Comparison of bone marrow mesenchymal stem cells and tendon progenitor cells cultured on collagen surfaces

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ABSTRACT

Tendon injuries are a significant cause of morbidity in performance horses with superficial digital flexor tendon injury reported to represent up to 43% of overall Thoroughbred racehorse injuries. Natural repair is slow and results in inferior structural organization and biomechanical properties and, therefore, reinjury is common. The inability of tendon to regenerate after injury, or to heal with mechanical properties comparable to the original tissue, is likely attributable to low vascularity and cellularity of the tissue, low number of resident progenitor cells, and healing under weight-bearing conditions.

Strategies to improve tendon healing have focused on enhancing the metabolic response of tenocytes, modulating the organization of the newly synthesized extracellular matrix, or administering progenitor cells to enhance repair. Significant research effort has been directed at the use of adult mesenchymal stem cells as a source of progenitor cells for equine tendon repair and recent clinical applications have utilized adult autologous stem cells derived either from adipose tissue or bone marrow aspirates. Isolation of a homogenous population of stem cells from bone marrow is time-consuming, and there is much variation in cell numbers, cell viability and growth rates among samples. Recently, a population of progenitor cells has been isolated from equine flexor tendons, thus providing an alternative source of progenitor cells from the target tissue for therapeutic intervention.

The interaction between cells and the extracellular matrix (ECM) is an important factor in regulation of cell function. Proliferation, migration, differentiation and gene expression of many cell types are altered by adhesion to and interaction with matrix proteins and the extracellular environment. Tendon progenitor cells reside within a niche that comprises primarily parallel collagen fibers, and this niche plays an important role in regulating their function and differentiation. Culture conditions replicating this environment could be beneficial for both cell growth and matrix gene expression.

The objectives of the study were to compare cell growth kinetics and biosynthetic capabilities of bone marrow mesenchymal stem cells (BMMSCs) and tendon derived progenitor cells (TPCs) cultured on commercially available bovine, highly purified bovine, porcine, and rattus collagen sources and standard tissue culture surfaces. We hypothesized that collagen type I matrix would preferentially support TPC proliferation and up regulate gene expression for collagens and organizational components of tendon and therefore provide a culture system and progenitor cell type with advantages over the current practice of BMMSC expansion on standard cell culture plastic surfaces.

Cells were isolated from 6 young adult horses, expanded, and cultured on collagen-coated tissue culture plates, and no collagen control for 7 days. Samples were analyzed for cell

number on days 4 and 7, and for mRNA expression of collagen type I, collagen type III, cartilage oligomeric matrix protein (COMP), and decorin on day 7. Glycosaminoglycan (GAG) synthesis was analyzed on day 7. Differences of cell number between collagen groups and cell type, and in gene expression and GAG synthesis between collagen groups and cell types, were evaluated by use of mixed-model repeated measures ANOVA. Pair-wise comparisons were made on significant differences identified with ANOVA using Tukey's post hoc test. Statistical significance was set at $P \leq 0.05$.

A statistical significant (P=0.05) increase in cell number for TPCs grown on rattus collagen versus control on day 4 was observed. No difference in GAG synthesis or expression of collagen type I, collagen type III, COMP or decorin mRNA was observed between collagen groups and non-collagen controls for either cell type on day 7. TPCs cultured on all collagen types yielded more cells than similarly cultured BMMSCs on day 4, but only porcine collagen was superior on day 7. TPCs synthesized more GAG than BMMSCs when cultured on control surfaces only. BMMSCs expressed more collagen type I mRNA when cultured on control, porcine and highly-purified collagen, and more collagen type III when cultured on control, porcine, highly-purified collagen, and rattus collagen, than TPCs. Tendon-progenitor cells expressed significantly more COMP when cultured on control and all collagen types, and decorin when cultured on porcine, highly purified bovine and bovine collagen when compared to BMMSCs.

The results of this study revealed an advantage to culturing TPCs on randomly organized rattus collagen during the early growth phase. The beneficial effects of collagen-coated surfaces on cell proliferation is likely related to increased surface area for attachment and expansion provided by the random collagen matrix, and/or collagen-cell interactions. Tendon progenitor cells showed superior growth kinetics and expression of the matrix organizational components, COMP and decorin, than similarly cultured BMMSCs that expressed more collagen types III and I. TPCs synthesize more GAG compared to BMMSCs when cultured on plastic surfaces and there was no induction by collagen. Tendon progenitor cells should be considered as an alternative source of progenitor cells for injured equine tendons. Further in vitro studies characterizing factors that influence gene expression of both cell types is warranted.

Acknowledgements

I would like to acknowledge the help and support of my committee members, Dr. Jennifer Barrett, Dr. Nat White, and Dr. Linda Dahlgren, provided to me throughout the duration of my project. In particular, Dr. Barrett has been an invaluable mentor and leader in this project and without her, the project would not have been possible. I would also like to acknowledge the technical support of Zijuan Ma who greatly facilitated the completion of the laboratory work and Dr. Steven Werre for performing statistical analyses, and the American College of Veterinary Surgeons for their funding of the project through the Surgeon-in-Training grant.

To my dear wife (Theresa) and sons (Hugh & Jeremy), thank you for supporting me through this project; I simply could not have done this without you. Lastly, I would like to dedicate this thesis to my late father, Paul Hugh Brown, who was an inspiration to me throughout my life. He would be a very proud person if he were here today.

Attributions

Several colleagues and coworkers aided in the research and writing of this thesis. A brief description of their background and their contributions are included here.

Jennifer G. Barrett – DVM, PhD Diplomate ACVS (Marion duPont Scott Equine Medical Center, Virginia-Maryland Regional College of Veterinary Medicine) is the primary advisor and committee chairperson. Dr. Barrett's primary research interest is regenerative medicine and tissue engineering. Dr. Barrett has earned a PhD in molecular biology and has extensive experience in stem cell research. She played a vital role in the overall project design, laboratory work and writing of the thesis.

Nathaniel A. White II – DVM, MS Diplomate ACVS (Marion duPont Scott Equine Medical Center, Virginia-Maryland Regional College of Veterinary Medicine) is a committee member. Dr. White has extensive clinical experience in the treatment of equine tendon and ligament injury. He contributed significantly to the review of the thesis.

Linda A. Dahlgren – DVM, PhD Diplomate ACVS (Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine) is a committee chairperson. Dr. Dahlgren's primary research interest is equine tendon injury and she has earned both a Master's degree and PhD while focusing on this interest. Dr. Dahlgren provided thoughtful contribution to the planning of the project and review of the thesis.

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Introduction

Thesis Organization

This thesis is presented in a format that contains a journal publication as the central portion of the document. The publication is entitled "Comparison of bone marrow mesenchymal stem cells and tendon progenitor cells cultured collagen coated surfaces" and contains its own introduction, materials and methods, results, discussion, and references. The following introduction provides a brief overview of the research topic. The literature review is an expansion of the introduction to the manuscript and provides a summary of pertinent literature background information. The thesis is concluded by final comments that outline future directions for research.

Introduction

Tendons are specialized tissues that connect bone to muscle, transmitting the forces generated by these structures to allow for body movement. Tendon injuries due to overuse or age-related degeneration are a common clinical problem and are a significant cause of morbidity in both man and veterinary species. Damaged tendon tissue heals very slowly with the defect being replaced by scar tissue that rarely attains the structural organization and biomechanical properties of normal undamaged tendon.[1] Standard treatment for tendon injury is conservative, involving prolonged confinement and controlled exercise for up to 12 months post injury. Reinjury rates of 23-67% of horses treated conservatively have been reported with many sustaining re-injury within 2 years of the original injury.[2,3] Despite improvements in the early detection and serial evaluation of damaged tendons using ultrasonography and advances in rehabilitation techniques and treatments, a consistently successful treatment regimen has yet to be developed.

The poor clinical outcome associated with tendon injury and the limited capacity for regeneration of injured tendon have resulted in a growing interest in the use of tissue engineering and regenerative medicine approaches for tendon therapy in horses.[4] This approach is based on the hypothesis that there are insufficient resident progenitor cells available to regenerate tendon. Therefore, a technique of transplantation of large numbers of mesenchymal stem cells into injured tendon lesions has been developed and has been shown to promote healing not only in laboratory animal tendon injury models[5] but also in horses.[6,7] These studies have focused predominantly on bone marrow- and adipose-derived mesenchymal stem cell sources. There are inherent problems with obtaining sufficient numbers of stem cells from these tissue sources and/or the purity of cell samples collected has been questioned. Furthermore, there is a lack of understanding of how to influence differentiation of these cells to become tenocytes.

The recent discovery of tendon progenitor cells (TPCs) that possess regenerative capabilities has opened exciting new possibilities for treating tendon injuries, as well as challenges in understanding the basic biology of tendon. Stem cells by nature are pluripotent; however, we are now starting to recognize that some progenitor cells may be pre-programmed and have 'positional memory' and thus there is potential benefit to

exploiting the natural stem cell niche that exists in tendon.[8] New data continues to mount on the influence of the extracellular matrix on stem cell fate through physical interactions with cells. The tendon stem cell niche is highly specialized and consists predominantly of collagen type I. Combining TPCs with their natural niche *in vitro* lead to the original impetus for this project; TPCs were shown to engraft and thrive in acellular tendon explants[9] which then lead us to attempt culture on collagen-coated surfaces. Culture on collagen-coated plates is facile, and may offer an alternative to standard uncoated tissue culture plates. Pilot study data from our laboratory showed improved cell growth kinetics of TPCs cultured on collagen-coated surfaces compared to standard culture conditions. We wanted to expand this concept and test various species' sources of collagen type I that were commercially available.

The aims of this master's project were:

1. To compare cell growth kinetics and biosynthetic capabilities of BMMSCs and tendon progenitor cells
2. To investigate the effects of collagen type I on BMMSCs and TPC growth and tendon-related biosynthetic capabilities

Chapter 1: Literature Review

Clinical significance of tendon injury

Tendon injury in the horse is a significant problem and is reported to represent up to 43% of overall Thoroughbred racehorse injuries.[10-15] The majority (97-99%) of tendon injuries in Thoroughbred racehorses occur to the forelimb tendons,[10,13] with the superficial digital flexor tendon (SDFT) predominantly being involved and representing 75-93% of cases.[13,16] A 12 year retrospective study of Thoroughbreds racing in Hong Kong revealed tendon injury was the single most common veterinary related reason for retirement, representing a median of 13.7% of all retirements.[10] This same study identified an annual cumulative incidence of 2.3-4.3%; however, many horses did not undergo ultrasonographic examinations and therefore the incidence was likely underestimated. Studies of racing and training Thoroughbred horses where ultrasonography was used to diagnose SDFT injury, revealed an incidence of 11% in racehorses in Japan over a 1-year period and 24% in UK National Hunt horses over two race seasons.[13,15]

Racing career length is also reduced in racehorses due to tendon injury. A retrospective study of racehorses in Hong Kong reported a reduction in racing career length by a median of 1 year and the median period in training decrease of 25.6%.[10] The lay-up time for rehabilitation translated into a reduction in race starts (41.2%) and earnings (53.3%). Perhaps the most alarming finding in this study was that there was an upward trend in the incidence of tendon injury. The annual percentage of horses retiring from racing increased from 18.5% (193/1045) in 1992-93 to 28.8% (412/1432) in 2003-04.[10]

Tendon injury is not limited to racehorses. The incidence of tendon injury in sport horses in training has recently been reported to account for 43% of all injuries with a 2:1 distribution of superficial to deep digital flexor injury (DDFT).[14] Elite show jumpers have been reported to have a high risk of injury to the forelimb SDFT and DDFT, whereas horses competing in dressage have a high risk of injury to the hindlimb suspensory ligament.[17]

Flexor tendon anatomy and function

The flexor group of tendons of the equine forelimb arises from the caudomedial aspect of the humerus and occupies the caudal part of the forearm. The two flexors that are of greatest clinical significance in the equine are the SDFT and the DDFT (Figure 1.1). The SDFT occupies a central position within the flexor group and a purely tendinous accessory (check) ligament with origin from the caudal surface of the radius joins the main tendon in the lower part of the forearm. The superficial and deep flexor tendons share a common synovial sheath, the carpal sheath, during their passage through the carpal canal. The SDFT is superficial in the metacarpus but distal to the fetlock it bifurcates and obtains a deeper position that enables it to insert on neighboring parts of the first and second phalanges. The DDFT is the largest of the flexor group and in addition to the humeral head origin there are lesser heads from the upper parts of the radius and ulna. The tendon passes through the carpal canal and continues down the palmar aspect of the limb to finally insert upon the palmar surface of the third phalanx. In the metacarpus, the tendon is joined by a stout accessory (check) ligament that arises

from the thick fibrous joint capsule on the palmar aspect of the carpal joint. This ligament is an important element of the passive stay apparatus and has relatively greater significance than the analogous contribution of the superficial flexor tendon.[18]

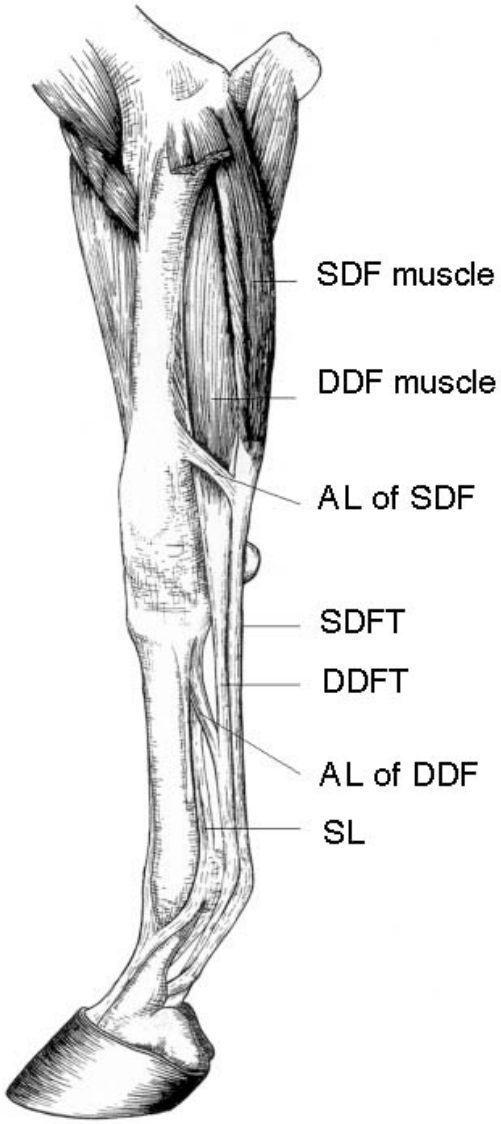

Figure 1.1. Equine distal forelimb (medial view) showing flexor tendon anatomy. AL, accessory ligament; DDF deep digital flexor; SDF, superficial digital flexor; SL, suspensory ligament.[19] (used under fairuse guidelines)

Tendons in the equine forelimb act mainly to position the limb correctly during locomotion support the limb and provide a passive stay apparatus. The SDFT of the horse transmits forces between the muscle (located above the carpus) and the lower part of the limb, and in doing so acts as a flexor of the metacarpophalangeal and other joints. It receives mainly tensional forces but where the tendon passes over joints, such as the metacarpophalangeal joint, it is subjected to compressive forces.

