IN VITRO COMPARISON OF EQUINE TENDON- AND BONE MARROW-DERIVED CELLS EXPANDED WITH FGF-2 PRIOR TO CULTURING WITH TENDON MATRIX AND IGF-I

BY

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THESIS

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ABSTRACT

This study was performed to determine the effects of fibroblast growth factor-2 (FGF-2) on monolayer expansion of equine tendon- and bone marrow-derived cells prior to culture with autogenous acellular tendon matrix and insulin-like growth factor-I (IGF-I). Progenitor cells were isolated from six young adult horses, expanded in monolayers with FGF-2, and cultured with autogenous acellular pulverized tendon and IGF-I for seven days. Initial cell isolation and subsequent monolayer proliferation were assessed. In the cell: pulverized tendon cultures, cell viability, expression of collagen types I and II, and cartilage oligomeric matrix protein (COMP) mRNAs, collagen and glycosaminoglycans (GAG) syntheses were assessed. Tendon-derived cells proliferated significantly more rapidly in the initial monolayer expansion cultures in comparison to bone marrow-derived cells. Further, monolayer expansion with FGF-2 significantly increased the cell numbers of tendon-derived cells. Expression of collagen type I, collagen type III and COMP mRNAs was higher in tendon-derived cell groups than bone marrow-derived cell groups. However, IGF-I supplementation significantly increased collagen type I and type III mRNA expression in only the bone marrow-derived cell groups. IGF-I supplementation significantly increased collagen synthesis of bone marrow-derived cells. Monolayer expansion with FGF-2 followed by IGF-I supplementation significantly increased proteoglycan synthesis in tendon-derived cells. In summary, tendon-derived cell cultures generated more cells and showed increased matrix synthesis following monolayer expansion with FGF-2 when compared to bone marrow-derived cells. *In vivo* experiments using FGF-2 expanded tendon-derived cells are warranted to evaluate the effects on tendon healing.

To My Grandfather

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CHAPTER 1

INTRODUCTION

Flexor tendon injuries are among the most common musculoskeletal injuries that contribute to the loss of athletic use of horses (Dowling et al. 2000, Dyson 2004, Ely et al. 2009, Genovese et al. 1996). In addition, the risk of tendon injury increases with age. The healing response is prolonged following tendon injury and the resultant repair tissue is usually of inferior mechanical strength. Consequently, the prognosis for return to previous levels of performance is poor (Dowling et al. 2000, Lam et al. 2007). A variety of surgical and conservative treatments have been developed, however none have succeeded in returning the healed tendon to its original strength (Bramlage 1991, Alves et al. 2001, Crowe et al. 2004, Hawkins, Ross 1995, Gibson, Burbidge & Pfeiffer 1997). Re-injury, despite prolonged and costly rehabilitation, is common.

The inability of equine tendon to regenerate after injury, or to heal with mechanical properties comparable to the original tissue is likely due to the low vascularity and cellularity of the tissue, the low number of progenitor cells within the tissue, and healing under weight-bearing conditions (Birch 2007a, Birch et al. 2008, Hosaka et al. 2005, O'Brien 1997). Additionally, it is thought that tendinitis is due to a long-term degenerative process rather than one episode of trauma (Birch, Bailey & Goodship 1998, Birch, Wilson & Goodship 2008). Strategies to improve tendon healing have aimed at enhancing the metabolic response of the injured tenocytes, modulating the organization of the extracellular matrix produced, or administering progenitor cells to enhance repair tissue (Krampera et al. 2006, Lacitignola et al. 2008, Richardson et al. 2007a). The use of mesenchymal stem cells (MSCs) for tissue repair, regeneration and engineering has been an extremely dynamic area of research over the

past decade. The majority of these studies have focused on the use of MSCs derived from bone marrow population. However more recent studies indicate that alternative sources of progenitor cells (fat, synovium, cartilage, and muscle) might also be beneficial for specific therapeutic applications (Ju et al. 2008, Nixon et al. 2008). The most prominent avenues of investigation have been directed at repair of bone, cartilage, myocardium and CNS tissues (Caplan 2007a, Caplan 2005a).

Bone marrow-derived MSCs have been used for tendon repair in horses, rabbits, and rats (Lacitignola et al. 2008, Pacini et al. 2007, Smith 2008a, Smith et al. 2003). To date, the studies performed in horses using bone marrow derived MSCs for tendon repair have been subjective with no clear, objective evidence of efficacy. Despite the lack of objective evidence, over 1,000 horses have been treated with intratendinous injections of direct bone marrow aspirates or adipose-derived progenitor cells over the last four years (Nixon et al. 2008, Smith 2008a). However, none of these studies used a homogeneous population of stem cells to repair tendon and one study documented bone formation within the tendon repair tissue (Dressler, Butler & Boivin 2005). A recent equine study using adipose-derived source of progenitor cells for treatment of a collagenase-induced model of tendinitis showed only histological improvement of repair over the untreated control, with no biomechanical data to substantiate improved outcomes (Nixon et al. 2008). Currently, stem cell therapy in veterinary medicine has generated a vast amount of revenue from clientele with minimal scientific follow-up on clinical cases.

Alternate sources of progenitor cells have been described for tendon repair in other species (Ju et al. 2008, Cao et al. 2002, Liu et al. 2006). However, there are only a few studies utilizing an equine model of tendinitis treated with alternate sources of progenitor

cells (Nixon et al. 2008). Recent lines of evidence suggests the presence of multipotent, clonogenic population of progenitor/stem cells in tendons, within an extra cellular matrix (ECM)-rich niche (Bi et al. 2007, Salingcarnboriboon et al. 2003, Rui et al. 2010)). One study showed tendon and muscle-derived cells proliferated more rapidly in monolayer cultures and had better viability and matrix production, compared to bone marrow-derived cells (Stewart et al. 2009a). A differential adherence preplating technique has been used in this study to isolate autogenous equine tendon-derived progenitor cells from the lateral digital extensor tendon. This method has been adapted from a technique described to isolate myogenic cells, a progenitor cell population in skeletal muscles (Gharaibeh et al. 2008, Jankowski et al. 2001).

Growth factors function as anabolic signaling peptides, resulting in cell proliferation and differentiation, up-regulation of cell metabolism, and synthesis of extracellular matrix. Fibroblast growth factor-2 (FGF-2), also known as basic fibroblast growth factor is a potent mitogen that binds to heparan-sulfate proteoglycans in the tendon extracellular matrix and is released with matrix degeneration (Dahlgren, Mohammed & Nixon 2005, Duffy et al. 1995, Chan et al. 2000). Mesenchymal stem cell cultures supplemented with FGF-2 proliferate more rapidly and exhibit an increased capacity for self-renewal and differentiation (Solchaga et al. 2010, Stewart et al. 2007). The authors have evaluated the effects of FGF-2 on equine bone marrow-derived MSCs at concentrations of 1, 10, and 100 ng/mL (Stewart et al. 2007) Optimal effects were reported at 100 mg/mL (the concentration used in this study). In addition, several *in vitro* and *in vivo* murine and lupine studies of tendon healing have shown enhanced angiogenesis, tendon fibroblast proliferation, and collagen type III expression in response to FGF-2 administration (Chan et al. 2000, Chan et al. 1997, Wang et al. 2005).

The role of insulin-like growth factor-I (IGF-I) in equine tendon healing has generated a considerable body of research in the last few years. *In vitro*, IGF-I stimulates mitogenesis, matrix gene expression, and collagen synthesis by tenocytes(Abrahamsson, Lundborg & Lohmander 1991)(Costa et al. 2006). Further, exogenous injections and gene therapy delivery of IGF-I have resulted in histological improvement in tendon healing following collagenase-induced tendinitis (Dahlgren et al. 2002, Schnabel et al. 2009). The dose-dependent effects of IGF-I on *in vitro* tenocyte matrix synthesis were evaluated by Abrahamsson et al (Abrahamsson, Lundborg & Lohmander 1991). Collagen synthesis was significantly increased at 100 and 250 ug of IGF-I/mL doses. A concentration of 100ug of IGF-I/mL was selected for use in the current study, on the basis of published results and the outcomes of previous stem cell-focused studies completed in the author's laboratory. The beneficial effects of IGF-I are increased mitogenesis, tendon gene expression, and collagen synthesis (Abrahamsson, Lundborg & Lohmander 1991, Olesen et al. 2006).

For clinically viable applications of cell-based therapies, the *in vitro* expansion of putative progenitor cell populations needs to be optimized to reduce the time required for generation of adequate cell numbers. Additionally, the biosynthetic activities of re-implanted cells need to be augmented to promote healing through effective tissue repair. Therefore, this study addressed two major objectives. Firstly, the mitogenic effects of FGF-2 on tendon- and bone marrow-derived cell populations were assessed during *in vitro* monolayer expansion. Secondly, the synthetic and phenotypic responses of monolayer-expanded cells to exogenous IGF-I were assessed in an *in vitro* "powdered matrix" model. Pulverized tendon derived from an autogenous source has been used as substrate material to provide a three-dimensional plane for cell adhesion/proliferation and matrix synthesis.

The goals of this Master's project are two-fold:

(1) To determine whether FGF-2 supplementation during monolayer expansion can enhance the proliferative capacity and matrix synthesis of both tendon- and bone marrow-derived cells.

(2) To evaluate whether IGF-I supplementation alone or following FGF-2 monolayer expansion would have a beneficial effect on cell proliferation, tendon gene expression, and matrix synthesis of tendon- and bone marrow-derived cells cultured with tendon matrix.

The overall objective of this study was to determine whether sequential administration of FGF-2, during monolayer expansion, and IGF-I in culture with pulverized tendon, would improve cell expansion and subsequent matrix synthesis. My hypothesis was that tendon-derived cells expanded with FGF-2 and cultured with pulverized tendon and IGF-I supplementation will have increased cell viability/ proliferation, matrix gene expression, and matrix synthesis in comparison to bone marrow-derived cells cultured under similar conditions.

CHAPTER 2

LITERATURE REVIEW

Flexor tendon injuries are amongst the most common musculoskeletal injuries that contribute to the loss of athletic use of horses (Dowling et al. 2000, Thorpe, Clegg & Birch 2010). Further, the healing process following tendon injury is prolonged and the resultant repair tissue is of inferior mechanical strength. Consequently, the prognosis for return to previous level of performance is poor (Patterson-Kane, Firth 2009). Recent management and treatment strategies do not return the healed tendon to its original strength. Re-injury, despite prolonged and costly rehabilitation, is common. Cell-based approaches with or without growth factor enhancement have been a recent focus in tendon research with an ultimate goal of better histological organization and biomechanical strength (Lacitignola et al. 2008, Schnabel et al. 2009, Richardson et al. 2007b, Smith 2008b).

Structure and Function of Tendons

Tendons are dense collagenous tissues that connect muscles to bones. Based on their function, equine tendons can be classified as 'weight-bearing' tendons that store and release elastic strain energy, increasing the efficiency of locomotion (e.g., digital flexor tendons) **(Figure 1)** and 'positional' tendons that transmit muscle generated forces to bones resulting in movement around joints (e.g., digital extensor tendons) (Birch 2007a). Tendons are complex organs, composed of a hierarchical arrangement of smaller subunits **(Figure 2)**. Morphologically, in cross-section tendons are divided into a number of fascicles, which are in turn composed of collagen fiber bundles and then fibrils (O'Brien 1997, Benjamin, Kaiser

& Milz 2008). The fascicles are held together by a loose areolar connective tissue, the endotenon, which becomes confluent with the outer epitenon. The epitenon is surrounded by the paratenon, a fine connective tissue sheath which functions as an elastic sheath to permit free movement of the tendon against the surrounding structures. Tendons consist primarily of collagen fibers oriented in the direction of force application. The collagen molecules are stabilized by intermolecular chemical crosslinks resulting in high tensile strength (Avery, Bailey 2005).

Molecular composition

Tendons are composed predominantly of water (70%) and cells, extracellular matrix, enzymes (30%) (Fu et al. 2002). The matrix is composed mainly of collagen and some elastin embedded in a proteoglycan-water matrix. Collagen accounts for 65-80% and elastin about 1-2% of the dry mass of the tendon (Hosaka et al. 2010). These components are produced by tenoblasts and tenocytes, which are elongated fibroblasts interspersed between the collagen fibers (Kannus 2000).

Collagen type I is predominant and accounts for about 90% of total tendon collagen, with a small portion comprised of collagen type III, about 4-5%, thought to form smaller and weaker fibrils (Patterson-Kane, Firth 2009). In the fibro-cartilaginous regions, collagen types II, IX, X and XI, may also be found (O'Brien 1997). Collagen type IV is found in the basement membrane with a trace of type V collagen (Dowling et al. 2000, O'Brien 1997). The structural unit of collagen is tropocollagen or a microfibril, consisting of three polypeptide chains wound together to form an alpha triple helix. These alpha chains are composed primarily of proline and glycine, present as every third residue. Several of these

microfibrils unite to form a fibril, and many fibrils are embedded in the extracellular matrix as collagen fibers.

Proteoglycans and glycoproteins combine to form a ground substance, accounting for 1-2% of the dry mass of the tendon (O'Brien 1997, Kannus 2000). The ground substance surrounds the collagen fibers and plays an important role in collagen fibrillogenesis and cellular interactions (Patterson-Kane, Firth 2009). Proteoglycans are composed of a core protein associated with four main glycosaminoglycans; dermatan sulfate, chondroitin sulfate, keratan sulfate and heparan sulfate (O'Brien 1997). Proteoglycans in tendons can be classified as, small leucine-rich proteoglycans such as decorin, biglycan and lumican, and large aggregating proteoglycans such as aggrecan and versican. The most abundant tendon proteoglycan is decorin, followed by biglycan, and others include, fibromodulin, lumican, aggrecan and versican (Yoon, Halper 2005). Decorin is considered as a key regulator of matrix assembly as it limits collagen fibril formation and directs tendon remodelling in relation to tensile forces (Danielson et al. 1997).

Cartilage oligomeric matrix protein (COMP) is an abundant non-collagenous glycoprotein in tendons that increase with growth and decline after maturation (DiCesare et al. 1994). It is a calcium binding pentamer, with each subunit shown to bind collagen type I, II and IX. Higher levels of COMP are reported in superficial digital flexor tendons (SDFT) as compared to deep digital flexor tendons (DDFT) (Sodersten et al. 2005). COMP concentrations are correlated with weight bearing and mechanical properties of the superficial digital flexor tendon (Dowling, Dart 2005). Further, higher COMP levels are found in association with small diameter collagen fibrils in the tensional area of the flexor tendons. It has been proposed that COMP functions to provide structural integrity to the

extracellular matrix by binding to multiple collagen fibrils in collagen fibrillogenesis (Sodersten et al. 2005, Dowling, Dart 2005).

The cellular compartment of tendons constitutes a very small portion of the total bulk of the tissue. Further, the cell-to-matrix ratio gradually decreases over age. Tenocytes are fibroblastic cells that are responsible for synthesis and turnover of the tendinous extracellular matrix. Tenocytes are arranged in parallel rows along the longitudinal axis of tendons, with gap junctions linking flattened cytoplasmic processes that extend through the extracellular matrix between cells (Stanley et al. 2007). Type 1, 2 and 3 tenocytes have been defined in equine tendons (Stanley et al. 2008). Type 1 tenocytes referred to as 'tenocytes' have long, thin spindle-shaped nuclei whereas type 2 tenocytes referred to as 'tenoblasts' have plump, cigar-shaped nuclei (Patterson-Kane, Firth 2009). Type 3 tenocytes are chondrocyte-like with round nuclei and are located at sites exposed to compressive forces, especially where tendons wrap around joints (Stanley et al. 2008). Type 2 cells are metabolically more active and have a higher biosynthetic capacity than type I cells and are present in a higher proportion in fetal and neonatal tendon. With increasing age, the proportion of type 1 tenocytes increases, along with a reduction in total cellularity and cellular activity (Patterson-Kane, Firth 2009, Dowling, Dart 2005, Stanley et al. 2008).

