Adipose-Derived Adult Stem Cells as Trophic Mediators of Tendon Regeneration

Shelley L. Stewart

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Biomedical and Veterinary Sciences

Linda A. Dahlgren, Chair
Jennifer G. Barrett
Willard H. Eyestone
William R. Huckle

June 26th, 2012

Blacksburg, Virginia

Keywords: Trophic, Stem Cells, Tendon, Growth Factors, Horse

Adipose-derived Adult Stem Cells as Trophic Mediators of Tendon Regeneration

Shelley L. Stewart

Abstract

The adipose-derived stromal vascular fraction (SVF) is a promising new therapy for equine flexor tendonitis. This heterogeneous population of cells may improve tendon healing via the production of growth and chemotactic factors capable of recruiting endogenous stem cells and increasing extracellular matrix production by tendon fibroblasts (TFBL). The purpose of this study was to evaluate the ability of adipose-derived cells (ADC) culture expanded from the SVF to act as trophic mediators *in vitro*. We hypothesized that ADCs would produce growth and chemotactic factors important in tendon healing and capable of inducing cell migration and matrix protein gene expression. Superficial digital flexor tendons and adipose tissue were harvested from eight adult horses and processed to obtain SVF cells, ADCs and TFBLs. Adipose-derived cells and TFBLs were grown in monolayer culture for growth factor quantification, to produce conditioned media for microchemotaxis, and in co-culture for quantification of matrix protein gene expression by TFBLs. Growth factor gene expression by SVF cells was significantly greater than in ADCs or TFBLs. Co-culture of TFBLs and ADCs resulted in modest up-regulation of matrix protein expression (collagen types I and III, decorin, and cartilage oligomeric matrix protein) by TFBLs. Media conditioned by ADCs induced ADC migration in a dose dependent manner. These findings support the role of both SVF and ADCs as trophic mediators in tendon regeneration. The differences detected in gene expression between SVF cells and ADCs indicate that additional studies are needed to evaluate the changes that occur during culture of these cells.

Acknowledgements

First and foremost I want to thank the Lord for His wisdom, abundant grace, and mercy. Philippians 4:13 “I can do all things through Christ which strengtheneth me”. James 1:5 “If any of you lack wisdom, let him ask of God, that giveth to all men liberally, and upbraideth not; and it shall be given him”.

Many thanks are due to my committee for their guidance and intellectual contributions to this project: Dr. Linda A. Dahlgren, Dr. Jennifer G. Barrett, Dr. Willard H. Eyestone, and Dr. William R. Huckle. Dr. Stephen Werre contributed heavily to the data analysis. My thanks also go out to Elle Donnini for her assistance with the growth factor gene expression assay, and to Anne Nichols for her considerable work on the microchemotaxis assay.

I would like to thank my family (Mom, Dad, my sister Lauren, and my Grandma Ruth) for their continued support and guidance, and my grandparents who have gone on to be with the Lord – Papaw Clyde and Grandma Doris, and Grandpa Earl. I especially would like to thank my family at Gateway Baptist Church – Dr. Scott and Kay Souther, Roger and Shelia St. Clair, Joe and Millie Ryan, and the Wright Family. My ‘horse family’ deserves considerable credit in helping me reach this milestone – Catherine Williams, the Countiss Family, Dr. Jacob R. Haught, Dr. Christie J. Boyd, and my farrier, Chris Harman. Finally, I would like to thank the Lord for blessing me with wonderful ‘therapists’ – my horse Cheyenne, and my dogs Max and Lionel.

Table of Contents

List of Figures and Tables

Introduction

Flexor tendon injuries are common in horses and are associated with significant morbidity, loss of use, and loss of revenue [1, 2]. The prognosis for return to previous athletic level is poor to good, and varies with the severity of the injury and other factors such as the age of the horse, its disposition, and owner compliance with the prescribed treatment plan [5]. Rehabilitation can take 12 months or longer [6]. Despite the high frequency of flexor tendon injuries, and continued industry and scientific efforts to improve case management, currently available treatment options result in less than optimal healing. Healed, or 'repaired', tendon is biomechanically inferior to normal tendon, predisposing the patient to re-injury. Therefore, there is an urgent need for the discovery of novel treatment modalities that result in regeneration of a biomechanically normal tendon.

The superficial digital flexor tendon has a narrow margin of safety, with strains experienced during high speed exercise being within the range at which the tendon fails [1, 7, 8]. Horses reach skeletal maturity at two to three years of age, which coincides with the beginning of their athletic careers; however, once skeletal maturity is reached, their flexor tendons may not be capable of functional adaptation [6]. Clinical tendonitis is thought to be preceded by microdamage to the tendon, with subsequent failure of tendon fibroblasts to maintain homeostasis [2]. Cross-links between collagen fibrils are damaged, and microdamage to the extracellular matrix (ECM) and collagen fibrils occurs. This microdamage accumulates over time, with repeated overuse episodes, until the point at which the extent of the damage is sufficient to result in clinical tendonitis [1, 6].

Traditional treatments for tendonitis are suboptimal and ultimately result in a high rate of re-injury. The initial goal of treatment is to control inflammation with cold hosing, icing, or cold compression therapy, in addition to bandaging, non-steroidal anti-inflammatory drugs, and stall rest. Ultrasound exams are performed at regular intervals to monitor the progression of healing and prescribe increasing increments of exercise. After the initial inflammatory period has subsided, the horse is started on a program of controlled hand walking, followed by walking under saddle, and then a slow return to regular work. The problem with these traditional treatment methods is that they result in healed tendon that is biomechanically different from a normal tendon. After injury, there is a disturbance in the ratio of the main collagenous components, type I and type III collagen, and this disturbance leads to a decrease in tendon tensile strength [9]. The ratio of type I to type III collagen rarely returns to the levels found in a normal tendon, contributing to the high rate of re-injury [10]. Additional factors contributing to high injury (and re-injury) rates include the low vascularity of tendon and an inherently small population of tendon fibroblasts that are responsible for tendon homeostasis.

There is, therefore, a need for a therapeutic technology that promotes tendon regeneration, rather than repair with poor quality scar tissue. The term regeneration implies healing with a tissue identical to that of normal tendon. One such treatment option currently being studied in horses is the use of cells isolated from the stromal vascular fraction (SVF) of adipose tissue (adipose-derived cells (ADC)). These SVF cells presumably include a subpopulation of adult stem cells, are readily isolated from adipose tissue with minimal patient morbidity, and are ready for implantation in 48 hours following harvest of adipose tissue. The low morbidity and speed of processing represents a significant advantage over stem cells from

other tissues, such as bone marrow, which can take two to three weeks or longer to expand to clinically significant numbers [11].

Despite the lack of information regarding the contribution of SVF cells to equine flexor tendon healing, this heterogeneous mixture of cells is currently being used to treat clinical cases of soft-tissue injury [12, 13]. Nixon and colleagues found that treatment with SVF cells resulted in significantly better histological scores and collagen fiber alignment, as compared to PBS-injected control tendons using a collagenase-induced model of superficial digital flexor tendon (SDFT) injury [13]. Treatment with SVF cells also resulted in increased gene expression of cartilage oligomeric matrix protein (COMP), an important mediator of collagen fibrillogenesis [14]. Clinically, over 4,000 US horses suffering from tendon injuries have been treated with SVF technology, with 78% of those horses returning to their previous athletic endeavors [12, 15].

It has been suggested that adult stem cells have two possible mechanisms of action in the tendon healing process: differentiation into tendon fibroblasts and production of soluble factors, including growth factors and chemotactic agents [4, 16]. Differentiation of stem cells into tendon fibroblasts upon implantation into a healing tendon would increase the number of cells capable of contributing to the repair process. Production of chemotactic factors could recruit endogenous mesenchymal stem cells (MSCs) to the site of injury. Anabolic growth factor production could affect cell proliferation, cell differentiation, cell metabolism, and angiogenesis, as well as direct the synthesis of ECM proteins and up-regulate the expression of tendon-related genes.

Further study of the mechanism by which ADCs contribute to flexor tendon healing is warranted. The *in vitro* experiments reported in this thesis were designed to investigate the anabolic effects of ADCs on tendon healing and further our understanding of how ADCs might direct a regenerative response in healing flexor tendons. We hypothesized that ADCs are trophic mediators, capable of secreting anabolic growth factors (Objective #1) and chemotactic factors to recruit endogenous stem cells (Objective #2), and secreting soluble factors that increase gene expression of matrix proteins in tendon fibroblasts (Objective #3).

Chapter 1: Literature Review

1.1 Flexor Tendon Biology

The equine flexor tendon is exquisitely designed. Imagine a horse galloping across country, navigating large fences over varied terrain, or picture a horse executing a reining spin or a canter pirouette. The many athletic activities at which the horse excels are possible in part thanks to the nature of their tendons. Flexor tendons have many functions, including attaching muscle to bone, transferring force, offering support, storing energy, and absorbing shock [6, 17]. The flexor tendons are analogous to a spring that stores energy and then releases it. To propel a galloping horse, tendons must be exceedingly strong. This strength is based on several specific structural properties, namely cross sectional area, collagen content, and collagen fibril cross-linking and organization [1, 18]. Ultimately it is the combination of a strong, elastic, energy-storing tendon that makes the horse capable of wonderfully athletic feats.

When studying flexor tendons, one must consider the multiple levels of organization that begin with the smallest unit, the tropocollagen molecule, and gradually increase through collagen fibrils, collagen fibers, subfascicles, fascicles, and tertiary fiber bundles forming the tendon itself (Figure 1) [18]. This complex organizational hierarchy of tendon is difficult to recapitulate following tendon injury using traditional treatment methods. Tropocollagen is a right handed superhelix, 300 nm in length and 1.5 nm in diameter [1, 19]. Collagen fibrils are an aggregate of soluble tropocollagen molecules, cross-linked to form insoluble collagen molecules, and are the units of tensile strength [2, 18]. Type I collagen, the predominant type of collagen found in the tensile region of flexor tendons, is characterized by covalent cross-linking between its hydroxylysine and lysine residues [20]. The enzyme lysyloxidase acts on the lysine and

hydroxylysine residues to form cross-links in the telopeptide region of the collagen molecules, forming aldehydes that spontaneously react with residues in neighboring collagen molecules to form immature cross-links [21]. These immature cross-links undergo further spontaneous chemical reactions to form mature cross-links, namely hydroxylysyl-pyridinoline and pyrrole [21]. The strength afforded by these mature cross-links is critical to the suitability of horses for athletic endeavors. In fact, there is a positive correlation between the amount of pyrrole cross-linking and the ultimate strength and stiffness of a tendon [21]. Flexor tendon failure results in damaged or ruptured cross-links, decreasing the ultimate strength and elasticity of the tendon and negatively impacting the horse's athletic potential.

The process of collagen fibril formation occurs extracellularly via cleavage of terminal procollagen peptides by specific metalloproteinases [19]. Collagen molecules are exocytosed as triple helical procollagen, the propeptides are cleaved in the pericellular space, followed by self-assembly of the molecules into fibrils and the formation of covalent intermolecular cross-links [6]. Collagen molecules within the fibrils have a staggered arrangement, or periodicity, of 62 nm [1, 22]. This quarter stagger arrangement of the triple helical collagen molecules allows the molecules to slide past one another during loading and unloading, as well as allowing a large number of collagen molecules to be packaged into a small cross sectional area. The small cross sectional area is necessary for efficient locomotion.

The endotenon, a network of connective tissue, covers each fascicle [18]. The tendon itself is covered by the epitenon, a loose connective tissue sheath housing the vascular, lymphatic, and nerve supply, which is then covered by a final connective tissue covering called the paratenon [18].

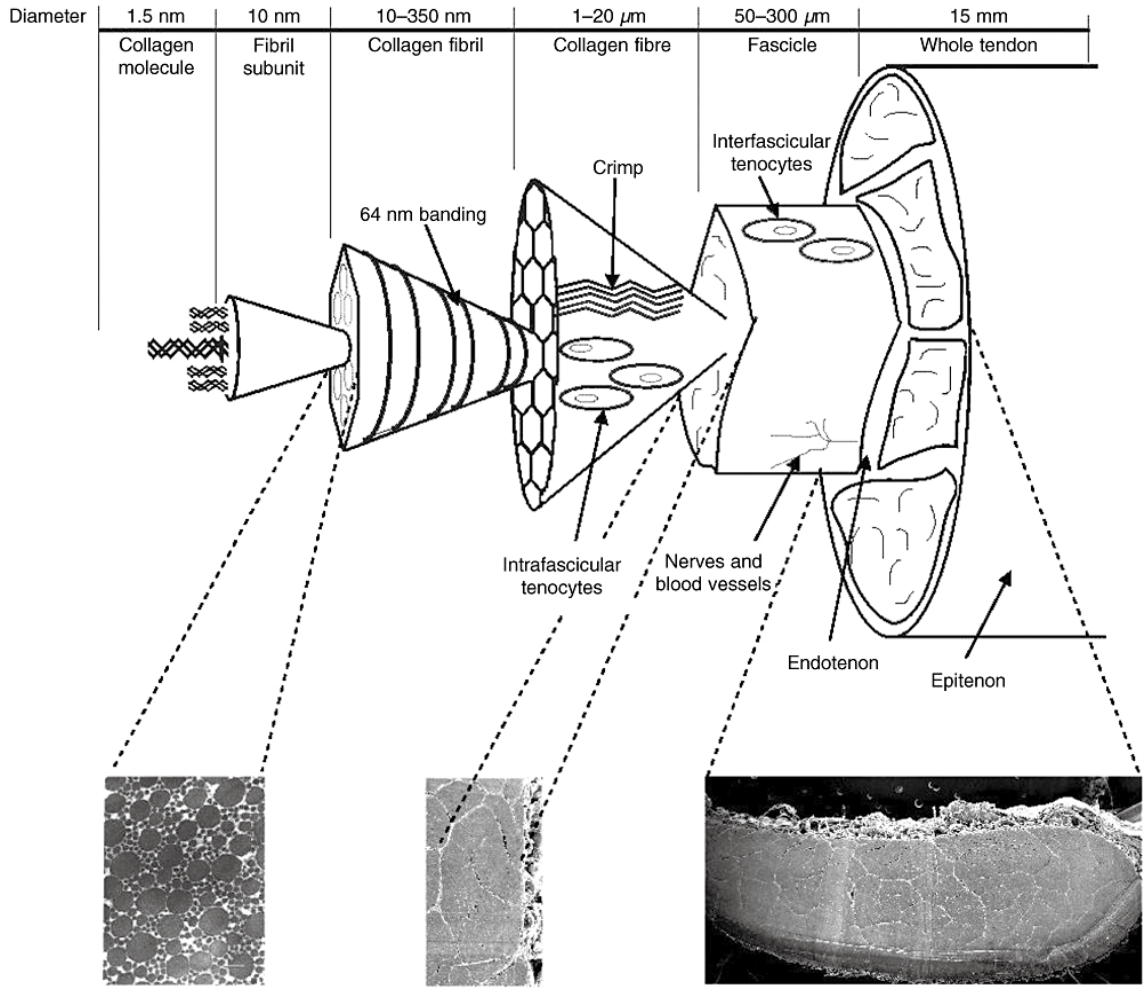

Figure 1. Representation of hierarchical structure of equine superficial digital flexor tendon [3]. Thorpe, C.T., P.D. Clegg, and H.L. Birch, *A review of tendon injury: why is the equine superficial digital flexor tendon most at risk?* Equine Vet J, 2010. **42**(2): p. 174-80. Used under fair use.

Flexor tendons receive their blood supply via perfusion proximally at the musculotendonous junction, distally at the osseous insertion, and via vessels originating in the endotenon [6]. The blood flow to flexor tendons is affected by age, exercise, and injury [23].

Microscopically, collagen fibers exhibit a phenomenon known as crimp. Crimp describes the wavy appearance of the collagen fibers that follow a zigzag pattern along the longitudinal tendon axis, seen as dark and light banding on picosirius red-stained tissues [6, 24, 25]. Crimp is only present when the tendon is unloaded, or when it is in the toe region of the stress-strain curve (less than 4% strain). The crimp straightens as the tendon is loaded, allowing the tendon to withstand higher stress without failing [24, 25]. As horses age, their tendons undergo a natural decrease in crimp angle. This decrease means that the collagen fibers straighten sooner during loading and the tendon experiences increased strains [24, 25]. Therefore, older horses may be more susceptible to flexor tendon injury.

The predominant differentiated cell type found in flexor tendons is the tendon fibroblast, or tenocyte [26]. Fibroblasts are mesenchymal in origin and are arranged in parallel with the long axis of the tendon, embedded in the collagenous fibrils they produce [18]. The appearance of the cells varies with the age and specific anatomic location of the tendon, but in general the cells have an oval or spindle-shaped nucleus. These cells actively produce proteins (including ECM proteins), as evidenced by the presence of an extensive rough endoplasmic reticulum and golgi apparatus [27, 28]. There are three phenotypic variations of fibroblasts described within the tendon parenchyma. Type I fibroblasts are thin with spindle-shaped nuclei, and are the predominant cell type in the tendons of aged horses [1, 27]. Type II cells have ‘cigar-shaped’ nuclei, and are most often seen in the tendons of young horses that have not yet reached skeletal maturity [1]. Type III fibroblasts are found in areas of compression, such as where a tendon

passes over a joint, and are more cartilage-like with round nuclei and visible nucleoli [1, 6]. The overall number of fibroblasts and their activity decrease with age as a result of cellular senescence, a contributing factor to the increased susceptibility of older horses to tendon injury [29]. Fibroblasts in the endotenon may coexist with a resident population of MSCs [30]. The exploitation of these MSCs to improve tendon healing is currently under investigation by multiple laboratories and their importance in the promotion of tendon regeneration over tendon repair will be discussed at length later in this review [16, 31-33].

Tendon fibroblasts have a low metabolic rate that decreases with increasing age of the horse [18]. This low metabolic rate, combined with the inherently low numbers of tendon fibroblasts, likely contributes to less than optimal healing after injury. Fibroblasts have the ability to generate energy anaerobically, which reflects their need to function during exercise, when decreased oxygenation to distal tissues prevents aerobic metabolism [18]. Fibroblasts using anaerobic processes to generate adenosine triphosphate may experience altered metabolic rates and as a result may be unable to perform their function of maintaining a balance between matrix synthesis and degradation [34]. On the other hand, it has been suggested that low metabolic rates after skeletal maturity may be normal and necessary [35]. High fibroblast metabolic rates would contribute to increased matrix remodeling, and constant matrix remodeling would weaken the tendon [35].

Tendon fibroblasts reside within the tendon matrix, or ECM, within and between collagen fascicles. They are characterized by cytoplasmic extensions that allow the formation of gap junctions, a vital method of communication that allows cells to coordinate biosynthesis and respond to mechanical stimulation [36]. Gap junctions also allow metabolites, ions, and small molecules (<1kDa) to pass between the sparsely placed tendon fibroblasts [37, 38]. Tendon

fibroblasts are responsible for synthesizing the components of the ECM: collagen, non-collagenous proteoglycans (PGs), and glycosaminoglycans (GAGs) [18]. Tendon is composed primarily of water (70% of the wet weight) [18]. The other major tendon component is collagen, (80% of the dry weight), the majority of which is type I with a minor component of type III collagen [1, 6]. Type III collagen is found mainly in the endotenon. The ratio of type III to type I collagen increases with age and injury [6]. The increase in type III collagen fibrils may decrease the mechanical strength of the tendon, due to the smaller diameter of the type III fibrils, and may, in part, explain the increased susceptibility of aged horses to tendon injury.

Proteoglycans are the predominant non-collagenous protein in tendon. They consist of a hyaluronic acid backbone with multiple aggregated protein cores with their associated sugar side chains (GAGs) [6]. Proteoglycans help maintain the structural integrity of tendons, as well as regulating ECM metabolism by binding to collagen via non-covalent cross-links [18]. Their hydrophilic nature, resulting from the negatively charged GAG side chains, assists in trapping water within the ECM, imparting a certain amount of resistance to the compression that occurs during locomotion and thus preventing damage to the ECM [6, 18]. The specific biochemical content of tendon varies with the anatomic location and the mechanical forces that predominate in that region. In the tensile region, the predominant GAG is dermatan sulphate, which accounts for 0.2% of the dry weight of the tendon [22]. In the sesamoid area, where the tendon contains more type II collagen, the predominant GAG is chondroitin sulfate [22].

Decorin, biglycan, fibromodulin, and lumican are small PGs characterized by having only one or two GAG side chains [6]. These PGs have structural and regulatory roles and bind other components of the ECM [6]. Decorin binds and coats type I collagen fibrils via electrostatic cross-links, and helps regulate collagen fibril diameter [39]. The small PGs are also known to

bind growth factors, such as transforming growth factor-β (TGF-β), which can assist in tendon homeostasis, adaptation, and response to injury via paracrine mechanisms [6]. Small PGs may sequester growth factors in the ECM, which are then available for release in response to injury, contributing to a 'regenerative microenvironment' [40]. The strength and elasticity of the tendon is partially dependent upon small PGs interacting with collagen fibrils [41].

Cartilage oligomeric matrix protein (COMP) is a pentameric glycoprotein that aids with the organization of the collagen network by influencing collagen fibrillogenesis [14]. Halasz and colleagues determined that COMP has a stimulatory effect on the formation of type I collagen fibrils by facilitating microfibril formation and the assembly of intermediate fibrillar structures [14]. By binding fibrillar collagens, such as types I and III, COMP helps the tendon resist load [6, 42, 43]. Levels of COMP in tendon decrease after two years of age, but may increase in response to mechanical load or exercise [35]. Another glycoprotein found in the ECM is tenascin-C, which is known to interact with other ECM proteins, including fibronectin, aggrecan, and versican, to modulate cell adherence to the ECM [19]. Tenascin-C also functions in modulating cell differentiation, migration, proliferation, and apoptosis. Tenascin-C is not expressed in normal adult tissues, but is expressed in pathological conditions and has been shown to be controlled by mechanical strain, up-regulated in tendonitis, and to play a critical role in collagen fiber alignment and orientation [19, 44, 45].

Just as there are control mechanisms for ECM production, there are mechanisms for ECM degradation and remodeling. Matrix metalloproteases (MMPs) are integral to tissue remodeling, repair, and inflammation, and control cell to cell, cell to ECM, and cell to growth factor interactions [19]. The bioavailability of growth factors sequestered in the ECM by PGs can be increased by the proteolytic activity of MMPs [19]. Expression of MMPs is low in the

resting state and elevated in the disease state [19, 46]. The activity of MMPs is tightly controlled, an obvious safeguard for maintaining the balance between anabolism and catabolism in the tendon ECM [19].

Understanding flexor tendon biology is requisite to understanding flexor tendon disease and how the disease affects the horse at the clinical level. By design, flexor tendons are prone to injury; however, their design also allows for the incredible athletic feats we ask our horses to perform. Those with a vested interest in equine welfare would benefit from a familiarity with tendon biology.

1.2 Flexor Tendon Disease

Through an appreciation for tendon biology, one is better able to understand the etiology of tendon disease and how to improve clinical outcomes. Flexor tendon injury is a common ailment of the performance horse and can be characterized as intrinsic injury (strain) or extrinsic injury (trauma). Injury is more often the result of intrinsic strain than the result of trauma [6]. In the case of intrinsic strain, the acute clinical lameness associated with tendon injury is preceded by subclinical degeneration of the tendon matrix, which can be bilateral [6]. Endogenous tendon fibroblasts are responsible for tendon homeostasis, but are sometimes unable to keep up with continuous microdamage due to the frequency of overuse episodes, as well as being hindered by inherently low cell numbers [2]. The result is disruption of covalent cross-linking between collagen fibrils and damage to the fibrils themselves, which alters the biomechanical integrity of the tendon. Over time, microdamage accumulates and, with continued loading, the tendon becomes damaged to the point that tendonitis becomes clinically apparent [1, 6].

Physical and mechanical parameters play a major role in tendon injury. The SDFT loads preferentially at the early stage of the horse's stride [1, 47]. Loading applies strain to the tendon; however, the amount of strain experienced depends on a number of factors: the specific activity being performed (over fences vs. flat work), the terrain, the rider's weight, and the speed at which the horse is traveling [6]. Foot conformation, age, exercise history, and physical fitness of the horse also affect tendon loading and strain [6]. Loading leads to stress relaxation of tendons, known as conditioning or creep [48, 49]. The amount of strain or stretch a tendon experiences depends on the force applied (loading), the properties of the tendon, and the cross sectional area [50]. Due to its small cross sectional area and lower yield strain, the mid-metacarpal region of the SDFT is more prone to lesions than the sesamoid region [51, 52].

In galloping horses, the SDFT operates close to its maximal strain limit, with strains of up to 16% having been recorded [1, 7, 8]. Flexor tendons fail at strains of between 12-20%; therefore, there is a narrow margin of safety when the horse is exercising at high speeds [1]. The strain experienced by flexor tendons can be described by a simplified stress-strain curve divided into four regions: toe, linear deformation, yield, and rupture [1]. The toe region represents 3-4% strain, or progressive loading of collagen fibers and relaxation of the crimp [53-55]. The linear region represents increasing strain, where crimp straightens as tensile force is transferred to adjacent overlapping fibrils [56]. This linear region can be used to calculate Young's modulus. As the strain or load continues to increase, the covalent cross-links between collagen fibrils reach their yield point and rupture, followed by rupturing of the collagen fibrils themselves, resulting in tendon failure [6, 57, 58].