Another important function of the SDFT is to act as an energy store for efficient locomotion. The SDFT acts as a spring to store and release elastic energy as it stretches and recoils during the stance and swing phase of each stride and in doing so it decreases the energetic cost of locomotion.[19] The SDFT along with the suspensory ligament are the main energy storing structures in the equine forelimb and are subjected to higher strains than the deep digital flexor tendon (DDFT) and common digital extensor tendon, which do not contribute significantly to energy storage.[19]

Tendon morphology

Tendons are composed of a densely packed collagen-rich connective tissue able to withstand high tensile forces. Collagen type I is predominant, but other collagens and proteoglycans are also deposited into the tendon extracellular matrix. Tendons have a longitudinally oriented, hierarchical structure with the largest subunits termed fascicles (Figure 1.2). These fascicles are separated by the endotenon, which is comprised of loose connective tissue carrying blood vessels, nerves and lymphatics.[20-22] Within the fascicles are fibers that contain collagen fibrils, the submicroscopic units of tensile strength of tendon. Fibrils are round in cross-section (20-300 nm diameter), presumed to measure millimeters to centimeters in length and comprised of cross-linked collagen molecules.[23] The fibrils and fibers are aligned in the longitudinal axis of the tendon, following an in-phase zigzag waveform termed ‘crimp’. Crimp is a mechanical and elastic buffer that straightens as the tendon is stretched. In theory, a tendon with a high crimp angle straightens and eventually fails at higher stress levels than a fibril with a low crimp angle.[24] Proteoglycans are important for spacing and lubrication of tendon fibrils and regulate lateral collagen fibril growth (Figure 1.3).[25-29]

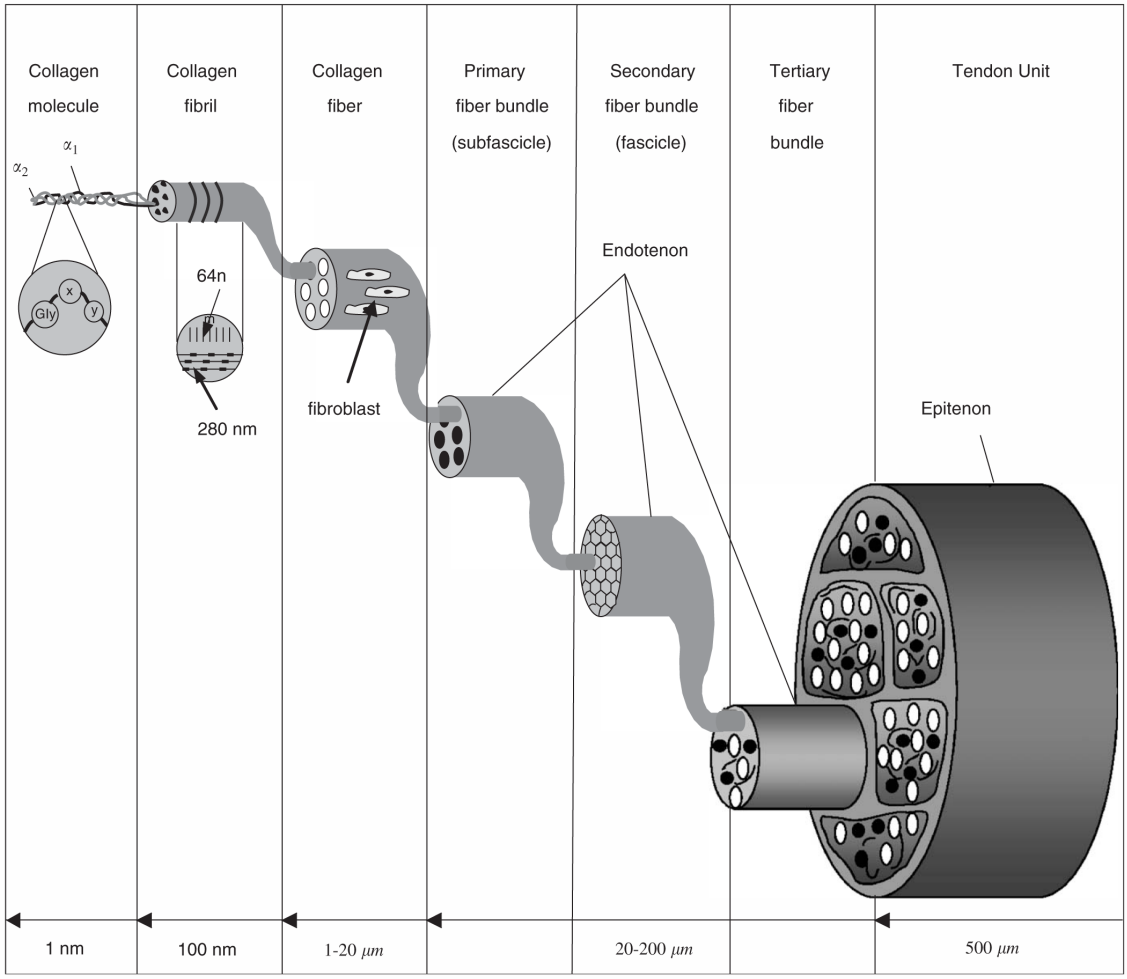

Figure 1.2. A schematic of the multi-unit hierarchical structure of tendon.[30] (used under fairuse guidelines)

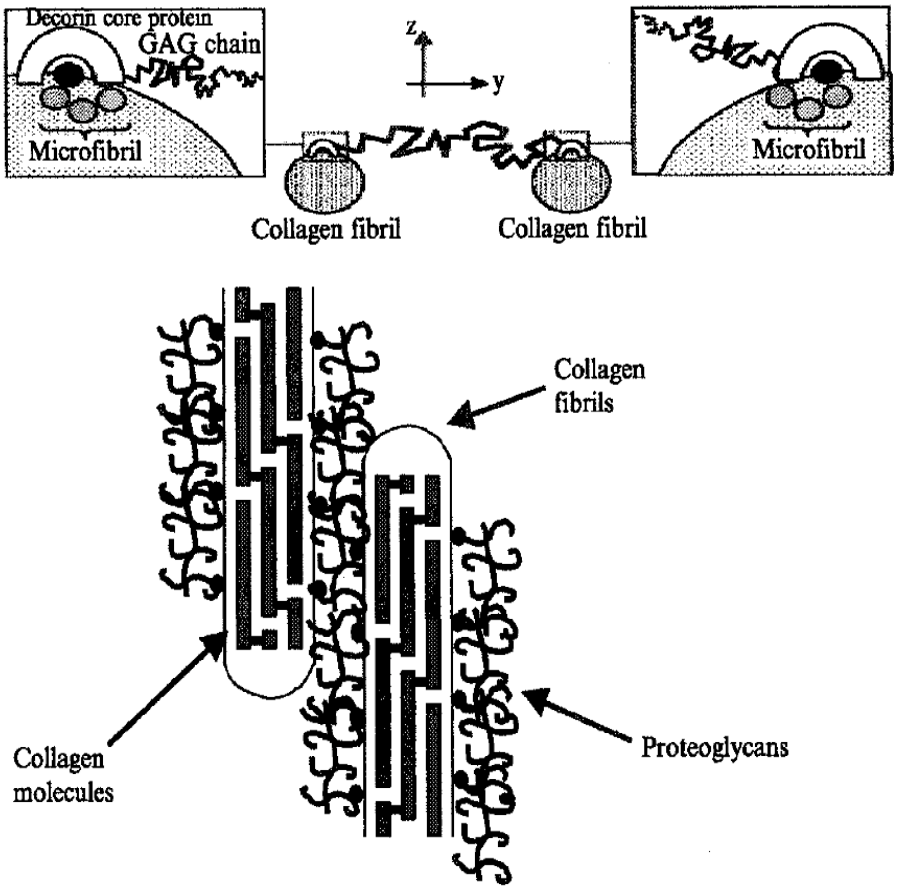

Figure 1.3 The possible way in which proteoglycan and GAG chains provide interfacial shear between collagen fibrils.[31] (used under fairuse guidelines)

The specialized molecular composition and organization of tendon results in a high strength structure that is able to resist unidirectional forces. The dense fibrous extracellular matrix of tendon is synthesized and maintained by a small population of tenocytes. Tenocytes are fibroblastic cells responsible for synthesis and turnover of the matrix. They are arranged in parallel rows between collagen fiber bundles within fascicles, where most lack direct contact with the vasculature located in the endotenon. The tenocytes extend long cytoplasmic processes within and between rows and are linked into complex three-dimensional networks by gap junctions and actin-associated adherens junctions that facilitate coordinated responses to mechanical loading.[32-34] Gap junctional intercellular communication may also facilitate transfer of nutrients between cells within the avascular matrix.[35]

Tendon extracellular matrix (ECM) is composed primarily of collagen, accounting for approximately 75% of the dry mass of adult tendon, with collagen type I predominating.[36] Type III collagen comprises only 4-5% of total collagen in the metacarpal region of normal adult equine SDFT and tends to be restricted to the endotenon and epitenon, and smaller, less well organized fibrils.[36-39] Type II collagen

occurs in fibrocartilaginous areas that are subjected to compression, such as where tendons wrap around joints. Small amounts of collagen types IV, V, IX, X and XII have been identified in various regions including insertion sites.[40,41]

The properties of fibril-forming collagens (such as collagen types I and III) are attributable to the rod-like triple helical structure formed by three tropocollagen α-chains constituting the collagen monomer (figure 1.2). The collagen molecules are produced as procollagens with propeptides at their N and C termini upon secretion into the extracellular matrix these propeptides are removed by N- and C-proteinases, producing the native triple helical molecule with retained short telopeptides of only a few amino acids.[42]

Collagen molecules are stabilized by intermolecular chemical crosslinks that determine the strength of the collagen fibril, and ultimately the tendon.[43] Some of the crosslinks are formed between adjacent amino acids after modification by the enzyme lysyl oxidase. Not all the mature crosslinks have been identified, although the best characterized are hydroxylysylpyridinoline and lysylpyridinoline.[44]

In the tendon, the development of mature mechanical properties is dependent on the assembly of a tendon-specific ECM. The composition of the ECM of the tendon differs with location along its length. The mid-metacarpal region, which is subjected to high tensional loads, contains mainly collagen type I, whereas the fetlock region, which receives compression forces, has a more chondroid matrix containing collagen type II.[45] The matrix is synthesized by the tenocytes and is composed of collagen fibrils organized as fibers, as well as fibril-associated collagenous and non-collagenous proteins. All of these components are integrated, during development and growth, to form a functional tissue. During tendon development, collagen fibrillogenesis and matrix assembly progress through multiple steps where each step is regulated independently, culminating in a structurally and functionally mature tissue.

Collagen fibrillogenesis occurs in a series of extracellular compartments where fibril intermediates are assembled and mature fibrils grow through a process of post-depositional fusion of the intermediates.[46] Linear and lateral fibril growth occurs after the immature fibril intermediates are incorporated into fibers, and the processes are regulated by the interactions of extracellular macromolecules with the fibrils. Interactions with quantitatively minor fibrillar collagens, fibril-associated collagens and proteoglycans influence different steps in fibrillogenesis. Each step of fibrillogenesis takes place in a unique compartment, both cytoplasmic and extracellular.[47] This provides a mechanism for compartmentalizing the regulatory interactions involving extracellular macromolecules.[47]

Collagen fibril thickness varies with age, with the tendons of the fetus having a fibril population that is homogenous in diameter and thinner than adult tendons.[48] The ratio of thin and thick fibrils increases with age. Exercise has a minimal effect on fibril thickness in young horses, suggesting that fibril diameter is dependent on growth and development rather than activity.[48]

Proteins present in the extracellular matrix (e.g. proteoglycans and other collagens) influence fibril growth and regulate fibril thickness. The functional features of collagen type I fibrils depend on their orientation, size and length; hence, fibrillogenesis is crucial for the form and final function of tendon *in vivo*. Cartilage oligomeric matrix protein, collagen III and the proteoglycans decorin, biglycan, fibromodulin, and lumican, all have been shown to play a role in regulation of collagen type I fibril formation.[27,39,49-53]

Cartilage oligomeric matrix protein (COMP) is a non-collagenous glycoprotein prominent in cartilage and adult tendon.[54-56] COMP is also known as thrombospondin 5[57] and has a pentameric protein structure with its subunits bound via disulfide bonds at their N termini and globular C terminal domains.[42,54] The pentameric nature of COMP allows high binding affinity with collagens types I and II that facilitates collagen fibrillogenesis by promoting early association of collagen molecules and leading to increased rate of fibrillogenesis and more distinct organization of the fibrils.[42,58] COMP binds to a collagen molecule at four separate sites at the C-terminal domains via a zinc-dependent mechanism. Once the fibril is formed, COMP becomes displaced from its molecular attachments.[59] Somewhat in contradiction to the importance of COMP in fibrillogenesis is the report of normal skeletal development in COMP-null mice that did not exhibit any tendon abnormalities.[60]

Equine tendon contains very little COMP at birth; however, while COMP accumulates in all weight bearing tendons with growth, the tensional (i.e. metacarpal) region of the SDFT contains the highest level by age 2 years (~3% of dry weight), during which time collagen fibrils undergo maturational changes.[58] These changes include a decrease in the reducible collagen crosslinks and an increase in the mature hydroxylysylpyridinoline crosslink, an increase in the mass average collagen fibril diameter and a decrease in the collagen crimp angle and length.[61] After 2 years of age, COMP levels decline rapidly in the tensional regions, whereas they are maintained in the compressed regions.[58] It has been hypothesized that high levels of COMP during development are important for the formation of 'higher quality', stronger tendon matrix.[58] COMP is more abundant in the equine SDFT compared to its concentration in the DDFT of adult horses and it has been suggested that COMP is expressed as a response to mechanical load.[58] Higher COMP immunolabelling has been correlated with increased small diameter collagen fibrils in the tensional area of the flexor tendon.[62]

In addition to COMP, the interactions of collagen fibrils with small leucine-rich proteoglycans (SLRPs) have been implicated as important regulators of collagen fibrillogenesis. SLRPs are a family comprising structurally related, but genetically distinct proteoglycans/glycoproteins that can be grouped into three classes.[63] The members of each class have high protein sequence similarity and show related biological roles. Decorin and biglycan (class I) and fibromodulin and lumican (class II) are found in tendon.[28,46,64] These four SLRPs bind to fibrillar collagens via two distinct sites. Decorin and biglycan compete for the same site on collagen type I, which is distinct from the fibromodulin/lumican-binding site.[53,65-67]

Decorin is produced by a variety of cells, including fibroblasts, myocytes, and smooth muscle cells.[68] In connective tissue, decorin participates in the regulation of collagen fibrillogenesis by binding collagen fibrils in cooperation with other SLRPs and a variety

of growth factors.[68] The arch-shaped structure of decorin permits interaction with the collagen triple helix[69] and also allows anchorage to d-bands within several collagen fibrils by non-covalent binding.[70] Regulation of fibrillogenesis through binding to collagen and limiting the lateral assembly of fibers has been demonstrated.[27,65,66,71-73] Morphological and mechanical *in vitro* analyses have also demonstrated that decorin significantly influences collagen fibril diameter and mechanical strength.[68]

In contradiction to its importance in fibrillogenesis, inhibition of decorin expression has been shown to increase the diameter of collagen fibers in healing ligaments, thus offering a potentially promising treatment for tendon injury by improving the mechanical properties of the scar tissue.[74] Decorin also plays a role in regulating interfibrillar spaces between collagen fibrils and promotes longitudinal growth of collagen.[75-77] Decorin is able to regulate the spacing among collagen fibrils by altering the size of it's own dermatan sulfate side chains, in healing skin.[76,78,79] Regional differences in amount and size of decorin correlate with regional differences in collagen fibril distribution and density of collagen fibrils in equine SDFT, that provides circumstantial evidence for a regulatory role in fibrillogenesis.[77]

Gene-targeting studies using mice deficient in decorin indicate that SLRPs/glycoproteins are involved in determining the mature collagen fibril structural phenotype and tissue function.[27] Decorin-deficient mice have defects in different connective tissues including dermis and tendon. The fibril profiles in the dermis and tail tendons are irregular relative to the wild-type controls, and subsequent biomechanical studies performed demonstrate a decrease in tensile strength of the decorin-deficient dermis.[27]

Biglycan-deficient mice have abnormal fibril structures in a variety of tissues including skin, bone, and tendons, analogous to those seen in the decorin-deficient mice.[80] Mice double deficient in both decorin and biglycan contain a population of fibrils that are markedly aberrant in structure, suggesting an interaction between the two closely related SLRPs.[28] The synergistic effects in double-deficient animals and more severe phenotypes in double compared to single mutant animals suggests a rescue and/or compensation mechanism in the single deficient animals and provides evidence for the existence of functional overlap between SLRPs.[28,64,81]

Pathobiology of tendon injury

The equine SDFT operates close to its mechanical failure limits and has low tolerance for over-strain. Tendon rupture *in vitro* has been reported to occur at between 12-20% strain, which overlaps with reported *in vivo* measured strains of up to 16% at the gallop.[82-84] Fatigue, poor conformation, lack of fitness, uncoordinated muscle activity are all contributors that act to produce excessive biomechanical forces that may lead to acceleration of degenerative changes by disrupting the matrix or may induce full clinical injury by exceeding the mechanical properties of the tendon.[12] Within a population of horses, the mechanical properties of the tendon vary considerably. These observations suggest that not only are horses with weaker tendons likely to be more prone to injury, but also that any small changes in tendon biomechanical properties dramatically affect the incidence of injury. Variation in SDFT strength and stiffness arise from variation in the structure and molecular composition of the tendon matrix.[85] Experimental work and

epidemiological data indicate that, while tendons are sensitive to mechanical load during growth when they are able to adapt to forces placed on them, tendon has limited ability to adapt to loads applied after skeletal maturity.[86] 'Natural' levels of exercise, while causing accumulation of fatigue damage, would not likely weaken the tendon sufficiently within the animal's lifetime and tendon injury would be a rare event in the wild. In contrast, athletic endeavor accelerates this ageing change by the imposition of a greater number of high strain loading cycles that can initiate clinical injury when the tendon is weakened sufficiently.

