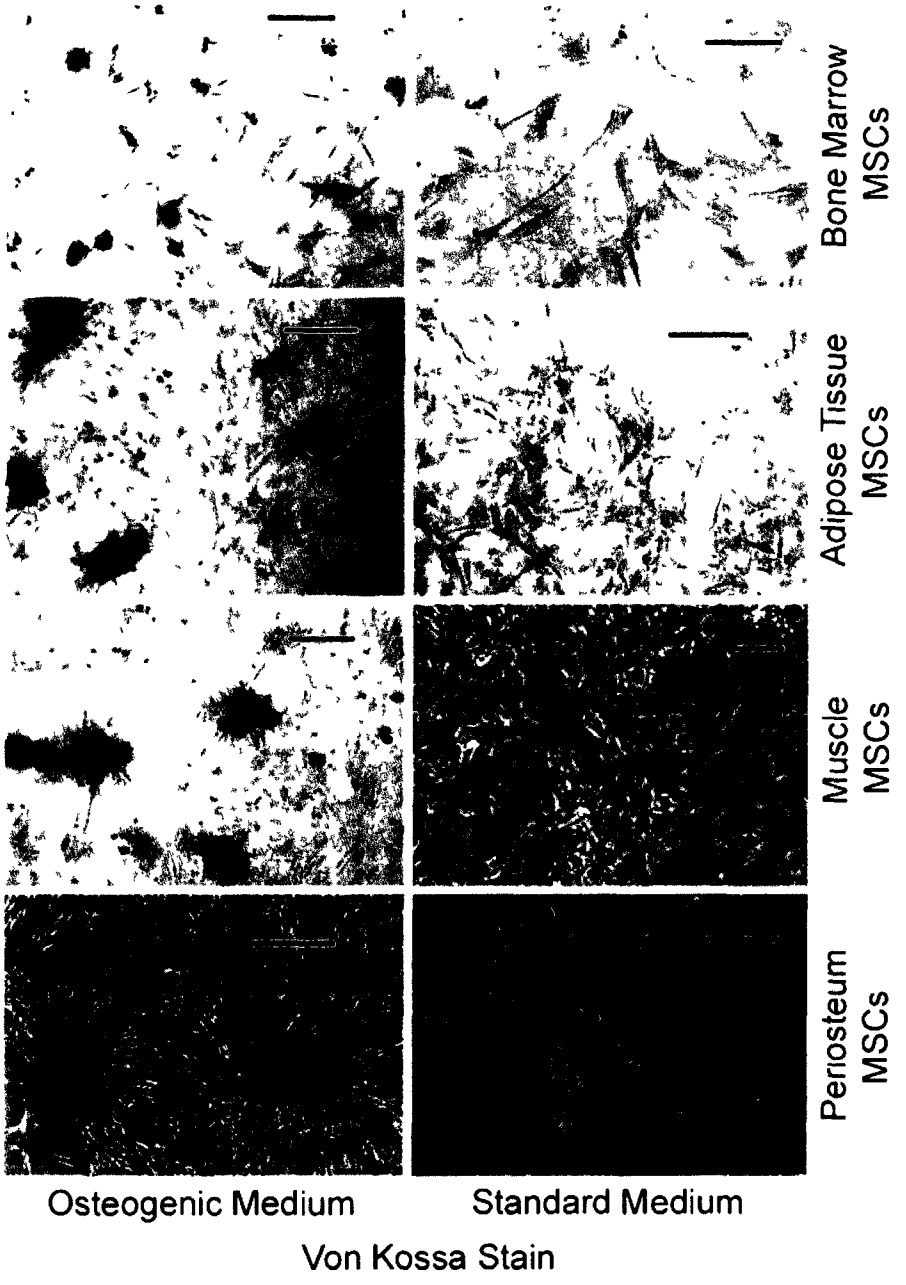

Figure 8. Light microscopic images of positive osteogenic differentiation of BMSCs, AMSCs, MMSCs, and PMSCs are presented. The MSCs subjected to culture with osteogenic medium for eight weeks demonstrate nodules that stain positively with Von Kossa. The control group shows a population of spindle-like cells that neither formed nodules nor stained positively with Von Kossa.
Magnification is 20 x. Scale bar = 40 μm

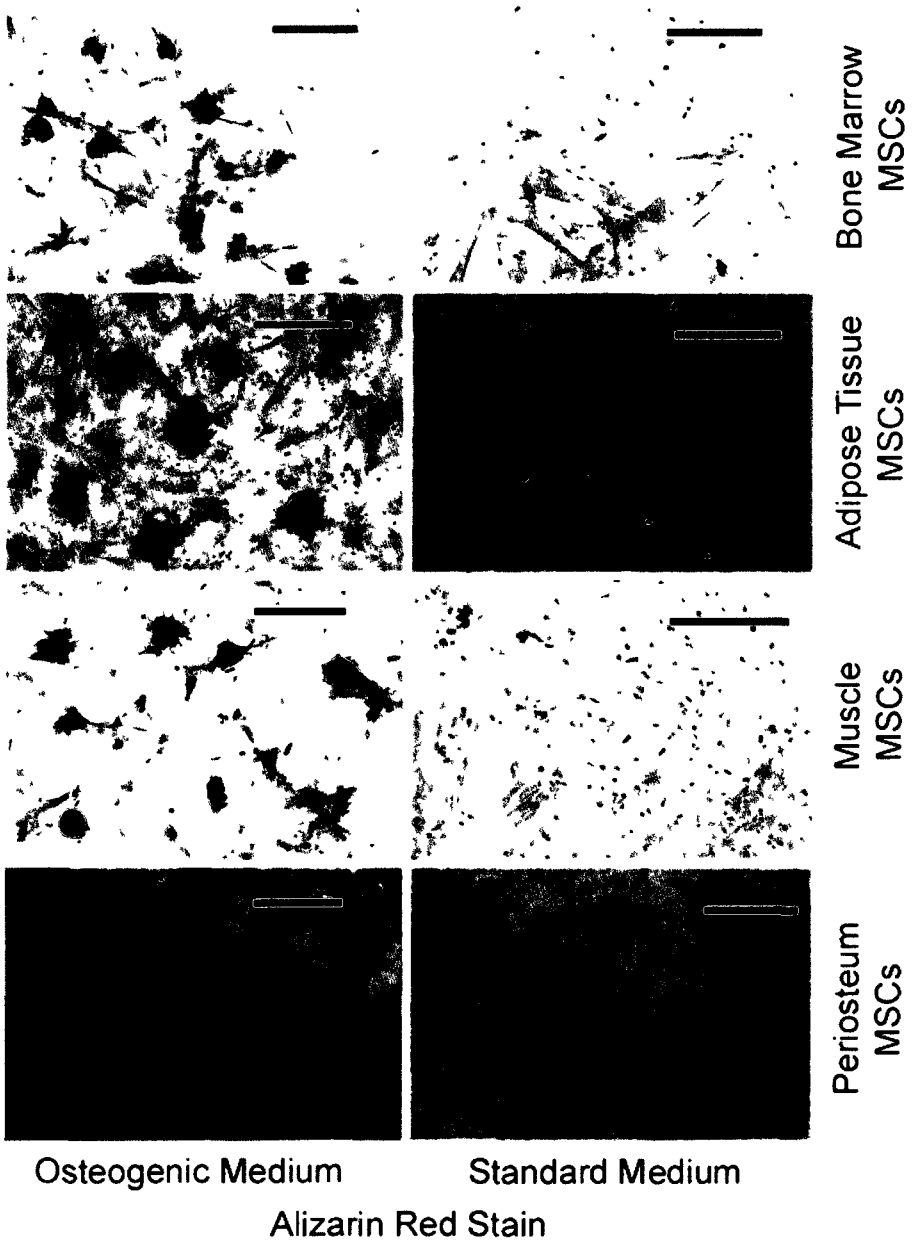

Figure 9. Light microscopic images of osteogenic differentiation of the four donor tissue- derived MSCs is demonstrated by nodule formation and positive Alizarin red staining for calcium deposits in the cells cultured in osteogenic medium. The MSCs grown in standard medium for eight weeks did not show evidence of mineral deposition. Magnification is 20 x. Scale bar = 40 μm

