Porcine Urinary Bladder Matrix in an *In Vitro* Equine Model of Tenogenesis

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Academic Abstract

Extracellular matrix (ECM) is responsible for tendon strength and elasticity. Healed tendon ECM lacks structural integrity, leading to reinjury. Porcine urinary bladder matrix (UBM) provides a scaffold and source of bioactive proteins to improve tissue healing, but has received limited attention for treating tendon injuries. The objective of this study was to evaluate the ability of UBM to induce matrix organization and tenogenesis using a novel *in vitro* model. We hypothesized that addition of UBM to tendon ECM hydrogels would improve matrix organization and cell differentiation. Hydrogels seeded with bone marrow cells (n = 6 adult horses) were cast using rat tail tendon ECM ± UBM, fixed under static tension and harvested at 7 and 21 days for construct contraction, cell viability, histology, biochemistry, and gene expression. By day 7, UBM constructs contracted significantly from baseline, whereas control constructs did not. Both control and UBM constructs contracted significantly by day 21. In both groups, cells remained viable over time and changed from round and randomly oriented to elongated along lines of tension with visible compaction of the ECM. There were no differences over time or between treatments for nuclear aspect ratio, DNA, or glycosaminoglycan content. Decorin, matrix metalloproteinase 13, and scleraxis expression increased significantly over time, but not in response to UBM treatment. Mohawk expression was constant over time. Cartilage oligomeric matrix protein expression decreased over time in both groups. Using a novel ECM hydrogel model, substantial matrix organization and cell differentiation occurred; however, the addition of UBM failed to induce greater matrix organization than tendon ECM alone.

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Public Abstract

Tendon injuries are common in horses and are painful and can be career- and life-ending. Tendons have a special structure and organization that enables them to withstand high tensile forces without permanent deformation. Injured tendons heal by forming stiff, disorganized scar tissue that makes the tendon more prone to re-injury. The lining of urinary bladders from pigs (UBM) provides a physical mesh and signaling factors that help heal injuries in a variety of tissues to a more normal state. However, UBM has not been evaluated in a laboratory model of tendon tissue formation to determine how it can help heal tendon injuries. Three-dimensional models of new tendon tissue formation (neotendons) were made with rat tail tendon matrix and stem cells collected from horse bone marrow. The neotendons were placed under steady tension for 3 weeks. The models were collected after 1 and 3 weeks to measure their width, numbers of live cells, cell and matrix organization, levels of tendon matrix components and expression of genes found in tendons. Most cells in the neotendons remained alive during the study period. Over time, UBM-treated and untreated neotendons became narrower compared to their starting width. The width of UBM-treated neotendons decreased faster than non-treated neontendons in the first week of the study. Cells became longer, narrower, and oriented along lines of tension. Expression of genes important in tendon development and structure either increased or was constant over time. UBM treatment did not change cell shape or increase levels of tendon-associated genes, DNA, or tendon matrix components. Our novel tendon model successfully created organized tendon-like tissue when placed under tension. However, UBM treatment did not improve formation of tendon-like tissue to a greater extent than controls.

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Attributions

Several colleagues aided in the writing and research behind Chapter 3: Urinary bladder matrix does not improve tenogenesis in an *in vitro* equine model. All colleagues listed are co-authors of the manuscript and brief descriptions of their contributions are below.

Linda A. Dahlgren, DVM, PhD, DACVS, is a Professor of Large Animal Clinical Sciences at the Virginia-Maryland College of Veterinary Medicine. Dr. Dahlgren oversaw study design, secured funding for the project, supervised data analysis and interpretation, and contributed to manuscript preparation.

Bruno C. Menarim, DVM, MS is a veterinarian and doctoral candidate in Dr. Dahlgren's laboratory. Dr. Menarim conceived the study with Dr. Dahlgren, helped procure funding, developed the model used, and contributed to data acquisition and manuscript preparation.

Anne E.C. Nichols, PhD, is a Postdoctoral Research Associate at the University of Rochester Medical Center and is a graduate of the doctoral program in Dr. Dahlgren's laboratory. Dr. Nichols contributed to study design and manuscript preparation.