Histological structure

Histologically, tendons have a highly organized structure **(Figure 3)**. Linear fibers of collagen are aligned along the longitudinal axis in each fascicle. Tenocytes are located both intrafascicularly and interfascicularly, arranged in rows along the direction of the collagen fibers (Thorpe, Clegg & Birch 2010). Vascular channels are present in the endotenon. A characteristic crimp pattern of collagen fibers is a typical ultra-structural feature of tendons

(Figure 4). The crimps function as a buffer to allow longitudinal elongation in response to physiological tensile loads (O'Brien 1997). In addition, on tensile loading the central fibers straighten first and therefore receive a higher load. As age increases, a generalized reduction in crimp angle occurs, with a greater degree of reduction in central fibers (Patterson-Kane et al. 1997).

Physiologic matrix turnover

Native tenocytes are involved in constant matrix remodelling under normal circumstances by synthesizing collagen, proteoglycans, and enzymes responsible for degradation, such as matrix metalloproteinases (MMPs). Tendons have low oxygen consumption values in comparison to other tissues. The low metabolic rate with well-developed anaerobic energy production is adapted for load-bearing for prolonged periods of time (Kannus 2000). However, the rate of recovery after activity is consequently slow.

Synthesis of collagen occurs intracellularly in tenocytes, with the formation of mRNA for each alpha chain which are then assembled on the polyribosomes bound to rough endoplasmic reticulum, and stored as preprocollagen (Zhang et al. 2005). Soluble procollagen is formed from the preprocollagen, followed by hydroxylation of proline and lysine. The procollagen is secreted extracellularly to form insoluble tropocollagen, which aggregates to form collagen fibrils (Zhang et al. 2005).

Synthesis of proteoglycans also occurs intracellularly, in the rough endoplasmic reticulum of tenocytes, where the core protein is assembled and glycosylation is initiated. The process is completed in the Golgi complex, where sulfation takes place (O'Brien 1997). The continual process of matrix remodelling is primarily mediated by proteases acting in the extracellular environment. These include MMPs and aggrecanases from the “a disintegrin

and metalloproteinase with thrombospondin motifs" (ADAMTS) family. The activity of MMPs is highly regulated by tissue inhibitors of metalloproteinase (TIMPs) (Riley, Medscape 2008). Gelatinases, MMP-2 and -9, and collagenases, MMP-1 and -13, are thought to be involved in tendon metabolism, through their broad proteolytic capacity. Their activity is reversibly inhibited by TIMPs-1 and -2. A balance between the activities of MMPs and TIMPs regulates tendon remodeling (Karousou et al. 2008). ADAMTS-2, -3, and -14 are pro-collagen peptidases, and function as regulators of collagen fibril assembly. ADAMTS-1 and -4 are capable of cleaving matrix proteoglycan versican and glycoprotein COMP (Hosaka et al. 2002).

Energy-storing flexor tendons experience higher strains during physiological activity than positional extensor tendons (Birch, Wilson & Goodship 2008). Also, functionally distinct tendons differ in matrix composition, with a higher glycosaminoglycan, COMP, water content and cellularity in SDFTs when compared to common digital extensor tendon (CDET) (Birch et al. 2008). Historically, tendon was assumed to be a relatively inert tissue, however, a recent study reported active tendon matrix turnover (Birch 2007b). The same study reported a difference in the rate of matrix turnover between functionally distinct tendons in the absence of disease process, with a lower level of collagen gene expression and MMP activity in SDFT when compared to DDFT.

Superficial Digital Flexor Tendinitis in Horses

Musculoskeletal injuries involving tendons and ligaments are a major cause of career-ending lameness in performance horses. A recent study in National Hunt horses showed that 46% of the injuries involved tendons and ligaments (Ely et al. 2009). Some tendons are more

prone to injuries than the others, with the majority occurring in the forelimbs (97-99%) as they bear 60% of the total body weight during rest and more during galloping. Superficial digital flexor tendons are commonly affected due to its small cross-sectional area, small margin of safety and high tensile loads experienced at maximal exertion. The same study reported superficial digital flexor tendon injuries in about 89% of all tendon and ligament injuries and the remainder involved the suspensory ligament. Another study described the prevalence of SDF tendinitis in Thoroughbred racehorses in Hong Kong to range from 25-53% with 97% of these occurring in the forelimbs (Dyson 2004). Superficial digital flexor tendinitis was described as the most common cause of retirement in this population of equine athletes. There is a high risk of re-injury following prolonged rehabilitation. Studies report successful return to racing in 20-60% of the affected horses but up to 80% of these horses sustained re-injury (Dowling et al. 2000, Genovese et al. 1996). Results of another study revealed 96% of the horses returned to previous function, but the re-injury rate was 42.5-44%, with a higher rate in horses used for flat racing (Dyson 2004).

Pathophysiology

The cross-sectional area of the SDFT is smallest at the mid-metacarpal region. Consequently, this site is highly susceptible to injury, as the central collagen fibers take more load than the peripheral fibers (Patterson-Kane, Firth 2009). One study, evaluated the incidence of tendinitis in the proximal aspect of the SDFT in older performance horses (Chesen et al. 2009). Tendinitis or more accurately termed 'tendinopathy' is a result of chronic overuse rather than a single event to failure. Localized microdamage to collagen fibrils can occur when the SDFT is exposed to high strains up to 19%. This isolated fibrillar damage causes an alteration in cell-matrix interactions leading to increased matrix

breakdown in relation to cellular repair mechanisms (Patterson-Kane, Firth 2009). This 'tendinopathy' cycle results in further weakening due to repeated and cumulative microtrauma, where insufficient time is provided to effect repair between traumatic episodes.

Limb motion generates considerable heat in the peripheral tissues. An increase in core temperature of up to 5.4^{0}C has reported in the mid-cannon SDFT as a consequence of kinetic energy being released as heat. Some evidence suggests that this hyperthermia can result in damage to extracellular matrix and cell death (Wilson, Goodship 1994). However, a contradictory outcome was reported by Hosaka et al, with 70-90% cell survival following heating of tenocyte suspensions for up to 10 minutes (Hosaka et al. 2005, Birch, Wilson & Goodship 1997). Another study reported apoptosis of tenocytes in inflamed SDFT via a caspase-3-dependent pathway as a cause of cell death (Hosaka et al. 2006).

An inflammatory cascade associated with cytokine release takes place following injury. Increased expression of IL-1α, IL-1β, TNF-α, and IFNγ has been reported in inflamed SDFTs (Hosaka et al. 2002). Both the expression and activity of MMPs are stimulated by pro-inflammatory cytokines. There is increased expression of MMPs-2 and -9, along with a down-regulation of TIMP-1 in chronic tendinopathy (Karousou et al. 2008, Riley 2005). A local imbalance in MMPs and TIMP activities can cause progressive degeneration and weakening of the extracellular matrix of tendons. A study conducted in human ruptured supraspinatus tendons showed increased MMP-1 levels, causing degradation of the collagen fibril network (Riley et al. 2002). Expression of ADAMTS-1, -4, and -5 has also been shown in tendon (Riley et al. 2002). However, their levels of expression and activity are still unclear in tendinopathies. As a consequence to the inflammatory process and proteolytic activities, intra- and inter-molecular cross-links within collagen fibrils break down, leading to fibril

elongation at the microscopic level followed by macroscopic changes (Patterson-Kane, Firth 2009).

Degeneration of the SDFT is characterized by discoloration of the central core region accompanied by swelling. Fibril diameter also changes, with a predominance of small-diameter collagen fibrils which ultimately decreases the tensile strength of injured tendons, and increases the susceptibility to re-injury. Microscopically, increased cellularity with inflammatory cell infiltrates, cell rounding, and disruption of collagen fiber organization is observed (Riley, Medscape 2008). Collagen type III mRNA expression increases following injury with a sequential increase in collagen type III content (Dahlgren, Brower-Toland & Nixon 2005, Samiric et al. 2009). Some studies report a decrease in mRNA expression of collagen type I in diseased tendon, with no difference in collagen type I content (Samiric et al. 2009). Parallel studies conducted in injured human Achilles tendons show increased collagen type I mRNA levels with a sequential increase in collagen turnover (de Mos et al. 2007b). However, the percentage of denatured collagen was higher with a poor quality collagenous matrix. In contrast, a recent study showed no significant increase in expression of collagen type I mRNA in acute tendinopathy (Taylor et al. 2009). In addition, no significant change in total collagen content was seen in equine SDF tendinopathy (Birch, Bailey & Goodship 1998). An increase in sulfated glycosaminoglycans (sGAGs) and water content occurs in tendinopathies which gives rise to tissue swelling (Samiric et al. 2009). A significant increase in large aggregating (proteoglycans, versican, and aggrecan) and small proteoglycans (biglycan and fibromodulin) has been reported, with no changes in levels of decorin (Samiric et al. 2009).

Process of Tendon Healing

There are three phases in tendon healing; the reactive inflammation phase, the acute reparative or proliferative phase, and the chronic remodelling or maturation phase.

Reactive inflammation

This phase lasts for about a week and is characterized by a marked increase in cross-sectional area at and around the site of injury with local hemorrhage and edematous swelling. This is followed by inflammatory cell infiltration, initially by neutrophils, with predominant macrophage accumulation after 24 hours to phagocytize debris and necrotic cells. Consequently, this causes a release in proteolytic enzymes and results in further mechanical disruption.

Changes in growth factor expression occur immediately following injury. Studies show increased expression of transforming growth factor- β1 (TGF-β1) in early stages of tendon healing. The large round cells within the endotenon have a higher expression of TGF- β1 when compared to those within the lesional tissue (Dahlgren, Mohammed & Nixon 2005, Berglund et al. 2006). In addition, expression of connective tissue growth factor (CTGF) significantly decreases in the tendon sheath following injury with no change in the tendon tissue (Berglund et al. 2006). The same study reported an increased expression of basic fibroblastic growth factor (bFGF) in tendon tissue at the same time point. Studies also indicate minimal increase in expression of insulin-like growth factor-I (IGF-I) in acute stages of tendon healing (Dahlgren, Mohammed & Nixon 2005), (Berglund et al. 2006). During the reactive inflammatory phase there is a marked increase in mRNA expression of collagen type III and type I in a collagenase-induced tendinitis (Dahlgren, Mohammed & Nixon 2005). The percentage composition of collagen type III begins to increase in this early phase.

Proliferation

This phase begins within a few days of injury and peaks for about 2 to 3 weeks. Neovascularization is a dominant feature of this phase, slowly replacing the inflammatory cell infiltrates. Proliferation of fibroblasts takes place from surrounding endotenon and epitenon. Tenocytes in the peri-lesional tissue may also undergo cell division, leading to increased cellularity at the site of injury. Fibroblastic cells that are proliferating have a rounded appearance with plump nuclei, with a tenoblast-like appearance (Dahlgren, Mohammed & Nixon 2005).

Growth factors responsible for angiogenesis and mitogenesis regulate tissue activities during this phase. Vascular endothelial growth factor (VEGF), a prototypic angiogenic growth factor is up-regulated in early tendon healing (Dahlgren, Mohammed & Nixon 2005, Patterson-Kane, Firth 2009, Fenwick, Hazleman & Riley 2002), (Patterson-Kane, Firth 2009, Dahlgren, Brower-Toland & Nixon 2005). Expression of bFGF increases during this phase. bFGF also has angiogenic effects in healing tendons (Duffy et al. 1995). IGF-I mRNA expression levels are reported to peak around 4 weeks following injury in a collagenase-induced model of tendinitis (Dahlgren, Mohammed & Nixon 2005). IGF-binding proteins (IGFBPs) regulate the activity of IGF-I by restricting its bioavailability to IGF-I receptors. In addition, IGFBPs are also thought to protect IGF-I from proteases. Increased expression of IGFBP-2, -3 and -4 occurs between 2-4 weeks following tendon injury and decreased expression of IGFBP-5 and -6 is present throughout healing period for up to 24 weeks (Dahlgren, Mohammed & Nixon 2006). The mitogenic effect of IGF-I plays an important role in stimulating cell proliferation in this phase of healing (Dahlgren, Mohammed & Nixon 2005).

In a collagenase-induced tendonitis study, mRNA expression for collagen type I was six-fold higher than collagen type III. Collagen type III mRNA expression was localized to cells within the endotenon in early stages of healing followed by intralesional tenocytes during later stages of healing (Riley et al. 2002). Synthesis of collagen type III peaked 3-6 weeks post-injury, comprising up to 35% of total collagen by 1-2 weeks and remained high for up to 4 weeks post-injury. Collagen type I fell to approximately 66% by 1 week following injury. A larger proportion of small fibrils began to form in the healing response with inferior mechanical properties. One study showed increased mRNA expression of versican around 6 days post-injury and of aggrecan around 24 days post-injury in rabbit flexor tendons (Berglund et al. 2006). A decreased expression of decorin, and a relative increase in expression of biglycan were also evident in injured tendons when compared to normal tendons.

Remodelling

The remodelling or maturation phase is characterized by formation of fibrous tissue. During this stage, the healing tissue undergoes changes in size and shape. This phase is divided into consolidation and maturation processes (Sharma, Maffulli 2006). The consolidation stage begins at about 6 weeks and continues for up to 10 weeks post injury. The repair tissue changes from cellular to fibrous. However, increased vascularity and cellularity persists for up to 3 months post-injury (Patterson-Kane, Firth 2009, Fenwick, Hazleman & Riley 2002). Tenocyte nuclei gradually become more spindle-like with an increased synthetic activity early on which gradually decreases.

Persistent increases in TGF- β1 and IGF-I mRNA levels remained up to 6 months post-injury. These increases were restricted to mature fibroblasts within the healing tendons

(Dahlgren, Mohammed & Nixon 2005). In addition, mRNA expression of collagen type I and type III was increased up to 6 months post-injury. A higher proportion of collagen type I was synthesized during this stage. However, collagenous tissue may still be randomly oriented with thickened areas of endotenon due to increased numbers of blood vessels (Patterson-Kane, Firth 2009).

The maturation stage begins after approximately 10 weeks, marked by a gradual change from fibrous tissue to scar-like tendon tissue. This transition continues for up to a year (Sharma, Maffulli 2006). The newly formed collagen fibrils begin to orient longitudinally along tensile forces. The larger diameter collagen fibrils are not replaced following healing. The tendon cross-sectional area often remains increased, along with increased tissue mass. Healed tendons have decreased elastic properties and do not regain the original mechanical strength. Peritendinous fibrosis often occurs following healing with adhesions to adjacent structures that can significantly interfere with normal function. Together, the abnormal composition and arrangement of fibers, poor biomechanical properties, and prolonged period of healing are responsible for high occurrence of re-injury.

Treatment of Tendinitis

Many treatment strategies have been advocated for equine tendonitis, depending on phase of healing. Also, therapeutic approaches differ depending on the duration of time since the original insult.

Acute tendon injuries

Acute tendon injuries constitute a medical emergency that require rapid reduction of inflammation. Failure to reduce tendon inflammation can lead to further damage. Physical

therapies, which include ice application, cryotherapy, and compression bandaging, and immediate controlled mobilization are important for reducing inflammation and limiting the action of proteolytic enzymes on tendon extracellular matrix (Bramlage 1991). Cryotherapy exerts a beneficial effect through local vasoconstriction, decreased enzymatic activity, and reduced formation of inflammatory mediators. Cryotherapy for 20 minutes at frequent intervals is advised. One study reported reduction in core temperature of equine SDFT to 21.8^{0}C with no effects on the viability of tenocytes following cryotherapy for 1 hour (Petrov et al. 2003). Pressure applied by compression bandaging with coaptation also reduces inflammation and edema formation. Coaptation by application of a palmar splint or heel support may be carried out to provide further support to the tendon. Administration of systemic non-steroidal anti-inflammatory drugs (NSAIDs) such as phenylbutazone and flunixin meglumine has been advocated in the acute stages to reduce inflammation and to provide analgesia (Dowling et al. 2000, Bramlage 1991).

Chronic tendinopathies

Both, medical and surgical treatment options have been described for chronic tendinopathies with little objective evidence for continuing beneficial effects (Dowling et al. 2000). A brief review of various therapeutic options is provided below.

Medical Management of Tendinitis

Controlled exercise programs

Earlier studies have extensively evaluated controlled exercise programs as a conservative approach for rehabilitation of SDFT injuries with the goal of maintaining gliding function, and promoting optimal collagen healing. It involves gradual increase in

exercise regimen over a protracted period of time, up to about a year, with careful monitoring with diagnostic ultrasound at 3 month intervals (Gills 1997). One study showed 71% of injured horses returned to performance following controlled exercise when compared to 25% of injured horses with pasture rest (Gills 1997). Other studies reported similar results with 59-75% of injured horses returned to performance following controlled exercise (Sawdon, Yovich & Booth 1996, Marr et al. 1993). However, these studies failed to document the severity of the initial lesions, which may have an important influence on the outcome.

