OPTIMIZING CHONDROGENESIS OF CANINE MESENCHYMAL STEM CELLS FOR FUTURE USE IN TREATMENT OF OSTEOCHONDRAL DEFECTS

A Thesis

by

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ABSTRACT

Osteochondrosis and traumatic osteochondral defects are debilitating disorders affecting articular cartilage of millions of human and veterinary species. As articular cartilage is highly specialized and lacks reparative capacity, focal osteochondral defects (FODs) lead to a chronic progressive disease state and subsequent widespread osteoarthritis. While medical and surgical management options are available, they are not without complications. To address these limitations, recent work in the field of regenerative medicine has focused on the use of mesenchymal stem cells (MSCs) and three-dimensional (3D) scaffolds to develop tissue engineering constructs to replace injured articular cartilage restoring function and eliminating clinical signs. While much work has been performed in human MSC tissue engineering, canine MSC (cMSC) tissue engineering for the treatment of osteochondral defects remains in its infancy. The objective of the work presented herein was to develop an improved 3D serum free system for cMSC chondrogenesis and determine the effect of dose and combination of three important chondrogenic growth factors (TGF-β3, BMP-2 and bFGF) using bone marrow-derived cMSCs.

During phase 1, the effect of ten growth factor combinations on cMSC chondrogenesis was determined using construct morphometry, live/dead staining, and histology. High doses of BMP-2 and bFGF produced heavier, larger, consistently shaped constructs when compared to lower doses. Based on these results, in phase 2, the effect

of dexamethasone, TGF-β3, and high concentrations of BMP-2, and bFGF were assessed using quantitative assays of cytotoxicity, glycosaminoglycan (GAG) accumulation, and gene transcription. In the presence of bFGF, evidence of reduced cell stress suggested a potential role in cell survival during initial stages of chondrogenesis when the cells initiate a condensation event. Moreover, the presence of BMP-2 and bFGF in chondrogenic media resulted in the highest accumulation of GAG. Gene expression studies demonstrated that upregulation of genes associated with chondrogenesis and osteogenesis occurred in a time-dependent manner. Ultimately, this work demonstrates that the 3D serum-free collagen type I system described is useful for assessing canine chondrogenic differentiation. Collectively, this work describes the effect of chondrogenic growth factors on cMSCs in combination with a 3D collagen type I scaffold and represents an important advance in canine chondrogenesis.

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NOMENCLATURE

3D	Three-dimensional
ACI	Autologous chondrocyte implant
AUP	Animal use protocol
BMP-2	Bone morphogenetic protein-2
bFGF	Basic fibroblast growth factor
CCM	Complete culture medium
cDNA	complimentary DNA
CT	Threshold level for qPCR
cMSC	Canine mesenchymal stem cells
Dhh	Desert hedgehog
DMEM	Dulbeco's Modified Essential Medium
DMMB	1,9-dimethyl-methylene blue
ECM	Extracellular matrix
FBS	Fetal bovine serum
FOD	Focal osteochondral defect
GAG	Glycosaminoglycan
HA	Hyaluronan
H&E	Hematoxylin and eosin
hMSC	Human mesenchymal stem cells
Ihh	Indian hedgehog

LDH	Lactate dehydrogenase
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MSCs	Mesenchymal stem cells
NBF	Neutral buffered formalin
OA	Osteoarthritis
OATS	Autologous osteochondral transplants
OC	Osteochondrosis
OCD	Osteochondritis dissecans
PBS	Phosphate buffered saline
PGA	Polyglycolic acid
PTHRP	Parathyroid hormone related peptide
qPCR	Real-time PCR
rER	Rough endoplasmic reticulum
SD	Standard deviation
Shh	Sonic hedgehog
TGF-β3	Transforming growth factor beta -3
TIMPs	Tissue inhibitors of MMPs

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

ARTICULAR (HYALINE) CARTILAGE

Articular cartilage, also referred to as hyaline cartilage, is a specialized connective tissue located in diarthrodial joints. The function of articular cartilage is to facilitate locomotion by providing a lubricated surface with a very low coefficient of friction for joint articulation and load transmission (Sophia Fox et al., 2009). To accomplish this task, both the cellular arrangement and extracellular matrix (ECM) composition and arrangement are highly specialized. A detailed discussion of the cellular and ECM arrangements will be provided below. Importantly, while the cellular and ECM arrangements are unique, the reader should note that normal articular cartilage lacks blood vessels, lymphatics and nerves (Sophia Fox et al., 2009). Recent studies have demonstrated that the in vivo cartilage thickness in the stifle joint (knee joint in human beings) is 0.6-1.3 mm in dogs and 2.2-2.5 mm in human beings (Frisbie et al., 2006).

The ECM is a major structural component of articular cartilage. The ECM of articular cartilage consists of components including collagen, proteoglycans other non-collagenous proteins, glycoproteins, and water (Sophia Fox et al., 2009). Water contributes up to approximately 80% of the weight of healthy articular collagen, with approximately 30% of water content tightly associated with the intrafibrillar space of collagen. The remaining water is located within physical pore spaces located within the

ECM. (Maroudas et al., 1991; Torzilli, 1985). In healthy articular cartilage, water content decreases as the depth of cartilage increases (Buckwalter and Mankin, 1997), and it is this flow of water that is involved in the transport and distribution of nutrients to chondrocytes (Sophia Fox et al., 2009).

A critical component of articular cartilage ECM is the fibrillary network of structurally supporting collagen, of which collagen type II accounts for approximately 80% of the articular cartilage ECM (Gelse et al., 2003). Similar to all helical collagens, collagen type II is composed of a triple helix of three collagen monomer chains. Each collagen type varies in tissue-specific expression and synthesis of structural proteins and glycoprotein components, which result in the unique functional and biologic characteristics of the specific cartilage at distinct locations (Gelse et al., 2003). Collagen type II is composed of three identical α-chains (referred to as a homotrimer) and is found not only in articular cartilage but in the vitreous body and nucleus pulposus (Gelse et al., 2003).

Compared to collagen type I, the most abundant collagen in the body, collagen type II chains interact with proteoglycans and thus have higher contents of hydroxylysine, glucosyl and galactosyl residues (Mayne, 1989). In addition, the self-assembly of collagen type II has been suggested to be more complex than that of collagen type I (Fertala et al., 1994). In addition to collagen type II, articular cartilage also contains lower amounts of collagen type III, VI, IX, X, XI, XII and XIV that contribute to the mature matrix (Eyre, 2002).

In addition to the collagens detailed above, proteoglycans account for 10-15% of the wet weight of the ECM and are the second largest group of ECM macromolecules present in articular cartilage (Sophia Fox et al., 2009). Proteoglycans are composed of a protein core with a varying number of linear glycosaminoglycan chains covalently attached which can be composed of more than 100 monosaccharides (Sophia Fox et al., 2009). Important proteoglycans of healthy articular cartilage include: aggrecan, decorin, biglycan, and fibromodulin (Sophia Fox et al., 2009). Aggrecan is a highly negatively charged molecule that maintains water content and provides osmotic properties, allowing cartilage to resist repetitive compressive loads. Additionally, aggrecan binds to the large glycosaminoglycan hyaluronan (HA) to form large proteoglycan aggregates (Sophia Fox et al., 2009). Decorin and fibromodulin contribute to interfibril interactions and normal collagen fibrillogenesis through their interactions with collagen type II (Sophia Fox et al., 2009).

A final group of articular cartilage ECM proteins include the non-collagenous proteins which constitute a smaller proportion of articular cartilage. Some examples of these include fibronectin, anchorin CII, oligomeric protein, fibronectin and tenascin. Their role has not been fully characterized however, it has been suggested that they play a role in organization and maintenance of the ECM (Sophia Fox et al., 2009).

Chondrocytes are the specialized cartilage cells responsible for both synthesis and digestion of the extracellular matrix through the secretion of cytokines, which is crucial for cartilage homeostasis (Pearle et al., 2005). Importantly, although a critical component of articular cartilage, chondrocytes account for only approximately 5% of

the wet weight of articular cartilage. Chondrocyte injury, apoptosis, or necrosis commonly results in a progressive loss of normal cartilage ECM due to a poor inherent reparative capacity of this unique tissue.

CARTILAGE INJURY

Developmental and traumatic joint diseases, such as osteochondrosis (OC), osteochondritis dissecans (OCD) and traumatic focal osteochondral defects (FODs), are debilitating articular cartilage lesions affecting millions of humans and veterinary species. These conditions specifically affect articular cartilage and, due to the inherently poor reparative capacity of articular cartilage, result in a chronic progressive disease state and subsequent widespread osteoarthritis (OA) of the affected joint. OC/OCD and FODs thus lead to a reduced quality of life for patients while representing a tremendous economic and clinical burden to both the veterinary and human health-care industries. The economic impact of osteoarthritis in humans approaches 60 billion dollars per year (Buckwalter et al., 2004) affecting more than 20 million people in the United States (Pearle et al., 2005). Although the actual economic impact on veterinary species is unknown, it was recently shown that pet owners spend 1.32 billion dollars annually on the treatment from cranial cruciate ligament ruptures, of which OA is an important component of the disease process (Wilke et al., 2005).

OC/OCD was originally described in veterinary medicine in the 1960s (Ytrehus et al., 2007). OC is defined as a multifocal disease of epiphyseal cartilage with many risk factors including: rapid growth, heredity, anatomic characteristics, trauma and dietary

factors (Ytrehus et al., 2007). With regards to repair, articular cartilage defects that do not penetrate the subchondral bone are unable to spontaneously repair (Fuller and Ghadially, 1972). Even with full thickness defects, multiple predisposing factors such as age, defect size and location may dictate whether or not repair occurs. Furthermore, repair of small defects usually occurs through the production of hyaline cartilage, however, larger defects repair with fibrous tissue (Coventry et al., 1972). This change in tissue type is biomechanically and biochemically different from hyaline cartilage and thus, further degeneration occurs (Shelbourne et al., 2003) leading to progression of OA.

Unfortunately, the etiology of osteochondrosis is not fully understood due to a lack of knowledge of the pathogenesis of the primary lesion. However, we do recognize that OC/OCD results from altered or abnormal endochondral ossification. Several main hypotheses have been formulated with regards to the pathogenesis of the abnormal endochondral ossification: the first is centered upon apoptosis of the endothelium lining cartilage canal vessels, the second is centered upon microtrauma to the vessels of the metaphysis, and the third is centered on altered cellular signaling between the molecules parathyroid hormone related peptide (PTHRP), indian hedgehog (Ihh), and TGF-β3 (Ytrehus et al., 2007). With regards to the first hypothesis, during skeletal development the blood supply to the physeal plate (i.e. the growth plate) closest to the metaphysis is somewhat limited. If this limited blood supply is focally interrupted, an area of focal ischemic necrosis results. As the bone continues to elongate, this focal area of cartilage will resist blood vessel invasion thus preventing the normal ossification process of the cartilage above the defect (Ytrehus et al., 2007). The second hypothesis centers around

microtrauma to the vessels of the metaphysis and could, in a similar manner to the first hypothesis, cause interruption of blood supply and a failure of differentiation of the hypertrophic cartilage leading to a persistent thickened physeal cartilage (Ytrehus et al., 2007). This thickened necrotic cartilage can fissure resulting in a flap of cartilage exposing the underlying bone and leading to the pain and lameness observed clinically. This separation of the flap of cartilage from the underlying bone is then termed an OCD lesion (Ytrehus et al., 2007).

Articular cartilage can also be traumatically injured. Initially, traumatic injury to articular cartilage often leads to small, focal osteochondral defects (FODs). These FODs can be caused by high impact athletic activities that lead to localized blunt trauma to a specific region of the articular cartilage. While some of these injuries induce temporary cartilage injury and subchondral bone bruising, other athletic injuries lead to thinning of articular cartilage, apoptosis of chrondrocytes and progressive OA.

OA is a multifactorial disease with an incompletely characterized etiopathogenesis. The disease is generally characterized by aberrant repair and eventual degradation of articular cartilage in association with alterations in subchondral bone metabolism, periarticular osteophytosis and a variable degree of synovial inflammation (Innes, 2012). Macroscopic changes in osteoarthritis can include softening (chondromalacia), fibrillation, and erosions (ulceration) (Noyes and Stabler, 1989). Idiopathic or primary osteoarthritis refers to that caused by aging (most common in humans but rare in veterinary species) and secondary osteoarthritis occurs to that caused by a preceding event (Innes, 2012). The two types do not differ from a pathologic basis

with the exception of primary cause. Proteolytic enzymes known as matrix metalloproteases (MMPs) are responsible for cartilage matrix degradation, mainly MMP-13 and MMP-1 (interstitial collagenases), MMP-3 (stromelysins), and MMP-2 and MMP-9 (gelatinases) (Martel-Pelletier, 2004; Reboul et al., 1996). Usually MMPs are kept controlled in a homeostatic state by tissue inhibitors of MMPs (TIMPs) however, in osteoarthritis an imbalance occurs leading to the destruction of collagen type II (Martel-Pelletier, 2004) and proteoglycans such as aggrecan. The precise cause of this imbalance is not completely understood however, there is evidence that acute mechanical overload, cyclic/fatigue loading, and cytokines (such as TNF-α, IL-1β and IL-6) lead to the disruption of cartilage homeostasis (Lin et al., 2004; Martel-Pelletier, 2004).

As detailed above, both human beings and veterinary species are affected by OC/OCD and athletically induced joint injuries, both of which result in FODs. In both species, the end result is progressive OA. The dog is an excellent translational model for many human diseases (Hoffman and Dow, 2016). OA is included as one of these disease models along with intervertebral disc degeneration, dilated cardiomyopathy, inflammatory bowel diseases, Crohn's fistulas, meningoencephalomyelitis (multiple sclerosis-like), keratoconjunctivitis sicca (Sjogren's syndrome-like), atopic dermatitis and chronic (end stage) kidney disease (Hoffman and Dow, 2016). Using companion animals, such as dogs, in clinical studies allows scientists to evaluate feasibility, safety and efficacy of novel treatment strategies, for example cell-based therapies, for

subsequent translation into human beings (Hoffman and Dow, 2016). At present, treatment options for OA are very similar between canines and human beings.

TREATMENT OF OSTEOARTHRITIS

Broadly, there are two primary categories of OA treatment: medical and surgical management. Medical management of OA typically involves a multimodal approach. This approach includes a combination of the following: activity control, weight management, nutritional support, physical therapy, and administration of a nonsteroidal anti-inflammatory drug, analgesic medications, nutraceuticals and purported slow-acting drugs of osteoarthritis (Aragon et al., 2007). While medical management is often used to treat patients with early FODs, medical management is often life long and requires the addition of new treatments or drugs as time progresses. In addition to life-long treatment, the strongest evidence for the alleviation of clinical signs is limited to non-steroidal anti-inflammatory medications (Aragon et al., 2007). These medications are not without adverse effects some of which include gastrointestinal disturbances, liver injury, and/or kidney failure (Doig et al., 2000; Forsyth et al., 1998; Moreau et al., 2003; Nell et al., 2002).

When medical management does not alleviate symptoms, surgical treatment is often pursued. A fundamental principle of treating focal cartilage defects is that early intervention is essential to the success of the procedure. There are a myriad of surgical treatment options for articular cartilage defects which can be divided into two broad categories: palliative versus restorative treatments. Palliative treatments focus on both

removing sources of irritation and inflammation (arthroscopic removal of fragments) (Cole et al., 2009) as well as enhancing the formation of a fibrocartilage membrane over the defect (which, importantly, does not restore structure or function). This is accomplished through procedures such as debridement, abrasion arthroplasty, and microfracture. With regards to debridement and fragment removal, clinical outcome may be poor for up to 25% of cases (Rudd et al., 1990). Microfracture is particularly advantageous due to the minimally invasive nature of the procedure, the short postoperative recovery time and the cost effectiveness (Clair et al., 2009). However, success is based upon early surgical intervention and the young age of the patient (Clair et al., 2009). Unfortunately, in veterinary patients, such as dogs, early diagnosis and intervention are often not possible due to the stoic nature of veterinary patients and the inability of most veterinary clients to recognize early clinical signs associated with OC/OCD or FODs and early OA. These important considerations often result in poor long-term function when microfracture is used to treat veterinary patients. Furthermore, depressed and thinned repair cartilage, incomplete peripheral integration and subchondral bony overgrowth have been observed in 25-40% of cases (Brown et al., 2004; Mithoefer et al., 2005).