In addition to high strain, flexor tendons in the galloping horse experience temperatures of 40 to 45°C [59]. Exposure of tendon fibroblasts to these temperatures *in vitro* resulted in the

synthesis of the pro-inflammatory cytokine tumor necrosis factor alpha, which in turn up-regulated production of MMPs [60]. An increase in MMP synthesis may lead to remodeling of the ECM, potentially weakening the tendon and predisposing the horse to microdamage and/or outright tendon failure. While it is conceivable that tendon fibroblasts can adapt to these temperatures without negative effects on cell viability, hyperemia may alter fibroblast synthetic activity or directly denature ECM proteins [6].

Changes in fibroblast synthetic activity with increasing age, as well as changes in ECM composition, predispose the tendon to microdamage, and may help explain the increased incidence of tendonitis with increasing age [50]. Stable inter- and intra-fibrillar cross-links may change over time, affecting the resistance of collagen to enzymatic digestion [1]. Increased levels of matrix-degrading MMPs have also been found in older tendons [50]. The SDFT is thought to reach maturity between two and three years of age in the horse based on the knowledge that collagen fibril diameter, crimp morphology, and mature collagen cross-links are stabilized by this age [24, 61]. In horses older than two years, an increase in stiffness and concomitant decrease in crimp angle may lead to the ability to withstand lower ultimate stress and strain [24]. Crimp angle is a safety mechanism of sorts, allowing tendons to withstand higher strains without failure. Collagen fibrils with a smaller crimp angle straighten sooner and thus fail at lower strains. In other words, tendons of skeletally mature horses may be more likely to fail at a lower strain [24]. There is, therefore, little room for adaptation through forced exercise, as continued loading in the adult horse leads to microdamage [6, 24].

Investigations into the effects of modern equine husbandry and training practices on parameters pertinent to normal tendon biochemistry have contributed to the understanding of how management practices may influence the frequency of tendon injuries. Cherdchutham and

colleagues found that foals allowed free pasture exercise had stronger, more elastic SDFTs with higher cellularity, GAG, hyaluronic acid, and COMP levels than foals confined to stalls or foals stall-rested and then subjected to forced exercise [62-64]. These studies led the authors to suggest that foals living on pasture, by exercising freely, were increasing ECM protein production and fibroblast cell numbers, thereby decreasing the incidence of tendon injury after skeletal maturity.

Tendonitis and tendon healing can be divided into four progressive and overlapping phases: subclinical injury, clinically apparent acute inflammatory, reparative, and remodeling. The subclinical phase, as previously discussed, is characterized by matrix degeneration, but is not accompanied by lameness or swelling. Birch and colleagues studied post-mortem SDFTs from horses not known to have clinical tendonitis, and found that the tendons exhibited macroscopic degeneration characteristic of subclinical tendonitis, including damaged collagen fibers, increased chondroitin sulfate content, and increased type III collagen in relation to type I collagen [65]. These findings indicated a change in tendon fibroblast synthesis of matrix components, particularly as the predominant GAG of the tensile region is dermatan sulfate, not chondroitin sulfate [22]. However, as chondroitin sulfate may promote fibrillogenesis, an increase in chondroitin sulfate levels may represent a beneficial response to injury [1]. It was proposed that microdamage to the matrix resulted in a loss of ECM integrity, thereby increasing the likelihood of clinical tendonitis [65].

When sufficient strain is encountered to cause partial or complete tendon failure, large numbers of collagen fibrils rupture and the tendon enters the inflammatory phase [66, 67]. It should be noted that inflammation is not thought to be a cause of flexor tendonitis in the horse, but rather is a response to acute injury. The inflammatory phase generally lasts for one to two

weeks or more after the onset of clinical disease, and is characterized by intratendinous hemorrhage, edema, heat, and the infiltration of leukocytes, including macrophages [6]. A platelet-containing blood clot forms at the site of injury, followed by platelet degranulation, which releases growth factors such as platelet derived growth factor BB (PDGF-BB), transforming growth factor-β (TGF-β), insulin like growth factor-I (IGF-I), and fibroblast growth factor-2 (FGF-2) [2, 68-70]. One of the many functions of growth factors is to stimulate the collagen synthesis and fibroblast proliferation that are necessary for tendon healing [18]. Fibrin protein in the blood clot cross-links with fibronectin to make a temporary matrix for cell attachment and migration [71].

The initial area of rupture, or lesion, may enlarge transiently through the action of proteases and collagenases, particularly MMP-13 and MMP-3, released by fibroblasts at the site of injury. Enlargement of the lesion removes damaged ECM and sets the stage for new collagen synthesis to fill the defect [2, 46]. Gene expression for collagen types I and III are up-regulated, and TGF-β1 expression increases, suggesting that fibroblasts modulate their gene expression profile in response to injury [9]. Gradually, as the inflammatory phase comes to a close, immune cells decrease in number and fibroblasts migrating from the epitenon and endotenon begin to infiltrate the site of injury, a hallmark of the reparative phase of healing [2].

The reparative phase begins a few days after injury, overlapping with the inflammatory phase, and peaking several months post-injury [6, 9, 72]. Type III collagen levels peak three to six weeks after initial injury [9]. The new tissue produced at this time has higher levels of water, fibronectin, and GAGs, and the collagen fibrils are randomly oriented [9, 10, 67, 73, 74]. Stall rest with controlled exercise is critical to decrease inflammation, prevent further damage, and promote parallel alignment of collagen fibrils [1]. Angiogenesis promotes accumulation of

fibroblasts within the damaged tissue by increasing the amount of vasculature available for cell migration. Fibroblasts then produce scar tissue, predominantly comprised of type III collagen [1]. As the scar tissue matures, fibroblasts exhibit higher levels of apoptosis [2]. Increased apoptosis could be a normal biological response, or it could represent an inappropriate biological response in which fibroblast numbers in the tendon are depleted to the point that the cells can no longer appreciably contribute to the healing process [2].

Remodeling begins several months after the onset of injury and can continue for 15-18 months or longer [6]. In normal tendon, the ratio of type I to type III collagen is 3:1 [10]. This ratio is altered post-injury and remains imbalanced following repair [10]. The increase in collagen type III results from an increase in fibroblast synthesis of this collagen in response to injury. The persistence of these smaller diameter type III collagen fibrils contributes to a mechanically weaker tendon [52, 67, 75, 76]. After levels of collagen type III peak, the amount of type III collagen decreases at the same time that the amount of type I collagen present increases [6, 10]. As remodeling progresses, collagen fibrils begin to align in the direction of stress, which might provide a biomechanical cue to the fibroblasts to increase synthesis of type I collagen and decrease synthesis of type III collagen [10]. Early in the healing process, low level loading via controlled exercise, such as hand walking, is critical for the promotion of correct fiber alignment [1].

Collagen fibril maturation during tendon remodeling may be controlled by GAGs [75]. It has been suggested that tendon fibroblasts alter the rate at which they synthesize GAGs in response to mechanical cues such as stress and that in turn the GAGs alter collagen fibril diameter [75]. Birch and colleagues found that the SDFT of horses subjected to high intensity exercise had lower levels of sulfated GAGs (chondroitin sulfate) than horses subjected to low

intensity exercise [76]. This finding differs from reports by the same laboratory for naturally occurring subclinical tendonitis [65]. The difference in these results is likely due to the difference in pathogenesis between naturally occurring tendonitis and tendonitis induced via forced exercise. If sulfated GAGs do indeed control fibrillogenesis, then a decrease in levels of sulfated GAGs during the reparative and remodeling phases of tendonitis would impair the creation of type I fibrils.

As the remodeling process begins to conclude, there is a reduction in collagen and GAG synthesis, cellularity, and vascularity [10, 73]. Collagen fibrils re-align along lines of stress, and the crimp pattern is re-established [10, 73]. The number of stable cross-links between collagen fibrils increases, causing an increase in tendon tensile strength [1]. 'Healed' tendons rarely regain their pre-injury biochemical or mechanical properties, contributing to a high incidence of re-injury [52, 77]. Decreased elasticity increases the chance of re-injury in adjacent areas [11]. As remodeling progresses, fibroblasts regain their typical spindle shape, which has been correlated with a concurrent decline in growth factor levels and cellular synthetic activity [9]. The time frame during which fibroblasts return to their basal metabolic rate may have critical effects on the quality of tendon repair and clinical outcome, including re-injury rate.

The high likelihood of SDFT re-injury necessitates the need for treatment options that promote recapitulation of normal tendon architecture. In racing Thoroughbreds, the incidence of SDFT injury is 8-43%, with 20-60% of those horses returning to their racing careers [78, 79]. However, up to 80% of those horses returning to racing will re-injure their SDFT [79, 80]. Treatment modalities are being investigated that aim to improve clinical outcomes by directing a regenerative response that mimics the events occurring during embryonic development.

Traditional treatments for flexor tendonitis include the application of ice to reduce inflammation, bandaging and poulticing to control edema, and confinement to minimize movement and prevent further injury. A controlled exercise program is developed by the attending veterinarian based on the results of serial ultrasound exams. Key parameters recorded during the ultrasound exams include tendon and lesion cross sectional area, the grade of severity of the lesion and its location, and most importantly, fiber alignment [80-82]. There is a fine line between the exercise necessary to promote a change from disorganized collagen fibrils to fibrils organized along lines of tension, and over-exercising and increasing the size of the lesion. An increase in cross sectional area during healing may indicate that exercise was increased too quickly and should be scaled back to avoid increasing the size of the tendon and prolonging the healing process [80]. Other traditional treatments that have been used to improve clinical outcomes with limited success include counterirritation, steroids, administration of polysulfated glycosaminoglycans, β-aminopropionitrile fumarate, and hyaluronic acid.

The goal of any treatment program should be to reduce inflammation during the acute phase and to improve collagen fiber alignment and other biomechanical properties during the reparative phase [83]. In light of the lack of improvement in tendonitis outcomes using traditional treatments, veterinarians have searched for other, non-traditional, treatment modalities to encourage regeneration.

1.3 Strategies for Tendon Healing – Growth Factors

The outcome of tendon healing is a repair process with highly variable results. The biological response to tendon injury depends on a number of factors, including the nature of the lesion, the response of the endogenous cell population, the presence of growth factors, and

environmental biomechanical cues, such as those from the ECM [84, 85]. Repair, as frequently results from traditional treatments, may not restore the original characteristics of the tissue. The goal of regenerative medicine is to return the injured tissue to a normal or disease-free state and ultimately restore structure and function [86, 87].

In order to recapitulate normal tendon architecture, researchers have sought to recreate aspects of embryonic tissue development using a combination of cells, bioactive proteins, and scaffolds [11]. The reparative phase of flexor tendon healing may be the ideal time to intervene with regenerative therapies, the idea being to direct a regenerative response before the development of biomechanically inferior scar tissue. If the goal is to direct the production of type I collagen over type III collagen and promote normal fibrillogenesis and the parallel alignment of collagen fibrils, then the ideal time for intervention may be when inflammation is subsiding and fibroblast proliferation and synthetic activity is increasing. Both MSCs and growth factors are being used clinically, and represent exciting new therapies for the treatment of flexor tendonitis.

Growth factors are signaling molecules that have the ability to influence a wide range of tissue effects including cell proliferation, ECM synthesis, and vascularization within healing tendon [88-92]. There is considerable overlap or redundancy in the functions of growth factors and in their cellular targets [93]. Interaction between growth factors in a synergistic or antagonistic manner can enhance or moderate a biological response [93, 94]. Growth factors exert their effects through three different signaling mechanisms or target cell populations: autocrine (same cell is the producer and the target), paracrine (neighboring cell is the target), and endocrine (cell in a remote location is the target). This multiplicity of signaling mechanisms allows growth factors to affect a variety of cell types and produce a diversity of responses [95].

One way in which growth factors are administered clinically is intralesionally as bone marrow aspirate or platelet rich plasma, both of which contain an assortment of growth factors [11]. Numerous reports documenting the beneficial effects of growth factors in promoting tendon regeneration exist in the literature [9, 91, 96-104].

Growth factor signaling is complex. Growth factors affect downstream changes in gene expression by binding to specific membrane receptors on target cells. Receptor binding induces an intracellular signal transduction pathway that, upon reaching the nucleus, initiates changes in gene expression and protein production, ultimately altering the biological response of the cell such as cell proliferation and differentiation and/or ECM production [95, 105-107]. There are multiple growth factors and cytokines considered important in tendon healing: TGF-β1 and -β3, FGF-2, IGF-I, PDGF-BB, and stromal cell derived factor-1α (SDF-1α).

Transforming growth factor-β belongs to a superfamily of related proteins bearing the same name, including activins, inhibins, bone morphogenetic proteins, growth and differentiation factors, and five isoforms of TGF-β [107, 108]. Proliferation and differentiation of MSCs, as well as ECM synthesis, can be influenced by TGF-β1 and -β3 [108]. Gene expression of collagen and other ECM proteins is up-regulated by TGF-β1 and -β3 [91, 109-112]. The ability of TGF-β family proteins to increase gene expression of matrix proteins may prove useful as a tool to promote tendon regeneration over tendon repair. High concentrations of several isoforms of TGF-β are normally found in bone, platelets, and cartilage, and TGF-β3 is found in healing wounds [108, 113, 114]. In the simplest sense, a tendon lesion is a wound and the infiltration of platelets during the acute phase is a likely source of TGF-β3.

Transforming growth factors -β1 and -β3 are important in tendon healing [9, 99, 100, 115-117]. Cell proliferation and the formation of collagen-like fibers in equine tendon fibroblasts *in vitro* were stimulated by TGF-β1 [118]. The ability of TGF-β1 to enhance cell proliferation and stimulate ECM production may, however, be a double edged sword. Overproduction of TGF-β1 in healing tendon has been implicated in scar tissue formation [119-121]. The administration of anti-TGF-β1 antibody increased postoperative flexor tendon range of motion after complete resection and repair of rabbit flexor digitorum profundus tendons [119]. The authors speculated that neutralizing TGF-β1 increased range of motion by limiting tendon adhesion formation [119]. Equine skin wounds exhibit reciprocal regulation of TGF-β1 and TGF-β3; when expression of one is high, expression of the other is low [113]. Elevating the local levels of TGF-β3 in relation to TGF-β1 may reduce scar tissue formation [122]. It appears then that TGF-β expression can be manipulated to promote the formation of type I collagen fibrils over the formation of the type III collagen fibrils that are characteristic of healing tendon [122].

Fibroblast growth factor has multiple isoforms that are produced by macrophages, MSCs, chondrocytes, and osteoblasts [108]. Fibroblast growth factor-2 has been shown to be important in tendon healing [102, 123]. Low levels of FGF-2 increased proliferation of human bone marrow-derived stem cells (BMSC) and up-regulated gene expression for collagen types I and III [102]. A study using canine flexor tendons fibroblasts demonstrated increased cell proliferation and collagen synthesis following culture with FGF-2 for 24 hours [123]. It is also known that FGF-2 upregulates granulation (scar) tissue formation, which would be detrimental with respect to clinical outcomes [124]. Other studies have found FGF-2 to be both up-regulated and down-

regulated in healing flexor tendons, depending on the model [117, 125]. These conflicting results suggest that more information is needed as to the exact role of FGF-2 in healing tendon.

Insulin like growth factor has two isoforms, IGF-I and IGF-II. Both are critical for proper skeletal development and fracture healing. Insulin like growth factor-I has received the most attention with respect to tendon healing. Osteoblasts and chondrocytes are sources of IGF-I, as well as the liver and tendon [96, 108, 126]. Insulin like growth factor-I regulates multiple biological functions, including cell growth and differentiation. It acts as a circulatory second messenger to growth hormone, exerting both paracrine and autocrine effects. The autocrine and paracrine effects of IGF-I are particularly important with respect to cartilage homeostasis [126]. Cartilage matrix degradation initiated by pro-inflammatory cytokines can be counteracted by the anabolic effects of IGF-I [126]. Insulin like growth factor-I also stimulates the production of collagen and GAGs [105, 127]. Avian flexor tendon cells express IGF-I at the message and protein levels, and IGF-I protein expression increases in response to trauma and mechanical load [96, 128, 129]. Insulin like growth factor-I mRNA was isolated from the tensile region of superficial digital flexor tendons, where it is presumably produced by tendon fibroblasts [130]. These findings indicate that IGF-I could be important in tendon healing, particularly since an increase in GAG production may influence collagen fibril diameter and fibrillogenesis [1, 75].

Insulin like growth factor-I can potentially improve tendon healing directly by binding cell surface receptors and inducing cell proliferation and ECM synthesis or indirectly by stimulating IGF-I production in neighboring cells [131]. In a collagenase-induced model of equine flexor tendonitis, IGF-I-treated tendons were characterized by increased DNA and total collagen (hydroxyproline) content, as well as decreased core lesion size on ultrasound [91]. Dahlgren and colleagues also found that IGF-I protein levels decreased in the first two weeks

after injury, then increased, peaking at about four weeks following injury [9]. Exogenous IGF-I administration undertaken within the first one to two weeks following injury could be used to supplement the naturally low endogenous levels present during that time period. Despite these encouraging findings using a model of flexor tendonitis, a subsequent retrospective study of Thoroughbred race horses treated with intralesional injection of IGF-I found no significant improvement in the prognosis for return to racing [132].

Insulin like growth factor-I has a short half-life and administration via transfected MSCs may provide an advantage over direct injection by providing sustained growth factor release [133]. Injection of BMSCs overexpressing IGF-I improved tendon histological scores as compared to untreated control tendons in a collagenase-induced model of equine flexor tendonitis. Interestingly, the results were not superior to injection of control BMSCs [134]. There was no difference in IGF-I gene expression between transfected and control BMSCs when the tendons were analyzed at eight weeks, suggesting that the lack of expected differences between transfected and control BMSCs was the result of a lack of continued up-regulation of IGF-I by the transfected cells. This conclusion is supported by the fact that adenovirus gene delivery typically results in upregulation for a four week period, and the fact that there was a significant difference in IGF-I gene expression between control and transfected cells immediately post-transfection. With further optimization this technology might be clinically viable [134].

Platelet derived growth factor has three isoforms: AA, BB, and AB. Platelet derived growth factor-BB is secreted by platelets and osteoblasts and is a component of bone marrow aspirate and platelet rich plasma [135]. It is a mitogen/chemoattractant for osteoblasts, fibroblasts, and MSCs, and has been studied for its effects on tendon healing [103, 104, 108].

Platelet derived growth factor-BB has been reported to increase proliferation and matrix synthesis by canine fibroblasts, and increase load to failure in an *in vivo* rabbit medial collateral ligament model [123, 136, 137]. A canine study using sustained release of PDGF-BB from a fibrin matrix delivery system found increased cell proliferation and matrix remodeling [138]. Only a modest stimulatory effect by PDGF-BB on equine tendon explants was reported as an increase in type I collagen gene expression after six days of culture in PDGF-BB [103]. In a rabbit tendon explant model, PDGF-BB increased the rate of DNA synthesis in a dose dependent manner, as measured by the uptake of radioactive thymidine [104]. A rat tendon healing model showed that exogenous administration of PDGF-BB seven days after injury increased the peak load, cross sectional area, and pyridinoline (a mature collagen cross-link used as a marker of collagen maturation) content of the tendons 14 days after injury [139]. More importantly, cells expressing the PDGF-BB receptor were found at the injury site six months later, indicating that healing tendon is capable of responding to PDGF-BB long term [139].

The stromal cell derived factor family contains many isoforms. Stromal cell derived factor-1α (also known as CXCL12) is a chemoattractant for MSCs, and as such, has generated interest in its potential role in regenerative medicine [140]. Stromal cell derived factor-1α and its receptor, CXCR4, play a role in the homing of circulating stem cells and in the engraftment of hematopoietic stem cells in ischemic tissue [141-143]. In a rat Achilles tendon injury model, exogenous SDF-1α in a silk-collagen scaffold reduced the influx of inflammatory cells and increased the influx of CXCR4-expressing fibroblasts [144]. Expression of collagen types I and III was up-regulated in the SDF-1α-treated group compared to the control group. The activity of SDF-1α may be controlled in part by MMPs [145, 146]. Activation of MMPs during the acute phase of tendon healing may increase SDF-1α expression by tendon fibroblasts. Since SDF-1α

is a chemoattractant for MSCs, this increased expression may in turn increase recruitment of MSCs from the endotenon.

Growth factors and cytokines have the potential to drive tendon regeneration and restore normal tendon architecture. Their administration as purified proteins, bone marrow aspirate, platelet rich plasma, or through genetically modified cells shows tremendous promise as a treatment for equine flexor tendonitis. The use of regenerative cell technologies alone, such as MSCs, may provide a means by which to exploit the favorable effects of growth factors on tendon healing by taking advantage of the capability of these stem cells to produce endogenous growth factors in a physiologic manner.

1.4 Strategies for Tendon Healing – Stem Cells

Mesenchymal stem cells are an exciting new regenerative therapy becoming available for the treatment of flexor tendonitis. These multipotent cells can be derived from many adult tissues and are generally harvested with limited patient morbidity [147-149]. While MSCs have been used clinically in veterinary medicine for the better part of a decade, there is limited scientific information to validate their use, specifically with respect to the mechanism by which they may improve clinical outcomes for flexor tendonitis. Stem cells are excellent candidates for regenerative medicine strategies because of their documented ability to exhibit trophic effects (produce soluble factors that have an indirect effect on other cells) [16]. These trophic effects can modulate chemotaxis, mitosis, and differentiation without the need for *in vitro* manipulation to direct differentiation of the stem cells [16]. The production of soluble factors also creates a local 'regenerative microenvironment' (Figure 2) [150].

Stem cells are undifferentiated progenitor cells of varying degrees of plasticity or potency. By definition, stem cells must be capable of undergoing many population doublings

without oncogenic (cancerous) transformation, and a single stem cell should be capable of creating more stem cells (clonogenicity) [151]. Generally, stem cells reside in a state of quiescence until intrinsic or extrinsic factors trigger self-renewal or differentiation [152]. Definitively designating cells as stem cells is complicated by the fact that cell surface markers differ between species and vary between tissues [153]. Using flow cytometry and polymerase chain reaction, stem cells from equine bone marrow and adipose tissue have been characterized as CD29, CD90 $^{+}$, CD34, CD44, CD45, CD73, and CD 105 $^{-}$ [154]. Conversely, other authors have characterized equine ADCs as being $CD44^{+}$ [155]. The negative results for certain markers may be due to a lack of antibody cross reactivity, rather than representing a truly negative result. Conflicting results may be due to differences in culture conditions used to propagate cells prior to analysis.

Stem cells can be harvested from adult or embryonic tissue. Embryonic stem cells (ESC) are derived from the inner cell mass of pre-implantation blastomeres, and are pluripotent, or capable of forming all three germ layers (mesoderm, ectoderm, and endoderm) [156]. High telomerase activity, as well as the expression of Oct4, Sox-2, Nanog, Rex-1, and SSEA-1, is typical of ESCs [157, 158]. Some researchers consider ESCs to be more useful than adult stem cells, which are only multipotent, or capable of differentiating into all cell types of the tissue from which they are derived [159, 160]. Selected work with ESCs has shown promise for various clinical applications (e.g. development of dopamine-making cells in an animal model of Parkinson's disease, and derivation of neural precursors and endothelial progenitor cells [161-164]). However, research developing ESCs for clinical use have generally been disappointing for a number of reasons [165].

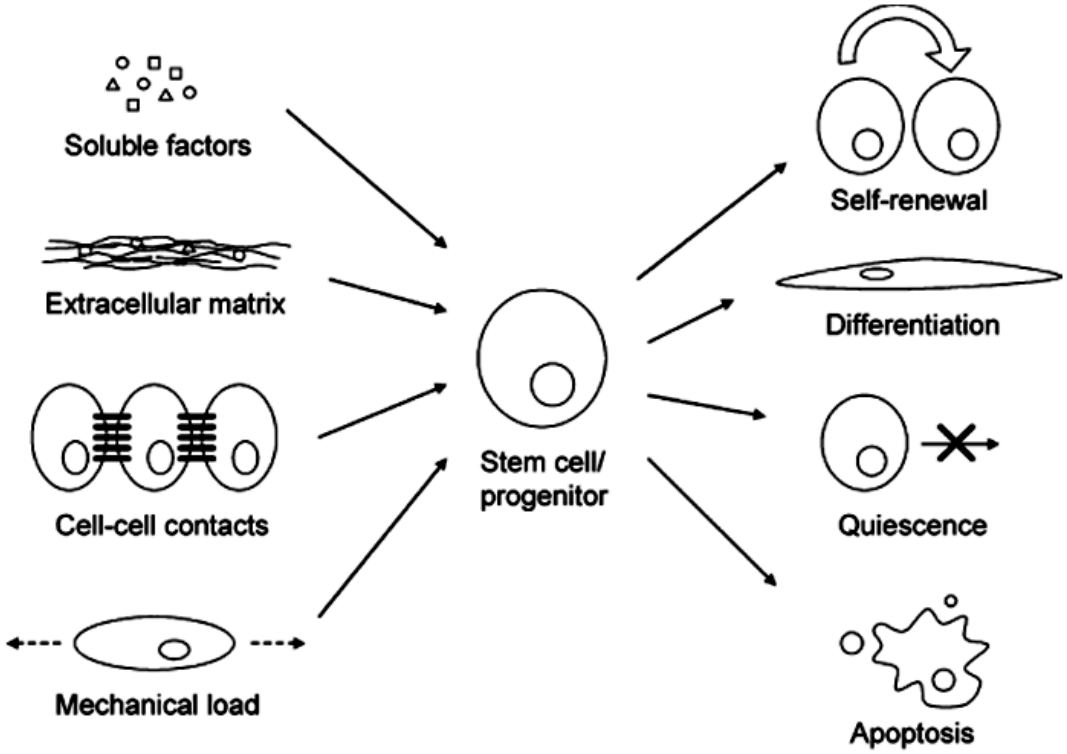

Figure 2. Stem cell interactions with various inputs from the microenvironment. Soluble factors, ECM, intercellular contacts, and biophysical forces synergize to influence stem cell fate. The plasticity of progenitor cells enables them to self-renew, differentiate, remain quiescent, or enter apoptosis [4].