Extracorporeal shock wave therapy

Extracorporeal shock waves are pressure waves generated outside the body. These pressure waves cause high local stresses and analgesic effects on sensory nerves, possibly through demyelination of nerves. One study reported a return to performance in 50% of the horses with injured proximal suspensory ligaments following shock wave therapy administered 3 times at 2-week intervals (Crowe et al. 2004). Another study reported increased neovascularization with no ultrasonographic improvement in collagenase-induced SDF tendinitis in horses at 12 weeks post-injury treated with three treatments of shock wave therapy at 3-week intervals (Kersh et al. 2006). A recent study showed increased gene expression of collagen type I and MMP-14 with disorganization of matrix structure in normal tendons 6 weeks post-exposure to shock waves, suggesting a potential harmful effect on surrounding normal tissue (Bosch et al. 2009).

Therapeutic ultrasound

A few studies have reported the effect of therapeutic ultrasound for tendinopathies in human athletes. Beneficial effects have been shown to be due to increased cell migration and gene expression, along with histologic improvement in entheseous insertions of tendon to

bone (Tsai et al. 2008, Moraes et al. 2009). However, there are no scientific reports of its use in equine tendinitis.

Intralesional medications

(1) Polysulfated glycosaminoglycans (PSGAGs) – PSGAGs have anti-inflammatory effects through inhibition of collagenases, MMPs and macrophage infiltration. A recent study evaluating intra-lesional PSGAG injection in a collagenase-induced SDF tendinitis model in horses showed histological improvement in collagen fiber organization at 5 months post-injury (Moraes et al. 2009). However, a clinical retrospective study showed no difference in re-injury rates in horses treated with PSGAGs when compared to control horses (Ely et al. 2009).

(2) Hyaluronan – sodium hyaluronan has been administered peritendinously and intralesionaly in tendinitis. One study showed histologic improvement and gross reduction in adhesion formation between DDFT and tendon sheath in collagenase-induced model of intra-synovial tendinitis (Gaughan et al. 1991). However, a collagenase-induced model of mid-cannon SDF tendinitis reported no improvement in ultrasonographic properties, biochemical parameters or biomechanical strength, with increased inflammation histologically following treatment with hyaluronan (Foland et al. 1992). In addition, a clinical retrospective study showed no significant difference between re-injury rates of horses treated with intralesional hyaluronan when compared to those treated conservatively (Ely et al. 2009).

(3) Beta-aminopropionitrile fumerate (BAPN) – intralesional BAPN inhibits lysyl oxidase, to prevent the formation of crosslinks between collagen fibers and promote better alignment of newly formed collagen. One study showed improved

ultrasonographic appearance and histological collagen alignment in BAPN-treated horses in a collagenase-induced model of SDF tendinitis (Dahlgren, Nixon & Brower-Toland 2001). In addition, long term follow-up results of horses with SDF tendinitis treated with BAPN showed reduced risk of reinjury. However, a recent *in vitro* study reported altered morphology of tendon fibroblasts in addition to decreased collagen synthesis in tendon explants following culture with BAPN, suggesting a delay in tendon healing (Dahlgren, Nixon & Brower-Toland 2001).

(4) Platelet-rich plasma (PRP) - platelet-rich plasma, an autologous concentrate of blood platelets has been introduced recently in humans for treatment of tendon injuries. Platelets are thought to influence healing by releasing growth factors such as platelet-derived growth factor (PDGF), VEGF, TGF-β and IGF-I at the site of injury, influencing cell migration, proliferation, and matrix synthesis (Anitua et al. 2005). An *in vitro* study reported increased expression of collagen type I, type III and COMP mRNAs in equine SDFT explants cultured with PRP when compared to whole blood or serum (Schnabel et al. 2007). Increased concentrations of TGF-β1 and PDGF were also measured. A recent *in vivo* study in horses showed improved biomechanical properties, histologic organization and increased biochemical composition, 6 months post-injury, in a mechanically-induced SDF tendonitis model treated with PRP (Bosch et al. 2010).

Surgical Management of Tendinitis

Superior/Proximal check ligament desmotomy

Desmotomy of the accessory ligament of the SDFT (DALSDFT) as a treatment for SDF tendinitis is performed to increase the length of the myotendinous unit and reduce strain

on the SDFT. Initial results demonstrated 70% of horses with SDF tendinitis treated with DALSDFT competed in at least two races without recurrent tendinitis. One study conducted in Standardbred racehorses reported 86% of horses treated with DALSDFT for SDF tendinitis returned to performance with 75% completing at least 5 starts (Hogan, Bramlage 1995). Another study conducted in Standardbred racehorses showed similar results, however also reported the 12.5% incidence of suspensory desmitis as a consequence of this procedure (Hawkins, Ross 1995). A prospective study conducted in 127 Thoroughbred racehorses showed no significant difference between DALSDFT and conservative management with reference to return to performance and recurrence of injury (Gibson, Burbidge & Pfeiffer 1997).

Annular ligament desmotomy

Transection of the palmar annular ligament (PAL) is recommended when enlargement of the distal SDFT occurs and causes constrictive impingement by the PAL, preventing gliding function and further exacerbating the tendinitis.

Tendon splitting

Early reports have described tendon splitting to improve blood flow to damaged tendon lesions that are relatively avascular (Stromberg, Tufvesson & Nilsson 1974). The aim of tendon splitting in acute tendinitis is to evacuate serum or hemorrhage. One study showed faster resolution of the core lesion, revascularization and increased collagen deposition in a collagenase-induced acute tendinitis treated with tendon splitting when compared to controls (Henninger et al.1992).

Novel Therapeutic Approaches for Tendon Regeneration

Complete regeneration is never achieved after tendon injury. More recent therapeutic developments are aimed at influencing the process of healing towards formation of physiologic and functional tissue. This has been attempted through the manipulation/application of various growth factors, use of cell-based approaches and gene therapy.

Use of growth factors in tendon healing

Growth factors and other cytokines stimulate cell proliferation and chemotaxis, influence angiogenesis, and induce cell differentiation. They also regulate metabolic activities of cells, leading to synthesis and secretion of ECM components. Several growth factors influence tendon healing and have been used in both, *in vitro* and *in vivo* models of tendon repair. These factors include VEGF, IGF-I, PDGF, FGF-2 and TGF-β.

VEGF regulates angiogenesis by breaking down vascular basement membranes, expression of α-integrins, vasodilatation and increased vascular permeability, and endothelial cell proliferation and monocyte migration. In tendons, VEGF is expressed in tendon sheath fibroblasts and its expression increases in early healing process (Jackson et al. 1997, Bidder et al. 2000). Therapeutically, intralesional injections of VEGF in a murine model of Achilles tendinopathy resulted in significant increases in tensile strength during the early course of healing (Zhang et al. 2003). In addition, VEGF also caused a significant increase in expression of TGF-β1 in the early repair stages when compared to the controls.

Transforming growth factor- β1 is released from platelets, lymphocytes, macrophages, endothelial cells and fibroblasts and stimulates chemotaxis, angiogenesis and transcription of extracellular matrix genes. Despite its beneficial effects, it is implicated in fibrous tissue

formation from excessive deposition of disorganized collagen. *In vitro*, TGF- β1 induces collagen type I production by tenocytes (Klein et al. 2002). Further, inhibition of TGF- β activity reduces scar formation by limiting excessive collagen formation and improves tendon healing. The addition of TGF- β neutralizing antibodies in healing flexor tendons of rabbits increased the range of motion when compared to controls (Chang et al. 2000). Current studies are investigating the efficacy of gene therapy strategies to cause sustained neutralization of TGF- β.

Insulin-like growth factor- I has been studied for its effects on tendon healing. The primary effect of IGF-I on tendon healing is through its mitogenic effect, stimulating tenocyte proliferation at the site of injury. Exogenously applied IGF-I stimulates replication, collagen and proteoglycan synthesis in healthy rabbit flexor tendons (Abrahamsson, Lundborg & Lohmander 1991). In an equine collagenase-induced SDF tendonitis model, intralesional injections of IGF-I resulted in reduced lesion size, increased cell proliferation and collagen synthesis, and a trend toward increased mechanical strength in treated tendons when compared to control tendons (Dahlgren et al. 2002). A detailed description of IGF-I, its function and effects, and clinical use in tendon healing is provided below.

PDGF acts as chemoattractant and mitogen for fibroblasts and endothelial cells. PDGF may exert some effects through IGF-I as it up-regulates IGF-I and its receptors in target cells. An *in vitro* study showed PDGF stimulates collagen, proteoglycan, and DNA syntheses in tenocytes (Yoshikawa, Abrahamsson 2001). In addition, a recent study utilizing a canine flexor tendon injury model showed improved functional properties and increased collagen synthesis with the use of controlled delivery of PDGF when compared to controls (Thomopoulos et al. 2009).

Fibroblast growth factor-2 stimulates angiogenesis and proliferation of fibroblasts. Application of exogenous FGF-2 accelerated healing of human patellar tendon in an *in vitro* model (Chan et al. 1997). This was consistent with *in vivo* studies showing FGF-2 injected intralesionally into healing patellar tendons in rats increased cell proliferation and collagen type III synthesis (Chan et al. 2000). A more detailed description of FGF-2 and its effects is provided further on.

Cell-based therapies for tendon regeneration

Stem cell therapy, especially the use of postnatal/adult tissue-derived mesenchymal stem cells (MSCs), is a recent focus of research in tendon healing. Following tendon injury, a cascade of inflammatory cells takes place. Cells involved in the synthesis of new tissue are present locally but comprise a very small component of the tissue (Richardson et al. 2007a). Further, this progenitor cell population varies with the functional properties of tendon and decreases with the age of tendon. Multiple sources of MSCs have been used for tendon regeneration. A brief review of various sources of MSCs used for the treatment of equine SDF tendinitis is provided below.

Empirically, MSCs from bone marrow have been used in equine SDF tendinitis with documented beneficial outcomes (Frisbie, Smith 2010, Koch, Berg & Betts 2009). Implantation of autogenous bone marrow MSCs (BMMSCs) as a potential therapeutic option for spontaneously occurring SDF tendinitis core lesions in performance horses was first described in 2003 (Smith et al. 2003). This case demonstrated the feasibility of the technique and reported no adverse reactions up to 6-weeks post injection. A later study determined the fate of these injected cells in experimentally-induced tendon lesions and reported successful integration into adjacent healthy tendon (Guest, Smith & Allen 2008). Since then, a number

of experimental and case-based studies have been performed to determine the usefulness of MSCs in horses. One study evaluated the effect of BMMSCs in 11 spontaneously occurring lesions of the SDFT in race horses in comparison to control horses (Pacini et al. 2007). In MSC-injected cases, there was an improvement in ultrasonographic scores and return to performance in 81% of horses with no incidence of re-injury up to 2 years following treatment. In contrast all control horses re-injured the SDFT within 4 to 12 months. Another retrospective study evaluated the use of BMMSCs in spontaneously occurring SDF tendinitis in 168 horses followed by rehabilitation with long term follow-up (Smith 2008a). This study reported successful return to function in all treated horses with occurrence of re-injury in only 13% of horses up to one year, and 23-43% in more than 1 year follow-up. Overall, the re-injury rate for the MSC-treated horses was 24%, which was significantly reduced compared to 56% incidence of re-injury in conventionally managed horses (Dyson 2004)

Adipose derived-nucleated cells have also been evaluated for treatment of equine tendon regeneration due to ease of collection, minimal donor-site morbidity and faster recovery of cells due to absence of a cell culture step (Richardson et al. 2007a). One study reported histologic improvement of collagenase-induced SDF tendinitis in horses treated with adipose-derived cells, when compared to control horses (Nixon et al. 2008). An immunomodulatory function of these cells was suggested in the healing process due to reduced infiltration of inflammatory cells. Another study evaluated the effects of autogenous adipose-derived cells dispersed in PRP as a biologic scaffold in spontaneously occurring SDFT core lesions in horses (Del Bue et al. 2008). This study reported favorable results with return to function in 87% of treated horses.

Alternative sources of cell-based therapies for tendon regeneration have been described in other species in experimental models. Synovial MSCs have been described in Achilles tendinopathy studies and were shown to stimulate superior healing of tendon-bone interfaces with improved fiber alignment (Ju et al. 2008). Another study reported improvement in histologic structure and tensile strength with use of dermal fibroblasts used to treat a porcine model of SDF tendon lesion (Liu et al. 2006). Autologous tenocytes engineered onto polyglycolic acid fibers were shown to provide superior healing in avian model of SDF tendon defect with improved histologic structure (Cao et al. 2002). More recently, embryonic stem cells have been described as a potential source of cells. A recent study, demonstrated better mechanical strength and histologic structure in a murine model of patellar tendinopathy treated with human embryonic stem cells, when compared to controls (Chen et al. 2009).

Gene therapy

The delivery of exogenous genes to injured tendon is a relatively new approach of enhancing tendon healing. Transfer of growth factor cDNAs provides a means of sustained, prolonged expression of therapeutic proteins. *In vitro* adeno-associated virus-mediated gene transfer of bFGF in tendon explants showed effective delivery to tenocytes and significantly increased expression of collagen type I and type III gene expression (Wang et al. 2005). Modification of murine tenocytes with VEGF expression constructs has been shown to increase the levels of expression of TGF-β, collagen type I and type III (Wang, Liu & Tang 2005). Similar results were obtained with exogenous PDGF gene transfection (Wang, Liu & Tang 2004).

In addition, this technique can be used as a tool for delivering growth factor-enhanced MSCs. One study showed IGF-I-enhanced MSCs resulted in histologic and biomechanical improvement in a collagenase-induced SDF tendinitis in horses, when compared to control (Schnabel et al. 2009). Another study evaluating the effect of BMMSCs transfected with TGF-β1 gene in a lupine model of Achilles tendinopathy showed higher concentrations of collagen type I protein and larger fiber bundle formation at the site of injury (Hou et al. 2009, Fu, Wong & Chan 1999). Bone morphogenetic protein-12 is involved in tenogenesis and tendon healing, and stimulates increased collagen type I expression (Seeherman et al. 2008). Recently, an *in vitro* BMP-12 transfection study conducted in equine BMMSCs and SDF tenocytes resulted in high BMP-12 and COMP expression (Murray, Santangelo & Bertone 2010). This study suggested the possible use of BMP-12-tranduced BMMSCs for tendon repair.

Fibroblast Growth Factor-2 (FGF-2)

FGF-2, also known as basic fibroblast growth factor (bFGF), is a member of the heparin-binding growth factor family. It is an 18-kd, 146-amino acid, single chain polypeptide, although larger forms are also present. FGF-2 binds to heparin and heparan-sulfate proteoglycans on cell surfaces and in the extracellular matrix. Following ECM degradation, FGF-2 is released and then binds to one of four specific cell surface receptors (FGFRs 1-4). FGFRs are cell surface tyrosine kinase receptors, which then initiate further signaling (Hsu, Chang 2004). Physiologically, FGF-2 is produced by endothelial cells, fibroblasts, smooth muscle cells, chondrocytes and mast cells, and acts on a wide variety of cells involved in biological processes, including development, differentiation, cell

proliferation and angiogenesis. In wound healing, FGF-2 causes proliferation and migration of keratinocytes. It causes fibroblasts to produce collagenase and stimulates proliferation of capillary endothelial cells, which are important for initiation of angiogenesis. Finally, it also helps in the formation of granulation tissue.

Mechanisms of FGF-2-induced responses in MSCs

The molecular mechanisms involved in the regulation of MSC proliferation and differentiation by bFGF have been addressed in several studies. Long term sub-culture of MSCs is decreased *in vitro* due to increased mRNA expression level of TGF-β. TGF- βs arrest the cell growth of epithelial cells and blood cells in G1 phase through inhibition of G1 cyclin-dependent kinases (CDKs). FGF-2 suppresses cellular senescence of human MSCs by down regulating TGF- β2 (Ito et al. 2007, Ito et al. 2008).

