Exploration of Canine Mesenchymal Stromal Cells and Cell Reprogramming for Future Immunotherapy and Cartilage Repair

by

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ABSTRACT

EXPLORATION OF CANINE MESENCHYMAL STROMAL CELLS AND CELL REPROGRAMMING FOR FUTURE IMMUNOTHERAPY AND CARTILAGE REPAIR

Keith Andrew Russell Advisor:
University of Guelph, 2017 Thomas G. Koch

There are an estimated 6.4 million dogs living in 34% of Canadian households. Dogs suffer from many conditions where only poor to no treatments exist. For example, cartilage injury, osteoarthritis, and other inflammatory disorders have been inadequately remedied by traditional medical care. Increasing effort has been put toward the innovation and optimization of cell-based therapies for tissue regeneration and immune modulation. Approaches include the use of mesenchymal stromal cells (MSC), induced pluripotent stem cells (iPSC), and other cell engineering techniques such as gene over-expression in somatic cells.

The hypothesis was that canine cells with immunomodulatory and chondrogenic properties can be isolated from adipose tissue (AT), bone marrow (BM), or articular cartilage (AC) and utilized to generate canine iPSC. The objectives were: 1) to assess the immunomodulatory and chondrogenic properties of canine AT- and BM-MSC and 2) to generate iPSC from canine MSC or chondrocytes and verify their pluripotency and chondrogenesis.

Canine MSC were derived from AT and BM. Isolation success and proliferation rates were compared when using two different culture supplements: fetal bovine serum (FBS) and platelet lysate (PL). Despite the fact that PL is slowly displacing use of the increasingly expensive and xenogeneic FBS in human MSC culture, PL proved detrimental to canine
MSC. It did not support isolation or long-term culture of MSC and showed evidence of inducing spontaneous adipogenesis. MSC source was also investigated. Few differences were found pertaining to differentiation and immunomodulation. Both MSC populations have good adipogenic and osteogenic, but poor chondrogenic potential, and have proficient lymphocyte suppressive properties. What distinguished AT- from BM-derived MSC were higher proliferation rates.

Since MSC were found to be poorly chondrogenic, both canine MSC and chondrocytes were used to generate iPSC. Some signs of reprogramming were apparent. However, the conditions needed to stabilize reprogrammed cells were not yet identified.

In conclusion, AT is the recommended source of canine MSC for immunotherapy. A practical source of chondrogenic cells for canine cartilage engineering strategies has not yet been established, but this work is a promising starting point for further study.
Declaration of Work Performed

I declare that the work that went into this thesis is wholly my own with the exceptions listed below.

Canine (and equine) tissue samples were collected with the help of Dr. Thomas Koch, Dr. Thomas Gibson, and Dr. Lynn Williams. Cell culture establishment, maintenance, and differentiation studies were assisted at different times over the years by Carmon Co, Andrew Chong, Crystal Tse, Amir Haji Alizadeh, Ritesh Briah, and Dr. Sarah Lepage. Natalie Chow helped with immunophenotyping. David Dukoff was primarily responsible for RNA isolation. Dr. Abrams-Ogg and the Small Animal Clinic at the Ontario Veterinary College donated canine platelet concentrate. Complete blood counts were run through the Animal Health Laboratory, University of Guelph. Jing Zhang performed real-time PCR. Laura Furness irradiated cells for immunomodulatory studies. Statistical analysis was aided by William Sears and Gabrielle Monteith. MSC reprogramming was performed by Courtney Brooks in the lab of Dr. Dean Betts. Chondrocyte reprogramming studies were performed under the guidance of Maria Mileikovskaia in the lab of Dr. Andras Nagy.
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I would like to thank the many lab mates and colleagues I have had the pleasure to know over the years in Guelph, Toronto, and London.

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2i Two small molecule inhibitors of mitogen-activated protein kinase kinase and glycogen synthase kinase 3β
5-mC 5-Methylcytosine
ABAM Antibiotic antimycotic solution
ALP Alkaline phosphatase
AT Adipose tissue
AT-MSC Adipose tissue-derived mesenchymal stromal cells
BM Bone marrow
BM-MSC Bone marrow-derived mesenchymal stromal cells
BMP Bone morphogenetic protein
cAC Cells derived from canine articular cartilage
CB Cord blood
CB-MSC Cord blood-derived mesenchymal stromal cells
CD Cluster of differentiation
CEBPA CCAAT/enhancer-binding protein alpha
CFU-F Colony forming unit(s)-fibroblast
COL Collagen
COMP Cartilage oligomeric matrix protein
DMEM Dulbecco’s Modified Eagle Medium
EdU 5-Ethynyl-2’-deoxyuridine
EGF Epidermal growth factor
EM Expansion medium
EpiSC Epiblast-derived stem cells
ERK Extracellular signal-regulated kinase
ESC  Embryonic stem cell
FABP  Fatty acid binding protein
FGF  Fibroblast growth factor
FBS  Fetal bovine serum
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GSK  Glycogen synthase kinase
HLA  Human leukocyte antigen
HGF  Hepatocyte growth factor
hTERT  Human telomerase reverse transcriptase
ICM  Inner cell mass
IDO  Indoleamine 2,3-dioxygenase
IFN  Interferon
iNOS  Inducible nitric oxide synthase
IL  Interleukin
iPSC  Induced pluripotent stem cells
ISCT  International Society for Cellular Therapy
LIF  Leukemia inhibitory factor
LPL  Lipoprotein lipase
MEF  Mouse embryonic fibroblast
MHC  Major histocompatibility complex
MSC  Mesenchymal stromal cell
OPN  Osteopontin
OSKM  Oct4-Sox2-Klf4-c-Myc iPSC reprogramming factors
OSN  Oct4-Sox2-Nanog transcriptional network
P  Passage
PBMC  Peripheral blood mononuclear cells
PBS  Phosphate buffered saline
PC  Platelet concentrate
PDGF  Platelet-derived growth factor
PGE2  Prostaglandin E2
PL  Platelet lysate
PPAR  Peroxisome proliferator-activated receptor
PPP  Platelet-poor plasma
PRP  Platelet-rich plasma
RUNX  Runt-related transcription factor
SF  Serum-free
SOX  Sry-related HMG box
SSEA  Stage-specific embryonic antigen
STAT  Signal transducer and activator of transcription
TERT  Telomerase reverse transcriptase
TGF  Transforming growth factor
TNF  Tumour necrosis factor
VEGF  Vascular endothelial growth factor
WBC  White blood cells
Preface

This thesis is presented in chapter format with chapters 2 and 3 taken directly from published manuscripts (Russell et al. 2015, 2016). In an effort to avoid unnecessary repetition, I have removed or shortened the abstracts and introductions from these manuscripts. What has been omitted has been adapted and incorporated into the thesis’ introductory chapter. Acknowledgements and references have also been moved to their appropriate sections. The remaining text of each manuscript has been unchanged from the published versions. Figures from these articles have been adapted to better suit the thesis format and 2 figures have been added to the method sections of chapters 2 and 3 to more clearly illustrate the key methods used. Appendix 1 contains an original manuscript in its entirety save for the acknowledgements and references (Russell and Koch 2015).
Chapter 1

Introduction
There are an estimated 6.4 million dogs living in 34% of Canadian households according to a 2014 Canadian Animal Health Institute survey (http://www.cahi-icsa.ca/companion-animal-health/). Dogs suffer from many conditions where only poor to no treatments exist. For example, it has been reported that as much as 20% of dogs one year or older suffer from joint cartilage injury or disease (Johnston 1997). Joint cartilage defects are a significant source of pain in dogs and can lead to chronic osteoarthritis if left untreated. Similarly, other inflammatory disorders have been inadequately remedied by traditional medical care. Over the last 15 years, increasing effort has been put toward the innovation and optimization of cell-based therapies for tissue regeneration and immune modulation. Approaches include the use of mesenchymal stromal cells (MSC), induced pluripotent stem cells (iPSC), and other cell engineering techniques such as gene over-expression in somatic cells. The purpose of this project was to provide a basis to develop novel cell-based therapies that will allow improved animal welfare through new effective treatments for currently hard-to-treat or incurable conditions in dogs such as osteoarthritis.

The hypothesis was that canine cells with immunomodulatory and/or chondrogenic properties can be isolated from adipose tissue (AT), bone marrow (BM) aspirates, or articular cartilage (AC) and utilized to generate canine iPSC. The objectives were: 1) to assess the immune-modulatory and chondrogenic properties of MSC cultures derived from canine AT and BM aspirates and 2) to generate canine iPSC from AT- and BM-derived MSCs or chondrocytes and to verify and compare their pluripotency and chondrogenesis.

Canine MSC were derived from the two most popular sources, AT and BM aspirate. Isolation success and proliferation rates were compared when using two different culture supplements: fetal bovine serum (FBS) and platelet lysate (PL). Despite the fact that PL is slowly displacing use of the increasingly expensive and xenogeneic FBS in human MSC culture, canine PL proved detrimental to canine MSC. It did not support isolation or long-
term culture of MSC and showed evidence of spontaneously differentiating the cells along the adipogenic lineage.

Few differences were found between the MSC sources pertaining to adipogenesis, osteogenesis, chondrogenesis, and immunomodulatory qualities. Both MSC populations have good adipogenic and osteogenic potential, but poor chondrogenic potential. Both MSC populations have proficient lymphocyte suppressive properties. What distinguished AT- from BM-derived MSC were their higher rates of cell growth and the option for a less invasive procurement procedure.

Since MSC do not easily become cartilage-producing chondrocytes, it was decided to also explore chondrocytes themselves as a starting cell for tissue regeneration therapies. Historically, chondrocyte use has been hampered by their low numbers within cartilage that required the cell proliferation in a laboratory prior to their clinical use. Culture expansion unfortunately alters the cells so they no longer bear a primary chondrocyte phenotype. Recent advances in the area of molecular and genetic techniques can likely overcome these limitations. The purpose was to generate chondrocytes that can be cryopreserved for long-term storage and provide an unlimited source of cells for effective cartilage repair therapies in the dog.

Both canine MSC and chondrocytes were reprogrammed using a cocktail of transcription factors to generate iPSC. With future clinical application in mind, the non-integrative Sendai virus and the excisable piggyBac transposon systems were used to reprogram. Progress was made with signs of reprogramming taking place. However, the conditions needed to stabilize reprogrammed cells in the pluripotent state were not yet identified.

Canine cell therapies could one day find a central role in the effective treatment of immune and inflammatory disorders, as well as become the key approach in regenera-
tive medicine via tissue engineering. Beyond their potential utility in the veterinary clinic, study of their treatment of canine conditions could contribute substantially to translational medicine as well. Our aim to identify the best source for cellular therapies resulted in AT as the recommended source of canine MSC to be used as an immunomodulatory agent. A practical source of chondrogenic cells for canine cartilage engineering strategies has not yet been established, but this work is a promising starting point for further study.

1.1 Background

Stem cells

Stem cells have become a focus of increasing study over the past two decades because of the tantalizing promise they hold for regenerative medicine and other areas of medical and veterinary research such as disease modelling and drug screening. From the discovery of MSC multipotency and immunomodulatory properties, to the controversial research involving embryonic stem cells (ESC) and somatic cell cloning, to the breakthrough of iPSC, each has been regarded as having the potential to revolutionize medicine.

A stem cell is defined by two characteristics: self-renewal and cell potency. Self-renewal is achieved by the division of a stem cell to produce at least one daughter cell that maintains identical phenotype and potency to the original cell. Cell potency refers to the differentiation potential of the stem cell. There are four levels of potency: totipotency, pluripotency, multipotency, and unipotency. Totipotent stem cells are only found within the first couple of cell divisions and give rise to the embryo and extra-embryonic tissues. At the blastocyst stage of development, pluripotent cells from the inner cell mass (ICM) can develop into all three germ layers of the embryo. Multipotent cells refer to more restricted stem cells that retain the potential to become multiple cell types, and unipotent cells are
precursor cells to a single cell type (Jaenisch and Young 2008).

The mechanisms involved in maintaining pluripotency have been investigated ever since ESC were first derived from the ICM of mouse embryos in 1981 (Evans and Kaufman 1981; Martin 1981). It was soon discovered that the cytokine leukemia inhibitory factor (LIF) is essential to maintaining self-renewal and inhibiting differentiation in mouse ESC (Smith et al. 1988). LIF activates signal transducer and activator of transcription 3 (STAT3) signalling, which in turn regulates transcriptional targets that are still largely unknown (Niwa et al. 1998; Matsuda et al. 1999). Regulation of pluripotency and indefinite self-renewal potential also involves the Oct4-Sox2-Nanog (OSN) transcriptional network (Niwa et al. 2000; Rodda et al. 2005; Masui et al. 2007). Oct4, Sox2, and Nanog form an interrelated autoregulatory loop in which they assemble at the promoters of their own genes. Together, these transcription factors initiate expression of hundreds of genes associated with pluripotency while silencing those associated with differentiation through histone modification and DNA methylation (Muchkaeva et al. 2012).

Although Oct4, Sox2, and Nanog also serve as master regulators in human pluripotent cells, there are key species differences that exist. In vitro, human ESC appear more loosely packed and flattened than the tightly packed and domed appearance of mouse ESC. In this way, human ESC more closely resemble pluripotent stem cells derived from the epiblast of postimplantation mouse embryos (EpiSC) rather than the preimplantation mouse embryo (mESC) (Jaenisch and Young 2008). The EpiSC is a product of its developmental environment in that the epiblast becomes elongated after implantation and is exposed to different factors including fibroblast growth factor (FGF), which prime the cells for commitment to different lineages. Accordingly, these two separate stages of pluripotency have been termed primed for the EpiSC phenotype and naïve for the mouse ESC phenotype (Nichols and Smith 2009). It follows that human ESC are insensitive to LIF found abundant in
the ICM and are instead dependent on FGF to maintain pluripotency (Amit et al. 2000; Vallier et al. 2005). FGF works through a signalling cascade on the extracellular signal-regulated kinase (ERK) pathway to drive conversion from the naïve to primed state, but also to maintain pluripotency through stabilization of the primed state (Lanner and Rossant 2010). Conversely, using two small molecule kinase inhibitors (2i) of the ERK pathway and glycogen synthase kinase 3β (GSK3β) can not only serve to stabilize mouse ESC, but also to transition primed EpiSC and human ESC back to a naïve state (Silva and Smith 2008; Ying et al. 2008; Bao et al. 2009; Hanna et al. 2010; Gu et al. 2012; Ware et al. 2014). When FGF/ERK signalling is blocked or lacking, lineage commitment does not take place (Kunath et al. 2007).

Lineage commitment or cell differentiation is, ultimately, a transition in gene expression profile from one cell type to another with a corresponding loss of potency. The niche concept of stem cells dynamically responding to and being regulated by their specific physiological microenvironment may explain how this transition occurs. Differentiation can be induced by a change in the physical space and paracrine signalling (as in the pre to postimplantation example above) or by other interactions with endocrine signals, metabolites, or other niche components (Scadden 2006). It is one of the key roles of a stem cell biologist to mimic the stem cell niche in order to both propagate the cells and direct their cell fate through modulation of culture conditions. Mastery of these manipulations will lead to the realization of regenerative medicine.

Regenerative medicine

Recently, two researchers from the University of Toronto proposed the following definition of regenerative medicine (Daar and Greenwood 2007):
Regenerative medicine is an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause, including congenital defects, disease, trauma and ageing. It uses a combination of several converging technological approaches, both existing and newly emerging, that moves it beyond traditional transplantation and replacement therapies. The approaches often stimulate and support the body’s own self-healing capacity. These approaches may include, but are not limited to, the use of soluble molecules, gene therapy, stem and progenitor cell therapy, tissue engineering and the reprogramming of cell and tissue types.

Regardless of the particular research problem or clinical aim, the choice of starting cell is rarely straightforward with each having unique sets of trade-offs. For tissue engineering approaches, it also may not be clear what potency of cell will be most amenable to producing near native-quality and functional tissue in the lab.