Metallo, C.M., et al., *Engineering the stem cell microenvironment.* Biotechnol Prog, 2007. **23**(1): p. 18-23. Used under fair use.

There are several disadvantages to using ESCs for clinical applications. First, ESCs are derived from human embryos via a process that by necessity destroys the embryo. Many people feel that this is unethical, based on the idea that life begins at conception [166]. This ethical issue is of reduced importance with regard to equine embryos and probably does not preclude the use of ESCs in horses as a result. Second, ESCs may promote uncontrolled cell proliferation, raising concerns of oncogenic transformation after clinical application [156]. Upon injection into immunocompromised mice, ESCs form teratomas, one of the basic definitions of an ESC [167]. Third, immune rejection may be a concern when using ESCs, as all ESCs are by definition allogeneic. In contrast, adult stem cells can be autologous or allogeneic and have immunosuppressive properties [156, 168]. Lastly, ESCs may need to be cultured on feeder cells, a possible source of xenogeneic contamination [169].

Despite the challenges in bringing ESC technology to the clinics, several companies are developing equine ESC lines for the treatment of equine tendon and ligament injuries [156]. Lines of equine embryonic-like stem cells derived from inner cell mass dissection in the laboratory failed to form teratomas upon injection into mice, calling into question their pluripotency [170]. Equine embryonic-like stem cells injected into SDFT lesions did not form teratomas or initiate an immune response [171]. After 90 days these green fluorescent protein-labeled cells were found not only at the injection site but at other sites of injury, and the survival numbers were quite high compared to BMSCs [171]. If the above mentioned hurdles can be overcome, including the issues of oncogenicity and immune rejection, ESCs may be a viable allogeneic cell source for the treatment of equine tendon injuries.

Induced pluripotent stem cells (iPS) are similar to ESCs, but are created via nuclear reprogramming of adult somatic cells [156]. Adult cells are harvested and forced via retrovirus

transfection to express embryonic transcription factors, including some combination of Oct4, Sox2, Klf4, c-Myc, Nanog, and Lin28 [156, 172]. Expression of these factors changes the epigenetic state of the cell from differentiated to pluripotent [172]. There are many similarities between iPSs and ESCs, including morphology, cell surface markers, and epigenetic state [172]. Induced pluripotent stem cells have the potential to be derived from an autologous cell source. This is an advantage over ESCs and the main difference between the two cell types. Induced pluripotent stem cells are, however, not currently available for use in regenerative medicine as retroviral delivery of reprogramming factors can cause tumor formation upon implantation [172]. New transfection techniques using small interfering RNAs may make retroviral delivery obsolete and move iPS technology closer to the clinic [173].

Considering the technical and ethical hurdles associated with the use of ESCs and iPSs, work in the field of tendon regeneration has focused on the use of adult stem cells. Adult stem cells are derived from non-embryonic tissues and should be capable of generating all the endogenous differentiated cell types found in the tissue from which they are derived [160]. Sources of multipotent adult stem cells include mesenchymal tissues such as bone marrow, adipose tissue, and umbilical cord blood. Cells from these sources can be harvested with limited morbidity, and unlike ESCs, are not associated with ethical issues [166]. They can be differentiated into adipose tissue, bone, cartilage, muscle, and neural tissue [147-149, 174]. Although adult stem cells have historically been considered multipotent, gene expression for Oct4 and Nanog was recently confirmed in equine ADCs, suggesting that adult stem cells may have greater plasticity than originally reported [175].

Adult stem cells are active during growth and development, but are generally quiescent thereafter [153]. Adult stem cells can be activated in response to injury or disease, and finding

ways to force them out of quiescence is a major hurdle to their usefulness in regenerative medicine. Mechanical signals are critical mediators of adult stem cell activity and differentiation, including apoptosis [153, 176-179]. Ultimately a combination of biochemical and mechanical signals probably controls stem cell fate.

For use in regenerative medicine techniques, adult stem cells should ideally be available in large numbers, easily harvested with limited morbidity, capable of differentiation along several pathways, and safe for use in an autologous or allogeneic manner [180]. In equine flexor tendons, the niche or home for these cells is thought to be the endotenon [181]. Ongoing research is investigating the use of MSCs derived from hind limb lateral digital extensor tendons to treat flexor tendon injuries [33, 182]. Currently, extensor tendons are used as the techniques available to harvest progenitor cells from flexor tendons result in further tendon damage, which is obviously undesirable. Tendon-derived MSCs may, in theory, be better able to stimulate tendon regeneration, as these cells are 'programmed' to withstand the cyclical loading and other biomechanical cues characteristic of the tendon ECM [33]. Theoretically, cells from sources other than tendon would be less desirable for tendon regeneration based on phenotypic and biochemical differences resulting from the niche from which they were isolated. However, because cells are capable of responding to their environment, the source of the cell may be of lesser importance than its ability to differentiate.

The use of tendon progenitor cells is still an investigative therapy, as a clinically appropriate source for these cells has yet to be identified. Clinical treatment of tendon injuries has focused on the use of more heavily researched MSCs from other sources, including bone marrow and adipose tissue. Equine MSCs cultured in a bioreactor with bone marrow supernatant synthesized a well-organized matrix, indicating the ability of these cells to contribute to ECM

renewal [159]. It has also been hypothesized that MSCs can contribute to healing via differentiation into tissue-specific cell types. This differentiation is controlled by the mechanical environment, soluble growth factors, the ECM, and contact with the native cell population [159]. When cultured on acellular equine flexor tendon matrix, MSCs began to align with the collagen fascicles [183]. In addition, COMP expression was up-regulated and collagen expression was down-regulated, as compared to a two dimensional culture system [183]. Equine bone marrow progenitor cells labeled with green fluorescent protein and injected into manually created SDFT lesions were detected within the lesions 34 days later. The presence of progenitor cells in healthy areas indicated less than 100% retention at the site of injury due to cell migration [184]. Sheets of human BMSCs cultured on frozen rabbit tendon grafts integrated into the tendon tissue and exhibited fibroblastic characteristics [185].

Despite evidence that MSCs do differentiate into tendon fibroblasts, it has been suggested that the primary mechanism by which stem cells contribute to tendon healing is via the indirect production of soluble factors that then orchestrate the healing response [33, 186]. Secretion of bioactive molecules (e.g. growth and chemotactic factors) instructs endogenous cells to participate in the healing process via autocrine and paracrine mechanisms [16, 150].

Evidence for the trophic capabilities of MSCs abounds. Mesenchymal cells nourish hematopoietic stem cells and can support full hematopoietic lineage progression *in vitro* [187]. When introduced into rat brains damaged by stroke, MSCs were shown to improve coordinated function, purportedly by inhibiting scar formation and apoptosis, increasing angiogenesis, and stimulating endogenous neural cells [188, 189]. These beneficial results were not associated with the differentiation of the MSCs into neural cells and therefore resulted from the MSCs acting in an indirect manner. Walter and colleagues showed that MSC-conditioned medium

caused fibroblasts to accelerate skin wound healing in a scratch assay [190]. Proliferation of the fibroblasts did not change; therefore, the accelerated wound healing was due to the presence of soluble factors in the MSC-conditioned medium having an indirect effect on the fibroblasts [190]. Other *in vitro* experiments have shown that MSC-conditioned medium supports endothelial cell migration and proliferation, as well as cardiac myocyte survival, and that MSCs condition their medium with soluble factors in a manner consistent with their trophic nature [191-198].

In addition to their trophic abilities, MSCs may play an immunomodulatory role in tendon healing [168]. Soluble factors secreted by MSCs have been shown to modulate both the innate and adaptive arms of the immune response, for example by blocking dendritic cell activation [168]. Mesenchymal stem cells lack expression of major histocompatibility complex class II molecules and T cell co-stimulatory molecules, including CD80, CD86, and CD40. It is therefore plausible that one positive effect of MSCs on tendon healing may be reduction of acute-phase inflammation by blocking T cell activation, which in turn might down-regulate the proteases and collagenases that enlarge the initial lesion. In addition, if MSCs do indeed possess immune modulating capabilities, there is an increased likelihood of the safe use of allogeneic stem cells to treat tendon injuries. The ability to use MSCs in an allogeneic manner would be beneficial in cases where the morbidity associated with cell harvest is unacceptable.

Stem cells can be isolated from umbilical cord blood at the time of foaling with the assistance of trained personnel to draw the blood. There is no morbidity to either the mare or foal and cord blood stem cell banking is commercially available [199]. However, the process of collecting the blood is not without technical difficulties, and sterility is a major concern [200, 201]. Umbilical cord blood stem cells may be more plastic than stem cells from other adult

tissues [202]. They can be differentiated into osteogenic, chondrogenic, and adipogenic lineages, but can also form hepatocytes [202, 203]. The most compelling evidence for their increased plasticity is the expression of Oct4, a protein marker for ESCs [202]. Stem cells have also been isolated from human umbilical cord blood and were shown to express bone, adipose tissue, and neural markers [204]. Like other non-embryonic stem cells, these cells represent a heterogeneous population of cells. In humans, adherent umbilical cord blood cells can be mesenchymal-like or osteoclast-like [205]. Both hematopoietic and mesenchymal progenitors can be harvested from umbilical cord blood, although hematopoietic cells predominate. Mesenchymal umbilical cord blood stem cells have been the focus of regenerative medicine research [206, 207]. Clinical trials investigating the use of umbilical cord blood stem cells for tissue engraftment, prevention of graft vs. host disease, treatment of osteoporosis, and treatment of articular cartilage injury are ongoing, while recent work has focused on the use of umbilical cord blood cells for cardiovascular therapy [208, 209].

Stem cells from multiple tissues are currently being used to treat tendon and cartilage injuries in the horse, including cells from bone marrow [15, 31, 32]. The use of BMSCs is supported by work in research animals showing an anabolic effect of BMSCs on tendon and ligament healing [210-212]. In a caprine model of induced osteoarthritis of the carpal joint, a single injection of BMSCs improved meniscal healing and retarded articular cartilage degeneration compared to controls [213]. In the horse, favorable results have been reported for re-injury rates after injection of BMSCs, as compared to traditional treatments [31]. Godwin and colleagues examined data from racehorses suffering from flexor tendonitis that were treated with BMSCs [32]. The horses were followed for a minimum of two years after return to full work and the percentage of horses suffering re-injury was compared to published data using other

treatment methods. The overall re-injury percentage (27.4%) was significantly lower than re-injury rates after treatment with traditional methods, providing further support for the clinical use of BMSCs to treat flexor tendonitis. Conversely, Caniglia and colleagues found that injection of BMSCs into surgically created SDFT core lesions had no effect on collagen fibril diameter, the parameter the authors had chosen as a measure of matrix quality [214]. The authors suggested that BMSCs do not promote matrix regeneration over matrix repair [214].

Equine BMSCs are aspirated from the sternum or tuber coxae under sedation [11]. When aspirating from the sternum care must be taken to avoid puncturing the heart, or the intersternebral space, which could cause the loss of negative pressure in the lungs, lung collapse, and death [11]. The aspiration procedure is also somewhat painful, and there is a risk of donor site hemorrhage and infection [215]. Because of these safety concerns, some veterinarians perform bone marrow aspiration only under general anesthesia; however, in the majority of cases it is safely done standing under sedation with the use of local anesthetic [216].

The frequency of BMSCs in aspirates from equine patients is approximately one in $4.2x10^3$ cells [217]. Bone marrow aspirate is thought to contain a heterogeneous population of nucleated cells, including hematopoietic cells and BMSCs [11]. The aspirate can be injected directly into the tendon lesion, subjected to density gradient centrifugation to increase the number of mononuclear cells, or cultured for several weeks to increase the number of stem cells [11]. Injection into the injured tendon immediately following harvest is possible with the use of pure bone marrow aspirate and concentrated bone marrow aspirate. These stall-side methods represent an advantage over ADCs, which have a 48 hour turnaround time between harvest and injection. The use of bone marrow aspirate carries the risk of mineralization at the injection site [11, 33]. On the other hand, while a culture period does increase the number and purity of

BMSCs, a delay of several weeks before injection may be a significant disadvantage, as scar tissue will have already begun to form in the healing tendon [6]. There is considerable disagreement as to the ideal time frame for implantation, with some researchers suggesting it should be performed between one and two weeks after the initial injury (after the inflammatory phase but before scar tissue begins to form), which is not possible when using BMSCs, while others suggest that implantation into scar tissue promotes stem cell viability and retention at the site of injury [11, 31, 32]. Other disadvantages of BMSCs are the small number of stem cells obtained from bone marrow aspirate and the potential for infection and localized acute inflammation from contaminated products [11, 33].

Adipose-derived cells are also being used to treat equine tendon and cartilage injuries [15, 33]. It has been suggested that ADCs are superior to BMSCs for tissue banking purposes, as BMSCs reach senescence after 30 or so population doublings *in vitro*, while ADCs undergo 60-80 doublings before reaching senescence [218]. Adipose-derived cells have been shown to differentiate into bone, cartilage, and adipose tissue [147-149]. Differentiation into a tendon fibroblast lineage has not yet been proven due to the lack of tenocyte-specific markers [147, 159, 219]. As mentioned previously, cell surface characterization is limited by the number of antibodies that cross react with equine tissues and depends in part on the stem cell environment (niche) and tissue culture conditions [152]. The development of equine specific reagents will allow for the complete characterization of equine ADCs.

The rights to the commercial veterinary application of ADC technology are owned by Vet-Stem, Inc. As marketed by Vet-Stem, Inc., the SVF cells are referred to as Vet Stem Regenerative Cells (VSRC). For clinical application, harvest of adipose tissue is performed in the standing horse under sedation and local anesthesia and is associated with minimal morbidity

[13]. The paraxial caudodorsal gluteal region is clipped and aseptically prepared bilaterally, followed by an injection of local anesthetic. Adipose tissue is then harvested aseptically via a surgical incision in the groove between the biceps femoris and semitendinosus muscles. An average of 20-30 grams of adipose tissue is harvested, with cell yields averaging 450,000 cells per gram of tissue, and cell viability ranging from 85-90% [12]. Following harvest, the adipose tissue is shipped overnight to Vet-Stem, Inc. where it is subjected to enzymatic digestion and cell isolation via centrifugation [13, 148, 220]. This process produces the SVF cells, or VSRCs, a heterogeneous population of the nucleated cells from the adipose tissue. The VSRC pellet consists of fibroblasts, mesenchymal stem cells, endothelial cells, mononuclear cells, neutrophils, lymphocytes, pericytes, eosinophils, and monocytes [220].

The cells are resuspended in physiologic solution containing antibiotics and shipped via overnight courier to the referring veterinarian with 48 hours of adipose tissue harvest. The VSRCs are then injected intralesionally under ultrasound guidance. Over 4,000 equine tendon injuries have been treated with VSRCs in the US, with 78% of sport horses reaching their previous level of work and 69% of race horses racing at least once [12, 15]. These regenerative cells are very safe to use, with a reported adverse event record of 0% systemic reactions and less than 0.5% local tissue reactions [221].

Vet Stem Regenerative Cells have been shown to differentiate along bone, adipose tissue, and cartilage pathways, and were positive for the cell surface markers CD44, CD90, CD133, and ABCG2 [220]. The results for surface markers are in agreement with the findings of Carvalho and colleagues, but differ from the findings of Ranera and colleagues [154, 155]. These differences are likely due to variables introduced during harvest and *in vitro* culture. The

positive result for the stem cell marker ABCG2 is significant as it supports the presence of cells of mesenchymal origin in the VSRC pellet [220].

A preliminary study using VSRCs to treat collagenase-induced SDFT lesions in the horse showed a significant improvement in histological scores compared to controls [13]. Specifically, tendons treated with the VSRCs showed improved fiber organization (uniformity and alignment) and fewer white blood (inflammatory) cells than controls treated with phosphate buffered saline. In addition, treatment with VSRCs resulted in increased gene expression for COMP. These results support the clinical use of ADC technology for treatment of tendonitis in the horse.

Despite the current clinical application of stem cell technology for tendon healing, there are several important questions that still remain to be answered. Which source of stem cells is best suited for promoting tendon regeneration? How many cells should be injected? What is the ideal solution for resuspension of the stem cells? Do stem cells incorporate into the native milieu or do they exert their effects through recruitment of other cells and secretion of growth factors? In pursuit of the answers to these questions, we have designed experiments to begin to examine the various purported mechanisms by which adipose-derived cells may contribute to tendon healing.

References

1. Goodship, A.E., H.L. Birch, and A.M. Wilson, *The pathobiology and repair of tendon and ligament injury.* Vet Clin North Am Equine Pract, 1994. **10**(2): p. 323-49.
2. Patterson-Kane, J.C. and E.C. Firth, *The pathobiology of exercise-induced superficial digital flexor tendon injury in Thoroughbred racehorses.* Vet J, 2009. **181**(2): p. 79-89.
3. Thorpe, C.T., P.D. Clegg, and H.L. Birch, *A review of tendon injury: why is the equine superficial digital flexor tendon most at risk?* Equine Vet J, 2010. **42**(2): p. 174-80.
4. Metallo, C.M., et al., *Engineering the stem cell microenvironment.* Biotechnol Prog, 2007. **23**(1): p. 18-23.
5. Marr, C.M., et al., *Factors affecting the clinical outcome of injuries to the superficial digital flexor tendon in National Hunt and point-to-point racehorses.* Vet Rec, 1993. **132**(19): p. 476-9.
6. Smith, R.K.W., *Pathophysiology of Tendon Injury*, in *Diagnosis and management of lameness in the horse*, M.W. Ross, Dyson, S.J., Editor. 2011, Elsevier Saunders: St. Louis. p. 694-706.
7. Stephens, P.R., D.M. Nunamaker, and D.M. Butterweck, *Application of a Hall-effect transducer for measurement of tendon strains in horses.* Am J Vet Res, 1989. **50**(7): p. 1089-95.
8. Riemersma, D.J. and H.C. Schamhardt, *In vitro mechanical properties of equine tendons in relation to cross-sectional area and collagen content.* Res Vet Sci, 1985. **39**(3): p. 263-70.
9. Dahlgren, L.A., H.O. Mohammed, and A.J. Nixon, *Temporal expression of growth factors and matrix molecules in healing tendon lesions.* J Orthop Res, 2005. **23**(1): p. 84-92.

10. Watkins, J.P., et al., *Healing of surgically created defects in the equine superficial digital flexor tendon: effects of pulsing electromagnetic field therapy on collagen-type transformation and tissue morphologic reorganization.* Am J Vet Res, 1985. **46**(10): p. 2097-103.

11. Fortier, L.A. and R.K. Smith, *Regenerative medicine for tendinous and ligamentous injuries of sport horses.* Vet Clin North Am Equine Pract, 2008. **24**(1): p. 191-201.

12. Dahlgren, L.A. *Fat-Derived Mesencymal Stem Cells for Equine Tendon Repair*. in *Veterinary Stem Cell Consortium* 2009. Leipzig, Germany: Regenerative Medicine Supplement 2.

13. Nixon, A.J., et al., *Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis.* Am J Vet Res, 2008. **69**(7): p. 928-37.

14. Halasz, K., et al., *COMP acts as a catalyst in collagen fibrillogenesis.* J Biol Chem, 2007. **282**(43): p. 31166-73.

15. Harman, R., Cowles, B., Orava, C., et al., *A Retrospective Study of 66 Cases of Tendon Injury in the Equine Treated with Adipose-Derived Stem and Regenerative Cell Therapy*. 2006, Vet-Stem Inc.

16. Caplan, A.I. and J.E. Dennis, *Mesenchymal stem cells as trophic mediators.* J Cell Biochem, 2006. **98**(5): p. 1076-84.

17. Wilson, A.M., et al., *Horses damp the spring in their step.* Nature, 2001. **414**(6866): p. 895-9.

18. Sharma, P. and N. Maffulli, *Tendon injury and tendinopathy: healing and repair.* J Bone Joint Surg Am, 2005. **87**(1): p. 187-202.

19. Bosman, F.T. and I. Stamenkovic, *Functional structure and composition of the extracellular matrix.* J Pathol, 2003. **200**(4): p. 423-8.

20. Woo, S.L. and J.A. Buckwalter, *AAOS/NIH/ORS workshop. Injury and repair of the musculoskeletal soft tissues. Savannah, Georgia, June 18-20, 1987.* J Orthop Res, 1988. **6**(6): p. 907-31.

21. Thorpe, C.T., et al., *Mechanical properties of the equine superficial digital flexor tendon relate to specific collagen cross-link levels.* Equine Vet J, 2010. **42 Suppl 38**: p. 538-43.

22. Gillard, G.C., et al., *The proteoglycan content and the axial periodicity of collagen in tendon.* Biochem J, 1977. **163**(1): p. 145-51.

23. Jones, A.J., *Normal and diseased equine digital flexor tendon: blood flow, biochemical and serological studies*. 1993, University of London.

24. Patterson-Kane, J.C., et al., *Age-related differences in collagen crimp patterns in the superficial digital flexor tendon core region of untrained horses.* Aust Vet J, 1997. **75**(1): p. 39-44.

25. Wilmink, J., A.M. Wilson, and A.E. Goodship, *Functional significance of the morphology and micromechanics of collagen fibres in relation to partial rupture of the superficial digital flexor tendon in racehorses.* Res Vet Sci, 1992. **53**(3): p. 354-9.

26. Hosaka, Y., et al., *Mechanism of cell death in inflamed superficial digital flexor tendon in the horse.* J Comp Pathol, 2005. **132**(1): p. 51-8.

27. Dowling, B.A., et al., *Superficial digital flexor tendonitis in the horse.* Equine Vet J, 2000. **32**(5): p. 369-78.

28. Dahlgren, L.A., B.D. Brower-Toland, and A.J. Nixon, *Cloning and expression of type III collagen in normal and injured tendons of horses.* Am J Vet Res, 2005. **66**(2): p. 266-70.

29. Ippolito, E., et al., *Morphological, immunochemical, and biochemical study of rabbit achilles tendon at various ages.* J Bone Joint Surg Am, 1980. **62**(4): p. 583-98.

30. Salingcarnboriboon, R., et al., *Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property.* Exp Cell Res, 2003. **287**(2): p. 289-300.

31. Smith, R.K., *Mesenchymal stem cell therapy for equine tendinopathy.* Disabil Rehabil, 2008. **30**(20-22): p. 1752-8.

32. Godwin, E.E., et al., *Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon.* Equine Vet J, 2012. **44**(1): p. 25-32.

33. Alves, A.G., et al., *Cell-based therapies for tendon and ligament injuries.* Vet Clin North Am Equine Pract, 2011. **27**(2): p. 315-33.

34. Birch, H.L., G.A. Rutter, and A.E. Goodship, *Oxidative energy metabolism in equine tendon cells.* Res Vet Sci, 1997. **62**(2): p. 93-7.

35. Smith, R.K., et al., *The influence of ageing and exercise on tendon growth and degeneration--hypotheses for the initiation and prevention of strain-induced tendinopathies.* Comp Biochem Physiol A Mol Integr Physiol, 2002. **133**(4): p. 1039-50.

36. McNeilly, C.M., et al., *Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions.* J Anat, 1996. **189 (Pt 3)**: p. 593-600.

37. Goodenough, D.A. and D.L. Paul, *Beyond the gap: functions of unpaired connexon channels.* Nat Rev Mol Cell Biol, 2003. **4**(4): p. 285-94.

38. Goldberg, G.S., P.D. Lampe, and B.J. Nicholson, *Selective transfer of endogenous metabolites through gap junctions composed of different connexins.* Nat Cell Biol, 1999. **1**(7): p. 457-9.

39. Brown, D.C. and K.G. Vogel, *Characteristics of the in vitro interaction of a small proteoglycan (PG II) of bovine tendon with type I collagen.* Matrix, 1989. **9**(6): p. 468-78.

40. Yamaguchi, Y., D.M. Mann, and E. Ruoslahti, *Negative regulation of transforming growth factor-beta by the proteoglycan decorin.* Nature, 1990. **346**(6281): p. 281-4.

41. Ezura, Y., et al., *Differential expression of lumican and fibromodulin regulate collagen fibrillogenesis in developing mouse tendons.* J Cell Biol, 2000. **151**(4): p. 779-88.

42. Hedbom, E., et al., *Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage.* J Biol Chem, 1992. **267**(9): p. 6132-6.

43. Morgelin, M., et al., *Electron microscopy of native cartilage oligomeric matrix protein purified from the Swarm rat chondrosarcoma reveals a five-armed structure.* J Biol Chem, 1992. **267**(9): p. 6137-41.

44. Mehr, D., et al., *Tenascin-C in tendon regions subjected to compression.* J Orthop Res, 2000. **18**(4): p. 537-45.

45. Mackie, E.J. and S. Ramsey, *Expression of tenascin in joint-associated tissues during development and postnatal growth.* J Anat, 1996. **188 (Pt 1)**: p. 157-65.