More recently, another study showed that FGF-2 induces transient activation of c-Jun N-terminal kinase (JNK). JNK signaling mediates FGF-2-induced stimulation of proliferation and maintenance of differentiation potential of human BMMSCs (Ahn et al. 2009). In brief, FGF receptor substrate 2 (FRS2) is a critical component of FGF signaling and is activated by FGF receptors. FRS2 recruits growth factor receptor-bound protein-2 (Grb2) and activates the Ras-Raf-mitogen-activated-protein kinase (MAPK) signaling pathway. MAPK signaling cascades are in turn mediated by JNK which is one of the signal transducers for growth factors.

Use of FGF-2 in progenitor cell culture systems

FGF-2 has been used in many cell culture systems to stimulate proliferation and maintain self renewal capacity of multiple types of stem/progenitor cell populations which

include BMMSCs, adipose-derived cells, and embryonic stem cells. Previous studies have shown that FGF-2 enhances the growth of MSCs and maintains their multilineage differential potential during *in vitro* expansion (Solchaga et al. 2005, Bianchi et al. 2003). Increased proliferation and maintenance of the undifferentiated state during *in vitro* expansion is important for MSC applications in cell-based tissue repair. Many studies have shown that *in vitro* expansion of bone marrow-derived MSCs with FGF-2 supplementation stimulates proliferation and delays loss of chondrogenic potential (Solchaga et al. 2010, Stewart et al. 2007). Similar effects on proliferation and maintenance of self-renewal by FGF-2 were demonstrated in human embryonic stem cells and adipose-derived cells (Eiselleova et al. 2009, Lee et al. 2009). Based on the available evidence, the effect of FGF-2 on monolayer expansion of equine tendon-derived cells was evaluated in this study and compared to FGF-2's effects on bone marrow-derived cells.

Insulin-like Growth Factor-I

Insulin-like growth factor-I (IGF-I), referred to originally as 'sulfation factor' and 'somatomedin', was later renamed IGF-I due to its structural homology with human proinsulin. IGF-I has autocrine and paracrine effects, stimulating anabolic responses in target cells by binding to type I IGF receptor (IGF-IR), a cell surface tyrosine kinase receptor. This leads to a complex signal transduction pathway, resulting in modulation of gene expression and anabolic responses within the cell (Dahlgren, Mohammed & Nixon 2006). The bioavailability of IGF-I is regulated by IGF binding proteins (IGFBPs), comprising six structurally related proteins with a high affinity for IGF-I. IGFBPs inhibit the activity of IGF-

I by restricting its access to IGF-IR, and also protect IGF-I from pericellular proteases and consequently increasing its extracellular half-life.

Cellular mechanisms of IGF-I activities

The primary physiologic role of IGF-I is mitogenesis. Some studies explained this mitogenic capacity through activation of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways. Both signaling pathways promote G_1/S cell cycle progression leading to increased proliferation (Mairet-Coello, Tury & DiCicco-Bloom 2009). Hypoxia/anoxia-induced apoptosis has been shown to be a cause of tenocyte death in chronic tendinopathy (Birch, Wilson & Goodship 1997). One study showed prosurvival effect of IGF-I on Achilles tendon cells by activation of protein kinase B (PKB) which prevents cell death by phosphorylating various cytoplasmic and nuclear targets (Scott, Khan & Duronio 2005)

Effects of IGF-I on tendon healing

IGF-I has been extensively studied over the last decade, with respect to its effects on tendon regeneration. Expression of IGF-I is increased in tendon healing as described earlier. A recent *in vivo* study showed increased expression of IGF-I and IGFBPs following mechanical loading in a murine model (Olesen et al. 2006). Further, this study also showed increased expression of mechano-growth factor (MGF), a splice variant of IGF-I, which is more rapidly up-regulated after loading when compared to IGF-I. Initial *in vitro* studies showed increased matrix synthesis and cell proliferation following IGF-I supplementation of tendon explant cultures (Abrahamsson, Lundborg & Lohmander 1991).

In vivo effects of IGF-I on tendon healing have also been evaluated. Intralesional injection of IGF-I into healing SDF tendon in an equine collagenase-induced tendinitis model

resulted in increased cell proliferation and collagen content, and a trend towards improved mechanical strength in treated tendons when compared to controls. This study also showed improvement in ultrasonographic scores and a reduced lesion size (Dahlgren et al. 2002). In addition, a gene therapy approach using IGF-I transfected BMMSCs in an equine collagenase-induced SDF tendinitis model showed improvement in histologic structure and a trend towards increased mechanical strength (Schnabel et al. 2009))

Mesenchymal Stem/Progenitor Cells

A stem cell is an unspecialized cell that is capable of replicating or self renewing itself and developing into specialized cells of a variety of cell types. Adult stem cells, like all stem cells, share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as self-renewal.
Second, they can give rise to mature cell types that have characteristic morphologies and specialized functions. Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Bone marrow consists of two populations of stem cells, which are hematopoietic stem cells, responsible for formation of all types of blood cells and the oldest recognized population of stem cells, and bone marrow stromal cells, which is a mixed cell population that generates bone, cartilage, fat, fibrous connective tissue, referred to as the mesenchymal stem cells, and was described shortly after. Since then, the presence of stem/progenitor cell populations has been described in a number of specialized tissues which includes, endothelium, nervous system, epithelial precursors in skin and digestive system, pancreas and liver, skeletal muscle, and dental pulp progenitor cells to name a few.

Bone marrow-derived MSCs

Bone marrow-derived MSCs undergoes physiologic differentiation through the mesengenic process into other phenotypes which includes cartilage, bone marrow, muscle, bone, tendon and ligament to maintain tissue homeostasis (Caplan 2005b, Caplan 2007b). Bone marrow-derived MSCs have been used extensively in cell-based reconstructive therapy in orthopedic injuries over the last decade with promising results (Wakitani et al. 2002b). Cartilage repair in osteoarthritis has been attempted in both human and veterinary patients with autogenous bone marrow-derived MSCs and has shown improvement in arthroscopic and histological grading scores (Wakitani et al. 2002a, Frisbie et al. 2009). Autogenous bone marrow derived-MSCs along with matrix composites have been used in promising resolution of critical segmental bone defects (Quarto et al. 2001).

Later studies have demonstrated the use of bone marrow-derived MSCs for tendon repair with beneficial effects. *In vivo* case-based studies in horses treated with autogenous bone marrow-derived MSCs have shown return to performance and reduced incidence of re-injury as described earlier (Pacini et al. 2007, Smith 2008a). However, bone marrow-derived MSCs are shown to be heterogeneous group of cells that contain subpopulations of tissue-committed progenitor cells or primitive pluripotent stem cells which may have a harmful effect on its use in tissue specific regeneration (Kucia, Ratajczak & Ratajczak 2005). One study reported formation of ectopic bone in a lupine model of tendon defect treated with autogenous bone marrow-derived MSCs demonstrating faulty repair (Dressler, Butler & Boivin 2005). In addition to this a significant variation in bone marrow-derived MSCs exists between subjects among different age groups.

Tendon-derived Progenitor Cells

Recent studies have led to the discovery of progenitor/stem cell populations in tendons based on the fact that tendons are derived from mesenchymal cells that also give rise to bone, cartilage, fat and muscle. First report of existence of MSCs in tendon tissue was made in 2003 (Salingcarnboriboon et al. 2003). This study reported the development of tendon-derived cell lines in transgenic mice which had enhanced proliferation *in vitro* with FGF-2 treatment. In addition, these cell lines expressed tendon-phenotype related genes, which included scleraxis, COMP, and type I collagen. More recently, another study identified a unique cell population, termed tendon stem/progenitor cells (TSPCs) within the tendon extracellular matrix-niche and demonstrated universal stem cell characteristics, which include clonogenecity, multipotency and self-renewal capacity from both mouse and human tendon samples (Bi et al. 2007). A previous study from our lab compared equine tendon-, muscle-, and bone marrow-derived cells and showed that tendon-derived cells yielded higher cell numbers following isolation in comparison to bone marrow-derived MSCs with significantly greater biosynthetic capacities (Stewart et al. 2009a).

Characterization of tendon-derived progenitor cells

Charaterization of tendon-derived progenitor cells have been performed in mouse and human samples and shown to express higher amounts of tendon-lineage specific markers like scleraxis, a twist-related bHLH transcription factor, COMP, the transcription factor SOX9 (Sox9), and osteogeneic transcription factor runt-related transcription factor 2 (Runx2) and tenomodulin, a cell surface marker for tenocyte proliferation and maturation when compared to bone marrow-derived MSCs (Bi et al. 2007). A recent attempt to characterize rat tendon-derived progenitor cells, performed flow cytometric analysis for MSC markers and showed

88-99% of these cells were positive for stem cell marker, CD44 and fibroblastic marker, CD90 (Rui et al. 2010). In addition, these cells were negative for hematopoietic stem cell marker, CD34 and for endothelial cell marker, CD31 confirming absence of contaminating hematopoietic cells and endothelial cells. The same study also showed expression of tenogenic markers like α-SMA, tenascin C, tenomodulin and aggrecan in rat tendon-derived cells. In addition, these cells did not express collagen type I at passage 0 (P0) and P3 and collagen type II at P0. Immuno-histochemical staining of human tendon-derived cells was positive for D7-FIB, a fibroblast-marker that maintained through multiple passages (de Mos et al. 2007a).

Multipotency of tendon-derived progenitor cells

Multipotency has been confirmed by differentiation towards osteogeneic, chondrogenic and adipogenic lineages. Many studies have performed differentiation experiments in mouse, rat and human species and reported successful differentiation towards all three lineages (Bi et al. 2007, Salingcarnboriboon et al. 2003, Rui et al. 2010, de Mos et al. 2007a). In addition, the differentiation capacity of tendon-derived was greater in comparison to bone marrow-derived MSCs with a higher osteogeneic and adipogenic capacity (Bi et al. 2007).

***In vivo* effects of tendon-derived progenitor cells**

Tendon-derived progenitor cells have been evaluated minimally in two studies in experimental murine models (Bi et al. 2007, Salingcarnboriboon et al. 2003). One study implanted sheets of tendon-derived cells in an experimentally created murine patellar tendon defect (Salingcarnboriboon et al. 2003). Successful incorporation was determined on histology 3 months after implantation. Another study evaluated *in vivo* osteogenesis of BMP-2 expanded tendon-derived cells by injecting with hydroxyapatite/tricalcium phosphate

carrier subcutaneously in immune-suppressed mice (Bi et al. 2007). Bone formation was observed after 8 weeks, along with tendon-like tissue in adjacent areas confirmed by the presence of unique collagen fibers when the tissue was visualized under polarized light.

Preplating Technique

Currently several methods are used to isolate and purify mesenchymal stem/progenitor cells for use in cell-based tissue repair due to their heterogeneous nature. Some of them include magnetic cell sorting, fluorescent activated cell sorting (FACS) and the preplating technique. Magnetic cell sorting and FACS are both cell surface marker-dependent techniques that isolate homogenous populations of stem/progenitor cells based on presence of MSC specific marker proteins on the cell surface. Immunomagnetic isolation technique has been reported to be used in isolating osteoprogenitors from human BMMSCs (Encina, Billotte & Hofmann 1999). FACS has been use to isolate mesenchymal progenitor cells from human, rat and mouse bone marrow (Bi et al. 2007, Scutt, Rolf & Scutt 2008).

However marker profile-dependent isolation methods are limited as they rely on the expression of cell surface marker proteins that are variable and change under cell culture conditions. These techniques also do not take into consideration behaviors such as cell survival, proliferation rates and multipotency *in vivo* and *in vitro*. Further, marker profile-dependent isolation methods are not feasible to isolate progenitor cell populations in species that do not have specified cell surface markers and established genotype databases.

A marker profile-independent method, known as the preplating technique has been described and evaluated for isolating myoblasts, a progenitor cell population in skeletal muscles (Gharaibeh et al. 2008). This technique involved culturing digested muscle tissue for

a set period of time to allow the fibroblastic cell fraction to attach while transferring the supernatant containing the myogenic fraction onto a new plate, thus separating the desired cell fraction. This step was repeated at 12-24 hour intervals for 5 consecutive days to give rise to cells fractions in preplate 0 (PP0) on day 0 to preplate 6 (PP6) on day 5.

Progenitor cells obtained via the preplating technique have been characterized and the technique validated by comparing with FACS and magnetic cell sorting. The first cells to adhere during the early stages of the preplating technique are known as the rapidly adhering cell (RAC) fraction and have been shown to be comprised of mostly fibroblast-like and myoblast cells. Cells isolated from the later stages of preplating contain muscle-derived stem cells and are known as slowly adhering fraction (SAC). One study showed increased immunochemical expression of desmin, a marker specific to myogenic cells in later preplates, PP4-PP6 ranging from 75-94% as compared to 5-37% in PP1-PP3 (Jankowski et al. 2001). Flow cytometric analysis of the PP6 cell fraction in the same study showed expression of surface proteins like Sca-1, CD34 and c-Kit, markers all specific to myogenic population. Another study compared magnetic cell sorting and preplating to purify human myoblasts (Park, Moon & Kim 2006). This study reported 83% increase in desmin positive cells from primary culture to PP5, as compared to 21% primary culture and positive selection of myoblasts by magnetic cell sorting increased from 30% to 42%.

Based on the evidence summarized above, this study aimed at isolating a progenitor cell population from the lateral digital extensor tendon of horses for cell-based therapies in tendon regeneration using the preplating technique and comparing with bone marrow-derived mesenchymal cells with reference to cell viability, tendon gene expression and matrix synthesis in an *in vitro* three dimensional model of tendon healing.

***In vitro* Models of Tendon Regeneration**

Lack of successful outcomes with existing treatment options for equine tendinitis has led to constant efforts in developing novel approaches of tendon regeneration with cell-based therapies to improve biomechanical strength. Various *in vitro* and *in vivo* models of tendinitis are described for evaluating the efficacy for these novel approaches. Most widely used *in vivo*-models of equine tendinitis include the collagenase-induced model and the mechanical model (Nixon et al. 2008, Dahlgren et al. 2002, Schnabel et al. 2009, Bosch et al. 2010). However, *in vivo* models are expensive, more labor intensive and involve ethical concerns to research animals. In addition, an *in vitro* screening step is omitted in many new therapies being evaluated, and assessing the need to carry out an *in vivo* evaluation in only promising therapies.

Acellular tendon has been used in tissue-engineering studies to develop a construct for an appropriate scaffold material for reseeding in cell-based approaches for flexor tendon injuries in lupine models with a goal of therapeutic use in humans (Zhang et al. 2009, Chong et al. 2009). Acellularization of tendons was carried out in these studies by freeze-thaw cycles followed by treatment with trypsin. One study comparing tenocytes and mesenchymal stem cells for reseeding of lupine acellular flexor tendon showed adherence of both cell types used and viability of cell-acellular construct up to 1 week *in vitro* (Kryger et al. 2007). A previous study from our lab used autogenous acellular tendon prepared as sheets of 1 cm X 1 cm from equine superficial digital flexor tendon as an *in vitro* model to compare tendon-, muscle- and bone marrow-derived cells with reference to cell adherence and viability, tendon gene expression and viability (Stewart et al. 2009a). Although viability of the cell types used to the acellular tendon was confirmed on histology, adherence and proliferation was possible

only to the surface of the acellular tendon, which does not simulate conditions present *in vivo* where cells are dispersed in the tendon matrix that contains bundles of collagen fibers. Hence, autogenous acellular pulverized superficial digital flexor tendon is used in this study to provide a 3-dimensional matrix model to evaluate adherence and viability of equine tendon- and bone marrow-derived cells expanded in monolayers with or without FGF-2 supplementation.

Objectives and Hypothesis

The objective of this study was to determine whether FGF-2 supplementation during monolayer expansion can enhance the proliferative capacity and matrix synthesis of both tendon- and bone marrow-derived cells. Secondly, this study evaluated whether IGF-I supplementation alone or following FGF-2 monolayer expansion would have a beneficial effect on cell viability, tendon gene expression, and matrix synthesis of tendon- and bone marrow-derived cells cultured with tendon matrix. For this study our laboratory utilized differential adherence pre-plating technique to isolate tendon-derived cells previously described to isolate progenitor cells from muscle. Bone marrow-derived progenitor cells were used as the current source for cell-based tendinitis therapy. My hypothesis was that tendon-derived cells expanded with FGF-2 followed by IGF-I supplementation with tendon matrix will have an increased cell viability/ proliferation, tendon gene expression, and matrix synthesis when compared to bone marrow-derived cells with or without growth factor cultured under similar conditions.