In contrast, restorative treatments attempt to repair or replace the cartilage defect and fully restore structure and function through procedures such as autologous osteochondral transplants (i.e. OATS), autologous chondrocyte implantation (ACI), or placement of allogenic osteochondral tissue grafts or synthetic cartilage substitutes. Osteochondral autograft transplantation involves harvesting osteochondral grafts (both

articular cartilage and underlying subchondral bone) from areas of limited weight-bearing and placing them into FODs (Fitzpatrick et al., 2010). OATS procedures have been used in knee FODs of human beings with a superior outcome compared to microfracture (Gudas et al., 2005). In one study by *Fitzpatrick et al*, osteochondral grafts were obtained from joints other than the affected joint. For example, using a stifle osteochondral graft to treat an osteochondral lesion in the shoulder (Fitzpatrick et al., 2010). Whether or not this causes further lameness has not yet been determined. The benefit however of autologous chondrocyte implantation is the ability to expand chondrocytes to fill defects up to at least 10cm^2 (Knutsen et al., 2004). In one randomized control trial in humans, a significant difference was not noted between autologous chondrocyte implantation compared with microfracture with the exception of significantly more improvement in multipurpose health survey (SF -36) in the first two years in the microfracture group versus the autologous chondrocyte implantation group (Knutsen et al., 2004). There was no difference between the treatment groups in terms of macroscopic or histological results however, the number of biopsy specimens examined was low (Knutsen et al., 2004). Some limitations noted with autologous chondrocyte implantation include: limited availability of autologous chondrocytes, de-differentiation of these cells during culture, graft failure and the cost and morbidity associated with two separate surgical procedures (one for harvest and a second for implantation) (Brittberg et al., 1994; Knutsen et al., 2004; Matsumoto et al., 2010; Redman et al., 2005). In conclusion, while a number of medical and surgical treatments exist for the management of OA secondary to FODs, more consistently effective treatments are needed.

REGENERATIVE MEDICINE

Although restorative treatments are performed in both human and veterinary medicine, they are not without complication, such as: donor site morbidity (Feczkó et al., 2003; Reddy et al., 2007; Szerb et al., 2005b), cartilage thickness discrepancies (Breur and Lambrechts, 2012), poor graft survival, and progression of OA (Breur and Lambrechts, 2012). To navigate these obstacles, much recent work has focused on regenerative medicine as a potential restorative treatment for FODs.

Regenerative medicine is defined as an interdisciplinary field of research and clinical application 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 aging (Daar and Greenwood, 2007). One method by which regenerative medicine may be used to treat patients with focal articular cartilage defects is the direct injection of mesenchymal stem cells (MSCs) into the affected joint (Emadedin et al., 2012). Mesenchymal stem cells, also known as multipotent stromal cells or marrow stromal cells, are progenitor cells of mesenchymal origin which are capable of differentiating within the mesenchymal line into a variety of specialized tissues including bone, fat, and cartilage (Caplan and Correa, 2011; Dominici et al., 2006). MSCs have been defined by *Dominici et al.* as spindle-shaped cells that are adherent to plastic, have specific cell surface antigen expression and have multipotent differentiation potential (Dominici et al., 2006). The cells must be positive for CD105, CD73 as well as CD90 and be negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR (Dominici et al., 2006). Although injecting MSCs in solution into

the joint is relatively straightforward and the most common method by which human and veterinary patients currently receive stem cell therapy, studies have shown that few cells remain at the injection site 50 days after multiple deliveries at different time points (Krause et al., 2010). Additionally, engraftment and differentiation into specialized cells needed for restoration of cartilage is limited due to lack of a matrix or signaling allowing the majority of cells to die or migrate away from the site of interest (Krause et al., 2010). In contrast to this finding, *Horie et al.* illustrated that synovium and bone marrow derived mesenchymal stem cells adhered and promoted regeneration of a meniscal lesion without the cells mobilizing to organ sites (Horie et al., 2009). These Lacz+ tagged MSCs produced measurable photon readings could be detected up to 28 days. However, similar to the findings by *Krause et al*., no photons could be detected at a longer time point (Horie et al., 2009) and thus cells did not remain at the site of injury. Whether positive short- or medium-term clinical outcomes are achieved after intra-articular MSC delivery remains controversial and thus systems to overcome this limitation have been extensively studied.

In order to use MSCs to treat FODs, it is imperative to confirm that preparations of MSCs are capable of undergoing transformation into cartilage (chondrogenesis) and to mechanistically define this process. The micromass pellet system was performed by *Sekiya et al.* as described in *Johnstone et al.* for human MSCs from bone marrow aspirates (Johnstone et al., 1998; Sekiya et al., 2002). Briefly, MSCs are trypsinized, counted and cell aliquots are spun down at 500xG in 15 mL polypropylene conical tubes and cultured for up to 21 days (Johnstone et al., 1998). This system is unique as it

represents a 3D assay and the fetal bovine serum (FBS) containing medium is replaced with a serum free medium. This method has been utilized to evaluate chondrogenesis in equine and canine MSC studies (Kisiel et al., 2012; Vidal et al., 2008). Unfortunately, *Kisiel et al.*, was unable to differentiate MSCs into chondrocytes using the protocol described by *Vidal et al.* and their attempt resulted in central necrosis and undifferentiated cells on histopathology (Kisiel et al., 2012). Using human MSCs, similar findings of central necrosis (Liangming Zhang et al., 2010) have occurred with the additional finding that only the outside layer of the pellets underwent chondrogenesis (Mueller and Tuan, 2008; Murdoch et al., 2007).

Kisiel et al., also noted that a proteoglycan matrix could be present in spite of the undifferentiated cells as the pellets stained with Alcian blue stain. Without supportive results on H&E and Safranin O staining however, it is difficult to form a conclusive statement on whether the matrix was present (Kisiel et al., 2012). Thus, mRNA expression of chondrocyte markers would be useful to confirm or deny differentiation of the MSCs down the chondrogenic lineage.

In addition to the concerns described above, traditional micromass pellet cultures are extremely small (1 mm – 3 mm diameter) in size depending on the species and induction recipe utilized (Shirasawa et al., 2006). Thus, using these cultures in translational studies is not feasible due to the inability to provide sufficient quantity of reparative cultures (Grande et al., 1999). Unpublished observations from our laboratory have also suggested that attempting to induce chondrogenesis of canine MSCs using the traditional/classic micromass pellet method is often unsuccessful. When canine MSCs

are placed in control culture medium with fetal bovine serum (CCM), the canine MSCs do not maintain micromass aggregates and undergo apoptosis. Given the fact that the long-term goal of regenerative medicine is to develop clinically relevant treatment options, chondrogenic cultures need to be large enough to fill in vivo defects in humans and veterinary species, and the histologic characteristics of these pellets must be improved. Tissue engineering is a sub-specialty of regenerative medicine that is attempting to address these limitations.

TISSUE ENGINEERING

Tissue engineering is a sub-specialty of regenerative medicine in which regenerative cells (i.e. MSCs) are combined with specialized scaffolds and/or growth factors to repair, regenerate and/or improve the structure and function of diseased tissue (Lijie Zhang et al., 2009). Focusing on tissue engineering constructs that combine cells, growth factors and a scaffold, both biologic and synthetic scaffolds with reparative cells such as chondrocytes or mesenchymal stem cells (MSCs) have been examined (Martin et al., 2001; Neu et al., 2009; Zscharnack et al., 2010). Ideally a scaffold should include the following characteristics: three dimensional, porous with an interconnected pore network, biocompatible, predictably bioresorbable, suitable for cell attachment, proliferation and differentiation, and lastly scaffolds must possess mechanical properties approaching those of the tissues to be replaced (Hutmacher, 2000). Using these scaffolds, MSCs, osteoblasts and chondrocytes can be harvested from the patient's tissues and expanded in vitro. Scaffold materials such as: hydroxyapatite, poly(α-

hydroxyesters) and natural polymers such as collagen and chitin have been described (Gunatillake and Adhikari, 2003; Guo and Ma, 2014).

Unfortunately, substantial limitations exist with current articular cartilage tissue engineering scaffolds. First, the ability of the scaffold to adhere to the surrounding native cartilage when placed within a defect has not been refined. Small defects have been repaired using a press-fit system however, larger lesions (as present in vivo) of 1 cm diameter are often not amenable to this repair (Grande et al., 1999). Without the ability to bond and integrate with adjacent tissues, tissue engineering scaffolds placed within the lesion are unable to remain in position to obtain a long-term biologic interlock. Second, current tissue engineering constructs provide an incomplete restoration of normal tissue architecture. Long term follow-up of cartilage tissue engineering constructs composed of polyglycolic acid (PGA) placed in a rabbit model illustrated that centers of calcification formed within the upper zones of the construct (Kandel et al., 1997). The authors concluded that in order to achieve long-term clinical success, current constructs must more closely resemble normal articular cartilage in regards to histologic appearance and biomechanical function. Biomechanical function is an important characteristic of a cartilage tissue engineering construct fundamental to in vivo success. A study by *Neu et al.*, used magnetic resonance imaging (MRI) to look at the biomechanics of cartilage constructs when placed under mechanical stress. This study illustrated that mechanical stress is not distributed equally between the repair and the surrounding tissue and thus further integration strategies are necessary (Neu et al., 2009). Unless the above limitations are addressed, disease progression is likely to continue even

with construct implantation. Furthermore, development of consistent cartilage tissue-engineering constructs capable of treating cartilage lesions is not only important for human patients, but may prove essential in veterinary patients as their clinical presentation is often delayed compared to their human counterparts (thus disease is often progressed) (Breur and Lambrechts, 2012). Despite the limitations of previous attempts at cartilage tissue engineering, the benefits of cartilage tissue engineering far outweigh current limitations. For this reason, cartilage tissue engineering efforts continue.

COLLAGEN AS A SCAFFOLD

Collagen is the most abundant protein in the extracellular matrix. Collagens are structural proteins composed of three independent left-handed polypeptide α-chains (Pietz, 1984) creating a unique right-handed triple helix (Gelse et al., 2003). Collagen is most commonly synthesized by fibroblasts by transcribing genes in the nucleus for mRNA processing; after splicing it is transported to the rough endoplasmic reticulum (rER) and golgi complex. Here the triple helix structure is formed from individual α-chains (Gelse et al., 2003). Each triple helix contains an integral repeat of amino acid motifs, Glycine-X-Y (GXY), enabling for the rotating helical structure around the central glycine axis (Hofmann et al., 1978). Glycine is the pivotal amino acid because of its size and repetitive location in the sequence allowing for bulkier side chains on the periphery. While glycine is necessary in the first position the subsequent amino acids, X and Y, can be any other sequence, but these positions are most commonly occupied by proline and hydroxyproline (Gelse et al., 2003). After post-translational modification,

chains may be assembled as homotrimers (collagen types: II, III, VII, and X) or as heterotrimers (collagen types: I, IV, V, VI, IX, and XI) before secretion as pro-collagen into the extracellular space (Gelse et al., 2003). Once secreted specific members of metalloproteinase family, procollagen *N*- and *C*- proteinase, cleave the N- and C-terminals, respectively (Prockop et al., 1998). Afterwards, collagen fibrils spontaneously assemble through hydrophobic and electrostatic interactions creating staggered arrangements of monomers, which aggregate to fibrils, and eventual fibrous tissue (Nimni, 1988; Silver et al., 1992).

In addition to the maintenance of structural integrity of tissues and organs, the extracellular matrix serves an important role in providing instruction through biomechanical and biochemical cues. Usually cells establish an extracellular matrix strength to fit the need of that specific cell during development and tend to maintain that strength over a lifetime (Humphrey et al., 2014). However, in diseased conditions this matrix can render the extracellular matrix more compliant and cause increases in stress and strain (Humphrey et al., 2014). A feedback mechanism then causes increased extracellular matrix stiffening, often referred to as fibrosis, mechanical failure or other pathologies (Humphrey et al., 2014).

While true that the composition of articular cartilage is composed of collagen type II, collagen type I scaffolds are commonly used for tissue engineering. Collagen type I is frequently used due to its abundance, availability and biocompatibility (Glowacki and Mizuno, 2008). Canine chondrocytes were placed in collagen type I and collagen type II scaffolds to be investigated in vitro however, and results favored using a

collagen type II scaffold (Nehrer et al., 1997). Unfortunately, in our experience collagen type II has been quite difficult to obtain in reliable formats, particularly in the quantities required for articular cartilage tissue engineering. Using hMSCs, *Yokoyama et al.*, combined collagen type I with human synovium MSCs and found that a combination of 5×10^7 to 1×10^8 MSCs combined with 30 mg/mL collagen type I produced constructs which illustrated increased mRNA expression of COL2A1 indicating progressive differentiation of cartilage with expression of aggrecan, decorin and biglycan (Yokoyama et al., 2005). With regards to animal models, *Zscharnack et al.,* utilized collagen type I gel combined with ovine MSCs or pre-differentiated chondrocytes to create a construct capable of integrating into two osteochondral defects with a diameter of 7 mm and a depth of 2 mm (Zscharnack et al., 2010). This study determined that constructs in which pre-differentiated chondrocytes were combined with collagen type I lead to an improved histologic appearance with regards to surface integrity, adhesion to adjacent cartilage, hypocellularity and mineralization when compared to constructs with undifferentiated MSCs and control groups (Zscharnack et al., 2010). Even when using collagen type I scaffolds, integration into surrounding tissues still occurred.

GROWTH FACTORS

Growth factors that promote chondrogenesis have been extensively studied for decades in humans however, canine research is still in its infancy (Figure 1.1). According to the reference *Mesenchymal Stem Cells Methods and Protocols*, an effective medium for inducing chondrogenesis in human MSCs includes: high glucose DMEM, 50 μg/mL of

L-ascorbic-2-phosphate, 40 μg/mL of L-proline, 100 μg/mL of sodium pyruvate, 5 mL ITS+ Culture Supplement (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, 5.35 mg/mL linoleic acid) (Reger et al., 2008). The specific cytokines/growth factors added immediately prior to use to this solution for chondrogenic differentiation include 10 ng/mL rhTGF-β3, 10^{-7} M dexamethasone and 500 ng/mL rhBMP-2 (Reger et al., 2008). Prior to the publication by *Reger et al.,* many publications by multiple investigators helped to define the induction medium detailed above (Martin et al., 2001; Puetzer et al., 2010; Sekiya et al., 2002; 2001; 2005; Shintani and Hunziker, 2011; Yokoyama et al., 2005). With respect to the optimum combination of growth factors that induce maximal chondrogenic differentiation of canine MSCs, less research has been conducted. A study by *Shirasawa et al*., determined that the combination of 100 nM dexamethasone, 500 ng/mL BMP-2 and 10 ng/mL TGF-β3 was optimum for chondrogenic differentiation using hMCSs (Shirasawa et al., 2006). This combination was compared with 1) TGF-β3 and dexamethasone alone, 2) TGF-β3, dexamethasone and retinoic acid, 3) TGF-β3, dexamethasone, insulin growth factor 1, 4) TGF-β3, dexamethasone, bFGF2, and 5) TGF-β3, dexamethasone and insulin (Shirasawa et al., 2006). In addition to producing the most chondrogenic cultures as assessed by histology, the combination of TGF-β3, dexamethasone and BMP-2 produced the largest diameter cultures after 21 days of incubation (Shirasawa et al., 2006).