Multipotent mesenchymal stromal cells (MSC) have attracted arguably the most attention in this field owing to the cells’ versatility. They are able to differentiate into various tissue lineages and are also able to modulate immune cells (Caplan 1991; Pittenger 1999; Dominici et al. 2006; Bieback et al. 2012). MSC are, therefore, not only suitable for ex vivo tissue engineering, but as an injectable cellular therapy. MSC do have certain limitations that must be acknowledged. They have variable differentiation and regenerative abilities, which could be due to donor age or culture conditions. They also undergo replicative aging meaning they lose their potency over time in culture and have limited expansion potential before reaching senescence (Caplan 1991; Bieback et al. 2012).

For the pluripotent cell option, induced pluripotent stem cells (iPSC) have largely re-
placed ESC and are being explored as a patient-specific source for cellular therapies. An iPSC is a somatic cell, most commonly the fibroblast, that has been reprogrammed to return to an ESC-like level of pluripotency. This approach circumvents any ethical issues involved in using cells derived from early embryos. However, iPSC have serious limitations in that they carry risk of tumorigenicity when applied in vivo in an undifferentiated state (Takahashi and Yamanaka 2006; González et al. 2011; Robinton and Daley 2012).

A population of cells derived from the desired tissue is another approach that may be taken. For cartilage engineering, chondrocytes are the only cell-type available from cartilage explants and exist in very low numbers. Culturing primary chondrocytes to reach clinically-relevant numbers rapidly changes the cells’ phenotype in a process called dedifferentiation. As their morphology changes from a cuboidal shape to a spindle shape, the chondrocytes also lose their chondrogenic gene expression profile (Schnabel et al. 2002). Chondrocytes, like MSC, also have a restricted window for expansion before losing all proliferative potential (Khan et al. 2009).

A combination of molecular and genetic techniques may offer a solution. Transdifferentiation is the conversion of one terminally-differentiated cell to another. Recently, dermal fibroblasts, a much more abundantly available cell type, were transdifferentiated into chondrocytes by stimulation with cartilage-derived morphogenetic protein 1 and elsewhere by ectopic expression of c-Myc, Klf4, (2 factors used in iPSC induction) and Sox9 (a master regulator of chondrogenesis) (Yin et al. 2010; Hiramatsu et al. 2011; Outani et al. 2013). Much work in this area remains as the former technique could only maintain the chondrogenic phenotype short-term, while the latter technique produced tumours when injected in mice.

The ideal starting cell and how they must be cultured, expanded, and induced has
not yet been determined and will likely depend on the required application. In the case discussed here, the ideal cell would be a fusion of the best features of each cell type: the chondrogenic potential of the primary chondrocyte for functionality, the immortality of the iPSC for expansion, and the non-immunogenicity of the MSC for enhanced grafting after transplantation.

**MSC history and characterization**

The concept of the MSC can be traced back to the work of A J Friedenstein who isolated colony forming units-fibroblast (CFU-F) or plastic-adherent clonogenic cells from BM. Through studies of these fibroblast-like cells from the 1960s through to the 1980s, Friedenstein and colleagues demonstrated they were highly proliferative in vitro and could differentiate into multiple different cell types of the mesenchymal lineage after single cell re-transplantation (Friedenstein et al. 1966, 1974, 1976, 1987; Afanasyev et al. 2009). Friedenstein and his colleagues called these cells ‘bone marrow osteogenic stem cells’ (Friedenstein et al. 1987). Cells with similar attributes appear to be ubiquitous throughout the body with populations since derived from most neonatal and adult tissues including fat, muscle, cord and peripheral blood (Williams et al. 1999; Zuk et al. 2001; Bieback et al. 2004; Chong et al. 2012). These cell populations from such a diverse range of tissues all share similar cell surface marker expression, differentiation potential, and immunomodulatory properties. While BM-MSC are thought to have a role in supporting hematopoiesis in the BM niche (Hematti and Keating 2013), the same physiological functions certainly cannot be ascribed to MSC found in the fat or muscle. The in vivo identity of these cells has yet to be illuminated.

Arnold Caplan brought the phrase mesenchymal stem cell and the acronym MSC into common use in the early 1990s (Caplan 1991), but ultimately this did not settle the matter of
nomenclature. Over the years, MSC have been called first pluripotent then multipotent, as their limitations compared to embryonic stem cells were realized. In 2006, the International Society for Cellular Therapy (ISCT) recommended they be called ‘multipotent mesenchymal stromal cells’ in a position paper in which they also posited minimal criteria (detailed below) for defining the cells (Dominici et al. 2006). Controversy over the naming continues with the late Paulo Bianco preferring the more restrictive term ‘skeletal stem cell’, and even Caplan returning to the conversation decades later to suggest his original acronym now represent our current clinical intentions assigning them ‘medicinal signaling cells’ (Bianco et al. 2006; Caplan and Correa 2011). All of this debate reflects the slow shift in perception of what MSC actually are.

Cell potency, or more specifically, in vitro differentiation potential into adipocytes, osteocytes, and chondrocytes, is included as one of the ISCT’s three criteria. Along with plastic adherence and positive or negative expression of a specific panel of cell surface markers (Fig. 1.1), these three criteria were laid out to try and standardize the practice of MSC study between labs in order to compare and contrast results with more validity (Dominici et al. 2006).

Notably, self-renewal was not on the list of ISCT criteria, which explains the recommendation for using the term *stromal* in place of *stem* in their designation (Dominici et al. 2006). Self-renewal has not been demonstrated in MSC (Bianco et al. 2006). While it is true that MSC have a high proliferative potential in vitro, all MSC cultures reach a limit from what is known as replicative aging. Low telomerase activity and shortening telomere length lead all MSC to eventually senesce (Banfi et al. 2002). Unlike hematopoietic stem cells, in which serial transplantation into irradiated mice has proven their capacity for self-renewal, no similar in vivo assay has proven the same for MSC (Rosendaal et al. 1979; Bianco et al. 2006).
Figure 1.1
MSC isolation and culture

Colonies of MSC can be derived from many sources, the most commonly used in research today being BM aspirate and AT. MSC are rare cells found at a concentration of less than 0.1% of marrow cells in human newborns—a concentration that decreases with patient age (Caplan 2007). While AT is reported to yield a 500-fold increase in MSC per millilitre when compared to marrow, *ex vivo* expansion is still required before the amount and concentration necessary for clinical application is acquired (Fraser et al. 2006; Bieback et al. 2008).

Whether in human or canine research, a lack of standardized protocols is a key barrier to clinically safe and reliable MSC-based therapies (Bieback et al. 2011). One of the known causes of variability in current protocols is the inclusion of fetal bovine serum (FBS) in the culture medium used. FBS is the most widely-used cell culture serum-supplement because of its high growth factor content and low levels of antibodies due to its fetal origin. FBS is typically added between 5% and 30% to supplement the basal Dulbecco’s Modified Eagle’s Medium (DMEM) in MSC expansion media. In addition to the problem of undefined and variable content from batch to batch, there are risks of complications from bovine antigens (Spees et al. 2004; Bieback et al. 2009; Allan and Strunk 2012). Exploration into possible alternatives to FBS has been taking place for years, and finding alternatives is becoming more pressing from a financial perspective as supply and demand issues have significantly increased the price of FBS (Brindley et al. 2012).

The most suitable alternative to FBS from a clinical standpoint would be a serum-free, non-xenogeneic, defined medium, which would satisfy all criteria for inclusion in a standardized good manufacturing practice (GMP) protocol. Such an approach involves either a long and iterative process to define the ideal formulation or the use of commercially available solutions (Lennon et al. 1995; Meuleman et al. 2006; Liu et al. 2007; Parker et
al. 2007; Lindroos et al. 2009; Chase et al. 2010; Rajala et al. 2010; Jung et al. 2010; Hareendran et al. 2010; Hudson et al. 2011; Mimura et al. 2011; Schwarz et al. 2012; Clark et al. 2015). A survey of the literature exploring this approach reveals that serum-free does not always mean xenogen-free (Liu et al. 2007; Mimura et al. 2011). However, the main factor for these media not being more widely adopted is likely that they are not cost effective.

Another factor, more relevant to our study, is that the efficacy of these media is species-dependent as can be seen in the only study to date involving canine cells to test FBS-free substitutes (Schwarz et al. 2012). In the study, equine, canine, and porcine adipose-derived MSC were cultured in FBS-supplemented DMEM or a commercially available serum-free (SF) medium supplemented with serum substitute. It was found that the SF medium effected a decrease in capacity for proliferation compared with the FBS media while the capacity for differentiation remained the same (Schwarz et al. 2012). It might be expected that any SF media commercially available would be optimized for use with human MSC due to a much higher demand. Indeed, the same SF media used above was shown in an earlier study with human MSC to produce better cell expansion while maintaining differentiation potential (Meuleman et al. 2006). As more SF media are developed and brought to market, proper testing will need to take place for every relevant species.

Autologous and allogeneic human serums have also been tested as alternative supplements to FBS. Autologous serum has been found to be a capable replacement for FBS (Stute et al. 2004; Shahdadfar et al. 2005; Mannello and Tonti 2007; Sato et al. 2016). However, the volumes needed for long expansion procedures might be too demanding for some human patients or for some companion animals as in the case here. Pooled allogeneic serum, which solves the problem of limited available volumes, has shown mixed results in the literature (Shahdadfar et al. 2005; Mannello and Tonti 2007; Hattori et al. 2008; Bieback et al.
2009, 2012; Poloni et al. 2012). Notably, one study looked at allogeneic serum alongside FBS, activated platelet rich plasma (PRP), and pooled human platelet lysate (PL) and found that PL was the closest substitute to FBS (Bieback et al. 2009).

PL has been explored as a source of growth factors for cell culture media since the 1970s (Eastment and Sirbasku 1980). PL is a concentrated solution of growth factors released from the platelet fraction by a freeze-thaw procedure (Fig. 1.2) that ruptures the platelet membranes (Schallmoser and Strunk 2013). Factors within the PL include platelet-derived growth factor (PDGF), transforming growth factor-β (TGFβ), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) (Rendu and Brohard-Bohn 2001). PL is non-xenogeneic and inexpensive. It can be derived from a patient’s own blood or generated from expired platelet concentrate (PC) fractions. PC is routinely prepared in medical or veterinary clinics to be at hand at all times for the treatment of bleeding disorders. The PC fractions are only suitable for clinical use within five days of production and are then discarded. This repurposed waste product has proven to be a rich and convenient source for the production of PL. Pooled human PL has been found to be the most satisfactory substitute to FBS for clinical-scale expansion and lays the framework for GMP-compliant production of MSC (Capelli et al. 2007; Bieback et al. 2009; Allan and Strunk 2012). Pooled human PL has been extensively studied in the last decade and has repeatedly been proven to outperform FBS in support of MSC isolation as well as the cells’ proliferative, progenitor, and non-progenitor functions (Doucet et al. 2005; Bernardo et al. 2007; Schallmoser et al. 2007; Capelli et al. 2007; Copland et al. 2013). According to a recent review of MSC culture methods, PL was used in 11% of protocols employed in recent MSC-therapy clinical trials second only to FBS at 73% (Ikebe and Suzuki 2014).
Figure 1.2
Schematic of fetal bovine serum (FBS) and platelet lysate (PL) production. (A) Commercially generated FBS: Blood is taken from the fetus of a cow at the time of slaughter. The blood is kept chilled and allowed to clot before it is centrifuged to separate the serum. It is filtered and then pooled. Reprinted and adapted from Cytotherapy, 16(2) Hemeda H, Giebel B, Wagner W., Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells. p. 172, © 2014, with permission from Elsevier. (B) Method to generate PL: Platelet-rich plasma (PRP) or expired platelet concentrate (PC) is centrifuged to pellet platelets. The pellet is resuspended minimally in platelet-poor plasma (PPP), counted, and resuspended to a concentration of $1 \times 10^6$ platelets/μL. Platelets are lysed by a freeze/thaw cycle to release growth factors. Adapted from Russell and Koch 2015.
MSC heterogeneity

The heterogeneity of MSC populations makes definitive characterization inherently challenging. At present, there is no single surface antigen or combination of surface antigens that can be used to identify a MSC specifically or to fully purify a population of cells (Bianco et al. 2006). In fact, expression of these markers may change depending on time in culture, on culture conditions, or on MSC harvest source (Lin et al. 2012b; Ka et al. 2014). Even within a single culture, there can be a range of cell morphologies and subpopulations with varying gene expression profiles and differentiation potential (Wagner et al. 2006; Ho et al. 2008; Bieback et al. 2012). These differences have produced challenges for comparing outcomes between MSC studies. As mentioned above, it is for this reason that the ISCT established three criteria to define the MSC: 1) plastic-adherence, 2) positive expression for cell surface markers CD105, CD73 and CD90, and negative expression for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR, and 3) trilineage differentiation potential into bone, cartilage, and fat (Dominici et al. 2006).

It should be noted that MSC of species other than human do not necessarily conform to this particular pattern of surface marker expression. Immunophenotyping of canine MSC, in particular, has been far less extensive, and to date, no universal set of markers has been outlined. Progress is being made with canine MSC markers such as CD44 and CD90 showing consistent positive and CD45 consistent negative expression, but a corresponding panel for canine MSC is yet to be established (Bakker et al. 2014). Accordingly, for canine MSC, other means of characterization must be employed. A quick visual check will determine the ability of canine MSC to adhere to plastic, and will show that they share a similar spindle-shaped morphology with human MSC. But, the burden of proof for many scientists working with non-human and non-rodent species falls onto the cells’ progenitor functions.
MSC progenitor functions

Progenitor function refers to the cells’ multipotency, and it is in the April 1999 report by Pittenger et al. that we first see the template for the ISCT proposal and for MSC studies going forward (Pittenger 1999). It details the isolation of human BM-MSC and includes immunophenotypical analysis and trilineage differentiation. It also reports on what are still standard induction reagents: 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin for adipogenesis, transforming growth factor–β3 for chondrogenesis, and dexamethasone, β-glycerol phosphate, and ascorbic acid for osteogenesis (Pittenger 1999).

The first report of canine MSC was in 1997 describing their isolation from BM aspirate from the iliac crest (Kadiyala 1997). The paper noted some differences from human MSC in that they were more abundant according to colony counts, smaller, and faster growing. To assess in vivo differentiation, two dogs received autologous implants of porous ceramics loaded with MSC, which yielded evidence of chondrogenic and osteogenic differentiation. To assess in vitro differentiation, only an osteogenic assay was run, which also yielded positive results (Kadiyala 1997).

Adipogenesis and osteogenesis are frequently shown in canine MSC studies most often validated with histological staining and sometimes with mRNA expression data of induced versus non-induced MSC populations (Bakker et al. 2014). Chondrogenic induction of canine MSC has proven challenging using standard protocols and robust chondrogenic differentiation remains to be shown (Csaki et al. 2007; Neupane et al. 2008; Eslaminejad and Taghiyar 2010; Vieira et al. 2010; Zuconi et al. 2010; Volk et al. 2012; Hodgkiss-Geere et al. 2012; Reich et al. 2012; Kisiel et al. 2012; Choi et al. 2013; Guercio et al. 2013; Bertolo et al. 2014; Lee et al. 2015; Sullivan et al. 2016). However, all of this may be less damaging to the clinical utility of MSC as a paradigm shift directs focus to their
non-progenitor functions (da Silva Meirelles et al. 2009; Griffin et al. 2010).

MSC non-progenitor functions

Non-progenitor function refers to the cells’ more recently discovered ability to influence resident cells and tissue functions through their secretome and direct cell-cell contact, including regenerative and immune modulatory effects (Uccelli et al. 2008). An immunoassay commonly used to assess immunomodulation is the lymphocyte proliferation assay or mixed lymphocyte reaction, which cultures stimulated lymphocytes with and without mitogenic proteins or irradiated allogeneic cells. A marked proliferative response is expected only in the stimulated cells. Early in this century, reports began to emerge of the ability of BM-MSC to suppress proliferation of these stimulated T-lymphocytes (Bartholomew et al. 2002; Di Nicola 2002). Soon after, AT-MSC were shown to have similar immunomodulatory properties as their BM-derived counterparts (Puissant et al. 2005).