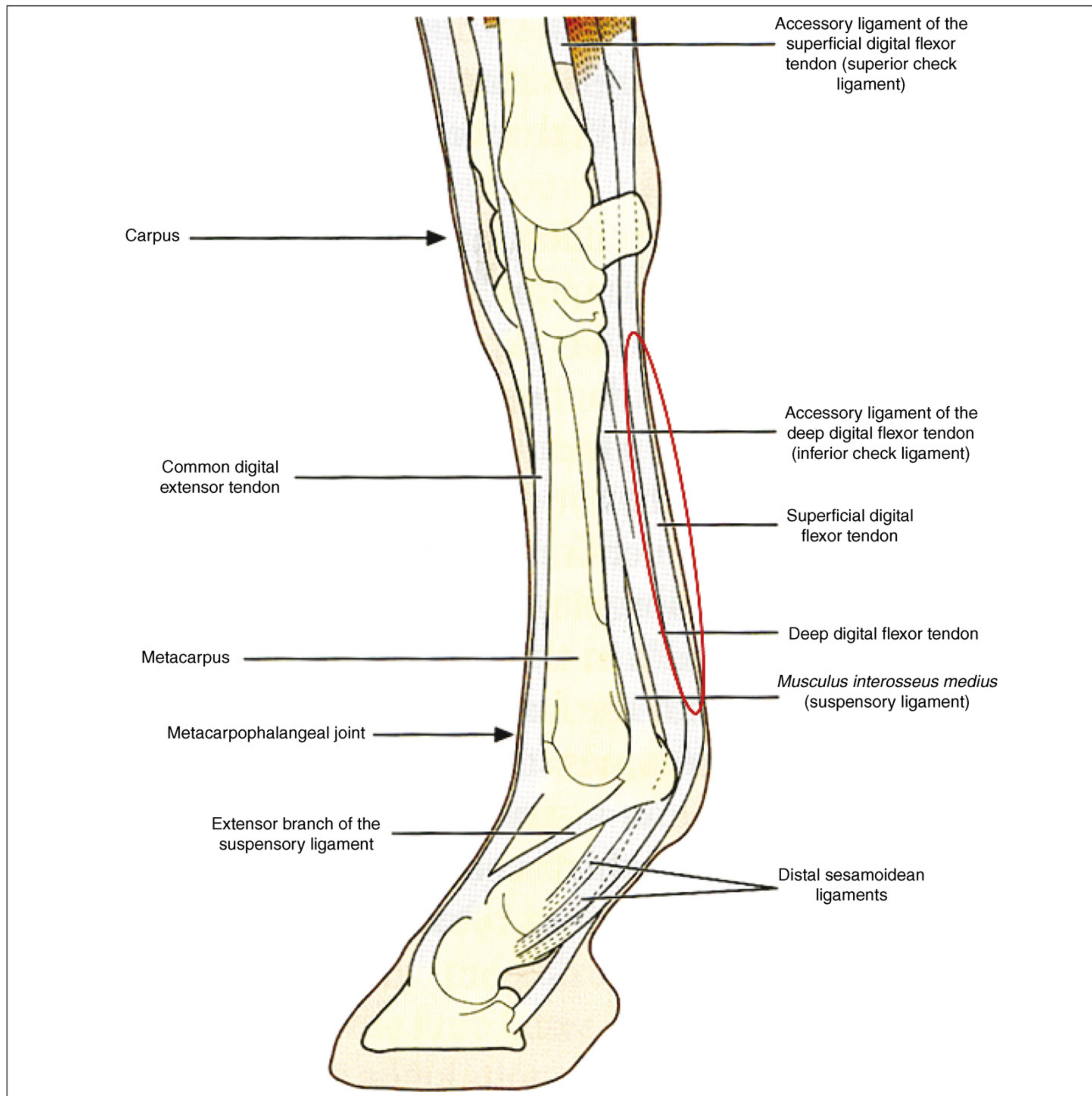

Figure 1: Equine distal limb anatomy (Richardson et al. 2007a).

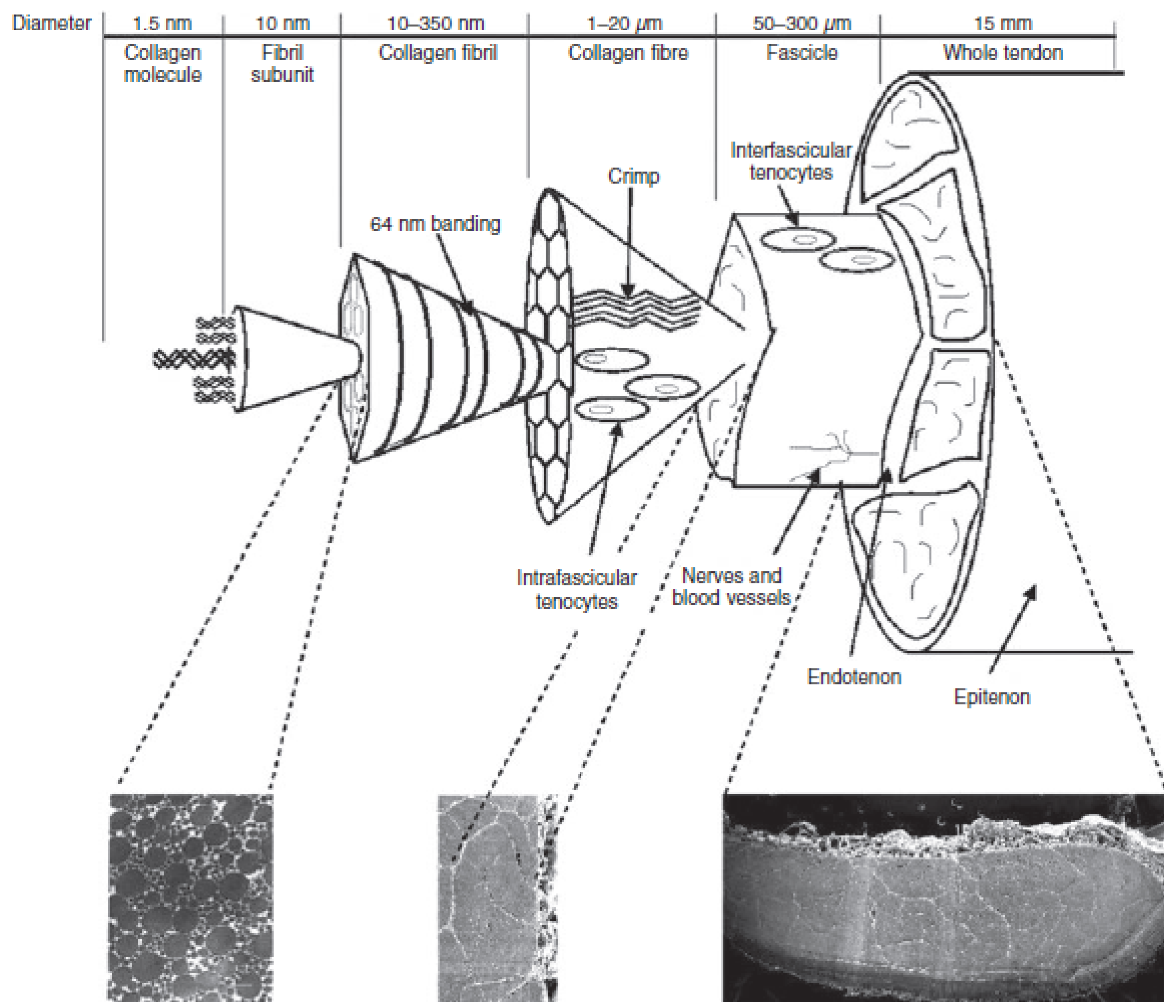

Figure 2: Representation of hierarchical structure of equine superficial digital flexor tendon (Thorpe, Clegg & Birch 2010).

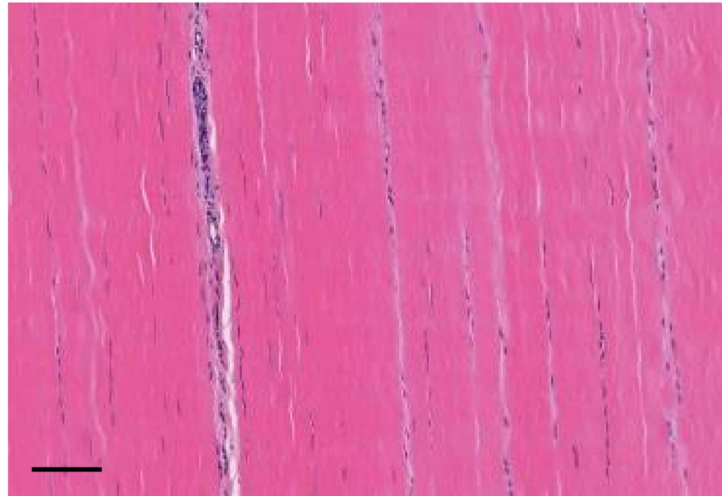

Figure 3: Photomicrograph of equine superficial digital flexor tendon stained with hematoxilin and eosin. Bar = 100 μm

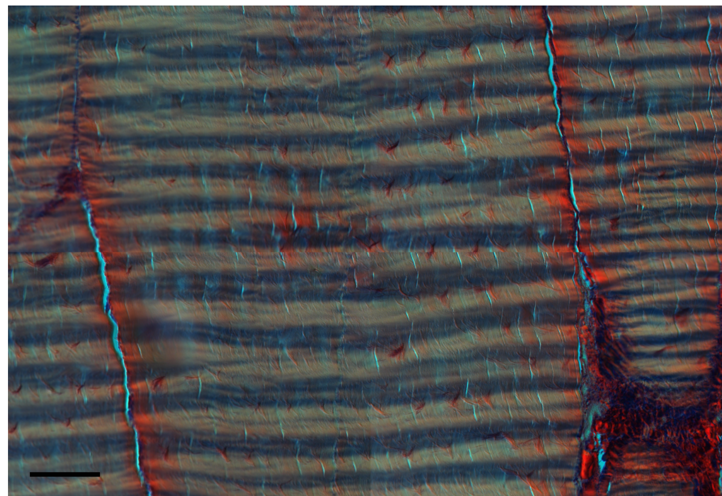

Figure 4: Photomicrograph of equine superficial digital flexor tendon stained with picro-sirius red under polarization light showing characteristic crimp pattern of collagen fibers. Bar = 100 μm

CHAPTER 3

MATERIALS AND METHODS

Collection of Samples

Bone marrow and tendon were collected aseptically from six horses (two to four years of age) euthanized for reasons unrelated to musculoskeletal disease. All samples were obtained in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee. All horses were sedated with 0.01 to 0.03 mg/kg of detomidine administered IV via a jugular catheter. Following collection of the bone marrow aspirates, all horses were euthanized by an intravenous injection of sodium pentobarbital (104mg/kg). The tendon specimens were collected immediately following euthanasia.

Progenitor Cell Culture

Processing of bone marrow-derived cells

Sternal bone marrow aspirates were collected using Jamshidi needles [a] (Schnabel et al. 2009). Approximately 15-20 mLs of bone marrow was aspirated into syringes containing 1,000 units of heparin. Each bone marrow aspirate was diluted with 15 mL of PBS solution and centrifuged at 300 X *g* for 10 minutes. The supernatant was removed, the pellet was resuspended in PBS solution, and centrifugation was repeated. Pelleted cells were resuspended in 12 mL of low-glucose DMEM[b] supplemented with 10% fetal bovine serum[c], 300 μg of L-glutamine[d]/mL, 100 U of sodium penicillin[e]/mL, and 100 μg of streptomycin sulfate[e]/mL. Resuspended cells were placed in a 75 cm^2 flask[f] and incubated at 37°C in a 5% carbon dioxide atmosphere with 90% humidity. The bone marrow-derived cells were

passaged after they reached focal confluence. The cells were monolayer expanded in culture medium supplemented with or without 100 ng FGF-2[g]/mL for two passages which provided sufficient cells for subsequent experiments. Time to confluence and cell counts at trypsinization were recorded.

Processing of tendon-derived cells

The lateral digital extensor tendon from a hind limb was harvested aseptically from each horse. A 4-cm x 1-cm sample of tendon was reserved for cell isolation. The specimen for cell isolation was diced into 0.25 cm^3 pieces and digested for 16 hours at 37°C in 0.2% collagenase[h] high-glucose DMEM supplemented with 1% FBS, 100 U of sodium penicillin/mL, and 100 µg of streptomycin sulfate/mL. Following digestion, the isolated cells were passed through a 40 µm filter[i]. The isolated cells were collected by centrifugation at 300 X *g* for 5 minutes. The supernatant was removed and the cell pellet was resuspended in media. Cell viability was determined by the use of exclusion of trypan blue dye[j] (O'Brien, Gottlieb-Rosenkrantz 1970).

Tendon-derived cell culture

Progenitor cells were collected from tendon by use of a previously described protocol (Stewart et al. 2009a, Gharaibeh et al. 2008). Tendon-derived cells were seeded at 13,300 cells/ cm^2 in culture flasks in high-glucose DMEM supplemented with 20% FBS, 300 ug of l-glutamine/mL, 100 U of sodium penicillin/mL, and 100 ug of streptomycin sulfate/mL. The slowly adherent, tendon-derived cells were preferentially isolated from the rapidly adherent, fibroblast-like cells by differential attachment. The culture medium and unattached cells were serially transferred to fresh culture flasks every 24 hours during the first 6 days of culture (Stewart et al. 2009a). The tendon-derived cells that adhered on day 6 of the transfer protocol

were maintained until confluence and expanded in monolayers with or without supplementation of FGF-2 (100ng/mL) for two passages to generate sufficient cells for subsequent experiments.

Tendon Matrix Culture Model

Superficial digital flexor tendons were collected from donor horses and pulverized in a freezer mill[h] under liquid nitrogen. The pulverized tendon was subjected to four rounds of freeze-thaw cycles at -80°C and 4°C to kill the resident tenocytes. A 1 percent (mass/volume) acellular tendon matrix suspension was prepared with tenogenic medium (high-glucose DMEM supplemented with 10% fetal bovine serum, 300 µg of L-glutamine/mL, 100 U of sodium penicillin/mL, 100 µg of streptomycin sulfate/mL, and 37.5µg/mL ascorbic acid). The tendon matrix suspension was maintained in culture without additional cells to serve as a negative control (matrix only), or seeded with 250,000-cell aliquots of the expanded tendon- and bone marrow-derived cells, supplemented with or without 100 ng IGF-I[i] /mL, in 24-well ultra-low attachment culture plates.[j] This experimental design **(Figure 5)** comprised nine treatment groups. Each treatment group contained eighteen replicates. Twelve replicates were used for RNA isolation, three replicates were used for collagen synthesis, and three replicates were used for GAG synthesis. The replicates for each treatment group were averaged as one data point. This was repeated for each of the 6 horses included in the study. Each treatment well was supplemented with one mL of tenogenic medium containing 0 or 100 ng/mL of IGF-I. Fresh medium was added to all wells every two days after removal of the exhausted medium. All culture samples were collected on day 7 by separating the medium from the matrix with cells by centrifugation.

Cell Number

Three replicates of each treatment group were used to measure cell number on day 7, by use of an mitochondrial metabolic assay[k] as per manufacturer's instructions. In brief, 50 μL of the assay reagent containing tetrazolium was added to fresh medium in each well and the cells were incubated at 37°C for 2.5 hours. One hundred μL of medium from each well was transferred to a 96-well plate and absorbance was measured at 492nm in a microplate reader[l] to detect concentrations of the metabolic product, formazan. All samples were assayed in duplicates, and a mean value was calculated to provide a single data point. The optical density data were converted to 'cell number' by reference to standard curves generated from known numbers of tendon- and bone marrow-derived cell cultures.