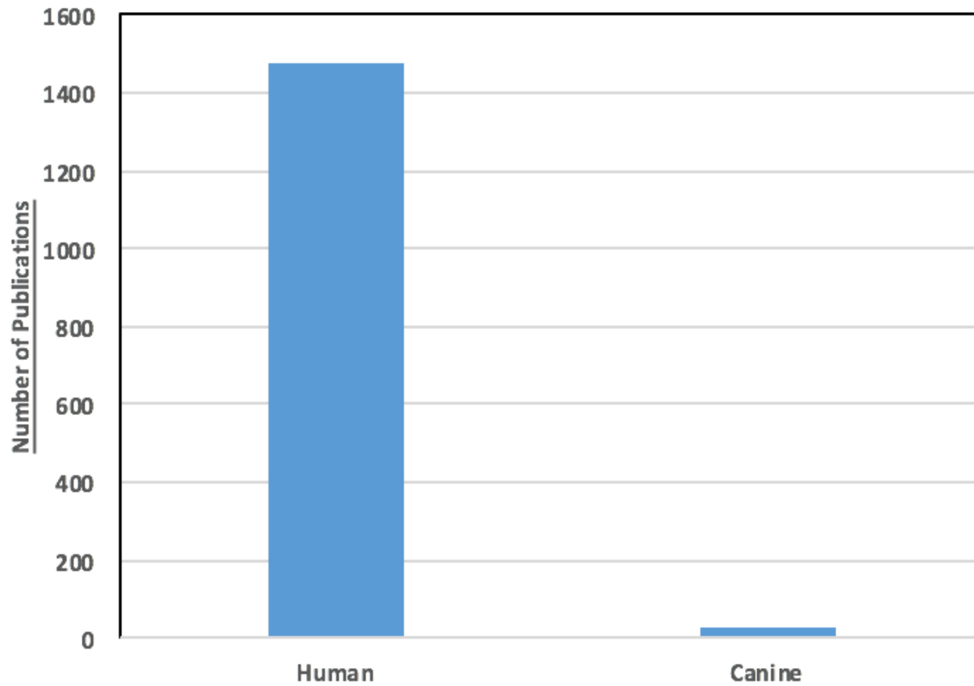


Figure 1.1: *PubMed literature search for primary research articles focused on chondrogenesis in human beings versus canines*. Using the search terms “human”, “chondrogenesis”, and “mesenchymal stem cells”, 1478 articles were identified demonstrating the robust foundation of literature that exists on the subject of human MSC chondrogenesis. In contrast, using the search terms “canine”, “chondrogenesis”, and “mesenchymal stem cells”, only 30 articles were identified. Further screening of this list of publications resulted in identification of 4 publications that attempted to perform chondrogenesis with canine MSCs using the traditional micromass assay. This literature search demonstrates the disparity between our fundamental understanding of human and canine MSC chondrogenesis.

Dexamethasone is a glucocorticoid that is used commonly in MSC cultures for adipogenic, osteogenic and chondrogenic differentiation (Derfoul et al., 2006; Sekiya et al., 2002; Shintani and Hunziker, 2011; Shirasawa et al., 2006; Yokoyama et al., 2005). Although required for multipotent differentiation, the exact mechanism of action is unknown (Derfoul et al., 2006).

Interestingly, *Shintani et al*., demonstrated that dexamethasone suppresses the effect of BMP-2 almost completely and had little impact on the effect of TGF-β1 during chondrogenic differentiation of synovial explants (Shintani and Hunziker, 2011). Furthermore, when dexamethasone was added to aggregates of isolated synovial MSCs instead of synovial explants, it had no influence on either the TGF-β1 or BMP-2 thus indicating the importance of the microenvironment to which dexamethasone is added (Shintani and Hunziker, 2011). In contrast to the findings on synovial implants, dexamethasone was added to bone marrow derived MSC aggregates and was shown to greatly enhance chondrogenesis induced by TGF-β1 (Shintani and Hunziker, 2011). This effect is consistent with other previously reported studies (Derfoul et al., 2006; Johnstone et al., 1998). This research not only demonstrates the importance of dexamethasone and various growth factor combinations in chondrogenesis of MSCs, but further indicates the importance of the origin of the MSCs when considering their interaction with various growth factors.

Transforming growth factor β (TGF-β) is a member of the transforming growth factor-β superfamily which also includes bone morphogenetic proteins and activins. TGF-β is produced and recognized by almost every cell in the body, regulates the

proliferation and differentiation of cells and is involved in embryonic development, wound healing and angiogenesis (Blobe et al., 2000). Three isoforms of TGF-β exist in vertebrates: TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is expressed in endothelial, hematopoietic and connective tissue, TGF-β2 is expressed in epithelial and neuronal cells and TGF-β3 is expressed in mesenchymal cells (Blobe et al., 2000). Most chondrogenic differentiation studies have focused their efforts on TGF-β1 or TGF-β3 (Derfoul et al., 2006; Reger et al., 2008; Sekiya et al., 2005; 2002; 2001; Shintani and Hunziker, 2011; Yokoyama et al., 2005). In order to differentiate between the three isoforms, *Barry et al.*, directly compared the addition of TGF-β1, TGF-β2 and TGF-β3 to chondrogenic pellets (Barry et al., 2001). This specific experiment examined chondrogenic differentiation of human MSCs. Interestingly, TGF-β3 and TGF-β2 pellets accumulated significantly more glycosaminoglycans when compared to TGF-β1. Additionally, with regards to differentiation to a chondrocytic phenotype on histologic examination, TGF-β1 consistently produced inferior collagen type II staining. Thus, although previous studies have shown chondrogenic differentiation using TGF-β1, TGF-β3 may produce higher quality chondrogenic pellets for future clinical use.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily with the exception of BMP-1 (Wozney et al., 1988). The structure of the TGF-β superfamily members include an amino-terminal signal sequence, a pro-domain and their carboxy-terminal mature peptide which is released by furin-mediated cleavage (Dijke et al., 2003). The mature domain is characterized by a 7-cysteine motif

and forms homodimers and heterodimers which are covalently linked by one di-sulphide bond (Dijke et al., 2003). BMP has the ability not only to induce bone by stimulating differentiation into the osteoblast lineage, but it also inhibits differentiation toward myoblasts (Katagiri et al., 1994). Further studies have also demonstrated the role of BMP in the development and function of pulmonary, cardiovascular, reproductive, urogenital organs and the nervous system (H. Chang et al., 2002; Goumans and Mummery, 2002).

With regards to mechanism of action, BMPs bind to specific serine/threonine kinase receptors (type I and type II) (Kawabata et al., 1998; Massagué, 1998). Once the receptor is bound, intracellular signaling via phosphorylation of specific proteins, including the Smad proteins, occurs (Kawabata et al., 1998; Massagué, 1998). BMP receptors phosphorylate Smad1, Smad5, and Smad8. R-Smad/Smad4 complexes are formed by activation by type-I receptors. These complexes accumulate in the nucleus where they regulate target genes (Kawabata et al., 1998; Massagué, 1998). Direct binding of R-Smads and Co-Smads to DNA is by MH1 domains and BMP-specific R-Smads interact with GC-rich containing sequences (Ishida et al., 2000; Korchynskyi and Dijke, 2002).

More importantly, mice genetically deficient in BMP transcription factor targets such as: Runx2, Dlx5, osterix and Msx2 develop skeletal abnormalities (Korchynskyi et al., 2004). The induction of Id proteins, inhibitors of basic helix-loop-helix proteins (Korchynskyi and Dijke, 2002) by BMPs may block the differentiation of mesenchymal

stem cells toward adipocytes and myoblast and thus support osteoblast differentiation (Dijke et al., 2003).

In addition to promoting osteogenic induction, BMPs have the ability to induce endochondral ossification and chondrogenesis (Leboy et al., 2001). The effect of BMP in chondrogenesis appears to be mediated by Sox 9 which is important to chondrogenesis and the fabrication of collagen type II and X (Semba et al., 2000). BMPs also affect Ihh, Sonic hedgehog (Shh) and Desert hedgehog (Dhh). Ihh and Shh enhance chondrogenesis and endochondral ossification (Grimsrud et al., 2001; Krishnan et al., 2001). It is important to realize however, that other effects of Ihh/Shh on cartilage are independent of BMPs. Ihh/Shh induces Nkx3.2, which induces Sox 9 which, in the presence of BMP induces chondrogenesis (Murtaugh et al., 2001; Zeng et al., 2002). In the presence of BMP, Sox 9 and Nkx3.2 establish a positive feedback loop to initiate chondrogenesis (Zeng et al., 2002).

Specifically, BMP-2 has been shown to enhance chondrogenesis using human iPS cells using qPCR expression analysis of Sox9, Col2a1, aggrecan, Col2b, Runx2, collagen type I and ColXa1 on cell micromasses (Guzzo et al., 2013). Additionally, in one study, BMP-2 increased collagen type II expression under leptin stimulation and suggested that BMP-2 may help repair damaged tissues undergoing OA in obese individuals (Chang et al., 2015). Based on this work, BMP-2 has been recognized as an important growth factor for chondrogenesis in humans and may be beneficial when translating these methods to canine chondrogenesis.

In addition to BMP-2 and TGF-β3, bFGF has been specifically implicated in increasing the proliferation rate of chondrocytes (Solchaga et al., 2005). The exact mechanism as to how this occurs is not fully understood however, it has been shown that bFGF downregulates a large percentage of known positive and negative regulators of proliferation (Solchaga et al., 2005). Even with this downregulation, the ultimate outcome of bFGF on human cells is increased proliferation. Furthermore, the gene expression profile performed in this study suggests that bFGF regulates chondrogenic differentiation through MAPK and Wnt signaling. With regards to MAPK, bFGF upregulates DUSP 4 and 6 which are negative regulators of the MAPK pathway, and thus, bFGF promotes chondrogenic differentiation (Solchaga et al., 2005). Similarly, bFGF downregulates signaling in the Wnt pathway which has been linked to the MAPK pathway (Tufan et al., 2002; Tuli et al., 2003) and is important for chondrogenesis and development in general (Solchaga et al., 2005). Alternatively, in a similar study in horses, bFGF did not compromise chondrogenesis, however, it did significantly accelerate synovial fluid chondroprogenitor cell expansion (Bianchessi et al., 2016). The above evidence combined with other work from our lab (WB et al., 2017), indicate that bFGF may be beneficial to chondrogenesis and should be examined during initial evaluation of canine chondrogenesis.

PURPOSE

The long-term goal of the lab is to mechanistically define the process of canine chondrogenesis and to develop a viable tissue engineering construct capable of restoring

normal joint structure and function for the treatment of FODs in veterinary and human patients. As a first step toward that goal, this thesis describes the development of an improved in vitro system to perform chondrogenic differentiation assays with canine MSCs. Secondly, we describe the use of this system to evaluate the effect of the growth factors/cytokines dexamethasone, TGF-β3, BMP-2, and bFGF on chondrogenesis of canine bone marrow-derived MSCs. This work is of clinical significance as once a combination of growth factors that consistently promote chondrogenesis is determined for canine MSCs, mechanistic and therapeutic efforts may be initiated.

CHAPTER II

DEVELOPMENT OF AN IMPROVED CHONDROGENIC DIFFERENTIATION ASSAY AND ASSESSMENT OF GROWTH FACTOR COMBINATIONS ON CANINE MESENCHYMAL STEM CELL (MSC) CHONDROGENESIS

SUMMARY

Degenerative and developmental joint disorders affect millions of human beings and veterinary species resulting in chronic pain, immobility, and loss of economic and personal productivity. Due to the fact that articular cartilage does not readily heal, attempts at treating articular cartilage lesions either medically or surgically are often met with frustration. The dog is an excellent translational model for humans affected by focal osteochondral defects (FOD) allowing scientists to evaluate feasibility, safety and efficacy of novel treatment strategies for subsequent translation into human beings (Hoffman and Dow, 2016). Much effort has focused on using mesenchymal stem cells (MSCs) to develop tissue engineering constructs to treat FODs. Chondrogenesis, or the differentiation process by which MSCs transition to chondrocytes, has been refined for decades in human beings. In contrast, our understanding of the chondrogenic process of canine mesenchymal stem cells (cMSCs) is in its infancy. A barrier to understanding the mechanistic processes governing canine chondrogenesis is the current inability to consistently induce chondrogenesis in vitro using protocols effective for human MSCs (hMSCs). Previous work using hMSCs has demonstrated that dexamethasone, TGF-β3 and BMP-2 are the optimal growth factors involved in influencing hMSCs down the

chondrogenic lineage. Recently, it was discovered that the addition of bFGF was necessary for cMSC invasion using 3D collagen type I invasion assays. Thus, the objectives of this study were to: first develop an improved in vitro assay to induce chondrogenesis of cMSCs; and, second to evaluate the effects of dexamethasone (D), TGF-β3 (T), BMP-2 (B), and bFGF (F) on cMSC chondrogenesis using the improved assay. Based on our laboratories substantial experience with serum-free three-dimensional (3D) collagen type I assays and existing literature on chondrogenesis of human and ovine MSCs in collagen type I scaffolds, we hypothesized that the inclusion of bFGF in chondrogenic differentiation medium would result in improved chondrogenesis when compared to media containing dexamethasone, and various combinations of TGF-β3 and BMP-2.

In phase 1, cMSCs were suspended in 5 mg/ml collagen type I gels in a serum-free environment with different combinations of these growth factors at two concentrations. Chondrogenic media was added to the cultures. Cultures were incubated for 21 days and evaluated with morphometry, live/dead staining, and with histopathology. Using the results of phase 1, four growth factor combinations were selected to be further assessed for chondrogenesis using quantitative assessment measures. In phase 2, cultures were established as for phase 1 and again cultured for 21 days. Cultures were evaluated for early cytotoxicity and apoptosis using a Lactate Dehydrogenase (LDH) assay, glycosaminoglycan (GAG) content using a colorimetric GAG assay, and the expression of chondrogenic and osteogenic genes using quantitative (real-time) PCR (qPCR). Cultures provided with high concentrations of BMP-2 and

bFGF were larger, heavier, and demonstrated more uniform gross appearance. The combination of DTBF resulted in reduced stress in early stages of culture as assessed by LDH levels in conditioned media. When glycosaminoglycan levels were quantified, cultures containing DTBF and DTB media exhibited significantly higher GAG levels as compared to other treatment groups. Interestingly, both osteogenic and chondrogenic gene expression significantly increased over the 21 day time course. Histologically, cultures treated with DTB and DTBF exhibited focal areas of primitive mesenchymal cells organizing into chondrogenic arrangements. These areas stained positive for proteoglycan using Toluidine blue and Safranin O.

Overall, the 3D collagen type I serum-free system consistently allowed chondrogenic assessment of cMSCs and both subjective and quantitative assays revealed important differences in the various treatment groups. Cultures treated with media containing DTB and DTBF appear to be the most promising for inducing chondrogenesis of canine bone marrow MSCs. This work serves as an initial, important advance in the field of canine chondrogenesis and regenerative medicine for treatment of articular cartilage injuries.

INTRODUCTION

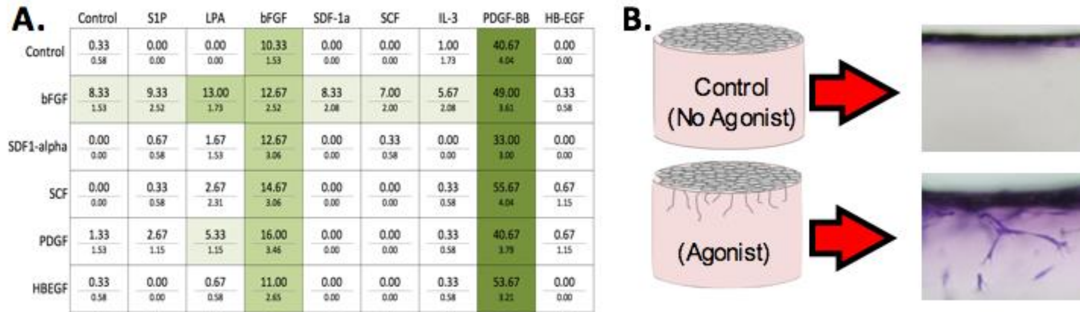
Osteochondrosis and traumatic joint diseases, such as osteochondrosis (OC), osteochondritis dissicans (OCD) and traumatic focal osteochondral defects (FODs), are articular cartilage lesions leading to osteoarthritis (OA) in millions of human and veterinary species. Broadly, the two primary categories of osteoarthritis treatment

include medical and surgical management. While medical management is often used to treat patients with early FODs, management is often life long and requires the addition of new treatments and drugs over time. When medical management fails to alleviate symptoms, surgical treatments are often pursued. Surgical treatment options for FODs can be divided into palliative and restorative treatments. Palliative treatments attempt to remove the source of irritation/inflammation enhancing the formation of fibrocartilage over the defect. This is accomplished through procedures such as: debridement, abrasion arthroplasty and microfracture. Restorative treatments aim to repair or replace the cartilage defect in order to fully restore structure and function (Cole et al., 2009). This is accomplished through procedures such as: autologous osteochondral transplants (OATS), autologous chondrocyte implantation (ACI), or placement of allogenic osteochondral tissue grafts or synthetic cartilage substitutes (Arinzeh et al., 2003; Breinan et al., 2001; Cook et al., 2008; Freed et al., 1993). Although restorative treatments are performed in both human and veterinary medicine, they are not without complication, including donor site morbidity (Feczkó et al., 2003; Reddy et al., 2007; Szerb et al., 2005a), cartilage thickness discrepancies (Breur and Lambrechts, 2012), poor graft survival and progression of OA (Breur and Lambrechts, 2012). In order to navigate these obstacles, recent work has focused the ability of regenerative medicine to restore the structure and function for FOD lesions.