5.5.4 Discussion

Mesenchymal stem cells derived from canine bone marrow, adipose tissue, muscle, and periosteum demonstrated bilineage differentiation down the adipogenic and osteogenic pathways. Chondrogenic differentiation was attempted but was not successfully demonstrated. In the literature, adipogenic, osteogenic, and chondrogenic differentiation are most commonly reported.[17,24,25,37] However, some authors preferred to substitute myogenic[18,22] or neurogenic[20] differentiation for chondrogenic differentiation, and another documented only bilineage differentiation.[12]

In our study, adipogenic differentiation was confirmed by the appearance of positively stained lipid vacuoles within cells subjected to an adipogenic medium, as previously reported with canine BMSCs and AMSCs.[16,22] The canine MMSCs appeared to have the greatest adipogenic potential based on a subjectively greater number of positively stained lipid vacuoles when compared to other tissue-derived MSCs. This is in contrast to the study reported by Yoshimura, et al.[36] who found that rat synovium-derived MSCs (SMSCs) and AMSCs had a greater adipogenic potential compared to BMSCs, MMSCs, and PMSCs based on an objective assessment of Oil Red O positive colony rate. Human SMSCs and AMSCs showed a similar superiority to BMSCs, MMSCs, and PMSCs for adipogenic differentiation.[21] However, the culture techniques differed between the three studies; canine MSCs in our study underwent adipogenic induction for a period of three weeks in comparison to four days for rat MSCs and two weeks for human MSCs.

All tissue-derived MSCs treated with an osteogenic medium differentiated into

osteoblasts. This is supported by the morphological appearance of bone nodules that stained positively with Alizarin red and Von Kossa.[12,17,21,96] Osteogenic differentiation has not been previously published for canine MMSCs and PMSCs. Csaki, et al.[16] demonstrated successful osteogenic differentiation of canine BMSCs using Von Kossa stain. Neupane and colleagues[17] used Alizarin red and Von Kossa to show the osteogenic potential of canine AMSCs. Although morphological assessment with histochemistry stains is a common method for confirming lineage differentiation, many researchers are providing stronger evidence by reporting upregulated gene expression in MSCs induced down the osteogenic lineage.[17,22,85] Researchers in our lab have evaluated mRNA expression of osteoblast markers in osteoinduced MSCs derived from each of the four tissues; ALP, RUNX2, OSTERIX, and OSTEOPONTIN expression was found in all osteo-induced MSCs. Basal expression of these markers was not seen in MSCs treated with standard culture medium, confirming differentiation down the osteogenic lineage. ALP and RUNX2 are early stage transcription factors of osteogenesis, whereas OSTERIX is a late stage transcription factor. OSTEOPONTIN, also known as bone sialoprotein 1, is expressed in other tissues but is considered an osteoblast specific marker.[17,85]

In addition to evaluating osteogenic differentiation for confirmation of MSC identity, MSCs are being evaluated for their ability to differentiate down the osteogenic lineage for applications in bone healing.[97] Optimal tissue sources and culture conditions are being evaluated. Toupadakis and colleagues[98] reported equine BMSCs to have a greater osteogenic potential in comparison to AMSCs; however, AMSCs were still successfully differentiated into osteoblasts. They showed that equine BMSCs had

greater calcium deposition and ALP activity compared to AMSCs, as assessed with Alizarin red and a commercial ALP activity staining kit. mRNA expression of RUNX2 and OSTERIX was also greater in osteoinduced BMSCs after four days in culture; however this was not the case after ten days. In a study evaluating various tissue sources from human-derived MSCs, BMSCs, SMSCs, and PMSCs had greater osteogenic potential in comparison to AMSCs and MMSCs based on a greater number of colonies stained with Alizarin red.[21] This is in contrast to Kern, et al.[99] who report no significant difference in osteogenic differentiation between human BMSCs, AMSCs, and umbilical cord blood-derived MSCs. The findings in the literature are not consistent.[75] Although this study did not compare the osteogenic potential between MSCs derived from four tissue sources, a difference was not apparent using subjective evaluation.

The time required for MSCs to undergo osteogenic differentiation is another interesting consideration. In this study, MSCs required at least seven to eight weeks in osteogenic culture medium to show mature nodules and satisfactory staining of mineralized extracellular matrix. Neupane, et al.[17] also cultured canine MSCs for six to eight weeks. Other studies report osteogenesis of canine MSCs after just three weeks in culture, despite culture media being similar.[16,18,22] A possible hypothesis for this short induction period is that cells were grown to confluency prior to induction; however others have shown rapid induction without waiting for cells to become confluent first.[82] Rapid osteoinduction has been demonstrated by supplementing media with BMP-2.[12,85] Canine periosteum derived cells also had better osteogenic potential when cultured in DMEM, rather than RPM1 1640.[100] The authors attributed this finding to the greater calcium content found in DMEM.

The other consideration is the outcome measure. For instance, if osteogenesis is supported by ALP activity, cells can be evaluated after two weeks of induction. This is because ALP activity is maximally increased during the first 12 days of culture.[96] However, to evaluate mineralized extracellular matrix with Von Kossa or Alizarin red, one would have to wait at least three weeks.[18,101] Others suggest that if mRNA expression is the outcome measure, samples can be evaluated as early as seven days since most changes in mRNA levels occur during that time period; in samples cultured for a longer duration, mRNA expression reaches a plateau.[81]

Our attempts at differentiating MSCs down the chondrogenic lineage were unsuccessful based on morphological and histochemical assessments, despite using a standard pellet culture system [21,20,24,35,102-106] with an induction technique similar to Csaki, et al.[16] Others have identified difficulty in obtaining cartilage tissue in a pellet culture and report similar central necrosis and undifferentiated cells.[107] The blue stain uptake seen with Alcian blue may convince a reader that a proteoglycan matrix exists, but this was not supported with H&E and Safranin O staining, or histologic appearance. Similar images to ours, demonstrating unclear chondrocyte morphology, exist in the literature and are not convincing of chondrogenic differentiation. Other studies clearly reveal chondrogenic differentiation using histology.[35,104,108,109] mRNA expression of chondrocyte markers collagen type II, aggrecan, and SOX9 would have been useful to evaluate differentiation down the chondrogenic lineage in this study.[22]

Technique differences could explain the chondrogenic differentiation results of our study in comparison to successful pellet cultures. For instance, our group used a higher initial cell plating number and a longer culture period than Csaki, et al.[16] Even

though successful differentiation using similar methodologies to ours is reported,[20,25,36,109-111] each of these factors may have contributed to deficient nutrient diffusion and resultant cellular necrosis.[35,107] Others may speculate that the isolated MSCs were incapable of chondrogenic differentiation because only a low percentage of a MSC population has the capacity to differentiate down three or more lineages.[9,112] Many protocols for chondrogenic differentiation are not standardized, making comparison among studies difficult. Initial cell numbers, growth factors, time in culture, and plating techniques vary among them.[16,17,22,25,35,36,113]

A three dimensional culture system for chondrogenesis is superior to the monolayer technique.[114] Micromass culture was recently shown to induce a larger amount of homogenized cartilage tissue with increased Toluidine blue staining and expression of collagen type II in comparison to the pellet culture.[107] Dexamethasone, FGF-2, TGF-β,[106] and BMP-2[103] have also shown to enhance MSC chondrogenesis *in vitro*. Although our study utilized dexamethasone and hTGF-β in the culture media, successful differentiation may have occurred if we exploited the effects of FGF-2 or BMP-2.

Pellets stained similarly regardless of whether hTGF-β1 was within the chondrogenic medium. This finding is in agreement with Neupane, et al.[17] who demonstrated positive Alcian blue staining in both treated and control micromass culture of cells, and disagrees with the findings of another study[16] which found that untreated pellets had reduced staining compared to those induced with chondrogenic media. Neupane, et al.[17] concluded that MSC culture in a three dimensional construct was sufficient to stimulate early chondrogenesis. However, addition of a growth factor from

the TGF-β family is reported to be necessary for chondrogenic differentiation of MSCs. Improved chondrogenesis has been shown with TGF-β2 and TGF-β3 in comparison to TGF-β1.[78] In our study, culture of canine BMSCs, AMSCs, MMSCs, and PMSCs in a three dimensional construct, alone or with the addition of hTGF-β1, did not result in chondrogenic differentiation.

5.5.5 References

1. Dominici M, LeBlanc K, Meuller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 2006;8:315-317.

2. Lange C, Cakiroglu F, Spiess A, et al. Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. *J Cell Physiol* 2007;213:18-26.