Stephen R. Werre, PhD, is a Research Assistant Professor of Veterinary Medicine Experimental Statistics and the Director of the Laboratory for Statistical Design and Study Analysis at the Virginia-Maryland College of Veterinary Medicine. Dr. Werre contributed to study design, performed statistical analysis on all data and was critical to data interpretation, and assisted with manuscript preparation.

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Chapter 1: 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 "Urinary bladder matrix does not improve tenogenesis in an *in vitro* equine model" and contains its own introduction, materials and methods, results, discussion, and references. The following 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.

Introduction

Tendon injuries are common in athletic horses and are slow to heal, requiring prolonged rest and rehabilitation. Injured tendons heal primarily by formation of fibrosis (scar tissue) that is weaker and less elastic than native tendon tissue. As such, tendon injuries often recur and lead to retirement or euthanasia of affected horses. These losses of athletic horses with tendon injuries leads to substantial economic losses in the equine industry. Treatment of tendon injuries in horses therefore represents a clinical challenge in veterinary medicine. Conservative treatment of tendon injuries consists of rest, anti-inflammatory administration, and cold therapy.[1] Graduated exercise and physical therapy are guided by documentation of tendon healing on repeat ultrasonography.[1-3] Regenerative therapies, including intralesional injection of mesenchymal stem cells, platelet-rich plasma, or both show promise in improving tendon healing;[4-9] however, evidence of their efficacy in a controlled clinical setting is limited. Surgical therapies, including tendon-splitting, desmotomy of the accessory ligament of the superficial

digital flexor tendon, and tenoscopy can be performed to stimulate tendon healing, reduce the risk of re-injury, and encourage soundness and return to work, although post-operative complications and/or the site and severity of the tendon lesion can limit surgical success.[10-16]

Given the limitations of currently available treatments and the burden that tendon injuries impose on equine welfare and the equine industry, a treatment that consistently supports physiologic tendon healing and reduces the risk of re-injury is needed. Extracellular matrix (ECM) scaffolds have been demonstrated to support site-specific tissue formation and remodeling in multiple tissues and species. ECM is readily isolated from several tissue sources and provides a three-dimensional physical scaffold, structural components, and bioactive signaling molecules.[17-20 21-24] Biodegradation of ECM by mononuclear cells releases bioactive factors from the scaffold.[25-27] ECM also encourages macrophage polarization toward an anti-inflammatory phenotype.[26,28-30] By all these mechanisms, ECM encourages local progenitor cell migration, differentiation, proliferation, and matrix organization. Porcine urinary bladder matrix (UBM) is one type of ECM that is isolated from the basement membrane of the urinary bladder, and is then decellularized, and lyophilized.[20,31] UBM is commercially available as a sheet for topical application (MatriStem™ MicroMatrix, ACell®, Columbia, Maryland) and a particulate form (MatriStem™, ACell®) that can be resuspended and injected into a tendon lesion under ultrasound guidance.[32] Decellularization and high conservation of the structural components of UBM across species minimize the risk of immunologic reaction with xenogeneic application of UBM.[33,34]

ECM from multiple tissue sources has been demonstrated to improve healing of experimental musculoskeletal injuries. Application of small intestinal submucosa (SIS) sheets improved the quality of tendon healing in animal models of collateral ligament,[35] Achilles

tendon,[36,37] and rotator cuff injury.[38,39] Tendon-derived ECM improved tenogenic differentiation of human adipose-derived stem cells seeded into three-dimensional collagen hydrogels.[40] *In vivo,* stem cell-seeded collagen hydrogels supplemented with tendon-derived ECM improved the mechanical strength of healed experimental rotator cuff injuries in rats.[41]

Literature on UBM treatment of musculoskeletal injuries is sparse; however, UBM has improved tendon healing in experimental and clinical tendon injuries in veterinary species. Circumferential application of stacked UBM sheets to Achilles tenotomy sites in mice increased progenitor cell chemotaxis to the site compared to application of an autologous tendon graft.[42] In one case series of horses with tendon or suspensory injuries treated with intralesional UBM injection, 85.9% of horses were sound and in work one year after treatment.[32] UBM has also successfully supported healing in other tissues. In people, application of a UBM sheet improved healing of complex and chronic wounds, urethral reconstruction, and rectopexy sites.[43-47] In veterinary medicine, UBM has been successfully used to treat urinary incontinence in dogs and for full-thickness corneal reconstruction in horses, dogs and cats.[48-50]