Results such as these are encouraging for the future potential allogeneic use of MSC in clinical cellular therapies particularly in the treatment of inflammatory conditions. Indeed, preclinical and clinical trials have already shown that allogeneic MSC are able to prevent acute graft-versus-host disease in the majority of cases (Blanc et al. 2004; Leto Barone et al. 2013). However, research in this area is ongoing with some questions unsettled. For example, the potency of immunomodulation depending on the MSC source is actively being investigated (Yoo et al. 2009; Melief et al. 2013).

It has been suggested that MSC effect this immunosuppression through cell-cell contact and secreted soluble factors (Fig. 1.3) (Nauta and Fibbe 2007; Uccelli et al. 2008; Yagi et al. 2010; Le Blanc and Mougiakakos 2012; Najar et al. 2016). For example, indoleamine 2,3-dioxygenase (IDO) inhibits T cell proliferation by catalyzing the tryptophan necessary for lymphocyte proliferation (Meisel et al. 2004). Remarkably, the mechanisms of im-
munosuppression by MSC do see species differences. While IDO is found to be a mediator in human MSC-lymphocyte interactions, it is inducible nitric oxide synthase (iNOS) that mediates in the mouse model (Ren et al. 2008, 2009). Other cytokines produced by MSC discovered to be involved in immunomodulatory effects include transforming growth factor beta (TGFβ1), prostaglandin E2 (PGE2), interleukin 10 (IL-10), and hepatocyte growth factor (HGF). Typically, proof of a factor’s involvement is shown when immune cell proliferation is restored through the inhibition of the candidate and/or the addition of recombinant proteins to replace what is lost in the reaction (Hwu et al. 2000; Di Nicola 2002; Aggarwal and Pittenger 2009).

Some factors like TGFβ1, PGE2, IL-10, and HGF are constitutively expressed, while others like IDO and iNOS are induced by pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), which are produced by activated T cells (Krampera et al. 2006; Uccelli et al. 2008; Ren et al. 2008). Pre-licensing or priming MSC with these inducers in culture promote their immunosuppressive properties (Meisel et al. 2004; Krampera et al. 2006; English et al. 2007; Ryan et al. 2007; Ren et al. 2008).

Few articles on canine MSC immune modulation have been published (Kang et al. 2008; Lee et al. 2011b; Kol et al. 2014). Kang et al. concluded that TGFβ, HGF, PGE2, and IDO are involved in cAT-MSC immunomodulation with lymphocyte proliferation restored in the presence of PGE2 and IDO inhibitors (Kang et al. 2008). The importance of PGE2 was further supported by other labs as well, one of which looked at cBM-MSC (Lee et al. 2011b; Kol et al. 2014). At the time of publishing our study (Russell et al. 2016), no comparison of canine AT- and BM-MSC with regard to their immunomodulatory function had been reported.
Figure 1.3

Immunomodulatory properties of mesenchymal stromal cells MSC. MSC secrete soluble factors to inhibit proliferation and activation of immune cells such as T cells, NK cells, dendritic cells (DC), and macrophages. MSC can be activated toward an immunosuppressive phenotype by interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α). IDO, indoleamine 2,3-dioxygenase; NO, nitric oxide; PGE2, prostaglandin E2; TGFβ, transforming growth factor beta; HGF, hepatocyte growth factor; IL-6, interleukin 6; IL-10, interleukin 10; and HLA-G5, human leukocyte antigen G.

Reprinted and adapted from Cytotherapy, 18(2) Najar et al. Mesenchymal stromal cells and immunomodulation: A gathering of regulatory immune cells. p.163, © 2016, with permission from Elsevier.
Influence of MSC harvest site

As it was discovered that MSC can be derived from almost all post-natal adult tissues and organs (Zuk et al. 2001; Romanov et al. 2003; da Silva Meirelles 2006), comparison studies of MSC from two or more sources have become common. The search for an alternative to BM-MSC has clinical justification owing to the painful and invasive nature of BM aspirate collection. AT-MSC have been shown to be comparable to BM-MSC when phenotype and differentiation capacity was considered, but with the advantage of having a higher proliferative potential (Romanov et al. 2005; Wagner et al. 2005; Kern et al. 2006; Rebelatto et al. 2008; Strioga et al. 2012; Vishnubalaji et al. 2012; Dmitrieva et al. 2012; Webb et al. 2012; Stanko et al. 2013).

For the canine, comparative investigation into cell surface marker and gene expression (Takemitsu et al. 2012; Screven et al. 2014; Sullivan et al. 2016), and at least one of adipogenic, osteogenic, or chondrogenic differentiation potential (Kang et al. 2012; Takemitsu et al. 2012; Spencer et al. 2012; Reich et al. 2012; Kisiel et al. 2012; Screven et al. 2014; Sullivan et al. 2016) can be found with varying quality. Some authors acknowledge the difficulty they had to meet the ISCT criteria for MSC (Kisiel et al. 2012; Sullivan et al. 2016). Kisiel et al. discuss the fact that their chondrogenic protocol did not yield satisfactory evidence of chondrocyte morphology and that the same lack of evidence can be seen elsewhere in the literature (Kisiel et al. 2012).

In our lab, we have recently compared equine MSC source and determined that greater variation is found between donors than source (unpublished), which again speaks to the problem of MSC heterogeneity. Alternatively, if consistency of MSC function cannot be found in a particular tissue harvest site, it may have to be sought from a clonal source of pluripotent cells.
Induced pluripotent stem cells

Yamanaka and Takahashi first announced the generation of iPSC in their landmark article in 2006 (Takahashi and Yamanaka 2006). The Yamanaka group proved that terminally differentiated cells could be transformed to become ESC-like by introducing four transcription factors (OCT4, SOX2, KLF4, and c-Myc, commonly referred to as OSKM or Yamanaka factors) that are highly expressed in ESC. Mouse and human fibroblasts were reprogrammed to show traits of ESC including the ability to self-renew indefinitely and to differentiate into cell types from all three germ layers. Successful reprogramming towards a pluripotent cell state was supported by mRNA expression of embryonic stem marker genes, embryoid body and teratoma formation, and maintenance of a normal karyotype (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007).

Since its publication, a new field of research has grown around understanding the process of cellular reprogramming, optimizing the Yamanaka method or testing alternative methods that improve on some of the shortcomings of the Yamanaka method. Among those shortcomings, the two that have attracted the most attention are problems inherent in the use of retroviruses for gene delivery and the very low efficiency of the technique.

Yamanaka used either a Moloney murine leukemia virus or a lentivirus delivery vector for transfection of the fibroblasts with OSKM (Takahashi and Yamanaka 2006; Takahashi et al. 2007). These retroviruses by nature integrate the genes into the genome of host cells. Introduction of OSKM is meant only to induce endogenous expression of pluripotency genes before ideally the transgenes are silenced. Genomic integration poses a risk in that it could lead to tumorigenesis through disrupted expression of tumour suppression genes or through the continued expression or reactivation of the oncogene c-Myc, which is a concern if ever iPSC are to be used for transplantation therapy (González et al. 2011).
One common method used to avoid these risks is to use the Sendai virus (SeV). SeV are RNA-based viruses that circumvent the need for DNA reverse transcription or for genome integration whatsoever. Since the SeV never enters the nucleus, the transgenes are expressed within the cytoplasm leaving the genome intact. Another benefit of using SeV over the retroviruses discussed is that reprogramming efficiency is significantly improved when compared to the reported efficiencies of the Yamanaka method (Fusaki et al. 2009; Robinton and Daley 2012; Lakshmipathy and Vemuri 2013). Another approach was published in 2009 by the Nagy lab at the University of Toronto. They published about their use of a piggyBac transposon system to deliver the Yamanaka factors necessary for reprogramming (Woltjen et al. 2009). The advantages of this system are that transgene expression can be made inducible, and the transgenes can be completely removed before the clinical use of the reprogrammed cells. Neither approach has been used in canine cell reprogramming studies.

Only a few research groups have generated canine iPSC to date (Shimada et al. 2009; Lee et al. 2011a; Luo et al. 2011; Whitworth et al. 2012, 2014; Gonçalves et al. 2012, 2017; Koh et al. 2013; Nishimura et al. 2013; Baird et al. 2015). Shimada et al. (2010) was the first group to do so using retroviral delivery of canine OSKM to embryonic fibroblasts (Shimada et al. 2009). Since then, all groups have used retroviruses as vectors, although the cell sources varied slightly including fibroblasts from various harvest sites and AT-MSC. There is one instance of canine chondrocytes being transduced with OSKM and Sox9, but the cells were not sorted and were soon used in pellet culture for chondrogenic assays, and pluripotency was not confirmed (Gurusinghe et al. 2015). With so few studies dealing with canine iPSC, there is much work left to contribute especially in the area of using reprogramming methods with non-integrating or excisable transgenes to produce more clinically safe and relevant cells.
Reprogramming MSC

The generation of iPSC has most commonly come from fibroblasts as donor cells dating back to the original Yamanaka paper. Even so, there is a recent accumulation of articles detailing the use of MSC as donor cells (Cai et al. 2010; Yan et al. 2010; Aoki et al. 2010; Oda et al. 2010; Ohnishi et al. 2012; Yulin et al. 2012; Diederichs and Tuan 2014). The first group to generate iPSC from MSC transfected human AT-MSC without c-Myc and accomplished a maximum of 0.02% reprogramming efficiency (Aoki et al. 2010). Human BM-MSC have also been reprogrammed with OSKM with the addition of p53 siRNA, valproic acid, and vitamin C for an efficiency of 0.1% (Yulin et al. 2012). Another group ran a study comparing induction of cryopreserved AT- and BM-MSC. The sample size was too small for efficiency rates to be significant, but they did find equal differentiation potentials between the two cell sources (Ohnishi et al. 2012). All three of these groups used retroviral delivery of transgenes.

Around the same time, other research teams were looking to go in the other direction: to derive MSC from iPSC, which several groups have achieved (Lian et al. 2010; Teramura et al. 2010; Fu et al. 2012; Jung et al. 2012; Guzzo et al. 2013; Diederichs and Tuan 2014; Hynes et al. 2014). Even more promising, one study showed that iPSC-derived MSC (iPSC-MSC) had higher regenerative potential than their BM-MSC counterparts for attenuating limb ischemia in mice. This enhanced regenerative ability is attributed to superior post-transplantation survival of the iPSC-MSC (Lian et al. 2010). The same lab in 2012, also showed that iPSC-MSC possess similar immunomodulatory properties to BM-MSC with regard to the inhibition of lymphocyte proliferation (Fu et al. 2012). At the time of writing, only one paper (Diederichs and Tuan 2014) has looked at comparing iPSC-MSC with MSC from the same donor. Using human BM-MSC from a single subject, they established a
single iPSC line from which they derived iPSC-MSC and compared them to the original BM-MSC. The results suggest that the iPSC-MSC do not share the same gene expression pattern, nor do they produce as high quality tissue as the original BM-MSC according to histological evaluation (Diederichs and Tuan 2014).

Reprogramming chondrocytes

A number of articles have shown that iPSC derived from other sources than MSC can be directed to form articular cartilage matrix (Medvedev et al. 2011; Diekman et al. 2012; Kuboth et al. 2012; Wei et al. 2012; Craft et al. 2013, 2015). However, other groups have sought to prove that the strategy for cartilage tissue engineering from pluripotent cells should begin with the desired cell type: chondrocytes. Reprogramming of human chondrocytes has been achieved by lentiviral means (Olee et al. 2011; Wei et al. 2012) as well as through mRNA reprogramming (Borestrom et al. 2014). The latter study went even further to show that chondrocyte-derived iPSC had increased chondrogenic differentiation capacity compared to their fibroblast-derived counterparts suggesting that so-called “epigenetic memory” could play a key role (Borestrom et al. 2014). The term epigenetic memory refers to the idea that reprogrammed adult cells are more likely to differentiate back down their original lineage owing to residual DNA methylation patterns (and other chromatin modification) characteristic of the cell’s tissue of origin. Epigenetic memory is often thought of as a limitation of current iPSC technologies since the cells, regardless of their reprogramming state, often retain a preference for reverting back to their original somatic cell state. However, from a clinical perspective epigenetic memory may be advantageous since these partially reprogrammed cells may be less likely to form unwanted tissues or become cancerous. This residual memory of cell fate was a key justification in pursuing reprogramming of MSC and chondrocytes in addition to overcoming their limited expansion potential.
1.2 Rationale

Recently, the dog has emerged as an increasingly useful preclinical animal model to study the development and safety of stem cell–based therapies (Kol et al. 2015). Comprehensive characterization and validation of the utility of canine MSC will provide far-reaching benefit in both the veterinarian field as well as in translational medicine. This PhD project aims to answer some of the pressing questions regarding the isolation, culture, and preferred tissue source of canine cells for clinical application as well as to explore the feasibility of using reprogramming technologies to develop an infinite source of canine immunomodulatory and chondrogenic cells. It is becoming increasingly clear that many veterinary clinicians are not waiting for the accumulation of evidence before applying cell-based therapies on their patients (Fortier and Travis 2011). This makes it all the more urgent to perform the foundational research to equip practitioners with the data necessary to make evidence-based decisions.

Hypothesis

Canine cells with immunomodulatory and/or chondrogenic properties can be isolated from adipose tissue (AT), bone marrow (BM) aspirates, or articular cartilage (AC) and utilized to generate canine iPSC.

Objectives

1. To isolate and expand canine MSC from AT and BM.

   a. To compare FBS and PL as media supplements in the isolation and expansion of AT- and BM-derived MSC.
b. To characterize and compare MSC derived from AT and BM with regard to their trilineage differentiation and immunomodulatory capacities.

2. To generate iPSC from canine MSC and chondrocytes.

Objective 1a was to isolate and expand canine MSC from the two most common sources: AT and BM. To make this objective more of a stand-alone project, it was decided to look at different culture supplements, the conventionally used FBS and an emerging alternative PL for the isolation and expansion of MSC. Since it took longer than expected to acquire the necessary canine samples, investigation began with established equine cord blood MSC cultures, a study which is detailed in appendix A (Russell and Koch 2015). Chapter 2 represents the evaluation of PL versus FBS with canine MSC (Russell et al. 2015). We expanded the canine study in an attempt to resolve some of the questions left by the findings of the equine study, the results of which were equally unexpected. We are the first to examine the effects of canine PL in MSC culture.

Objective 1b was to characterize and compare the canine MSC sourced from AT and BM. Chapter 3 reports on this comprehensive comparison study with a focus on, among other assays, trilineage differentiation potential and immunomodulatory capacity (Russell et al. 2016). At the time of submission, we were the first to compare the immune suppressive traits between canine AT- and BM-MSC.

Objective 2 changed as we frequently re-evaluated the direction the project should take and the accumulating results guided our decisions. The original ambition was to derive an induced pluripotent cell (iPSC) line from canine MSC to overcome the key shortcomings of the MSC, namely those attributed to limited replicative capacity. With future clinical application in mind, the non-integrative Sendai virus was used to reprogram these cells. After it was clear that the chondrogenic potential of MSC was lacking, the decision was
made to change the focus of reprogramming using the chondrocyte. Chapter 4 gives an account of the preliminary work on reprogramming canine MSC with the Sendai virus and chondrocytes with the excisable piggyBac transposon system. In chapter 5, there is a general discussion about the overall findings of this work and future directions that could be explored.
Chapter 2

Canine platelet lysate is inferior to fetal bovine serum for the isolation and propagation of canine adipose tissue- and bone marrow-derived mesenchymal stromal cell

This chapter is a modified version of Russell et al. (2015).
2.1 Introduction

Research into a FBS-free medium for the culture of canine MSC is extremely limited at this time. PL has proven to be a suitable alternative to FBS for expansion of human MSC.

2.2 Hypothesis

Canine AT- and BM-MSC can be isolated and expanded equally in PL and FBS at conventionally-used concentrations with differentiation of these MSC unaffected by choice of supplement.

2.3 Objectives

To evaluate the use of canine PL in comparison with FBS in MSC culture regarding:

- Isolation
- Proliferation
- Spontaneous differentiation
- Directed differentiation

2.4 Materials and methods

Ethics statement

This study adhered to the guidelines by the University of Guelph Animal Care Committee with regard to the procedures of collection of canine AT and BM samples. Collection of such tissue samples post-mortem and subsequent research conducted using specimens of this kind does not require review by the Animal Care Committee (falls under CCAC Category of Invasiveness A). The studies presented in this manuscript can be considered to have been
conducted in accordance with the institutional ethics guidelines. Dogs were sacrificed for reasons unrelated to the studies prior to collection of AT and BM.