In equine tendon injury, most of the damage occurs in the mid-metacarpal region of forelimb SDFT, which is under tensional force.[10,13,16,87-89] The cross-sectional area of the SDFT is smallest in the mid-metacarpal region and this has been suggested as a reason for this particular region's susceptibility to injury. However, the total amount of collagen at that level is similar to that of most other sites, which implies that it is not necessarily significantly weaker.[90,91]

In addition to the contribution of mechanical stresses to the pathophysiology of tendon injury, the relatively poor vascularity of the tendon has also been implicated as a potential factor. It has been suggested that blood supply to the mid-metacarpal region of the SDFT is deficient during exercise, leading to ischemia and reperfusion injury, and tenocyte anoxia.[12] A good vascular network and blood flows similar to that of resting skeletal muscle and an increased flow subsequent to exercise has been reported, suggesting that *in vivo* hypoxia may not occur.[12,92] However, there have been no reports of direct measurements of functional blood flow comparing different regions or relating it to tissue oxygen levels.[92,93] Exercise-induced hyperthermia has also been suggested as a potential player in the pathogenesis of tendon injury.[94] Temperatures of up to 45^{o}C have been induced in the tendons at the gallop; however, these temperature rises do not induce cell death in tenocytes *in vitro*, although adverse effects on the tendon matrix may be induced.[95]

Although the role of inflammation in tendon injury is still debated, it has long been known that tendon injuries are primarily degenerative conditions and therefore most authors refer to the condition as 'tendinopathy' instead of 'tendinitis' as the former term does not imply an etiology.[96] The absence of inflammatory cells in or around the lesion in chronic tendinopathies does not mean that inflammatory mediators are not involved in the process. Peritendinous tissue levels of inflammatory mediators such as prostaglandin E2, thromboxane, bradykinin and interleukin (IL)-6, have been shown to be increased after prolonged exercise, and increased expression of cyclo-oxygenase 2 is associated with patellar tendinopathy in humans.[96]

Tendon injury is frequently preceded by degenerative changes in the ECM rather than a single overloading event.[97] The 'tendinopathy cycle' is believed to begin when matrix breakdown overwhelms the cellular repair mechanisms.[35,98,99] It is generally agreed that tendon injury in many cases follows failure to adapt to a variety of stresses, resulting in an undefined period of accumulation of age- and exercise-related microdamage.[11] Matrix turnover, involving both the synthesis and degradation of matrix components, is important for the maintenance and repair of all connective tissue, including tendon. Resident tenocytes are constantly repairing damage under normal circumstances, but the

level of tenocyte synthetic activity in the SDFT under normal circumstances appears to be low.[35] Excessive repetitive loading may cause direct damage to the matrix that cannot be repaired by tenocytes for various reasons including insufficient time between episodes, or a frequency of repeated and/or non-uniform overstrain that overwhelms cellular capacity. An age-related reduction in collagen turnover *in vivo* and in strain-induced collagen synthesis of *in vitro*-cultured tenocytes has been reported, which is consistent with tendon injury incidence increasing with increasing age and accumulated exercise.[34,85,100,101]

Major molecular changes associated with remodeling activity in human Achilles tendinopathy include increased expression of type III collagen, fibronectin, tenascin C, aggrecan and biglycan genes.[96] There is evidence of increased proteolytic enzyme activity and changes in the expression and activity of various metalloproteinases, and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs).

The continual process of matrix remodeling is a constitutive (albeit slow) activity in normal tendons, affecting proteoglycans in addition to collagen,[102,103] and is thought be primarily mediated by metalloproteinases (MMPs) acting in the extracellular environment. Collagenases are members of the MMP superfamily and are some of the few enzymes capable of cleaving the intact type I collagen molecule in the extracellular environment.[104-106] Several MMPs (MMP1 (collagenase-1), MMP2 (gelatinase A), MMP8 (neutrophil collagenase), MMP13 (collagenase-3), and MMP14) have been shown to have activity against fibrillar collagen.[96] Cleavage occurs at a specific locus in the collagen triple helix, between residues 775 and 776,[105] which generates three-quarter- and one-quarter-length fragments that are in turn susceptible to other proteinases, such as the gelatinases.

Proteoglycans are turned over much more rapidly than the fibrillar collagens, and are primarily degraded by a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). Aggrecanases, which include ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS8, and ADAMTS9, are believed to be responsible, although precisely which enzyme is involved in turnover of tendon proteoglycan is currently unknown. The activity of MMPs is highly regulated at multiple levels, including transcription, activation and inhibition by TIMPs.[107] In addition to their roles in collagen degradation, both MMPs and ADAMTS have been shown to be important in regulating cell activity, growth and development, and repair and also pathologic processes, including inflammation and degeneration.[108]

Once a tendon has suffered clinical injury, a sequence of events is activated, similar to that occurring in other soft tissues such as skin, that results in healing with fibrous scar.[6] The sequence of events begins with an inflammatory response that will be clinically evident as heat, swelling, and pain response in most horses. The inflammatory phase is short lived and lasts from a few days to 2 weeks in most instances. Very soon after clinical injury, fibroplasia begins which overlaps with the inflammatory reaction and peaks anywhere between 3-6 months after injury. The fibroplasia phase is associated with pronounced vascular ingrowth and the result is the production of fibrous scar tissue that replaces the normal tendon matrix. This disorganized pathological matrix can, if given

long enough, reach similar ultimate tensile strength as the original tendon, but it has reduced elasticity resulting in reduced performance and a substantial risk of reinjury.[12]

Natural repair is slow and reinjury is common with rates of up to 80% reported in racehorses.[3,12] After tendon injury, the initial cellular infiltrate is dominated by blood-borne cells involved in local debridement of damaged tissue that is later substituted by cells from the epitenon and endotenon, that migrate to the lesion and synthesize new matrix.[109] The new matrix produced by the fibroblast cell population results in enlargement of the tendon and loss of the normal histological appearance of tendon. Cells and matrix are arranged in a much more random fashion as opposed to the normal 'crimp' pattern. The scar tissue contains an increased amount of collagen type III (20-30% vs. ~1% in normal tendon) and has less cross-linking than normal tendon.[110] Collagen type III tends to form smaller fibrils than collagen type I, and therefore, the equine tendon scar has a decrease in overall tensile strength.[110] Despite this fact, most of the reinjury occurs at the proximal or distal interface with the more 'normal' tendon, which suggests that these regions are the 'weak link' in the repair.[111,112]

From a practical viewpoint, regeneration of tendon from differentiated tenocytes secreting type I collagen in parallel bundles would constitute a more satisfactory healing response than the development of scar tissue from altered fibroblasts secreting inferior type III collagen.

Tissue engineering and regenerative medicine

Regenerative medicine and tissue engineering are rapidly growing fields that seek to repair or regenerate damaged or diseased tissues and organs through the implantation of combinations of cells, scaffolds and soluble mediators.[113,114] In order to achieve these goals, a readily available cell source(s) that, under controlled conditions, can provide the appropriate function is needed, and therefore, there has been significant research interest in both adult and embryonic stem cells. Adult MSCs are capable of self-renewal and their progeny are further capable of differentiating into one of several phenotypes such as osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, and adipocytes.[115] MSCs secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities. They also been shown to have decreased immunogenicity and possess immunosuppressive properties. [116,117]

Mesenchymal stem cells

The concept of stem cells originated at the end of the 19th century as a theoretical postulate to account for the ability of certain tissues (blood, skin, *etc*.) to self renew for the lifetime of an organism even though they are comprised of short-lived cells. Tissues retain a population of cells capable of replenishing stocks throughout life. Most notable is the blood cell systems, where immunity relies on a population of white blood stem cells capable of responding specifically to renewed infection. In addition to the rich source of hematopoietic stem cells responsible for replenishing the cells within the blood, bone marrow contains a different group of cells that form the fibrous 'stroma' within the marrow. While these cells play a role in maintaining the hematopoietic stem cells, they

have also been proven as a source of mesenchymal progenitor cells that contribute to general repair by accessing the vascular system.[118]

The early work of Friedenstein and colleagues demonstrated ectopic bone and marrow formation following transplantation of bone marrow. Subsequent to this work, they were able to isolate a subpopulation of bone marrow cells responsible for the osteogenesis.[119] These cells were distinguishable from the majority of hematopoietic cells by their rapid adherence to tissue culture plastic and the fibroblast-like appearance of their progeny in culture. These same authors were also able to demonstrate the clonal nature of the isolated cells and the development of multiple skeletal tissues following *in vivo* transplantation.[120]

In most studies, self-renewal is equated to sustained growth in culture or, in some scenarios, is assumed based on retention of *in vitro* differentiation after multiple population doublings. Stem cells can be broadly grouped into two categories based on their origin from either the embryo or the adult. Stem cells can be obtained from tissues of endodermal, mesodermal or ectodermal lineages. Beyond the bone marrow, adherent cells capable of density-independent growth are found in most tissues such as muscle[121], adipose tissue[122], synovium[123], and periosteum.[124] Like bone marrow derived stem cells, they can form colony-forming units (CFU-F) with fibroblast-like morphology. It is more than likely that adult stem cell systems exist in all tissues so that when cells naturally expire, these cells can be expediently replaced to create physiological balance in the organism.[115]

The potency of progenitor cells has not been compared systematically by *in vivo* assays, and prevailing evidence suggests that stem cells from different tissues are not the same.[125] For example, when grown and transplanted *in vivo* under conditions identical to those used for bone marrow-derived mesenchymal stem cells (BMMSCs), progenitor cells from dental pulp form dentin rather than bone.[126] Thus rather than a uniform, single class of ubiquitous MSCs, the evidence points to a varied class of clonogenic progenitors found in different tissues but endowed with tissue-specific potency.[125] In addition, there is growing evidence that the differentiation process is not so rigid and there is some capability of cells differentiating along a certain pathway either to return to a less differentiated state or 'trans-differentiate' to another line.[118] However, there appears to be some truth to the principle that cells lose a degree of 'multipotentiality' as they become more differentiated.

Bone marrow mesenchymal stem cells

The postnatal bone marrow contains marrow stromal cells, also known as mesenchymal stromal or stem cells.[127] Mesenchymal stem cells obtained from bone marrow[128-130] have the ability to differentiate along multiple lineage pathways *in vitro*, including cell types such as those in muscle[131], fat[130], bone[132,133], cartilage[129], liver[134], lung[135], and nerve.[128,130,136-138] Previous studies indicate that BMMSCs can form tendon- or ligament-type structures *in vitro*.[139] *In vivo* studies using a rabbit experimental tendon injury model have shown that BMMSCs can contribute to improved biomechanical characteristics.[139,140]

Stem cells represent a very small fraction of the total population of nucleated cells from the bone marrow, with only an estimated 0.001-0.01% of mononuclear cells isolated from a ficoll density gradient of bone marrow aspirate being MSCs.[130,141] Recent work in young horses (≤ 5 years-old) reported a CFU-F frequency of 0.024% within the nucleated cell pool, which translates to an average yield of 1.515 x 10^4 BMMSCs per 10 mL of bone marrow aspirate.[142] Donor age is a factor influencing human MSC frequency. Numbers have been reported to decline in an age-related manner from 1 in 10,000 (newborns) to 1 in 2,00,000 (an 80-year-old person).[143] However, it has been reported that multiple harvests stimulate the bone marrow, which may partially overcome the effect of age.[144]

Limited data are currently available concerning the number of cells required for repair of tendon defects and strain injuries. Multiple studies document the need for sufficient numbers of MSCs to optimize musculoskeletal tissue repair.[127,145-147] Canine models have shown that 15 million cells/mL of implant volume were sufficient to obtain improved bone production and regeneration.[148] Using a mathematical model, Muschler and colleagues estimated approximately 70 million osteoblasts would be required to produce a cubic centimeter of bone.[149] The number of MSCs required to treat equine tendon and ligament injuries is unknown; however, between 10-50 million MSCs was suggested by Richardson and colleagues.[4] *In vitro* studies using a rabbit model found that greater MSC seeding densities positively affected cellular morphology and contraction kinetics in collagen scaffolds.[150] In order to generate the requisite number of cells required for tendon repair, it is necessary to expand the numbers of cells isolated prior to transplantation. The cell doubling time for equine BMMSCs has been reported to be in the range of 1.4 -1.8 days.[142,151] It is feasible to obtain 1.5 x 10^4 primary MSCs following aspiration of bone marrow. Given a 23-day culture period (equivalent to 14 cell doublings), thus results in a 16,384-fold cell expansion, yielding ~250 million progenitor cells/10 mL of bone marrow aspirate.[142]

Variability of MSC yield has been reported for both horses and rats.[152,153] Comparison of the number of osteoprogenitor cells obtained from various commonly used cancellous bone graft sites in the horse revealed that sternal and tibial sites were inconsistent. The tuber coxae was the most reliable site, yielding on average 1.9% osteoprogenitor cells within the osteogenic cell population. [153] In a study evaluating the effect of MSC concentration on bone repair, significant variability was observed in the concentration of MSCs within bone marrow aspirates obtained from donor rats. This variation did not correlate with the age or sex of the individual.[152] Pittenger and colleagues reported yields of 50-375 million second passage cells following standard isolation protocols of 10 mL marrow aspirates obtained from 19-57 year-old human donors.[130] Factors that determine the variability of MSC concentration in bone marrow aspirates are unknown and there is need for further investigation into this area.

The identification of stem cells within the nucleated cell pool is somewhat controversial and there is no current consensus on a gold standard assay to isolate or identify BMMSCs.[154,155] It is generally accepted that bone marrow cells that adhere to tissue culture plastic are stem cells but the validity of this statement depends on the definition of stemness.[146] The most common test for MSCs have been *in vitro* assays for bone,

cartilage, adipose, and marrow stroma (hematopoietic support cells) using induction media. Some authors have reported the use of an *in vivo* porous calcium phosphate-ceramic cube implantation assay.[156-158] The problem with *in vitro* differentiation assays for MSCs is that the distinctive differentiated cells such as adipocytes or chondrocytes can transdifferentiate into completely different lineage phenotypes such as osteoblasts and therefore lead to erroneous conclusions.[117]

Use of cell surface markers (immunophenotyping) has been useful for determining isolation and lineage-specific differentiation of most somatic stem cells[123,128,130,141], but application has been hindered by lack of specificity and the need for immunohistochemical staining and automated flow cytometry to isolate the identified cells. Direct comparisons between human adipose-derived stem cells (ASC) and MSC immunophenotypes, show that they are >90% identical.[159] Many positive stem cell marker antibodies so far described in other species show little or no cross-reactivity in the horse and thus cannot be used.[160,161] In addition, change in the expression profile of stem cells with time in passage and plastic adherence has been reported.[162,163]

Gene specific DNA markers and their expression patterns are proving to be useful for identification purposes and recently a panel of markers was described for equine adult BMMSCs.[151] In this study, expression of the stem cell-related genes Oct4, Nanog and Sox-2 was identified. Oct4 and Nanog, which were previously considered exclusive markers of embryonic stem cells, were identified on the cell surface via immunohistochemistry (Oct4) and gene expression was detected via real time-PCR (Nanog). This observation and similar observations in murine MSCs[164] suggests that the distinction between embryonic and adult stem cells may no be strict. In fact, a comprehensive comparison using Affymetrix gene chips of human ASCs and MSCs revealed that these two cell types share a common transcriptosome.[165]

There have been no published studies identifying the optimum time to implant stem cells into injured equine SDFT lesions. Commonly cells are transplanted after the initial inflammatory phase but before fibrous tissue formation.[4] Richardson and colleagues hypothesized that the presence of mature fibrous tissue within the tendon would make implantation difficult and reduce the benefits of stem cell therapy because of its persistence. Their argument was supported by their clinical experience that indicated more successful outcomes when the interval between injury and implantation was on average 44 days versus 83 days for horses that reinjured their tendons.

Current studies of horses with natural occurring SDFT injuries with follow-up of ~1 year that were treated with autologous BMMSCs and that entered full training, reported a re-injury rate of 18%,[4] which compares favorably with a 56%[3] re-injury rate in previous analyses for the same category of horse treated conservatively. However, there is a lack of long-term follow-up (i.e. >2 years) and control studies that allow valid comparisons against horses treated conservatively with prolonged rehabilitation and controlled exercise. There are experimental studies using autologous BMMSCs transfected with green fluorescent protein that demonstrated engraftment of stem cells within mechanically induced SDFT lesions.[166] Another experimentally controlled study showed an improvement in structural aspects and histological scores following injection of BMMSCs into collagenase-induced SDF tendonitis lesions.[111] Interestingly, in this later

study, there were no differences in gene expression or biochemical analysis between MSC-treated and controls for tendon-related anabolic genes (Collagen type I & III), growth factor (IGF-I), or catabolic genes such as matrix metalloproteinases (MMP-3, -13) or aggrecanase-1 (ADAMTS-4).[111] Biomechanically, BMMSC-treated tendons were stiffer than controls, but this was not statistically significant.[111]

Adipose-derived stem cells

Adipose tissue derives from the mesodermal layer of the embryo and develops both pre- and postnatally.[167,168] Adult adipose tissue is a source of MSCs capable of multipotential differentiation in many species. In the human field of regenerative medicine, these cells have gained popularity because they are readily accessible in large quantities.[169] The microscopic location of the adipogenic progenitor cells is controversial, as it remains to be proven whether the origin of the cells correlates with the endothelial, pericyte, or stromal compartments.[170,171] Some authors hypothesize that MSCs are associated with perivascular locations. This hypothesis is supported by findings from morphological[172-175] and immunohistochemical[176,177] observations and from experiments demonstrating the differentiation of pericytes into mesenchymal cell types *in vitro*.[178-180] Results of a recent investigation into the correlation between MSC frequency and blood vessel density in horses showed that colony forming unit-fibroblast numbers were directly proportional to blood vessel density in adipose tissue, thus providing indirect evidence of MSC association with blood vessels.[181]

In humans, differences in stem cell recovery have been noted between subcutaneous white adipose depots, with the greatest numbers recovered from the arm as compared to the thigh, abdomen and breast.[182] The isolation of ASCs involves collagenase digestion of minced adipose tissue, centrifugation of the digest, and separation of the pelleted stromal vascular fraction (SVF) from the floating population of mature adipocytes.[169] The SVF consists of a heterogenous cell population, including circulating blood cells, fibroblasts, pericytes and endothelial cells as well as "preadipocytes" or adipocyte progenitors.[169] The final isolation step involves selection of the plastic adherent population within the SVF cells, which are enriched for ASCs. This step is not utilized in clinical practice and therefore the cell population used to treat tendon injury is not enriched for ASCs.