3. Sarraf C, Otto W, Eastwood M. *In vitro* mesenchymal stem cell differentiation after mechanical stimulation. *Cell Prolif* 2011;44:99-108.

4. Manini I, Gulino L, Gava B, et al. Multi-potent progenitors in freshly isolated and cultured human mesenchymal stem cells: a comparison between adipose and dermal tissue. *Cell Tissue Res* 2011;344:85-95.

5. Bourzac C, Smith L, Vincent P, et al. Isolation of equine bone marrow-derived mesenchymal stem cells: a comparison between three protocols. *Equine Vet J* 2010;42:519-527.

6. Prockop D. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;276:71-74.

7. da Silva Meirelles L, Fontes A, Covas D, et al. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev* 2009;20:419-27.

8. Horn P, Bork S, Diehlmann A, et al. Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. *Cytotherapy* 2008;10:676-685.

9. Javazon E, Beggs K, Flake A. Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* 2004;32:414-425.

10. Chamberlain G, Fox J, Ashton B, et al. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007;25:2739-2749.

11. Baddoo M, Hill K, Wilkinson R, Gaupp D, et al. Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem* 2003;89:1235-1249.

12. Kamashina H, Farese J, Storm J, et al. The frequency, growth kinetics, and osteogenic/adipogenic differentiation properties of canine bone marrow stromal cells. *In vitro Cell Dev Biol Anim* 2008;44:472-479.

13. Schuh W, Friedman M, Carrade D. Identification of variables that optimize isolation and culture of multipotent mesenchymal stem cells from equine umbilical cord. *Am J Vet Res* 2009;70:1526-1535.

14. Jarocha D, Lukasiewicz E, Majka M. Adventage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105+ and CD271+ cells. *Folia Histochem Cytobiol* 2008;46:307-314.

15. Kassis I, Zangi L, Rivkin R, et al. Isolation of mesenchymal stem cells from G-CSF-mobilized human peripheral blood using fibrin microbeads. *Bone Marrow Transplant* 2006;37:967-976.

16. Csaki C, Matis U, Mobasheri A, et al. Chondrogenesis, osteogenesis, and adipogenesis of canine mesenchymal stem cells: a biochemical, morphological, and ultrastructural study. *Histochem Cell Biol* 2007;128:507-520.

17. Neupane M, Chia-Cheng C, Kiupel M, et al. Isolation and characterization of canine adipose-derived mesenchymal stem cells. *Tissue Eng Part A* 2008;14:1007-1015.

18. Martinello T, Bronzini I, Maccatrozzo L. Canine adipose-derived-mesenchymal stem cells do not lose stem cell features after a long term cryopreservation. *Res Vet Sci* 2010; doi:10.1016/j.rvsi.2010.07.024.

19. Kamashina H, Deng J, Oji T, et al. Expression of neural markers on bone marrow-derived canine mesenchymal stem cells. *Am J Vet Res* 2006;67:1921-1928.

20. Seo M, Jeong Y, Park J, et al. Isolation and characterization of canine umbilical cord blood-derived mesenchymal stem cells. *J Vet Sci* 2009;10:181-187.

21. Sakaguchi Y, Sekiya I, Yagishita K, et al. Comparison of human stem cells derived from various mesenchymal tissues. *Arthritis Rheum* 2005;52:2521-2529.

22. Vieira NM, Brandalise V, Zucconi E, et al. Isolation, characterization, and

differentiation potential of canine adipose-derived stem cells. *Cell Transplant* 2010;19:279-289.

23. Pountos I, Corscadden D, Emery P, et al. Mesenchymal stem cell tissue engineering: techniques for isolation, expansion, and application. *Injury* 2007;38S4:S23-S33.

24. Beyer Nardi N, da Silva Meirelles L. Mesenchymal stem cells: isolation, *in vitro* expansion and characterization. *Handb Exp Pharmacol* 2006;174:249-282.

25. Braun J, Hack A, Weis-Klemm M, et al. Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. *Am J Vet Res* 2010;71:1228-1236.

26. Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. *Exp Hematol* 2000;28:875-884.

27. Schugar RC, Chirieleison SM, Wescoe KE, et al. High harvest yield, high expansion, and phenotype stability of CD146 mesenchymal stromal cells from whole primitive human umbilical cord tissue. *J Biomed Biotechnol* 2009:1-11.

28. Chandler H, Colitz C. Molecular biology for the clinician: Understanding current methods. *J Am Anim Hosp Assoc* 2006;42:326-335.

29. Polak J, Van Noorden S. *An introduction to immunocytochemistry: current techniques and problems.* Revised ed. New York: Oxford University Press, 1987.

30. Kawamura A. *Fluorescent antibody techniques and their applications.* 2nd ed. Tokyo: University of Tokyo Press,1977.

31. Goding J. *Monoclonal antibodies: principles and practice.* 3rd ed. London, Academic Press, 1996.

32. Hayat M. *Microscopy, immunohistochemistry, and antigen retrieval methods for light and electron microscopy.* Electronic resource. Kluwer Academic/Pluner Publishers, 2002.

33. Green K. Immunohistochemistry basics. Eastern Health CIHRT Exhibit P-2168, page 1. http://www.cihrt.nl.ca/exhibits/Ken%20Green%20Exhibits/P-2168_Immunohistochemistry%20Basics.pdf, 2011.

34. Fortier L. Stem cells: classifications, controversies, and clinical applications. *Vet Surg* 2005;34:415-423.

35. Vidal MA, Kilroy GE, Johnson JR, et al. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal

stromal cells: adipogenic and osteogenic capacity. Vet Surg 2006;35:601-610.

36. Yoshimura H, Muneta T, Nimura A, et al. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* 2007;327:449-462.

37. Arnhold SJ, Goletz I, Klein H et al. Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. *Am J Vet Res* 2007;68:1095-1105.

38. Ren G, Michael L, Entman M, et al. Morphological characteristics of the microvasculature in healing myocardial infarcts. *J Histochem Cytochem* 2002;50:71-79.

39. Sorrentino A, Ferracin M, Castelli G, et al. Isolation and characterization of $CD146^{+}$ multipotent mesenchymal stromal cells. *Exp Hematol* 2008;36-1035-1046.

40. Rallapalli, S, Bishi DK, Verma RS, et al. A multiplex PCR technique to characterize human bone marrow derived mesenchymal stem cells. *Biotechnol Lett* 2009;31:1843-1850.

41. Pierantozzi E, Gava B, Manini I, et al. Pluripotency regulators in human mesenchymal stem cells: expression of NANOG but not of OCT-4 and SOX-2. *Stem Cells Develop* 2010 DOI: 10.1089/scd.2010.0353.

42. Chambers I, TomLinson S. The transcriptional foundation of pluripotency. *Develop* 2009;136:2311-2322.

43. Johansson H, Simonsson S. Core transcription factors, Oct4, Sox2 and Nanog, individually form complexes with nucleophosmin (Npm1) to control embryonic stem (ES) cell fate determination. *Aging* 2010;2:815-822.

44. Carlin R, Davis D, Weiss M, et al. Expression of early transcription factors Oct-4, Sox-2 and Nanog by porcine umbilical cord (PUC) matrix cells. *Reprod Biol Endocrinol* 2006;6:8.

45. Lee J, Kim H, Rho J, et al. The human OCT-4 isoforms differ in their ability to confer self-renewal. *J Biol Chem* 2006;281:33554-33565.

46. Jung M, Peterson H, Chavez L, et al. A data integration approach to mapping OCT4 regulatory networks operative in embryonic stem cells and embryonal carcinoma cells. *PloS ONE* 2010;5:e10709.

47. Karantzali E, Lekakis V, Ioannou M, et al. Sall1 regulates embryonic stem cell differentiation in association with Nanog. *J Biol Chem* 2011;286:1037-1045.

48. de Jong J, Stoop H, Gillis AJM, et al. Differential expression of SOX17 and SOX2 in germ cells and stem cells has biological and clinical implications. *J Pathol* 2008;215:21-30.

49. Pan G, Thomson J. Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Res* 2007;17:42-49.

50. Keramari M, Razavi J, Ingman K, et al. Sox2 is essential for formation of trophectoderm in the preimplantation embryo. *PLoS ONE* 2010;5:e13952.

51. Lengerke C, Fehm T, Kurth R, et al. Expression of the embryonic stem cells marker SOX2 in early-stage breast carcinoma. *BMC Cancer* 2011;11:42-52.