While ample literature exists to support the use of UBM in healing injuries in multiple tissues, objective data on the efficacy of UBM in supporting tendon tissue formation (tenogenesis) are lacking. In particular, there is a need for *in vitro* evaluation of the mechanisms by which UBM may guide tenogenesis, including stimulation of cellular and matrix organization, synthesis of tendon ECM components, and expression of tendon-associated genes. One way to evaluate tenogenesis *in vitro* is to construct ECM hydrogels, which are three-dimensional, cell-seeded models of tendon tissue formation. The improvement in markers of tenogenesis with addition of tendon ECM to collagen hydrogels under strain suggests that UBM may support tenogenesis in a similar fashion.[40] The aim of this Master of Science project was to evaluate the

ability of UBM to guide matrix organization and tenogenesis using a novel *in vitro* hydrogel model.

Chapter 2: Literature Review

Anatomy of Tendons

Tendons connect muscles to bones to provide skeletal support and translate the energy of muscular contraction into movement. Tendons are conserved across mammalian and non-mammalian species and consist of sparse, specialized tendon cells (tenocytes) embedded in a highly organized, relatively avascular extracellular matrix (ECM).[51-53] There are two general types of tendons: positional and energy-storing. Positional tendons, such as the common digital extensor tendon in horses, support and maintain stance of the distal limb.[54,55] Energy-storing tendons elongate and store kinetic energy when loaded in the stance phase of the stride.[56,57] The stored energy is released as the limb is advanced, propelling the limb forward and improving the efficiency of locomotion.[56,57] The equine superficial digital flexor tendon is one of the most widely-studied energy-storing tendons due to its propensity for injury.[58-62] While tendons share some general properties, their composition and structure are *anisotropic*: they change during development, following exercise, and by anatomic location to adapt to the load placed on them.[54,55,63-70]

Gross anatomy

Tendons are divided into three functional regions: the musculotendinous junction, the tensional region, and the insertion onto bone (Figure 2.1).[53,71] The musculotendinous junction serves as the transition from the muscle to its tendon of insertion. The tensional (middle) region elongates and stores elastic energy during the weight-bearing phase of the stride.[1,61] The insertion anchors the tendon to the bones distally on the limb. As tendons course distally on the limb, they undergo changes in direction as they cross over bony protuberances and joints. In

areas of directional change, tendons are encased in synovial sheaths that enable the tendon to glide without friction as the underlying joint flexes and extends.[53] Over high-motion joints, tendons are surrounded by fibrous connective tissue bands (retinacula) that prevent the tendon from displacing as the underlying joints move.[1]

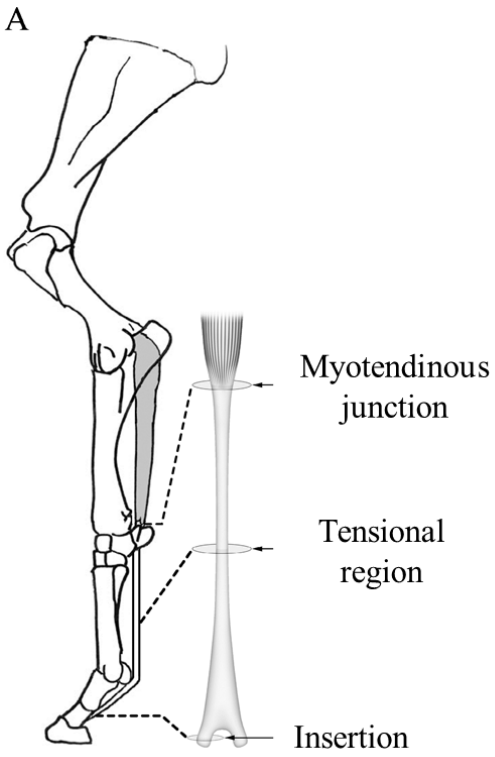

Figure 2.1 Regions of the equine superficial digital flexor tendon. From Wantabe et al Connect Tissue Res 2007.