Regenerative medicine is defined as an interdisciplinary field of research and clinical application 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 aging (Daar and Greenwood, 2007). The direct injection of mesenchymal stem cells (MSCs) is one method by which regenerative medicine may be used to treat FODs. Unfortunately, studies have shown that few cells remain at the injection site after delivery (Krause et al., 2010). To address these limitations, tissue engineering, a sub-specialty of regenerative medicine in which MSCs are combined with scaffolds and/or growth factors, has been extensively studied. The goal of this treatment is to repair, regenerate and/or improve the structure and function of diseased articular cartilage (Lijie Zhang et al., 2009). Unfortunately, even tissue engineering as a treatment option has substantial limitations that exist including: the ability of the scaffold to integrate with surrounding native bone and cartilage (Grande et al., 1999), failure to provide complete restoration of normal tissue architecture (Kandel et al., 1997), an inability to restore the biomechanical properties of native cartilage (Neu et al., 2009) and the lack of knowledge of the ideal chondrogenic media that would contribute to the production of cartilage-like tissue engineering constructs.

Although culture conditions resulting in consistent chondrogenesis of human MSCs (hMSC) have been known for decades, the ideal chondrogenesis medium remains unknown for canine MSCs (cMSCs) (Kisiel et al., 2012). As detailed in Chapter I, bFGF has been implicated in increasing the proliferation rate of chondrocytes (Solchaga et al., 2005) and inclusion of bFGF in expansion medium has resulted in increased chondrogenic culture size and proteoglycan content (Solchaga et al., 2005). Interestingly, bFGF has also been shown to improve cMSC survival, attachment and invasion (WB et al., 2017) in 3D collagen type I invasion assays (Figure 2.1).



A.

	Control	S1P	LPA	bFGF	SDF-1a	SCF	IL-3	PDGF-BB	HB-EGF
Control	0.33 0.58	0.00 0.00	0.00 0.00	10.33 1.53	0.00 0.00	0.00 0.00	1.00 1.73	40.67 4.04	0.00 0.00
bFGF	8.33 1.53	9.33 2.52	13.00 1.73	12.67 2.52	8.33 2.08	7.00 2.00	5.67 2.08	49.00 3.61	0.33 0.58
SDF1-alpha	0.00 0.00	0.67 0.58	1.67 1.53	12.67 3.06	0.00 0.00	0.33 0.58	0.00 0.00	33.00 3.00	0.00 0.00
SCF	0.00 0.00	0.33 0.58	2.67 2.31	14.67 3.06	0.00 0.00	0.00 0.00	0.33 0.58	55.67 4.04	0.67 1.15
PDGF	1.33 1.53	2.67 1.15	5.33 1.15	16.00 3.46	0.00 0.00	0.00 0.00	0.33 0.58	40.67 3.79	0.67 1.15
HBEGF	0.33 0.58	0.00 0.00	0.67 0.58	11.00 2.65	0.00 0.00	0.00 0.00	0.33 0.58	53.67 3.21	0.00 0.00

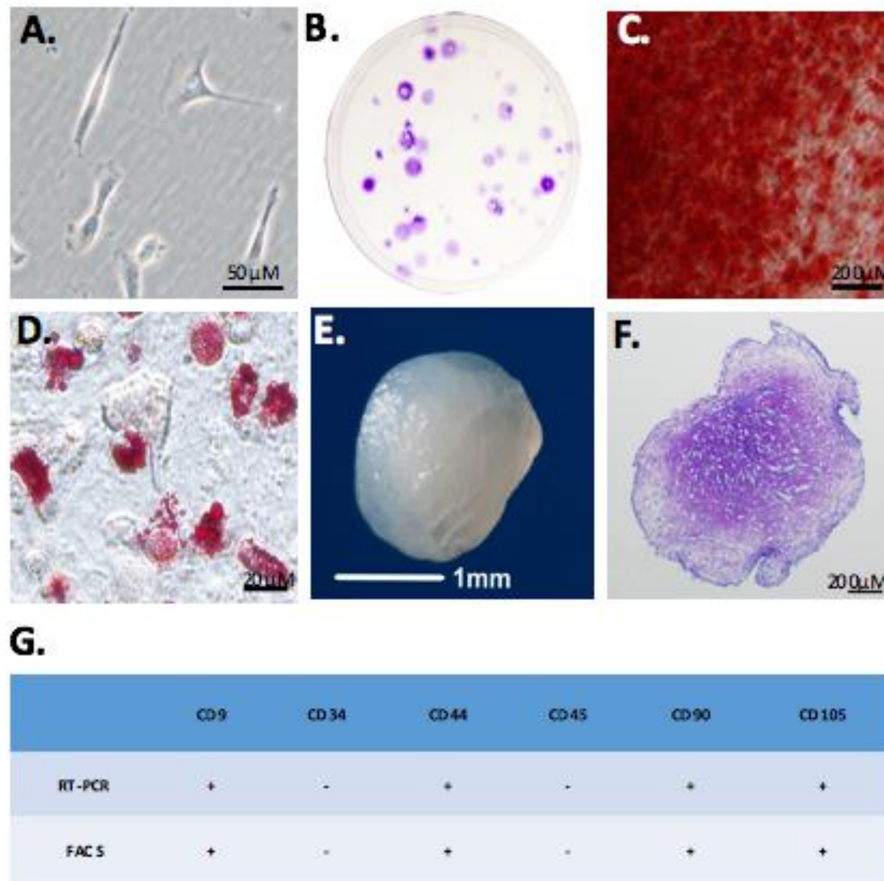
Figure 2.1: *Basic fibroblast growth factor (bFGF) is an important survival factor for the invasion of cMSCs in 3D collagen matrices.* In a related study in our laboratory, P2 marrow cMSCs were suspended on 3.75 mg/ml collagen type I gels under serum-free conditions and assessed for invasion over a 48 hour time course. **A)** Quantification of cell invasion. The invasion agonist mixed within the collagen gels is provided on the top row of the table, while supplemental growth factors added to culture media are listed in the left hand column. Number of invading cells (mean +/- SD) are reported per high powered field (n= 3 wells/condition). Note the effect of bFGF on overall cell invasion, regardless of the agonist mixed within the collagen gels. **B)** Graphic illustration of the 3D cMSC invasion assay in which control gels remain untreated with invasion agonists (top) whereas invasion agonists of interest (in this experiment PDGF-BB) are polymerized within the gel (bottom). Cells proteolytically invade the collagen matrices in response to relevant agonists. A side view of cMSC invasion cultures is provided demonstrating the appearance of cMSC invasion in response to PDGF-BB (agonist in gel) and bFGF (survival factor in medium).

Thus, given our lab's long term goal of developing viable tissue engineering constructs capable of restoring normal joint structure and function, the objectives of this study were to: first develop an improved in vitro assay to induce chondrogenesis of cMSCs; and, second to evaluate the effects of dexamethasone (D), TGF-β3 (T), BMP-2 (B), and bFGF (F) on cMSC chondrogenesis using the improved assay. Based on our laboratories substantial experience with serum-free three-dimensional (3D) collagen type I assays and existing literature on chondrogenesis of human and ovine MSCs in collagen type I scaffolds, we hypothesized that inclusion of bFGF in chondrogenic differentiation medium would result in improved chondrogenesis when compared to media containing dexamethasone, and various combinations of TGF-β3 and BMP-2.

MATERIALS AND METHODS

Cell culture

Canine MSCs (cMSCs) were isolated, from the iliac crest of an adult Walker Hound as a component of an unrelated study (TAMU AUP 2011-149). Cells were confirmed to meet criteria for MSCs as defined by the position paper by *Dominici et al* (Dominici et al., 2006). This included morphological assessment, colony forming capacity, flow cytometry profile, and in vitro tri-lineage differentiation (Figure 2.2). Cells were cryopreserved in 30% FBS (PS-FBS: Atlanta Biological, Inc., Flowery Branch, GA) and 5% DMSO (Sigma, St. Louis, MO) and stored in liquid nitrogen. Passage 1 (P1) cMSCs (1×10^6 total cells) were thawed, plated overnight into two 150 cm^2 tissue culture plates in CCM (αMEM containing 100 units/mL penicillin, 100



	CD9	CD34	CD44	CD45	CD90	CD105
RT-PCR	+	-	+	-	+	+
FACS	+	-	+	-	+	+

Figure 2.2: *Characterization of canine bone marrow-derived mesenchymal stem cells (cMSCs).* Bone marrow cMSCs were isolated using gradient centrifugation and adherence to tissue culture plastic. P1 cells were expanded at 100 cells/cm^2 and cryopreserved in 5% DMSO and 30% FBS. Cells were thawed and expanded to P2 cells for subsequent experiments. **A)** Phase contrast microscopy image of cMSCs in culture. **B)** cMSC colony forming unit (CFU) assay from the primary cell population (bone marrow aspirate) demonstrating the presence of CFUs within the nucleated fraction of bone marrow. **C)** 21 day Alizarin Red Staining (ARS) of osteogenic cMSCs. **D)** 21 day Oil Red O staining of adipogenic cMSCs. **E)** A gross photograph of a 21 day chondrogenic micromass culture. **F)** Micromass cultures from (E) were fixed, sectioned, and stained with Toluidine blue to screen for proteoglycan deposition. **G)** cMSC demonstrated flow cytometry and RT-PCR patterns consistent with MSCs in other species.

μg/mL streptomycin, 0.292 mg/mL glutamate (Invitrogen, Carlsbad, CA) and 10% premium select fetal bovine serum (PS-FBS: Atlanta Biological, Inc., Flowery Branch, GA)) at 37°C and 5% humidified CO_2. The following day cells were washed with PBS, trypsinized, and re-plated as P2 cells for expansion at 250 cells/cm^2. Cells were incubated as noted above with media changes occurring every other day until cells reached 70% confluence. Prior to each experiment, P2 cMSCs were washed with PBS, trypsinized, and centrifuged at 500xG for 5 minutes. Cells were washed in an additional 10 mL volume of Dulbecco's Modified Eagle Medium (DMEM) in an effort to remove residual serum from the medium. Cells were manually counted via hemocytometer.

Collagen type I isolation

Collagen type I was prepared from commercially acquired rat tail tendons by non-proteolytic isolation using 0.1% acetic acid (Bayless et al., 2009; Rajan et al., 2006). After lyophylization, collagen type I was re-suspended at 7.1 mg/mL and maintained at 4°C.

Suspension of cMSCs in 3D collagen type I gels

cMSCs were combined at 10 x 10^6 cells/mL into a collagen type I gel (5 mg/mL final collagen type I concentration) and maintained at 4°C as previously described (Salazar et al., 1999). The cell and gel mixture was divided into the treatment groups noted in Figure 2.3 for phase 1. TGF-β3 (rhTGF-β3, R&D Systems, Minneapolis, MN) (10 ng/mL) and dexamethasone (Sigma) (10^{-7} M) were combined with a low (50 ng/mL)

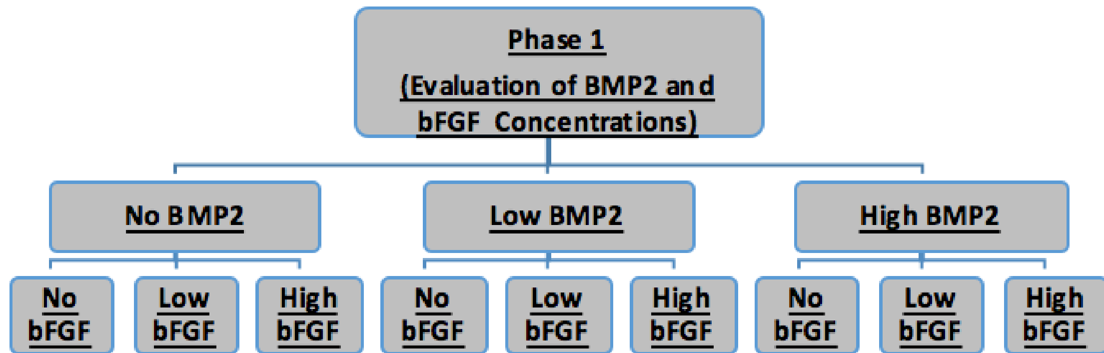


Figure 2.3: *Phase 1 experimental approach.* In order to screen a large number of growth factor doses and combinations, an initial set of experiments was performed with cMSCs suspended in 3D collagen type I gels. In these experiments, the concentrations of dexamethasone (10^{-7} M) and rhTGF-β3 (10 ng/mL) remained constant. Recombinant human BMP-2 (rhBMP-2) was supplemented at 0 ng/mL (No BMP-2), 50 ng/mL (Low BMP-2) or 500 ng/mL (High BMP-2). Recombinant human basic fibroblast growth factor (rhbFGF) was supplemented at 0 ng/mL (No bFGF), 10 ng/mL (Low bFGF) or 100 ng/mL (High bFGF). Cultures were thus treated with ten varying media conditions for 21 days and evaluated subjectively using live/dead staining, gross photography and assessment of culture weight, and histopathology. Results of Phase 1 studies were used to select the most promising growth factor conditions for Phase 2 studies.

and a high dose (500 ng/mL) of BMP-2 (rhBMP-2; R&D Systems), with a low (10 ng/mL) and a high (100 ng/mL) dose of bFGF (rhbFGF, R&D Systems) and the combination of a low and a high dose of BMP-2 with a low and a high dose of bFGF. Once the cell, collagen type I gel, and growth factor combinations were manually pipetted, 100 µL of the cMSC/collagen gel mixture was pipetted into individual wells of a 24 well plate. The plate was then placed in the incubator at 37°C and 5% humidified CO_2 for 30 minutes to allow the collagen to gel (i.e. to set or solidify). Next, 750 µL of chondrogenic medium was added to each well. This chondrogenic medium consisted of DMEM containing 100 units/mL of penicillin, 100 µg/mL of streptomycin, 10^{-7} M dexamethasone, 50 µg/mL ascorbate-2-phosphate (Sigma), 40 µg/mL L-proline (Sigma), 100 µg/mL sodium pyruvate (Sigma) and 10 ng/mL TGF-β3 (R&D Systems) added as described (Reger et al., 2008; Sekiya et al., 2002). Media were also supplemented with BMP-2 and bFGF as noted in Figure 2.3. Once all pellets were covered in chondrogenic medium, they were incubated for up to 21 days with media changes performed twice weekly. Cultures were photographed and weighed at day 3 and day 21. At 21 days, cultures were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for histologic evaluation using H&E, Toluidine blue and Safranin O.

The same protocol was followed for phase 2 using four combinations of growth factors (Figure 2.4).