52. Moretto-Zita M, Jin H, Shen Z, et al. Phosphorylation stabilizes Nanog by promoting its interaction with Pin1. *Proc Natl Acad Sci U S A* 2010;107:13312-13317.

53. Riekstina U, Cakstina I, Parfejevs V, et al. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart, and dermis. *Stem Cell Rev and Rep* 2009;5:378-386.

54. Violini S, Ramelli P, Pisani L, et al. 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* 2009;10:29-39.

55. Erlich HA. *PCR technology: principles and applications for DNA amplification.* New York, Stockton Press, 1989.

56. Darbre P. *Basic molecular biology: essential techniques*. Chichester, John Wiley & Sons, 1999.

57. Hunt M. Real Time PCR. Microbiology and Immunology On-line. Class notes. http://pathmicro.med.sc.edu/pcr/realtime-home.htm, 2010.

58. McPherson MJ, Quirke P, Taylor GR. *PCR: a practical approach.* Oxford, IRL Press at Oxford University Press, 1991.

59. McPherson MJ, Hames BD, Taylor GR. *PCR 2: a practical approach.* Oxford, IRL Press at Oxford University Press, 1995.

60. Freifelder D. Essentials of molecular biology. Boston, Jones and Bartlett Publishers, Inc. 1985.

61. Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; 29: 2002-2007.

62. Chen L, Daley GQ. Molecular basis of pluripotency. *Hum Mol Genet* 2008;17:R23-R27.

63. Izadpanah R, Joswig T, Tsien F, et al. Characterization of multipotent mesenchymal stem cells from the bone marrow of rhesus macaques. *Stem Cells Dev* 2005;14:440-451.

64. Pacini S, Carnicelli V, Trombi L, et al. Constitutive expression of pluripotency-associated genes in mesodermal progenitor cells (MPCs). *PLoS ONE* 2010;5:e9861.

65. Luyckx A, De Somer L, Jacobs S, et al. Oct4-negative multipotent adult progenitor cells and mesenchymal stem cells as regulators of T-cell alloreactivity in mice. *Immunol Lett* 2011;137:78-81.

66. Karaoz E, Okcu A, Gacar G, et al. A comprehensive characterization study of human bone marrow MSCs with an emphasis on molecular and structural properties. *J Cell Physiol* 2010;226:1367-1382.

67. Greco S, Liu K, Rameshear P. Functional similarities among genes regulated by Oct4 in human mesenchymal and embryonic stem cells. *Stem Cells* 2007;25:3143-3154.

68. Fakhr Taha, Hedayati V. Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells. *Tissue Cell* 2010;42:211-216.

69. Seo K, Lee S, Bhandari D, et al. OCT4A contributes to the stemness and multi-potency of human umbilical cord blood-derived multipotent stem cells (hUCB-MSCs). *Biochem Biophys Res Commun* 2009;384:120-125.

70. Ambady S, Malcuit C, Kashpur O, et al. Expression of NANOG and NANOGP8 in a variety of undifferentiated and differentiated human cells. *Int J Dev Biol* 2010;54:1743-1754.

71. Alt E, Yan Y, Gehmert S, et al. Fibroblasts share mesenchymal phenotypes with stem cells but lack their differentiation and colony-forming potential. *Biol Cell* 2011;103:197-208.

72. Kadiyala S, Young RG, Theide MA. Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential *in vivo* and *in vitro*. *Cell Transplant* 1997;6:125-134.

73. Salem H, Theimermann C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 2010;28:585-596.

74. Lee JY, Zhou Z, Taub P, et al. BMP-12 treatment of adult mesenchymal stem cells *in vitro* augments tendon-like tissue formation and defect repair *in vivo*. *PLoS ONE* 2011;6:e17531.

75. Chung D, Hayashi K, Toupadakis C, et al. Osteogenic proliferation and differentiation of canine bone marrow and adipose tissue derived mesenchymal stromal cells and the influence of hypoxia. R*es Vet Sci* 2010, doi: 10.1016/j.rvsc.2010.10.012.

76. Bosnakowski D, Mizuno M, Kim G, et al. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. *Biotechnol Bioeng* 2006;93:1152-1163.

77. Malgieri A, Kantzari E, Patrizi M, et al. Bone marrow and umbilical cord blood human mesenchymal stem cells: state of the art. *Int J Clin Exp Med* 2010;3:248-269.

78. Barry F, Murphy J. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 2004;36:568-584.

79. Solchaga LA, Penick KJ, Welter JF. Chondrogenic differentiation of bone marrow-derived mesenchymal stem cells: tips and tricks. In: *Mesenchymal stem cell assays and applications*. 2011;698:253-278.

80. Williams JT, Southerland SS, Souza J, et al. Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. A*m Surg* 1999;65:22-26.

81. Karlsen T, Mirtaheri P, Shahdadfar A, et al. Effect of three-dimensional culture and incubator gas concentration on phenotype and differentiation capability of human mesenchymal stem cells. *J Cell Biochem* 2011;112:684-693.

82. Neuhuber B, Swanger S, Howard L, et al. Effects of plating density and culture time on bone marrow stromal cell characteristics. *Exp Hematol* 2008;36:1176-1185.

83. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. *J Cell Sci* 2000;113:1161-1166.

84. Kraus K, Kirker-Head C. Mesenchymal stem cells and bone regeneration. *Vet Surg* 2006;35:232-242.

85. Volk SW, Diefenderfer DL, Christopher SA, et al. Effects of osteogenic inducers on cultures of canine mesenchymal stem cells. *Am J Vet Res* 2005;66:1729-1737.

86. Jang J, Lee S, Park S. Combined effects of surface morphology and mechanical straining magnitudes on the differentiation of mesenchymal stem cells without using biochemical reagent. *J Biomed Biotechnol* 2011; Article ID 860652, doi:10.1155/2011/860652.

87. Chayen J, Bitensky L. *Practical Histochemistry*. 2nd ed. Chichester, John Wiley & Sons, 1991.

88. Vacca L. Laboratory manual of histochemistry. New York, Raven Press, 1985.

89. IHCWORLD Life Science Information Network. Oil Red O Staining Protocol. http://www.ihcworld.com/_protocols/special_stains/oil_red_o_ellis.htm, 2011.

90. IHCWORLD Life Science Information Network. Hematoxylin and Eosin Staining Protocol. http://www.ihcworld.com/_protocols/special_stains/h&e_ellis.htm, 2011.

91. IHCWORLD Life Science Information Network. Safranin O Staining Protocol. http://www.ihcworld.com/_protocols/special_stains/safranin_o.htm, 2011.

92. Camplejohn K, Allard S. Limitations of Safranin O staining in proteoglycan-depleted cartilage demonstrated with monoclonal antibodies. *Histochem* 1988;89:185-188.

93. Bonewald LF, Harris SE, Rosser J, et al. Von Kossa staining alone is not sufficient to confirm that mineralization *in vitro* represents bone formation. *Calcif Tissue Int* 2003;72:537-547.

94. IHCWORLD Life Science Information Network. Von Kossa Staining Protocol. http://www.ihcworld.com/_protocols/special_stains/von_kossa.htm, 2011.

95. IHCWORLD Life Science Information Network. Alizarin Red Staining Protocol. http://www.ihcworld.com/_protocols/special_stains/alizarin_red_s.htm, 2011.

96. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell based therapies. *Tissue Eng* 2001;7:211-228.

97. Granero-Molto F, Weis J, Miga M, et al. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells* 2009;27:1887-1898.

98. Toupadakis CA, Wong A, Genetos DC, et al. 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* 2010;71:1237-1245.

99. Kern S, Eichler H, Stoeve J, et al. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;24:1294-1301.

100. Wu X, Lin M, Li Y, et al. Effects of DMEM and RPMI 1640 on the biological behaviour of dog periosteum-derived cells. *Cytotechnol* 2009;59:103-111.

101. Baptista L, do Amaral R, Carias R, et al. An alternative method for the isolation of mesenchymal stromal cells derived from lipoaspirate samples. *Cytotherapy* 2009;11:706-715.

102. Ichinose S, Tagami M, Muneta T, et al. Morphological examination during *in vitro* cartilage formation by human mesenchymal stem cells. *Cell Tissue Res* 2005;322:217-226.

103. Sekiya I, Larson BL, Vuoristo JT, et al. Comparison of effect of BMP-2,-4, and -6 on *in vitro* cartilage formation of human adult stem cells from bone marrow stroma. *Cell Tissue Res* 2005;320:269-276.