46. Clegg, P.D., S. Strassburg, and R.K. Smith, *Cell phenotypic variation in normal and damaged tendons.* Int J Exp Pathol, 2007. **88**(4): p. 227-35.

47. Riemersma, D.J., et al., *Tendon strain in the forelimbs as a function of gait and ground characteristics and in vitro limb loading in ponies.* Equine Vet J, 1996. **28**(2): p. 133-8.

48. Majima, T., et al., *Biomechanical effects of stress shielding of the rabbit patellar tendon depend on the degree of stress reduction.* J Orthop Res, 1996. **14**(3): p. 377-83.

49. Atkinson, T.S., B.J. Ewers, and R.C. Haut, *The tensile and stress relaxation responses of human patellar tendon varies with specimen cross-sectional area.* J Biomech, 1999. **32**(9): p. 907-14.

50. Birch, H.L., *Tendon matrix composition and turnover in relation to functional requirements.* Int J Exp Pathol, 2007. **88**(4): p. 241-8.

51. Crevier, N., et al., *Segmental variations of in vitro mechanical properties in equine superficial digital flexor tendons.* Am J Vet Res, 1996. **57**(8): p. 1111-7.

52. Crevier-Denoix, N., et al., *Mechanical properties of pathological equine superficial digital flexor tendons.* Equine Vet J Suppl, 1997(23): p. 23-6.

53. Evans, J.H. and J.C. Barbenel, *Structural and mechanical properties of tendon related to function.* Equine Vet J, 1975. **7**(1): p. 1-8.

54. Woo, S.L., *Mechanical properties of tendons and ligaments. I. Quasi-static and nonlinear viscoelastic properties.* Biorheology, 1982. **19**(3): p. 385-96.

55. Cribb, A.M. and J.E. Scott, *Tendon response to tensile stress: an ultrastructural investigation of collagen:proteoglycan interactions in stressed tendon.* J Anat, 1995. **187 (Pt 2)**: p. 423-8.

56. Birk, D.E., Silver, F.H., Trelstad, R.L., *Matrix Assembly*, in *The Cell Biology of the Extracellular Matrix*, E.D. Hay, Editor. 1991, Academic Press: New York. p. 221-254.

57. Pins, G.D., et al., *Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties.* Biophys J, 1997. **73**(4): p. 2164-72.

58. Kotha, S.P. and N. Guzelsu, *Tensile damage and its effects on cortical bone.* J Biomech, 2003. **36**(11): p. 1683-9.

59. Wilson, A.M. and A.E. Goodship, *Exercise-induced hyperthermia as a possible mechanism for tendon degeneration.* J Biomech, 1994. **27**(7): p. 899-905.

60. Hosaka, Y., et al., *Effect of heat on synthesis of gelatinases and pro-inflammatory cytokines in equine tendinocytes.* Biomed Res, 2006. **27**(5): p. 233-41.

61. Patterson-Kane, J.C., et al., *Comparison of collagen fibril populations in the superficial digital flexor tendons of exercised and nonexercised thoroughbreds.* Equine Vet J, 1997. **29**(2): p. 121-5.

62. Cherdchutham, W., et al., *Age-related changes and effect of exercise on the molecular composition of immature equine superficial digital flexor tendons.* Equine Vet J Suppl, 1999(31): p. 86-94.

63. Cherdchutham, W., et al., *Effects of exercise on the diameter of collagen fibrils in the central core and periphery of the superficial digital flexor tendon in foals.* Am J Vet Res, 2001. **62**(10): p. 1563-70.

64. Cherdchutham, W., et al., *Effects of exercise on biomechanical properties of the superficial digital flexor tendon in foals.* Am J Vet Res, 2001. **62**(12): p. 1859-64.

65. Birch, H.L., A.J. Bailey, and A.E. Goodship, *Macroscopic 'degeneration' of equine superficial digital flexor tendon is accompanied by a change in extracellular matrix composition.* Equine Vet J, 1998. **30**(6): p. 534-9.

66. Knorzer, E., et al., *New aspects of the etiology of tendon rupture. An analysis of time-resolved dynamic-mechanical measurements using synchrotron radiation.* Arch Orthop Trauma Surg, 1986. **105**(2): p. 113-20.

67. Oakes, B.W., *Tissue Healing and Repair: Tendons and Ligaments*, in *Rehabilitation of Sports Injuries: Scientific Basis*, W.R. Frontera, Editor. 2003, Blackwell Science: Oxford. p. 56-98.

68. Ross, R., E.W. Raines, and D.F. Bowen-Pope, *The biology of platelet-derived growth factor.* Cell, 1986. **46**(2): p. 155-69.

69. Martin, P., *Wound healing--aiming for perfect skin regeneration.* Science, 1997. **276**(5309): p. 75-81.

70. Eppley, B.L., J.E. Woodell, and J. Higgins, *Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing.* Plast Reconstr Surg, 2004. **114**(6): p. 1502-8.

71. Mosesson, M.W., *Fibrinogen and fibrin structure and functions.* J Thromb Haemost, 2005. **3**(8): p. 1894-904.

72. Fackelman, G.E., *The nature of tendon damage and its repair.* Equine Vet J, 1973. **5**(4): p. 141-9.

73. Williams, I.F., K.G. McCullagh, and I.A. Silver, *The distribution of types I and III collagen and fibronectin in the healing equine tendon.* Connect Tissue Res, 1984. **12**(3-4): p. 211-27.

74. Sharma, P. and N. Maffulli, *Biology of tendon injury: healing, modeling and remodeling.* J Musculoskelet Neuronal Interact, 2006. **6**(2): p. 181-90.

75. Parry, D.A., G.R. Barnes, and A.S. Craig, *A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties.* Proc R Soc Lond B Biol Sci, 1978. **203**(1152): p. 305-21.

76. Birch, H.L., A.M. Wilson, and A.E. Goodship, *Physical activity: does long-term, high-intensity exercise in horses result in tendon degeneration?* J Appl Physiol, 2008. **105**(6): p. 1927-33.

77. Williams, I.F., A. Heaton, and K.G. McCullagh, *Cell morphology and collagen types in equine tendon scar.* Res Vet Sci, 1980. **28**(3): p. 302-10.

78. Genovese, R.L. *Prognosis of superficial flexor tendon and suspensory ligament injuries*. 1993.

79. Silver, I.A., et al., *A clinical and experimental study of tendon injury, healing and treatment in the horse.* Equine Vet J Suppl, 1983(1): p. 1-43.

80. Genovese, R.L., et al. *Superficial digital flexor tendonitis. Long term sonographic and clinical study of racehorses*. 1996.

81. Gillis, C., et al., *Effect of maturation and aging on material and ultrasonographic properties of equine superficial digital flexor tendon.* Am J Vet Res, 1995. **56**(10): p. 1345-50.

82. Gillis, C., et al., *Ultrasonography as a method to determine tendon cross-sectional area.* Am J Vet Res, 1995. **56**(10): p. 1270-4.

83. Ross, M.W., Genovese, R.L., Dyson, S.J., Jorgensen, J.S., *Superficial Digital Flexor Tendonitis,* in *Diagnosis and Management of Lameness in the Horse*, M.W. Ross, Dyson, S.J., Editor. 2011, Elsevier Saunders: St. Louis. p. 706-726.

84. Gurtner, G.C., et al., *Wound repair and regeneration.* Nature, 2008. **453**(7193): p. 314-21.

85. Grzesik, W.J. and A.S. Narayanan, *Cementum and periodontal wound healing and regeneration.* Crit Rev Oral Biol Med, 2002. **13**(6): p. 474-84.

86. Poss, K.D., *Advances in understanding tissue regenerative capacity and mechanisms in animals.* Nat Rev Genet, 2010. **11**(10): p. 710-22.

87. Lin, L., et al., *Guided tissue regeneration in periapical surgery.* J Endod, 2010. **36**(4): p. 618-25.

88. Molloy, T., Y. Wang, and G. Murrell, *The roles of growth factors in tendon and ligament healing.* Sports Med, 2003. **33**(5): p. 381-94.

89. Tang, J.B., et al., *Tendon healing in vitro: promotion of collagen gene expression by bFGF with NF-kappaB gene activation.* J Hand Surg Am, 2003. **28**(2): p. 215-20.

90. Zhang, F., et al., *Effect of vascular endothelial growth factor on rat Achilles tendon healing.* Plast Reconstr Surg, 2003. **112**(6): p. 1613-9.

91. Dahlgren, L.A., et al., *Insulin-like growth factor-I improves cellular and molecular aspects of healing in a collagenase-induced model of flexor tendinitis.* J Orthop Res, 2002. **20**(5): p. 910-9.

92. Kang, H.J. and E.S. Kang, *Ideal concentration of growth factors in rabbit's flexor tendon culture.* Yonsei Med J, 1999. **40**(1): p. 26-9.

93. Werner, S. and R. Grose, *Regulation of wound healing by growth factors and cytokines.* Physiol Rev, 2003. **83**(3): p. 835-70.

94. Barrientos, S., et al., *Growth factors and cytokines in wound healing.* Wound Repair Regen, 2008. **16**(5): p. 585-601.

95. Barnes, G.L., et al., *Growth factor regulation of fracture repair.* J Bone Miner Res, 1999. **14**(11): p. 1805-15.

96. Tsuzaki, M., et al., *Insulin-like growth factor-I is expressed by avian flexor tendon cells.* J Orthop Res, 2000. **18**(4): p. 546-56.

97. Banes, A.J., et al., *Mechanical load stimulates expression of novel genes in vivo and in vitro in avian flexor tendon cells.* Osteoarthritis Cartilage, 1999. **7**(1): p. 141-53.

98. Banes, A.J., et al., *PDGF-BB, IGF-I and mechanical load stimulate DNA synthesis in avian tendon fibroblasts in vitro.* J Biomech, 1995. **28**(12): p. 1505-13.

99. Sakai, T., et al., *Effects of combined administration of transforming growth factor-beta1 and epidermal growth factor on properties of the in situ frozen anterior cruciate ligament in rabbits.* J Orthop Res, 2002. **20**(6): p. 1345-51.

100. Hildebrand, K.A., et al., *Exogenous transforming growth factor beta 1 alone does not improve early healing of medial collateral ligament in rabbits.* Can J Surg, 2002. **45**(5): p. 330-6.

101. Moreau, J.E., et al., *Growth factor induced fibroblast differentiation from human bone marrow stromal cells in vitro.* J Orthop Res, 2005. **23**(1): p. 164-74.

102. Hankemeier, S., et al., *Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: potential implications for tissue engineering of tendons and ligaments.* Tissue Eng, 2005. **11**(1-2): p. 41-9.

103. Haupt, J.L., B.P. Donnelly, and A.J. Nixon, *Effects of platelet-derived growth factor-BB on the metabolic function and morphologic features of equine tendon in explant culture.* Am J Vet Res, 2006. **67**(9): p. 1595-600.

104. Yoshikawa, Y. and S.O. Abrahamsson, *Dose-related cellular effects of platelet-derived growth factor-BB differ in various types of rabbit tendons in vitro.* Acta Orthop Scand, 2001. **72**(3): p. 287-92.

105. Zapf, J., C. Schmid, and E.R. Froesch, *Biological and immunological properties of insulin-like growth factors (IGF) I and II.* Clin Endocrinol Metab, 1984. **13**(1): p. 3-30.

106. Massague, J., *TGFbeta signaling: receptors, transducers, and Mad proteins.* Cell, 1996. **85**(7): p. 947-50.

107. Roberts, A.B.a.S., M.B, *The Transforming Growth Factor-Bs*, in *Peptide Growth Factors and Their Receptors I*, R.A. Sporn M, Editor. 1990, Springer-Verlag Inc: New York. p. 419-472.

108. Lieberman, J.R., A. Daluiski, and T.A. Einhorn, *The role of growth factors in the repair of bone. Biology and clinical applications.* J Bone Joint Surg Am, 2002. **84-A**(6): p. 1032-44.

109. Murphy, D.J. and A.J. Nixon, *Biochemical and site-specific effects of insulin-like growth factor I on intrinsic tenocyte activity in equine flexor tendons.* Am J Vet Res, 1997. **58**(1): p. 103-9.

110. Klein, M.B., et al., *Flexor tendon healing in vitro: effects of TGF-beta on tendon cell collagen production.* J Hand Surg Am, 2002. **27**(4): p. 615-20.

111. Dahlgren, L.A., A.J. Nixon, and B.D. Brower-Toland, *Effects of beta-aminopropionitrile on equine tendon metabolism in vitro and on effects of insulin-like growth factor-I on matrix production by equine tenocytes.* Am J Vet Res, 2001. **62**(10): p. 1557-62.

112. Murphy, P.G., et al., *Influence of exogenous growth factors on the synthesis and secretion of collagen types I and III by explants of normal and healing rabbit ligaments.* Biochem Cell Biol, 1994. **72**(9-10): p. 403-9.

113. Theoret, C.L., et al., *Expression of transforming growth factor beta(1), beta(3), and basic fibroblast growth factor in full-thickness skin wounds of equine limbs and thorax.* Vet Surg, 2001. **30**(3): p. 269-77.

114. Frank, S., M. Madlener, and S. Werner, *Transforming growth factors beta1, beta2, and beta3 and their receptors are differentially regulated during normal and impaired wound healing.* J Biol Chem, 1996. **271**(17): p. 10188-93.

115. Anaguchi, Y., et al., *The effect of transforming growth factor-beta on mechanical properties of the fibrous tissue regenerated in the patellar tendon after resecting the central portion.* Clin Biomech (Bristol, Avon), 2005. **20**(9): p. 959-65.

116. Chang, J., et al., *Gene expression of transforming growth factor beta-1 in rabbit zone II flexor tendon wound healing: evidence for dual mechanisms of repair.* Plast Reconstr Surg, 1997. **100**(4): p. 937-44.

117. Chen, C.H., et al., *Tendon healing in vivo: gene expression and production of multiple growth factors in early tendon healing period.* J Hand Surg Am, 2008. **33**(10): p. 1834-42.

118. Arai, K., et al., *TGF-beta alters collagen XII and XIV mRNA levels in cultured equine tenocytes.* Matrix Biol, 2002. **21**(3): p. 243-50.

119. Chang, J., et al., *Studies in flexor tendon wound healing: neutralizing antibody to TGF-beta1 increases postoperative range of motion.* Plast Reconstr Surg, 2000. **105**(1): p. 148-55.

120. Letterio, J.J. and A.B. Roberts, *Regulation of immune responses by TGF-beta.* Annu Rev Immunol, 1998. **16**: p. 137-61.

121. Letterio, J.J. and A.B. Roberts, *TGF-beta: a critical modulator of immune cell function.* Clin Immunol Immunopathol, 1997. **84**(3): p. 244-50.

122. O'Kane, S. and M.W. Ferguson, *Transforming growth factor beta s and wound healing.* Int J Biochem Cell Biol, 1997. **29**(1): p. 63-78.

123. Thomopoulos, S., et al., *Effect of several growth factors on canine flexor tendon fibroblast proliferation and collagen synthesis in vitro.* J Hand Surg Am, 2005. **30**(3): p. 441-7.

124. Okumura, M., et al., *Effect of basic fibroblast growth factor on wound healing in healing-impaired animal models.* Arzneimittelforschung, 1996. **46**(5): p. 547-51.

125. Chang, J., et al., *Molecular studies in flexor tendon wound healing: the role of basic fibroblast growth factor gene expression.* J Hand Surg Am, 1998. **23**(6): p. 1052-8.

126. Nixon, A.J., B.D. Brower-Toland, and L.J. Sandell, *Primary nucleotide structure of predominant and alternate splice forms of equine insulin-like growth factor I and their gene expression patterns in tissues.* Am J Vet Res, 1999. **60**(10): p. 1234-41.

127. Rechler, M.M.a.N., S.P., *Insulin-like Growth Factors,* in *Peptide Growth Factors and Their Receptors I,* R.A.B. Sporn M.B., Editor. 1991, Springer, Verlag: New York. p. 263-367.

128. Hansson, H.A., et al., *Transiently increased insulin-like growth factor I immunoreactivity in tendons after vibration trauma. An immunohistochemical study on rats.* Scand J Plast Reconstr Surg Hand Surg, 1988. **22**(1): p. 1-6.

129. Hansson, H.A., et al., *Somatomedin C immunoreactivity in the Achilles tendon varies in a dynamic manner with the mechanical load.* Acta Physiol Scand, 1988. **134**(2): p. 199-208.

130. Dahlgren, L.A. and A.J. Nixon, *Cloning and expression of equine insulin-like growth factor binding proteins in normal equine tendon.* Am J Vet Res, 2005. **66**(2): p. 300-6.

131. Nixon, A.J., R.A. Saxer, and B.D. Brower-Toland, *Exogenous insulin-like growth factor-I stimulates an autoinductive IGF-I autocrine/paracrine response in chondrocytes.* J Orthop Res, 2001. **19**(1): p. 26-32.

132. Witte, T.H., A.E. Yeager, and A.J. Nixon, *Intralesional injection of insulin-like growth factor-I for treatment of superficial digital flexor tendonitis in Thoroughbred racehorses: 40 cases (2000-2004).* J Am Vet Med Assoc, 2011. **239**(7): p. 992-7.

133. Haupt, J.L., Nixon, A.J. *Gene Enhanced Mesenchymal Stem Cells for the Treatment of Tendonitis*. in *Trans 52nd Annual Meeting of the ORS*. 2007. San Diego (CA).

134. Schnabel, L.V., et al., *Mesenchymal stem cells and insulin-like growth factor-I gene-enhanced mesenchymal stem cells improve structural aspects of healing in equine flexor digitorum superficialis tendons.* J Orthop Res, 2009.

135. McCarrel, T. and L. Fortier, *Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression.* J Orthop Res, 2009. **27**(8): p. 1033-42.

136. Woo, S.L., et al., *Engineering the healing of the rabbit medial collateral ligament.* Med Biol Eng Comput, 1998. **36**(3): p. 359-64.

137. Hildebrand, K.A., et al., *The effects of platelet-derived growth factor-BB on healing of the rabbit medial collateral ligament. An in vivo study.* Am J Sports Med, 1998. **26**(4): p. 549-54.

138. Thomopoulos, S., et al., *PDGF-BB released in tendon repair using a novel delivery system promotes cell proliferation and collagen remodeling.* J Orthop Res, 2007. **25**(10): p. 1358-68.

139. Chan, B.P., et al., *Supplementation-time dependence of growth factors in promoting tendon healing.* Clin Orthop Relat Res, 2006. **448**: p. 240-7.

140. Son, B.R., et al., *Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases.* Stem Cells, 2006. **24**(5): p. 1254-64.

141. Lapidot, T., *Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice. The role of SDF-1/CXCR4 interactions.* Ann N Y Acad Sci, 2001. **938**: p. 83-95.

142. Askari, A.T., et al., *Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy.* Lancet, 2003. **362**(9385): p. 697-703.

143. Yamaguchi, J., et al., *Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization.* Circulation, 2003. **107**(9): p. 1322-8.

144. Shen, W., et al., *The effect of incorporation of exogenous stromal cell-derived factor-1 alpha within a knitted silk-collagen sponge scaffold on tendon regeneration.* Biomaterials, 2010. **31**(28): p. 7239-49.

145. McQuibban, G.A., et al., *Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1.* J Biol Chem, 2001. **276**(47): p. 43503-8.

146. Lau, T.T. and D.A. Wang, *Stromal cell-derived factor-1 (SDF-1): homing factor for engineered regenerative medicine.* Expert Opin Biol Ther, 2011. **11**(2): p. 189-97.

147. Caplan, A.I. and S.P. Bruder, *Mesenchymal stem cells: building blocks for molecular medicine in the 21st century.* Trends Mol Med, 2001. **7**(6): p. 259-64.

148. Zuk, P.A., et al., *Human adipose tissue is a source of multipotent stem cells.* Mol Biol Cell, 2002. **13**(12): p. 4279-95.

149. Berg, L., et al., *Chondrogenic potential of mesenchymal stromal cells derived from equine bone marrow and umbilical cord blood.* Vet Comp Orthop Traumatol, 2009. **22**(5): p. 363-70.

150. Caplan, A.I., *Adult mesenchymal stem cells for tissue engineering versus regenerative medicine.* J Cell Physiol, 2007. **213**(2): p. 341-7.

151. Melton, D.A., Cowen, C. , *"Stemness: Definitions, Criteria, and Standards*, in *Essentials of Stem Cell Biololgy*, R. Lanza, Editor. 2009, Academic Press: San Diego. p. xxiii-xxix.

152. Kuhn, N.Z. and R.S. Tuan, *Regulation of stemness and stem cell niche of mesenchymal stem cells: implications in tumorigenesis and metastasis.* J Cell Physiol, 2010. **222**(2): p. 268-77.

153. Castillo, A.B. and C.R. Jacobs, *Mesenchymal stem cell mechanobiology.* Curr Osteoporos Rep, 2010. **8**(2): p. 98-104.

154. Ranera, B., et al., *Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue.* Vet Immunol Immunopathol, 2011. **144**(1-2): p. 147-54.

155. de Mattos Carvalho, A., et al., *Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine species adipose tissue.* Vet Immunol Immunopathol, 2009. **132**(2-4): p. 303-6.

156. Fortier, L. *Equine Embryonic Stem & Induced Pluripotent Stem Cells*. in *Veterinary Stem Cell Consortium*. 2009. Leipzig, Germany: Regenerative Medicine Supplement 2.

157. Pan, G. and J.A. Thomson, *Nanog and transcriptional networks in embryonic stem cell pluripotency.* Cell Res, 2007. **17**(1): p. 42-9.

158. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.

159. Richardson, L.E., et al., *Stem cells in veterinary medicine--attempts at regenerating equine tendon after injury.* Trends Biotechnol, 2007. **25**(9): p. 409-16.

160. Verfaillie, C.M., Crabbe, A. , *Multipotent Adult Progenitor Cells*, in *Essentials of Stem Cell Biology*, R. Lanza, Editor. 2009, Academic Press: San Diego. p. 233-241.

161. Bjorklund, L.M., et al., *Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model.* Proc Natl Acad Sci U S A, 2002. **99**(4): p. 2344-9.

162. Zhang, S.C., et al., *In vitro differentiation of transplantable neural precursors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1129-33.

163. Yamashita, J., et al., *Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors.* Nature, 2000. **408**(6808): p. 92-6.

164. Kim, J.H., et al., *Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease.* Nature, 2002. **418**(6893): p. 50-6.

165. Weiss, M.L. and D.L. Troyer, *Stem cells in the umbilical cord.* Stem Cell Rev, 2006. **2**(2): p. 155-62.

166. Farley, M.A., *Stem Cell Research: Religious Considerations*, in *Essentials of Stem Cell Biology*, R. Lanza, Editor. 2009, Academic Press: San Diego. p. 609-617.

167. Heng, B.C., T. Cao, and E.H. Lee, *Directing stem cell differentiation into the chondrogenic lineage in vitro.* Stem Cells, 2004. **22**(7): p. 1152-67.

168. Bassi, E.J., et al., *Exploring the Role of Soluble Factors Associated with Immune Regulatory Properties of Mesenchymal Stem Cells.* Stem Cell Rev, 2011.

169. Rossant, J., *Embryonic Stem Cells in Perspective*, in *Essentials of Stem Cell Biology*, R. Lanza, Editor. 2009, Academic Press: San Diego. p. 13-15.

170. Paris, D.B. and T.A. Stout, *Equine embryos and embryonic stem cells: defining reliable markers of pluripotency.* Theriogenology, 2010. **74**(4): p. 516-24.

171. Guest, D.J., M.R. Smith, and W.R. Allen, *Equine embryonic stem-like cells and mesenchymal stromal cells have different survival rates and migration patterns following their injection into damaged superficial digital flexor tendon.* Equine Vet J, 2010. **42**(7): p. 636-42.

172. Hockemeyer, D., Soldner, F., Jaenisch, R., *Direct Reprogramming of Somatic Cells to a Pluripotent State*, in *Essentials of Stem Cell Biology*, R. Lanza, Editor. 2009, Academic Press: San Diego. p. 29-31.

173. Pfaff, N., et al., *miRNAs involved in the generation, maintenance, and differentiation of pluripotent cells.* J Mol Med (Berl), 2012.

174. Gonzalez, M.A. and A. Bernad, *Characteristics of adult stem cells.* Adv Exp Med Biol, 2012. **741**: p. 103-20.

175. Raabe, O., et al., *Further insights into the characterization of equine adipose tissue-derived mesenchymal stem cells.* Vet Res Commun, 2011. **35**(6): p. 355-65.

176. Haudenschild, A.K., et al., *Pressure and distortion regulate human mesenchymal stem cell gene expression.* Ann Biomed Eng, 2009. **37**(3): p. 492-502.

177. Huang, C.H., et al., *Interactive effects of mechanical stretching and extracellular matrix proteins on initiating osteogenic differentiation of human mesenchymal stem cells.* J Cell Biochem, 2009. **108**(6): p. 1263-73.

178. Sen, B., et al., *Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable beta-catenin signal.* Endocrinology, 2008. **149**(12): p. 6065-75.

179. Wagner, D.R., et al., *Hydrostatic pressure enhances chondrogenic differentiation of human bone marrow stromal cells in osteochondrogenic medium.* Ann Biomed Eng, 2008. **36**(5): p. 813-20.

180. Gimble, J.M., A.J. Katz, and B.A. Bunnell, *Adipose-derived stem cells for regenerative medicine.* Circ Res, 2007. **100**(9): p. 1249-60.