Subjective assessment of viability in early cMSC cultures using live/dead staining

At day 0, 1, and day 3 media were removed from the 3D cultures and two PBS

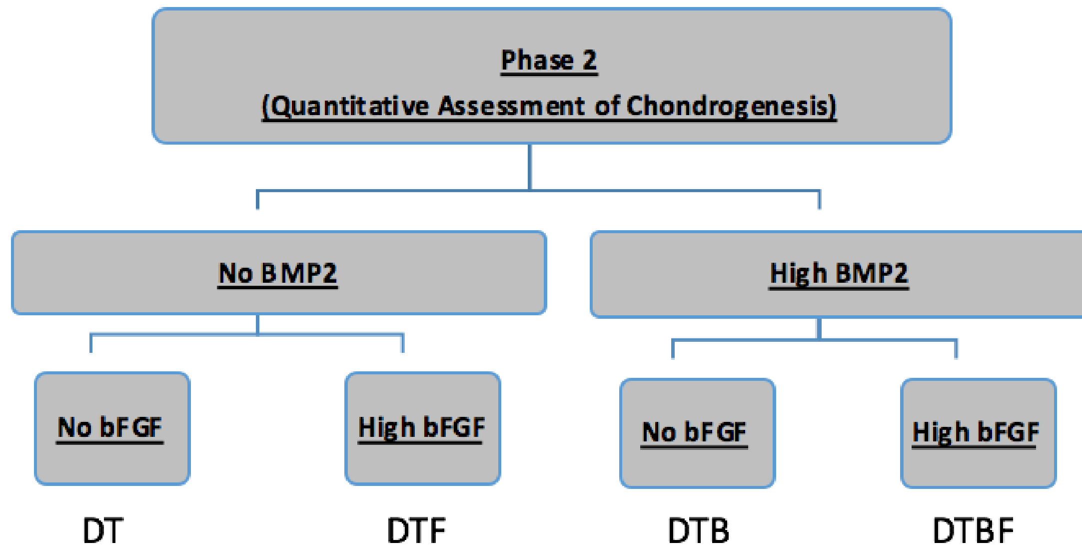


Figure 2.4: *Phase 2 experimental approach.* Based on the results of Phase 1 studies, four promising growth factor combinations were selected for quantitative analysis. As with Phase 1 studies, the concentrations of dexamethasone (10^{-7} M) and rhTGF-β3 (10 ng/mL) remained constant. Recombinant human BMP-2 (rhBMP-2) was omitted from culture medium (No BMP-2), resulting in the treatment referred to as DT. BMP-2 was supplemented at 500 ng/mL (High BMP-2) resulting in the treatment group DTB. Recombinant human basic fibroblast growth factor (rhbFGF) was either then omitted from culture medium (No bFGF) or added to culture medium at 100 ng/mL (High bFGF), resulting in the treatment groups DTF and DTBF respectively. Cultures were thus treated with 4 media conditions for 21 days and quantitatively assessed using the Lactate Dehydrogenase (LDH) assay, quantitative glycosaminoglycan (GAG) assay, and qPCR for transcriptional changes associated with chondrogenesis or osteogenesis.

washes performed. After the final PBS wash, 500 µL of fresh PBS containing 0.2 µM calcein (Sigma) and 100 µg/mL propidium iodide (Botinium, Hawyard, CA) were added to each construct (n=3 constructs/treatment condition). Constructs were incubated at 37°C for 30 minutes and evaluated for live/dead staining using an Olympus microscope and fluorescent microscopy. Individual fluorophore images were acquired. Subsequently, images were merged and overlays produced using SPOT software (version 5.1; Sterling Heights MI).

Quantitative assessment of cytotoxicity/apoptosis with the Lactate Dehydrogenase (LDH) assay

As noted above, 3D constructs containing collagen type I and P2 cMSCs were cultured for either 24- or 72 hours with media exchange occurring on day 0, day 1 (after sample acquisition) and day 3 (after sample acquisition). Conditioned media (n=3) from each treatment group were collected in 200 µL aliquots and stored at -20°C. Upon completion of all experiments, media were allowed to thaw on ice and assessed for LDH levels following manufacturer's instructions using the colorimetric plate reader assay "Determination of Optimum Cell Number for LDH Cytotoxicity Assay kit" (ThermoScientific, Rockford, IL). LDH levels in cultures were quantified against known LDH standards and reported as ng/mL LDH in conditioned media.

Quantitative assessment of Glycosaminoglycan (GAG) content

3D constructs containing collagen type I and P2 cMSCs were cultured for 3, 10

and 21 days to assess glycosaminoglycan content. At each time point, media were removed and n=3 constructs per treatment condition were placed in 500 µL of papain solution based on manufacturer's instructions (Biocolor Life Science Assays, County Antrim, UK). The cultures were heated at 65°C and manually agitated every 15-30 minutes until the spherical collagen cultures were not visibly identified. Samples were then centrifuged at 10,000 RPM for 10 minutes and the supernatant (approximately 500 µL) collected and stored at -20°C. Upon completion of the experiment, samples were allowed to thaw on ice and were assessed for glycosaminoglycan content following manufacturer's instructions for Blyscan Sulfated Glycosaminoglycan Assay (Biocolor Life Science Assays, County Antrim, UK) with sample volumes of 30 µL. Glycosaminoglycan content levels in cultures were quantified against known glycosaminoglycan standards and reported as an amount in ug/mL.

Assessment of cMSC gene expression using quantitative (q) PCR

3D constructs containing collagen type I and P2 cMSCs were cultured for 3, 10 and 21 days to assess gene expression changes over the 21 day time course. At each time point, n=6 cultures from each treatment group were incubated at 37°C and 5% humidified CO_2 in 2.5 mg/mL bacterial collagenase (Sigma) in DMEM for two hours. Constructs were manually agitated every 30 minutes until completely dissolved. Once dissolved, the digests were centrifuged at 1,000 RPM for 10 minutes to pellet the liberated cells. After removal of supernatants, messenger RNA (mRNA) samples were isolated using the Dynabeads ® mRNA Direct™ Purification kit (ThermoFisher), treated

with DNase to remove any contaminating DNA, and quantified using a Qubit Fluorometer 2.0 (ThermoFisher). Complimentary DNA (cDNA) was synthesized from 30 ng mRNA (normalized across all samples) using random hexamer primers and Superscript III reverse transcriptase (Invitrogen) following the SuperScript III RT kit instructions.

Canine qPCR primers for known osteogenic and chondrogenic genes were commercially synthesized as follows: Col1A1 (Nicholson et al., 2007) Forward: GCCGCTTCACCTACAGTGTCA, Reverse: GAGGTCTTGGTGGTTTTGTATTCG; COL2A (Nicholson et al., 2007) Forward: CAGCAGGTTCACATATACTGTTCTGA, Reverse: CGATCATAGTCTTGCCCCACTT; SOX9 (Neupane et al., 2008) Forward: GCTCGCAGTACGACTACACTGAC, Reverse: GTTCATGTAGGTGAAGGTGGAG; AGGRECAN (Neupane et al., 2008) Forward: ATCAACAGTGCTTACCAAGACA, Reverse: ATAACCTCACAGCGATAGATCC; OSTERIX (Neupane et al., 2008) Forward: ACGACACTGGGCAAAGCAG, Reverse: CATGTCCAGGGAGGTGTAGAC and OSTEOCALCIN (Neupane et al., 2008) Forward: GAGGGCAGCGAGGTGGTGAG, Reverse TCAGCCAGCTCGTCACAGTTGG. Housekeeping genes RPL13A and RPL32 were used and the genes were commercially synthesized as follows: RPL13A (Peters et al., 2007) Forward: GCCGAAGGTTGTAGTCGT, Reverse: GGAGGAAGGCCAGGTAATTC and RPL32 (Peters et al., 2007) Forward: TGGTTACAGGAGCAACAAGAAA , Reverse: GCACATCAGCAGCACTTCA

PCR reactions (20 μL) were prepared with 10 μL of SYBR Green Master Mix

(ThermoFisher), 0.6 µL of Forward Primer, 0.6 µL of Reverse Primer, 6.8 µL of water, 0.4 µL of cDNA, and 1.6 µL of transfer RNA. Cycling conditions were performed using the CFX96 Real-Time System (Bio-Rad, Hercules, CA) with an initial SYBR® Green PCR Master Mix enzyme activation at 95°C for 10 minutes, followed by 40 cycles of: denature at 95°C for 15 seconds, anneal/extend at 60°C for 1 minute. A melt curve was performed immediately after qPCR. Cycling conditions were performed at 55°C for 30 seconds followed by 55°C for 5 seconds. Following this, the temperature was increased by 0.5°C per cycle per second for a total of eighty cycles.

cDNA was used in two independent qPCR reactions measuring the six genes of interest using RPL13A and RPL32 as housekeeping genes. mRNA was also isolated from the same cMSCs at day 0 after they were lifted off the expansion plates prior to their establishment in a 3D culture. The expression for each gene of interest was averaged over the two independent qPCR reactions. The threshold (CT) levels were normalized to both the housekeeping genes and the gene expression at day 0 for relative gene expression. Relative gene expression was graphed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Statistical analysis

Descriptive statistics were generated and presented as mean ± standard deviation for all data sets. Analytical statistics included Two-way ANOVA with Tukey's post-hoc test. All statistics were performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Significance was established as $p \leq 0.05$.

RESULTS

Phase 1

Gross photography

Gross photographs for cMSC cultures treated with the ten growth factor combinations described in Figure 2.3 are shown in Figure 2.5. At day 1 (24 hours after initiation of culture), constructs remain somewhat similar in shape and size to their appearance immediately upon gel polymerization. By day 3, the majority of cMSC constructs underwent a marked condensation event, in which the cell/matrix constructs were physically condensed by the cMSCs. Note the consistent size of the no cells treatment group at day 1 and day 3, indicating that the condensation event requires the presence of cMSC. Interestingly, cultures containing TGF-β3 underwent the most consistent and dramatic condensation. Cultures treated with serum-free chondrogenic basal medium containing dexamethasone alone did not undergo contraction. Control constructs treated with 10% FBS also underwent condensation, which is consistent with our experience with the use of 10% serum in 3D collagen assays. These results demonstrate that cells are required for the condensation of 3D collagen gels, and furthermore that the presence of TGF-β3 and not dexamethasone alone drives this process. Gross photographs of phase 1 constructs at 21 days are provided in Figure 2.6.

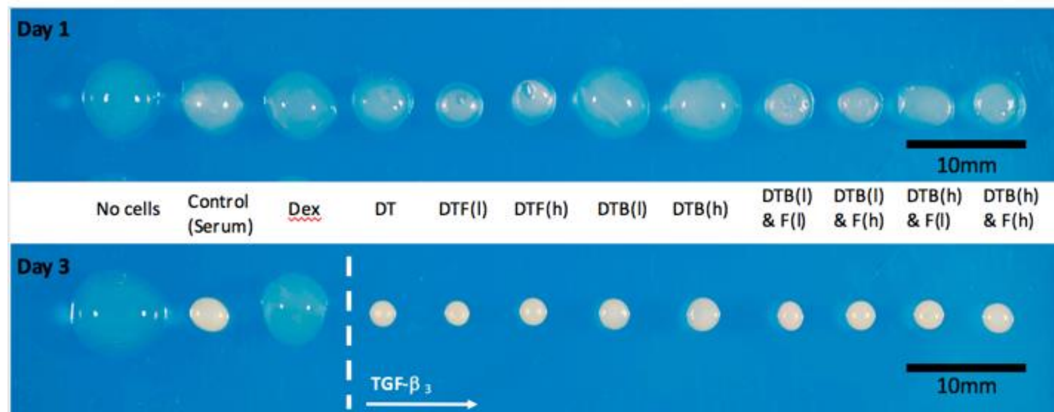


Figure 2.5: *Gross photographs of cMSC/collagen type I constructs at day 1 and day 3.* At the time points indicated, culture media were removed, constructs were washed with PBS, and transferred to a photography station to document changes in construct size in response to the ten Phase 1 treatment conditions. In the absence of cells, collagen type I gels do not experience any notable change in size (left construct – No cells). Interestingly, cMSC cultures treated with dexamethasone also failed to undergo changes in the size of cultures. In the presence of rhTGF-β3 (dashed white line), marked condensation of cultures occurs between day 1 and day 3, consistent with what is known about early mesenchymal condensation during chondrogenesis and other tissue morphogenesis events. A cMSC construct treated with Complete Culture Medium (CCM) which contains 10% FBS is also provided (second construct from left). This construct also underwent condensation, consistent with prior experience adding FBS to 3D collagen cultures. The fact that FBS induced condensation is not surprising as the FBS contains a diverse and incompletely characterized milieu of growth factors (including TGF-β3), cytokines, and other proteins.

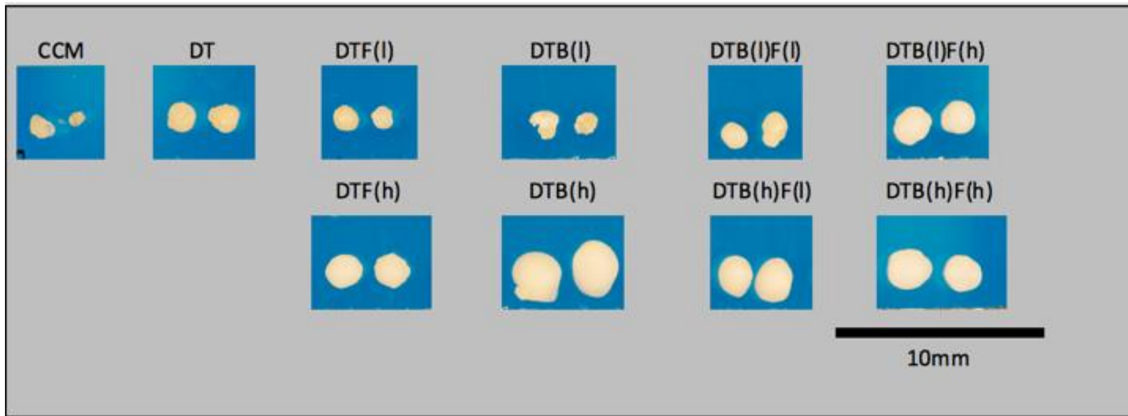


Figure 2.6: *Gross photographs of cMSC/collagen type I constructs at day 21.* Additional cMSC cultures from the experiment shown in Figure 2.3 were incubated for 21 days with media exchange performed twice weekly. At that time, constructs were washed with PBS and transferred to a photography station to document changes in construct size at the cessation of cultures. With high doses of BMP-2 and bFGF (noted by the presence of "h" in the media treatment conditions above), constructs possessed a smooth and regular appearance and were larger in size as compared to their low dose counterparts (noted by the presence of "l" in the media treatment conditions). Cultures treated with Complete Culture Medium (CCM) are shown. Interestingly, while treatment with CCM resulted in early condensation of cultures (Figure 2.5), long term culture in CCM resulted in cultures with substantial fragmentation, surface irregularity, and in contrast to all other treatment conditions, colonization of the 2D plastic monolayer with cMSCs.

At 21 days, constructs treated with CCM, dexamethasone and TGF-β3 (DT) and the lower doses of BMP-2 and bFGF were consistently smaller in size and irregular in shape (Figure 2.6, top row). In contrast, cultures treated with dexamethasone, TGF-β3 and higher dosing combinations of BMP-2 and bFGF were larger in size and more uniformly spherical in size (Figure 2.6, bottom row). These subjective observations indicate that in order to produce larger long-term cMSC/collagen gel cultures, TGF-β3 and higher doses of BMP-2 and bFGF may be required. Quantitative assessment of these cultures at day 3 and day 21 is provided in Figure 2.7. These results demonstrate the effect of early condensation on long-term construct weight for most of the treatment conditions. Importantly, cultures treated with DTB(h), DTB(h)F(l) and DTB(h)F(h) maintained culture weights across the 21 day time course. In fact, cultures treated with the medium DTB(h) were the only treatment condition to result in an increase in construct weight over time.