104. Mastrogiacomo M, Derubeis AR, Cancedda R. Bone and cartilage formation by skeletal muscle derived cells. *J Cell Physiol* 2005;204:594-603.

105. Tuli R, Tuli S, Nandi S, et al. Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone. *Stem Cells* 2003;21:681-693.

106. Stewart AA, Byron CR, Pondenis HC, et al. Effect of dexamethasone supplementation on chondrogenesis of equine mesenchymal stem cells. *Am J Vet Res* 2008;69:1013-1021.

107. Zhang L, Su P, Xu C, et al. Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. *Biotechnol Lett* 2010;32:1339-1346.

108. Tsutsumi S, Shimazu A, Miyazaki K, et al. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun* 2001;288:413-419.

109. Berg LC, Koch TG, Heerkens T, et al. Chondrogenic potential of mesenchymal stromal cells derived from equine bone marrow and umbilical cord. *Vet Comp Orthop Traumatol* 2009;22:363-370.

110. Eslaminejad MB, Taghiyar L. Study of the structure of canine mesenchymal stem cell osteogenic culture. *Anat Histol Embryol* 2010;39:446-455.

111. Giovannini S, Brehm W, Mainil-Varlet P, et al. Multilineage differentiation potential of equine blood-derived fibroblast-like cells. *Differentiation* 2008;76:118-129.

112. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 2009;284:143-148.

113. Weir C, Morel-Kopp M, Gill A, et al. Mesenchymal stem cells: isolation, characterisation and *in vivo* fluorescent dye tracking. *Heart Lung Circ* 2008;17:395-403.

114. Erickson GR, Gimble JM, Franklin DM, et al. Chondrogenic potential of adipose tissue-derived stromal cells *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 2002;290:763-769.

Chapter 6: Proliferation Potential

6.1 Introduction

Mesenchymal stem cells have been targeted as a novel resource in the field of tissue engineering and regenerative medicine. This stems from their relatively easy isolation, proliferative capacity, pluripotency and paracrine effects. However, the ideal cell number required and ideal tissue source, with respect to proliferative capacity, is not clearly defined. This knowledge would optimize cell based therapies and harvesting methodologies.[1]

Currently, high cell numbers are being utilized in therapeutic trials and experiments. Some studies report using as much as 10^6 to 10^9 MSCs.[2-4] However, contradictory reports regarding this requirement exist in the literature; thus making firm guidelines difficult to establish. One group demonstrated a dose dependent therapeutic effect when administering MSCs in a brain injury model; administration of 3×10^6 cells provided a greater benefit than 1×10^6 cells.[3] This was in contrast to another brain injury model in which higher doses of MSCs failed to provide any additional advantage.[5] In Black, et al.'s[6] study, clinical improvements were seen despite using non-culture expanded AMSCs. But on the other hand, formation of one cubic centimetre of bone is reported to require 70 million progenitor cells.[7] The frequency of MSCs is low in bone marrow (1 human BMSCs per 10^4 to 10^5 bone marrow mononuclear cells)[2] and not as low in adipose tissue (1 human AMSC per 30 stromal vascular cells).[8] However, to obtain numbers in the order of 10^9 MSCs, culture expansion is required.

Factors shown to affect MSC proliferative potential include: donor age and body mass index,[9] species strain,[10] tissue source and location,[11-13] isolation method,[14] oxygen

tension,[15]cell seeding density,[7] serum source and lot number,[4,16] use of growth factors like basic fibroblast growth factor and heparin-binding epidermal growth factor,[17] and cryopreservation.[4]

In humans, reduced proliferation potential has been observed in BMSCs obtained from older patients[9,18] and AMSCs from female patients with a greater body mass index.[19] Some studies have demonstrated that hyperoxic conditions (21%) result in poorer proliferation compared to hypoxic conditions (2-5%), whereas others report the opposite finding.[9,15] Grayson and colleagues[15] speculated that the hypoxic conditions resulted in greater cell yields because of sustaining MSC growth in later passages rather than increasing their growth rate. Greater proliferation has been documented in MSCs seeded at a lower density.[4] For instance, a 74 fold increase in expansion was seen in canine BMSCs seeded at 10 cells/cm^2 and only a 48 fold increase when seeded at 1000 cells/cm^2.[20] This has proven true for human and rat MSCs.[10] Literature describing the superiority of either FCS or autologous serum is not consistent.[16] Sotiropoulou and colleagues[4] even showed that the type of plastic flasks used to culture MSCs can significantly affect the growth kinetics of MSCs. A yield of 553.07 ± 26.26 cells/10^6 bone marrow mononuclear cells were obtained in Falcon flasks compared to 178.06 ± 24.90 to 287.04 ± 10.31 cells/10^6 bone marrow mononuclear cells in Greiner, Nunc, and Costar flasks ($p<0.01$). Although the flasks were made of the same material, the effect was thought to be from differences in manufacturing. The addition of fibroblast growth factor-2 to human BMSCs resulted in a significantly greater MSC yield without significantly affecting their immunomodulation properties.[21]

Colony forming unit-fibroblast (CFU-F) assay and the cumulative population doubling time are the most common methods reported to evaluate MSC proliferation potential.[2,4,13,22-24] With the CFU assay, MSCs are plated at a low density and cultured for a period of time (for example, ten to fourteen days).[25] The dishes are then stained with 0.5% Crystal violet, and the number of colonies present, with a predetermined minimum number of MSCs (ie.>20)[1] or minimal diameter (>2mm),[4] are counted. The number reported is typically number of colonies per number of cells seeded. Mesenchymal stem cell expansion potential can be predicted by the CFU assay.[25]

With the population doubling time, cells are seeded and counted with a haemocytometer at designated times, and the numbers are entered into a formula[1] and statistically analyzed:

$CD = \ln (N_f/N_i) / \ln(2)$

$DT = CT/CD$

CD: cell doubling number
N_f: final number of cells counted
N_i: initial number of cells seeded
DT: cell doubling time
CT: culture time

To get the cumulative population doubling time (level), the population doubling from each passage is added to the cell doubling number of the previous passages.[23]

As initially stated, two of the objectives of this study were to determine which of the four donor tissues has the greatest proliferation potential and to determine the MSC yield/gram of donor tissue after cells are grown to confluency in passage one. This information could potentially define a superior tissue source for clinical applications and provide specific guidelines for tissue collection requirements.

6.2 Methods

Initial Cell Seeding in Passage 0

The initial cell seeding densities of MSCs in passage zero (P0) were recorded for adipose tissue, muscle, and periosteum from six dogs. This was evaluated because cell seeding density has been identified as a factor affecting proliferation.[7]

Proliferation Assay

Passage one (P1) MSCs derived from bone marrow, adipose tissue, muscle, and periosteum from seven dogs were cultured at a seeding density of 3,100 cells/cm^2 in six well dishes containing SM. The cells were plated in triplicate for 24, 48, 72, and 96 hours. At each time point, the cells were washed with PBS and trypsinized with 0.05% trypsin (Invitrogen, Toronto, ON) for 30 minutes. The reaction was stopped with adding SM. Viable cells, as determined with the use of 0.04% Trypan blue, were counted using a haemocytometer. The culture medium was changed every two days.

Mesenchymal Stem Cell Yield/Gram of Donor Tissue

Data was collected from seven dogs to determine the mean MSC yield obtained per gram of tissue of bone marrow, adipose tissue, muscle, and periosteum after the cells were grown to 80-100% confluency in passage one. The volume of bone marrow and the weight of the three other tissue sources was recorded during the isolation and culture procedures. However, in order to statistically compare all four donor tissue sources the volume of bone marrow required conversion to a unit of weight. We justified a 1:1 (g:mL) conversion factor in a three step process. First, a thorough search of the literature did not identify the mass density of canine bone marrow. However, it is

reported that human bone marrow has a mass density of 0.98-1.03 g/mL.[26] Our laboratory also confirmed that equine bone marrow has a mass density of 1 g/mL and that canine blood has a mass density of 1 g/ml (unpublished data). Although not ideal, if we believe that canine bone marrow has a similar composition to canine blood and that canine bone marrow may have a similar mass density to human and equine bone marrow, we can consider the conversion factor to be a reasonable assumption. Thus from this point forward, any reference to the quantity of bone marrow will be reported in grams.