181. da Silva Meirelles, L., P.C. Chagastelles, and N.B. Nardi, *Mesenchymal stem cells reside in virtually all post-natal organs and tissues.* J Cell Sci, 2006. **119**(Pt 11): p. 2204-13.

182. Durgam, S.S., et al., *Comparison of equine tendon- and bone marrow-derived cells cultured on tendon matrix with or without insulin-like growth factor-I supplementation.* Am J Vet Res, 2012. **73**(1): p. 153-61.

183. Richardson, L.E. *Extracellular Matrix Cues for Mesenchymal Stem Cell Differentiation*. in *Proceedings of the 53rd Orthopaedic Research Society*. 2007.

184. Guest, D.J., M.R. Smith, and W.R. Allen, *Monitoring the fate of autologous and allogeneic mesenchymal progenitor cells injected into the superficial digital flexor tendon of horses: preliminary study.* Equine Vet J, 2008. **40**(2): p. 178-81.

185. Ouyang, H.W., et al., *Mesenchymal stem cell sheets revitalize nonviable dense grafts: implications for repair of large-bone and tendon defects.* Transplantation, 2006. **82**(2): p. 170-4.

186. Ribitsch, I., et al., *Basic Science and Clinical Application of Stem Cells in Veterinary Medicine.* Adv Biochem Eng Biotechnol, 2010.

187. Majumdar, M.K., et al., *Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells.* J Cell Physiol, 1998. **176**(1): p. 57-66.

188. Chen, J., et al., *Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat.* J Neurosci Res, 2003. **73**(6): p. 778-86.

189. Li, Y., et al., *Gliosis and brain remodeling after treatment of stroke in rats with marrow stromal cells.* Glia, 2005. **49**(3): p. 407-17.

190. Walter, M.N., et al., *Mesenchymal stem cell-conditioned medium accelerates skin wound healing: an in vitro study of fibroblast and keratinocyte scratch assays.* Exp Cell Res, 2010. **316**(7): p. 1271-81.

191. Gnecchi, M., et al., *Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement.* FASEB J, 2006. **20**(6): p. 661-9.

192. Kinnaird, T., et al., *Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms.* Circ Res, 2004. **94**(5): p. 678-85.

193. Gruber, R., et al., *Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells.* Tissue Eng, 2005. **11**(5-6): p. 896-903.

194. Kinnaird, T., et al., *Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms.* Circulation, 2004. **109**(12): p. 1543-9.

195. Kamihata, H., et al., *Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines.* Circulation, 2001. **104**(9): p. 1046-52.

196. Nagaya, N., et al., *Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis.* Am J Physiol Heart Circ Physiol, 2004. **287**(6): p. H2670-6.

197. Tang, Y.L., et al., *Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction.* Ann Thorac Surg, 2005. **80**(1): p. 229-36; discussion 236-7.

198. Potapova, I.A., et al., *Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro.* Stem Cells, 2007. **25**(7): p. 1761-8.

199. Equstem. *Umbilical Cord Blood*. 2009 [cited 2011 February 9th]; Available from: http://www.equstem.com/umbilical-cord-blood.html.

200. Haught, J., *Harvesting Stem Cells from Equine UCB*, S. Stewart, Editor. 2010.

201. Dahlgren, L.A., *Equine UCB Stem Cell Harvesting*, S. Stewart, Editor. 2010.

202. Reed, S.A. and S.E. Johnson, *Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types.* J Cell Physiol, 2008. **215**(2): p. 329-36.

203. Koch, T.G., et al., *Isolation of mesenchymal stem cells from equine umbilical cord blood.* BMC Biotechnol, 2007. **7**: p. 26.

204. Goodwin, H.S., et al., *Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers.* Biol Blood Marrow Transplant, 2001. **7**(11): p. 581-8.

205. Park, K.S., Y.S. Lee, and K.S. Kang, *In vitro neuronal and osteogenic differentiation of mesenchymal stem cells from human umbilical cord blood.* J Vet Sci, 2006. **7**(4): p. 343-8.

206. Peault, B., *Hematopoietic stem cell emergence in embryonic life: developmental hematology revisited.* J Hematother, 1996. **5**(4): p. 369-78.

207. Tavassoli, M. and J.J. Minguell, *Homing of hemopoietic progenitor cells to the marrow.* Proc Soc Exp Biol Med, 1991. **196**(4): p. 367-73.

208. Arien-Zakay, H., P. Lazarovici, and A. Nagler, *Tissue regeneration potential in human umbilical cord blood.* Best Pract Res Clin Haematol, 2010. **23**(2): p. 291-303.

209. Roura, S., J.M. Pujal, and A. Bayes-Genis, *Umbilical cord blood for cardiovascular cell therapy: from promise to fact.* Ann N Y Acad Sci, 2012. **1254**: p. 66-70.

210. Young, R.G., et al., *Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair.* J Orthop Res, 1998. **16**(4): p. 406-13.

211. Awad, H.A., et al., *Autologous mesenchymal stem cell-mediated repair of tendon.* Tissue Eng, 1999. **5**(3): p. 267-77.

212. Awad, H.A., et al., *Repair of patellar tendon injuries using a cell-collagen composite.* J Orthop Res, 2003. **21**(3): p. 420-31.

213. Murphy, J.M., et al., *Stem cell therapy in a caprine model of osteoarthritis.* Arthritis Rheum, 2003. **48**(12): p. 3464-74.

214. Caniglia, C.J., M.C. Schramme, and R.K. Smith, *The effect of intralesional injection of bone marrow derived mesenchymal stem cells and bone marrow supernatant on collagen fibril size in a surgical model of equine superficial digital flexor tendonitis.* Equine Vet J, 2011.

215. Giovannini, S., et al., *Multilineage differentiation potential of equine blood-derived fibroblast-like cells.* Differentiation, 2008. **76**(2): p. 118-29.

216. Hovanessian, N., *Bone marrow aspiration*, S.L. Stewart, Editor. 2010.

217. Vidal, M.A., et al., *Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity.* Vet Surg, 2006. **35**(7): p. 601-10.

218. Vidal, M.A., et al., *Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue, and umbilical cord tissue.* Stem Cells Dev, 2012. **21**(2): p. 273-83.

219. Vidal, M.A., et al., *Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells.* Vet Surg, 2007. **36**(7): p. 613-22.

220. Sand, T. *Preliminary Characterization of Canine and Equine Cell Preparations Obtained by Enzymatic Digestion of Adipose Tissue*. in *International Federation for Adipose Therapeutics and Science*. 2006.

221. Harman, R., Sand, T. *Safety of Administration of Adipose-Derived Stem and Regenerative Cells in the Horse and the Dog*. in *International Federation for Adipose Therapeutics and Science*. 2006.

Project Summary

Purpose

The purpose of these studies was to find scientific support for the clinical use of equine adipose-derived cells (ADC) as a treatment for flexor tendon injuries in the horse by demonstrating their trophic capabilities.

Objectives

Objective #1: Demonstrate that equine ADCs produce an appropriate profile of growth factors and cytokines to direct a tissue regenerative response.

Objective #2: Test the hypothesis that equine ADCs produce chemotactic factors that induce migration of adult stem cells to the site of injury.

Objective #3: Document that equine ADCs produce soluble factors capable of inducing a regenerative response in equine tendon fibroblasts grown in co-culture.

Hypotheses

Hypothesis #1: Equine ADCs produce a profile of growth factors and cytokines appropriate to direct a tissue regenerative response in tendon. Protein levels for growth factors relevant to tendon healing will be higher for ADCs compared to tendon fibroblasts.

Hypothesis #2: Equine ADCs produce soluble factors that stimulate migration of adult stem cells in a dose dependent manner *in vitro*. Media conditioned by ADCs will induce migration of greater numbers of ADCs than media conditioned by tendon fibroblasts.

Hypothesis #3: Co-culture of ADCs and tendon fibroblasts will result in the production of soluble factors by the ADCs that will cause up-regulation of matrix protein gene expression by tendon fibroblasts as well as an increase in cell proliferation and GAG and collagen content.

Chapter 2: Manuscript

Adipose-derived Adult Stem Cells as Trophic Mediators of Tendon Regeneration

Shelley L. Stewart, BS; Elle Donnini; Anne C. Nichols, BS; Stephen R. Werre, PhD; Jennifer G. Barrett, DVM, PhD; William R. Huckle, PhD; Willard H. Eyestone, PhD; Linda A. Dahlgren, DVM, PhD

Abstract

The adipose-derived stromal vascular fraction (SVF) is a promising new therapy for equine flexor tendonitis. This heterogeneous population of cells may improve tendon healing via the production of growth and chemotactic factors capable of recruiting endogenous stem cells and increasing extracellular matrix production by tendon fibroblasts (TFBL). The purpose of this study was to evaluate the ability of adipose-derived cells (ADC) culture expanded from the SVF to act as trophic mediators *in vitro*. We hypothesized that ADCs would produce growth and chemotactic factors important in tendon healing and capable of inducing cell migration and matrix protein gene expression. Superficial digital flexor tendons and adipose tissue were harvested from eight adult horses and processed to obtain SVF cells, ADCs and TFBLs. Adipose-derived cells and TFBLs were grown in monolayer culture for growth factor quantification, to produce conditioned media for microchemotaxis, and in co-culture for quantification of matrix protein gene expression by TFBLs. Growth factor gene expression by SVF cells was significantly greater than in ADCs or TFBLs. Co-culture of TFBLs and ADCs resulted in modest up-regulation of matrix protein expression (collagen types I and III, decorin, and cartilage oligomeric matrix protein) by TFBLs. Media conditioned by ADCs induced ADC

migration in a dose dependent manner. These findings support the role of both SVF and ADCs as trophic mediators in tendon regeneration. The differences detected in gene expression between SVF cells and ADCs indicate that additional studies are needed to evaluate the changes that occur during culture of these cells.

Introduction

Flexor tendon injuries are common in equine athletes, causing significant numbers of days out of training and loss of revenue [1, 2]. Treatments for tendonitis have traditionally included cold therapy, bandaging, stall rest, and controlled exercise. Despite the high frequency of flexor tendon injuries, and continued industry and scientific efforts to reduce the incidence of injury, currently available treatment options result in less than optimal healing. Healed, or 'repaired', tendon is biomechanically inferior to normal tendon, predisposing the tendon to re-injury [1, 3].

There is, therefore, a need for novel treatments that result in regeneration of a biomechanically normal tendon, restoring normal tendon structure and function. Adult stem cells are an exciting new technology that has been advocated for the promotion of tendon regeneration. These multipotent cells can be harvested from many adult tissues, including adipose and bone marrow [4-6]. There are advantages and disadvantages associated with the different sources. Stem cells from adipose tissue are harvested with limited morbidity, are found in greater numbers than stem cells from bone marrow, and are available to the clinician for implantation within 48 hours of harvest [7]. The frequency of stem cells from equine adipose tissue has been reported to be as high as 43% of the nucleated cells [8]. By contrast, the frequency of stem cells in bone marrow has been reported as 1 in $4.2x10^3$ cells [9]. Bone marrow aspirate is advantageous in that it has stall-side applications; it can be injected into tendons immediately following harvest in either its unprocessed state or as a cell concentrate following removal of the red blood fraction. Bone marrow aspirate can also be cultured to enrich for bone marrow derived stem cells, although this increases the interval between harvest and

implantation by several weeks. However, all bone marrow products carry the risk of mineralization at the injection site [7, 10].

Adult stem cells likely influence the tendon healing process by two possible mechanisms: differentiation into tendon fibroblasts that contribute directly to healing and the production of soluble factors that indirectly influence the healing process by signaling to other nearby cells [11, 12]. This latter mechanism of action is referred to as "trophic", consistent with bioactive factors produced by one cell that may either directly cause intracellular signaling or indirectly by causing other cells in the area to produce additional bioactive factors [10, 13-15]. The soluble factors produced by adult stem cells include chemotactic factors capable of recruiting endogenous mesenchymal stem cells to the site of injury and growth factors and cytokines capable of modulating cell proliferation and differentiation and extracellular matrix (ECM) synthesis. Growth factors and cytokines relevant to tendon healing include transforming growth factor (TGF) -β1 and -β3 ,stromal cell-derived factor 1α (SDF)-1α, fibroblast growth factor (FGF)-2, insulin like growth factor (IGF)-I, and platelet derived growth factor (PDGF)-BB. Transforming growth factor-β1 and -β3, FGF-2, and IGF-I are all important mediators of cell growth and differentiation, as well as the synthesis of matrix proteins [16, 17]. Platelet derived growth factor-BB is a mitogen and chemoattractant for adult mesenchymal stem cells [18]. Stromal cell-derived factor 1α is also an important mesenchymal cell chemoattractant [19].

The use of autologous nucleated cells isolated from the stromal vascular fraction (SVF) of adipose tissue (adipose-derived cells (ADC)) has become a popular treatment for tendon and ligament injuries in the horse over the past 10 years [7]. These cells represent a heterogeneous mixture of the nucleated cells found in adipose tissue, including fibroblasts, mesenchymal stem cells, endothelial cells, mononuclear cells, neutrophils, lymphocytes, pericytes, eosinophils, and

monocytes [20-22]. The intralesional injection of ADCs under ultrasound guidance in a collagenase-induced model of superficial digital flexor tendons (SDFT) resulted in significant improvements in histological scores, including improved fiber organization and decreased numbers of inflammatory cells compared to saline-treated controls [21]. In addition, ADC treatment resulted in increased gene expression for cartilage oligomeric matrix protein (COMP), an important mediator of collagen fibrillogenesis [23]. In the US, over 4,000 equine tendon injuries have been successfully treated with ADCs. Seventy-eight percent of sport horses returned to their previous athletic endeavors and 69% of race horses raced at least once [20, 24]. These results support the clinical use of ADCs for the treatment of equine tendon injuries; however, there is limited information available regarding the exact mechanism by which ADCs may promote tendon regeneration.

Traditionally, differentiation of stem cells into tendon fibroblasts following implantation was viewed as the primary mechanism by which adult stem cells contributed to tendon healing. More recently, the idea that ADCs contribute to tendon healing through indirect, or trophic, mechanisms, has gained support. However, there is no direct evidence that ADCs may have the capacity to function in this manner in the horse [10, 14, 25]. Further investigation of the mechanism by which ADCs may contribute to soft tissue healing will provide information critical for the successful application of adult stem cell therapy in the horse. The purpose of these studies was to gain insights into the mechanism by which ADCs may participate in promoting a regenerative response in healing soft tissues. We hypothesized that ADCs are capable of secreting growth factors relevant to tendon healing, chemotactic factors for the recruitment of stem cells, and soluble factors capable of stimulating tendon fibroblasts (TFBL) to participate in tissue healing.

Materials and Methods

Sample Harvest and Cell Culture:

Paired samples of tendon and adipose tissue were harvested from eight young adult horses (age 2-8 years) representing various light breeds (3 Thoroughbreds, 1 Quarter Horse, 1 Standardbred, 3 Warmbloods) that were donated to the Veterinary Teaching Hospital for reasons unrelated to tendon injury. Horses were examined clinically and ultrasonographically to rule out existing tendonitis and were euthanized via barbiturate overdose prior to harvesting tissues. All protocols were approved by the Institutional Animal Care and Use Committee. All cell culture reagents were purchased from Mediatech, Inc., Manassas, VA unless otherwise noted. Horses were placed in lateral recumbency and the paraxial caudodorsal gluteal region was clipped and aseptically prepared bilaterally. Adipose tissue was harvested aseptically via a surgical incision in the groove between the biceps femoris and semitendinosus muscles [21]. Twenty to thirty grams of adipose tissue from each horse was placed in 50 ml conical tubes containing sterile phosphate buffered saline (PBS) , stored at 4˚C, and shipped by overnight courier to a commercial laboratory for isolation of the nucleated cell fraction (SVF) (Vet-Stem, Inc., Poway, CA).

Briefly, adipose tissue was processed via modified collagenase and hyaluronidase digest [21]. Following isolation, the nucleated cell pellet was resuspended in sterile PBS, cryopreserved in aliquots of 4-8x10^6 cells, and shipped by overnight courier on dry ice to our lab. Cell aliquots were thawed rapidly in a 37˚C water bath, placed in standard tissue culture coated polystyrene flasks in ADC growth medium (Mesenchymal Stem Cell Medium (MSCM) with 5% (v/v) fetal bovine serum (FBS)) (ScienCell Research Laboratories, Carlsbad, CA) at 37˚C, 5%

CO_2, and 95% humidity, and expanded for use in described assays. Cells were passaged routinely using trypsin (0.25% trypsin/2.21 mM EDTA) prior to reaching 70% confluence and cryopreserved at sequential passages in freeze medium (90% FBS with 10% dimethylsulfoxide). Cultured cells from the nucleated cell fraction are termed ADCs to distinguish them from the SVF (cryopreserved but not cultured). Cells were used at passages 2-5 for the experiments described.

The tensile region of the SDFT from both forelimbs was harvested using aseptic technique and transported to the lab in ice cold Hank's Balanced Salt Solution [26]. The paratenon was removed and the central portion of the tendon was minced into 1–2 mm pieces, digested in 0.075% w/v collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ) in digest medium (high-glucose DMEM (Dulbecco's Modified Eagle's Medium), 10% (v/v) FBS , 300 µg/ml L-glutamine, 25 mM HEPES, 20 units/ml penicillin G, 20 µg/ml streptomycin sulfate, and 50 µg/ml ascorbic acid (Fisher Scientific, Pittsburgh, PA), and 30 µg/ml α-ketoglutaric acid (MP Biomedicals, LLC., Solon, Ohio)) overnight at 37°C, 5% CO2, and 95% humidity with stirring. Following overnight digestion, tendon digests were filtered through a layer of cheesecloth (grade 50) and 40 mm nylon mesh (Small Parts, Inc, Miami Lakes, FL) and centrifuged at 405 x g for 10 minutes at 25°C. The cell pellet was resuspended in TFBL growth medium (DMEM, 10% (v/v) FBS, 300 µg/ml L-glutamine, 25 mM HEPES, 20 units/ml penicillin G, 20 µg/ml streptomycin sulfate, and 5 mg/ml ascorbic acid) and fibroblasts were plated in $75cm^2$ tissue culture polystyrene flasks for expansion at $37^{\circ}C$, 5% CO_2, and 95% humidity. Cells were incubated until confluent, trypsinized, and replated as above until they reached 100% confluence. Cells from sequential passages were cryopreserved in freeze medium at concentrations of $2\text{-}10x10^6$/ml. Tendon fibroblasts subcultured for 2 to 6 passages were used

for all experiments. A murine fibroblast cell line (NIH/3T3, ATCC, Manassas, VA) was used as an additional control population and were grown by standard methods in DMEM containing 10% FBS, 300 µg/ml L-glutamine, 20 units/ml penicillin G, and 20 µg/ml streptomycin sulfate.

Growth Factor Quantification:

Paired TFBL and ADCs from 8 horses at passages 2-6 and NIH/3T3 cells were seeded at $1.0x10^4/cm^2$ in 12 well tissue culture plates in cell appropriate growth media (ADC or TFBL growth media) for 3 and 6 days. Growth media without cells were incubated for the same time periods as controls. Cell monolayers were trypsinized and cell number determined via trypan blue exclusion and hand counting via hemocytometer. Monolayers were combined from each of the 12 wells within each treatment group and the average number of cells per well was determined. Conditioned media for each treatment group was harvested, combined as above, centrifuged at 300 x g for 5 minutes, removed from any residual cell pellet, and frozen at -20°C in 500 µl aliquots. Growth factor protein levels in conditioned media were determined using enzyme linked immunosorbent assay (ELISA) for IGF-I, TGF-β3, PDGF-BB, SDF-1α (R&D Systems, Inc., Minneapolis, MN), FGF-2 (RayBiotech, Inc., Norcross, GA), and TGF-β1 (Promega Corporation, Madison, WI) according to the manufacturers' instructions [16-19]. Protein levels were normalized to cell number from the trypan blue counts from the matching set of wells. Because PDGF-BB and TGF-β3 levels were below the detectable limit of these ELISA kits, conditioned media samples were concentrated by filter centrifugation using pore sizes selected based on protein size for PDGF-BB (3 kDa filter) and TGF-β3 (10 kDa filter) (Amicon Ultra, Millipore Corporation, Darmstadt, Germany) and these ELISAs repeated [27, 28].

Tendon fibroblasts and ADCs at passages 4 and 5 were grown in various basal media and reduced serum conditions to test the feasibility of using a single basal medium and low serum formulation for both cell types. This was necessary as a result of confounding results from having grown TFBLs and ADCs in different basal media and in the presence of moderate amounts of FBS. Paired TFBLs and ADCs from 4 horses were seeded in separate 24 well plates at $1.1x10^4/cm^2$ in either MSCM or DMEM with 1, 2, 5, or 10% FBS or 1% FBS/insulin transferrin selenium (ITS, 10mL/L) and grown for 6, 48, and 96 hours. At harvest, media from duplicate wells were harvested for glycosaminoglycan (GAG) content and, monolayers were trypsinized and centrifuged at 300 x g for 10 minutes. Cell pellets were lyophilized (Virtis AdVantage Lyophilizer, SP Industries, Gardiner, NY) and digested in 0.5% papain (MP Biomedical LLC, Solon, OH) for 4 hours at 65°C. Glycosaminoglycan content of the cell pellets and conditioned media was determined using the dimethylmethylene blue dye binding assay and spectrophotometric analysis [29]. The DNA content of the cell pellets was determined using bisbenzamide binding and fluorometric assay following digestion in 0.5% papain for an additional 16 hours at 65°C [30]. Conditioned media were also used for IGF-I and SDF-1α quantification via ELISA as previously described.

As an alternative approach to determining growth factor expression in TFBLs and ADCs, gene expression for the growth factors described was quantified in the place of protein production. Paired tendon fibroblasts and ADCs at passages 3-4 were cultured in MSCM with 5% FBS for 24 hours in 6 well plates at $1.0x10^4/cm^2$. Gene expression of SVF cells was quantified using cryopreserved aliquots of the uncultured nucleated cells from the SVF ($4\text{-}8x10^6$ cells/aliquot) (n=10; 6 of which were common to the 8 used for the other assays described in this study). Total RNA was isolated via guanidinium chloride-phenol extraction (Trizol®, Invitrogen

Corporation, Carlsbad, CA), column purification (RNeasy® spin columns, Qiagen, Valencia, CA), quantified via fluorescent dye binding (cultured cells; Quant-iT™ Ribogreen® RNA Reagent, Invitrogen Corporation) or spectrophotometry (SVF cells; NanoDrop, NanoDrop Products, Wilmington, DE), and reverse transcribed to cDNA using a commercial kit (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA) [26]. Replicate wells were analyzed individually. Complementary DNA was amplified by fluorescent real time PCR (ABI PRISM 7300 Sequence Detection System, Applied Biosystems). Equine specific primers and MGB probes were designed using equine specific sequence information. Sequence information for PDGF-BB and SDF-1α was obtained by comparing published bovine sequence to the equine genome to identify the correct equine sequence from the equine genome project (EquCab2, BLAST search, Ensembl, Wellcome Trust, Hinxton, UK; http://useast.ensembl.org/equus_caballus/info/index). Primers and probes were then designed using commercial software (Primer Express v 3.0, Applied Biosystems) (Table 4) [26]. Fibroblast growth factor-2 is a proprietary TaqMan® Gene Expression Assay from Applied Biosystems (Assays-by-Design, Ec03470033_m1). Relative gene expression was calculated using the comparative threshold cycle method ($\Delta\Delta C_t$) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA expression as the endogenous control [31, 32]. Samples were normalized by TFBL expression levels.

Co-Culture of Tendon Fibroblasts and Adipose-derived Cells:

Tendon fibroblasts at passages 4-5 were seeded in 24 well plates at $2.5x10^4/cm^2$ and ADCs at passages 2-3 were seeded in 0.4 μm pore size Transwell® inserts (Corning Inc, Corning, NY) at $3x10^3$-$1.2x10^4/cm^2$. Both cell types were grown for 73 hours in MSCM with 5% FBS (n=8) [33, 34]. As an additional control, inserts were seeded with $12x10^4$ NIH/3T3 cells per

insert. The negative control consisted of TFBLs incubated with MSCM with 5% FBS (0 ADC control) in the inserts for 73 hours. Inserts allowed TFBLs to have contact with ADC-conditioned medium, but not the ADCs themselves. Triplicate monolayers of TFBLs were harvested as previously described, combined, and used for quantification of collagen (Sircol™ Collagen Assay, Biocolor Ltd, Carrickfergus, UK), GAG, and DNA content. Triplicate medium samples were combined from each treatment group, centrifuged at 300 x g for 5 minutes, and stored at -20°C for GAG quantification as previously described. Soluble collagen content in conditioned medium and cell pellets was quantified spectrophotometrically using the sirius red dye-binding method [16]. Cell pellets were digested in 200 µl of pepsin-acid solution (Sigma-Aldrich Co, St. Louis, MO) for 7 days with stirring at 4°C [16]. Soluble collagen and GAG content for medium and cell pellets were normalized to DNA content. A separate set of wells were harvested for gene expression of ECM proteins (collagen types I and III, COMP, and decorin; Table 4) as previously described [26]. Both GAPDH and 18s ribosomal RNA were used as endogenous controls to determine which gene was most stable under our experimental conditions. Both genes were found to be acceptable candidates and GAPDH was selected based on previously published literature [35]. Samples were normalized by expression levels for TFBLs.