Visual assessment of viability

In order to assess cell viability and apoptosis during the initial stages of culture, viability was assessed with live/dead staining at day 0 (Figure 2.8) and day 3 (Figure 2.9). Representative 10X objective overlay images of live and dead fluorescence at day 0 demonstrated that a majority of the cells stained with calcein (green = calcein positive) shortly after collagen gel polymerization. This figure demonstrates that the trypsinization and processing methods did not subjectively lead to substantial cell stress or apoptosis at the time of initiation of cultures. Representative 10X objective overlay images of

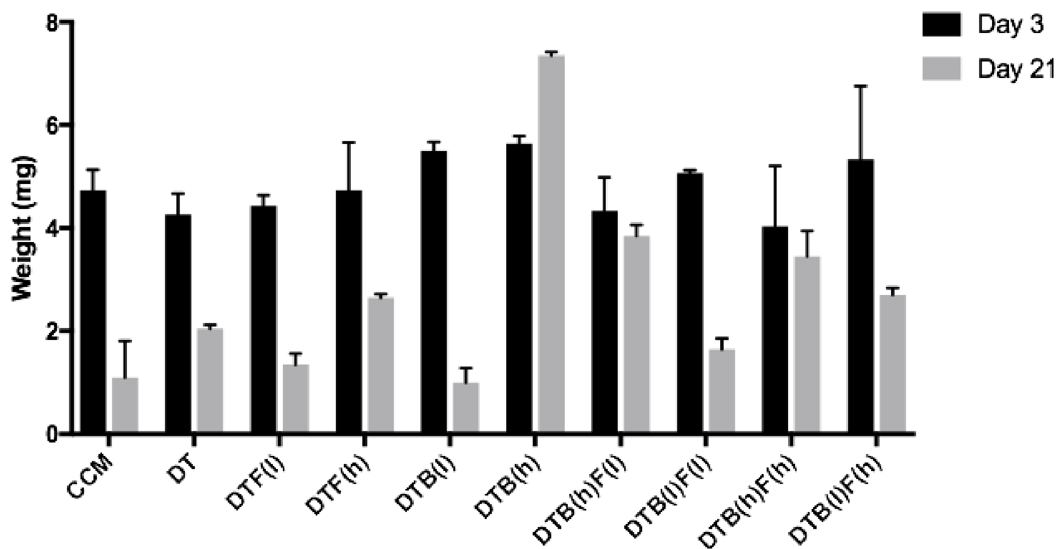


Figure 2.7: *cMSC/collagen type I culture weight at day 3 and day 21.* Constructs from Phase 1 experiments (n=3 constructs/treatment condition) were removed from media at day 3 or day 21. Residual moisture from media was removed from the surface of constructs using filter paper. Construct weight was next determined. Data were reported as mean +/- S.D. for day 3 (black bars) or day 21 (gray bars) for each treatment condition. Note that day 21 weights of DTB(h), DTB(h)F(l) and DTB(h)F(h) were similar or higher compared to day 3 culture weights for each respective treatment group.

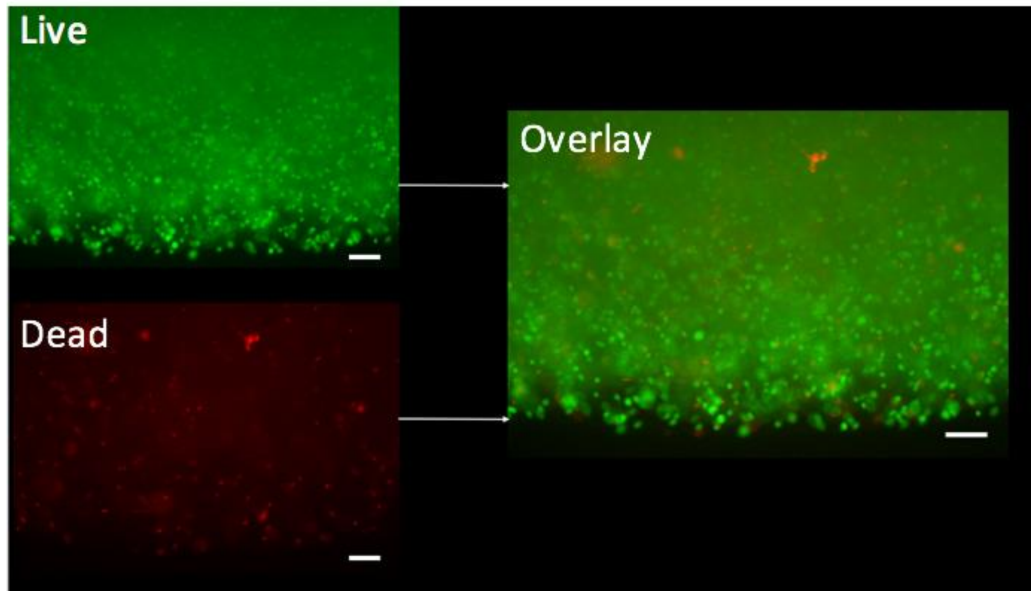


Figure 2.8: *Subjective cytotoxicity assessment of cMSC/collagen type I constructs via live/dead staining at day 0.* P2 cMSCs (approximately 70-80% confluence) were washed twice with PBS, trypsinized, lifted, and centrifuged at 500xG for 5 minutes. A second wash using medium without serum was performed to remove residual serum. Cells were counted and suspended in a 5 mg/ml collagen type I gel at 10 x 10^6 cells/mL in 100 µL volumes. Cultures were incubated at 37°C and 5% CO_2 for 30 minutes to induce collagen gel polymerization. Upon polymerization, two PBS washes were performed and cultures were incubated at 37°C for 30 minutes in 500 µL of PBS containing 0.2 µM calcein (Sigma) and 100 µg/mL propidium iodide (Botinium, Hawyard, CA). Constructs were subjectively evaluated for live/dead staining using an Olympus microscope and fluorescent microscopy. Individual images of live or dead staining were obtained and representative images of day 0 constructs are provided above (left panels). Images were merged and an overlay produced for each construct using SPOT software (version 5.1; Sterling Heights MI). At completion of cell processing and collagen gel polymerization (day 0), constructs contained a large proportion of live (green= calcein positive) cells, with a relatively low number of dead cells (red = propidium iodide positive) (Bar = 100 µm, Obj. = 10X). Note the relatively round or spherical appearance of cells at the time of gel polymerization. These images demonstrate the cell processing and construct fabrication did not lead to acute necrosis of cMSCs and represents a strong starting point for initiation of Phase 1 experiments.

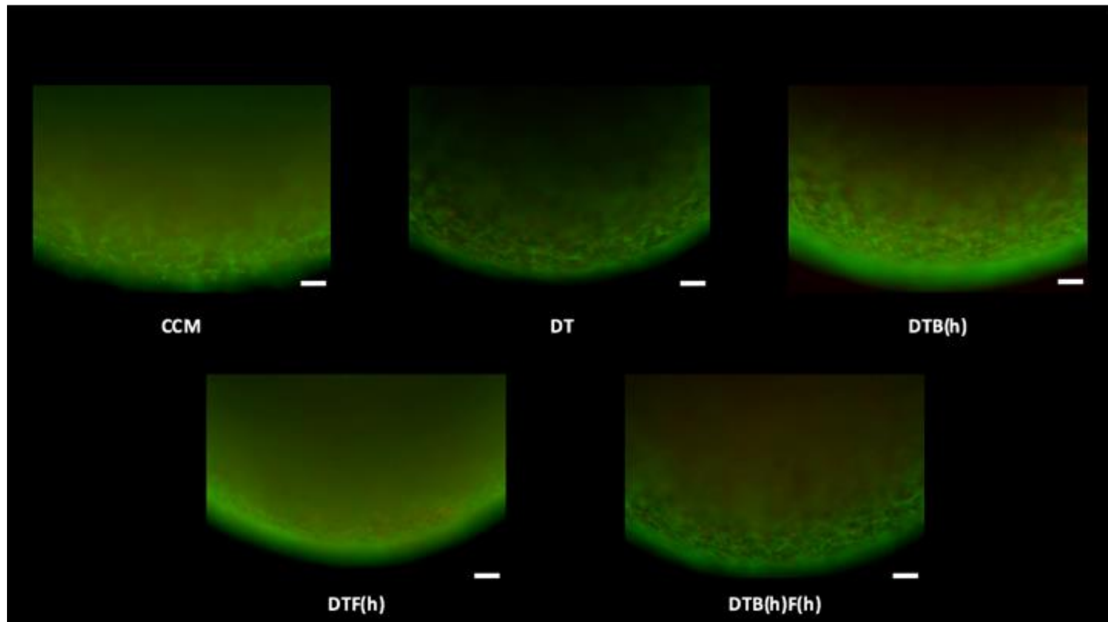


Figure 2.9: *Subjective cytotoxicity assessment of cMSC/collagen type I constructs via live/dead staining at day 3.* cMSC cultures were incubated for 3 days at 37°C and 5% CO_2. Cultures were washed with PBS twice and were incubated at 37°C for 30 minutes in 500 µL of PBS containing 0.2 µM calcein (Sigma) and 100 µg/mL propidium iodide (Botinium, Hawyard, CA). Constructs were subjectively evaluated for live/dead staining using an Olympus microscope and fluorescent microscopy. Individual images of live or dead staining were obtained. Live and dead images of each construct were merged and an overlay produced using SPOT software (version 5.1; Sterling Heights MI). Representative examples of Phase 1 treatment group overlay images are shown above. At day 3, constructs contained a large proportion of live (green= calcein positive) cells, with a relatively low number of dead cells (red = propidium iodide positive) (Bar = 100µm, Obj. = 10X) with transition of the most superficial cells to a spindle or mesenchymal phenotype. There is some propidium iodide uptake near the center of the pellets (top of each image) which indicates some degree of apoptosis within the deeper region of the cultures. Subjectively, no major differences in live/dead staining were identified between treatment conditions.

live/dead staining for five of the growth factor combinations evaluated in Phase 1 are shown in Figure 2.9. Three days after initiation of culture, there was a predominant staining with calcein (green). In contrast, cells staining with propidium iodide (red) were only occasionally noted. While the results presented in Figures 2.5-2.7 demonstrate the effect of growth factor combination and dose on initial condensation and long term construct shape and size, using live/dead staining there was subjectively no detectible difference in viability of the cells at day 3 of culture in response to the growth factor combinations described in Figure 2.9. These results indicate that at the initiation of cultures (day 3), growth factor treatment group does not appear to have a major effect on the proportion of live or dead cells for cells located within the periphery of our cultures.

Histopathology

Representative histopathology of select growth factor combination is shown in Figure 2.10. Due to the size of the evaluated constructs, the histological appearance of the 21 day cultures was quite diverse and uniform chondrogenic differentiation was not noted throughout any of the evaluated constructs. In a number of conditions, groups of primitive mesenchymal cells were organizing as though they were differentiating down a chondrocyte lineage. In these areas, the extracellular matrix (ECM) stained blue to purple with Toluidine blue and pink to orange with Safranin O. These special stains suggest that the ECM in these regions contains proteoglycans. These findings are particularly clear in Figure 2.10A, DTB at a low dose of BMP-2 (50 ng/mL). Cultures treated with high doses of BMP-2 demonstrated similar findings, these cultures

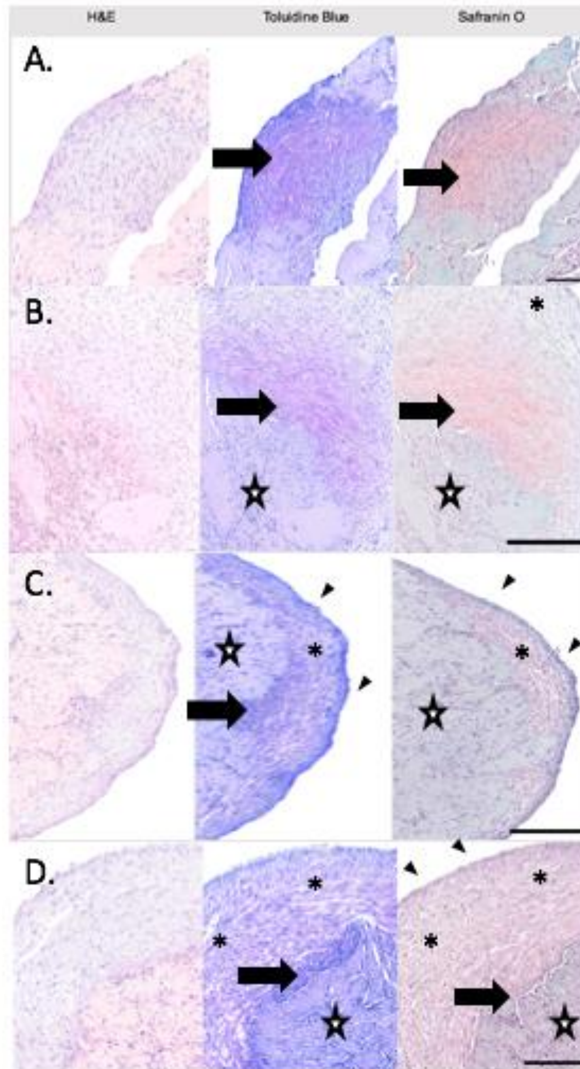


Figure 2.10: *Histologic assessment of cMSC/collagen type I constructs at 21 days*. Phase I cMSC cultures were prepared as described and incubated in the described media combinations for 21 days with media exchange twice weekly. Cultures were gently washed with PBS and fixed in 10% neutral buffered formalin (NBF). Cultures were embedded, processed for histology, and assessed using H&E, Toluidine blue, and Safranin O. **A)** DTB(l). The black arrows highlight the primitive mesenchymal cells moving towards a chondrocyte phenotype with supporting positive Toluidine blue and Safranin O stain. (bar=100µm). **B)** DTB(h). The black arrows highlight the primitive mesenchymal cells moving towards a chondrocyte phenotype staining purple with supporting positive Toluidine blue and Safranin O stain. The asterisk indicates a loosely interwoven myxomatous arrangement of cells that do not stain with Toluidine blue or Safranin O. The white stars denote the appearance of a zone of deep necrosis within the construct (bar = 500µm). **C)** DTB(h)F(l). The black arrows highlight the primitive mesenchymal cells moving towards a chondrocyte phenotype with supporting positive Toluidine blue and Safranin O stain. The asterisks indicate a loosely interwoven myxomatous arrangement of cells that do not stain with Toluidine blue or Safranin O. The white stars denote the appearance of a zone of deep necrosis within the construct. Arrowheads denote a fibrous capsule that forms in the presence of bFGF (bar = 400µm). **D)** DTB(h)F(h). The black arrows highlight the primitive mesenchymal cells moving towards a chondrocyte phenotype. Asterisks indicate a loosely interwoven myxomatous arrangement of cells that do not stain with Toluidine blue or Safranin O. The white stars denote the appearance of a zone of deep necrosis within the construct. Arrowheads denote the presence of a fibrous capsule in the presence of bFGF (bar = 200µm).

contained a peripheral rim of poorly organized myxomatous tissue with a distribution of mesenchymal cells that did not stain for proteoglycans as evidence in Figure 2.10C (asterisks). In contrast to the loosely interwoven myxomatous matrix present in cultures containing BMP-2, cultures treated with low and high doses of bFGF demonstrated a spindle shaped, organized fibrous peripheral rim of tissue (Figure 2.10C,D, arrowheads). Importantly, regardless of the growth factor treatment group, the cells within the center of the constructs appeared non-viable, with evidence of either apoptosis or necrosis (loss of cell structure, pyknosis). Central regions of presumptively non-viable cells are shown in Figure 2.10 (white stars).

In Phase 1, construct morphometry, live dead staining, and histopathology were used to identify potentially promising chondrogenic growth factor conditions for cMSCs cultured in a 3D collagen type I scaffold and treated with ten various combinations of growth factors. Based on the results of Phase 1 experiments, we selected the following conditions for additional quantitative evaluation in Phase 2 experiments: dexamethasone, TGF-β3 (DT), dexamethasone, TGF-β3, BMP-2 (500 ng/mL) (DTB), dexamethasone, TGF-β3 , bFGF (100 ng/mL) (DTF) and dexamethasone, TGF-β3 , BMP-2 (500 ng/mL) and bFGF (100 ng/mL) (DTBF).