Platelet concentrate and platelet lysate

Platelet concentrate (PC) was collected by the Ontario Veterinary College Small Animal Clinic through methods previously described for reasons unrelated to this study (Abrams-Ogg et al. 1993). After 5 - 7 days, canine PC was released for inclusion in this study, transferred to our lab, and processed immediately. PC was added to 50 mL tubes and centrifuged at 2000 g for 10 minutes. The pellets were resuspended in a minimal volume of platelet-poor plasma (PPP) and a complete blood count was run. Once the platelet concentration was known, the PC was diluted with platelet-poor plasma (PPP) to $1 \times 10^6$ platelets/µL according to convention (Schallmoser and Strunk 2013). The PC was frozen to -80°C and thawed in a 37°C water bath to lyse the platelets. For use in medium, PL from 10 dogs were pooled, spun at 4000 g for 15 minutes, and filtered through a 0.22 µm filter (Millex-GP Filter Unit, Millipore, Etobicoke, Ontario) before addition.

MSC isolation and expansion

Matched samples of BM aspirate and AT were obtained from a total of 8 dogs. All dogs used weighed a minimum 30 kg, were of unknown age, and were to be euthanized for reasons unrelated to this study. The dogs were euthanized by intravenous injection of pentobarbital (Euthanyl Forte, 540mg/5 Kg, Biomeda-MTC Animal Health, Cambridge, Ontario) before BM was extracted from the humerus with a 13 gauge Jamshidi needle (Kendall, Tyco Healthcare, Pointe-Claire, Quebec) and expelled into a 10 mL glass tube coated with heparin (Sandoz, Boucherville, Québec). Approximately 10 g of subcutaneous AT were surgically removed from the abdomen and placed in phosphate buffered saline (PBS, Roche,
Indianapolis, Indiana) with 2% penicillin/streptomycin (Life Technologies, Grand Island, New York).

The AT was minced in a Petri dish and washed with equivalent volume of PBS with penicillin/streptomycin until the solution ran clear. The tissue was then digested in an equivalent volume of collagenase I (Sigma-Aldrich, St. Louis, Missouri) in PBS solution (1 mg/mL) for 60 - 180 minutes shaking at 200 rpm on a heat block set at 37°C. When digested, the solution was filtered twice through a 70 µm falcon strainer (BD, Franklin Lakes, New Jersey). The nucleated cell fraction was pelleted out by centrifugation at 400 g for 15 minutes before being resuspended in PBS. These steps were repeated 4 times with the volume separated into 2 tubes for the final spin to prepare for resuspension in the different treatment media. BM aspirates were plated directly in one of either FBS or PL treatment groups. All isolated cells were plated in 12 well plates with MSC expansion medium consisting of low glucose DMEM (Lonza, Walkersville, Maryland), 10% pooled FBS (Life Technologies, Grand Island, New York) or 10% pooled PL, 1% penicillin/streptomycin, and 1% L-glutamine (Lonza, Walkersville, Maryland). All cell cultures were incubated at 38°C in a humidified environment containing 5% CO₂.

All cultures had media completely replaced daily for the first 3 days to remove red blood cell contamination, after which they were changed every 2 days until colonies were large enough for passage. Colonies were considered large enough when they surpassed 0.75 mm in diameter. Cells were passaged by washing with PBS and incubating at 38°C in cell detachment solution (Accupax, Innovative Cell Technologies, San Diego, California) for 12 minutes before seeding at 5000 cells/cm². Only cultures that expanded to a minimum of 5 million cells were cryopreserved for further experiment.
MSC proliferation

AT-MSC and BM-MSC isolated in FBS (n = 10) were thawed from cryopreservation and plated in MSC medium with 10% FBS. After 5 days, they were passaged and seeded at 5000 cells/cm² into 96 well plates again with 10% FBS medium. After cells attached (overnight), all medium was removed and replaced by either FBS or PL treatment medium at concentrations 5, 10, 20, 30, 40, 50 or 60% in quadruplicate wells (Fig. 2.1). Proliferation was analyzed by resazurin assay (Sigma-Aldrich, St. Louis, Missouri) after removal of all treatment media, addition of PBS with 10% resazurin (i.e. no FBS or PL), and incubation for 4 hours at 37°C. Plates were read at 585 nm using an excitation wavelength of 555 nm according to the manufacturer’s directions by plate reader (SpectraMax i3 Multi-Mode microplate reader, Molecular Devices, Sunnydale, California).

![Figure 2.1]

**Schematic of the resazurin (AlamarBlue) proliferation assay.** Mesenchymal stromal cells (MSC) are cultured for 1 week in expansion medium with 10% fetal bovine serum (FBS). One day before treatment, MSC are passaged into 96 well plates. MSC are treated with media containing 0, 5, 10, 20, 30, 40, 50 or 60% FBS or platelet lysate (PL) for 4 days before assayed.

**Spontaneous differentiation**

The same AT-MSC and BM-MSC cultures used for the proliferation assays were also seeded at 5000 cells/cm² into 48 well plates for spontaneous differentiation testing by trilineage
staining. Adipogenesis was assessed by Oil Red O (Sigma-Aldrich, St. Louis, Missouri) staining of lipids. Osteogenesis and chondrogenesis were assessed using a von Kossa staining protocol, which involved the addition of 1% silver nitrate (Sigma-Aldrich, St. Louis, Missouri) and exposure to UV light for 60 min, before counter-staining with Toluidine Blue (Sigma-Aldrich, St. Louis, Missouri). A plate from each culture was stained at days 4, 8, and 12 after treatments were added.

Parallel 6 well plates of the same cultures were grown and analyzed for adipogenic mRNA expression (Table 2.1). Cells were scraped on day 12 and flash frozen in lysis buffer for subsequent RNA extraction (RNeasy kit, Qiagen, Hilden, Germany). cDNA was synthesized from 500 ng RNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, New York) following manufacturers’ instructions. PCR reactions were performed using PerfeCta SYBR Green FastMix, ROX (Quanta BioScience, Gaithersburg, Maryland) with an Applied Biosystems 7300 Real Time PCR system. Data were analyzed with the \(2^{-\Delta\Delta CT}\) method. Gene expression fold-change is presented as the PL-treated cultures relative to the FBS-treated cultures with GAPDH as reference gene.

Directed differentiation

Trilineage differentiation was performed through chemical induction into adipocytes, osteocytes, and chondrocytes in parallel with non-induced controls. For adipogenesis and osteogenesis, 6 AT- and 4 BM-MSC isolated in 10% FBS and expanded between 3 and 5 passages were seeded at 5000 cells/cm² and grown to 90% confluence in 10% PL or 10% FBS expansion medium detailed above. To induce adipogenesis, the cells were cultured for 14 days in DMEM-LG with 1 µM dexamethasone (Sigma-Aldrich, St. Louis, Missouri), 0.5 mM 3-isobutyl-1- methyl-xanthine (Sigma-Aldrich, St. Louis, Missouri), 10 µg/mL recombinant human (rh) insulin (Sigma-Aldrich, St. Louis, Missouri), 0.2 mM indomethacin
Table 2.1
Primers used in qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEBPA</td>
<td>AGTCAAGAAGTCGGTGAGCAAG</td>
<td>GCGGTCAATTGTCACTGGTGAG</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td>FABP4</td>
<td>ATCAGTGTAACGGGGATGTG</td>
<td>GACCTTTCTGTCATCCGCAGTA</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td>Leptin</td>
<td>CTATCTGTCTGTGGTTGAAGCTG</td>
<td>GTGTGTGAAATGTCAATTGATCCTG</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td>LPL</td>
<td>ACACATTCAAGAGGGTCAACC</td>
<td>CTCTGCAATCACACGGATGGC</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td>PPARG2</td>
<td>ACGATGGCCCGTTGGATTGATG</td>
<td>TGGCTCCATGAAGTCACCAAAGG</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGTCCCACCCCCCAATGTATC</td>
<td>CTCCGATGCTGCTTCACCTACCTT</td>
<td>(Brinkhof et al. 2006)</td>
</tr>
</tbody>
</table>
(Sigma-Aldrich, St. Louis, Missouri), 15% rabbit serum (Sigma-Aldrich, St. Louis, Missouri), 1% L-glutamine, and 1% ABAM. To induce osteogenesis, the cells were cultured for 14 days in DMEM-LG with 0.1 µM dexamethasone, 10 mM glycerol 2-phosphate, 0.05 mM ascorbic acid, 10% FBS, 1% L-glutamine, and 1% antibiotic antimycotic solution (ABAM, Life Technologies, Grand Island, New York). For chondrogenesis, 250,000 cells per well in a 96-well plate were spun down (200 g, 10 min, RT) to form a pellet and cultured for 14 days in DMEM-HG (Lonza, Walkersville, Maryland), 0.1 µM dexamethasone, 0.1 mg/mL ascorbic acid (Sigma-Aldrich, St. Louis, Missouri), 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, Minnesota), 200 mM Glutamax (Life Technologies, Grand Island, New York), 10 mg proline (Sigma-Aldrich, St. Louis, Missouri), 40 µg/mL ascorbic acid, 100 mM sodium pyruvate (Life Technologies, Grand Island, New York), 1% ITS (Life Technologies, Grand Island, New York), 1% L-glutamine, and 1% ABAM. Adipogenesis was assessed by Oil Red O staining of lipids and osteogenesis was assessed using Alizarin Red S (Sigma-Aldrich, St. Louis, Missouri) stain for calcium as previously reported (Koch et al. 2007). Chondrogenesis was histologically evaluated using Toluidine Blue stain for glycosaminoglycan content as previously reported (Co et al. 2014).

Data analysis

All data were analyzed using R statistical software (version 3.1.3., The R Foundation for Statistical Computing, Vienna, Austria). For the proliferation data, results were modelled as a 3-factor factorial in a randomized complete block design (RCBD) treating dog as a blocking factor. A log transform of the plate readings minus 7% more than the smallest reading (due to some negative values) was performed for a more normal distribution. Least squares means were determined and back-transformed for readability. For the qPCR data, results were modelled as a 3-factor factorial in a RCBD treating dog as a blocking factor.
MSC source was removed from the model as it showed no evidence of effect \((p = 0.74)\). We formally tested residuals for normality and plotted them against the predicted values and explanatory variables to assess ANOVA assumptions and to look for unequal variance and outliers. Based on this analysis, the data were fundamentally normal except for outliers, which were kept in the data set. Least squares means were determined and converted to fold-difference by using base 2 to the power of the \(-\Delta\Delta CT\). Data are presented as mean ± confidence interval. Statistical difference was assessed at \(P<0.05\).

2.5 Results

MSC isolation and expansion

Isolation success was based not only on colony formation, but also each population’s ability to expand to sufficient numbers of 5 million cells, as outlined above, within 4 passages for cryopreservation. Based on these criteria, while 7 out of 16 populations cultured in PL-supplemented medium formed colonies, growth of these colonies arrested before sufficient numbers could accumulate (Table 2.2). In contrast, 14 of the 16 FBS-supplemented cultures met isolation criteria. Elements unrelated to typical MSC morphology can be seen among the PL-supplemented colonies compared to their FBS-supplemented counterparts (Fig. 2.2).
Table 2.2
Isolation success of canine AT- and BM-MSC in 10% FBS or 10% PL.

<table>
<thead>
<tr>
<th>Source</th>
<th>Treatment</th>
<th>Colony formation</th>
<th>Expanded to 5 × 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose tissue</td>
<td>FBS</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>3/8</td>
<td>0/3</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>FBS</td>
<td>8/8</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>4/8</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Figure 2.2
Primary canine adipose tissue (AT)- and bone marrow (BM)-derived mesenchymal stromal cell (MSC) colonies isolated in 10% fetal bovine serum (FBS) or 10% platelet lysate (PL). Scale bar = 50 µm.
MSC proliferation

AT-MSC (n = 6) and BM-MSC (n = 4) established in the presence of FBS, but expanded in PL exhibited maximal proliferation at 10% PL (Figs. 2.3A and 2.3B). In contrast, the same MSC cultures expanded in FBS exhibited increased proliferation rates with increased FBS concentration until a plateau was reached. There were no significant differences between the treatment groups up to 20% in the AT-MSC (Fig. 2.3A) and 30% in the BM-MSC (Fig. 2.3B). Significant differences were found at 30% in the AT-MSC (P = 0.043) and in both AT-MSC and BM-MSC at 40%, 50%, and 60% concentrations (P < 0.001).

Morphological differences can be seen in cells cultured for 21 days without passage with FBS-cultured cells maintaining tight spindle shapes and PL-cultured cells clumping together and detaching showing little resemblance to MSC (Fig. 2.4).

Spontaneous differentiation

Common to AT- and BM-MSC, both FBS and PL treatment groups stained negative for chondrogenesis and osteogenesis when combining Toluidine Blue and von Kossa after 4, 8, and 12 days in culture (Fig. 2.5). When stained for adipogenesis with Oil Red O, lipid droplets can be seen forming only in the PL-treated cells as early as day 4 and progressively more in days 8 and 12 (Fig. 2.6).

Adipogenic mRNA expression of *CEBP4* and *FABP4* was up-regulated in both 10% and 30% concentrations of PL-treated 12-day cultures when compared to the FBS-treated cultures (Fig. 2.7). For *CEBP4*, there was close to 4-fold expression in both 10% (3.86, P < 0.001) and 30% (3.92, P = 0.002) PL-treated MSC. For *FABP4*, there was 2.35-fold expression at 10% PL (P = 0.026) and 6.56-fold expression at 30% PL (P < 0.001) compared to the same concentration FBS. There were no significant differences found between the
Figure 2.3
Proliferation assays (resazurin) of (A) adipose tissue-derived mesenchymal stromal cells (MSC) (n=6) and (B) bone marrow-derived MSC (n=4) in fetal bovine serum (FBS)– or platelet lysate (PL)-enriched medium from 5% to 60%. Base medium (DMEM) was used as a negative control. (*P<0.05, ***P<0.001; error bars=CI.)
treatments for the expression of Leptin, \textit{LPL}, or \textit{PPARG2}.

\textbf{Directed differentiation}

A minimum number of 4.6 million cells were necessary for trilineage (adipogenesis, osteogenesis, and chondrogenesis) differentiation studies. While all 10 FBS cultures (6 AT- and 4 BM-MSC) yielded enough cells, only 3/10 PL cultures (3 AT-MSC) expanded sufficiently for trilineage differentiation. After 14 days in induction medium, MSC previously grown in either PL or FBS stained positive for adipogenesis (Fig. 2.8) and osteogenesis (Fig. 2.9). Chondrogenesis was unsuccessful under the conditions provided in this study.