Tendon are surrounded externally and divided internally by a connective tissue network (Figure 2.2).[52,61] The paratenon is a loose connective tissue layer that overlies the tendon and enables it to move independently from the overlying skin as the joints flex and extend.[72] The epitenon directly encases the tendon[72] and is separated from the paratenon by a thin layer of fluid that allows the tendon to glide smoothly within the paratenon.[73] Within the tendon parenchyma, the epitenon continues as the endotenon, the loose connective tissue layer that separates and

surrounds collagen fiber bundles (fascicles).[72] The endotenon allows fascicles to glide past each other when the tendon is loaded, allowing the tendon to deform elastically (without permanent structural change) under physiologic loads, and delivers the neurovascular supply to the tendon parenchyma.[1,61] The vascular supply to the tendon arises from the paratenon and the connective tissues surrounding the musculotendinous junction and osseous insertion.[74] Vessels and their associated nerves course in the paratenon, then enter the epitenon and ultimately the endotenon, where they run parallel to the collagen bundles and supply the interior of the tendon.[74] In regions where the tendon is encased in a synovial sheath, gases and nutrients also diffuse between the synovial membrane and the tendon through the synovial fluid.[75]

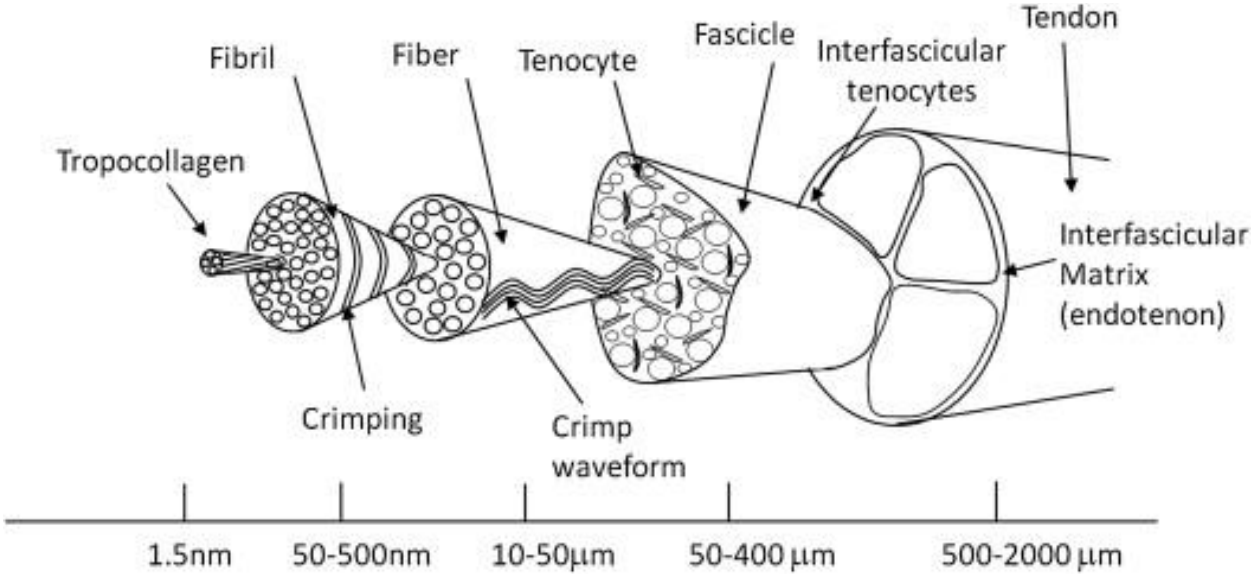

Figure 2.2 Structural hierarchy of collagen in tendon. From Screen HR et al Proc. Inst Mech. Eng H. 2004.