Phase 2

Quantitative assessment of cytotoxicity/apoptosis with the Lactate Dehydrogenase (LDH) assay

Cytotoxicity of the cMSC/collagen gel constructs was quantitatively assessed by

assaying LDH levels in conditioned media at day 1 and day 3 (Figure 2.11). There was a significant increase in overall LDH levels at day 3 as compared to day 1 ($p<0.0001$). When comparing growth factor combinations within each day, there were no detectible differences in growth factor combinations at day 1. In contrast, there was a significant decrease in LDH levels in cultures treated with the combination of DTBF as compared to DTF ($p=0.0024$), DTB ($p=0.0067$) and DT ($p=0.0024$). While one interpretation of these data is that cell stress and apoptosis increases at day 3 as compared to day 1. It is important to remember that at day 1, media were conditioned for 24 hours. For day 3 samples, media were conditioned for 48 hours. As such, the amount of time in which LDH was allowed to accumulate was longer for the day 3 time point. This confounding variable makes it difficult to definitively state that day 3 cultures were experiencing more stress/apoptosis than day 1 cultures. Regardless, results of this experiment suggest that the growth factor combination DTBF leads to reduced cell stress during early stages of culture when assessed using the LDH assay.

Quantitative assessment of total glycosaminoglycan (GAG) content

Due to the fact that cartilage is a GAG and proteoglycan rich tissue, we proceeded to evaluated total GAG content using a colorimetric, 1,9-dimethyl-methylene blue (DMMB) based assay at day 3, 10 and 21 (Figure 2.12). There was a significant difference in total GAG content when evaluated over time (days in culture) ($p=0.0096$). At day 3, GAG levels were significantly higher in DTBF constructs than DT ($p=0.0083$), DTB ($p=0.0056$) and DTF ($p=0.0019$). At day 10, GAG levels were significantly higher

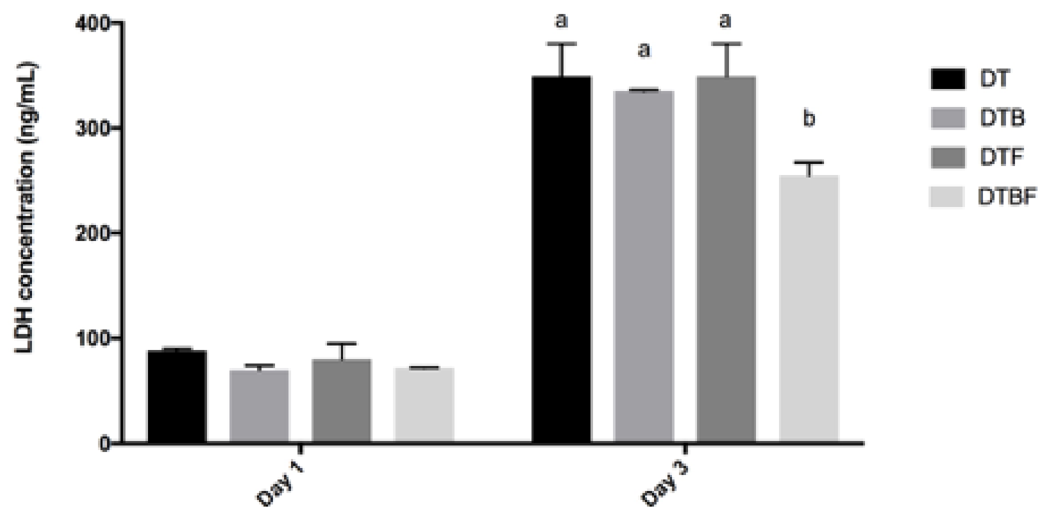


Figure 2.11: *Quantitative assessment of cMSC cytotoxicity in early cMSC/collagen type I cultures using a Lactate Dehydrogenase (LDH) assay*. Based on the results of phase 1 studies, four media treatment conditions were selected for quantitative analysis. Cultures were established as described and were incubated for 1 or 3 days. At each time point, conditioned media were collected for evaluation (n=3 wells/treatment group) and stored at -20°C. At completion of the experiment, media were thawed and LDH quantified using a colorimetric LDH assay. LDH levels (ng/mL) were determined by plotting absorbance against an LDH standard curve. Data are reported as mean ± S.D. LDH levels for each treatment condition at day 1 or day 3 are reported. Letters denote significant differences between growth factor combinations ($p<0.05$). Additionally, there was a significant difference between the LDH levels at day 1 versus day 3 ($p<0.0001$).

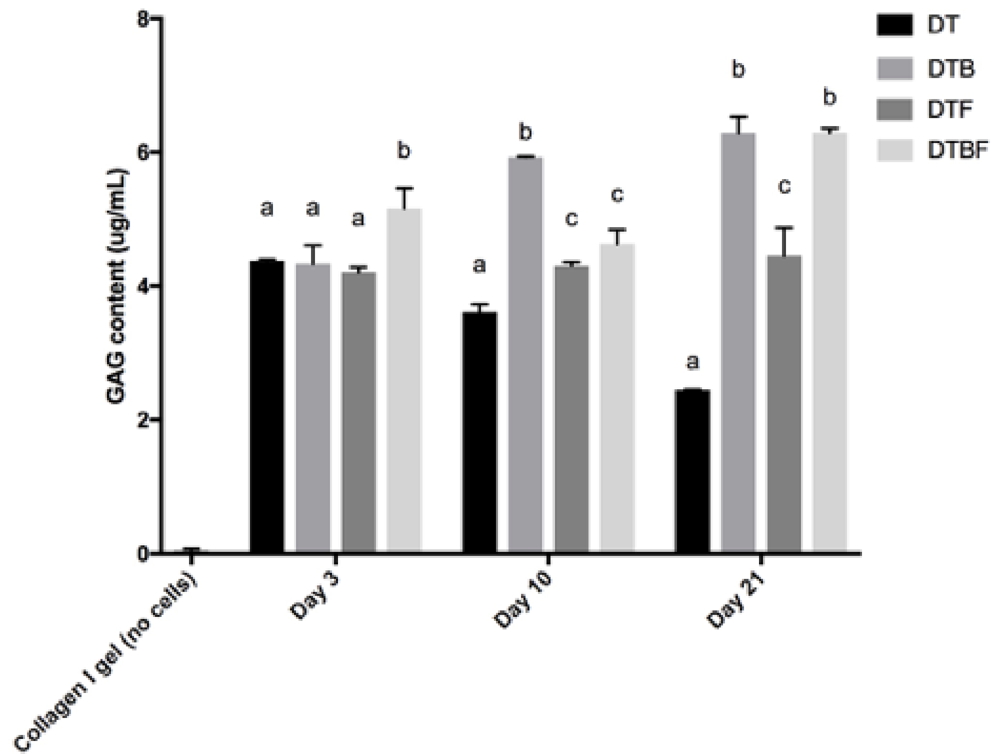


Figure 2.12: *Quantitative assessment of glycosaminoglycan (GAG) in cMSC cultures.* Based on the results of phase 1 studies, four media treatment conditions were selected for quantitative analysis. Cultures were established as described and were incubated for 3, 10, or 21 days. At each time point, constructs were removed from media, and digested in 500 μL of papain digestion buffer (n=3 constructs/condition) for 3 hours with gentle mechanical agitation. Eppendorf tubes containing digested constructs were centrifuged and the supernatant was transferred to new tubes and stored at -20°C. Upon completion of the experiment, samples were thawed at room temperature and total GAG was determined using a colorimetric GAG assay. To determine the basal GAG levels in our collagen type I, equal volumes of blank collagen type I gel (5 mg/mL) were assayed in the absence of cMSCs (far left). Absorbance values were determined and total GAG content (μg/mL) was determined using a known GAG standard curve. GAG levels were reported as mean ± S.D. for each media condition and time point. Letters denote significant differences between growth factor combinations ($p<0.05$) within each time point. There was a significant difference between GAG content at day 3, day 10 and day 21 ($p=0.0096$).

in DTB constructs than DT ($p<0.0001$), DTF ($p<0.0001$) and DTBF ($p=0.0001$).

Interestingly, in the absence of BMP-2 or bFGF, cultures (DT) had lower GAG content as compared to DTB ($p<0.0001$), DTF ($p=0.0206$) and DTBF ($p=0.0012$). At day 21, there was a significant increase in GAG content in both DTB and DTBF groups as compared to DT ($p<0.0001$) and DTF ($p<0.0001$). As was the case at day 10, there was a significant decrease in GAG content in cultures treated with DT as compared to DTB ($p<0.0001$), DTF ($p<0.0001$), and DTBF ($p<0.0001$). These results suggest that GAG content increased over time in the cMSC/collagen gel constructs, and that the growth factor combinations DTB and DTBF produced the highest total GAG levels over time. To our knowledge this is the first instance in which GAG levels have been quantitatively assessed in chondrogenic differentiation of cMSCs.

Quantitative assessment of gene expression during chondrogenesis of cMSCs using qPCR

Finally, real-time (q)PCR was used to assess transcriptional changes of cMSC cultures using a panel of six genes of interest (Figure 2.13). cMSC/collagen gel constructs were evaluated at day 3, day 10, and day 21 for transcriptional changes of the chondrogenic genes collagen type II, alpha chain (COL2A), aggrecan and the chondrogenic transcription factor SOX9. Constructs were assessed for transcriptional changes of the osteogenic matrix protein osteocalcin as well as the osteogenic

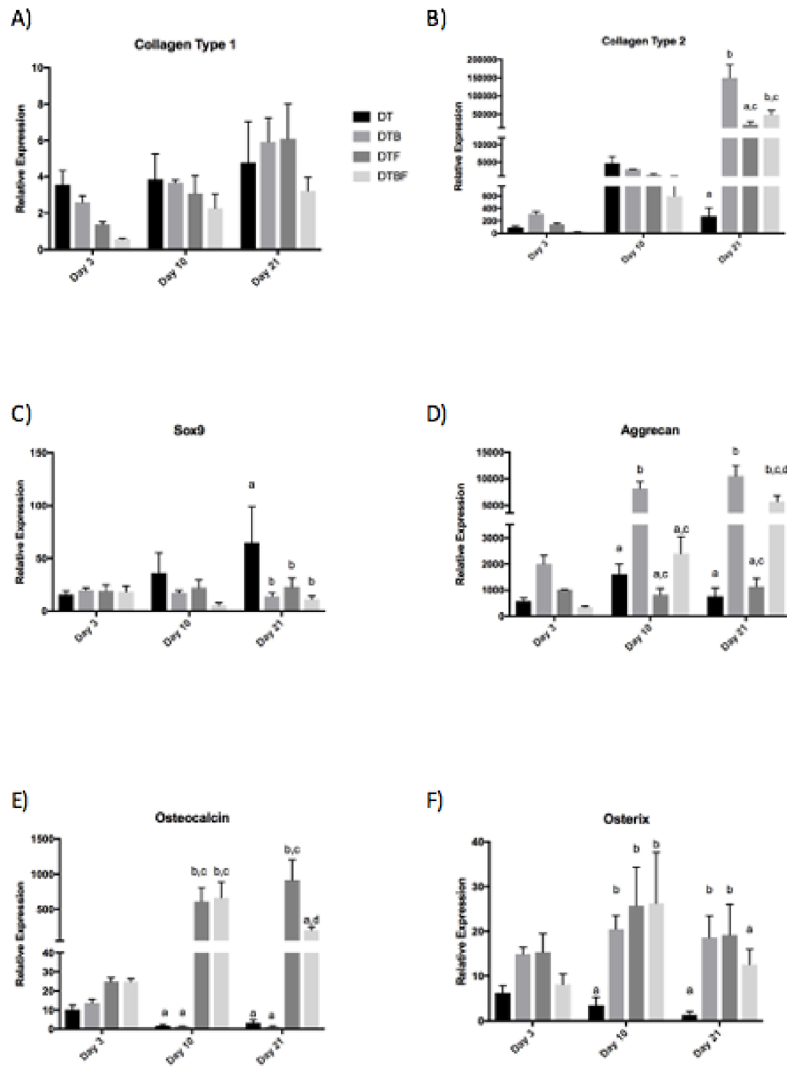


Figure 2.13: *Quantitative assessment of gene transcription in cMSC/collagen type I cultures.* Based on the results of phase 1 studies, four media treatment conditions were selected for quantitative analysis. Cultures were established as described and were incubated for 3, 10, or 21 days. At each time point, constructs (n=6 constructs/condition) were digested in a 2.5 mg/mL bacterial collagenase solution for up to 3 hours in 1.7 mL Eppendorf tubes with gentle manual agitation. Upon successful digestion, tubes were centrifuged at 1,000xG for 10 minutes. Supernatant was removed and the pelleted cells were lysed using 660 µL of cell lysis buffer followed by mRNA isolation according to manufacturer instructions (Dynabeads mRNA Direct purification kit, Thermo-Fisher Sci., Rockford, IL). mRNA were quantified and 30 ng of mRNA from each sample were used to generate cDNA. Real-time PCR (qPCR) was performed for 6 canine genes of interest. All genes were normalized to two housekeeping genes (RPL32 and RPL13A) and expression was reported as mean +/- S.D. relative to mRNA isolated from cMSCs at day 0. Representative gene expression for: A) Collagen Type I, B) Collagen Type II, C) Sox 9, D) Aggrecan, E) Osteocalcin, and F) Osterix. Letters denote significant differences between growth factor combinations ($p<0.05$). There were significant differences in gene expression at day 3, day 10 and day 21 within the growth factors. P Values: Collagen Type I ($p=0.0001$), Collagen Type II ($p<0.0001$), Aggrecan ($p=0.0001$), Osteocalcin ($p=0.0004$) and Osterix ($p=0.0014$). We were unable to detect a significant difference between gene expression at day 3, day 10 and day 21 for Sox 9 ($p=0.0644$).

transcription factor osterix as previously described (Neupane et al., 2008). Collagen type I gene expression was also assessed, alpha chain (COL1A1).

There was a modest yet significant increase in the relative expression of collagen type I over time, regardless of the growth factor treatment condition ($p=0.0001$) (Figure 2.13A). There was no detectible effect of the treatment groups on expression of this gene ($p=0.2559$). This finding suggests that while collagen type I was used to create 3D constructs with cMSCs, suspension in collagen type I did not result in substantial increases in the expression of collagen type I as assessed by qPCR.

In contrast, there was a dramatic increase in collagen type II gene expression over time, with collagen type II expression increasing approximately 50,000 – 100,000 fold by 21 days of culture ($p<0.0001$) (Figure 2.13B). Although we did not detect significant differences in growth factor combinations at day 3 and 10, at day 21 there was a significant decrease in collagen type II expression in DT when compared to DTB ($p<0.0001$) and DTBF ($p=0.0036$). There was a significant increase in collagen type II expression in cultures treated with DTB as compared to DTF ($p<0.0001$), DTBF ($p<0.0001$) and DT ($p<0.0001$). These results demonstrate that our chondrogenic treatment conditions induced collagen type II expression over time, DTB resulted in the greatest increase in collagen type II expression, whereas, the absence of BMP-2 or bFGF resulted in the lowest expression of collagen type II over time.

There was no detectible difference in the relative expression of SOX9 gene expression over time ($p=0.0644$) (Figure 2.13C). However, at day 21, SOX9 expression was significantly increased in cultures treated with DT as compared to DTB ($p=0.0053$),

DTF ($p=0.0195$) and DTBF ($p =0.0036$). These results were surprising in the context of our collagen type II and aggrecan findings (see below). These results suggest that while BMP-2 and bFGF are important in up-regulation of the chondrogenic genes, collagen type II and aggrecan, they may also induce osteogenic as well as chondrogenic events (see osteogenic gene results below).

There was a significant increase in the relative expression of the proteoglycan aggrecan over time ($p=0.0001$) (Figure 2.13D). While we were unable to detect differences in relative expression of aggrecan at day 3, at day 10 there was a significant increase in aggrecan expression in response to DTB as compared to DT ($p<0.0001$), DTF ($p<0.0001$) and DTBF ($p<0.0001$). At day 21, there was a significant increase in the relative expression of aggrecan in culture treated with DTB as compared to DT ($p<0.0001$), DTF ($p<0.0001$) and DTBF ($p= 0.0003$). Moreover, cultures treated with the growth factor combination DTBF had statistically higher aggrecan expression as compared to DTF ($p=0.0004$) and DT ($p=0.0002$). These results closely followed the collagen type II expression profile presented in Figure 2.13B and suggest that the growth factor combinations DTB and DTBF may be superior canine chondrogenic media conditions when assessed by qPCR.