Figure 2.4
\textbf{Long-term effect of 10\% FBS and 10\% PL expansion media on MSC over period of 21 days without passage.} Both cultures stained with Alizarin Red S. Scale bar = 250 \textmu m.
Figure 2.5

**Short-term effect of MSC in PL vs. FBS culture.** Effect of 10% platelet lysate (PL) and 10% fetal bovine serum (FBS) expansion media on canine adipose tissue (AT)- and bone marrow (BM)-derived mesenchymal stromal cells over 12 days. Cultures stained with the von Kossa protocol and Toluidine Blue for early markers of osteogenesis and chondrogenesis respectively. Scale bar = 50 µm.
Figure 2.6

Short-term effect of MSC in PL vs. FBS culture. Effect of 10% platelet lysate (PL) and 10% fetal bovine serum (FBS) expansion media on canine adipose tissue (AT)- and bone marrow (BM)-derived mesenchymal stromal cells (MSC) over 12 days. Cultures stained with Oil Red O for early markers of adipogenesis. Lipid droplets seen forming in the PL cultures only. Scale bar = 250 µm.
Figure 2.7

**Adipogenic gene expression of MSC in short-term culture of PL vs. FBS culture.**

Fold difference adipogenesis marker expression of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells cultured in fetal bovine serum (FBS)- or platelet lysate (PL)-enriched medium at 10% (yellow) and 30% (orange) after 12 days. Horizontal bar represents no change. (*P<0.05, **P<0.01, ***P<0.001; error bars=CI.)

of pellet cultures from both AT- and BM-MSC and both FBS and PL treatment groups (Fig. 2.10).
Figure 2.8

Directed adipogenesis of canine mesenchymal stromal cells (MSC) previously expanded in 10% platelet lysate (PL) or fetal bovine serum (FBS). Adipogenic potential of adipose tissue (AT)-derived (MSC) was assessed with Oil Red O staining after 14 days in induction medium. Adjusted for brightness. Scale bar = 50 μm. No BM-MSC cultured in PL expanded sufficiently for adipogenesis.
Directed osteogenesis of canine mesenchymal stromal cells (MSC) previously expanded in 10% platelet lysate (PL) or fetal bovine serum (FBS). Osteogenic potential of AT-MSC was assessed with Alizarin Red S staining after 14 days in induction medium. Adjusted for brightness. Scale bar = 250 µm. No BM-MSC cultured in PL expanded sufficiently for osteogenesis.
Figure 2.10
Directed chondrogenesis of canine mesenchymal stromal cells (MSC) previously expanded in 10% platelet lysate (PL) or fetal bovine serum (FBS). Chondrogenic potential of AT- and bone marrow (BM)-MSC was assessed with Toluidine blue staining after 21 days in induction medium. Control pellets cultured in expansion medium did not remain intact through histological processing. Adjusted for contrast. Scale bar = 100 µm.
2.6 Discussion

Our results show PL performing inadequately in the key areas of MSC isolation and long-term expansion. However, PL may yet prove feasible as an alternative to FBS in certain areas of canine MSC culture such as in transition medium just prior to clinical use or differentiation.

While colonies did emerge from digested AT and BM cultured in isolation medium with 10% PL, none of these colonies were robust enough to yield numbers high enough for long-term cell banking. Almost all (14 out of 16) MSC populations isolated in FBS grew to sufficient number for cryopreservation. Distinct morphological differences were seen in the primary colonies especially in the BM primary colonies (Fig. 2.2). It may be that other concentrations of PL could have improved on isolation success as only a concentration of 10% was used in the PL isolation medium.

Similar to our recent findings with equine cord blood-derived MSC (Russell and Koch 2015), PL supported canine AT- and BM-MSC proliferation similarly to FBS only at lower concentrations up to 20% (AT-MSC) or 30% (BM-MSC) before it became detrimental to cellular growth (Figs. 2.3A and 2.3B). When left long-term in PL culture for 21 days without passage, MSC were prone to clump together and lift off the plate, whereas MSC for the same length of time in FBS retained a tight formation of spindle-shaped cells (Fig. 2.4).

Spontaneous differentiation down the adipogenic line may offer an explanation to these findings. Adipogenic staining of MSC cultured in as low as 10% PL and for as briefly as 4 days show pervasive evidence of lipid droplet formation when compared to those grown in 10% FBS (Fig. 2.6). Lipid droplets were seen to accumulate increasingly over time only in the PL cultures (Fig. 2.6). Stronger mRNA expression of \( CEBPA \) and \( FABP4 \) in the PL cultures was also found to support these findings (Fig. 2.7). Expression of these two
markers are known to increase progressively through adipogenesis (Sekiya et al. 2003).

Directed differentiation appears unaffected by prior choice of expansion medium supplement. Both adipogenic and osteogenic induction show a comparable level of staining after 14 days with Oil Red O and Alizarin Red S respectively (Figs. 2.8 and 2.9). Chondrogenesis via pellet culture was also attempted using the lab-established protocol used for equine MSC (Koch et al. 2007; Berg et al. 2009; Buechli et al. 2013). However, this protocol did not produce convincing histological evidence of chondrogenesis with MSC grown in either PL or FBS (Fig. 2.10). Similar difficulties inducing chondrogenesis in canine MSC have been reported (Kisiel et al. 2012).

Overall, these findings were unexpected since PL is being increasingly used to support the propagation of various human MSC (Doucet et al. 2005; Bernardo et al. 2007; Schallmoser et al. 2007; Capelli et al. 2007). We noticed a similar dose-dependent effect of PL on equine cord blood-derived MSC (Russell and Koch 2015). Complement-induced lysis by the PL was ruled out due to diminished proliferation of MSC treated with heat-inactivated PL (data not shown).

Taken together, these results raise some concern about clinical use of platelet-derived products like platelet-rich plasma (PRP) with MSC. PRP should not be considered a neutral carrier solution for MSC. Activated platelets appear to compel adipogenic differentiation of MSC even when dilute. Therefore, co-culture or co-injection of these elements may significantly limit the regenerative capacity of the MSC. Further study into in vivo effects of MSC in combination with platelet-derived products is justified.
Chapter 3

Characterization and immunomodulatory effects of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells

*This chapter is a modified version of Russell et al. (2016).*
3.1 Introduction

In this study, we examined both the progenitor and non-progenitor functions of canine AT- and BM-MSC. Surface marker expression, population doubling times, and DNA methylation quantification were also compared for the purpose of explaining any differences between the cell sources with regard to their differentiation or immunomodulatory capacities.

3.2 Hypothesis

Donor paired canine adipose tissue (AT)- and bone marrow (BM)-derived MSC will have similar differentiation capacity and immune modulatory properties.

3.3 Objectives

To characterize AT- and BM-derived MSC with regard to their:

- Population doubling time
- Cell surface marker expression
- Global DNA methylation quantification
- Trilineage differentiation potential
- Immunomodulatory potency

3.4 Materials and methods

Ethics statement

Guidelines by the University of Guelph Animal Care Committee were closely followed with regard to the collection of canine blood, AT, and BM samples. Since collection of these tissue samples occurred post-mortem and dogs were sacrificed for reasons unrelated to the
studies, subsequent research conducted using these samples did not require review by the Animal Care Committee (falls under CCAC Category of Invasiveness A). Therefore, these studies were conducted in accordance with the institutional ethics guidelines. Blood and tissues were collected immediately after the dogs were euthanized by intravenous injection of pentobarbital (Euthanyl Forte, 540mg/5 Kg, Biomeda-MTC Animal Health, Cambridge, Ontario) at Hillside Kennels Animal Control, Innerkip, ON. Euthanasia was deemed necessary by the kennel as the dogs were aggressive/dangerous and not suitable for adoption.

MSC isolation

Cryopreserved AT- and BM-MSC were thawed from previously isolated and cryopreserved cultures from 8 dogs (Russell et al. 2015). The dogs used were of unknown age each weighing a minimum 30 kg.

MSC culture and proliferation

MSC were cultured in expansion medium (EM) composed of low glucose Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Walkersville, Maryland), 10% pooled FBS (Life Technologies, Grand Island, New York), 1% penicillin/streptomycin, and 1% L-glutamine (Lonza, Walkersville, Maryland) and incubated at 38°C in a 5% CO₂ humidified environment. Cells were harvested at 60-80% confluency using a cell detachment solution (Accumax, Innovative Cell Technologies, San Diego, California) and counted with an automated cell counter (Nucleocounter NC100, Mandel Scientific, Guelph, Ontario). Population doubling times were calculated from passage 2 through passage 5 (AT-MSC, n=8; BM-MSC, n=6). For all the following experiments, MSC from passages 3-6 were used for this study.
Immunophenotyping

Canine MSC (n≥3 for each cell source) were analyzed for surface marker expression using the Accuri C6 flow cytometer and software (BD, Mississauga, ON). Canine peripheral blood mononuclear cells (PBMC, n≥1) were used as controls. The antibodies used are listed in Table 3.1. All antibodies utilized were canine-specific except for MHC I (bovine) and CD73 (human), which were stated to cross-react with canine cells by the manufacturers and were validated with bovine and human PBMC respectively. Expression of CD105 antigens were not determined since the anti-human CD105 antibody (clone SN6, AbD Serotec) did not detect CD105 when validated against human mononuclear cells. Unstained samples of each MSC and PBMC were gated to determine surface marker expression of their stained counterparts.

Table 3.1
Cell surface marker list utilized to characterize MSC populations.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Target</th>
<th>Host</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC I</td>
<td>H58A</td>
<td>Bovine</td>
<td>Mouse</td>
<td>Kingfisher Biotech</td>
</tr>
<tr>
<td>MHC II</td>
<td>YKIX334.2</td>
<td>Dog</td>
<td>Rat</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>CD4</td>
<td>YKIX302.9</td>
<td>Dog</td>
<td>Rat</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD8</td>
<td>YCATE55.9</td>
<td>Dog</td>
<td>Rat</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>CD14</td>
<td>TüK4</td>
<td>Dog</td>
<td>Mouse</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>CD29</td>
<td>MEM-101A</td>
<td>Dog</td>
<td>Mouse</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>CD34</td>
<td>1H6</td>
<td>Dog</td>
<td>Mouse</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>CD44</td>
<td>YKIX337.8.7</td>
<td>Dog</td>
<td>Rat</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>CD45</td>
<td>YKIX716.13</td>
<td>Dog</td>
<td>Rat</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>CD73</td>
<td>7G2</td>
<td>Human</td>
<td>Mouse</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>CD90</td>
<td>YKIX337.217</td>
<td>Dog</td>
<td>Rat</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>
Global DNA methylation quantification

Genomic DNA was isolated from AT- and BM-MSC samples (AT-MSC, n=6; BM-MSC, n=6) using a column purification system (Quick-gDNA MiniPrep, Zymo Research, Irvine, California). Global DNA methylation levels were quantified with a 5-methylcytosine ELISA kit according to the manufacturer’s instructions (Zymo Research, Irvine, California).

Trilineage differentiation

Trilineage differentiation (AT-MSC, n=6; BM-MSC, n=4) was performed as previously described except where indicated (Russell et al. 2015). Briefly, for adipogenesis and osteogenesis, cells were cultured for 14 days with either EM as described above or induction medium. Adipogenesis induction medium consisted of low-glucose DMEM with 1 μM dexamethasone (Sigma-Aldrich, St. Louis, Missouri), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, Missouri), 10 μg/mL recombinant human (rh) insulin (Sigma-Aldrich, St. Louis, Missouri), 0.2 mM indomethacin (Sigma-Aldrich, St. Louis, Missouri), 15% rabbit serum (Sigma-Aldrich, St. Louis, Missouri), 1% L-glutamine, and 1% antibiotic antimycotic solution (ABAM, Sigma-Aldrich, St. Louis, Missouri). Osteogenesis induction medium consisted of low-glucose DMEM with 0.1 μM dexamethasone, 10 mM glycerol 2-phosphate, 0.05 mM ascorbic acid, 10% FBS, 1% L-glutamine, and 1% ABAM. For chondrogenesis, 250,000 cells were pelleted in a 96-well plate and cultured for 21 days in high-glucose DMEM (Lonza, Walkersville, Maryland), 0.1 μM dexamethasone, 0.1 mg/mL ascorbic acid (Sigma-Aldrich, St. Louis, Missouri), 10 ng/mL TGF-β3 (R&D Systems, Minneapolis, Minnesota), 200 mM Glutamax (Life Technologies, Grand Island, New York), 10 mg proline (Sigma-Aldrich, St. Louis, Missouri), 40 μg/mL ascorbic acid, 100 mM sodium pyruvate (Life Technologies, Grand Island, New York), 1% Insulin-
Transferrin-Selenium (Life Technologies, Grand Island, New York), 1% L-glutamine, and 1% ABAM. To promote better chondrogenesis, 0, 50, 100, or 200 ng/mL bone morphogenetic protein 2 (BMP-2) was added to the media.

Adipogenesis and osteogenesis samples were stained with Oil Red O and Alizarin Red S stains (Sigma-Aldrich, St. Louis, Missouri) respectively. Chondrogenesis samples were histologically evaluated with toluidine blue staining for glycosaminoglycan content and hematoxylin and eosin staining for general pellet structure as previously reported (Co et al. 2014). Adipogenic, osteogenic, and chondrogenic mRNA transcript abundance was analyzed by RT-qPCR using the primers listed in table 3.2 (Neupane et al. 2008; Figueroa et al. 2011). cDNA was synthesized from 500 ng RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, New York) using manufacturers’ instructions. PCR reactions were performed using the PerfeCta SYBR Green FastMix, ROX (Quanta BioScience, Gaithersburg, Maryland) with the Applied Biosystems 7300 Real Time PCR system. Data were analyzed using the $2^{-\Delta\Delta CT}$ method. Gene expression data is presented as the induction medium-treated cultures relative to the expansion medium-treated control cultures with GAPDH used as reference gene.

Immunomodulatory properties

Whole blood was obtained from the jugular vein of dogs with an 18-gauge needle attached to a 450 mL blood collection bag (Fenwal, Baxter, Deerfield, Illinois). PBMC were isolated using a density gradient media (Ficoll-Paque Plus, GE Healthcare, Mississauga, Ontario). In a 50 mL tube, 7.5 mL of Ficoll-Paque Plus was added to the bottom. A 10 mL 1:1 mix of whole blood and PBS was then added on top and spun at 400 g for 20 min with acceleration set to 1 and deceleration set to 0. The mononuclear layers were pooled and washed repeatedly with PBS before resuspension in EM. PBMC were counted and frozen
Table 3.2
Oligonucleotide primer list.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipogenic</td>
<td>CEBPA</td>
<td>AGTCAAGAAGTCGTTGAGCAG</td>
<td>GCGGTCATTTCGACTTGGAG</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>FABP4</td>
<td>ATCACTGTAAACGGGGATGTA</td>
<td>GACTTTTCTGCACTCCGATGA</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>CTTAGTCCTGTTGTAAGCTG</td>
<td>GTGTTGTAAGAATGCGATCCCTG</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>LPL</td>
<td>ACACATTCACAAGAAGGTCACC</td>
<td>CTCTGCAATCACACGGATGGC</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>PPARG2</td>
<td>ACACGGATGCTGCGTCCTGGAG</td>
<td>TGGCTCCATGAAACACACAAAGG</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td>Osteogenic</td>
<td>COL1A1</td>
<td>GTAGACACCACCACTCAAAGAC</td>
<td>TTCCAGTGGGTGGGACATC</td>
<td>(Neupane et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>RUNX2</td>
<td>AACCACGAAATGCGACTTCCA</td>
<td>GGACATGCTGAGGCTGACT</td>
<td>(Figueroa et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>Osteopontin</td>
<td>GCACCTGAGAAGGCGACAGC</td>
<td>AGTGGTTGCGCGCCCTGGTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALP</td>
<td>CCAACCTCCTGCGCAAACAAAAT</td>
<td>CTCTATCTTCTCCGAGCTACA</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>GAPDH</td>
<td>TGTCCCACCCCAATGTATC</td>
<td>CTCCGATGCGCTTCACTACCTT</td>
<td>(Brinkhof et al. 2006)</td>
</tr>
</tbody>
</table>
in EM with 10% DMSO (Sigma-Aldrich, St. Louis, Missouri) until use in the lymphocyte proliferation assays.

AT- and BM-MSC (AT-MSC, n=4; BM-MSC, n=4) were thawed and seeded 7 days prior to coculture with PBMC. On day 5, 4 treatments of each MSC were designated: 1) 200 ng/mL recombinant canine interferon-gamma (IFN-γ, Kingfisher Biotech, Saint Paul, Minnesota, Cat# RP0271D-025) added, 2) 50 ng/mL recombinant canine tumour necrosis factor-alpha (TNF-α, R&D Systems, Minneapolis, Minnesota, Cat#1507-CT-025) added, 3) both IFN-γ and TNF-α added, and 4) neither IFN-γ nor TNF-α added. Lymphocyte reaction plates (48 well) were set up on day 7 (Fig. 3.1). PBMC were seeded at 500,000 cells per well and stimulated with 5 μg/mL concanavalin A. MSC were irradiated with 20 Gy and seeded at 50,000 cells per well. Corresponding MSC treatments were continued accordingly in the reaction plates. After 72 hours, 5-ethynyl-2’-deoxyuridine (EdU, a modified thymidine analogue) was added at a concentration of 10 μM and left for 24 hours before cells were collected and processed according to manufacturer’s directions (Click-iT Plus EdU Flow Cytometry Assay Kit, Fisher Scientific, Ottawa, Ontario). EdU is incorporated during DNA synthesis and is used to quantify newly-synthesized DNA. Staining was completed the following day with Alexa Fluor 647 picolyl azide and analyzed using the Accuri C6 flow cytometer and software.