To validate that TFBLs are capable of responding to soluble factors and that diffusion occurs between the inserts and the lower culture wells, TFBLs from 4 horses at passages 4-6 were seeded in duplicate at $8.3x10^3/cm^2$ in 24 well plates and grown in MSCM with 5% or 15% FBS without Transwell® inserts and with MSCM with 15% FBS loaded in 3.0 µm pore size Transwell® inserts for 73 hours for determination of DNA content. Replicate wells were

processed as separate samples. Samples from an identical plate were harvested for later analysis of gene expression of matrix proteins (collagen types I and III, COMP, and decorin).

Microchemotaxis:

Tendon fibroblasts and ADCs at passages 3-5 from 6 horses were seeded separately in MSCM with 0.5% FBS in 12 well plates at $3.9x10^2/cm^2$ (low density) and $3.9x10^4/cm^2$ (high density) and grown for 72 hours to create conditioned media for use in the microchemotaxis assay. Media were harvested, centrifuged at 300 x g for 5 minutes to remove any residual cell pellet, and stored at -20˚C for later use. The lower wells of a microchemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD) were loaded with 25 µl of either low or high density TFBL- or ADC- conditioned medium [36-39]. Mesenchymal stem cell media with 20% FBS or 0.1% bovine serum albumin (BSA, Sigma Aldrich) were used as positive and negative controls respectively. An 8 µm polycarbonate filter (Neuro Probe, Inc.) was placed between the lower and upper wells, the chamber assembled, and the upper wells seeded with $5x10^3$ADCs per well in MSCM with 0.1% BSA (6 wells per treatment). The chamber was incubated for 16 hours at 37°C with 5% CO_2 and 95% humidity, disassembled, and the filter removed. The filter was washed with PBS, the upper side scraped, and the filter was washed again with PBS. This process was repeated three times to remove non-migrated cells. The filter was then fixed for 5 minutes in ice cold 100% methanol, stained for 15 minutes in propidium iodide (MP Biomedical, LLC), rinsed once with PBS, and migrated cells quantified using commercial software (Olympus CKX-41 ,Olympus, Center Valley, PA; Infinity 3 camera, Lumenera, Ottawa, Ontario; ImageJ, National Institutes of Health, Washington, DC) [40]. Images were imported into ImageJ as eight bit image stacks and converted to binary using the “threshold” function. The resulting objects,

representing cell nuclei, were counted using the "analyze particles" function. The mean of the 6 identical wells was used for statistical analysis.

Statistics:

Descriptive statistics were performed using a commercial software program (Statistix 9©, Analytical Software, Tallahassee, FL). ELISA data were analyzed using Kruskal Wallis one way ANOVA (Statistix 9©). All other data were analyzed using mixed model ANOVA with posthoc analysis (Tukey's (growth factor gene expression and biochemistry assays) or Bonferonni (microchemotaxis assay)) (SAS, SAS Institute Inc, Cary, NC). Significance was set at $p \leq 0.05$.

Results

Growth Factor Quantification:

Concentrations of PDGF-BB and TGF-β3 were below the detectable limits of the ELISAs at both 3 and 6 days for TFBL-, ADC-, and NIH/3T3- conditioned media and the media controls. Growth factor levels for TGF-β1, FGF-2, IGF-I, and SDF-1α were very low or below the detectable limits of the ELISAs. Detectable growth factor levels are reported in Table 1. Both TFBLs and ADCs were successfully grown in MSCM at low serum concentrations for up to 96 hours without negative effects on DNA or GAG content (Tables 2 and 3). At no time were there any statistically significant differences between treatment groups for DNA or GAG content. Mesenchymal Stem Cell Medium with 2% FBS was selected as the basal culture medium for both TFBLs and ADCs for the repetition of IGF-I and SDF-1α protein quantification. This concentration of serum was the lowest concentration that did not result in potentially undesirable morphological alterations of cells. Both IGF-I and SDF-1α ELISAs were run, and results showed that protein levels in TFBL- and ADC-conditioned media when using MSCM with 2% FBS were similar to those in TFBL and ADC growth media (data not shown). Thus we were unable to accurately measure growth factor protein levels despite the reduced FBS concentrations as compared to the higher amounts in the TFBL (10% FBS) and ADC growth media (5% FBS).

Gene expression for SDF-1α, IGF-I, PDGF-BB, FGF-2, TGF-β1, and TGF-β3 in SVF cells was significantly higher than in TFBLs and ADCs (Figures 1 and 2, $p \leq 0.0001$). Gene expression for TGF-β1was significantly higher in TFBLs than ADCs (Figure 2B, $p \leq 0.0001$). There were no significant differences for expression of the other growth factors and cytokines

between TFBLs and ADCs. The overall p-value resulting from the mixed-model ANOVA test of each growth factor (SDF-1α, IGF-I, PDGF-BB, FGF-2, TGF-β1, and TGF-β3) was $p \leq 0.0001$.

Table 1. Growth factor levels in conditioned media after 3 and 6 days of culture. Mean ± SD. ND = not detectable.

Source of Conditioned Medium	Units	Days in Culture	Transforming growth factor-β1	Fibroblast growth factor-2	Insulin like growth factor-I	Stromal cell derived factor-1α
Adipose-derived Cells	pg/cell	3	1.11 ± 0.26	2.97 ± 2.26	104 ± 1.44	ND
		6	1.24 ± 0.39	2.13 ± 1.13	16.1 ± 5.16	3.43 ± 2.16
Tendon Fibroblasts		3	1.91 ± 0.38	0.22 ± 0.22	287 ± 5.45	ND
		6	1.24 ± 0.33	0.16 ± 0.09	ND	ND
NIH/3T3 Cells		3	2.69 ± 2.0	0.01 ± 0.03	375 ± 16.3	3.72 ± 2.31
		6	2.84 ± 0.92	0.10 ± 0.09	ND	36.3 ± 15.2
Adipose-derived Cell Growth Medium (no cells)	pg/μl x 10^5	3	8.71 ± 1.31	53.2 ± 18.5	879 ± 2.20	ND
		6	9.23 ± 0.86	27.8 ± 7.33	70.9 ± 6.44	ND
Tendon Fibroblast Growth Medium (no cells)		3	3.44 ± 0.92	ND	610 ± 6.00	ND
		6	3.38 ± 1.00	ND	ND	ND

Table 2. DNA content after culture in various media formulations. DNA content (µg/ml) of monolayers of tendon fibroblasts and adipose-derived cells and grown in Mesenchymal Stem Cell Medium (MSCM) or Dulbecco's modified eagle's medium (DMEM) with 1, 2 or 10% fetal bovine serum (FBS) or 1% FBS plus insulin transferrin selenium (ITS) for 36, 48 or 96 hours. Mean ± SD.

Cell Type	Medium	Concentration of FBS	Time in Culture (hours)		
			36	48	96
Tendon Fibroblasts	DMEM	10%	18.6 ± 2.95	22.5 ± 4.07	29.2 ± 8.72
		2%	16.5 ± 4.44	16.4 ± 3.93	22.7 ±6.54
		1%	17.4 ± 5.53	15.5 ± 2.43	19.5 ± 2.79
		1% + ITS	22.3 ± 8.13	23.5 ± 4.70	32.4 ± 11.6
Adipose-derived Cells		10%	35.8 ± 7.68	45.1 ± 2.19	130.6 ± 43.3
		2%	32.3 ± 8.14	41.9 ± 9.70	64.3 ± 15.1
		1%	29.6 ± 6.60	40.6 ± 10.5	49.4 ± 8.05
		1% + ITS	24.0 ± 10.8	38.0 ± 7.91	52.0 ± 12.6
Tendon Fibroblasts	MSCM	10%	23.2 ± 3.34	31.8 ± 7.94	76.8 ± 36.2
		2%	23.7 ± 2.44	29.9 ± 7.93	47.4 ± 1.46
		1%	18.8 ± 1.46	29.9 ± 6.41	48.9 ± 11.1
		1% + ITS	22.7 ± 5.73	26.2 ± 5.26	45.7 ± 8.80
Adipose-derived Cells		10%	30.9 ± 7.51	56.9 ± 18.2	80.1 ± 28.8
		2%	38.7 ± 11.2	41.0 ± 8.28	80.2 ± 28.3
		1%	28.2 ± 8.65	44.8 ± 16.6	61.0 ± 13.3
		1% + ITS	36.3 ± 9.88	50.2 ± 5.02	63.9 ± 15.0

Table 3. GAG content after culture in various media formulations. Glycosaminoglycan (GAG) content of monolayers of tendon fibroblasts and adipose-derived cells and grown in Mesenchymal Stem Cell Medium (MSCM) or Dulbecco's modified eagle's medium (DMEM) with 1, 2 or 10% fetal bovine serum (FBS) or 1% FBS plus insulin transferrin selenium (ITS) for 36, 48 or 96 hours. Mean ± SD.

Cell Type	Medium	Concentration of FBS	Time in Culture (hours)		
			36	48	96
Tendon Fibroblasts	DMEM	10%	3.95 ± 1.44	6.04 ± 5.47	7.89 ± 3.19
		2%	1.72 ± 1.16	1.50 ± 0.33	5.33 ± 5.16
		1%	1.07 ± 0.90	2.08 ± 0.96	2.27 ± 1.22
		1% + ITS	1.57 ± 0.23	1.38 ± 0.40	5.19 ± 1.52
Adipose-derived Cells		10%	4.48 ± 1.25	4.15 ± 0.94	10.4 ± 1.42
		2%	1.97 ± 0.76	2.11 ± 0.58	4.76 ± 0.62
		1%	1.04 ± 0.84	1.60 ± 0.40	3.71 ± 0.60
		1% + ITS	2.41 ± 2.49	1.70 ± 0.55	5.25 ± 1.53
Tendon Fibroblasts	MSCM	10%	2.69 ± 1.63	3.04 ± 1.47	6.50 ± 1.73
		2%	2.13 ± 0.84	2.07 ± 0.22	8.13 ± 6.06
		1%	1.31 ± 0.70	1.19 ± 0.22	3.80 ± 1.33
		1% + ITS	1.29 ± 1.13	1.45 ± 0.26	4.39 ± 1.48
Adipose-derived Cells		10%	3.87 ± 0.99	4.20 ± 1.20	8.02 ± 1.63
		2%	3.69 ± 2.23	4.00 ± 0.55	7.59 ± 1.50
		1%	1.52 ± 1.31	2.62 ± 0.80	6.53 ± 0.40
		1% + ITS	2.18 ± 0.77	2.83 ± 1.00	7.89 ± 3.19

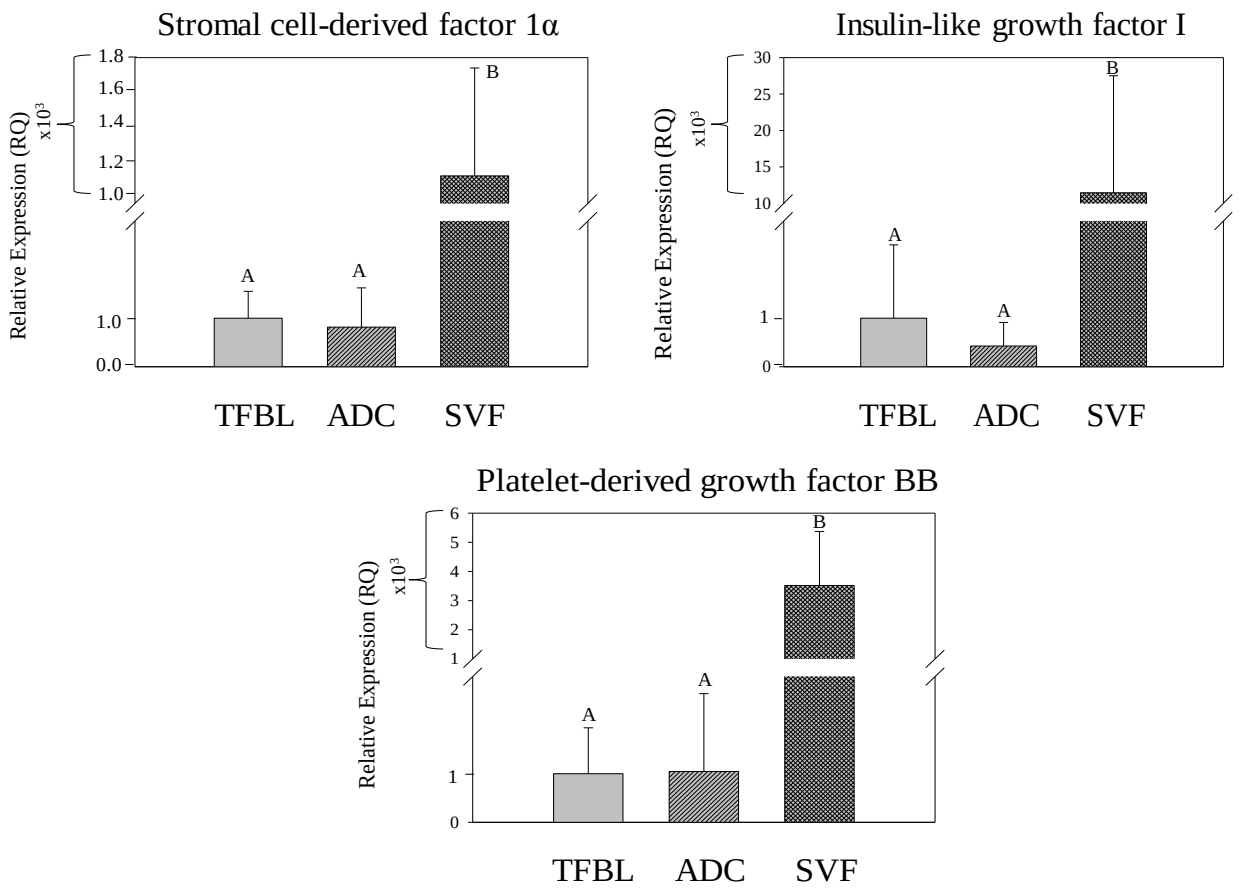

Figure 1. Relative gene expression of growth factors. Expression for stromal cell derived factor 1α (top left), insulin like growth factor I (top right), and platelet derived growth factor-BB (bottom) for tendon fibroblasts (TFBL) and adipose-derived cells (ADC) cultured for 24 hours and stromal vascular fraction (SVF) cells normalized to expression by TFBL. Letters indicate statistically significant differences between groups ($p \leq 0.0001$). Mean ± SD.

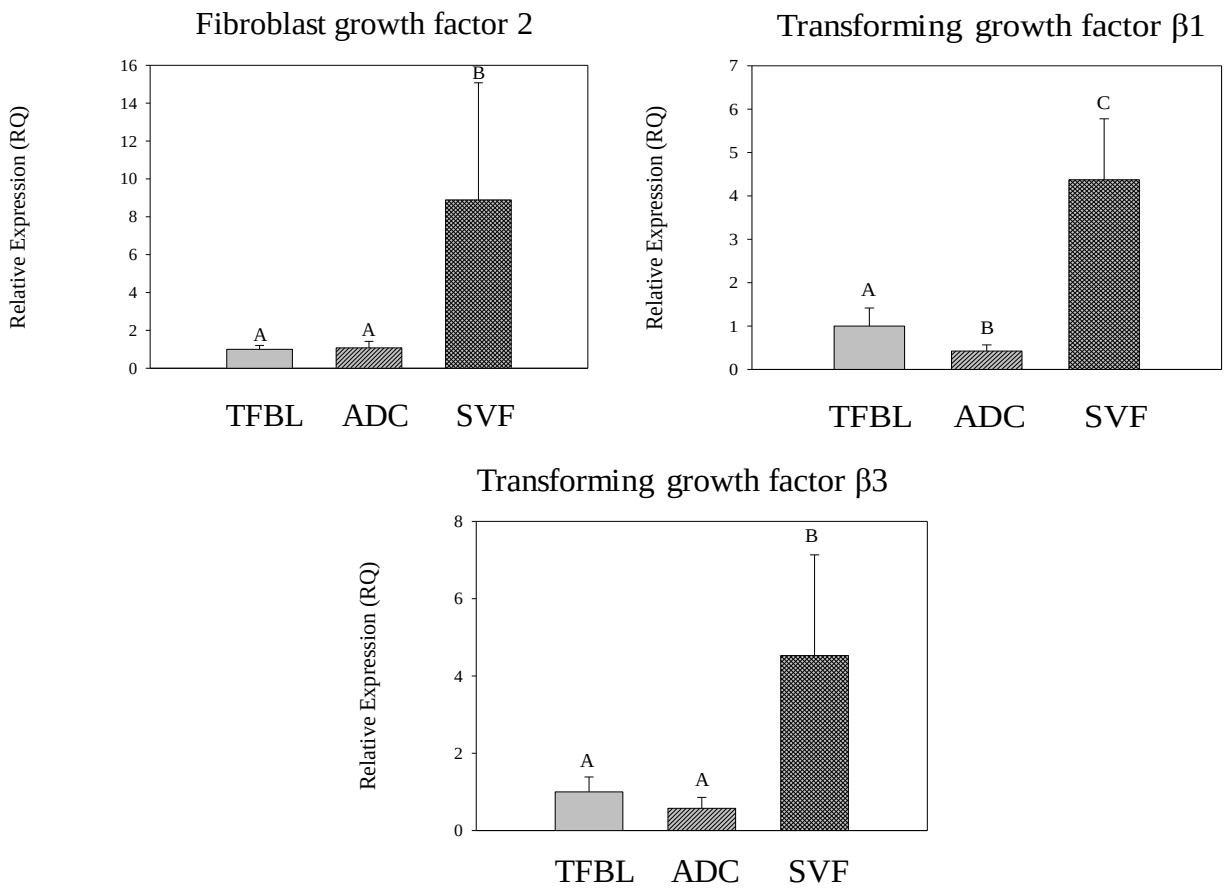

Figure 2. Relative gene expression of growth factors. Expression for fibroblast growth factor-2 (top left), transforming growth factor-β1 (top right), and transforming growth factor-β3 (bottom) for tendon fibroblasts (TFBL) and adipose-derived cells (ADC) cultured for 24 hours and stromal vascular fraction (SVF) cells normalized to expression by TFBL. Letters indicate statistically significant differences between groups ($p \leq 0.0001$). Mean ± SD.

Co-culture:

Gene expression for collagen types I and III, COMP, and decorin, varied significantly based on the cell type and seeding density in the Transwell® inserts (Figures 3 and 4). Gene expression for collagen type I was significantly greater in TFBLs co-cultured with 2-4x10^3 ADCs per insert compared to those cultured with 0 ADCs or 4x10^3 NIH/3T3 cells per insert (p=0.0001) (Figure 3A). Gene expression for collagen type III and COMP were significantly greater in TFBLs co-cultured with 1-4 x 10^3 ADCs per insert compared to those cultured with 0 ADCs per insert (p=0.0001 and p=0.0030, respectively) (Figures 3B and 4A). Expression of collagen type III and COMP by TFBLs co-cultured with 4x10^3 3T3 cells per insert was greater than in TFBLs co-cultured with 0 ADCs. There were no significant differences in gene expression for collagen types I and III, COMP, or decorin between the four seeding densities of ADCs. Co-culture of TFBLs with 1 and 4x10^3 ADCs per insert resulted in significantly higher expression of decorin than co-culture with 0 ADCs per insert (p=0.0005) (Figure 4B).

There were no significant differences between cell type or seeding density for DNA (p=0.8611), GAG (p=0.0383) or collagen (p=0.3189) content of cell monolayers and GAG (p=0.4169) or collagen (p=0.9656) content of culture media using the 0.4 µm pore size Transwell® inserts (Figures 5-7). DNA content was significantly higher in TFBLs co-cultured with high serum medium (MSCM with 15% FBS) than in TFBLs cultured directly in control medium (MSCM with 5% FBS) when using the 3.0 µm pore size Transwell® inserts (Figure 8) (p=0.05).

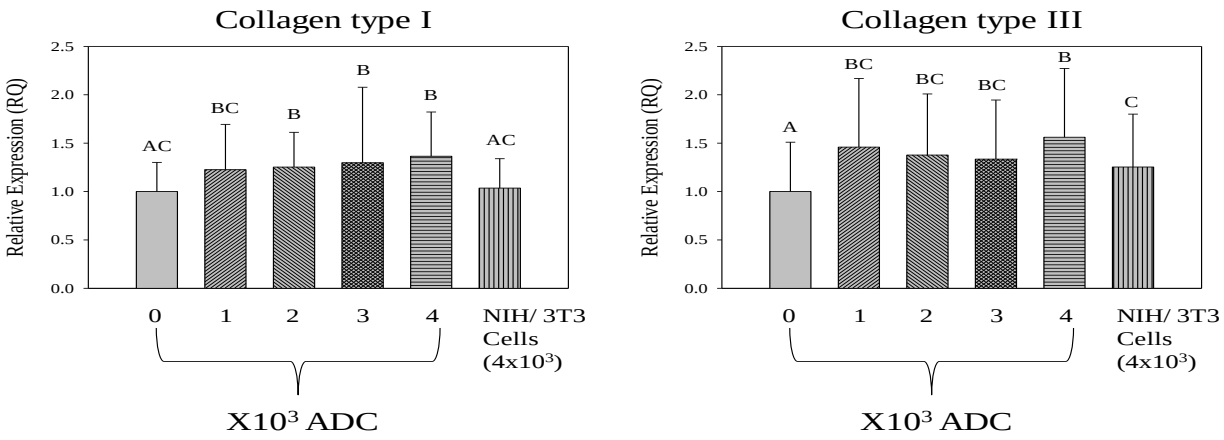

Figure 3. Relative gene expression of matrix proteins. Expression of collagen type I (left) and collagen type III (right) in monolayers of tendon fibroblasts co-cultured for 73 hours with 0-4x10^3 adipose-derived cells (ADC) or 4x10^3 NIH/3T3 cells seeded per insert. Expression normalized to 0x10^3 ADCs. Letters indicated statistically significant differences between groups (p=0.0001). Mean ± SD.

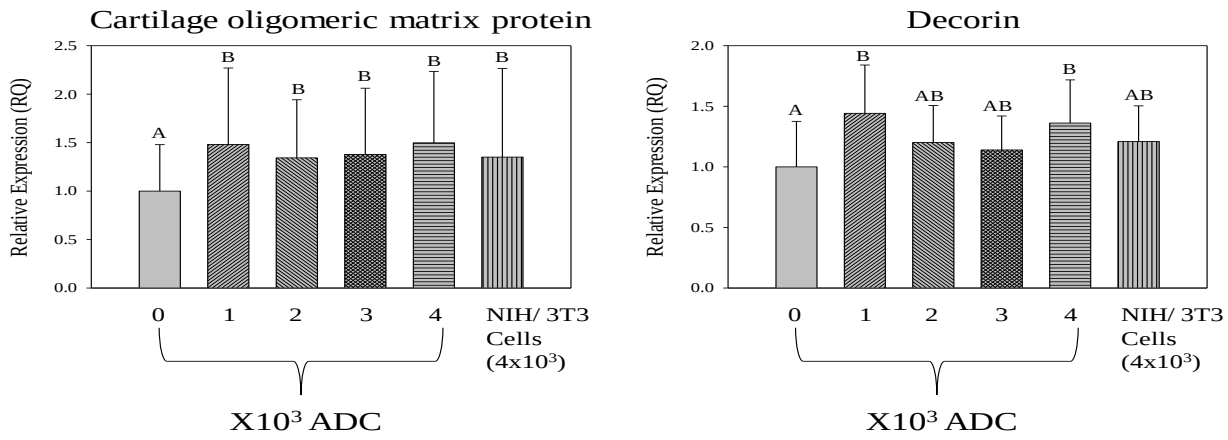

Figure 4. Relative gene expression of matrix proteins. Expression of cartilage oligomeric matrix protein (left) and decorin (right) in monolayers of tendon fibroblasts co-cultured for 73 hours with 0-4x10^3 adipose-derived cells (ADC) or 4x10^3 NIH/3T3 cells seeded per insert. Expression normalized to 0x10^3 ADCs. Letters indicated statistically significant differences between groups (p=0.0030 (COMP), p=0.0005 (Decorin)). Mean ± SD.