The number of primary SVF-nucleated cells in a population of middle-aged humans are reported to vary from ~300,000 to 404,000[183] cells/mL of lipoaspirate[82], which is comparable to equine data showing variability in numbers ranging from 140,000 to 538,000 cells/mL of adipose tissue.[184] The SVF contains a greater proportion of stromal/stem cells per unit volume relative to bone marrow, offering an advantage in horses, which tend to have a requirement for large numbers of cells given the size of common lesions in injured tendons.[184] Consistent tissue collection and laboratory techniques are pivotal for maximizing cell yields. The number of adherent human SVF cells was found to vary significantly depending on the collagenase digestion times, sampling size and sampling location.[185] The reason(s) for individual variability of ASC yields from SVF is yet unknown, however it is hypothesized that age or body mass may affect stromal cell numbers in adipose tissue. There are conflicting reports in the human literature concerning ASC yields and age, with one study failing to demonstrate a correlation[183] while another showed a very significant negative correlation with age.[186]

Recent investigation into equine ASCs revealed that approximately 1 in 2.3 ± 0.4 of the total SVF nucleated cells were MSCs, based on colony forming unit-fibroblast assays, and differentiation in response to adipogenic and osteogenic inductive conditions.[184] An average cell doubling time of 2.1 ± 0.9 days during the first 10 cell doublings was reported for these same ASCs harvested from young horses (≤5 years).[184] Furthermore, in this study, ASCs did not display a lag period in their initial expansion rates as was observed in BMMSCs in a companion study performed by the same group.[142,184] Human and laboratory animal ASCs display a cell doubling time of 2-4 days depending on the culture medium and passage number.[169] Prolonged passage (i.e. >4 months) of human ASCs have been observed to undergo malignant transformation but it is not known if the same is true for equine ASCs.[187]

One major advantage of SVF cells over BMMSCs is that the former has a higher initial yield of cells and therefore has the advantage of cheaper cost and speed of preparation (cells are returned to the practitioner within 48h).[4] Based on the average yield of 300,000 nucleated SVF cells/mL of adipose tissue, and a frequency of 43% MSCs, on average 1.3 x 10^6 MSCs would be harvested from 10mL of adipose tissue, which if cultured for 21 days would result in approximately 1.8 x 10^9 cells.[184] The potential yield of MSCs for similar collection volume and culture time is therefore superior for ASCs when compared to BMMSCs. A recent experimental controlled study using SVF cells in an equine collagenase model showed benefits in terms of tissue organization, but there was no difference in biochemical and molecular analyses between treated and control tendons.[188] Despite widespread clinical use of ASCs to treat tendon injury in North America, there are no published results reporting the outcome following treatment in clinical cases.

Tendon-derived progenitor cells

Salingcarnboriboon and colleagues were the first to report establishment of murine tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like properties.[189] The clonal-derived cell lines exhibited fibroblastic morphology and expressed tendon phenotype-related genes encoding scleraxis, six-1, COMP, EphA4, and type I collagen. These same cells were then implanted into patellar tendon defects in adult mice and histological evidence of tissue consistent with tendon was observed. However, fibrocartilaginous tissue was also present. To further define the plasticity of the cell lines, gene expression and response to induction media for other mesenchymal tissues was investigated. Low level osteoblastic, chondrogenic and adipogenic phenotype-related gene expression was reported. In addition, an increase in gene expression and characteristic changes in phenotype were observed when the cells were exposed to osteogenic, chondrogenic and adipogenic induction media. These observations collectively provided circumstantial evidence for the existence of a stem cell population in tendon, that have the potential to differentiate into tendon and other mesenchymal derived tissues.

Subsequent investigations performed by Bi and colleagues successfully identified and isolated a unique cell population from human and murine tendon tissues that, on the basis of a number of different criteria, showed characteristics of stem cells.[190] The isolated TPCs demonstrated clonogenicity, multipotency, and self-renewal capacity, and could generate tendon-like tissue after extended expansion *in vitro* and transplantation *in vivo*.

Using a DNA labeling-retention assay, this study was able to show that the TPCs reside within a niche environment that is surrounded predominantly by ECM proteins, suggesting that the ECM plays a role in organizing the TPC niche. This hypothesis was supported by experiments using mice deficient in the ECM proteins biglycan and fibromodulin. Depletion of these two SLRPs adversely affected the differentiation of TPCs by modulating bone morphogenetic protein (BMP) signaling, leading to impaired tendon formation *in vivo*.[190]

Recently, tendon-derived progenitor cells have been isolated from equine SDFT explants and the multidifferentiation potential of expanded progenitor cells has been demonstrated.[191] The isolated cell population exhibited fibroblast-like morphology and was shown to be capable of differentiating along multiple lineages including adipocytic, osteocytic, and chondrocytic lines. Direct comparison of TPCs with BMMSCs are limited; however, one research group reported poorer differentiation capacity of cells recovered from adult tendon compare to BMMSCs.[192] Further work investigating the capacity of TPCs to produce tendon tissue *in vivo*, and the induction signals required to promote tendon matrix synthesis, is required.

Evidence of differentiation of MSCs toward a tenocyte lineage *in vitro* has mainly been based on expression of tendon-specific or tendon-related genes. The difficulty of this process is that a marker that is specific to the tendon cell has not been identified. While many candidate genes, and even a panel of markers can be used, each of the putative markers has disadvantages to use for identifying a specific tendon cell.

Tenomodulin (Tnmd) is a type II transmembrane glycoprotein that is predominantly expressed in tendons, ligaments and the eye.[193,194] Low levels of mRNA transcripts have also been identified in muscle, thymus, heart, liver, spleen, nervous tissue, lungs, and cartilage.[195] Besides the utility of Tnmd as a tenocyte marker, it is also a regulator of tenocyte proliferation and is involved in collagen fibril maturation.[193]

Scleraxis (Scx), a transcription factor, is specifically expressed in mesenchymal precursors of connective tissues in early development. In later development stages in mice, Scx transcripts are selectively expressed in tendons and their progenitors.[196-198] Scx has been shown to be an important transcription factor during tendon development as evidenced by Scx null mutants exhibiting severe defects in force transmitting tendons.[199] It has also been shown to be a good marker of the tendon cell lineage *in vivo*, but its use *in vitro* has been questioned due to its expression in skeletal lineage cells.[194] Scleraxis positively regulates the expression of Tnmd and it has been suggested that the use of a combination of Scx as an early marker and Tnmd as a late marker would enable clarification of the molecular mechanisms underlying tenocyte differentiation and tendon regeneration.[194]

Gene expression markers of tenocytes in normal and diseased equine SDFT, and in monolayer and three-dimensional (3D) tendon progenitor cell culture were recently investigated.[200] Of 12 genes representative of musculoskeletal tissues, high levels of collagen alpha-2(1) chain (COL1A2) and Scx, and low levels of tenascin C were found to be representative of adult tensional tendon phenotype. Tenascin C gene expression was significantly upregulated in acutely injured tendon, which is consistent with the

appearance of tenascin C during the inflammatory phase of wound healing.[201,202] Relative Scx gene expression levels in tenocyte monolayer and 3D cultures were significantly lower than in normal adult tendon, making it difficult to use as a marker specifically for cell type outside of a whole tissue environment.

Interestingly, Tnmd was not shown to be a good marker of equine tenocytes as similar levels were identified in both tendon and bone.[200] This study confirmed that a panel of 'marker' genes is required to identify tendon cell phenotype from other mesenchymal tissues, which is a similar to investigations into TPC markers in mice and humans.[190] There appears to be some similarity in expression of markers between TPCs and BMMSCs types in mice and humans but their patterns are not identical.[190] TPCs highly express tendon-related factors, such as Scx, Tnmd, COMP, and tenascin-C. Mouse TPCs expressed CD90.2, a fibroblast marker, but not CD18, a BMMSC marker. These data suggest that TPCs are closely related to BMMSCs, but are not identical.

A major challenge in the application of TPCs is identifying a readily available autologous tendon tissue that can be used to isolate cells for expansion. Unlike bone marrow aspirates, autologous flexor tendon tissue is not readily available for use, and the removal of sections of tendon so that cells can be recovered leads to the formation of a secondary lesion at the donor site, and such lesions are unacceptably in horses. It has been proposed that the lateral digital extensor tendon can serve as a source of tissue for TPC isolation (Dr. J Barrett, personal communication). One study has shown that tenocytes from flexor *versus* extensor tendons behave similarly to *in vitro* conditions, suggesting that cells are equivalent and functionally respond to mechanical load to generate differences between tendon phenotypes.[203] However, a different study contradicted this study and demonstrated that there were differences between the flexor and extensor tenocytes in response to TGF-β and cyclic load.[101]Thus, using tenocytes from positional tendons or from other areas of the tendon might not be ideal.[4] In principle, progenitor cells maintain the ability to differentiate; therefore, concerns about whether the source is flexor or extensor tendon are allayed by the plasticity of the progenitor cell population.

Stem cell niche

The stem cell niche has been defined as a specialized microenvironment that houses stem cells and maintains a balance of quiescence, self-renewal and cell-fate commitment (figure 1.4).[190] The stem-cell niche is a three-dimensional structure composed of cells, cytokines, and the ECM.[204,205] A number of stem cell niches have been identified within a variety of tissues and organs, such as the bulge of the hair follicle, crypt and perivascular region provide niche microenvironments for epidermal, intestinal, and neural stem cells, respectively.[190] As mentioned previously, the tendon stem cell niche has been shown to be composed predominantly of ECM and that alteration of ECM composition changes the TPC pool size. In murine tendon, altering the niche has been shown to detour TPC fate from tenogenesis to osteogenesis, leading to ectopic ossification in the tendon of biglycan and fibromodulin deficient mice.[190]

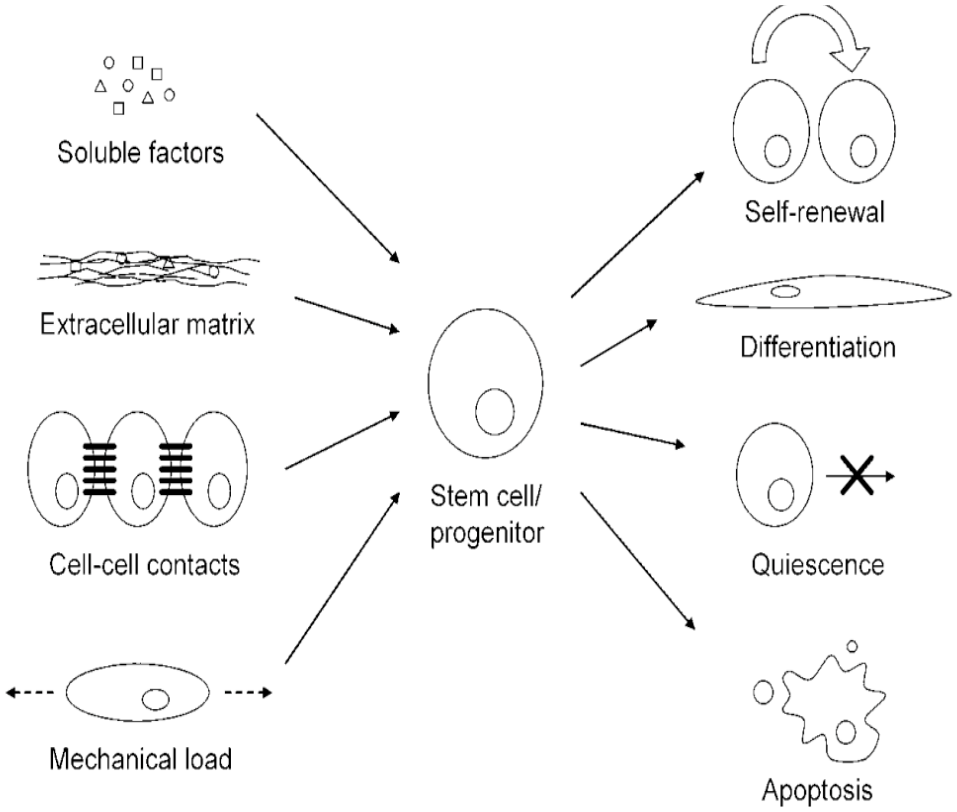

Figure 1.4. Stem cell interactions with various inputs from the microenvironment.[206] (used under fairuse guidelines)

MSCs as trophic mediators

Investigators have postulated a number of nonexclusive mechanisms through which mesenchymal stem cells can be used to repair and regenerate tissues. MSCs delivered into an injured or diseased tissue may secrete cytokines and growth factors that stimulate recovery in a paracrine and autocrine manner. Such functional secretions of bioactive factors can have profound effects on local cellular dynamics. For example, MSCs could modulate the stem cell niche of the host by stimulating the recruitment of endogenous stem cells to the site and promote their differentiation along the required pathway.[169] In a related manner, MSCs might provide antioxidants, free-radical scavengers, and chaperone/heat shock proteins at an ischemic site. As a result, toxic substances released into the local environment would be removed, promoting recovery of the surviving cells. Recent studies have suggested that transplanted BMMSCs can transfer mitochondria to damaged cells, rescuing aerobic metabolism.[207] MSCs may also differentiate along a desired lineage and directly participate in tissue regeneration.

The effects of MSC-secreted bioactive molecules can be either direct or indirect or both. Direct effects occur via intracellular signaling and indirect effects are the result of causing another cell in the vicinity to secrete a functionally active agent.[115] The indirect route is referred to as trophic by some authors and this term is adapted from the original

use of the word by neurobiologists to indicate the bioactive molecules released from nerve terminals that were not neurotransmitters.[115] Caplan and colleagues defined trophic effects as those chemotactic, mitotic, and differentiation-modulating effects that emanate from cells as bioactive factors exerting their effects primarily on neighboring cells and whose effects never result in differentiation of the producer cell.[115] An example of this trophic effect is the action of marrow stromal cells in the support of hematopoiesis via the production of cytokines such as granulocyte colony-stimulating factor (G-CSF), stem cell factor, myeloid colony-stimulating factor (M-CSF), and interleukin-6 (IL-6), while the MSCs do not themselves become hematopoietic.[208]

The results of tissue engineering experiments evaluating the incorporation of MSCs into tissue repair models have been mixed.[209] In many situations, MSCs are either not shown to be present in the repair or present in very low numbers.[210,211] Despite these results, there is often a measurable therapeutic effect, which has been attributed to the trophic effects of MSCs. In support of this theory, there is evidence of MSC-mediated trophic effects in stroke, myocardial infarct and meniscus repair models.[115] For example, in a rodent model of stroke where MSCs were systemically injected after permanent middle cerebral artery occlusion, treated rats showed reduced thickness of the scar wall that usually forms, and reduced numbers of reactive astrocytes in the scar boundary subventricular zones.[212] These effects were attributed to MSC-mediated inhibition of scar formation, decreased apoptosis, increased angiogenesis, and stimulation of intrinsic cells to support the re-establishment of the complex neurological pathways resulting in coordinated motor and neural activity.[115] All of these effects were observed in the absence of any evidence of integration of labeled MSCs within the neural tissue. Similarly, the trophic effects of MSCs on cardiac infarct models have shown that MSCs implanted into ischemic myocardium stimulated an increase in the production of vascular endothelial growth factor, increased vascular density and blood flow, and decreased apoptosis, all of which were influenced by the secretion of bioactive molecules.[213]