Statistical Analysis

The significant differences among the average of the natural logarithm of the initial cell seeding densities of AMSCs, MMSCs, and PMSCs in P0 were determined by one way ANOVA. The significant differences among the average of the natural logarithm from each triplicate MSC count between the four tissue-derived MSCs were determined by a linear mixed model.[27] The tissue and time effects were considered to be fixed effects. The contribution of each dog to the log transformed MSC count was considered a random effect. The significant differences among the square root of MSC yield per gram of tissue were determined by general linear model. Bonferroni procedure was used to adjust for multiple tissue comparisons.[28] Statistical analyses were performed using STATA 10.0 (StataCorp, College Station, TX) and Minitab 16 (Minitab Inc, State College, PA). Statistical significance was set at $P < 0.05$.

6.3 Results

Initial Cell Seeding in Passage 0

The initial cell seeding densities of AMSCs, MMSCs, and PMSCs in P0, including their standard deviation, were noted in Chapter 4. The mean and the standard deviation (mean ± SD) of the natural logarithm of the initial cell seeding density of AMSCs is 14.24 ± 0.41, MMSCs is 14.76 ± 0.33, and 14.45 ± 0.77 for PMSCs. Using one way ANOVA, the results indicate that no statistically significant difference exists for the initial MSC seeding numbers in P0 between these three donor tissue sources ($p=0.28$).

Proliferation Assay

The mean ± SD of the log transformed MSC counts for BMSCs, AMSCs, MMSCs, and PMSCs at each time point (24, 48, 72, and 96 hours) of the proliferation assay is presented in Table 2. The mean of the log transformed MSC counts was higher for MMSCs than for BMSCs, AMSCs, and PMSCs at each of the time points. The standard deviation of the log transformed MSC counts was higher for BMSCs than for AMSCs, MMSCs, and PMSCs at all time points. The standard deviation of the log transformed MSC counts was the lowest for AMSCs at 48, 72, and 96 hours. The mean of the log transformed MSC counts for BMSCs, AMSCs, MMSCs, and PMSCs increased at each time point.

Time had a significant effect on the mean of the log transformed MSC counts for BMSCs, AMSCs, MMSCs, and PMSCs ($P=0.00$). There was no statistically significant difference between the four donor tissue-derived MSCs on the mean of the log transformed MSC counts ($P=0.36$).

Table 2. Mean ± SD of log transformed MSC counts obtained from bone marrow, adipose tissue, muscle, and periosteum derived MSCs at 24, 48, 72, and 96 hours of proliferation assay one.

	Mean ± SD of Log Transformed Mesenchymal Stem Cell Counts at each Time Point (hours)			
Tissue	24	48	72	96
Bone Marrow	2.69 ± 0.63	3.58 ± 1.02	3.82 ± 0.92	4.69 ± 1.09
Adipose Tissue	2.76 ± 0.50	3.15 ± 0.65	4.17 ± 0.35	4.76 ± 0.33
Muscle	3.22 ± 0.45	3.60 ± 0.83	4.63 ± 0.62	5.30 ± 0.71
Periosteum	2.73 ± 0.59	3.22 ± 0.69	4.31 ± 0.64	4.90 ± 0.48

Mesenchymal Stem Cell Yield/Gram of Tissue

The mean ± SD of the MSC yield/gram of bone marrow, adipose tissue, muscle, and periosteum and the days for these cells to reach 80-100% confluency is presented in Table 3. The mean of the square root of the MSC yield/gram of tissue for each of the tissues is presented in Figure 10. The data shows that periosteum had the highest MSC yield/gram of tissue and bone marrow had the lowest MSC yield. BMSC counts had the highest standard deviation in the mean number of days taken to reach 80-100% confluency in passage one, whereas MMSC counts had the lowest standard deviation.

The effect of time for the cells to reach 80-100% confluency in P1 was not significantly different between BMSCs, AMSCs, MMSCs, and PMSCs ($P= 0.740$). There was also no statistical difference seen for the dog effect ($P=0.443$). There was a significant difference between the mean of the square root of MSC yield/gram of bone marrow, adipose tissue, muscle, and periosteum tissues ($P<0.001$). PMSCs provided the highest mean of the square root of the MSC yield/gram of periosteum in comparison to

the mean of the square root of the MSC yield/gram of bone marrow, adipose tissue, and muscle.

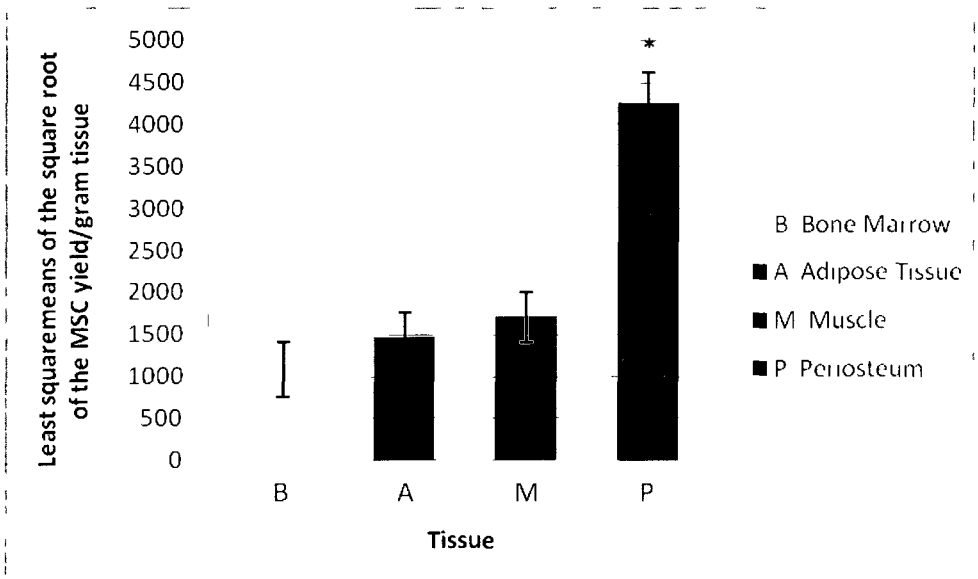

Figure 10. Comparison of the mean of the square root of the MSC yield/gram of tissue for BMSCs, AMSCs, MMSCs, and PMSCs after reaching 80-100% confluency in passage one. Periosteum-derived MSCs demonstrated a significantly higher mean of the square root of MSC yield/gram of tissue when compared to the other three tissue sources. * signifies statistical significance at $P < 0.05$.

Table 3. Mean ± SD of the mesenchymal stem cell yield/gram of tissue from each of the four donor tissue sources and mean ± SD days taken for those cells to reach 80-100% confluency in passage one of proliferation assay two.

Tissue Source	Mean ± SD of Mesenchymal Stem Cell Yield/gram of tissue	Mean ± SD Days to reach 80-100% Confluency
Bone Marrow	1,449,788 ± 1,198,602	16.17 ± 4.17
Adipose Tissue	2,334,463 ± 1,253,126	13.86 ± 2.04
Muscle	3,367,969 ± 2,088,825	15.00 ± 0.58
Periosteum	19,400,000 ± 12,800,000	13.2 ± 1.64

6.4 Discussion

The proliferation potential of canine MSCs derived from bone marrow, adipose tissue, muscle, and periosteum was evaluated using a proliferation assay and determining the MSC yield/gram of donor tissue for each of these tissue sources. We found that there was no significant difference in the proliferation potential of BMSCs, AMSCs, MMSCs, and PMSCs when cultured over a four day period in the proliferation assay. However, when evaluating MSC yield/gram of donor tissue, periosteum was a superior tissue source and provided MSCs with the greatest proliferative potential in comparison to bone marrow, adipose tissue, and muscle. A significantly greater number of PMSCs, compared to BMSCs, AMSCs, and MMSCs, could be obtained per gram of donor tissue when allowed to grow to confluency in P1.