When assessing the osteogenic associated genes osteocalcin and osterix, there was a significant increase in the expression of osteocalcin over time ($p=0.0004$) (Figure 2.13E). While we were unable to detect differences in expression of osteocalcin at day 3, there was a significant increase in osteocalcin expression at day 10 in cultures treated with DTBF as compared to DT ($p= 0.0007$) and DTB ($p= 0.0007$). Cultures treated with

DTF had significantly higher osteocalcin expression as compared to DT (p=0.0013) and DTB (p=0.0013). At day 21, there was a significant increase in osteocalcin expression in cultures treated with DTF as compared to DT (p<0.0001), DTB (p<0.0001) and DTBF (p=0.0003).

Lastly, there was a significant increase in osterix expression over time (p=0.0014) (Figure 2.13F). Although we were unable to detect differences in the relative expression of osterix at day 3, at day 10, cultures treated with DT had a significantly lower osterix expression as compared to DTB (p=0.0280), DTF (p=0.0049) and DTBF (p=0.0041). At day 21, cultures treated with DT had a significantly lower osterix gene expression as compared to DTB (p=0.0265) and DTF (p=0.0218). Although visually there was greater osterix expression in cultures treated with DTBF as compared to DT, these differences were not significantly different (p=0.1838). These results suggest that the presence of the growth factors BMP-2 and bFGF, while increasing expression of the chondrogenic genes, collagen type II and aggrecan, may also activate the osteogenic pathway (as assessed by osteocalcin and osterix qPCR).

DISCUSSION

The objective of this study was twofold: first, to develop an improved assay system to perform subjective and quantitative analysis of canine chondrogenesis and second, to identify the effect of dose and combination of three chondrogenic growth factors known to induce chondrogenesis of human MSCs. We selected collagen type I as our 3D scaffold due to its wide availability, biocompatibility, spontaneous self-assembly

(Glowacki and Mizuno, 2008), and the fact that collagen type I has been successfully used in human and ovine chondrogenic studies (Yokoyama et al., 2005; Zscharnack et al., 2010). It is well known that growth factors often induce dramatically different cellular responses depending on the dose or available concentration of the growth factor. In Phase I studies, we successfully developed a system to place high-density cMSC cultures in relatively large 3D collagen type I cultures (Figure 2.3). We next used this system to subjectively screen the growth factors TGF-β3, BMP-2, and bFGF at multiple concentrations or doses. Based on our results of phase 1, cultures treated with higher doses of bFGF and BMP-2 produced larger and consistently shaped constructs as compared to their lower dose counterparts. Long-term morphometry and photography suggested that the combinations DT, DTF (100 ng/mL), DTB (500 ng/mL) and DTBF (BMP-2 at 500 ng/mL and bFGF at 100 ng/mL) warranted further quantitative assessment. In phase 2 studies, we demonstrated that in the early phases of culture, the medium DTBF reduced cytotoxicity of cultures. Interestingly, the media conditions DTB and DTBF produced the highest glycosaminoglycan levels over a 21 day time course. With regards to gene expression, evidence of both chondrogenic and osteogenic activity was present in cultures containing BMP-2 and bFGF (DTB, DTF, and DTBF). These findings are supported by the diverse histologic appearances of our constructs, which contained regions of presumptive chondrogenic differentiation as well as transitional type cells that may represent the initiation of the endochondral ossification pathway.

In summary, this project has demonstrated the effectiveness of the system in performing subjective and quantitative assessment of canine chondrogenesis. Moreover, using a variety of subjective and quantitative assessment tools, the media conditions DTB and DTBF appear to be the most promising for future canine bone marrow-derived MSC chondrogenic studies. At the time of this writing, it is unable to be determined if DTB or DTBF is a superior chondrogenic culture medium.

Previous studies have demonstrated the difficulty in inducing repeatable chondrogenic differentiation using cMSCs and the traditional micromass culture method (Kisiel et al., 2012). Furthermore, our lab has demonstrated the difficulty working with the micromass system using cMSCs as the constructs fragment easily and are smaller than 1 mm in size making the future option of clinical translation difficult. In other *in vitro* model systems, bFGF was required for cMSC invasion and survival (WB et al., 2017). In human chondrocyte studies, bFGF was noted to enhance proliferation and delay the loss of chondrogenic potential.

Due to the biphasic effect of growth factors, both a low and high concentration of bFGF and BMP-2 were evaluated in combination with traditional concentrations of dexamethasone and TGF-β3. This effect of growth factor dose was noted when evaluating the size and shape of chondrogenic constructs (Figure 2.6). Media containing the higher concentrations of growth factors [DTB(h), DTF(h), DTB(h)F(h)] produced constructs with consistent shape and regular margins compared to those with low concentrations and the control medium. At day 3, all constructs with TGF-β3 underwent condensation. This is similar to that seen *in vivo* as TGF-βs have been implicated in the

modulation of N-cadherin expression levels (Tuli et al., 2003). These modulations likely control condensation–like cell-cell and cell-matrix interactions which then lead to chondrogenic differentiation (Chimal-Monroy and Díaz de León, 1999; Tuli et al., 2003).

Lastly, assessment of construct weight over time suggested that high doses of BMP-2 or bFGF were required to maintain or increase culture weight over the 21 day time course (Figure 2.7).

When quantitatively assessed, there was a statistically significant difference in cytotoxicity between day 1 and day 3 for all four growth factor combinations in phase 2. This suggests that the cells were undergoing more stress at day 3 when compared to day 1. However, it is important to note that at day 1, the cells were provided with 750 uL of medium. This medium was then removed after 24 hours for day 1 analysis and the constructs were provided with fresh media. After 48 hours (day 3), the medium was removed for day 3 analysis. Thus, the statistically significant increase between days could be accounted for by the extra 24 hours the media had in contact with the constructs. When evaluating the difference between growth factors at a fixed time point, at day 3, cultures treated with DTBF produced significantly lower LDH levels when compared to the other three growth factor treatments. This suggested that in early culture, the addition of bFGF may be somewhat protective. Admittedly, LDH levels were not continuously assessed over time and as such, the long-term effects of bFGF in cultures is still unknown. The timing of the delivery of bFGF is certainly worthy of additional investigation and will be the focus of future studies.

One component of articular cartilage which allows resilience is glycosaminoglycan (GAG) content. When evaluating the GAG content of the cMSC cultures at day 3, DTBF produced higher GAG levels as compared to the other three growth factor combinations. At day 10 DTB surpassed DTBF and DTF and finally by day 21 both DTB and DTBF produced the highest GAG levels. These results demonstrate that DTB and DTBF result in the greatest GAG accumulation over time. However, it is important to note that this assay measures total GAG content. Thus, the large increase in glycosaminoglycan content at day 3 for each growth factor combination compared to that of collagen type I should be interpreted with caution. GAGs are present within cells, on the cell surface and in the cell-produced ECM. Thus, this assay measures all three cell and matrix spaces and we are not able to distinguish between those deposited in the ECM against those produced in the cell and on the cell surface. Nevertheless, while ECM staining was not uniformly robust, our histology results suggest GAG accumulation within the ECM in various portions of the constructs (Figure 2.10).

One challenge of working with the canine species as a model system is the relative paucity of known working primer sets for genes of interest. A small number of osteogenic and chondrogenic genes of interest have been previously described by *Neupane et al* and others (Neupane et al., 2008; Nicholson et al., 2007; Peters et al., 2007). Using these sequences, qPCR was performed at early, middle, and late time points using a panel of six genes of interest. All gene expression was normalized to two housekeeping genes (RPL13A and RPL32), based on the stability of these genes in a

majority of tissues in a thorough paper evaluating housekeeping genes in various dog tissues (Peters et al., 2007). Expression was reported relative to the number of copies present within cells at the initiation of each experiment.

The presence of BMP-2 and bFGF in various combinations resulted in increased expression of collagen type II and aggrecan when compared to cultures treated with media containing dexamethasone and TGF-β3. Interestingly, the greatest increase in the expression of SOX9, a master transcription factor for chondrogenesis, occurred in cultures treated with medium containing dexamethasone and TGF-β3. The presence of BMP-2 or bFGF resulted in a dampening of SOX9 expression. These results suggest that while BMP-2 and bFGF certainly led to regional chondrogenesis as assessed by histology and supported by qPCR and GAG data, the presence of these growth factors may also dually activate osteogenic differentiation pathways. In support of this concept of dual differentiation, the presence of bFGF led to significant increases in osteocalcin expression and the presence of BMP-2 and/or bFGF led to significant increases in osterix gene expression. These results suggest that while chondrogenic activity is occurring, the addition of the growth factors BMP-2 and bFGF led to some degree of osteogenic activity. These results may account for some of the changes seen histopathologically and may indicate the possibility of endochondral ossification. Additional studies are warranted to evaluated this important finding.

While the histologic findings of focal/regional chondrogenic activity initially appear discouraging, to the author's knowledge, the methods described in this thesis are the first to successfully allow consistent assays of chondrogenesis with canine bone

marrow-derived MSCs. As such, this work represents a significant advance in the field of cMSC chondrogenesis. The finding of regional chondrogenesis may be due to the large increase in construct size when compared to the traditional micromass culture system initially described by *Sekiya et al* (Sekiya et al., 2002). Our initial studies concluded at 21 days. As such, it is possible that cultures may continue to undergo differentiation over time. Additional experiments would be necessary to evaluate this possibility.

Within the most promising areas of differentiation, primitive mesenchymal cells that morphologically adopted a cartilage phenotype produced ECM that stained positive with both Toluidine blue and Safranin O. Despite these findings, it is difficult to determine whether these areas are indicative of primitive chondrogenesis or other differentiation processes such as endochondral ossification where a cartilage intermediary is transformed into bone. With endochondral ossification, chondrocytes become terminally differentiated to a hypertrophic phenotype increasing their cellular fluid volume by almost 20 times (Goldring and Tsuchimochi, 2006). Morphologic evidence of chondrocyte hypertrophy in the histologic sections evaluated in this study were not noted. Once these chondrocytes undergo hypertrophy, they begin to express collagen type X and alkaline phosphatase (Goldring and Tsuchimochi, 2006). To further evaluate the possibility that our cultures contain early endochondral ossification, collagen type X gene expression or immunohistochemistry could be evaluated using qPCR. Although the canine genome is sequenced, functional primer sets for collagen type X are undescribed and would need to be validated prior to use in our system.

Lastly, treatment of our 3D cultures with each of the growth factor combinations resulted in the presence of a zone of necrosis in the central aspect of cultures. TUNEL staining or active caspase immunohistochemistry would be necessary to confirm this appearance. A reason for the central zone of necrosis may include a larger diffusion distance for nutrient and waste exchange. Zones of necrosis within the central area of chondrogenic cultures have been previously reported (Kisiel et al., 2012; Liangming Zhang et al., 2010) in both human and canine MSC studies. *Zhang et al*., hypothesized that these changes may be explained by the difference in oxygen tension in the environment with extremely high cell density resulting in poor nutrient diffusion (Liangming Zhang et al., 2010). As the optimum oxygen tension and cell density combination has yet to be determined for canine chondrogenesis, additional studies are likely warranted to test this hypothesis. Interestingly, pilot experiments were performed in which cMSCs were expanded under hypoxia and transitioned to normoxia during chondrogenesis while also performing the reverse treatment conditions (normoxia to hypoxia). These treatments did not dramatically alter the chondrogenic process when assessed histologically (unpublished observations).

Limitations of the work described in this thesis include the use of only one preparation of canine MSCs which were isolated from the bone marrow of a healthy intact Walker Hound. As such, the effect of donor age, sex, health, and tissue source of MSCs on canine chondrogenesis in this model system remain unknown. As noted above, the qPCR panel was somewhat limited as compared to other model systems. Admittedly, the ability to quantitatively assess early live and dead cells was prevented by the large

3D nature of the constructs and the inability to use fluorescence to image through these constructs without mechanically sectioning through the gels. Furthermore, the methods and growth factor combinations described in this study did not result in widespread chondrogenesis when the pellets were evaluated via histopathology, despite the fact that GAG content and qPCR results provided evidence for chondrogenic differentiation. While there are potentially an unlimited number of growth factors and growth factor combinations worthy of evaluation, we only focused on a select number of growth factors. Lastly, this system relies on collagen type I as opposed to the collagen type II found in articular cartilage.

Despite these limitations, the system defined in this report allows assessment of cMSC differentiation events using a variety of both subjective and quantitative assessment tools. This system is suggested by the authors to prove useful for additional mechanistic studies aimed at defining canine chondrogenesis. It may also prove useful as a foundation for translational work in which cMSCs are induced toward chondrogenesis in a collagen scaffold and utilized to treat focal cartilage defects in canine experimental or spontaneous disease models.

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis outlines the importance of addressing the clinical and economic burden accompanying focal articular cartilage defects, such as those caused by the developmental orthopedic disease, osteochondrosis, as well as by localized joint trauma. The purpose of the work presented within this thesis was to develop an *in vitro* serum-free collagen type I system to evaluate cMSC chondrogenesis using a number of subjective and quantitative assessment tools. Once established, the effect of the growth factors TGF-β3, BMP-2, and bFGF on cMSC chondrogenesis were determined. This work serves as a significant advance in canine chondrogenesis and may serve as a launching point for future translational studies *in vivo*.

In Chapter II, specific combinations of TGF-β3, BMP-2 and bFGF were combined with cMSCs and a collagen type I scaffold for evaluation of chondrogenic differentiation in two sets of experiments: Phase 1 (a screening experiment) and phase 2 (a quantitative assessment of cytotoxicity and chondrogenesis). Results of phase 1 demonstrated that chondrogenic media containing the higher dosages of BMP-2 and bFGF produced larger, heavier and consistently shaped constructs, which may prove to be more amenable to translation to the clinical setting. Importantly, cell handling and construct fabrication did not affect cMSC health at day 0 as assessed visually using live/dead staining. Subjectively, there was no difference in live/dead staining results across any of the growth factor combinations at day 3. Furthermore, histologic assessment revealed focal regions of mesenchymal cells organizing in a chondrogenic

phenotype, particularly in cultures treated with dexamethasone, TGF-β3, BMP-2 (DTB) and dexamethasone, TGF-β3, BMP-2, and bFGF (DTBF). In phase 2, cytotoxicity was evaluated during early stages of cultures and total GAG content was determined at 3, 10, and 21 days. Treatment of cMSC constructs with medium containing DTBF reduced cell stress and apoptosis early in culture when compared to the other growth factor combinations. These results suggest that the incorporation of bFGF may be advantageous during initiation of cMSC chondrogenic cultures. Moreover, constructs treated with media containing DTB and DTBF had significant increases in total GAG content at day 21, suggesting that, in this *in vitro* canine system, these growth factor combinations are capable of producing constructs that accumulate GAG over time.

Finally, assessment of gene expression using qPCR demonstrated an up-regulation of both osteogenic and chondrogenic genes during the course of our chondrogenic cultures. These results suggest that that multiple differentiation processes may be occurring within different regions of the constructs. These findings were supported by histopathology as small distinct regions were seen to undergo chondrogenic differentiation and stain with Toluidine blue and Safranin O. There were no areas indicative of hypertrophic chondrocytes and thus, endochondral ossification, given the osteogenic and chondrogenic gene expression on qPCR, was less likely. Additional assessment of histological sections using TUNEL staining and immunohistochemistry for collagens type II and X are currently under consideration. Collectively, the results presented in Chapter II demonstrate that the 3D serum-free collagen type I system is useful for cMSC differentiation assays. Both subjective and

quantitative assessment measures were developed to assess chondrogenesis representing an advance for the field of canine chondrogenesis.

Future studies however, are necessary to further elucidate the molecular mechanisms of canine chondrogenesis. From a clinical perspective, additional work is necessary to determine if tissue type or donor variability will impact chondrogenesis using this system. Additionally, in order to fabricate larger constructs for implantation in larger animals or human beings, future studies may be necessary to fuse early chondrogenic cultures as was recently demonstrated with hMSCs (Bhumiratana et al., 2014). The results of these future experiments may lead to novel regenerative medicine and tissue engineering treatments, which will ultimately address many of the disadvantages of the currently available medical and surgical treatment options.

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