Data analysis

Results were modelled as multi-factor factorials in a randomized complete block design treating dog as a blocking factor. Least squares means were determined. Log transformation of data was performed where necessary and back-transformed for readability. We tested residuals for normality and plotted them against the predicted values and factors to assess ANOVA assumptions and to look for unequal variance. We found that data were normal
except for outliers in the adipogenesis data, but no outliers were removed. For the gene expression data, least squares means were converted to fold-difference by using $2^{-\Delta\Delta CT}$. Data are presented as mean ± confidence interval with statistical difference assessed at P<0.05. All data analysis was performed using R statistical software (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria).
3.5 Results

MSC isolation

As reported (Russell et al. 2015), our criteria for isolation success was based not only on colony formation, but also the ability to expand to a minimum 5 million cells. Accordingly, 8/8 AT-MSC and only 6/8 BM-MSC met these isolation criteria.

MSC proliferation

AT-MSC proliferated faster than BM-MSC with significantly lower doubling times (P < 0.001) at all passages (P) between 2 and 5 (Fig. 3.2). Proliferation rate also decreased with increasing passage for MSC from both cell sources as significant differences were found between both P2 and P5 (P=0.02) and P3 and P5 (P=0.02). Mean (± 95% confidence interval) doubling time in days were P2: (AT)1.72 ± 0.23, (BM)3.57 ± 0.23; P3: (AT)1.75 ± 0.23, (BM)3.62 ± 0.23; P4: (AT)2.30 ± 0.23, (BM)4.75 ± 0.23; P5: (AT)3.28 ± 0.23, (BM)6.77 ± 0.23.

Immunophenotyping

Very similar cell surface molecule expression profiles were detected between AT- and BM-MSC (Table 3.3). MSC from both sources were highly positive for CD90, CD44, CD29, and MHC I while negative for CD45 and MHC II. Moderate expression of CD73, CD8 and mild expression of CD14 was also found in both cell types. The only differences seen were in the expression of CD4 (AT: moderate, BM: mild) and CD34 (AT: mild, BM: negative).
Figure 3.2

Adipose tissue (AT) derived mesenchymal stromal cells (MSC) proliferate faster than those derived from bone marrow (BM). Population doubling time of canine AT- and BM-derived MSC from passage 2 to 5. (*P<0.05, ***P<0.001; error bars=CI.)

Table 3.3

Surface marker expression of canine adipose tissue (AT)-, bone marrow (BM)-derived mesenchymal stromal cells (MSC), and peripheral blood mononuclear cells (PBMC). Numbers represent percent of populations showing positive expression by flow cytometry (± SD).

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>AT-MSC</th>
<th>SD</th>
<th>BM-MSC</th>
<th>SD</th>
<th>PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC I</td>
<td>97.6</td>
<td>1.8</td>
<td>98.5</td>
<td>1.6</td>
<td>95.4</td>
</tr>
<tr>
<td>MHC II</td>
<td>4.7</td>
<td>1.7</td>
<td>1.5</td>
<td>0.3</td>
<td>99.0</td>
</tr>
<tr>
<td>CD4</td>
<td>48.0</td>
<td>3.5</td>
<td>19.9</td>
<td>9.7</td>
<td>84.9</td>
</tr>
<tr>
<td>CD8</td>
<td>55.3</td>
<td>11.5</td>
<td>58.6</td>
<td>18.6</td>
<td>83.5</td>
</tr>
<tr>
<td>CD14</td>
<td>7.1</td>
<td>2.9</td>
<td>7.6</td>
<td>1.0</td>
<td>94.8</td>
</tr>
<tr>
<td>CD29</td>
<td>81.8</td>
<td>12.2</td>
<td>83.6</td>
<td>18.4</td>
<td>38.0</td>
</tr>
<tr>
<td>CD34</td>
<td>18.6</td>
<td>3.4</td>
<td>3.6</td>
<td>1.5</td>
<td>92.7</td>
</tr>
<tr>
<td>Surface marker</td>
<td>AT-MSC</td>
<td>SD</td>
<td>BM-MSC</td>
<td>SD</td>
<td>PBMC</td>
</tr>
<tr>
<td>---------------</td>
<td>--------</td>
<td>----</td>
<td>--------</td>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td>CD44</td>
<td>100</td>
<td>0</td>
<td>99.8</td>
<td>0.2</td>
<td>100</td>
</tr>
<tr>
<td>CD45</td>
<td>1.5</td>
<td>0.3</td>
<td>1.0</td>
<td>0.4</td>
<td>99.2</td>
</tr>
<tr>
<td>CD73</td>
<td>63.2</td>
<td>5.7</td>
<td>59.9</td>
<td>12.5</td>
<td>97.7</td>
</tr>
<tr>
<td>CD90</td>
<td>99.6</td>
<td>0.3</td>
<td>89.0</td>
<td>7.5</td>
<td>89.4</td>
</tr>
</tbody>
</table>

Global DNA methylation quantification

AT-MSC showed significantly higher (P<0.001) genome-wide DNA methylation levels (6.59 ± 0.52%) than BM-MSC (3.81 ± 0.30%) (Fig. 3.3).

Trilineage differentiation

After an induction period of 14 days, both AT- and BM-MSC stained positive for adipogenesis (Fig. 3.4) and osteogenesis (Fig. 3.5). Adipogenic mRNA transcript abundance (Fig. 3.6) of leptin was upregulated in AT-MSC (11.15-fold, P<0.001) and BM-MSC (12.40-fold, P<0.001) and lipoprotein lipase (LPL) was upregulated in AT-MSC only (6.02-fold, P=0.002). Osteogenic mRNA levels (Fig. 3.7) were upregulated for osteopon5-methylcytosinetin (OPN) in AT-MSC (13.21-fold, P<0.001) and BM-MSC (5.73-fold, P=0.004) and for Runt-related transcription factor 2 (RUNX2) in AT-MSC (4.30-fold, P=0.03) and BM-MSC (6.96-fold, P=0.002). A significant difference was discovered (P=0.03) between AT- and BM-MSC for alkaline phosphatase (ALP) mRNA with upregulation found only in AT-MSC (20.63-fold, P<0.001). Chondrogenesis was unsuccessful (Fig. 3.8) after a 21 day induction period regardless of the concentration of BMP-2 added. However, AT-MSC are BMP-sensitive as noted by increased Toluidine Blue staining and more heterogeneous tissue formation compared to TGF-β3 alone as well as BMP-supplemented BM-MSC.
Immunomodulatory properties

Lymphocyte proliferation assays were used to assess the lymphocyte-suppressive capacity of different canine MSC populations. AT- and BM-MSC equally suppressed stimulated PBMC proliferation when compared with stimulated PBMC alone (Fig. 3.9). Priming the MSC with treatments of IFN-γ, TNF-α, or both had no effect on MSC immunomodulatory capacity.
Figure 3.4

Adipogenic induction of AT- and BM-derived canine MSC. Adipogenic potential of both canine adipose tissue (AT) and bone marrow (BM)-derived mesenchymal stromal cells was indicated with positive Oil Red O staining after 14 days in induction medium. Control samples were negative for Oil Red O staining. Scale bars = 100 μm.
Figure 3.5

Osteogenic induction of AT- and BM-derived canine MSC. Osteogenic potential of both canine adipose tissue (AT)- and bone marrow (BM)-derived mesenchymal stromal cells was indicated with positive Alizarin Red S staining after 14 days in induction medium. Control samples were negative for Alizarin Red S staining. Scale bars = 200 μm.
Figure 3.6

**Upregulation of adipogenesis markers leptin and lipoprotein lipase (LPL).** Difference in adipogenesis marker expression of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells after 14 days in induction medium. Horizontal line represents no change. (**P<0.01, ***P<0.001; error bars=95% CI.)
Figure 3.7
Upregulation of osteogenesis markers alkaline phosphatase (ALP), osteopontin (OPN), and Runx-related transcription factor 2 (RUNX2). Difference in osteogenesis marker expression of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells after 14 days in induction medium. Horizontal line represents no change. (*P<0.05, **P<0.01, ***P<0.001; error bars=95% CI.)
Figure 3.8
Poor chondrogenic potential of both canine adipose tissue (AT)- and bone marrow (BM)-derived mesenchymal stromal cells. Induction time was 21 days in medium containing 10 ng/mL transforming growth factor beta 3 (TGF-β) and between 0 and 200 ng/mL bone morphogenetic protein 2 (BMP-2). Samples stained with (A) hematoxylin and eosin and (B) toluidine blue. Images were adjusted for brightness and contrast. Scale bars = 100 μm.
Figure 3.9

**Canine MSC inhibit T-cell proliferation.** Concanavalin A (ConA)-stimulated peripheral blood mononuclear cells (PBMC) were cocultured with adipose tissue- or bone marrow-derived mesenchymal stromal cells (MSC) treated with interferon-gamma (IFN), tumour necrosis factor-alpha (TNF), both, or neither. Stimulated and unstimulated PBMC were used as controls. Different letters between groups represent a significant difference (p<0.05).
3.6 Discussion

This is the first study to compare the immunomodulatory capacities of canine AT- and BM-MSC in addition to evaluating their general characterization and differentiation potentials. We found that both sources of MSC had proficient immunosuppressive properties. In characterizing AT- and BM-MSC, we found no profound differences between the cell types except for the significantly higher expansion rate of AT-MSC, which has been previously reported (Kang et al. 2012). Faster proliferation along with the potential for a less invasive method of their procurement makes them the preferred source for canine MSC.

We cocultured PBMC stimulated with Con-A with irradiated AT- or BM-MSC in order to determine whether they could suppress lymphocyte proliferation. We tested MSC cultured with and without proinflammatory factors IFN-γ, TNF-α, or both for 3 days leading up to, plus the 4 days of, coculture with PBMC. While all treatment groups successfully suppressed PBMC proliferation, no treatment significantly outperformed any other within each source group (Fig. 3.9). It is possible that a larger sample size would discern greater differences trending toward BM-MSC as PBMC proliferation is consistently lower in these wells across treatments.

If differences in immunomodulatory capacity were to emerge, the question of whether differences in surface marker expression might correlate with a more potent immunosuppressive phenotype becomes an interesting one. Our results are in agreement with the canine literature for those surface markers that show consistent expression across several studies (Kamishina et al. 2006; Csaki et al. 2007; Kang et al. 2008, 2012; Vieira et al. 2010; Lee et al. 2011b; Martinello et al. 2011; Takemitsu et al. 2012; Kisiel et al. 2012; Screven et al. 2014), in particular, positive CD90 and CD44 and negative CD45 expression. AT-MSC showed moderately higher expression of CD34 (18.6% ± 3.4) than BM-MSC (3.6% ± 1.5)
and CD4 (48.0% ± 1.7 versus 19.9 ± 9.7). All other markers fell within the same ranges of expression as seen in table 3.3. It has been recently reported that fat and BM harvest sites do have some influence on surface marker expression (Sullivan et al. 2016), and likely played a role here as well.

Our first attempt at chondrogenesis of canine MSC (Russell et al. 2015) was poorly demonstrated after 21 days in the induction medium we use routinely with equine MSC (Berg et al. 2009; Co et al. 2014). To enhance our induction medium, we added BMP2 at different concentrations based on several reports showing it was a potent driver of MSC chondrogenesis (Schmitt et al. 2003; Guilak et al. 2004; Toh et al. 2005; Shirasawa et al. 2006; An et al. 2010). Unfortunately, in spite of these efforts, chondrogenesis did not improve (Fig. 3.8). Pellets generally appeared necrotic with no evidence of lacunae formation across all samples. AT-MSC were BMP sensitive as evidence by altered pellet morphology and Toluidine Blue staining pattern. BMPs therefore remain candidates for aiding the chondrogenic differentiation, but more work is needed to determine their temporal and co-induction molecular interplays. It should be noted that canine chondrogenesis has not been robustly demonstrated in the literature as has been noted by others (Kisiel et al. 2012). Until an effective induction protocol is found, it appears that in vitro MSC chondrogenic differentiation is limited in the dog compared to other species.

Adipogenic potential was demonstrated with induced cells rich with lipid droplets stained with Oil Red O (Fig. 3.4). Histological data was supported by gene expression analysis showing upregulation of adipogenesis markers leptin in both AT- and BM-MSC and LPL in the AT-MSC samples (Fig. 3.6). Likewise, osteogenic potential was also demonstrated with evident mineralization stained with Alizarin Red S supported by upregulation of osteogenesis markers OPN and RUNX2 in both AT- and BM-MSC and ALP in AT-MSC (Figs. 3.5 and 3.7). It was thought that the reduced global DNA methylation levels of BM-
MSC (Fig. 3.3) might provide the cells stronger differentiation potential (Collas et al. 2008; Berdasco and Esteller 2011). However, at least with the three lineages induced, global DNA methylation levels had little effect. It would be interesting to examine the ability of these cells to differentiate outside the trilineage cell fates.

Ultimately, there were few differences detected between AT- and BM-MSC with regard to immunophenotyping, differentiation potential, or immunomodulatory capacity. Major differences between the sources of MSC were only found in DNA methylation levels and proliferation doubling time. This may seem counterintuitive, but this simple measure of global methylation accounts neither for specific patterns of DNA methylation nor other factors affecting gene expression like histone modification. While the difference in DNA methylation appears to have no detectable effect on differentiation potential, a higher rate of proliferation provides a key advantage to AT-MSC.

Regardless of cell source, the significant in vitro suppression of mononuclear cells warrants in vivo investigation of canine AT- and BM-MSC efficacy in modulating the immune system of inflammation-based conditions. As for their progenitor side, new protocols for chondrogenesis will need to be developed if canine MSC are to serve as chondroprogenitor cells. Failing that, other canine cells with chondrogenic potential should also be considered.
Chapter 4

Reprogramming of canine mesenchymal stromal cells and chondrocytes
Joint cartilage defects are a significant source of pain in dogs and can lead to chronic osteoarthritis if left untreated. No treatments exist that provide long-term pain relief or restore precondition levels of function. Cell replacement strategies, including the use of MSC and chondrocytes, are being explored for cartilage tissue regeneration.

Replicative aging remains an obstacle for wider MSC use (Fehrer and Lepperdinger 2005). A robust solution to cellular aging might be to “reset” the replicative age of MSC through their derivation from pluripotent cells such as iPSC. If the iPSC-MSC telomere length is restored, capacities for both proliferation and differentiation might be improved when compared to the original MSC. The generation of iPSC-MSC may provide the best of both the MSC and the iPSC, and would be a consistent source of cells for long-term single patient treatment. Further, if iPSC could consistently generate immunosuppressive MSC-like cells, allogeneic cell therapies could be obtained from a potentially infinite source.

Based on the results of our characterization studies showing the poor chondrogenic potential of canine MSC, we also decided to direct our research to re-examine chondrocytes, the principal cells found in healthy cartilage, as the starting cell for cartilage regeneration therapies. Although seemingly the most logical starting point, chondrocytes’ limited proliferative capacity has historically been the main deterrent for their use in tissue engineering strategies (Nesic et al. 2006). In addition, during *in vitro* expansion of chondrocytes, they dedifferentiate into a fibroblast-like cell (Fig. 4.1) with lower chondrogenic potential (Schulze-Tanzil 2009). Similar to MSC, generation of iPSC from canine chondrocytes could provide a perpetual source of cells that could retain epigenetic memory of their chondrogenic origin.
4.2 Hypothesis

An unlimited supply of canine chondroprogenitor cells can be generated from putative mesenchymal stromal cell- or chondrocyte-derived iPSC.