The effect of time on increasing cell numbers in culture was an expected finding in both the proliferation assay and the evaluation of MSC yield/gram of tissue; it is a reflection of the inherent proliferative capacity of MSCs *in vitro*.[12,29] It is reported that the cell seeding density within a passage significantly effects the proliferation potential of MSCs; typically, MSCs show greater proliferative capacity when cultured at lower seeding densities.[4,10] The initial cell seeding densities of MSCs in P0 were not the same for AMSCs, MMSCs, and PMSCs, and unfortunately were not evaluated for BMSCs. Although the numbers were not exactly the same, statistical analysis showed that the cell seeding densities of AMSCs, MMSCs, and PMSCs in P0 were not significantly different. However, the seeding densities for the proliferation assay were the same. It is interesting to note that Sotiropoulou and colleagues[4] demonstrated that the proliferative potential of BMSCs did not correlate with the initial plating density whereas the

passaging seeding density did. Peister, et al.[10] also reported that the plating density did not affect the CFU potential in the next passage. Therefore, it is possible that even if a significant difference existed in our initial cell seeding densities in P0, that it would not have correlated with a difference in MSC proliferative capacity as evaluated in the proliferation assay.

Comparisons among MSCs obtained from multiple tissue sources are limited in the literature, but reports for human and rat-derived MSCs exist. The proliferation assay findings in this canine MSC study were in agreement with a rat MSC study that showed similar proliferative potential between rat-derived AMSCs, MMSCs, and PMSCs up to 10 days in culture when plated at 100 and 500 cells/cm^2.[30] Rat SMSCs had the greatest proliferative potential and the highest cell yield when compared to MSCs derived from bone marrow, adipose tissue, muscle, and periosteum.[30] Rat PMSCs, MMSCs, and AMSCs had similar proliferative potential that was decreased in comparison to SMSCs, but higher in comparison to BMSCs. This is in contrast to our study that found no difference in proliferation potential between canine BMSCs, AMSCs, MMSCs, and PMSCs as assessed with the proliferation assay. It also differs in comparison to our study when we evaluate MSC yield/gram of donor tissue because PMSCs were shown to be superior in comparison to the other three donor tissue-derived MSCs in this respect. Human AMSCs and MMSCs had the lowest proliferative capacity in comparison to BMSCs, PMSCs, and SMSCs.[12] The authors stated that even though human AMSCs had the lowest proliferative capacity, the final cell yield in passage three provided 10^9 cells, and would suffice for current therapeutic and experimental requirements.

Mesenchymal stem cell yield/gram of tissue is also infrequently reported.[31] With a mean of sixteen days in culture, we could obtain a mean of 1.45 x 10^6 BMSCs, 2.33 x 10^6 AMSCs, 3.37 x 10^6 MMSCs, and 19.40 x 10^6 PMSCs per gram of bone marrow, adipose tissue, muscle, and periosteum, respectively. In another study, culture of canine AMSCs resulted in a yield of 0.53 x 10^6 AMSCs/g of tissue within five to six days.[13] Chung, et al.[9] obtained a yield of 4.2 x 10^5 AMSC/g of adipose tissue and 1.2 x 10^5 BMSCs/ml of canine bone marrow within six to eight days. Adipose-derived MSCs from rats, mice, and humans have shown superior proliferative potential when compared to BMSCs.[9,32] The differences in MSC yield is likely a factor of time in culture and passage number.[12] Other factors reported to influence cell yield include culture media and cell doubling times.[12,32]

Current therapeutic trials are utilizing high numbers of MSCs which require *in vitro* culture expansion. Even though MSCs can successfully proliferate up to forty population doublings,[33] concerns do exist with their expansion. Potential issues identified include loss of multipotential differentiation ability, senescence, genetic instability, and tumour formation.[24,34,35] Although these issues are not evaluated as part of this project, it is important to be aware of these concerns and address them in future studies.

6.5 References

1. Vidal MA, Kilroy GE, Lopez MI, et al. Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. *Vet Surg* 2007;36:613-622.

2. Deans RJ, Moseley AB. Mesenchymal stem cells: Biology and potential clinical uses. *Exp Hematol* 2000;28:875-884.

3. Bang O, Lee J, Lee P, et al. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol* 2005;57:874-882.

4. Sotiropoulou PA, Perez SA, Salagianni M, et al. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells* 2006;24:462-471.

5. Chanda D, Kumar S, Ponnazhagan S, et al. Therapeutic potential of adult bone marrow-derived mesenchymal stem cells in diseases of the skeleton. *J Cell Biochem* 2010;111:249-257.

6. Black LL, Gaynor J, Gahring D, et al. Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. *Vet Ther* 2007;8:272-284.

7. Vidal MA, Kilroy GE, Johnson JR, et al. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet Surg 2006;35:601-610.

8. Mitchell J, McIntosh K, Zvonic S, et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 2006;24:376-385.

9. Chung D, Hayashi K, Toupadakis C, et al. Osteogenic proliferation and differentiation of canine bone marrow and adipose tissue derived mesenchymal stromal cells and the influence of hypoxia. R*es Vet Sci* 2010, doi: 10.1016/j.rvsc.2010.10.012.

10. Peister A, Mellad JA, Larson BL, et al. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differential potential. *Blood* 2004;103:1662-1668.

11. Riekstina U, Cakstina I, Parfejevs V, et al. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart, and dermis. *Stem Cell Rev and Rep* 2009;5:378-386.

12. Sakaguchi Y, Sekiya I, Yagishita K, et al. Comparison of human stem cells derived from various mesenchymal tissues. *Arthritis Rheum* 2005;52:2521-2529.

13. Neupane M, Chia-Cheng C, Kiupel M, et al. Isolation and characterization of canine adipose-derived mesenchymal stem cells. *Tissue Eng Part A* 2008;14:1007-1015.

14. Bourzac C, Smith L, Vincent P, et al. Isolation of equine bone marrow-derived mesenchymal stem cells: a comparison between three protocols. *Equine Vet J* 2010;42:519-527.

15. Grayson WL, Zhao F, Bunnell B, et al. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2007;358:948-953.

16. Nimura A, Muneta T, Koga H, et al. Increased proliferation of human synovial mesenchymal stem cells with autologous human serum. *Arthritis Rheum* 2008;58:501-510.

17. Hashimoto J, Kariya Y, Miyazaki K. Regulation of proliferation and chondrogenic differentiation of human mesenchymal stem cells by laminin-5 (laminin-332). *Stem Cells* 2006;24:2346-2354.

18. Yu JM, Wu X, Gimble JM, et al. Age-related changes in mesenchymal stem cells derived from rhesus macaque bone marrow. *Aging Cell* 2011;10:66-79.

19. Aust L, Devin B, Foster SJ, et al. Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* 2004;6:7-14.

20. Kamashina H, Farese J, Storm J, et al. The frequency, growth kinetics, and osteogenic/adipogenic differentiation properties of canine bone marrow stromal cells. *In vitro Cell Dev Biol Anim* 2008;44:472-479.

21. Auletta JJ, Zale EA, Welter JF, et al. Fibroblast growth factor-2 enhances expansion of human bone marrow-derived mesenchymal stromal cells without diminishing their immunosuppressive potential. *Stem Cells Int* 2011; doi:10.4061/2011/235176.

22. Russell KC, Phinney DG, Lacey MR, et al. *In vitro* high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells* 2010;28:788-798.

23. Kern S, Eichler H, Stoeve J, et al. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;24:1294-1301.

24. Moussavi-Harami F, Duwayri Y, Martin JA, et al. Oxygen effects on senescence in chondrocytes and mesenchymal stem cells: consequences for tissue engineering. *Iowa Orthop J* 2004;24:15-20.

25. DiGirolamo CM, Stokes D, Colter D, et al. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* 1999;107:275-281.

26. ICRU (1992). International Commission on Radiation Units and Measurements. *Photon, electron, proton, and neutron interaction data for body tissues.*

ICRU Report 46. Maryland: International Commission on Radiation Units and Measurements, 1992, 13.

27. Dohoo I, Martin W, Stryhn H. Chapter Twenty One: Mixed models for continuous data. In: Dohoo IR., Martin W, Stryhn H. *Veterinary Epidemiologic Research*. 2nd ed. Charlottetown: VER Inc./Friesens, 2009, 553-565.

28. Christensen R. Chapter Six. In: Ronald Christensen. *Analysis of Variance, Design and Regression*. 1st ed. New York: Chapman & Hall / CRC Press, 2006, 152-155.

29. Vidal MA, Robinson SO, Lopez MJ, et al. Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow. *Vet Surg* 2008;37:713-724.

30. Yoshimura H, Muneta T, Nimura A, et al. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* 2007;327:449-462.

31. Schugar RC, Chirieleison SM, Wescoe KE, et al. High harvest yield, high expansion, and phenotype stability of CD146 mesenchymal stromal cells from whole primitive human umbilical cord tissue. *J Biomed Biotechnol* 2009:1-11.