MSC immunomodulatory effects

Several studies have reported on the *in vitro* and *in vivo* immunosuppressive properties of BMMSCs.[214-217] MSCs secrete a number of bioactive factors, some of which act to directly inhibit the effector functions of both B and T cells, the generation of dendritic cells, and the proliferation of natural killer (NK) cells in response to interleukin (IL)-2.[218] In bone marrow, it has been speculated that one of the main functions of MSCs is the protection of hematopoietic precursors from inflammatory damage.[219] This was supported by a study using a lethal cecal-puncture ligation murine model that showed MSCs were capable of inhibiting the inflammatory effects of septic shock.[220] Inhibition of chronic inflammatory processes such as models of autoimmune arthritis, diabetes, multiple sclerosis, and lupus have been documented by syngeneic, or in some cases allogenic MSCs.[221] Once at the site of inflammation, MSCs act to regulate inflammation through processes such as suppression of macrophage activation, inhibition of T-cell helper (Th)-1 & 17 generation, suppression of NK and T cytotoxic cell function, stimulating generation of Th2 cells, inducing generation of regulatory T cells (Tregs) and eliciting suppression of dendritic cell maturation.[221] Mechanistically, MSC are thought to suppress various immune functions through release of immune suppressive cytokines such as IL-

10, TGF-β and hepatocyte growth factor (HGF), and other inhibitory enzymes and inhibitory molecules.[221] The exact *in vivo* mechanism(s) are not clearly established; however *in vitro* studies indicate that direct contact between MSCs and lymphocytes may also be important.[222]

The immune phenotype of culture-expanded MSCs is widely described as major histocompatability complex (MHC) class I positive, MHC class II negative, CD40 negative, CD80 negative, and CD86 negative.[223] Because of difficulties in obtaining sufficient autologous stem cells from some patients, alternative allogenic sources of stem cells from healthy donors have attracted considerable interest. BMMSCs have been shown to escape the immune system because they do not express MHC class II or co-stimulatory molecule B7, and consequently, they do not induce allospecific T cell proliferative responses.[217] MSCs are therefore regarded as nonimmunogenic, suggesting that they are capable of trespassing donor immune barriers. Similar immunosuppressive properties have been described for human and rodent allogenic ASCs *in vitro*.[222] The ability to use allogeneic MSCs has been reported in other species.[224] More recently in the equine field, transplantation of allogeneic BMMSCs into experimentally created SDFT lesions of 2 horses was reported where there was no histological inflammatory response to transplantation.[166] Use of allogeneic BMMSCs may be of practical importance because MSCs become more difficult to isolate from bone marrow with increasing age and MSCs from aged patients have been shown decreased multipotency.[225,226]

Gene-enhanced MSCs

Growth factors are peptide-signaling molecules that regulate many aspects of cellular metabolism including the cell cycle, cell growth and differentiation, and the production and destruction of ECM products.[111] Given that the effects of growth factors are mediated primarily via autocrine and paracrine mechanisms, local sustained administration of exogenous growth factors is required and this has led to gene therapies using MSCs. Previous work has shown beneficial effects of particular growth factors such as insulin-like growth factor I (IGF-I), in stimulating cellular proliferation and matrix synthesis in an equine collagenase-induced model of tendon injury.[112] The short half-life of IGF-I and other potential growth factors makes their exogenous administration challenging and costly. A recent study utilizing IGF-I gene enhanced autologous BMMSCs demonstrated improved structural aspects of healing in equine collagenase-induced tendon injury model; however, the added value of IGF-I gene-enhanced MSC implantation was minimal compared to naïve MSC injection.[111]

The bone morphogenetic proteins (BMP) are a family of related proteins in the transforming growth factor-β superfamily known for osteoinductive capacity.[227] BMP12, a human analogue of murine growth and differentiation factor-7, is related to other BMPs involved in the developmental processes of the musculoskeletal system,[228] including regulating tissue differentiation,[229,230] tendon healing, and tenogenesis.[231] Unlike other BMPs, BMP12 does not have an obvious osteoinduction effect on tendon cells and is associated with accelerated healing in a variety of animal tendon repair models.[232] In rats, *in vivo* experimentation revealed that BMP12 induced formation of tendon- and ligament-like tissue,[233] and differentiated MSCs toward tenocytes *in vitro*.[229] In a recent *in vitro* study evaluating the influence of BMP12 gene enhancement on equine tenocytes and

BMMSC cultured on monolayer surfaces, tenocytes responded by demonstrating early morphological evidence of gene expression of tendon-related proteins such as collagen type I and COMP.[232] Further *in vivo* studies of BMP12 gene enhanced TPCs is warranted to evaluate the effects of BMP12 on tendon healing. BMP12 gene enhanced TPCs may perform a dual role by participation in tissue regeneration and as a vehicle for growth factors (i.e. BMP12) and thus provide a novel treatment for tendon injury.

Influence of extracellular matrix

The ECM is well known for its ability to provide structural support for organs and tissues. The role of the ECM in cell adhesion and signaling to cells through adhesion receptors such as integrins and fibronectin has received much attention. More recently, mechanical characteristics of the matrix (stiffness, deformability), as well as mechanical load, have been shown to provide inputs into cell behavior.[234] The interaction between cells and the ECM is an important regulatory factor of cell function. Proliferation, migration, differentiation and gene expression of many cell types may all be altered by adhesion to and interaction with matrix proteins and the extracellular environment.[235] Tendon progenitor cells reside within a niche that comprises primarily parallel collagen fibers, and this niche plays an important role in regulating their function and differentiation.[22,190,236]

Collagen-cell interactions

The prevalence of collagen in the ECM of the majority of tissues in the body underlies its ability to support growth, differentiation and function in a wide variety of cell types.[237] Normal cells must attach to a substrate in culture to survive and divide, and they deposit material on the surface to which they adhere.[238] The standard culture conditions that are used in stem cell culture are proprietary treated plastic surfaces and it has been shown that the plastic or glass surface is adequate without collagen. Although it is not clear how glass and plastic surfaces interact with the cell membrane, studies suggest that tissue culture plastic absorbs fibronectin and other glycoproteins from serum that the cells use for attachment. In this case, the plastic and glass would serve as a surrogate for the natural collagenous matrix. Cell-collagen adhesion is mediated by many glycoproteins, of which fibronectin has been extensively studied (figure 1.5).[238] One of the earliest demonstrations of a positive and direct effect of an ECM macromolecule on the differentiation process was the clonal conversion of myoblasts to myotubes in response to collagen.[238] Both native and denatured collagen support differentiation, but it was only when the collagen was used as a substrate and not when it was added to the medium, that an effect was observed.[238] Collagen has low immunogenicity[239] and different species collagen have been shown to have an equal effect in promoting differentiation of muscle.[240] Since collagen is a component of the substrate laid down by cells, is widely recognized for its ability to promote differentiation, and has minimal immunogenicity, it is logical to hypothesize theoretical benefits of collagen-coated tissue culture surfaces. Indeed, the beneficial properties of collagen are utilized when designing biologic scaffolds for tissue engineering purposes.[206]

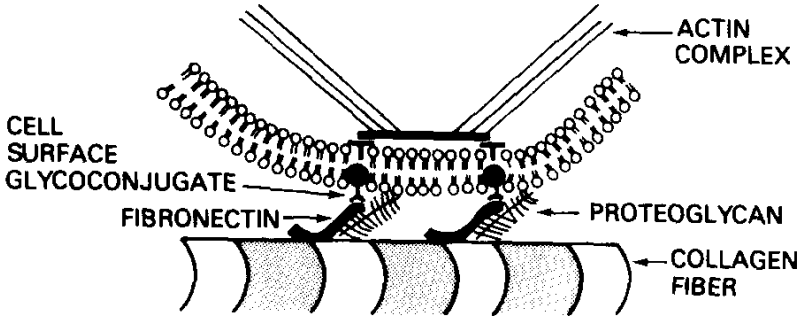

Figure 1.5 Schematic model of a cell-collagen adhesion site.[238] (used under fairuse guidelines)

Investigations into the fate and action of transplanted stem cells has led to development of *in vitro* systems that attempt to replicate or simulate important components of the native tendon ECM. Richardson and colleagues investigated culture of equine BMMSCs in 3D gels comprised of either hydrogel mixed with specific matrix proteins or bone marrow supernatant and found that the BMMSCs were able to produce abundant ECM consisting of linearly aligned extracellular matrix with some remarkable similarities to tendon.[241] They found that the addition of ECM proteins abundant in tendon (*e.g.* collagen type I) stimulated an increase in expression of this gene in the cells, consistent with the hypothesis that interaction with matrix proteins is an important stimulus for BMMSC matrix synthesis.

Studies investigating the effects of devitalized tendon matrix on BMMSC and TPC growth and tendon-related gene expression were recently performed.[9,241] Equine TPCs and BMMSCs cultured on an autologous acellular native tendon matrix demonstrated engraftment and alignment with the highly organized collagen network. TPCs had superior cell growth kinetics, with similar matrix biosynthetic capabilities to BMMSCs, suggesting a positive influence of the primarily collagen matrix.[9] The similar gene expression between the two cell types suggested that the matrix had influenced the differentiation of BMMSCs toward a more tenocytic nature. These studies provide circumstantial evidence that implanted cells can survive and induce the formation of new tendon in a similar fashion to tenocytes. Furthermore, the ECM plays a key role in influencing stem cell differentiation.

ECM and stem cell differentiation

With the inherent plasticity and multilineage potential provided by stem cells comes an increased need for regulating cell differentiation, growth, and phenotypic expression. Classically, the control of stem cell fate, either *in vivo* or *in vitro*, has been attributed principally to genetic and molecular mediators (*e.g.*, growth factors, transcription factors). However, increasing evidence has revealed that a diverse array of additional environmental factors contribute to the overall control of stem cell activity. There is strong evidence emerging that the ECM influences stem cell fate through interactions

between ECM ligands and cell surface receptors.[242] It is now clear that ECM-based control of the cell may also occur through multiple physical mechanisms, such as ECM geometry at the micro- and nano-scale, ECM elasticity, or mechanical signals transmitted from the ECM to the cells.[243] An improved understanding of the interaction of these mediators with classical signaling pathways may provide new insights into the regulation of self-renewal and differentiation of stem cells. The ability to better engineer artificial ECMs that can control cell behavior, through physical as well as molecular interactions, may further extend our capabilities in engineering substitutes from adult or embryonic stem cells.[206]

Birch and coworkers have shown marked differences in matrix composition and turnover in functionally distinct tendons. These differences relate to the magnitude of strain that tendon experiences during physiological function.[85] They hypothesized that tendon cells may be pre-set during embryological development to function at a specific strain suitable for their anatomical position. Alternatively, cells may simply respond to the imposed strain environment by expressing a phenotype suitable for the environment. Recent research in the field of dermatology has demonstrated that skin fibroblasts have 'positional memory' and express specific gene patterns that relate to their topographic identity.[8] Determining if cells are pre-programmed or respond to environmental cues is key to successful cell based therapies and tissue engineering strategies. Little is known about the capability of MSCs to differentiate into tissue-specific cell types *in vivo*. A combination of mechanical stimuli and proximity to tenocytes and tendon matrix are believed to be important stimuli for differentiation into tendon cells.[151]

Two- versus three-dimensional culture

Monolayer culture of TPCs provides a simple method to study cell phenotype. Recent investigations have highlighted difference in cellular gene expression in monolayer compared with 3D cultures.[244] The gene expression profile of tenocytes in monolayer culture has recently been shown to alter with progressive passaging.[245] Comparison of tendon cell markers in equine monolayer cultures and normal tendon has highlighted significantly less or complete loss of certain gene expression in monolayer and 3D culture.[200] Three dimensional cell culture methods are thought to more closely mimic the *in vivo* cellular environment; however, certain *in vitro* culture systems may not adequately mimic *in vivo* conditions.[244,246] Further work is warranted to identify culture systems that recreate the transcriptional profiles of normal tendon.

Conclusions

Equine SDF tendinopathy is a significant clinical problem for horse owners and equine veterinarians. Limited clinical and experimental research results suggest a significant improvement in the outcome of cases with the use of stem cell-based therapies that have been developed recently. Questions regarding the optimal progenitor cell type for use in tendon injury, how to consistently identify commonly used progenitor cells, how to optimize growth and differentiation of progenitor cells *in vitro*, and the most appropriate combination of progenitor cells, growth factors and scaffolds or extracellular matrix that can optimize tendon regeneration, remain. There is considerable hype regarding stem cell application in general and while tremendous advances have been made in recent years

that do provide convincing support for stem cells to be a clinically useful and effective treatment, there is much to learn about these cells and their application. Unrealistic expectations in the short term may lead to disappointments and failures that would be detrimental to the development of this important research area.

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Objectives and Hypothesis

Objectives

To compare cell growth kinetics and biosynthetic capabilities of bone marrow mesenchymal stem cells (BMMSCs) and tendon derived progenitor cells (TPCs) cultured on bovine, highly purified bovine, porcine, and rat collagen-coated tissue culture surfaces.

Hypotheses

We hypothesized that collagen type I matrix would preferentially support TPC proliferation and up regulate gene expression for collagens and organizational components of tendon and therefore provide a culture system and progenitor cell type with advantages over the current practice of BMMSC expansion on standard cell culture plastic surfaces.

Chapter 2: Manuscript

Comparison of bone marrow mesenchymal stem cells and tendon progenitor cells cultured on collagen surfaces

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Abstract

Objective - To compare cell growth kinetics and biosynthetic capabilities of bone marrow mesenchymal stem cells (BMMSCs) and tendon derived progenitor cells (TPCs) cultured on bovine, highly purified bovine, porcine, and rat collagen-coated tissue culture surfaces.

Sample Population - Cells from 6 young adult horses.

Procedures - Cells were isolated from sternal bone marrow aspirates and mid metacarpal superficial digital flexor tendon, expanded, and cultured on bovine, porcine and rat collagen type I-coated tissue culture plates for 7 days. Samples were analyzed for cell viability and number on days 4 and 7, glycosaminoglycan (GAG) synthesis on day 7, and mRNA expression of collagen type I, collagen type III, cartilage oligomeric matrix protein (COMP), and decorin on day 7.

Results – A statistically significant (P=0.05) increase in cell number was observed for TPCs grown on rat collagen on day 4. No difference in GAG synthesis or expression of collagen type I, collagen type III, COMP or decorin mRNA was observed between collagen groups and non-collagen controls for either cell type on day 7. TPCs cultured on all collagen types yielded more cells than similarly cultured BMMSCs on day 4, but only porcine collagen was superior on day 7. TPCs synthesized more GAG than BMMSCs when cultured on control surfaces only. BMMSCs expressed more collagen type I mRNA when cultured on control, porcine and highly-purified collagen, and more collagen type III when cultured on control, porcine, highly-purified collagen, and rat collagen, than TPCs. Tendon-progenitor cells expressed significantly more COMP when cultured on control and all collagen types, and decorin when cultured on porcine, highly purified bovine and bovine collagen when compared to BMMSCs.

Conclusions and Clinical Relevance - There is an advantage to culturing TPCs on randomly organized rat collagen during the early growth phase. Tendon progenitor cells showed superior growth kinetics, and expression of the matrix organizational components, COMP and decorin, than similarly cultured BMMSCs that preferentially

expressed more collagen types III and I. Further *in vitro* studies characterizing factors that influence gene expression of both BMMSCs and TPCs are warranted.

Introduction

Tendon injuries are a significant cause of morbidity in equine performance horses. Superficial digital flexor tendon (SDFT) injury is reported to represent up to 43% of overall Thoroughbred racehorse injuries leading to early retirement of approximately 14% of horses.[1-6] Natural repair is slow and results in inferior structural organization and biomechanical properties, therefore, reinjury is common with rates of up to 80% reported in racehorses.[3,7] The inability of tendon to regenerate after injury, or heal with mechanical properties comparable to the original tissue, is likely attributable to low vascularity and cellularity of the tissue, low number of resident progenitor cells, and healing under weight-bearing conditions.[2,3,8,9]

Strategies to improve tendon healing have focused on enhancing the metabolic response of tenocytes, modulating the organization of the newly synthesized extracellular matrix, or administering progenitor cells to enhance repair.[10-14] Significant research effort has been directed at the use of adult mesenchymal stem cells (MSCs) as a source of progenitor cells for equine tendon repair. Recent clinical applications have utilized adult autologous MSCs derived either from adipose tissue or bone marrow aspirates.[13,15-22] Isolation of a homogeneous population of progenitor cells from bone marrow is time-consuming, and there is much variation in cell numbers, cell viability and growth rates among samples.[23,24] Recently, a population of progenitor cells with multidifferentiation potential has been isolated from equine flexor tendons providing an alternative source of progenitor cells as well as a target cell for therapeutic intervention.[25]

Tendon is composed primarily of type I collagen arranged into fibers aligned along the longitudinal axis of the tendon.[26] Collagen type III is also present but only comprises approximately 4-5% of total collagen in the metacarpal region of normal adult equine SDFT.[27,28] Cartilage oligomeric matrix protein (COMP), and decorin are important extracellular matrix components produced by tenocytes, that together with collagen type III, have been shown to be integral in the regulation of fibrillogenesis and organization of tendon.[29-31] Collagen fibers are surrounded by ground substance composed of proteoglycans and glycosaminoglycans (GAGs) that help package the collagen fibrils. GAGs are negatively charged macromolecules, that are important in determining the water content of the extracellular matrix (ECM) of tendon.[32] Collagen, COMP and proteoglycan synthesis are all increased following tendon injury.[33-37]

The interaction between cells and the ECM is an important regulatory factor of cell function. Proliferation, migration, differentiation and gene expression of many cell types may all be altered by adhesion to and interaction with matrix proteins and the extracellular environment.[38] Tendon progenitor cells reside within a niche comprised primarily parallel collagen fibers that plays an important role in regulating their function and differentiation.[26,39,40] Two independent studies have evaluated the effects of acellular native tendon matrices on equine tenocytes (TCs) and BMMSCs, or TPCs and BMMSCs.[41,42] Both demonstrated engraftment and alignment with the highly organized collagen network. Positive effects of collagen type I-coated surfaces on BMMSC proliferation and gene expression of collagen types I and III, fibronectin, and decorin were reported; however, the effect of collagen on TPC proliferation has not been studied. It is unknown whether a collagen-rich extracellular environment could influence TPC

growth and whether there are any differences between species-specific type I collagens in their ability to influence cell proliferation and tendon matrix gene expression.