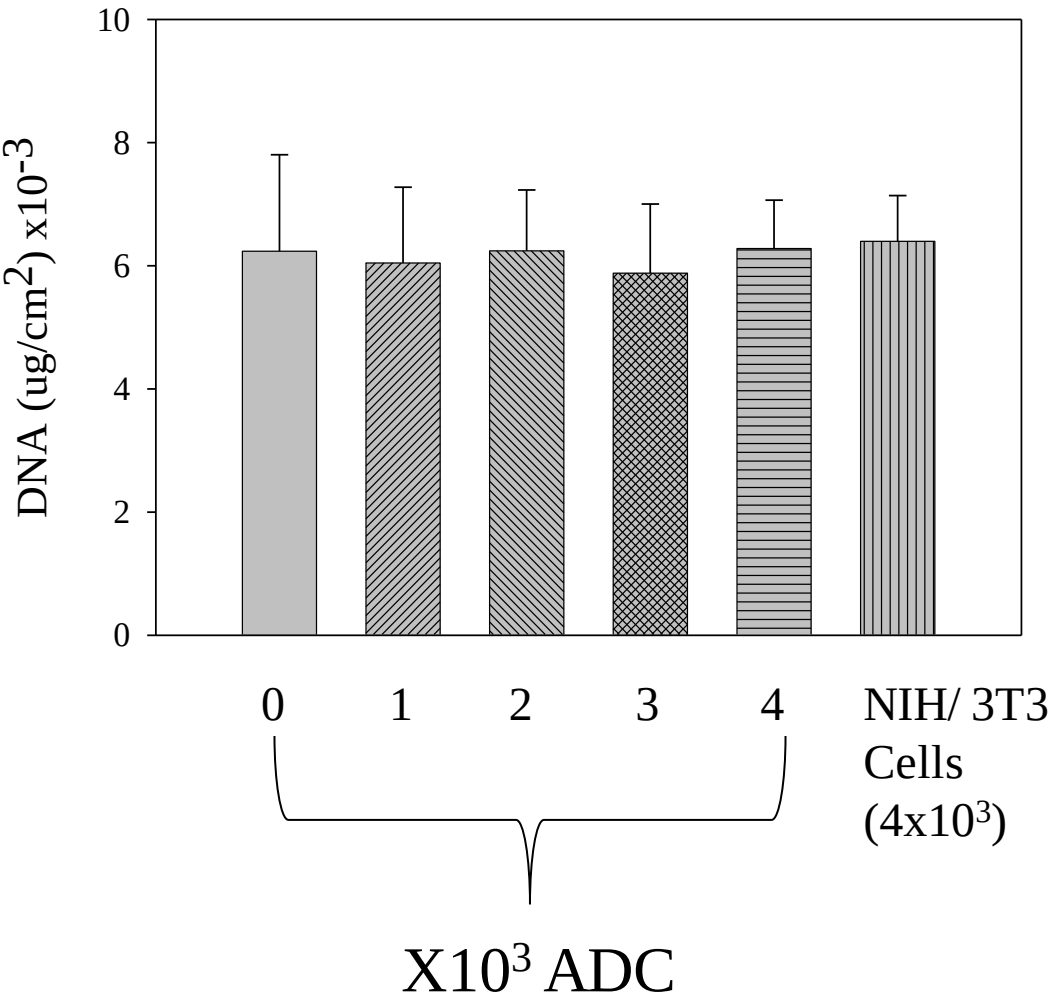

Figure 5. DNA content following co-culture. DNA content in monolayers of tendon fibroblasts co-cultured for 73 hours with 0-4x10^3 adipose-derived cells (ADC) or 4x10^3 NIH/3T3 cells seeded per insert. DNA content normalized to 0x10^3 ADCs. There were no statistically significant differences between treatment groups (p=0.8611). Mean ± SD.

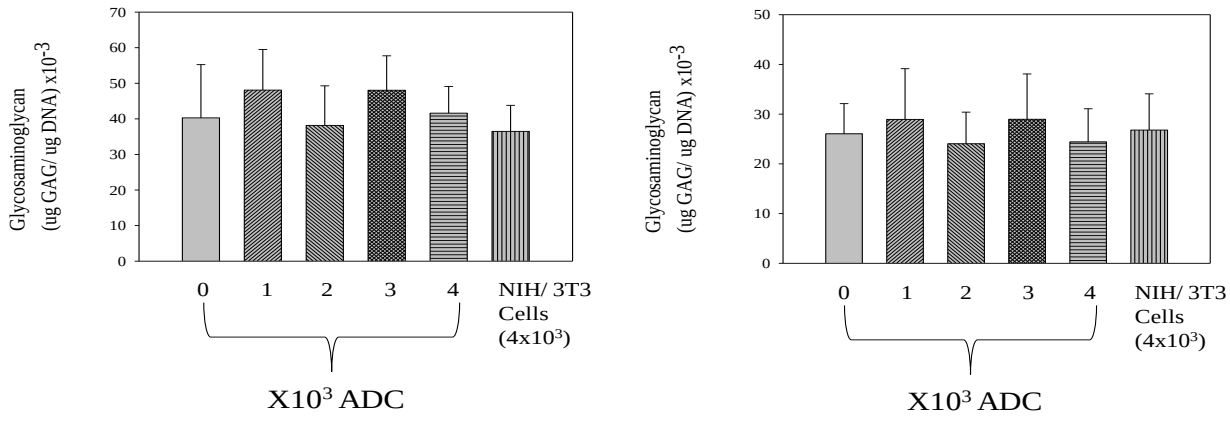

Figure 6. Glycosaminoglycan content following co-culture. Glycosaminoglycan production in monolayers of tendon fibroblasts (TFBL) (left) and their conditioned medium (right) co-cultured for 73 hours with 0-4x10^3 adipose-derived cells (ADC) or 4x10^3 NIH/3T3 cells seeded per insert. There were no statistically significant differences between treatment groups (p=0.0383 (monolayers), p=0.4169 (conditioned medium)). Mean ± SD.

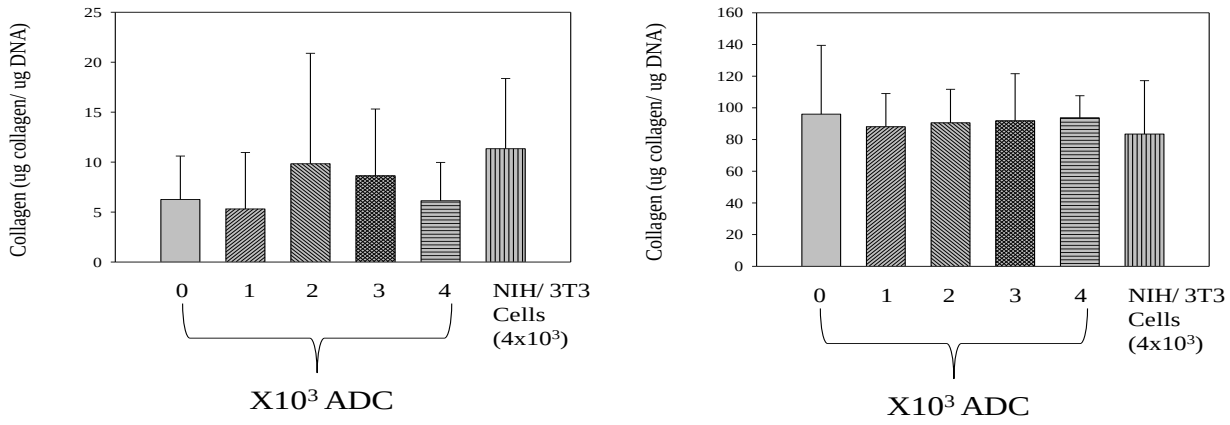

Figure 7. Collagen content following co-culture. Collagen production in monolayers of tendon fibroblasts (TFBL) (left) and their conditioned medium (right) co-cultured for 73 hours with 0-4x10^3 adipose-derived cells (ADC) or 4x10^3 NIH/3T3 cells seeded per insert. There were no statistically significant differences between treatment groups (p=0.3189 (monolayers), p=0.9656 (conditioned

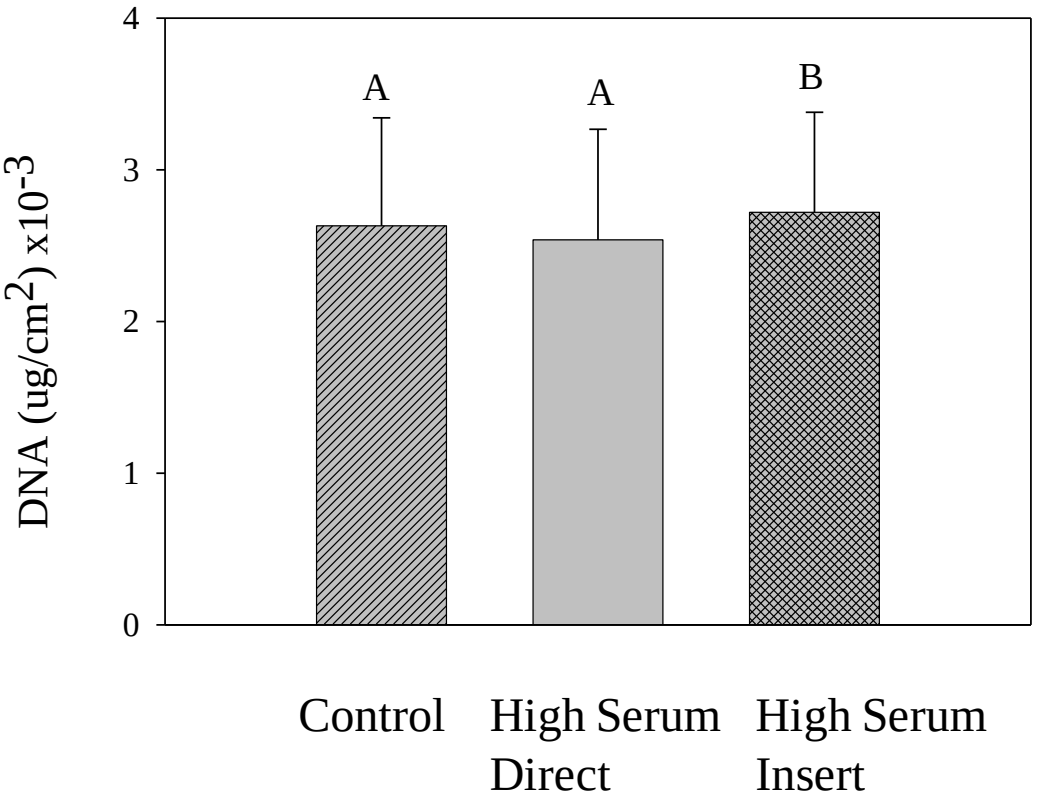

Figure 8. DNA content. DNA content in monolayers of tendon fibroblasts after 24 hours of culture directly in control medium (Mesenchymal stem cell media with 5% FBS), directly in high serum medium (Mesenchymal stem cell media with 15% FBS) or co-culture with high serum medium (Mesenchymal stem cell media with 5% FBS) in 3.0 μm pore inserts. Letters indicate statistically significant differences between groups (p=0.0431).

Microchemotaxis:

There were a significantly greater number of migrated cells following culture in the conditioned medium from the higher seeding density of ADCs compared to conditioned medium from the lower seeding density ($p=0.0027$) (Figures 9 and 10). There were no significant differences between the conditioned media from the high and low seeding densities of TFBLs or between the high or low density media from TFBLs and ADCs.

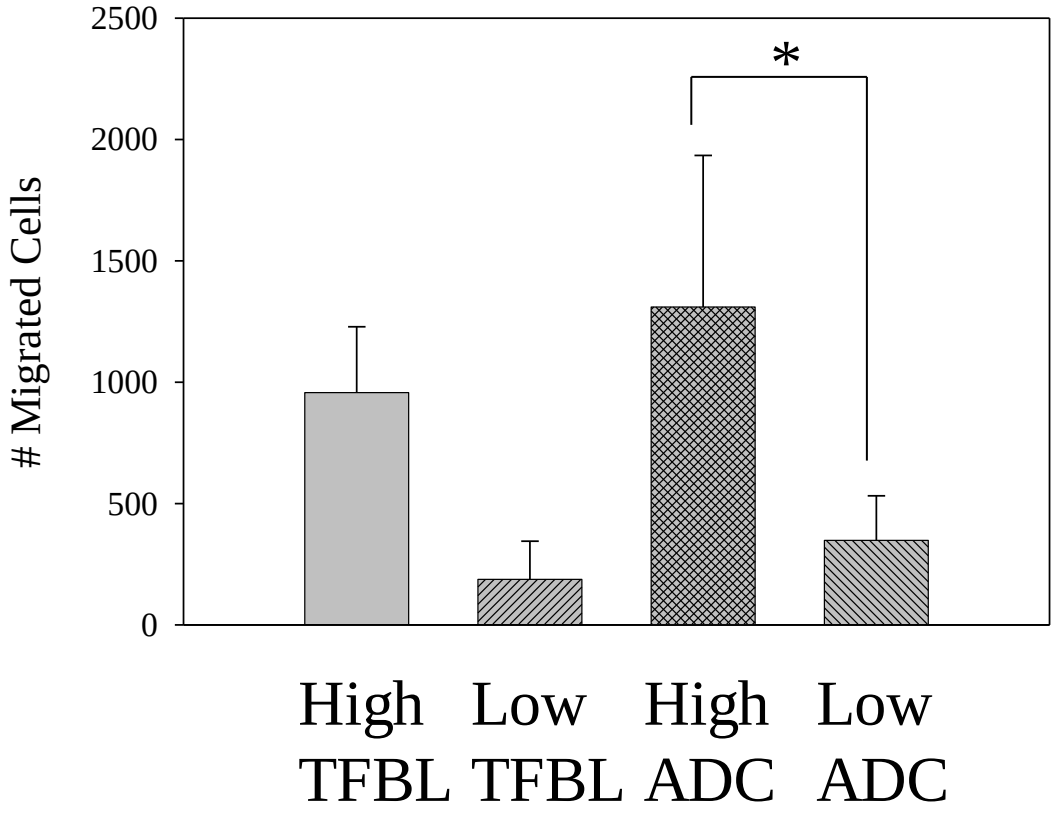

Figure 9. Cell Migration. Number of cells that migrated to tendon fibroblast (TFBL)- or adipose-derived cell (ADC)-conditioned media from high and low seeding densities. * indicates statistically significant difference between groups (p=0.0027).

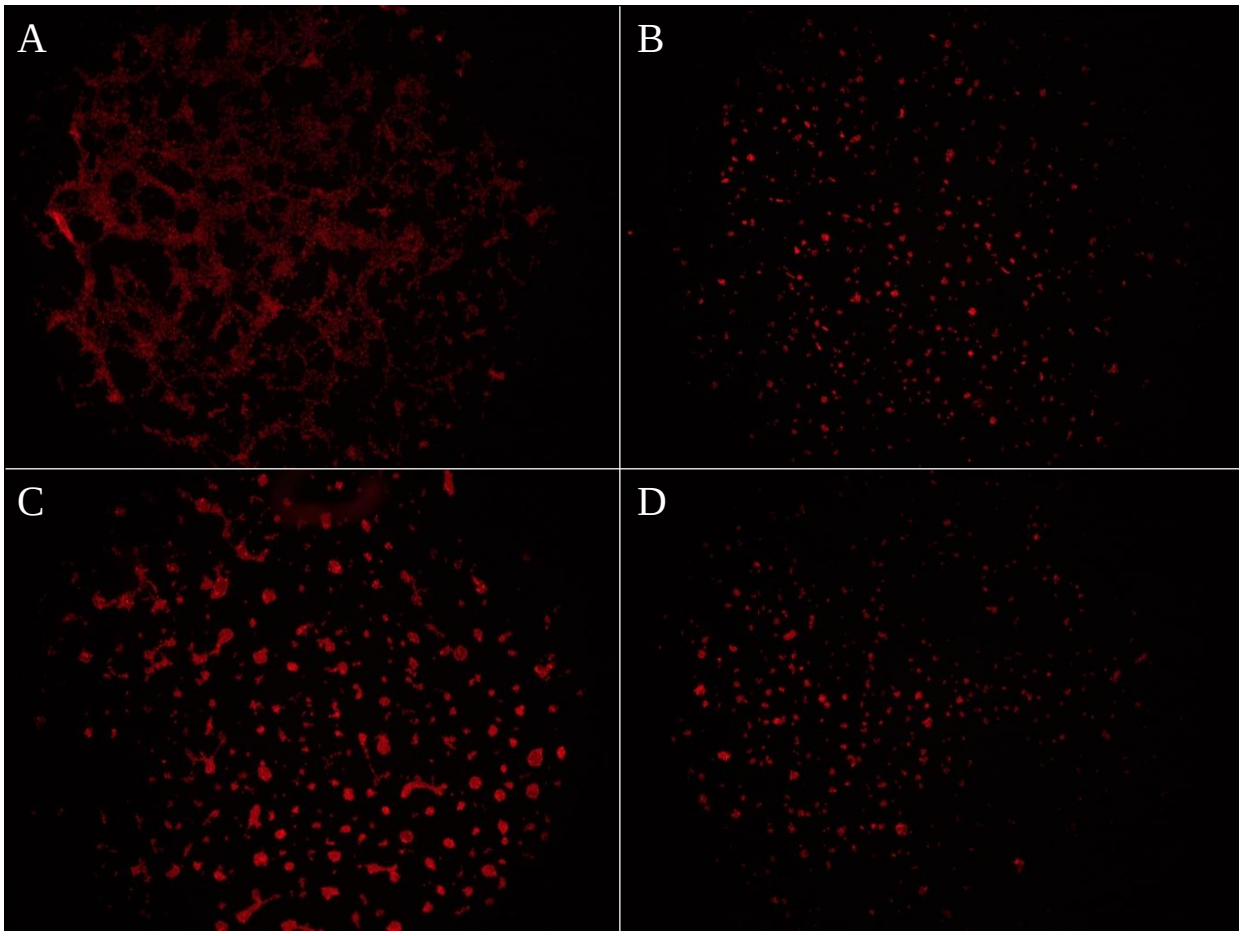

Figure 10. Representative images from microchemotaxis assay. Images of propidium iodide-stained polycarbonate membranes from microchemotaxis assay from a single horse demonstrating the seeding density-dependent cell migration. Left panels (A,C) are from high seeding density conditioned media and right panels (B,D) are from low seeding density conditioned media. Top panels (A,B) are adipose-derived cell-conditioned media and bottom panels (C,D) are tendon fibroblast-conditioned media.

Discussion

The overarching aim of this project was to provide evidence that ADCs contribute to tendon regeneration by producing soluble factors capable of indirectly affecting TFBLs in a trophic manner. Experiments were designed to examine growth factor, cytokine, and chemotactic factor production by ADCs, and to investigate the ability of soluble factors produced by ADCs to increase matrix protein production by TFBLs.

Quantification of growth factors in the conditioned media from the various cell types proved complicated because of the relatively large amounts of growth factors present in the basal media, the relatively small contribution that the nascent growth factors produced by the cells made to the media, and the consumption of growth factors by the growing cells. The media samples incubated without cell monolayers (cell-free media) were designed to provide baseline growth factor protein values that would be subtracted from the levels in the TFBL- and ADC-conditioned media. However, after completion of the assays, normalization in this manner was not possible due to the magnitude of the difference in growth factor levels between the cell-free media and the conditioned media, with the levels in the conditioned media being much lower. The magnitude of difference between the controls and the conditioned medium was especially noticeable after normalizing growth factor levels in the conditioned medium to cell number. There are several potential explanations for the low levels of growth factors present in the conditioned media. It is possible that there was a lack of cross-reaction between the human antibodies in the ELISA kits and our equine samples. However, several of the assays have previously been validated for use in the horse (IGF-I, PDGF-BB, TGF-β1) and there is considerable sequence homology between horses and humans for the growth factors and cytokines we evaluated [18, 41-43]. For example, equine TGF-B1 and IGF-I nucleotide

sequence is 91% and 88% homologous with the human nucleotide sequence, respectively [44, 45]. In addition, as a positive control serum samples were analyzed for each ELISA kit, and homogenized equine skin supernatant was analyzed for TGF-β3. Therefore, a lack of antibody cross-reaction does not represent a satisfactory explanation for the low levels of growth factors in the samples.

Growth factor protein levels in conditioned media were lower than expected at both three and six days, suggesting the possibility that the time points chosen for this study may have been too late for the detection of growth factors in the conditioned medium. However, these time points were selected based on published studies and were chosen to give the cells time to condition the media and allow for the accumulation of growth factors [46-48]. Tendon fibroblasts and ADCs do express growth factors relevant to tendon healing as shown by the gene expression data; however, the rate at which the cells use the growth factors for proliferation and matrix production may exceed that at which it is produced, precluding detection in conditioned media via ELISA. It is also possible that the presence of the growth factors in the media and in FBS in excess amounts initiated a negative feed-back loop, whereby the cells were able to obtain their needed growth factors from the media and thus did not produce the growth factors in the expected quantities. Additionally, the possibility exists that low level growth factor production is biologically appropriate for both TFBLs and ADCs. High levels of growth factors may not be necessary to promote tendon regeneration. Indeed, the level of growth factor production necessary to initiate biologically relevant anabolic effects is unknown, and likely varies based on the individual growth factor.

The use of specialized TFBL and ADC growth media for each cell type was designed to maintain the cells in their 'ideal' environment with the thought that they would be less likely to

be secreting anabolic growth factors if cultured in sub-optimal conditions. However, the use of two different formulations of basal media and percentages of serum made it difficult to draw comparisons between the TFBLs and ADCs. Both cell types require serum for growth, which represents a confounding factor when trying to quantify protein levels. Additionally, there is a high degree of variability between individual lots of fetal bovine serum and its various vendors with respect to growth factor concentrations [49]. With these facts in mind, the logical solution was to redesign the experiments so that both cell types were grown in the same type of medium and concentration of FBS to allow direct comparisons between groups.

Troubleshooting the ELISA assays and identifying the need for much lower basal concentrations of growth factors from the medium itself prompted the investigation of utilizing low serum culture conditions for the TFBLs and ADCs. The results of this media optimization experiment led us to choose MSCM with 2% FBS as the most appropriate medium for repeating the growth factor protein quantification. Adipose-derived cell basal medium (MSCM) was chosen because of concerns that the ADCs might differentiate in less than optimal medium, whereas this was not a concern with the fully differentiated TFBLs. Cells grown in 1% FBS and 1% FBS with ITS demonstrated undesirable morphological alterations, including partial detachment of the monolayer and colony formulation, indicating that these media formulations were potentially not suitable for growth of these cells. Specifically, after monolayer detachment, both cell types changed their morphology from spindle-shaped to an elongated, linear morphology, and appeared to stretch across the gap in the monolayer in a linear fashion. This morphology was not observed under any other culture conditions. Despite the decrease in serum to 2% FBS, determination of cell-sourced growth factor levels remained masked by the presence of excess growth factors in the serum. Accurate quantification of growth factors via ELISA may

require the development and optimization of serum-free culture conditions for TFBLs and ADCs.

As an alternative to quantifying growth factor protein levels, growth factor gene expression was quantified instead. Both TFBLs and ADCs expressed mRNA for SDF-1α, IGF-I, PDGF-BB, FGF-2, TGF-β1, and TGF-β3. Of particular interest was the vastly higher expression in SVF cells compared to either TFBLs or ADCs. These differences that exist between the SVF cells and the ADCs suggests that the process of cell expansion using standard *in vitro* tissue culture techniques, including the use of serum and a two-dimensional tissue culture system, results in metabolic changes in the cells and their patterns of gene expression. While it is standard practice to culture cells on tissue culture-coated plastic ware, these conditions do not replicate the *in vivo* environment, or niche, in which the cells normally reside nor do they provide the cells with similar biomechanical cues. This may be particularly important with respect to cells in the flexor tendon, which experiences cyclical loading as a major component of its daily function. The absence of biomechanical and biochemical signals could have a profound negative effect on multiple parameters and could explain the vast difference in growth factor gene expression between the cultured and un-cultured cells. Although it is not surprising that the mere culture of the cells, even for as few passages as possible, would alter gene expression, the magnitude to which the changes occurred is surprising. The clinical importance of this finding is difficult to fully ascertain based on the results of this study, and the mechanism(s) by which this may occur is unknown.

Clinically, fresh, uncultured, SVF cells are used for intralesional injection in the majority of cases because of their ease of procurement and the speed with which they are available for injection. In a smaller number of cases, SVF cells are being expanded in culture to increase the

number of cells available for injection in the short term or to provide additional doses of cells for later treatments without the need for repeated harvest and processing of adipose tissue. The use of culture-expanded ADCs from the SVF is occurring in an increasing number of cases and this, when considered in light of our gene expression data, imparts a critical need to further characterize the mechanism by which these changes may be occurring, whether these changes in gene expression are clinically important, and whether ADCs revert to the higher levels of growth factor expression following implantation into the mechanically, biologically, and structurally instructive environment of the *in vivo* tendon. A critical point of consideration for clinicians is that cells expanded in culture for multiple passages may differ in their ability to direct a regenerative response. In addition to differences in growth factor gene expression, cultured cells and uncultured SVF may differ in matrix protein gene expression and the production of chemotactic factors.

The co-culture assay was designed to determine if TFBLs up-regulate matrix protein production and gene expression in response to ADC-conditioned medium. Analysis of the co-culture data (0.4 μm pores) found that there were modest increases in gene expression for the matrix proteins investigated; however, these differences were inconsistent across the various seeding densities of ADCs and failed to demonstrate the expected dose response. There was no consistent pattern with respect to gene expression in comparison to response to conditioned medium from NIH/3T3 cells. Based on the results on the gene expression data, there is some minor evidence that ADCs may produce soluble factors that could influence the gene expression for extracellular matrix proteins important in tendon healing; however, the magnitude of the response suggests that additional studies using an alternative experimental design may be necessary to further define this question.

No statistically significant differences were found for DNA, GAG, or collagen content of either the monolayers of the culture medium. The failure to see the expected increase in cell proliferation could be due to a true lack of response in the cells or due to the specific parameters of the experimental design. The former would include the possibility that the hypothesized soluble factors were not produced by the ADCs and/or that the TFBLs were not responsive to those that were produced. It is also possible that the hypothesized bioactive factors were produced by the ADCs, but did not diffuse from the inserts to come in contact with the TFBLs as a result of the selection of an inappropriate pore size for the inserts. Based on the study design, it is also possible that a differences in cell proliferation was produced but that due to less than optimal seeding density and/or harvest time point this difference was not detected. Seeding the cells too densely and harvesting after the monolayers reached confluence could mean that the cells were no longer in the log phase of growth at the time of harvest and that early differences were negated by a static endpoint for detection. Using a shorter time point and seeding at a lower density would allow the monolayers to be harvested when the cells are still in the log phase and might detect the expected differences in cell proliferation. Co-culture for a longer period of time may be necessary to produce the expected difference in TFBL GAG and collagen production by allowing for greater accumulation of matrix proteins.