32. Toupadakis CA, Wong A, Genetos DC, et al. 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* 2010;71:1237-1245.

33. Salem H, Theimermann C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 2010;28:585-596.

34. Chagastelles PC, Beyer Nardi N, Camassola M. Biology and applications of mesenchymal stem cells. *Sci Prog* 2010;93:113-127.

35. Herberts CA, Kwa MS, Hermsen HP. Risk factors in the development of stem cell therapy. *J Transl Med* 2011;22:29-43.

Chapter 7: Conclusion

7.1. Conclusion

An ideal tissue source for use in regenerative therapies could be defined as a tissue that is in abundance, can be harvested with minimal invasiveness and morbidity to the patient, is economically advantageous, and one that provides a high number of effective MSCs within a short period of time. Clinically, obtaining muscle and periosteum from the dog would be more invasive in comparison to adipose tissue and bone marrow, but if a niche demanded their availability, post mortem collection and storage in a bank for allogenic purposes may be a possibility. The benefits of allogenic MSCs are still controversial,[1,2] but studies exist demonstrating the positive effects in models of canine myocardial infarction[3] and spinal cord injury.[2] Allogenic MSCs are an appealing option because they have the potential to avoid host immune rejection,[4] have immunosuppressive capabilities,[5] and can be obtained readily in large numbers without waiting the necessary time required for culture expansion of autogenous MSCs.

In conclusion, this study has shown that canine skeletal muscle and periosteum are sources of mesenchymal stem cells. Periosteum is a superior tissue source in providing the highest MSC yield/gram of tissue within a clinically relevant time period. We were able to successfully demonstrate the isolation, characterization, and proliferative capacity of MSCs from canine bone marrow, adipose tissue, muscle, and periosteum. This study contributes to the basic understanding of canine MSCs, which ideally should be sought prior to their clinical application in veterinary regenerative therapies and tissue engineering. Their current use may be better applied if we understand the tissue sources available, the ideal tissue source, cell transplantation

number, mode of administration, mechanisms of action, and long term safety and efficacy. We also need to be aware that information gathered from other species is important, but may not be applicable to companion animals at all levels.

7.2 References

1. Eliopoulous N, Stagg J, Lejeune L. Allogenic marrow stromal cells are immune rejected by MHC Class I- and Class II- mismatched recipient mice. *Blood* 2005;106:4057-4065.

2. Jung DI, Ha J, Kang B, et al. A comparison of autologous and allogenic bone marrow-derived mesenchymal stem cell transplantation in canine spinal cord injury. *J Neurol Sci* 2009;285:67-77.

3. Vela DC, Silva GV, Assad JA, et al. Histopathological study of healing after allogenic mesenchymal stem cell delivery in myocardial infarction in dogs. *J Histochem Cytochem* 2009;57:167-176.

4. Fortier L. Stem cells: classifications, controversies, and clinical applications. *Vet Surg* 2005;34:415-423.

5. Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy* 2003;5:485-489.

APPENDIX: RAW DATA

Proliferation Assay

Dog	Passage	Tissue	24h1	24h2	24h3	48h1	48h2	48h3	72h1	72h2	72h3	96h1	96h2	96h3
4	1	1	10	10	5	15	5	5	25	20	10	35	55	35
5	1	1	na	na	na	na	na	na	na	na	na	na	na	na
6	1	1	30	15	20	125	115	95	na	na	na	470	675	690
7	1	1	5	15	10	120	165	165	295	120	310	570	575	455
8	1	1	10	15	15	30	25	35	35	55	40	80	190	70
9	1	1	10	5	5	10	10	10	15	15	15	30	35	30
10	1	1	5	5	45	20	35	15	15	20	30	30	40	30
4	1	2	30	35	20	110	55	50	110	95	75	115	125	85
5	1	2	10	10	5	45	45	40	90	80	65	150	170	75
6	1	2	35	20	30	55	25	25	55	80	70	90	100	145
7	1	2	5	20	45	15	15	15	105	20	50	150	45	120
8	1	2	5	10	10	10	15	5	25	35	40	80	80	255
9	1	2	20	15	10	25	45	15	90	85	165	235	285	325
10	1	2	5	5	25	35	30	10	110	65	70	120	255	110
4	1	3	20	35	15	45	60	30	115	85	50	150	100	85
5	1	3	15	15	25	50	45	25	120	115	85	275	260	105
6	1	3	35	30	25	65	20	55	65	70	80	225	275	140
7	1	3	15	20	45	15	15	15	100	90	60	90	185	155
8	1	3	0	0	15	5	10	10	35	40	35	75	50	105
9	1	3	55	40	45	140	70	85	315	285	265	880	805	695
10	1	3	20	15	5	75	25	40	235	425	150	440	360	675
4	1	4	15	20	15	40	35	30	145	100	95	190	185	150
5	1	4	20	25	10	15	25	10	85	90	105	265	120	135

Dog	Passage	Tissue	24h1	24h2	24h3	48h1	48h2	48h3	72h1	72h2	72h3	96h1	96h2	96h3
6	1	4	10	0	10	60	15	25	170	70	140	60	165	270
7	1	4	15	30	20	40	15	25	80	45	80	130	80	115
8	1	4	0	5	5	5	10	5	15	15	20	45	45	55
9	1	4	na	na	na	na	na	na	na	na	na	na	na	na
10	1	4	na	na	na	na	na	na	na	na	na	na	na	na

APPENDIX: RAW DATA

Mesenchymal Stem Cell Yield/Gram of Tissue and Time to Confluency

Dog	Tissue	Weight (g)	# cells P0	# cells/g tissue P0	P0 days	% Confluency P0	# cells P1	P1 days	% Confluency P1	# cells/g tissue P1
4	BM	5	200 000	40 000	11	20	7 250 000	17	95	1 450 000
4	Fat	9.49	5 250 000	553 213	7	90	11 125 000	11	95	1 172 286
4	Muscle	8.09	950 000	117 428	8	50	9 625 000	15	100	1 189 740
4	Periosteum	3.48	28 875 000 used 10 000 000 for P1	8 297 413	7	100	32 750 000	11	100	9 410 919
5	BM	5	0				0			
5	Fat	5	9 750 000	1 950 000	8		18 000 000	15	100	3 600 000
5	Muscle	4.3	12 000 000	2 790 697	8		4 750 000	15	80	174 418
5	Periosteum	0.93	6 750 000	7 258 064	8		22 500 000	15	100	24 193 548
6	BM	5	2 700 000	540 000	8	90	15 500 000	11	95	3 100 000
6	Fat	8.87	5 000 000 used 2 500 000 for P1	563 697	6	95	4 300 000	11	95	484 780
6	Muscle	4.33	750 000	173 210	8	45	15 880 000	15	100	3 667 436
6	Periosteum	2.16	12 125 000 used 7 500 000 for P1	5 613 425	8	100	25 250 000	12	100	11 689814

Dog	Tissue	Weight (g)	# cells P0	# cells/g tissue P0	P0 days	% Confluency P0	# cells P1	P1 days	% Confluency P1	# cells/g tissue P1
7	BM	5	1 950 000	390 000	8	90	13 100 000	11	100	2 620 000
7	Fat	5.79	7 750 000	1 338 514	9	95	12 875 000	16	100	2 223 661
7	Muscle	4.6	11 500 000 used 6 900 000 for P1	2 500 000	9	75	25 375 000	16	100	5 516 304
7	Periosteum	0.5	4 425 000	8 850 000	9	85	19 880 000	14	100	39 760 000
8	BM	12.5					3 200 000	18	80	256 000
8	Fat	2.75					7 500 000	14	100	2 727 272
8	Muscle	2.81					15 250 000	14	100	5 427 046
8	Periosteum	1.14					13 750 000	14	100	12 061 403
9	BM	2					2 100 000	20	100	1 050 000
9	Fat	2.6	5 500 000	2 115 384	8		5 500 000	15	80	2 115 384
9	Muscle	3.4	4 000 000	1 176 470	8		16 250 000	15	80	4 779 411
9	Periosteum	1.03	na				na			
10	BM	27.5	1 800 000	65 454	11		6 125 000	20	100	222 727
10	Fat	2.8	3 500 000	1 250 000	8		11 250 000	15	80	4 017 857
10	Muscle	3.5	2 250 000	642 857	8		9 875 000	15	80	2 821 428
10	Periosteum	0.81	na				na			