RNA Isolation and Gene Expression

Twelve replicates from each treatment group were pooled, snap-frozen in liquid nitrogen, and stored at -80˚C for RNA isolation. Total RNA was extracted using Trizol[m] reagent according to the manufacturer's suggested protocol and purified in silica columns.[n] RNA concentration and purity were assessed by UV spectrophotometry and agarose gel electrophoresis respectively. One µg of RNA in each sample was converted to cDNA with a commercial reverse transcription kit[o] and oligo(dT) primers. Target cDNAs were amplified via real-time PCR using *Taq* DNA polymerase[p] and gene-specific primers designed from available published sequences in Genbank, and a multiple sequence alignment program.[q] Primer specificity was confirmed by cloning and sequencing the PCR products **(Appendix)**. Real-time quantitative PCR was performed in triplicate for collagen I, collagen III, and COMP mRNAs and the reference gene, elongation factor-1α (EF1α). A fluorescence

detection system[r] was used to measure PCR-generated cDNA and generate threshold cycle values. All reactions were run as singleplex and the relative gene expression was quantified by use of the $2^{-\Delta\Delta CT}$ method (Livak, Schmittgen 2001).

Collagen Synthesis

Collagen synthesis was determined via [^{3}H] proline incorporation according to a published protocol (Cechowska-Pasko, Surazynski & Bankowski 2009). On day 6, three wells of each treatment group were radiolabeled with 50 μCi of [^{3}H] proline[s]/mL of tenogenic medium and incubated for 24 hours. The samples were washed three times with 0.5mL of PBS containing 1mM Proline and stored at -80˚C. Radiolabeled samples were freeze-thawed three times, digested, and homogenized[t] prior to RNase treatment. The total protein was precipitated with tricholoroacetic acid and washed three times with L-proline to remove traces of unincorporated [^{3}H] proline. The resulting pellets were digested with purified collagenase,[u] and centrifuged at 3220X*g* for 10 minutes. The supernatant and pellets were separated and transferred to scintillation liquid. Radioactivity was measured in a scintillation counter.[v] Newly synthesized collagen was detected on the basis of radioactivity in the sample supernatants following collagenase-digestion.

Glycosaminoglycan (GAG) Synthesis

GAG synthesis was determined by measuring $^{35}SO_4$ incorporation into each sample. Three wells of each treatment group were radiolabeled with 10 μCi of ^{35}S labeled sodium sulfate/mL[w] during the last 24 hours of the experiments (Masuda, Shirota & Thonar 1994). The samples were washed three times with PBS and then digested in 1 mL of buffer

containing 0.5 mg of papain[x] at 65°C for 16 hours. Twenty-five μL aliquots of ^{35}S-labelled, papain-digested samples were placed in multiwell punch plates,[y] precipitated with alcian blue dye, and counted by scintillation(Masuda, Shirota & Thonar 1994, Stewart et al. 2009b). All counts per minute (CPM) values were adjusted for decay of ^{35}S-radioisotope from the time of radiolabeling to assay. Values were expressed as CPM per 250,000 cells.

Statistical Analyses

Mean $\pm$ SE for each statistic were calculated for each cell type and supplementation of FGF-2, IGF-1, and the combination of FGF-2 and IGF-1. Background values detected in the matrix-only group were subtracted from the values of the other groups for quantification of collagen and GAG synthesis. Cell number, collagen synthesis and GAG synthesis data were log-transformed for data normalization. The effect of cell type was analyzed using a Mixed Effects Model, with the subject as a random effect. Among the individual cell types (tendon- and bone marrow-derived cells), the effect of growth factor supplementations were evaluated using 2-way repeated-measures ANOVA to control for differences between horses. When group differences for growth factor supplementation were noted, pair-wise multiple comparisons were made using Holm-Sidak non-parametric test. A commercially available statistical program was used to perform statistical analyses.[z] Values of $P \leq 0.05$ were considered significant.

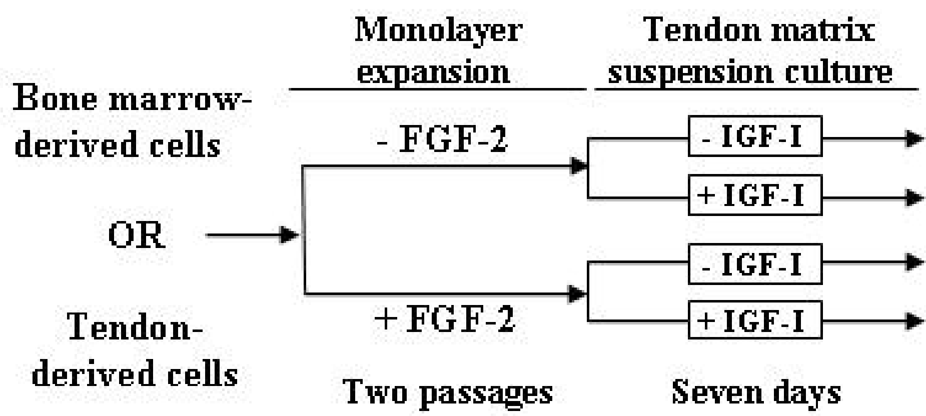

Figure 5: Experimental design

CHAPTER 4

RESULTS

Monolayer Cell Expansion

Overall, the mean cell number following monolayer expansion was significantly (P = 0.011) higher in tendon-derived cells when compared to bone marrow-derived cells **(Table 1)**. Monolayer expansion of tendon-derived cells with FGF-2 significantly (P = 0.027) increased cell number when compared to the unsupplemented tendon-derived cultures **(Figure 6)**. In contrast, monolayer expansion with FGF-2 did not significantly (P = 0.311) affect proliferation of bone marrow-derived cells.

Cell Number

After seven days in culture with pulverized tendon matrix and IGF-I, there was no significant effect of cell type on mean cell number, as determined by mitochondrial metabolic assay **(Figure 7)**. Monolayer expansion with FGF-2 did not significantly change the numbers of tendon- (P = 0.052) or bone marrow-derived (P = 0.096) cells cultured with tendon matrix. There was no significant (P = 0.178) effect of IGF-I supplementation on cell number for either cell type. The cell number of the “matrix only” control group was zero, confirming the absence of viable cells.

Extracellular matrix gene expression

As expected, no mRNA was isolated from the acellular matrix samples, verifying that no viable endogenous tenocytes remained. Therefore, the gene expression data derived from

bone marrow-derived cells cultured with tendon matrix without growth factor supplementation (bone marrow-derived cells only) were used as reference values for comparative analyses.

Collagen type I

Collagen type I mRNA expression in tendon-derived-cell groups and bone marrow-derived-cell groups was not significantly different ($P = 0.087$) **(Figure 8)**. Among the tendon-derived cell groups, supplementation with IGF-I did not significantly ($P = 0.095$) increase collagen type I mRNA expression. In the bone marrow-derived cell groups, IGF-I significantly ($P = 0.028$) increased collagen type I mRNA expression. Monolayer expansion with FGF-2 had no effect on subsequent collagen type I mRNA expression in either tendon- or bone marrow-derived cell types cultured with tendon matrix.

Collagen type III mRNA expression

Overall, tendon-derived cell groups cultured with tendon matrix expressed significantly ($P = 0.003$) more collagen type III mRNA than bone marrow-derived cell groups **(Figure 9)**. Within the tendon-derived cell groups, there was no significant effect of FGF-2 ($P = 0.623$) or IGF-I ($P = 0.119$) on collagen type III mRNA expression. In the bone marrow-derived cell groups, IGF-I supplementation significantly ($P = 0.048$) increased collagen type III mRNA expression**.** Monolayer FGF-2 expansion of bone marrow-derived cells had no effect ($P = 0.523$) on collagen type III expression.

COMP mRNA expression

Overall, tendon-derived cell groups cultured with tendon matrix expressed significantly ($P = 0.001$) more COMP mRNA than bone marrow-derived cell groups **(Figure**

10). However, there was no significant effect of FGF-2 or IGF-I on COMP mRNA expression in either tendon- or bone marrow-derived cells.

Collagen Synthesis

Although mean collagen synthesis of tendon-derived cell groups approached significance (P = 0.055), the synthesis rate was still less than that of bone marrow-derived cell groups **(Figure 11)**. Monolayer expansion of tendon-derived cells with FGF-2 did not affect their subsequent collagen synthesis (P = 0.367). Further, IGF-I did not affect collagen synthesis by tendon-derived-cell groups (P = 0.055). There was no significant (P = 0.532) effect of FGF-2 expansion on collagen synthesis by bone marrow-derived cells when compared to the unsupplemented controls. However, collagen synthesis in the bone marrow-derived cell grouos supplemented with IGF-I remained lower than collagen synthesis in the tendon-derived groups.

Collagen synthesis normalized to cell number

When values of collagen synthesis were normalized to cell number, no significant difference (P= 0.134) was seen in the per-cell collagen synthesis between tendon- and bone marrow-derived cell groups **(Figure 12)**. Among the tendon-derived cell groups, monolayer expansion with FGF-2 had no significant effect (P = 0.342) on sequential per-cell collagen synthesis. However, supplementation of IGF-I significantly (P = 0.030) increased the per-cell collagen synthesis of tendon-derived cells in comparison to the supplemented control. In the bone marrow-derived cell groups, monolayer expansion of bone marrow-derived cells with FGF-2 had a favorable effect (P = 0.071) on sequential per-cell collagen synthesis.

Supplementation of IGF-I had no significant (P = 0.671) effect on mean log collagen synthesis normalized to cell number in bone marrow-derived cell groups.

GAG Synthesis

GAG synthesis was significantly ($P = 0.0058$) higher in the tendon-derived cell groups than the bone marrow-derived **(Figure 13)**. Within the tendon-derived cell groups, monolayer expansion with FGF-2 significantly ($P = 0.030$) increased GAG synthesis. Further, IGF-I supplementation of tendon-derived cell:matrix cultures also increased GAG synthesis ($P = 0.016$). In the bone marrow-derived cell groups, monolayer expansion with FGF-2 had no effect ($P = 0.305$) on GAG synthesis. However, IGF-I significantly ($P = 0.022$) increased GAG synthesis by bone marrow-derived cells, in comparison to unsupplemented cultures.

GAG synthesis normalized to cell number

When GAG synthesis was normalized to cell number, overall, per-cell synthesis of tendon-derived cell groups approached significance (P = 0.066) in comparison to bone marrow-derived cell groups **(Figure 14)**. However, there was no significant effect of supplementation with FGF-2 or IGF-I on per-cell GAG synthesis of tendon- or bone marrow-derived cell groups cultured with tendon matrix.

Cell Type	(+) FGF-2	(-) FGF-2	P- value
Tendon-derived Cells	$15.34X10^6 \pm 2.597\ X10^6$ *‡	$9.14\ X\ 10^6 \pm 1.03\ X10^6$ *	0.027
Bone marrow-derived Cells	$5.87\ X\ 10^6 \pm 1.79\ X10^6$	$3.06\ X\ 10^6 \pm 0.85\ X10^6$	0.311

Table 1: Mean $\pm$ SE values for cell number following monolayer expansion with or without FGF-2 supplementation in tendon- and bone marrow-derived cells. *Significant effect based on cell type. ‡ Significant effect of monolayer expansion with 100 ng/mL of FGF-2.

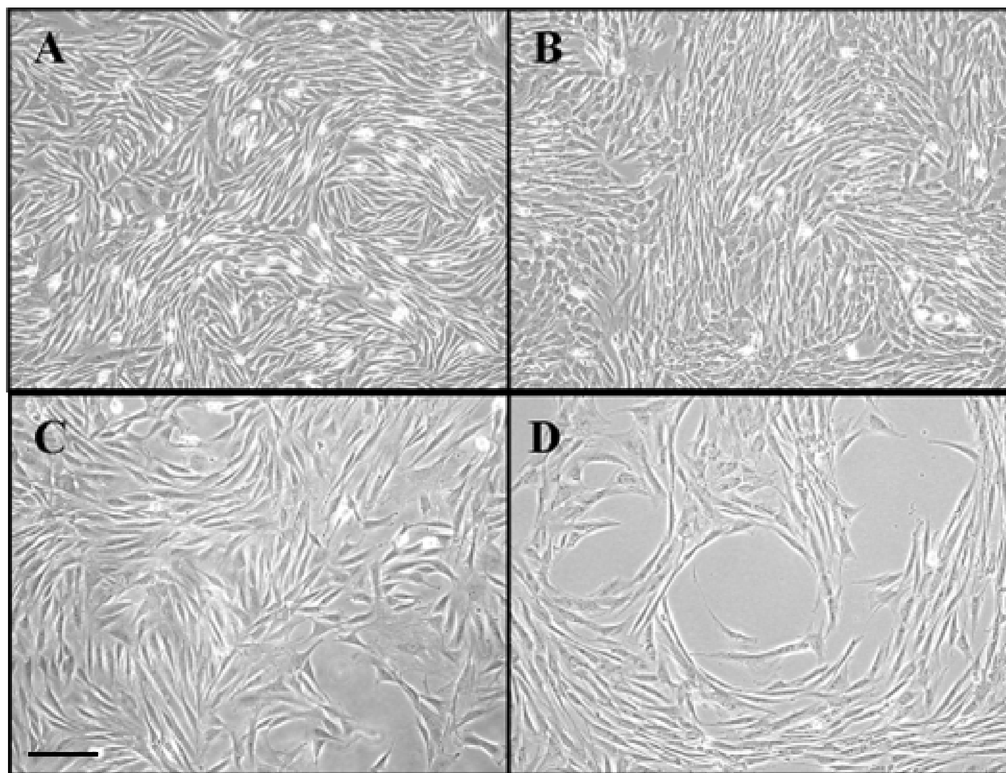

Figure 6. Bright field photomicrograph during monolayer expansion of tendon-derived cells with FGF-2 (**A**) and without FGF-2 (**B**), bone marrow-derived cells with FGF-2 (**C**) and without FGF-2 (**D**) supplementation. Bar = 50 µm

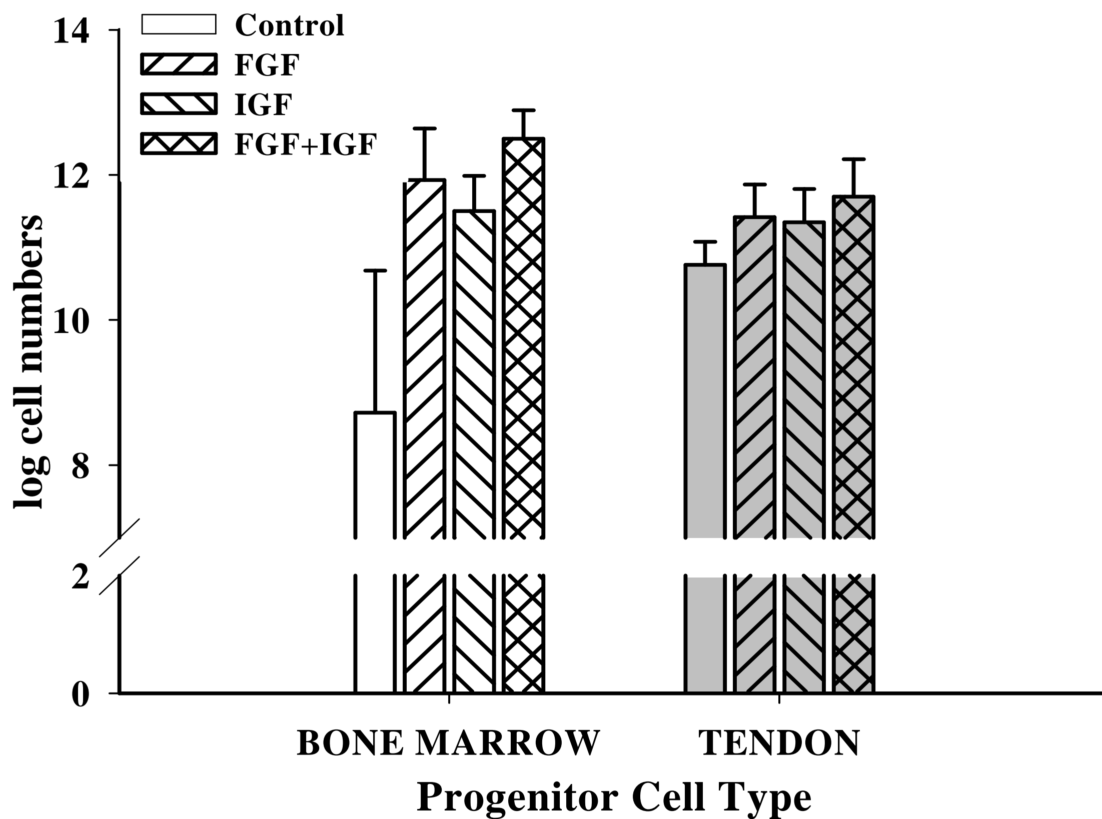

Figure 7: Log mean ± SE cell numbers present in each treatment group following expansion with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix.