4.3 Objectives

1. To generate MSC-derived iPSC via Sendai viral delivery of the c-Myc, Klf4, Oct4, and Sox2 transgenes.
2. To generate chondrocyte-derived iPSC via the piggyBac transposon system using doxycycline-inducible transgene expression of c-Myc, Klf4, Oct4, and Sox2.
3. To evaluate the chondrogenic potential of putative canine iPSC.
4.4 Materials and methods

MSC reprogramming

Canine AT- and BM-MSC were thawed and grown for 2 days prior to transduction (Fig. 4.2). Transduction of MSC was performed with the Cytotune 2.0 Sendai kit (Thermofisher) at 300,000 cells per well at a 5-5-6 MOI (h(Klf4, Oct4 and Sox2), hc-Myc, hKlf4). Cells were cultured on tissue culture plates in canine MSC expansion medium as above. A week following transduction with Sendai, the cells were passaged from their original well into 10 cm dishes with mouse embryonic fibroblast (MEF) feeder cells. On day 8, the medium was changed to ciPSC culture medium: KnockOut Dulbecco’s modified eagle medium with 15% KnockOut serum replacement, L-glutamine, nonessential amino acids, penicillin–streptomycin, beta-mercaptoethanol, human recombinant LIF (10 μg/mL) and basic FGF (4 ng/mL). After 6 weeks, non-permeabilized cell colonies were incubated for 90 minutes with a fluorophore-conjugated pluripotency marker stage-specific embryonic antigen 4 (SSEA-4, eBioscience) diluted in PBS for assessment and imaging.

Chondrocyte reprogramming

We isolated cells from explanted canine articular cartilage (cAC) from dogs euthanized for reasons unrelated to this study (age range: 6.8 - 9.8 years old). Articular cartilage was removed and stored overnight in a tissue culture dish containing PBS with 1% ABAM. Larger pieces were then cut into 1-2 mm squares, placed in a 1% collagenase solution, and incubated at 37°C overnight. After digestion, media were filtered through a 40 micron filter and spun down at 600 g for 6 minutes. The pellet was resuspended, counted, and seeded at 8000 cells/cm² in cAC expansion medium (EM) consisting of DMEM F12 with 5% FBS.

Transfections were performed (~50% efficiency, ~43% recovery) as previously de-
Figure 4.2
**Schematic of canine mesenchymal stromal cell (MSC) reprogramming.** MSC were cultured on tissue-culture plastic in MSC expansion medium for 2 days prior to transduction with Sendai virus containing human Oct4, Sox2, Klf4, and c-Myc (OSKM). One week after transduction, cells were passaged onto irradiated mouse embryonic fibroblasts (iMEF). The next day, the medium was changed to induced pluripotent stem cell (iPSC) medium. Courtesy of Courtney Brooks.

scribed (Parreno et al. 2016) after comparing other transfection kits commercially available (Fig. 4.3). Briefly, 2 million cAC were suspended in 100 μL of buffer containing 5 mM KCl, 15 mM MgCl₂, 120 mM Na₂HPO₄/NaH₂PO₄, and 50 mM mannitol and placed in the device-specific cuvette. Cells were transfected using the Nucleofector II device (Amaxa) with the program U024.

Expanded cells were transfected with the 4 Yamanaka factors (separate or polycistronic murine or separate canine c-Myc, Klf4, Oct4, Sox2) using the piggyBac transposon system (Fig. 4.4). Transfections also included eGFP to confirm transfection success except in one case when human telomerase reverse transcriptase (hTERT) was used. The addition of hTERT was to prolong cell divisions in an attempt to improve reprogramming efficiency (Lee et al. 2004; Nicholson et al. 2007). Immediately after transfection with factors, cells were resuspended and seeded on MEFs in high serum EM (20% FBS) for 4 hours before media was changed to cAC EM. On day 1, media was changed to EM with 1.5 μg/ml doxycycline. On day 2, media was changed to either SR media containing DMEM F12 with
Figure 4.3

Transfection efficiency test. Canine chondrocytes were transfected with eGFP using 3 transfection reagent kits, Fugene HD, Lipofection 3000, and jetPrime, or the Nucleofector II device. Scale bar = 100 µm.

15% KnockOut serum replacement, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 4 mM GlutaMAX, 50 U/ml penicillin/streptomycin (Invitrogen), 200 U/ml hLIF (Millipore), 5 µg/ml insulin or insulin growth factor (Sigma), and 4 ng/ml bFGF (Peprotech), SR with 1 µM mitogen-activated protein kinase inhibitor PD0325901 and 3 µM glycogen synthase kinase-3 inhibitor CHIR99021 (SR + 2i, both from StemGent), or NutriStem hPSC XF medium (NS, Stemgent) (Fig. 4.5). All OSKM-transfected cells were cultured for a minimum of 28 days before plates were discarded. Select colonies were manually cut and replated in 4 well plates.
4.5 Results

MSC reprogramming

Cell colonies formed only in the AT-MSC plates and stained positive for SSEA-4 despite not displaying typical cESC colony morphology (Fig. 4.6).

Chondrocyte reprogramming

After 6 - 12 days, colonies began to appear with a variety of morphologies (Fig 4.7). Some colonies were seen briefly containing cells with ESC-like morphology (Fig 4.8). Polycistronic expression of OKMS produced many more colonies than when using separate factors (Fig. 4.9). Use of 2i in media suppressed colony formation. No colonies could be proliferated long-term. Cell colonies would ceased to expand either before or after passaging.
Figure 4.5
**Media used for canine chondrocyte reprogramming protocol.** Three rounds of reprogramming were attempted. In round 1, the transfected cells were cultured in SR medium which consists of Dulbecco’s Modified Eagle’s Medium (DMEM) F12 with 15% KnockOut Serum Replacement (KSR), human leukemia inhibitory factor (hLIF), and basic fibroblast growth factor (bFGF). In round 2, 6 different treatments were used: SR; SR with inhibitors of mitogen-activated protein kinase kinase and glycogen synthase kinase 3β (2i) from day 0, 2, 4, or 6; or NutriStem (NS). In round 3, SR or NS was used.
Figure 4.6
SSEA-4 staining of 3 representative colonies of partially reprogrammed canine adipose tissue-derived MSC. Courtesy of Courtney Brooks.

Figure 4.7
Colony morphology of canine chondrocytes after transfection with Yamanaka factors and eGFP. Scale bar = 100 μm.
Figure 4.8
Cell morphology of select canine chondrocytes after transfection with Yamanaka factors and eGFP. Scale bar = 20 μm.
Figure 4.9
Wells containing canine chondrocytes after transfection with polycistronic and separate Yamanaka factors and eGFP. Many colonies can be seen as white specs in this macro view of a representative well of chondrocytes after transfection with polycistronic Yamanaka factors (left) versus those transfected with separate factors where few colonies can be seen (right).
4.6 Discussion

Some colonies appeared from the transfected AT-MSC that did not resemble typical colonies of the primed-type human ESC or the naïve-type mouse ESC. The cell colonies did stain positive for the pluripotency marker SSEA-4 suggesting that some level of reprogramming may have taken place. Although there is also some evidence that cAC are responsive to the reprogramming methods employed, the conditions necessary to sustain the cells with a pluripotent phenotype were not found. Transfection of cAC with hTERT in addition to OSKM did not appear to further reprogramming of the cells. Further trial and error with various media formulation may yet yield a truly pluripotent cell line.

Without exception, retroviral integration of transgenes has been the sole method used to deliver transgenes to canine cells for reprogramming thus far (Shimada et al. 2009; Lee et al. 2011a; Luo et al. 2011; Whitworth et al. 2012; Gonçalves et al. 2012, 2017; Koh et al. 2013; Nishimura et al. 2013; Koh and Piedrahita 2015; Baird et al. 2015). The Sendai viral or piggyBac transposon systems delivering non-integrative or excisable transgenes are implicitly safer for future cellular therapies and accordingly are alternatives worth pursuing. Nevertheless, these safer alternatives are evidently not as straightforward to use with canine cells, and more research is necessary. Perhaps, reprogramming fibroblasts with these methods would be a reasonable provisional step since all but one of the canine iPSC research groups used the fibroblast as a starting cell (Shimada et al. 2009; Lee et al. 2011a; Luo et al. 2011; Whitworth et al. 2012; Gonçalves et al. 2012, 2017; Koh et al. 2013; Nishimura et al. 2013). Other variables to explore include culturing the cells in hypoxic conditions (5% O₂) to simulate the oxygen tension of the stem cell niche, or the addition of small molecules such as histone deacetylase inhibitor valproic acid and vitamin C to aid in chromatin remodelling (González et al. 2011). It may also be that the objective
could have been achieved under the same conditions with cells derived from a younger donor (Mahmoudi et al. 2013). Indeed, the one canine iPSC line derived from a cell type other than the fibroblast was derived from AT-MSC collected from an 11 month old dog while undergoing surgery (Baird et al. 2015).

In conclusion, canine MSC and chondrocytes do appear receptive to reprogramming with the Sendai viral and piggyBac transposon systems utilized. Unfortunately, reprogramming was incomplete, and the cells produced were too unstable to proliferate long-term. The establishment of canine iPS cell lines using these methods will come when the right balance of conditions is achieved.
Chapter 5

General discussion
This project began with the overarching goal of answering some necessary questions about the culture and function of canine MSC and the possibility of using iPSC as a starting point for cellular therapies. The overall hypothesis of this PhD project was partially addressed by the first objective. Canine MSC can be isolated from AT and BM aspirate, and that they respond equally to our differentiation and immunomodulatory assays has strong evidence to support it. As expected, the intentionally ambitious second objective of reprogramming canine cells with methods better suited for future clinical application has been more challenging to fulfill. However, success is built on a scaffold of failure, and these contributions will guide the next researcher to take up the challenge.

Throughout this project, other questions have been answered as well. We now know that PL is not a sensible alternative to FBS in the culture of canine (or equine) MSC due to its adipogenic effects. The PL study from the first objective remains the only published investigation of PL as a canine MSC culture supplement and should caution clinicians against the combination of MSC with any platelet-derived product. We also contributed to the slowly growing literature of canine MSC characterization and MSC source comparison. We discovered that the current chondrogenesis protocols are not effective in producing credible chondrocyte morphology or cartilage tissue from canine MSC. It may be that canine MSC are not a plausible source for cartilage tissue engineering strategies, which is why we turned our attention to reprogrammed chondrocytes as a possible chondrogenic source. Finally, we found that both AT- and BM-MSC are effective mediators of lymphocyte proliferation in vitro.

At the end of a long journey such as this, questions often arise about how things could have been done better. In general, the use of fetal-sourced tissues would be preferred especially for reprogramming studies as this is known to increase reprogramming efficiency (Mahmoudi and Brunet 2012). Unfortunately, sourcing tissues as we did from random eu-
thanized dogs gave us little control over the ages. Even with the characterization studies, donor age may have played a role and should not be underestimated (Katsara et al. 2011). It would be interesting to rerun these same experiments with cells derived from fetal tissues to learn how much of an effect donor age does have in canine cell function and reprogramming.

For the PL study, the results were so unexpected and contradictory to human PL studies that many unanswered questions remain. Would human MSC grown in human PL at any of the doses we tested show signs of lipid droplet formation if histologically stained? Does canine PRP also induce adipogenesis in canine MSC? Would human PL have the same effect on canine MSC or, conversely, would canine PL have the same effect on human MSC? These are fascinating questions that should be answered.

Another unresolved question linking the spontaneous adipogenesis from the PL study to the directed adipogenesis from the characterization study is why adipocyte marker expression was so different between the two cases. While it is clear from histological examination that similar levels of adipogenesis is occurring, the PL-induced samples show upregulation of CEBPA and FABP4, and the induction medium-induced samples show upregulation of leptin and LPL. This difference may point to the relative stage of adipogenesis with the PL-induced samples revealing a less mature phenotype. CEBPA expression is known to occur early in adipogenesis, while leptin and LPL are expressed late in more mature adipocytes (Niemela et al. 2008). Exploring these genes’ expression at several time points would help clarify the progression of induction in both cases.

The biggest take-away message from the PL study is how severely culture methods can affect the character of a cell. There are two disparate ways of reacting to this message. First, standardization of methods across the discipline is key to advancing the field of MSC
study. This point, as has been discussed in the introduction, is the thrust behind the ISCT criteria for defining MSC—although, these strict criteria have their drawbacks also as discussed below. Secondly, this opens up research into different culture methods to produce MSC with different qualities for different applications. Culture medium composition is but one of several factors including cell source characteristics and differences in technical protocols discussed by Ksiazek that may affect MSC proliferation (Ksiażek 2009). Of course, the effects of these factors are not limited to proliferation. Cytokine production, trophic abilities, and differentiation potential can all be influenced. Research into these variables is ongoing with investigations into such approaches as growth factor media supplementing, coculturing with other cell types, and substrate surfaces (Tsuchiya et al. 2004; Coutu et al. 2012; Mahmoudi et al. 2013). That is not to suggest that we do not first need an accepted standard method of MSC culture to use as controls in these experiments, without which measurement and comparison of these factors are unreliable.

During the characterization study, the defects of the ISCT criteria soon became apparent. As stated in the introduction, the ISCT recommends that MSC be identified with positive expression of CD105, CD73 and CD90, and negative expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. According to our results, AT-MSC were not CD34 negative (18.6% ± 3.4). We are not the first group to report CD34 positive canine MSC (Kang et al. 2008; Sullivan et al. 2016). This speaks to the human-specific focus of the ISCT guidelines and the lack of a consensus surface marker panel for canine MSC. Moreover, even in human studies, it has been reported that AT-MSC express CD34 and gradually lose this expression over time in culture (Lin et al. 2012a). With immunophenotype so susceptible to change, what researchers are left with is little more than a vague map of questionable utility. In practice, until the cell surface proteome can provide predictive identification of desired characteristics in the resulting subset populations, the MSC
researcher must accept that they are dealing with a population of cells with varying levels of proliferative and differentiation capacity (Książek 2009; Sarugaser et al. 2009).

Overinterpreting of canine chondrogenesis may be another consequence of the ISCT criteria. Successful chondrogenesis of canine MSC had been reported elsewhere (Csaki et al. 2007; Neupane et al. 2008; Seo et al. 2009; Vieira et al. 2010; Reich et al. 2012). But, as was noted in our manuscripts and elsewhere (Kisiel et al. 2012; Russell et al. 2015, 2016), canine chondrogenesis has not yet been robustly demonstrated. The poor chondrogenesis of our canine MSC was puzzling since our lab has not experienced a similar global failure in our equine MSC work (Berg et al. 2009; Co et al. 2014). We do observe variability in chondrogenic potency among equine cell cultures (donor-donor variability), but we have not observed general failure as in these canine cultures. It therefore seems like MSC chondrogenic differentiation is associated with species differences between the dog and other species. It may be that some researchers may feel pressure to tick off the chondrogenesis box of the ISCT criteria in spite of poor evidence and feel justified in doing so since the focus of their study lies elsewhere.

It is for these reasons that the ISCT criteria should be used as a flexible guide to defining MSC with acknowledgement to their shortcomings. Other critiques of the ISCT have also appeared over the years (Bersenev 2012; Samsonraj et al. 2015). It is, at least, encouraging to see that the organization is beginning to adapt and expand their interpretation of MSC to include recommendations for the assessment of immunological characteristics of the cells (Krampera et al. 2013; Galipeau et al. 2015). However, the same focus on human MSC may limit the proposal’s usefulness in other species. For example, one of the recommendations is that IDO response be measured to assure successful priming. But, this mechanism is not found in mouse, and there have been contradictory findings about IDO in dogs (Kang et al. 2008; Saulnier et al. 2016).
Still, another suggestion they made recently helped in designing our own immunoassay: that MSC be primed with IFN-γ ± TNF-α. It is not clear why the priming agents we used in our lymphocyte proliferation assay did not increase the immunosuppressive effect of our MSC. Clues may be found in a recent review of the MSC licensing process. In it, Krampera outlines several variables that may hamper the immunoregulatory phenotype of MSC if improperly applied including agent dose and length and timing of exposure (Krampera 2011). Unfortunately, since our immunoassay was performed near the end of the characterization study, we did not have the luxury of testing a range of IFN-γ and TNF-α concentrations or durations of priming due to dwindling MSC stock. Regardless, even without the additive effects of priming, there is sufficient evidence of immunomodulatory qualities to warrant further in vivo investigation.