The objectives of this study were to compare cell growth kinetics and tendon matrix component biosynthetic capabilities of TPCs and BMMSCs cultured on commercially available bovine, porcine and rat type I collagen sources. We hypothesized that a randomly oriented collagen matrix would preferentially support TPC proliferation versus BMMSCs, and upregulate tendon-related gene expression and therefore provide a culture system and progenitor cell type with advantages over the current practice of BMMSC expansion on standard tissue culture surfaces. A culture system that is able to efficiently provide adequate cell numbers for cell therapy and direct progenitor cells to produce tendon matrix, would be beneficial to regenerative medicine efforts to improve the outcome of equine flexor tendon injury.

Materials and Methods

Collection of samples- Bone marrow aspirates and tendon samples were collected aseptically from six young horses (2-5 years) euthanatized for reasons unrelated to musculoskeletal disease. Samples were obtained in accordance with the guidelines reviewed and approved by the Institutional Animal Care and Use Committee of the Virginia Polytechnic Institute and State University. All horses were sedated with 0.5-1.0 mg/kg of xylazine intravenously prior to induction of anesthesia. Anesthesia was induced with 2.2 mg/kg of ketamine and 0.1 mg/kg of diazepam given intravenously. General anesthesia was maintained by intravenous infusion of 5% guaifenesin, 1 mg/mL ketamine and 1 mg/mL of xylazine. Following collection of bone marrow aspirates as previously described,[43] all horses were euthanatized with 104 mg/kg of pentobarbital sodium given intravenously. The tendon specimens were collected immediately following euthanasia, as detailed below.

Cell culture technique- All cell cultures (both BMMSCs and TPCs) were incubated at 37ºC in a 5% carbon dioxide atmosphere with 90% humidity for media supplementation every 48 hours. Once approaching 70% confluence, adherent cells were trypsinized using standard tissue culture technique, counted and plated at 500,000 cells per 75- cm^2 flasks to propagate adequate cell numbers. Time to confluence and cell counts at trypsinization were recorded. BMMSCs were grown in BMMSC medium: low-glucose Dulbecco's modified eagle medium (DMEM)[a] supplemented with 10% fetal bovine serum (FBS)[b], 300 μg of L-glutamine[c]/mL, 100 U of sodium penicillin and 100 μg of streptomycin sulfate[d]/mL. TPCs were grown in TPC medium: high-glucose DMEM supplemented with 10% FBS, 10% Horse Serum (HS), 37.5 μg/ml of ascorbic acid, 300 μg of L-glutamine/mL, 100 U sodium penicillin and 100 μg of streptomycin sulfate /mL. TPCs and BMMSCs were each tested for cell proliferation in both DMEM glucose concentrations and both serum concentrations (low-glucose DMEM *v*. high-glucose DMEM; 10% FBS *v*. 10% FBS 10% HS), and the above media were the optimal media tested for each cell type (data not shown).

Processing of bone marrow mesenchymal stem cells- The left tuber coxae was clipped, aseptically prepared and a bone marrow biopsy needle[e] was used to aspirate a total of 60 mLs of bone marrow into 2 syringes each containing 5,000 units of heparin diluted to a

volume of 10 mLs with phosphate buffered saline (PBS). Bone marrow aspirate was then transferred to centrifugation tubes, diluted with PBS solution (2:1) and centrifuged at 300 x *g* for 15 minutes at 4ºC. The cell pellets were resuspended in PBS solution, and centrifugation repeated. Pelleted cells were resuspended in 12 mL of BMMSC medium in 75-cm^2 flasks.

Processing of tendon-derived progenitor cells- The entire metacarpal SDFT was harvested aseptically from both forelimbs from each horse following euthanasia. A 2-cm^3 sample from the mid-metarcarpal core region was snap-frozen in liquid nitrogen for control RNA isolation. A 6-cm X 1-cm^2 sample of tendon from the mid-metacarpal tensional region was diced into 0.5-cm^3 pieces and digested in an orbital shaker for 16 hours at 37ºC in 0.1% collagenase[f] high-glucose DMEM supplemented with 1% FBS, 37.5 µg/mL of ascorbic acid[g], 100 U of sodium penicillin and 100 µg of streptomycin sulfate /mL. Following digestion, the suspensions were passed through 100µm sterile cell filters[h]. The isolated cells were collected by centrifugation at 300 x g for 5 minutes. The supernatant was removed, and the cell pellet was resuspended in TPC medium. The cells were then subjected to a differential adherence protocol as previously described.[42,44] Briefly, cells were plated and allowed to settle undisturbed for 2 days prior to the slowly adherent cells being removed and placed in a new tissue culture plate. The slowly adherent cell population, or TPCs, was expanded to obtain adequate numbers for experiments, all experiments used cells from passage 1. The isolated TPCs were cultured in 75-cm^2 flasks in TPC medium as described above until approximately 90% confluence. Time to confluence and cell counts at trypsinization were recorded. Cells were released from the flasks with trypsin (0.05%) and re-seeded at 5000 cells/cm^2.

Tendon progenitor cell and bone marrow mesenchymal stem cell culture- Once adequate cell numbers for each TPC and BMMSC culture were obtained, cells were trypsinized and resuspended at a concentration of 1.5 x10^7 cells in 1.5 mL of DMEM, 10% FBS, and 10% DMSO, and then stored in the vapor phase of liquid nitrogen. All cultures that were utilized for these experiments were from passage 1. The viability of all cryopreserved cells was assessed with trypan blue stain[i] immediately after thawing.

First passage TPCs and BMMSCs were seeded at 1 x10^3 cells/cm^2 in 24-well plates, and 25 cm^2 (T25) flasks. For the well plates and T25 flasks, experiments were equally divided between surfaces with no modification and wells and flasks pre-coated with bovine[j], highly purified bovine[k], porcine[l], and rat[m] collagen type I. Tissue origins for each collagen preparation were as follows: bovine: dermis, highly purified bovine: tendon, porcine: dermis, and rat: tendon. The porcine and rat collagens were dissolved in 0.02M acetic acid, and the bovine and highly purified bovine collagens were dissolved in 0.01M HCl. Diluted collagen solution was added to tissue culture surfaces to result in a final surface area concentration of 8 µg/cm^2 of the respective collagen, and washed with PBS to normalize pH. Experiments performed on twenty four-well plates were performed in triplicate and T25 flasks for mRNA analysis were performed in duplicate. Media was changed every 48 hours and cultures were monitored daily over the 7 day culture period. Photomicrographs were taken on day 5.

Cell proliferation- The CellTiter 96 Aqueous[n] assay was used to determined cell number of 3 replicates of each cell type and collagen group on 24-well plates on days 4 and 7. For

the tissue well plates, 50 μL of the CellTiter reagent was added to fresh ascorbate-free media in each well and the cells were incubated at 37ºC for 2.5 hours. A 100 μL sample of media from each test well were transferred to a 96-well plate and absorbance was measured at 490nm in a microplate reader[o]. All samples were assayed in triplicate, and a mean value was calculated to provide a single data point. The optical density values were converted to a cell number by reference to standard curves carried out on cells from each horse for each cell type. Specifically, the standard curve was generated by trypsinizing cells and counting using a hemacytometer. Cells were then plated as a serial dilution in 24-well plates, and the same procedure was performed on the standard curve wells as the sample wells after the cells equilibrated overnight.

RNA isolation and gene expression-The expression of selected genes characteristic of tendon fibroblast phenotype (collagen types I and III, COMP, decorin) was quantified on day 7 by real-time PCR. Duplicate 25-cm^2 flask samples from each experimental group were isolated using Trizol[p] method, and purified by use of a commercially available column-based protocol[q]. This protocol included an on-column DNase treatment to exclude genomic template contamination. The RNA from freshly collected, snap-frozen tendon was used as a reference control for gene expression analysis, to relate *in vitro* expression levels to *in vivo* expression.

Tendon tissue RNA was isolated by use of a protocol adapted from a technique for cartilage RNA isolation.[45] Briefly, tissues were pulverized under liquid nitrogen, then homogenized in guanidium isothiocyanate lysis buffer, extracted with phenol-chloroform, precipitated with isopropanol, and purified by use the column-based protocol (above).

One microgram of RNA in each sample was converted to cDNA with a commercial transcription kit and oligo (dT) primers[r]. Target cDNAs were amplified via real-time PCR by use of *Taq* DNA polymerase (TaqMan®)[s] and gene specific primers and MGB probes from available published equine sequences (**Appendix A**). Real time quantitative PCR assay was performed in triplicate for collagen type I, collagen type III, COMP, and decorin and as a reference, 18S RNA. A Real-Time PCR system[t] was used to perform the assay. All reactions were run as single-plex, and the relative gene expression was quantified by use of the $2^{-\Delta\Delta Ct}$ method.[46] Collagen type I, collagen type III, COMP and decorin mRNA values were normalized to expression of 18S RNA, and subsequently normalized to tendon tissue expression of each gene product of interest.

Glycosaminoglycan- Cell monolayers were collected for quantification of glycosaminoglycan production on day 7. Cell monolayers were released with 2mM EDTA at 37ºC for 10 minutes, and digested in papain[u] (0.15 mg/mL) at 65ºC overnight. The 1,9-dimethymethylene blue assay was performed by use of the direct spectrophotometric method to measure the total GAG content.[47,48] Results were compared with a chondroitin sulfate standard curve and standardized to relative cell number (DNA). Total DNA content was determined from the papain digest by use of fluorometric dye assay[v] and a microplate reader, as previously described.[49] Results were compared with a standard curve of calf thymus DNA.

Statistical Analysis- All cell count data were $\log_e$ transformed to obtain normal distribution. Differences of cell count, GAG synthesis and gene expression between

collagen groups and cell type, were evaluated by use of mixed-model repeated measures ANOVA. Pair-wise comparisons were made on significant differences identified with ANOVA using Tukey's post hoc test. A commercial statistical program[w] was used to perform analysis. Cell count data are reported as geometric least squares means and relative gene expression data are presented as mean ± SD. Values of $P \leq 0.05$ were considered significant.

Results

Cell isolation and expansion- Following differential adherence plating, the tendon progenitor cells proliferated in uniform monolayer cultures and adopted a tightly packed, fusiform morphology. BMMSCs grew in clonal expansion groups that had focal areas of tightly packed cells with fusiform morphology. Less time to confluence after initial plating was recorded for TPCs than BMMSCs (5-8 days and 12-14 days, respectively) but thereafter, subsequent passage times for both cell types were similar (4-6 days).

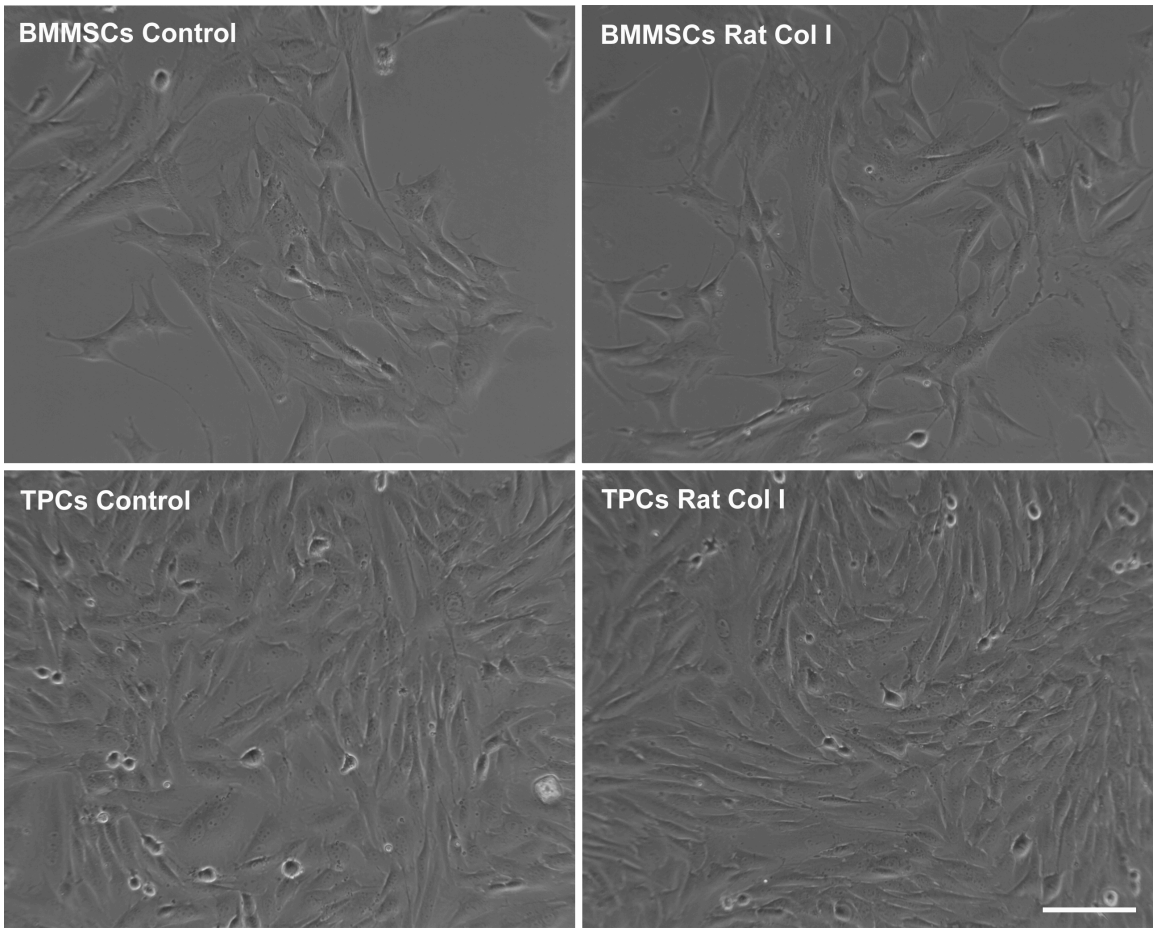

Figure 2.1 Representative phase contrast photomicrographs of cell morphology of TPCs and BMMSCs following 5 days of culture on control and rat collagen type I. Bar = 100μm

Cell morphology and number- Figure 2.1 shows representative images of the TPCs and BMMSCs growing on control (uncoated) wells *v*. collagen-coated wells on day 5. Cell

morphology after 5 days in culture on collagen coated plates was not subjectively different; however, a difference in cell number between cultures is apparent.

Increased cell growth was observed on all collagen coated plates for both BMMSCs and TPCs versus control on days 4 and 7; however, only TPCs cultured on rat collagen was significantly (P = 0.05) increased on day 4 (figures 2.2 & 2.3; table 1). When comparing between cell type, TPCs cultured on all collagen groups yielded significantly more cells than similarly cultured BMMSCs on day 4, but only when cultured on porcine collagen-coated surfaces on day 7.