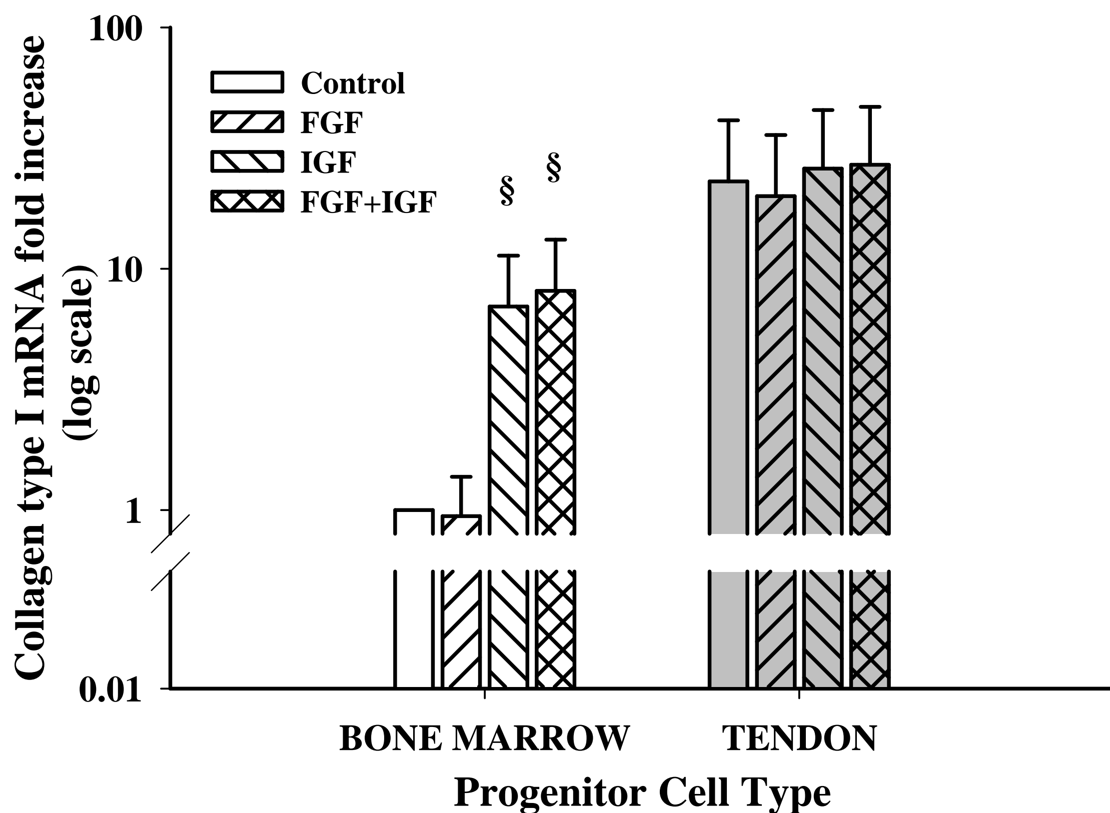

Figure 8: Mean ± SE values (log scale) for collagen type I mRNA expressionn normalized to EF1-α. The x- axis represents equine tendon- and bone marrow-derived cells expanded with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix. § Significant effect of supplementation of IGF-I when compared to no IGF-I. N=6.

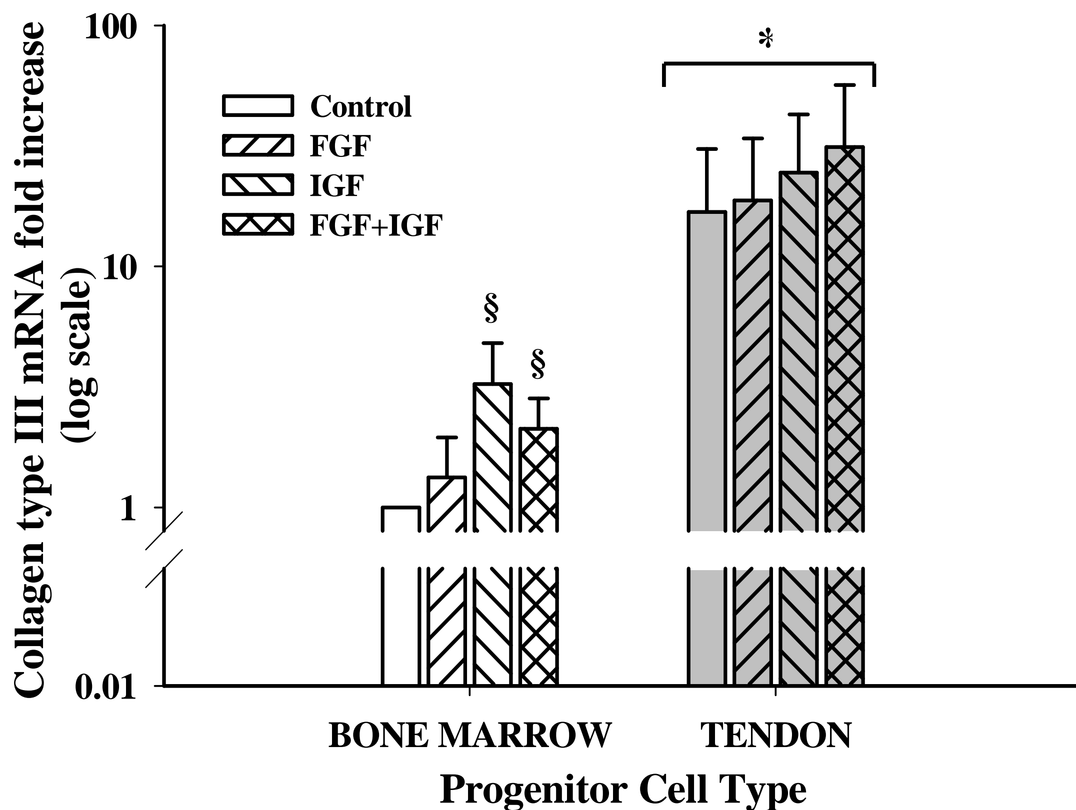

Figure 9: Mean ± SE values (log scale) for collagen type III mRNA expression normalized to EF1-α. The x- axis represents equine tendon- and bone marrow-derived cells expanded with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix. * Significant effect based on cell type. § Significant effect of supplementation of IGF-I when compared to no IGF-I. N=6.

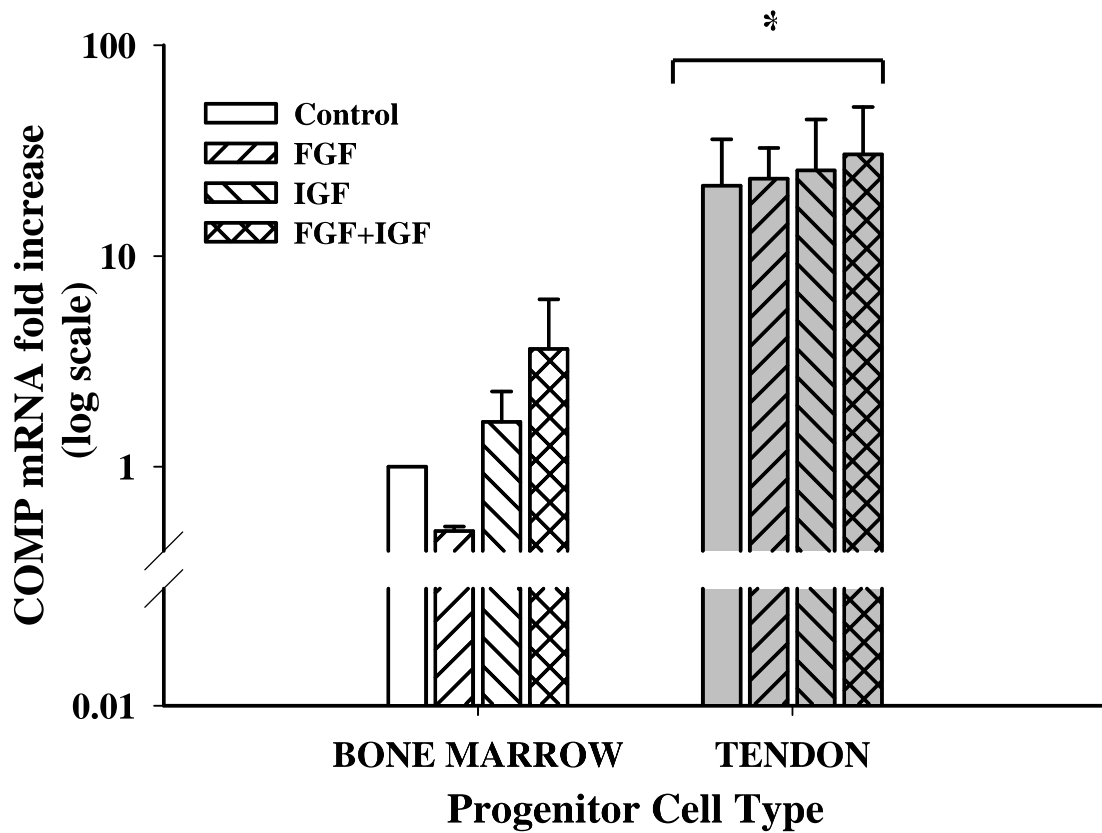

Figure 10: Mean ± SE values (log scale) for collagen type III mRNA expression normalized to EF1-α. The x- axis represents equine tendon- and bone marrow-derived cells expanded with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix. * Significant effect based on cell type.

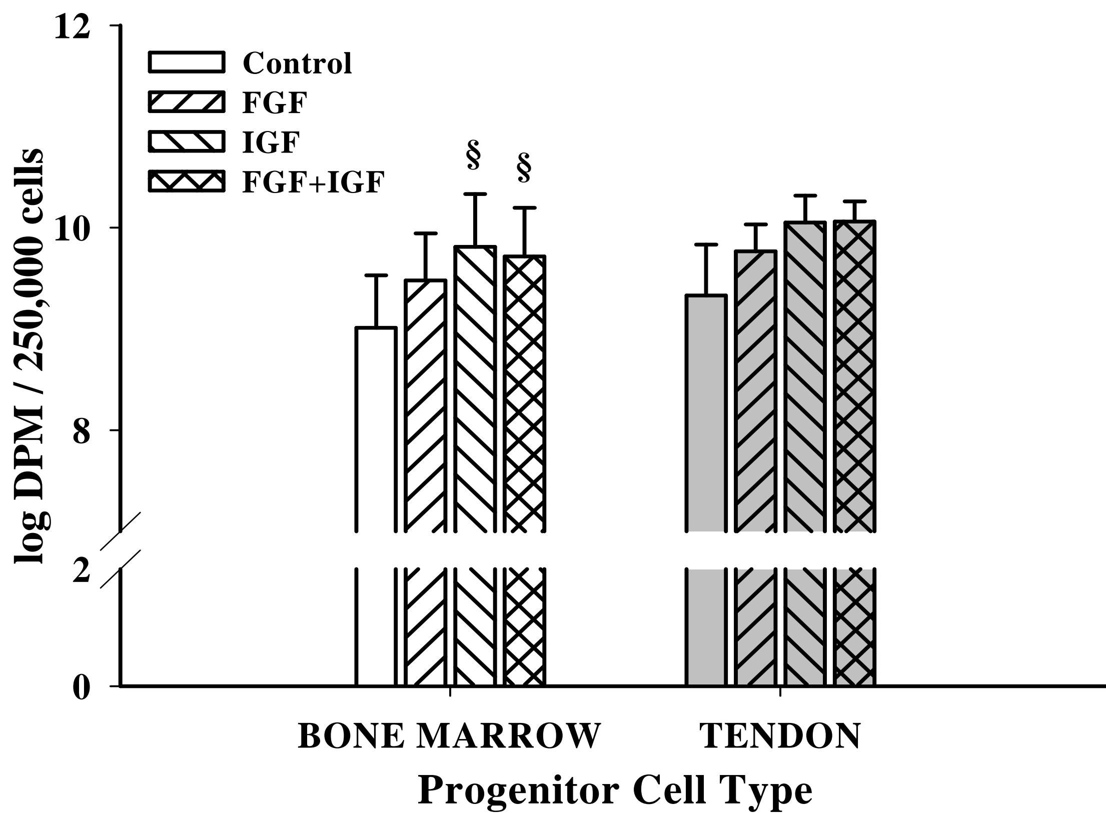

Figure 11: Log mean + SE (DPM- disintegrations per minute) incorporation of [^{3}H] proline into collagen of the matrix formed by the combination of cells and pulverized acellular tendon. Cells were expanded with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix. § Significant effect of supplementation of IGF-I in comparison to control. N=6.

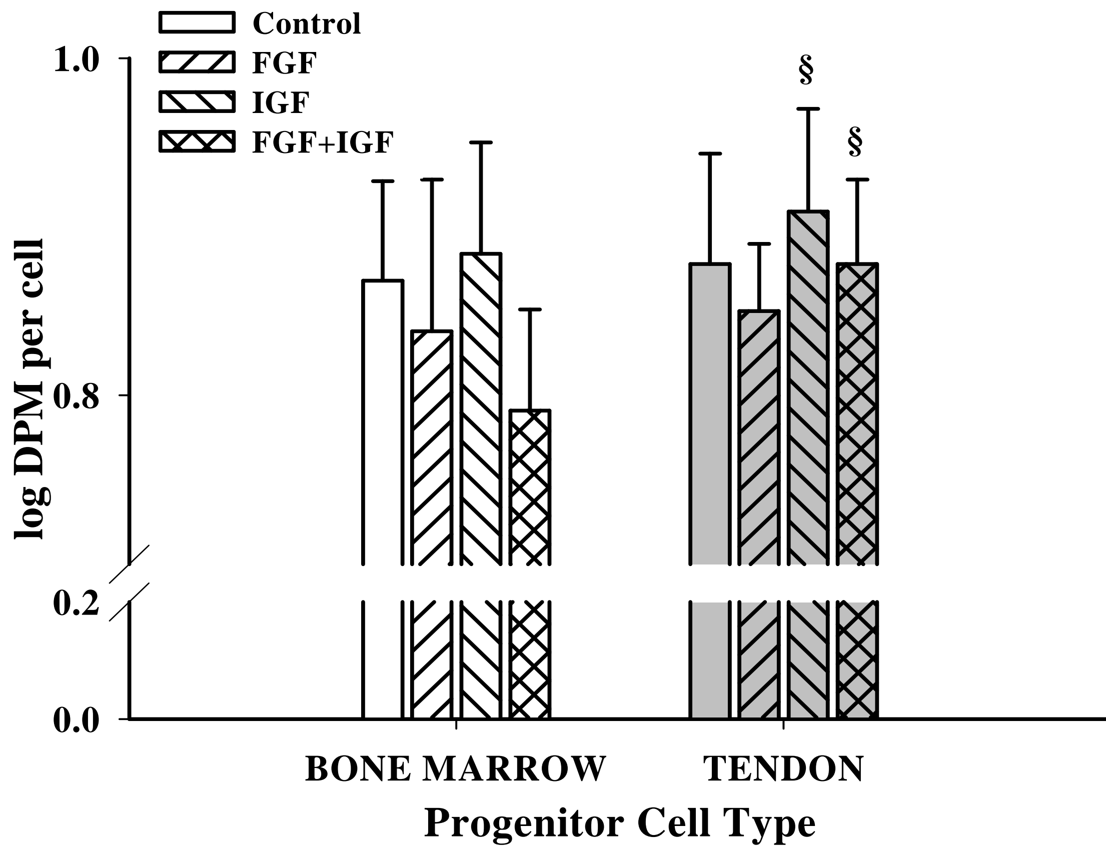

Figure 12: Log mean DPM (disintegrations per minute) normalized to cell number ± SE. Cells were expanded with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix.§ Significant effect of supplementation of IGF-I.