In the end, MSC source was not a significant contributor to the variation found among cultures’ response to our differentiation and immunomodulatory assays. That is not to say, however, that one source has no benefit over the other. Acquiring AT can be a less invasive process than BM aspirate if autologous applications are being considered. Moreover, AT-MSC’s faster proliferation is a clear advantage for shorter expansion times to clinically sufficient numbers for both autologous and allogeneic use. Ultimately, the results of these studies will contribute to safer and more efficient protocols necessary to bring the clinical use of canine MSC into common veterinary practice.

MSC are still one of the most promising cell types for regenerative medicine going forward, even if there has been a shift in their likely role. Much has been made about the diminishing relevance of MSC’ in vivo progenitor functions for clinical applications (Horwitz and Dominici 2008; Caplan and Correa 2011; Keating 2012). But, even though the focus of their non-progenitor functions has been their immunosuppressive capabilities, they also have trophic functions that have the potential to be exploited for therapeutic purposes in-
cluding promotion of angiogenesis and extracellular matrix formation as well as induction of progenitor cell differentiation (Wang et al. 2014). This last mode could help improve tissue engineering strategies through coculture with primary or passaged chondrocytes as an example, especially in species like the canine where MSC themselves have poor chondrogenic potential (Tsuchiya et al. 2004; Wang et al. 2013; Kubosch et al. 2016). In these cocultures, MSC’ progenitor functions could return to prominence in a role complementary to its trophic one. For example, native cartilage has a layered conformation, and using different ratios of MSC:chondrocytes in a similarly layered in vitro system may help engineer analogous constructs.

Cartilage engineering for the dog may have to rely more heavily on chondrocytes than in other species. However, the obstacles of limited numbers of cells that can be harvested, dedifferentiation, and limited propagation in culture must be overcome. This may be accomplished by culture or by transformation. The work that was started here has shown signs that these approaches are the right ones to follow. We made the serendipitous discovery that certain inhibitors added to the culture could revert dedifferentiated chondrocytes to a primary chondrocyte morphology (see Appendix B). We saw the brief appearance of cells with ESC-like morphology during reprogramming. These results are encouraging and should be taken forward to their conclusion, one which will yield a robust chondrogenic cell source that can be used to address any size defect in canine joints.

Many obstacles and gaps in our knowledge of canine cells remain. The results of this project will guide canine researchers and clinicians to optimal handling and culture of MSC, to better knowledge of the MSCs’ strengths and weaknesses, and to the production of transgene-free iPSC or immortalized cell lines, which will be safer for use in the clinic.
Appendix A

Equine platelet lysate as an alternative to fetal bovine serum in equine mesenchymal stromal cell culture - too much of a good thing?

This chapter is a modified version of Russell and Koch (2015).
A.1 Summary

Reasons for performing study:

Multipotent mesenchymal stromal cells (MSC) are often culture-expanded *in vitro*. Presently, expansion medium (EM) for MSC is supplemented with fetal bovine serum (FBS). However, increasing cost, variable composition, and potential risks associated with bovine antigens call for alternatives. Platelet lysate (PL) has shown promise as an alternative supplement.

Objectives:

To determine how equine umbilical cord blood (CB) MSC proliferate in EM enriched with PL or FBS at various concentrations.

Study Design:

A randomized dose escalation study was performed, and cell proliferation was determined using a resazurin semi-quantitative assay.

Methods:

Platelet concentrate was generated from 5 equine whole blood samples through a double centrifugation method and standardized to \(1 \times 10^6\) platelets/µL prior to a freeze/thaw cycle to produce PL. Pooled PL or pooled FBS was added to EM at concentrations of 5% to 60%. Proliferation of 4 equine CB-MSC cultures was determined after 4 days.
Results:

CB-MSC proliferated with a dose-dependent response with no significant difference found between PL and FBS up to a 30% concentration. Beyond 30%, proliferation fell in the PL-cultured cells, while continued dose-dependent proliferation was noted in the FBS-cultured cells. Despite reduced cell numbers in high PL concentrations, live/dead staining revealed that adherent cells remained viable.

Conclusions and potential relevance:

We determined that EM enriched with PL can support short-term equine CB-MSC proliferation at conventional culture concentrations. Based on the unexpected suppression of CB-MSC at higher PL concentrations, an in vivo dose study is indicated to investigate if combinational therapies of CB-MSC and PRP are associated with synergistic or antagonistic effect on CB-MSC function.

A.2 Introduction

Multipotent mesenchymal stromal cells (MSC), also known as mesenchymal stem cells, play a key role in many emerging equine therapies in the field of veterinary regenerative medicine (Koch et al. 2009). Common to all current equine MSC culture protocols is the use of fetal bovine serum (FBS) as a biological supplement to support MSC growth through a variety of largely unknown growth factors and cytokines. However, the use of FBS is associated with challenges such as high variability in batch-to-batch composition, risk of immune response to bovine antigens, and rising cost (Brindley et al. 2012; Bieback 2013).

Platelet lysate (PL) has been used as a FBS substitute in expansion medium (EM)
to support growth of human MSC with PL showing a higher mitogenic effect than FBS (Doucet et al. 2005). PL can be derived from whole blood through simple centrifugation steps and a freeze/thaw cycle to release growth factors from lysed platelets of the resulting platelet concentrate (PC) or platelet-rich plasma (PRP), thus providing a species-specific FBS alternative.

The influence of concentration and how equine PL and FBS compare is a current knowledge-gap. The hypothesis of this study was that PL and FBS equally support proliferation of equine cord blood- (CB-)MSC.

A.3 Materials and methods

Platelet concentrate and platelet lysate

Whole blood (800 - 900 mL) was collected from 5 adult horses from the University Equine Research Herd. All procedures complied with University of Guelph animal care committee protocols (Animal Utilization Protocol #1756). PC was generated as previously described, (Fig A.1) (Textor et al. 2011). Briefly, blood was centrifuged at 200 g for 15 minutes. The plasma was then centrifuged at 400 g for 15 minutes. The platelet pellet was then resuspended with aspirated platelet-poor plasma (PPP). The PC was diluted to $1 \times 10^6$ platelets/µL after a complete blood count was performed. PL was then generated by a single freeze/thaw cycle at -80°C overnight followed by a 37°C thaw as previously described (Schallmoser and Strunk 2013). Resulting products were pooled, centrifuged at 4000 g for 15 minutes, and filtered with 0.22 µm filters.
Growth factor immunoassays

FBS, PPP, PC, and PL samples after 1, 2, and 3 freeze/thaw cycles were assayed with the PDGF-BB immunoassay\(^b\) as a proxy for general growth factor content according to the manufacturer’s direction. Subsequently, FBS and a pool of 3 PL were assayed with both PDGF-BB and TGF-β immunoassays\(^b\). All samples were read at 450 nm (EL800 Universal Microplate Reader\(^c\)).

Thawing and expansion of CB-MSC

Equine CB-MSC isolated and cryobanked as previously described (Berg et al. 2009) from 4 unrelated foals were quick-thawed in 37°C water and plated in culture flasks at 5000 cells/cm\(^2\). The cells were incubated at 38.5°C, 5% CO2 in humidified atmosphere. EM consisted of low glucose (1 g/L) Dulbecco’s Modified Eagle Medium\(^d\), 30% FBS\(^e\), 1% penicillin/streptomycin\(^e\), 1% L-glutamine\(^d\), and 2 U/mL heparin\(^f\). All cells were expanded for 5 days before the proliferation assays.
Proliferation and viability assays

MSC were seeded in 96-well plates at 5000 MSC/cm², cultured in 100 µL of 10% pooled FBS EM and left overnight to allow for cell attachment. The following day, EM was substituted by 100 µL of treatment medium containing either pooled PL (n=5) or pooled FBS (5 independent batches) at concentrations between 5% and 60%. After 4 days, treatment media were completely replaced with 100 µL of assay master mix consisting of PBS with 10% resazurin. Plates were incubated at 38.5°C for 4 hours and read by plate reader (SpectraMax® i3 Multi-Mode microplate reader).

Calcein AM and propidium iodide were added at 2 and 5 µM respectively to representative PL and FBS wells for fluorescent imaging of cell viability (EVOS® FL cell imaging system).

Data analysis

All data were analyzed using R statistical software. Data from the ELISAs were evaluated with ANOVA. To compare treatment media at different concentrations, Fisher’s protected LSD was used to calculate unadjusted p-values. Data are presented as mean ± confidence interval. Statistical difference was assessed at P<0.05.

A.4 Results

Platelet concentrate and platelet lysate

Preliminary PL feasibility studies demonstrated that PDGF-BB was released by the freeze/thaw method to up to 20 times (p < 0.001) the concentration of what was found in the PC sample (Fig A.2). WBC concentrations of the PC samples used ranged from 0.2 – 1.3 WBC/µL with a mean platelet:WBC ratio of 1175 (data not shown).
Figure A.2
Relative concentration of platelet-derived growth factor BB (PDGF-BB) at all stages of equine platelet lysate (PL) generation. FBS = Fetal bovine serum, PPP = platelet-poor plasma, PC = platelet concentrate, F/T # = number of freeze/thaw cycles. ***P<0.001.

Growth factor immunoassays

Subsequent ELISAs found concentrations of PDGF-BB to be 5.2 ng/mL (p = 0.01) in the PL sample with no PDGF-BB found in the FBS sample. TGF-β levels were more than double in PL at 24.5 ng/mL than FBS at 11.8 ng/mL (p = 0.04) (Fig A.3).

Proliferation and viability assays

Equine CB-MSC showed a dose-dependent response with no significant difference between the pooled PL and FBS treatments up to a concentration of 30% (Fig A.4, Table A.1). Sig-
Platelet-derived growth factor BB (PDGF-BB) and transforming growth factor beta (TGF-β) concentrations in fetal bovine serum (FBS) and equine platelet lysate (PL).

*P<0.05.

Significant differences begin to emerge between the treatments beyond 30% with CB-MSC proliferative response to PL decreasing and response to FBS continuing to increase.
Resazurin proliferation assay of equine cord blood mesenchymal stromal cells cultured for 4 days in fetal bovine serum-(FBS-) or platelet lysate-(PL-)enriched medium from 5% to 60%. DMEM (base medium only) was used as negative control. n=4 with 4 technical replicates per treatment. ***P<0.001, *P<0.05, error bars represent confidence interval.

Table A.1
Multiple comparisons in CB-MSC proliferative response to PL and FBS treatment media at the same concentrations. P-values are unadjusted and were calculated by Fisher’s protected LSD. LCL = lower confidence limit; UCL = upper confidence limit.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Difference</th>
<th>Unadjusted p-value</th>
<th>LCL</th>
<th>UCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS.5 - PL.5</td>
<td>-1.40E+07</td>
<td>0.05</td>
<td>-2.80E+07</td>
<td>9.80E+04</td>
</tr>
</tbody>
</table>
Calcein AM fluorescent staining revealed that although there were CB-MSC lost in the higher concentrations of PL, remaining adherent cells were viable (Fig A.5).

### A.5 Discussion

For *in vitro* expansion of CB-MSC, PL was shown to perform equally to FBS at all conventionally-used concentrations. The bell-shaped curve of the CB-MSC proliferative response to PL medium was an unexpected finding. Similar studies on BM-MSC from human subjects have only tested PL to up to a 10% or 20% concentration, wherein a dose-dependent response is noted (Doucet et al. 2005; Horn et al. 2010). The identification of a PL threshold at 30% was in contrast to that of FBS-treated CB-MSC which exhibited a continued positive correlation with increased concentrations. The mechanism behind the loss of CB-MSC at higher PL concentrations are undetermined. Contribution of catabolic gene expression is unlikely since platelet:WBC ratios showed that PC were purified sufficiently prior to platelet lysis (McCarrel et al. 2012). Cell death may be involved in the cell loss although remaining adherent CB-MSC were viable cells. These findings raise questions about concurrent use of PRP/PC and MSC *in vivo* (Renzi et al. 2013). It may be that current preparations of PRP deliver too many platelets to the injury site negating therapeutic effects of MSC.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Difference</th>
<th>Unadjusted</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>p-value</td>
<td>LCL</td>
<td>UCL</td>
</tr>
<tr>
<td>FBS.10 - PL.10</td>
<td>-8.60E+06</td>
<td>0.2</td>
<td>-2.30E+07</td>
<td>5.40E+06</td>
</tr>
<tr>
<td>FBS.20 - PL.20</td>
<td>-1.30E+07</td>
<td>0.06</td>
<td>-2.70E+07</td>
<td>7.90E+05</td>
</tr>
<tr>
<td>FBS.30 - PL.30</td>
<td>-9.30E+06</td>
<td>0.2</td>
<td>-2.30E+07</td>
<td>4.70E+06</td>
</tr>
<tr>
<td>FBS.40 - PL.40</td>
<td>1.50E+07</td>
<td>0.03</td>
<td>1.20E+06</td>
<td>2.90E+07</td>
</tr>
<tr>
<td>FBS.50 - PL.50</td>
<td>3.00E+07</td>
<td>&lt;0.001</td>
<td>1.60E+07</td>
<td>4.40E+07</td>
</tr>
<tr>
<td>FBS.60 - PL.60</td>
<td>4.80E+07</td>
<td>&lt;0.001</td>
<td>3.40E+07</td>
<td>6.20E+07</td>
</tr>
</tbody>
</table>
Previous equine PL studies show little detail with regard to MSC proliferation (Del Bue et al. 2007, 2008; Seo et al. 2013). Del Bue et al. (2007, 2008) reported a dose-dependent response to PL in two separate experiments with equine AT-derived MSC (Del Bue et al. 2007, 2008). However, few biological samples were tested, and FBS was not included as a control group in these studies. One study that did perform a comparative study with FBS reported no significant difference between the two supplements at 10% which is in accordance with our findings (Seo et al. 2013).

Equine PL is an inexpensive, easy-to-make supplement that our data suggest is able to deliver high levels of growth factors to enhance the proliferation of equine CB-MSC
with an acknowledgement that there may be a threshold for its safe use with MSC. Future efforts should be directed at determining whether this effect on proliferation is similar in MSC derived from different sources such as AT or BM, or is sustained over longer terms and over multiple passages of the cells. In vivo PRP and MSC dose titration studies appear warranted to determine if co-injection has synergistic or antagonistic effects.

A.6 Manufacturers’ addresses

a. Millipore, Etobicoke, Ontario, Canada
b. R&D Systems, Minneapolis, Minnesota, USA
c. Bio-tek Instruments, Winooski, Vermont, USA
d. Lonza, Walkersville, Maryland, USA
e. Life Technologies, Grand Island, New York, USA
f. Sandoz, Boucherville, Québec, Canada
g. Roche, Indianopolis, Indiana, USA
h. Sigma-Aldrich, St. Louis, Missouri, USA
i. Molecular Devices, Sunnyvale, California, USA
j. R Foundation, Open Source Software
Appendix B

Culture media tests for reprogramming canine chondrocytes
Passage 3 cAC were seeded at 2 different densities (50,000 and 100,000) in various pluripotent cell culture media to determine which would provide the best environment for reprogramming. Media tested were NutriStem hPSC XF medium (NS, Stemgent), SR media (SR + FGF, described above), SR with pigment epithelium-derived factor (SR + PEDF) in place of bFGF, and SR with 2i medium (SR + 2i). Cells were also grown in cAC expansion medium (F12 + FBS, described above) as a control.

Results

The NS medium seemed to support cAC proliferation equally to that of F12 + FBS (Fig. D.1). SR + FGF also supports a high rate of proliferation, although the cells’ morphology were altered. The SR + PEDF medium had the most detrimental effect of cAC proliferation and morphology. The morphology of cells seeded at high density in 2i changed the most drastically.

Discussion

Based on these results, we chose to continue to use the SR + FGF as well as the NS media for the third round of reprogramming detailed above. What is especially remarkable, though, is that the densely-seeded cells in the SR + 2i medium appears to have reverted to a primary chondrocyte morphology. Further experiments should be a priority to determine if 2i medium can reverse dedifferentiation of cultured chondrocytes.
Figure B.1
Canine chondrocytes reprogramming media test.
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