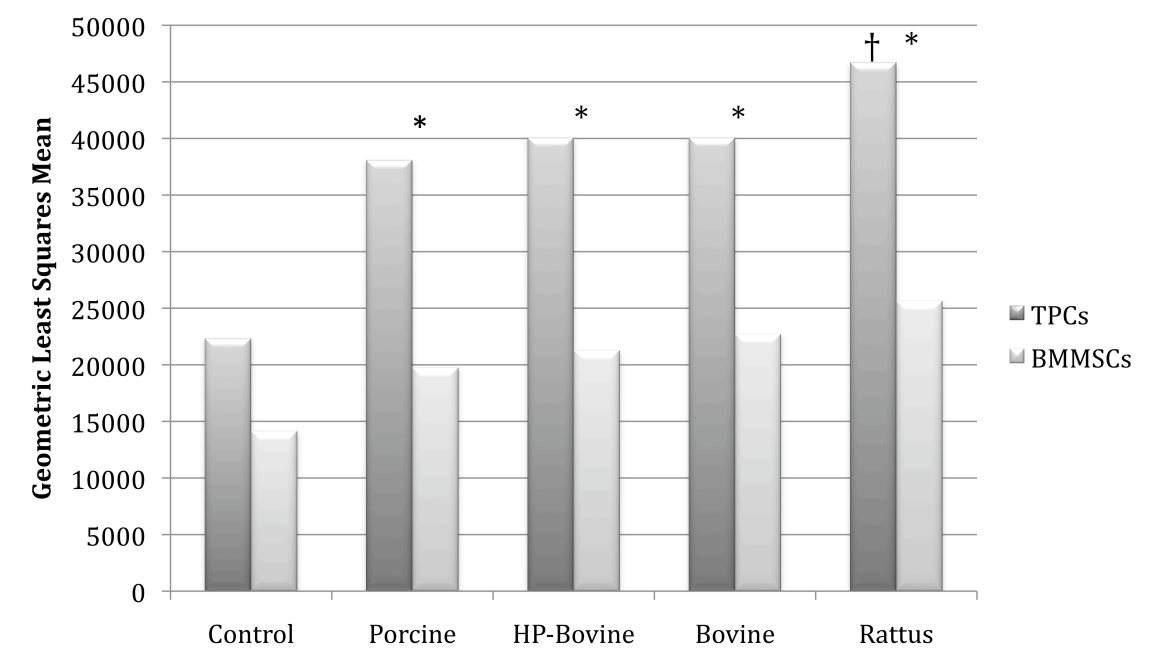

Figure 2.2 Cell count of TPCs and BMMSCs following 4 days of culture on control and collagen-coated plates. Asterisk (*) denotes differences ($P \leq 0.05$) between TPCs and BMMSCs within treatment group; dagger (†) indicates difference between collagen type and control for TPCs (P=0.05). TPCs; tendon progenitor cells, BMMSCs; bone marrow mesenchymal stem cells, HP-Bovine; highly purified bovine collagen.

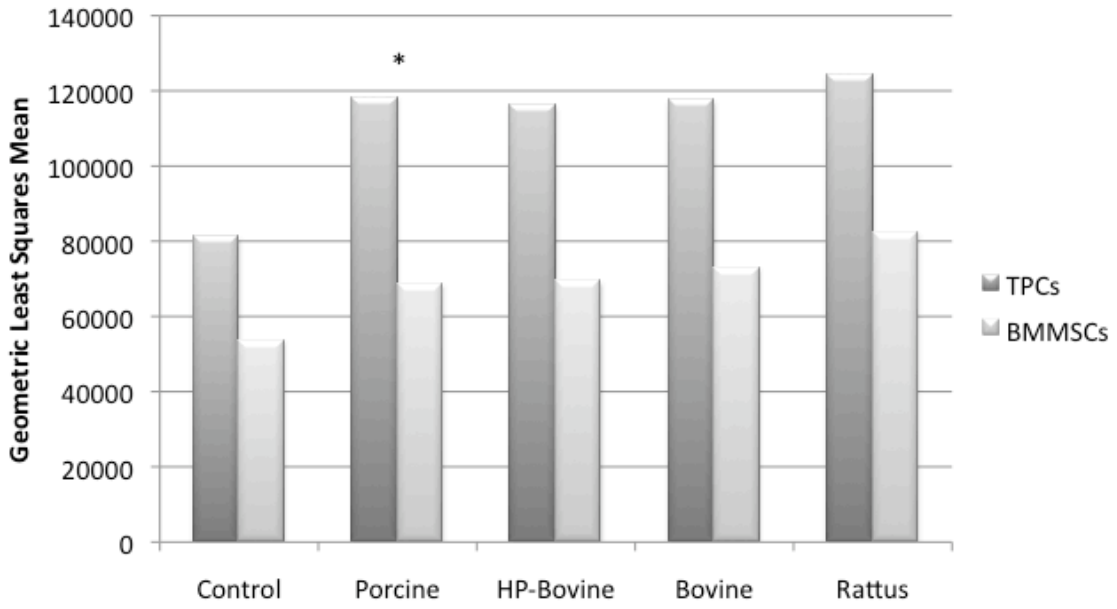

Figure 2.3 Cell count of TPCs and BMMSCs following 7 days of culture on control and collagen types. Asterisk (*) denotes differences ($P \leq 0.05$) between TPCs and BMMSCs within treatment group. TPCs; tendon progenitor cells, BMMSCs; bone marrow mesenchymal stem cells, HP-Bovine; highly purified bovine collagen.

Collagen group	Day 4				Day 7			
	TPCs		BMMSCs		TPCs		BMMSCs	
	95% Lower	95% Upper	95% Lower	95% Upper	95% Lower	95% Upper	95% Lower	95% Upper
Control	14544	34279	9212	21714	52828	124517	34965	82413
Porcine	24807	58472	12858	30306	77042	181589	44721	105409
Bovine	26095	61507	14797	34878	76481	180268	47477	111904
HP-bovine	26090	61494	13852	32650	75592	178189	45189	106511
Rat	30424	71711	16692	39344	81040	191014	53691	126551

Table 1. Cell number geometric 95% confidence interval for collagen groups for TPCs and BMMSCs on days 4 and 7.

Gene expression- No differences in collagen type I, collagen type III, COMP, or decorin gene expression were observed between collagen groups and non-collagen controls for TPCs or BMMSCs (figures 2.4, 2.5, 2.6 & 2.7). Relative to *in vivo* tendon gene expression, TPCs and BMMSCs expressed more collagen type I, collagen type III and decorin but less COMP. When comparing between cell types, BMMSCs expressed significantly more collagen type I when cultured on control, porcine and highly-purified collagen, and more collagen type III when cultured on control, porcine, highly-purified collagen, and rat collagen-coated surfaces. Tendon progenitor cells expressed

significantly more COMP when cultured on control and all collagen groups, and decorin when cultured on porcine, highly purified bovine and bovine collagen.

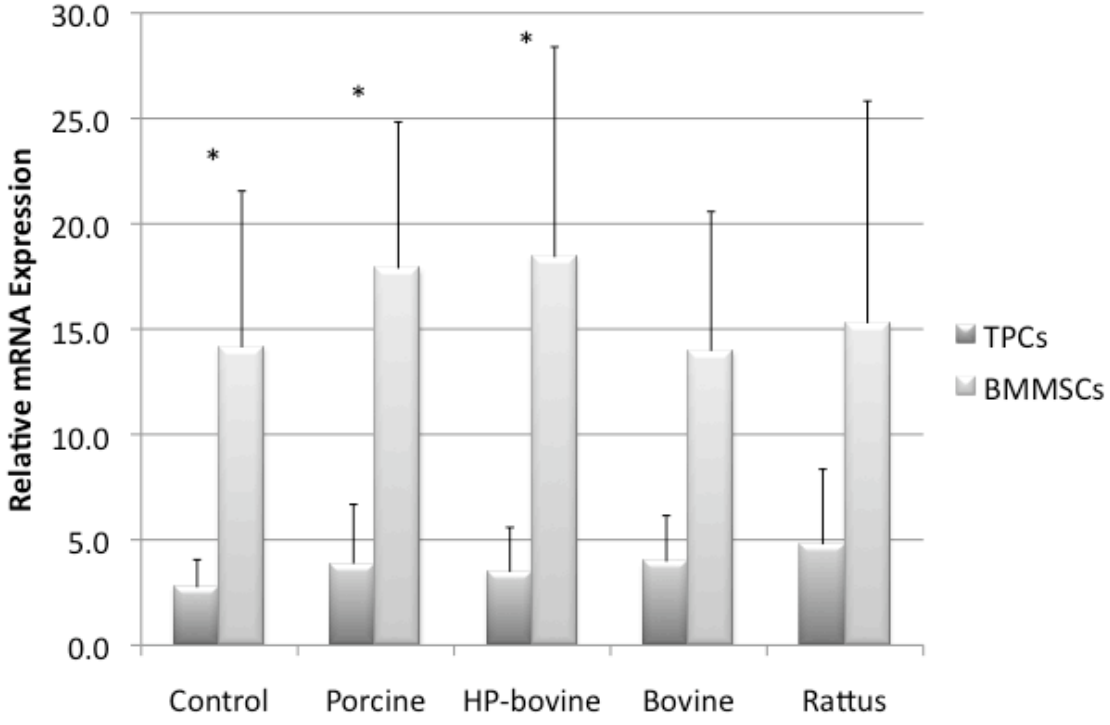

Figure 2.4 Mean ± SD relative gene expression of collagen type I for TPCs and BMMSCs cultured on each collagen group, determined for a 24 h period at 7 days of culture. Asterisk (*) denotes differences ($P \leq 0.05$) between TPCs and BMMSCs within treatment group. TPCs; tendon progenitor cells, BMMSCs; bone marrow mesenchymal stem cells, HP-Bovine; highly purified bovine collagen.

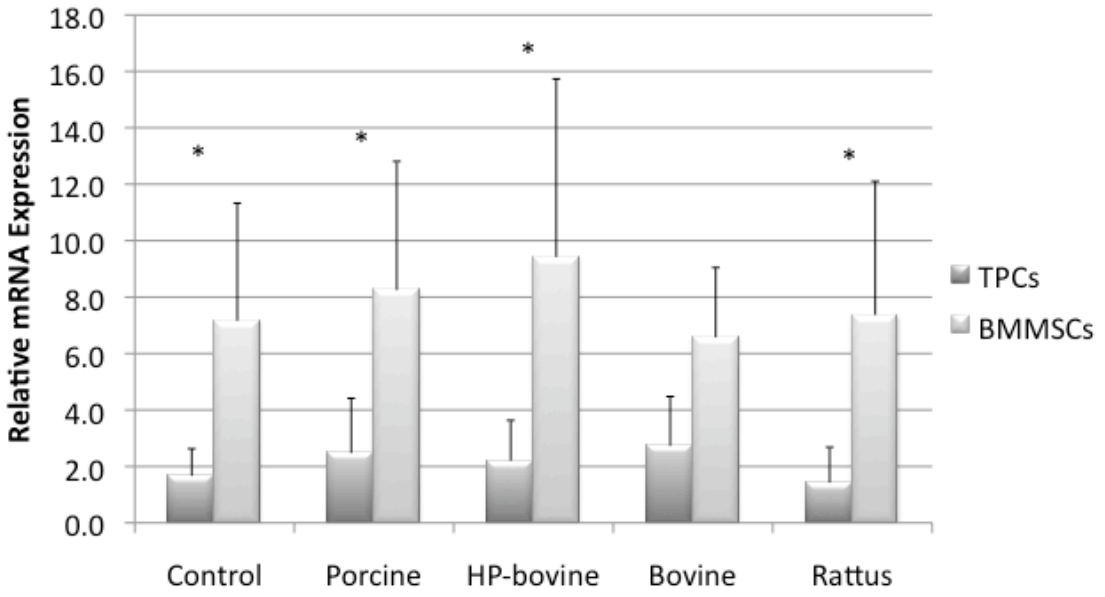

Figure 2.5 Mean ± SD relative gene expression of collagen type III for TPCs and BMMSCs cultured on each collagen group, determined for a 24 h period at 7 days of culture. Asterisk (*) denotes differences ($P \leq 0.05$) between TPCs and BMMSCs within treatment group. TPCs; tendon progenitor cells, BMMSCs; bone marrow mesenchymal stem cells, HP-Bovine; highly purified bovine collagen.

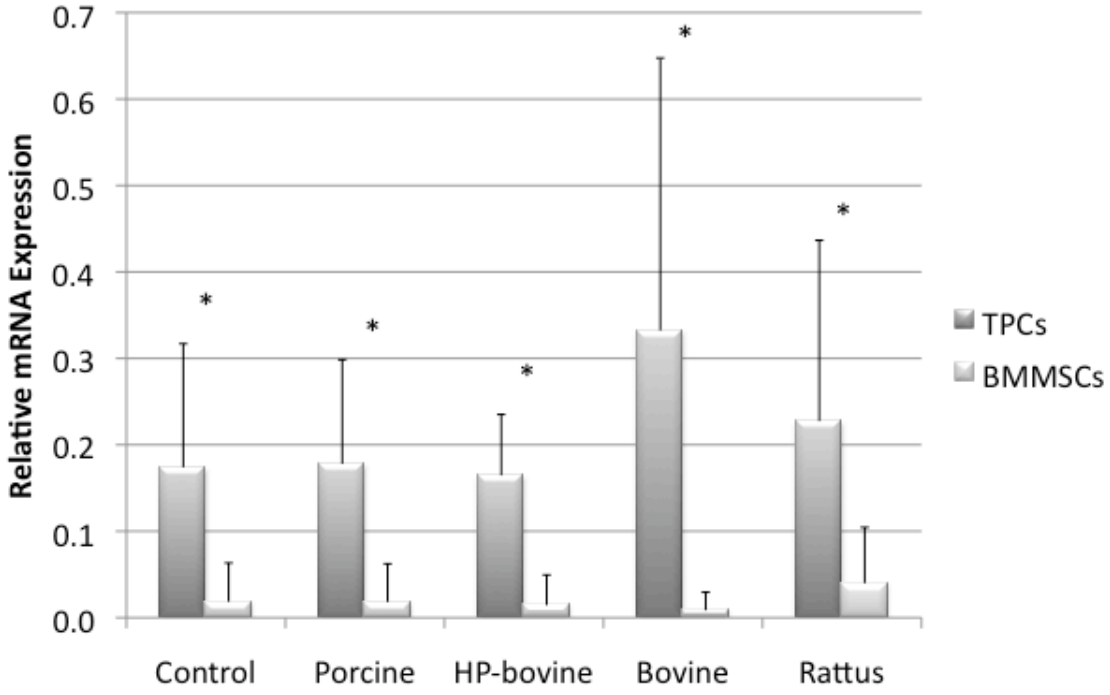

Figure 2.6 Mean ± SD relative gene expression of cartilage oligomeric matrix protein (COMP) for TPCs and BMMSCs cultured on each collagen group, determined for a 24 h period at 7 days of culture. Asterisk (*) denotes differences ($P \leq 0.05$) between TPCs and

BMMSCs within treatment group. TPCs; tendon progenitor cells, BMMSCs; bone marrow mesenchymal stem cells, HP-Bovine; highly purified bovine collagen.

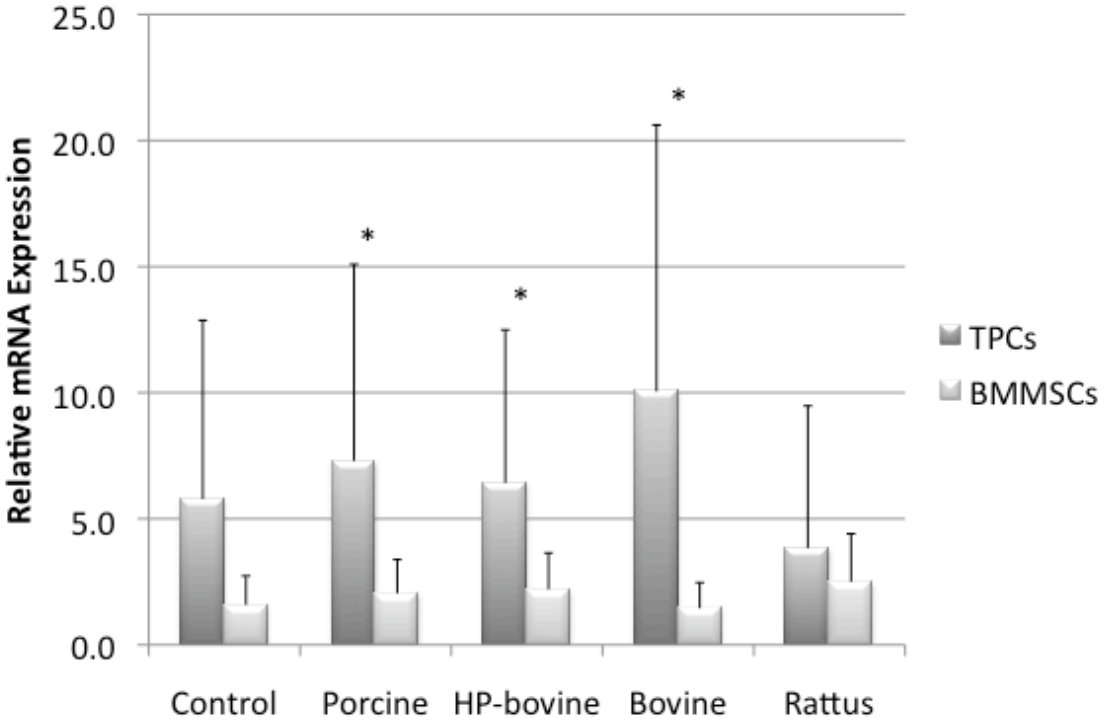

Figure 2.7 Mean ± SD relative gene expression of decorin for TPCs and BMMSCs cultured on each collagen group, determined for a 24 h period at 7 days of culture. Asterisk (*) denotes differences ($P \leq 0.05$) between TPCs and BMMSCs within treatment group. TPCs; tendon progenitor cells, BMMSCs; bone marrow mesenchymal stem cells, HP-Bovine; highly purified bovine collagen.