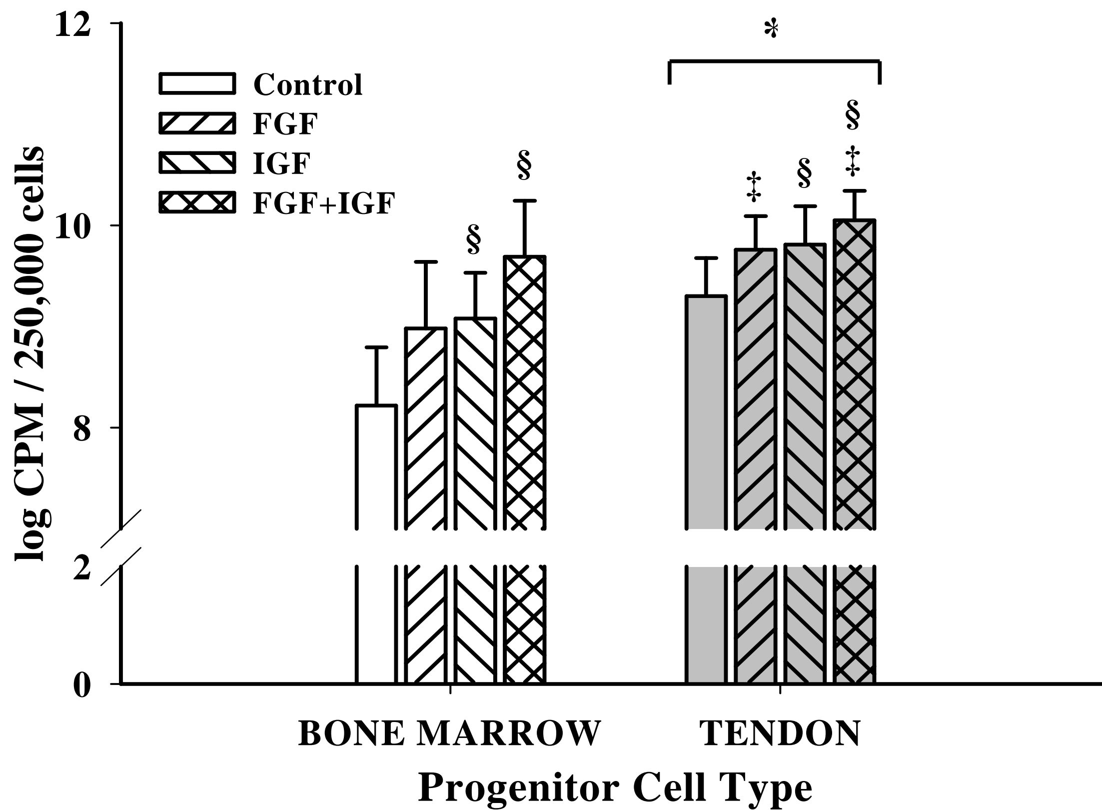

Figure 13: Log mean ± SE (CPM- counts per minute) incorporation of sulfur 35-labeled sodium sulfate into GAG of the matrix formed by the combination of cells and pulverized acellular tendon. Cells were expanded with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix. * Significant effect based on cell type. ‡ Significant effect of monolayer expansion with FGF-2 in comparison to control. § Significant effect of supplementation of IGF-I in comparison to control. N=6.

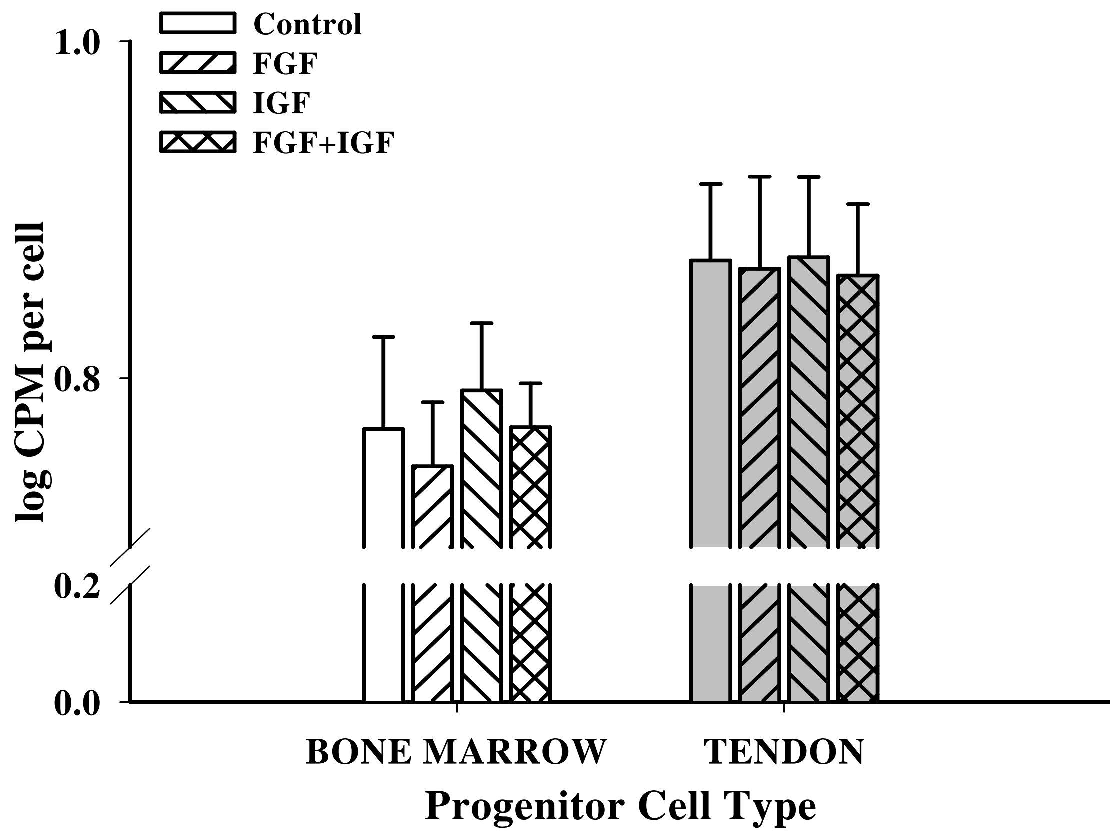

Figure 14: Log mean CPM (counts per minute) normalized to cell number ± SE. Cells were expanded with and without FGF-2 and cultured for 7 days with and without IGF-I and pulverized acellular tendon matrix.

CHAPTER 5

DISCUSSION

The objective of this study was to evaluate the effect of sequential growth factors, FGF-2 and IGF-I on tendon-derived cells as a therapeutic option for cell-based therapies for the treatment of tendinitis. Bone marrow-derived cells were used as the gold standard for comparison. Overall, FGF-2 increased cell proliferation during monolayer expansion and IGF-I increased subsequent matrix synthesis in the tendon-derived cells. Tendon-derived cells were more proliferative in culture *in vitro* and required a shorter duration of time to generate clinically relevant numbers than bone marrow-derived cells. In addition, less inter-animal variability was noted in statistics derived from tendon-derived cells when compared to bone marrow-derived cells. The results obtained in this study were derived from six young adult horses and the statistical power for significance ranged from 0.6 - 0.9.

This *in vitro* study suggests that tendon-derived cells can be obtained in sufficient numbers from an autogenous specimen for cell-based treatment of tendinitis in horses(Smith 2008b); (Nixon et al. 2008, Schnabel et al. 2009). Recent studies have evaluated the presence of a stem cell population within a tendon extracellular matrix niche(Bi et al. 2007, de Mos et al. 2007a, Scutt, Rolf & Scutt 2008). Moreover, differentiation towards adipogenic, osteogeneic and chondrogenic pathways have been reported, confirming the multipotential capacity of the tendon stem cell populations in mice, rats, rabbits, horses, and humans(de Mos et al. 2007a, Scutt, Rolf & Scutt 2008). One study showed increased chondrogenic capacity of caprine tendon-derived cells in comparison to bone marrow-derived MSC controls, suggesting that tendon-derived progenitor cells are feasible for cell-based therapies

directed towards tissues other than tendon itself (Funakoshi, Spector 2010). In this study, tendon-derived cells, overall, had an increased expression of collagen type III mRNA when compared to bone marrow-derived cells. An increased expression of collagen type III can alter the collagen type I/type III ratio and eventually affect the quality of the resulting structure. Determinations of collagen type I and III protein production or consequent extracellular matrix organization were not performed in this study. These analyses will require an *in vivo* study, to clarify the clinical benefits of sequential FGF-2 and IGF-I administration for cell-based repair of tendon injuries. In addition, following monolayer expansion with FGF-2, both tendon- and bone marrow-derived cells were cultured in tenogenic medium with pulverized tendon and IGF-I. It is possible that this medium may have provided optimum conditions for matrix synthesis of tendon-derived cells versus bone marrow-derived cells.

In this study, FGF-2 significantly increased proliferation of tendon-derived cells during monolayer expansion; however, this effect was not significant in the bone marrow-derived cells. This contrast may be due to the considerable "between donor" variation in bone marrow aspirate responses. FGF-2 had minimal effects on cell viability and adherence of both tendon- and bone marrow-derived cells on acellular pulverized tendon. In addition, FGF-2 did not influence ECM gene expression or matrix synthesis in either cell type. There have been no previous reports describing the effect of FGF-2 expansion on subsequent activities of tendon-derived cells. *In vitro* studies, evaluating the effect of FGF-2 on bone marrow-derived MSCs have shown mitogenic effects and a protective effect on subsequent multilineage potential during proliferation *in vitro* (Stewart et al. 2009a, Tsutsumi et al. 2001). Further, *in vivo* studies with intratendinous injections of FGF-2 in murine and canine

models demonstrated angiogenic stimulation in the early stages of tendon healing(Dahlgren, Mohammed & Nixon 2005, Chan et al. 2000)(Duffy et al. 1995, Chan et al. 1997). Based on this study, supplementation with FGF-2 should be considered to increase the proliferation rate of tendon-derived cells *in vitro*, without detrimental effects on subsequent matrix synthesis.

Previously, an *in vitro* study described IGF-I enhanced tenocyte proliferation and matrix synthesis in equine tendon explants (Murphy 1997). In the current study, IGF-I increased collagen and proteoglycan synthesis in both tendon and bone marrow-derived cells, although this effect was only seen at a transcriptional level in the bone marrow derived cells. In addition, sequential administration of IGF-I to FGF-2 expanded tendon-derived cells significantly increased GAG synthesis when compared to tendon-derived cells without growth factor supplementation. These variables may alter per cell biosynthetic rates among the different treatment groups. These increases in matrix synthesis suggest that IGF-I supplementation is justified for tissue regeneration applications of both cell types, as supported by two recent *in vivo* studies that utilized the equine collagenase model of tendinitis (Dahlgren et al. 2002, Schnabel et al. 2009). Both these *in vivo* studies showed improvement in biomechanical properties which is a critical outcome for successful tendon repair. In this study, the improvement in gene expression and matrix synthesis was apparent in bone marrow-derived cells with IGF-I supplementation and is similar to other *in vitro* studies (Dahlgren, Nixon & Brower-Toland 2001, Murphy, Nixon 1997). However, the increases in gene expression and matrix synthesis by bone marrow-derived cells supplemented with IGF-I remained lower than the corresponding activities of tendon-derived cells supplemented with IGF-I.

In this *in vitro* study, acellular pulverized tendon was used to provide a three dimensional substrate for cell adherence with the goal of simulating the *in vivo* micro-environment of a damaged tendon, in contrast to acellular tendon matrix explants which provide an intact, surface for attachment. Previous studies have shown tendon-derived cells had superior adherence to and viability on acellular tendon matrix explant than bone marrow-derived cells (Stewart et al. 2009a).[aa] In contrast, the results from this study show no difference in viability between tendon- and bone marrow-derived cells in co-culture with pulverized matrix suspension. This suggests that the powdered tendon used in the current study have cell adhesion and survival properties that differ from acellular tendon matrix explants used previously. Further, acellular tendon explants may provide topographic cues for cell attachment due to the organized fiber pattern as present *in vivo*. Further work needs to be done to assess the differential effects of specific substrate characteristics on progenitor cell colonization and survival.

Hind limb lateral digital extensor tenectomies were performed to obtain tendon-derived cells and tendon matrix. In this technique, a 4 cm X 1 cm tendon specimen provided sufficient numbers of tendon-derived cells for potential clinical applications. This procedure is used clinically for the treatment of refractory stringhalt; however, the long-term safety and morbidity associated with lateral digital extensor tenectomies needs further evaluation. In addition, a recent study demonstrated metabolic and homeostatic differences between equine flexor and extensor tenocytes (Hosaka et al. 2010). Common digital extensor tenocytes were less proliferative and had reduced matrix synthetic capacity *in vitro* when compared to superficial and deep digital flexor tenocytes. It is possible that lateral digital extensor tendon-

derived cells, although an expedient source of tendon-derived progenitors, are not biosynthetically ideal for the repair and/or regeneration of digital flexor tendon injuries.

In conclusion, the results of this study demonstrate that, *in vitro*, tendon-derived cells have increased matrix gene expression and matrix synthetic capacity when compared to bone marrow-derived cells. In general, growth factor supplementation had more pronounced effects on bone marrow-derived cells. However, tendon-derived cells proliferated more rapidly in monolayer culture than bone marrow-derived cells with FGF-2 supplementation. Also, GAG synthesis of tendon-derived cells was increased following FGF-2 supplemented monolayer expansion and IGF-I administration during cell:matrix suspension culture. Accepting these beneficial responses, these results were obtained in an *in vitro* model and require careful interpretation and *in vivo* assessment before translation into clinical therapies. Based on the results obtained from this study, further research in use of tendon-derived cells and growth factor enhancement of these cells for therapeutic applications in *in vivo* models of tendinitis is warranted.

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APPENDIX

Primers used for real-time PCR amplification of genes in this study of equine bone marrow- and tendon-derived cells

Gene	**Sequence**	**Amplicon (bp)**
Eq Col I (S)	GAA AAC ATC CCA GCC AAG AA	231
Eq Col I (A)	GAT TGC CAG TCT CCT CAT CC	
Eq Col III (S)	AGG GGA CCT GGT TAC TGC TT	215
Eq Col III (A)	TCT CTG GGT TGG GAC AGT CT	
Eq COMP (S)	TCA TGT GGA AGC AGA TGG AG	223
Eq COMP (A)	TAG GAA CCA GCG GTA GGA TG	
Eq EF1-α (S)	CCC GGA CAC AGA GAC TTC AT	328
Eq EF1-α (A)	AGC ATG TTG TCA CCA TTC CA	

bp = base pairs; S = sense; A = antisense

FOOTNOTES

a. Jamshidi bone marrow biopsy needle, Cardinal Health, Dublin, Ohio.

b. DMEM, Mediumtech Inc, Herndon, Va.

c. Gemini Bioproducts, Woodland, Calif.

d. L-glutamine, 200 mM, Invitrogen, Carlsbad, Calif.

e. Penicillin-streptomycin, BioWhittaker, Cambrex Bio Science, Walkersville, Md.

f. FGF-2, R & D systems, Minneapolis, MN.

g. Collagenase type II, Worthington Biochemical Corp, Lakewood, NJ.

h. Freezer mill, SPEX Certi Prep, Matuchen, NJ.

i. IGF-I, R & D systems, Minneapolis, MN.

j. 24 well ultra-low attachment plates, Fisher Scientific, Pittsburgh, PA.

k. Cell Titer 96 Aq_{ueous} One Solution Cell Proliferation Assay, Promega, Madison, WI.

l. Microplate reader, FLUOstar Optima, BMG Laboratories, Durham, NC.

m. Trizol, Invitrogen, Carlsbad, Calif.

n. Rneasy, Qiagen, Valencia, Calif.

o. Superscript II, Invitrogen, Carlsbad, Calif.

p. iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, Calif.

q. ClustalW, www.ebi.ac.uk.

r. iCycler iQ real-time PCR detection system, Bio-Rad Laboratories, Hercules, Calif.

s. [^{3}H] proline, Sigma Chemical Co, St Louis, MO.

t. Hand-held or post-mounted homogenizer, Pro Scientific, Oxford, CT.

u. Collagenase, purified, Worthington Biochemical Corp, Lakewood, NJ.

v. LS6500 Multi-purpose scintillation counter, Beckman Coulter Inc, Fullerton, Calif.

w. ^{35}S- labeled sodium sulphate, MP Biochemicals, Irvine, Calif.

x. Papain, Sigma-Aldrich, St Louis, Mo.

y. Multiwell punch plates, PDVF plate, Millipore, Bedford, Mass.

z. R 2.9.1 statistical software, online at www.r-project.org.

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