The lack of sizeable differences for the co-culture experiment using the 0.4 μm pore size Transwell® inserts prompted us to examine whether the TFBLs were indeed capable of responding to the soluble factors we hypothesized were present in the co-culture design. Additionally, because the original choice of pore size (0.4 μm) was potentially too small to allow for passage of soluble factors produced by the ADCs, a supplemental experiment was designed using PBS in the lower wells and methylene blue dye in the inserts (0.4 and 3.0 μm pores) to

visually check diffusion from the inserts to the lower wells. The 0.4 μm pore size is reportedly suitable for drug delivery applications and was therefore chosen for these studies to allow passage of soluble factors without allowing cell migration [33, 34]. Diffusion rates using the 3.0 μm pores appeared to be greater than when using the 0.4 μm pores, and equilibration between the lower wells and inserts appeared to be faster using the 3.0 μm pores. These differences in diffusion and equilibration rates supported the need for a larger pore size in the co-culture scenario.

A portion of the co-culture was repeated using 3.0 μm pores to test for improved diffusion by measuring DNA content of TFBLs. Medium with a high serum content (MSCM with 15% FBS) was chosen as a positive control because serum is a known source of anabolic growth factors. Our results using the 3.0 μm pores showed a significant difference in DNA content when TFBLs were incubated with high serum (MSCM with 15% FBS) in the 3.0 μm insert versus incubation directly in control serum (MSCM with 5% FBS). This indicates that the TFBLs are capable of responding to soluble factors and that there is diffusion from the insert using 3.0 μm pores, thereby validating the co-culture system. Validation of the co-culture assay using 3.0 μm pores suggests that the lack of expected response by the TFBLs with respect to DNA, GAG, and collagen content when using the 0.4 μm pores may very well have been due in part to lack of diffusion due to the small pore size. Repetition of the full co-culture experiment using 3.0 μm pore inserts is indicated.

Gene expression of collagen types I and III, COMP, and decorin was modestly increased in TFBLs co-cultured with ADCs as compared to co-culture without ADCs. Thus TFBLs are capable of modifying their gene expression in response to factors present in ADC-conditioned medium. While the increase in gene expression was modest, these results suggest that *in vivo*

application of ADCs might promote tendon regeneration via the production of soluble factors that direct TFBLs to up-regulate expression of matrix proteins. Changes in gene expression may have been more pronounced with a larger pore size for the inserts.

The use of NIH/3T3 cells as an additional control provided a useful point of comparison to the ADCs. Collagen type I gene expression was greater in TFBLs co-cultured with 2-4x10^3 ADCs than in TFBLs co-cultured with 4x10^3 NIH/3T3 cells. Co-culture with the highest dose of ADCs (4x10^3) resulted in the TFBLs having higher type III collagen gene expression than when cultured with the 3T3 cells, and co-culture of the TFBLs with the 3T3 cells increased TFBL gene expression as compared to co-culture with the cell-free control. These results indicate that co-culture with ADCs increases gene expression compared to co-culture with a differentiated cell type. This is an important point as it supports the idea that ADCs have a different secretory profile than differentiated cells. Based on the gene expression data, we feel that repeating the co-culture using TFBLs and the SVF cells would yield the expected results. The differences in growth factor gene expression between the cultured cells and the SVF cells support the idea of repeating these experiments with the SVF cells. As the primary clinical application of this technology for equine tendon injuries is SVF cells and not cultured ADCs, the effects of the SVF cells on TFBL matrix protein production is of particular relevance.

The microchemotaxis assay was designed to indirectly provide evidence that ADC-conditioned medium contains chemotactic factors capable of inducing ADC migration. The production of chemotactic factors by ADCs *in vivo* is clinically important, as these factors could recruit endogenous stem cells to the site of injury. The recruitment of endogenous stem cells could be a major mechanism by which ADCs could promote tissue healing. Endogenous cells might be recruited in substantial numbers in response to implantation of only a small number of

ADCs and the survival and retention at the site of injury for endogenous cells might be significantly higher than seen with implanted cells. The statistically significant difference in the number of migrated ADCs between the high and low dose ADC-conditioned medium demonstrates that ADC-conditioned media contains chemotactic factors that induce migration in a dose dependent manner. The ideal number of stem cells to inject in a clinical scenario has yet to be determined, but this result suggests that the production of chemotactic factors is proportional to cell number. The lack of a statistically significant dose response with the TFBL-conditioned medium was due to the fact that only three of the six horses had cells migrate to the low dose conditioned medium. If more horses had cells migrate to the low dose TFBL-conditioned medium, a significant difference may have been detected, as the p-value for the comparison between the high and low TFBL doses ($p=.0626$) was close to reaching statistical significance. Additionally, inclusion of a higher number of sampled horses may have also increased the power of the study to detect a true difference.

The absence of a significant difference in the number of ADCs that migrated to TBFL-conditioned medium versus ADC-conditioned medium raises the possibility that the production of chemotactic factors is similar between the two cell types. In these experiments TFBLs were included as a control cell population. Gene expression data indicates that TFBLs are producing mRNA for growth factors and cytokines relevant to tendon healing and the data from the microchemotaxis experiment shows that TFBLs are producing chemotactic factors. These results suggest that another cell type may have been a more optimal control for these studies than TFBLs and that TFBLs should be further characterized to investigate their potential roles in tendon healing. It is possible that the population of TFBLs isolated via collagenase digest from

the SDFT does contain tendon progenitor cells and/or that TFBLs are capable of making a greater than reported contribution to tissue healing.

Another possibility worth exploring is that the cells cultured for these experiments may not be stem cells. The application of flow cytometry to equine cells has been hampered by a lack of equine specific antibodies [50]. This means that while the cell surface marker profile of ADCs from other species has been fully characterized (e.g. humans and mice), this is not true for equine ADCs. Data from other species cannot be extrapolated directly to the horse as there is considerable inter-species variation and can only be used as an initial basis on which to evaluate the equine cells. Based on the current literature, the evidence suggests that the plastic-adherent cells cultured from the SVF for use in these experiments are likely to be ADCs [4, 22, 51]. However, further characterization of these cultured cells is necessary to confirm that they are indeed stem cells.

The assumption has been made by many researchers that the adipose-derived (stem) cells found in the SVF make a greater contribution to tendon healing than the other cell types present in the SVF. However, it is possible that the heterogeneity of the SVF cell population is an asset, with the various cell types working synergistically to contribute to tendon regeneration. The combination of ADSCs, pericytes, fibroblasts, immune cells, and endothelial cells interacting with the tendon ECM may be what results in improved clinical outcomes. Providing a physiologic mixture of cells may be critical to promoting regeneration over repair. It has also been suggested that the pericytes in the SVF may be the true 'power player' and MSCs may in fact be pericytes that have migrated from the vasculature to sites of injury [52]. These concepts provide intriguing questions for future investigations.

In summary, evidence has been presented to support the hypothesis that ADCs produce soluble factors in a trophic manner. It has been shown that ADCs do produce mRNA for SDF-1α, IGF-I, PDGF-BB, FGF-2, TGF-β1, and TGF-β3. Each of these growth factors and cytokines is capable of having an anabolic effect on tendon healing. Growth factors produced by ADCs could potentially modulate expression/production of these factors by TFBLs *in vivo*, which would be particularly beneficial when endogenous TFBL expression is low. Documentation has been provided that ADCs release chemotactic factors into their conditioned medium that induce migration of ADCs and that this migration follows a dose response. If this *in vitro* result reflects the response that occurs *in vivo*, then ADCs could contribute to tendon healing by recruiting endogenous stem cells to the site of injury. Finally, evidence has been provided that co-culture of TFBLs and ADCs results in a modest increase in expression of matrix protein genes by TFBLs. *In vivo* it is possible that ADCs could modulate the production of collagen by endogenous TFBLs and thus modulate the ratio of type I to type III collagen that is disturbed during injury and fails to return to normal. Returning type I collagen levels to those of normal tendon might increase the tensile strength of the tendon and decrease the risk of re-injury. Cartilage oligomeric matrix protein is an important mediator of collagen fibrillogenesis. If ADCs could increase expression of COMP by endogenous TFBLs this may promote fibrillogenesis, which would also increase the strength of the healing tendon. Taken together, the results of this study suggest that while considerable work is yet to be done to determine the mechanism by which equine ADCs and the SVF contribute to tendon healing, at least a portion of the beneficial effect of these cells appears to derive from the secretion of soluble factors in a trophic manner.

References

1. Goodship, A.E., H.L. Birch, and A.M. Wilson, *The pathobiology and repair of tendon and ligament injury.* Vet Clin North Am Equine Pract, 1994. **10**(2): p. 323-49.
2. Patterson-Kane, J.C. and E.C. Firth, *The pathobiology of exercise-induced superficial digital flexor tendon injury in Thoroughbred racehorses.* Vet J, 2009. **181**(2): p. 79-89.
3. Smith, R.K.W., *Pathophysiology of Tendon Injury*, in *Diagnosis and management of lameness in the horse,* M.W. Ross, Dyson, S.J., Editor. 2011, Elsevier Saunders: St. Louis. p. 694-706.
4. Zuk, P.A., et al., *Human adipose tissue is a source of multipotent stem cells.* Mol Biol Cell, 2002. **13**(12): p. 4279-95.
5. Caplan, A.I. and S.P. Bruder, *Mesenchymal stem cells: building blocks for molecular medicine in the 21st century.* Trends Mol Med, 2001. **7**(6): p. 259-64.
6. Smith, R.K., *Mesenchymal stem cell therapy for equine tendinopathy.* Disabil Rehabil, 2008. **30**(20-22): p. 1752-8.
7. Fortier, L.A. and R.K. Smith, *Regenerative medicine for tendinous and ligamentous injuries of sport horses.* Vet Clin North Am Equine Pract, 2008. **24**(1): p. 191-201.
8. Vidal, M.A., et al., *Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells.* Vet Surg, 2007. **36**(7): p. 613-22.
9. Vidal, M.A., et al., *Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity.* Vet Surg, 2006. **35**(7): p. 601-10.
10. Alves, A.G., et al., *Cell-based therapies for tendon and ligament injuries.* Vet Clin North Am Equine Pract, 2011. **27**(2): p. 315-33.

11. Caplan, A.I. and J.E. Dennis, *Mesenchymal stem cells as trophic mediators.* J Cell Biochem, 2006. **98**(5): p. 1076-84.

12. Metallo, C.M., et al., *Engineering the stem cell microenvironment.* Biotechnol Prog, 2007. **23**(1): p. 18-23.

13. Richardson, L.E., et al., *Stem cells in veterinary medicine--attempts at regenerating equine tendon after injury.* Trends Biotechnol, 2007. **25**(9): p. 409-16.

14. Ribitsch, I., et al., *Basic Science and Clinical Application of Stem Cells in Veterinary Medicine.* Adv Biochem Eng Biotechnol, 2010.

15. Richardson, L.E. *Extracellular Matrix Cues for Mesenchymal Stem Cell Differentiation*. in *Proceedings of the 53rd Orthopaedic Research Society*. 2007.

16. Dahlgren, L.A., H.O. Mohammed, and A.J. Nixon, *Temporal expression of growth factors and matrix molecules in healing tendon lesions.* J Orthop Res, 2005. **23**(1): p. 84-92.

17. Theoret, C.L., et al., *Expression of transforming growth factor beta(1), beta(3), and basic fibroblast growth factor in full-thickness skin wounds of equine limbs and thorax.* Vet Surg, 2001. **30**(3): p. 269-77.

18. Schnabel, L.V., et al., *Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons.* J Orthop Res, 2007. **25**(2): p. 230-40.

19. Menon, L.G., et al., *Differential gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells.* Stem Cells, 2007. **25**(2): p. 520-8.

20. Dahlgren, L.A. *Fat-Derived Mesencymal Stem Cells for Equine Tendon Repair*. in *Veterinary Stem Cell Consortium* 2009. Leipzig, Germany: Regenerative Medicine Supplement 2.

21. Nixon, A.J., et al., *Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis.* Am J Vet Res, 2008. **69**(7): p. 928-37.

22. Sand, T. *Preliminary Characterization of Canine and Equine Cell Preparations Obtained by Enzymatic Digestion of Adipose Tissue*. in *International Federation for Adipose Therapeutics and Science*. 2006.

23. Halasz, K., et al., *COMP acts as a catalyst in collagen fibrillogenesis.* J Biol Chem, 2007. **282**(43): p. 31166-73.

24. Harman, R., Cowles, B., Orava, C., et al., *A Retrospective Study of 66 Cases of Tendon Injury in the Equine Treated with Adipose-Derived Stem and Regenerative Cell Therapy*. 2006, Vet-Stem Inc.

25. Jung, H., et al., *Transforming growth factor-beta 1 in adipose derived stem cells conditioned medium is a dominant paracrine mediator determines hyaluronic acid and collagen expression profile.* Cytotechnology, 2011. **63**(1): p. 57-66.

26. Cissell, J.M., S.C. Milton, and L.A. Dahlgren, *Investigation of the effects of prostaglandin E on equine superficial digital flexor tendon fibroblasts in vitro.* Vet Comp Orthop Traumatol, 2010. **23**(6): p. 417-23.

27. Zvonic, S., et al., *Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis.* Mol Cell Proteomics, 2007. **6**(1): p. 18-28.

28. Chen, L., et al., *Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing.* PLoS One, 2008. **3**(4): p. e1886.

29. Farndale, R.W., D.J. Buttle, and A.J. Barrett, *Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue.* Biochim Biophys Acta, 1986. **883**(2): p. 173-7.

30. Rao, J. and W.R. Otto, *Fluorimetric DNA assay for cell growth estimation.* Anal Biochem, 1992. **207**(1): p. 186-92.

31. Leutenegger, C.M., et al., *Quantitative real-time PCR for the measurement of feline cytokine mRNA.* Vet Immunol Immunopathol, 1999. **71**(3-4): p. 291-305.

32. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

33. Lee, I.C., et al., *The differentiation of mesenchymal stem cells by mechanical stress or/and co-culture system.* Biochem Biophys Res Commun, 2007. **352**(1): p. 147-52.

34. van der Wal, B.C., et al., *Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model.* Int J Cancer, 1997. **73**(6): p. 885-90.

35. Taylor, S.E., et al., *Gene expression markers of tendon fibroblasts in normal and diseased tissue compared to monolayer and three dimensional culture systems.* BMC Musculoskelet Disord, 2009. **10**: p. 27.

36. Ruster, B., et al., *Induction and detection of human mesenchymal stem cell migration in the 48-well reusable transwell assay.* Stem Cells Dev, 2005. **14**(2): p. 231-5.

37. Schmidt, A., et al., *Basic fibroblast growth factor controls migration in human mesenchymal stem cells.* Stem Cells, 2006. **24**(7): p. 1750-8.

38. Millette, E., et al., *Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation.* Circ Res, 2005. **96**(2): p. 172-9.

39. Koyama, N., C.E. Hart, and A.W. Clowes, *Different functions of the platelet-derived growth factor-alpha and -beta receptors for the migration and proliferation of cultured baboon smooth muscle cells.* Circ Res, 1994. **75**(4): p. 682-91.

40. Menon, M.B., et al., *Fluorescence-based quantitative scratch wound healing assay demonstrating the role of MAPKAPK-2/3 in fibroblast migration.* Cell Motil Cytoskeleton, 2009. **66**(12): p. 1041-7.

41. De Kock, S.S., et al., *Administration of bovine, porcine and equine growth hormone to the horse: effect on insulin-like growth factor-I and selected IGF binding proteins.* J Endocrinol, 2001. **171**(1): p. 163-71.

42. Hidaka, C., et al., *Acceleration of cartilage repair by genetically modified chondrocytes over expressing bone morphogenetic protein-7.* J Orthop Res, 2003. **21**(4): p. 573-83.

43. Fortier, L.A., et al., *Age-related changes in serum insulin-like growth factor-I, insulin-like growth factor-I binding protein-3 and articular cartilage structure in Thoroughbred horses.* Equine Vet J, 2005. **37**(1): p. 37-42.

44. Nixon, A.J., B.D. Brower-Toland, and L.J. Sandell, *Primary nucleotide structure of predominant and alternate splice forms of equine insulin-like growth factor I and their gene expression patterns in tissues.* Am J Vet Res, 1999. **60**(10): p. 1234-41.

45. Nixon, A.J., B.D. Brower-Toland, and L.J. Sandell, *Molecular cloning of equine transforming growth factor-beta1 reveals equine-specific amino acid substitutions in the mature peptide sequence.* J Mol Endocrinol, 2000. **24**(2): p. 261-72.

46. Park, B.S., et al., *Adipose-derived stem cells and their secretory factors as a promising therapy for skin aging.* Dermatol Surg, 2008. **34**(10): p. 1323-6.

47. Rehman, J., et al., *Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells.* Circulation, 2004. **109**(10): p. 1292-8.

48. Doorn, J., et al., *Pro-osteogenic trophic effects by PKA activation in human mesenchymal stromal cells.* Biomaterials, 2011. **32**(26): p. 6089-98.

49. Schwarz, C., et al., *Effects of different media on proliferation and differentiation capacity of canine, equine and porcine adipose derived stem cells.* Res Vet Sci, 2011.

50. Ranera, B., et al., *Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue.* Vet Immunol Immunopathol, 2011. **144**(1-2): p. 147-54.

51. da Silva Meirelles, L., et al., *MSC frequency correlates with blood vessel density in equine adipose tissue.* Tissue Eng Part A, 2009. **15**(2): p. 221-9.

52. Caplan, A.I., *Why are MSCs therapeutic? New data: new insight.* J Pathol, 2009. **217**(2): p. 318-24.

Conclusions

The application of SVF technology to equine flexor tendon injuries results in improved clinical outcomes; however, the mechanism by which the SVF promotes tendon regeneration over tendon repair remains unknown. The aim of this project was to find support for the idea that ADCs cultured from the SVF contribute to equine flexor tendon regeneration via indirect, or trophic, mechanisms. The experiments contained herein examined the production of soluble factors by ADCs, including growth factors and chemokines, chemotactic factors, and factors capable of up-regulating matrix protein production by TFBLs.

To summarize the findings of this project, each of the experiments provided support for the trophic nature of the contribution of ADCs to tendon regeneration. Adipose-derived cells were shown to produce mRNA for growth factors and cytokines (SDF-1α, IGF-I, PDGF-BB, FGF-2, TGF-β1, and TGF-β3) that could have an anabolic effect on tendon healing. Soluble factors that induce migration of ADCs are found in ADC-conditioned medium. These chemotactic factors could promote migration of endogenous stem cells *in vivo*. Exposure of TFBLs to ADC-conditioned medium in a co-culture scenario modestly up-regulates TFBL matrix protein production. *In vivo* ADCs may modulate expression of collagen types I and III by TFBLs and return the ratio of these matrix components to pre-injury levels, thereby increasing tendon tensile strength and decreasing re-injury rates.

An interesting result from these experiments is that an important difference was noted in gene expression for growth factors and cytokines between SVF cells and cultured TFBLs and ADCs. It was demonstrated that gene expression by the SVF cells is many orders of magnitude greater than expression by or TFBLs or ADCs. Thus, *in vitro* culture conditions, including the use of serum and two-dimensional plastic adherent culture, appear to induce important changes

in cultured cells that may have implications for clinical use. While the primary clinical application of ADC technology is via fresh SVF, ADCs are being cultured from the SVF and used clinically or cryopreserved for future use. Bone marrow aspirate is also cultured and cryopreserved for clinical applications, and is presumably subject to similar culture-induced changes. Future experiments are needed to further investigate the differences between SVF cells and cultured ADCs by repeating the microchemotaxis and co-culture experiments using fresh, uncultured SVF cells. Additional future experiments to further characterize the SVF cells and the cultured ADCs are necessary to examine cell surface markers and more precisely determine cell phenotypes. The lack of equine specific flow cytometry antibodies to determine cell surface marker profiles has hampered this characterization and development of a broader set of equine antibodies would greatly benefit these cell characterizations and therefore our knowledge of how to most effectively apply SVF technologies to clinical disease. Use of flow cytometry to determine the profile of cells from fresh and frozen SVF and the percentage of each cell type contained in the heterogeneous SVF will need to be compared to the marker profile for cultured ADCs over sequential passages to define the stemness of the ADCs and to determine which cell component from the SVF has the potential to make the greatest contribution to tendon regeneration.

Optimization of *in vitro* culture conditions also requires further investigation. Examination of ADCs in a three-dimensional culture system and potentially the development of an *in vitro* culture system combining decellularized tendon matrices and cyclical strain will much more closely mimic the *in vivo* tendon environment and provide insights into how ADCs might interact with the tendon environment *in vivo*. Each of these future experiments will augment the

knowledge provided in this manuscript and provide further support for the hypothesis that the primary contribution of ADCs to tendon regeneration is via trophic mechanisms.

Appendix A

Table 4. Primer and probe sequence. Growth factor and housekeeping gene sequence information (5'-3') for equine specific primers and MGB probes for real time PCR.

Gene	Forward Primer	Reverse Primer	MGB Probe	Reference
SDF-1α	TTAAAATCCTCAACACTCCGAACTGT	CGGGTCAATGCACACTTGTCTATT	CAGCCTTGCCACGATCT	BC119979 (BOVINE)
TGF-β1	CCTGTGACAGCAAAGATAACACACT	CGGTTCATGCCATCAATGG	CATCAACGGGTTCAGTTC	NM_001081849
TGF-β3	CGCAGTGCGGACACAAC	GCAGATGCTTCGGGATTCAG	CACGGTGCTGGGACTGT	A.J. NIXON LAB
IGF-I	GCGCCACACCGATATGC	CACTCCCTCTACTTGTGTTCTTCAA	CAAGGCTCAGAAGGAAGTA	NM_001082498
PDGF-BB	CCTGCTGCACGGAGACT	GGGACCGCGTCAGATTCAG	CCCCGTCTTCATCTACG	NM_001017953 (BOVINE)
Collagen Type I	GCCAAGAAGAAGGCCAAGAAGAA	TGAGGCCGTCCTGTATGC	ACATCCCAGCAGTCACCT	[26]
Collagen Type III	CTGCTTCATCCCACTCTTATTCTG	ATCCGCATAGGACTGACCAAGAT	AACAGGAAGTTGCTGAAGG	[26]
COMP	GAGATCGTGCAAACAATGAACAG	GACCGTATTCACGTGGAACGT	CTGGCTGTGGGTTACA	[26]
Decorin	AAGTTGATGCAGCTAGCCTGAGA	GGCCAGAGAGCCATTGTCAGAA	ATTTGGCTAAATTGGGACTG	[26]
18s RNA	GAGGCCCTGTAATTGGAATGAG	CGCTATTGGAGCTGGAATTACC	CAAGTCTGGTGCCAGCA	[26]
GAPDH	CAAGTTCCATGGCACAGTCAAG	GGCCTTTCCGTTGATGACAA	CCGAGCACGGGAAG	NM_001163856

Appendix B

Draft 09/01/2009

(Questions? Concerns? Contact Gail McMillan, Director of the Digital Library and Archives at Virginia Tech's University Libraries: gailmac@vt.edu)

(Please ensure that Javascript is enabled on your browser before using this tool.)

Virginia Tech ETD Fair Use Analysis Results

This is not a replacement for professional legal advice but an effort to assist you in making a sound decision.

Name: Shelley Stewart

Description of item under review for fair use: Figure 1. Representation of hierarchical structure of equine superficial digital flexor tendon.

Report generated on: 05-31-2012 at : 16:53:46

Based on the information you provided:

Factor 1

Your consideration of the purpose and character of your use of the copyright work weighs: in favor of fair use

Factor 2

Your consideration of the nature of the copyrighted work you used weighs: in favor of fair use

Factor 3

Your consideration of the amount and substantiality of your use of the copyrighted work weighs: in favor of fair use

Factor 4

Your consideration of the effect or potential effect on the market after your use of the copyrighted work weighs: in favor of fair use

Based on the information you provided, your use of the copyrighted work weighs: in favor of fair use

Details about analysis of fair use (Opens in a new window)

Print this page or save as PDF.

Draft 09/01/2009

(Questions? Concerns? Contact Gail McMillan, Director of the Digital Library and Archives at Virginia Tech's University Libraries: gailmac@vt.edu)

(Please ensure that Javascript is enabled on your browser before using this tool.)

Virginia Tech ETD Fair Use Analysis Results

This is not a replacement for professional legal advice but an effort to assist you in making a sound decision.

Name: Shelley Stewart

Description of item under review for fair use: Figure 2. Stem cell interactions with various inputs from the microenvironment.

Report generated on: 06-11-2012 at : 21:03:28

Based on the information you provided:

Factor 1

Your consideration of the purpose and character of your use of the copyright work weighs: in favor of fair use

Factor 2

Your consideration of the nature of the copyrighted work you used weighs: in favor of fair use

Factor 3

Your consideration of the amount and substantiality of your use of the copyrighted work weighs: in favor of fair use

Factor 4

Your consideration of the effect or potential effect on the market after your use of the copyrighted work weighs: in favor of fair use

Based on the information you provided, your use of the copyrighted work weighs: in favor of fair use

Details about analysis of fair use (Opens in a new window)

Print this page or save as PDF.