Glycosaminoglycan- No difference in GAG production was observed between collagen groups within TPC and BMMSC types (figure 2.8). Increased GAG synthesis was observed for TPCs compared to BMMSCs cultured on control plates only.

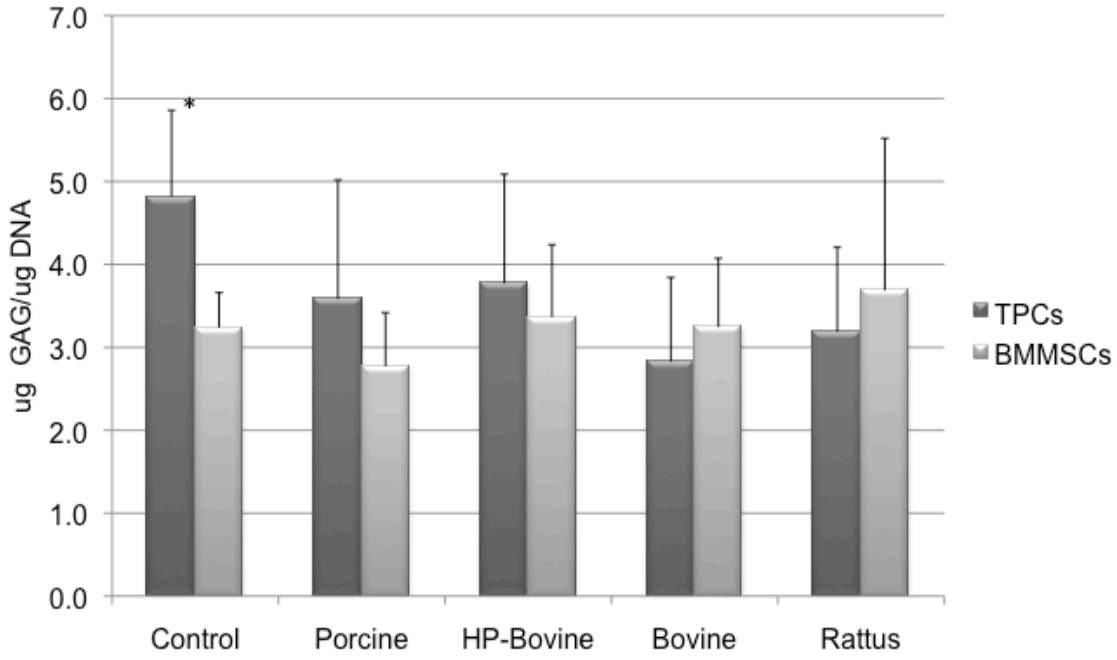

Figure 2.8 Glycosaminoglycan (GAG) synthesis (mean ± SD) normalized to total DNA content to account for cell number for TPCs and BMMSCs on day 7. Asterisk (*) denotes significant ($P \leq 0.05$) differences between groups and cell types. TPCs; tendon progenitor cells, BMMSCs; bone marrow mesenchymal stem cells, HP-Bovine; highly purified bovine collagen.

Discussion

The culture of TPCs on rat collagen type I increased TPC proliferation during the exponential growth phase (days 0-4) compared to uncoated control plates. By day 7, the cells were approaching confluence and therefore reaching the plateau phase of the growth curve and no significant difference was observed. There was a trend toward increased cell proliferation on other collagen-coated surfaces for both cell types; however, large inter-animal variation resulted in a lack of statistical significance. Improving cell proliferation could reduce the amount of time between acquiring cell biopsy and treating tendon lesions. Thus, culture of TPCs on rat collagen type I may be preferred over standard tissue culture plates alone. No adverse affects of the collagen -coated plates were noted and gene expression patterns for extracellular matrix components were similar to uncoated plates. Coating plates with randomly organized collagen is facile and therefore, may provide an improved method for cell culture for regenerative medicine to treat tendon lesions in the short term.

The beneficial effects of collagen-coated surfaces on cell proliferation is likely related to increased surface area for attachment and expansion provided by the random collagen matrix, and/or collagen-cell interactions. Cells in hollow fibers or other scaffold systems have been found to grow at very high densities.[50] The high cell density per unit volume of medium is due to the enormous increase in surface area provided by the fibers or struts, on which cells can spread, and by entrapment of cells between them.[50]

Due to the lack of availability of a commercial source of cell culture grade equine type I collagen, we did not investigate allogenic collagen. Collagen is known for its low immunogenicity due to the highly conserved simple repetitive amino acid sequence that differs little among animal species.[51] Differences in the telopeptides attached to each end of the collagen molecule that do not contain the G-X-Y amino acid sequence (where X & Y are usually proline and hydroxyproline) may be responsible for the superior properties of rat collagen compared to other types of collagen studied. Richardson and colleagues reported beneficial effects on proliferation of BMMSCs cultured on collagen type I-coated surfaces although they did not report the same effect for tenocytes.[41] In contrast to tenocytes, TPC proliferation was increased on rat collagen type I-coated surfaces. Differences in these studies could be the increased number of horses that were used in this study (6 rather than 3), as well as the selection process for progenitor cells rather than straight culture of tendon cells after digestion of the tissue.

Cell-matrix interaction is important for determining many aspects of cell function. Collagens contain specific cell binding amino acid motifs that interact with cell surface integrin adhesion receptors.[51] The integrin family of cell adhesion receptors is well recognized as providing anchorage for cells to the extracellular matrix and for having major effects on many aspects of cell behavior such as cell shape and polarization, cytoskeletal organization, cell motility, proliferation, survival, and differentiation.[52] Use of collagen matrices for support of cells *in vitro* was pioneered by Ehrmann and Gey in 1956 and collagen matrices have since been used to support the differentiation and expression of tissue-specific function of a variety of cell types in culture.[53-57] Nandi and colleagues reported superior growth of mammary tumor epithelial cells cultured on rat tail collagen gels compared to monolayer culture.[58] Beneficial effects of collagen on epithelial cell morphology and response to growth factors compared to culture on plastic has also been reported.[59] When cells interact with a proper substratum in culture, they are more likely to respond to growth factor stimuli as they would *in vivo*.[60] In addition to providing a surface composed of a natural ECM component, culturing on collagen allows the cells to maintain a more physiological association with each other. Collagen matrices and other extracellular proteins have been used to support phenotypes and tissue-specific functions *in vivo*.[61] Type I collagen contained in the surface of bioscaffolds has been shown to upregulate integrin expression of human mesenchymal stem cells seeded onto such surfaces.[62]

The optimal cell number for transplantation into equine tendon lesions is not currently known, but estimates of between 10 and 50 million have been proposed.[16] The observation of superior cell proliferation of TPCs in the present study is supported by the work of Stewart and colleagues who reported equine TPCs required less time to reach confluence than BMMSCs.[42] Both human and mouse TPCs have been shown to proliferate faster than BMMSCs isolated from the same individuals.[39] Collectively, these observations support the superior cell growth kinetics of TPCs over BMMSCs and therefore offer an advantage in terms of earlier attainment of cells for treatment of time-sensitive tendon injuries in both humans and equines.

This study did not address whether TPCs are more likely to be beneficial to treat tendon lesions than BMMSCs. It did demonstrate that there are significant differences between

the two cell types in terms of cell proliferation and gene expression patterns. The optimal ECM expression pattern for tendon regeneration is not currently known. Based upon expression patterns during tendon development, we suspect that high collagen type I, low collagen type III, and high COMP and decorin relative to normal tendon tissue would be beneficial to tendon regeneration. Relative to normal tendon tissue cell expression, the TPCs did express higher collagen types I and III, as well as decorin; however, statistical analysis was not performed since the tendon tissue was used as the calibrator tissue. BMMSCs expressed higher collagen types I and III compared with the TPCs, but lower COMP and decorin. GAG synthesis was increased only in TPCs grown on uncoated plates. Increased GAG synthesis corresponds well with increased decorin expression, since GAGs are an integral component of proteoglycans in the ECM milieu. It is unclear when assessing these differences, which would be preferred. Further studies investigating the effects that these expression patterns have on tendon lesions is needed.

Equine TPCs and BMMSCs cultured on acellular native matrices expressed less collagen type I and COMP, and more collagen type III relative to normal tendon.[42] In the same study, significantly increased expression of collagen type III and COMP by TPCs compared to BMMSCs was reported. Comparison of Stewart and colleagues results to the present study suggests that standard two dimensional culture conditions used in our experiments induces higher expression of collagen type I for both TPCs and BMMSCs with the later cell type expressing more collagen than TPCs. A similar effect of collagen type I-coated surfaces on BMMSC expression of collagens I and III was observed by Richardson and colleagues.[41] Tendon progenitor cells on the other hand expressed comparatively more COMP and decorin than BMMSCs, which may reflect differing constitutive gene expression patterns between the two progenitor cell types and their respective responses to *in vitro* culture conditions.

It is interesting to note that in our experiments, TPCs preferentially expressed higher levels of COMP and decorin genes, two constituents of tendon ECM involved in fibrillogenesis and structural organization, compared to BMMSCs. Our results add further to the notion that at least *in vitro*, BMMSCs and TPCs have different gene expression profiles and that each have certain elements desirable for synthesis of tendon matrix. Recent research indicates that adult MSCs coexpress genes specific for a number of mesenchymal lineages, including adipocytes, osteoblasts, fibroblasts, and muscle.[63] The factors that influence *in vitro* gene expression of MSCs are largely unknown and are currently an active area of research interest.

Inferences regarding differentiation of cells from gene expression profiles made from this experiment should not be made as none of the genes examined are specific for tenocytes. Collagen type I is the primary protein synthesized by tenocytes but this does not differentiate this cell type from fibroblasts producing connective tissue, including scar tissue.[64] Collagen types I and III are ubiquitous constituents of ECM and are not necessarily specific to ligament or tendon.[65] The synthesis of COMP provides a more discriminating analysis but it too is not specific to tendon, although it does have a restricted distribution in tissues primarily designed to withstand load (*e.g.* cartilage, tendon, and fibrocartilage).[16] Similarly, decorin mRNA is expressed by osteoblasts and is combined with other markers such as collagen type I, to delineate human MSC

differentiation toward osteoblasts.[66] Recently, Taylor and colleagues identified a panel of gene expression 'markers' that differentiate tendon fibroblasts from other mesenchymal cell types.[67] They suggested high expression of collagen alpha-2(I) chain gene and scleraxis and low expression of tenascin C are most representative of normal adult tendon phenotype.

Clearly a need to influence cell growth kinetics and gene expression *in vitro* in preparation for future transplantation exists. To this end, our experiments demonstrate an advantage in using TPCs cultured on rat type I collagen-coated surfaces as this cell type and culture system resulted in superior cell proliferation during the early growth phase. Induction of gene expression typical of resident tenocytes involved in the repair process was not demonstrated and further investigation into soluble factors, ECM cues, and signaling pathways to achieve this aim is warranted in order to improve tendon healing in both humans and equines.

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[a] DMEM, Mediatech Inc, Herndon, VA

[b] FBS, Invitrogen, Carlsbad, CA

[c] L-glutamine, Sigma Chemical Co, St Louis, MO

[d] Penicillin/Streptomycin, Invitrogen, Carlsbad, CA

[e] Jamshidi bone marrow biopsy needle, Cardinal Health, Dublin, OH

[f] Collagenase 2, Worthington Biochemical Corp., Lakewood, NJ

[g] Ascorbate, Sigma-Aldrich, St Louis, MO

[h] Cell Filters, BD Biosciences, Bedford, MA

[i] Trypan blue, Sigma Chemical Co. St Louis, MO

[j] Bovine Collagen Type I, BD Biosciences, Bedford, MA

[k] Apcoll Soluble Collagen, Devro Pty Ltd, Chryston, Scottland, UK

[l] Immunization Grade Porcine Type I Collagen, Chondrex Inc, Redmond, WA

[m] Rat Tail Collagen, Type I, BD Biosciences, Bedford, MA

[n] CellTiter 96 AQ Cell Proliferation Assay, Promega, Madison,WI

[o] Plate Chameleon V, Hidex, Turku, Finland

[p] Trizol, Invitrogen, Grand Island, NY

[q] RNA isolation kit ,Qiagen ,Valencia, CA

[r] Reverse transcriptase kit , Applied Biosystems, Foster City, CA

[s] TaqMan gene expression kit , Applied Biosystems, Foster City, CA

[t] 7500 Real-Time PCR System, Applied Biosystems, Foster City, CA

[u] Papain, Sigma-Aldrich, St Louis, MO

[v] Hoechst 33258, Sigma-Aldrich, St Louis, MO

[w] SAS PROC GLIMMIX, and JMP, SAS Institute Inc., Cary, NC

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Final comments

This study highlights *in vitro* properties of TPCs that make them an attractive alternative progenitor cell source for future tissue engineering and regenerative medicine applications in the treatment of equine tendon injury. We have demonstrated superior cell growth kinetics for TPCs compared to BMMSCs during the early growth phase, offering an advantage when culture-expanding autologous progenitor cells for transplantation. The lack of effect beyond the early growth phase may be related to cells reaching confluence by day 7 and/or new collagen production coupled with breakdown of the collagen by the cells. We did not investigate the fate of the collagen in cell cultures; however, mRNA expression of selected matrix metalloproteinases (MMPs) is pending which will give some indication of whether cells were actively producing proteases. Cells will make their own matrix over time, and one would expect collagen catabolism would occur in the process of matrix remodeling in our experiments. The differences in collagen-coated wells may not have been significant for the duration of the experiment due to this remodeling. Therefore, the collagen coating the plates may have only remained cell-instructive for a short period of time. Furthermore, optimization of collagen concentration for either TPCs or BMMSCs was not performed.

There may be an advantage to using TPCs that are perhaps partially differentiated toward tenocytes versus BMMSCs, which show a tendency to differentiate into osteoblasts. There is evidence that some 'pre-programming' or 'memory' capacity of progenitor cells occurs and therefore it makes sense to utilize TPCs in tendon regeneration.

Gene expression data did not show a clear benefit of either progenitor cell type. None of the genes examined in this study are specific for tendon and can only be considered 'tendon-related'. There are currently no panels of gene markers that are specific for tendon and therefore it is difficult (if not impossible) to determine whether either progenitor cell type examined truly differentiated into tenocytes. It was interesting to note that the TPCs expressed more organizational components of tendon rather than collagen. This observation may be circumstantial evidence that ECM interactions signaled the presence of collagen and TPCs responded by expressing genes for proteins involved in collagen fibrillogenesis. However, a similar response was observed on non-collagen control surfaces and thus it appears that the two progenitor cell types studied have differing constitutive gene expression profiles.

The influence of the culture medium on gene expression must also be considered. Currently, there is no defined tenocyte induction media, in contrast to bone, cartilage and adipose cell culture systems. The gene expression observed in our study probably reflects the influence of culture conditions (*i.e.* growth factors in the serum constituent of the medium). Culture conditions that more closely replicate *in vivo* conditions offer significant advantages for the study of gene expression and systems such as rotating bioreactors that provide improved oxygenation, availability of nutrients, and fluid shear forces are appealing.

The relative importance of expression of genes examined in this study can be debated. Clearly, preferential expression of collagen type I over type III is important for regeneration of 'normal' tendon as opposed to scar tissue. The role of the organization

components COMP, and decorin is not clear and there is emergent information that suggests that inhibition of some of these proteins can increase fibril diameter and therefore improve tendon strength. More research is required to determine which components are important, what the optimal concentrations are, and when they should be expressed. This information will likely come from an improved understanding of tendon development.

A major hurdle for the future application of TPCs in tendon tissue engineering and regenerative medicine is identifying a readily available source that causes minimal donor site morbidity. The use of autologous flexor tendons in horses is problematic and it may be that allogenic TPCs obtained from the tensional region of normal SDFT are the future. Allogenic BMMSCs have been injected into tendon lesions in horses and have been show to be non-immunogenic. Similar studies using TPCs need to be done in order to validate the use of allogenic TPCs, which would be a significant milestone for the advancement of this progenitor cell type.

Appendix A

Sequence information for primers and MGB probes for real time PCR analysis.

Gene of	Forward Primer	Reverse Primer	Probe
Collagen Type I	GCCAAGAAGAAGGCCAAGAA	TGAGGCCGTCCTGTATGC	ACATCCCAGCAGTCACC
Collagen Type	CTGCTTCATCCCACTCTTAT	ATCCGCATAGGACTGACCA	AACAGGAAGTTGCTGAA
COMP	GAGATCGTGCAAACAATGAA	GACCGTATTCACGTGGAAC	CTGGCTGTGGGTTACA
Decorin	AAGTTGATGCAGCTAGCCTG	GGCCAGAGAGCCATTGTCA	ATTTGGCTAAATTGGGA
18S RNA	GAGGCCCTGTAATTGGAATG	CGCTATTGGAGCTGGAATT	CAAGTCTGGTGCCAGCA