Tendon composition, organization, and developmental regulation

Tendon ECM consists of a fibrous component and amorphous, hydrophilic ground substance.[76] The fibrous component consists primarily of collagen, which makes up 78% of the total tendon dry weight.[52,76] Type I collagen is the primary collagen type in tendon and is organized in a hierarchy of longitudinally-aligned cylindrical units of increasing size.[52,72] This organization is essential for the specialized energy-storing function of tendons and requires that collagen is synthesized and processed in several precise steps. Collagen is initially synthesized as separate alpha chains on ribosomes within the rough endoplasmic reticulum of tenocytes.[77] Three alpha chains are assembled into a triple helix with hydrogen bonds between the N-terminus of glycine residues and carboxyl groups of the adjacent chain.[73] These helical molecules are released from the ribosome, packaged into vesicles within the endoplasmic reticulum, released into the cytoplasm, and secreted from the tenocyte as procollagen (Figure 2.3).[77] The amino- and carboxy-terminal propeptides of procollagen prevent premature organization of collagen molecules within the cell.[77] Once secreted and in the pericellular space, the propeptides are cleaved by procollagen C- and N-terminal proteases to form tropocollagen.[77,78] Tropocollagen molecules self-assemble into fibrils that are arranged in a longitudinal, tightly-packed "quarter stagger" fashion and are stabilized by intra- and intermolecular covalent crosslinks involving lysine and hydroxylysine residues (Figure 2.3).[76,77,79] The tight packing and covalent crosslinks of adjacent collagen molecules are crucial for maintaining tensile strength in energy-storing tendons.[76,80] Fibrils are grouped together to form fibers, and fibers are bundled into successively larger fascicles.[72,81] Fascicles are surrounded by endotenon and display a characteristic planar waveform pattern ("crimp") at rest.[52] The crimp straightens to absorb the initial energy of loading (Figure 2.2).[52,61] While

collagen assembly occurs extracellularly, tenocytes play an active role in creating compartments in the ECM where they regulate orientation of the newly-synthesized fibers and thereby regulate the orientation of the ECM.[81,82]

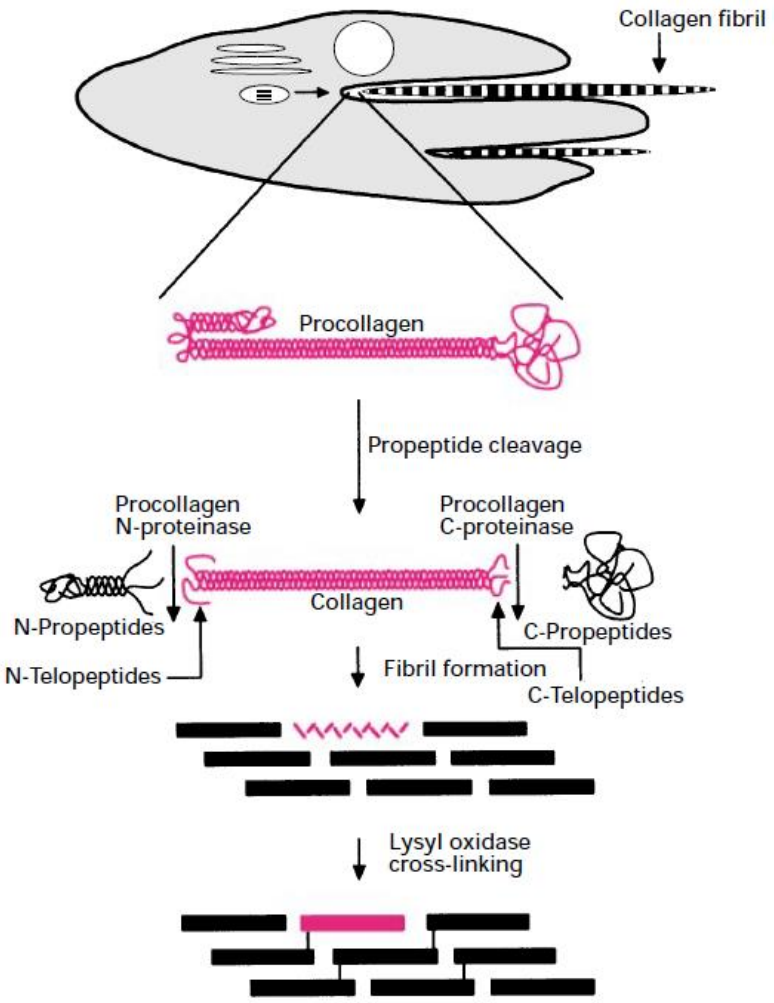

Figure 2.3 Fibrillar collagen synthesis pathway. Kadler KE et al J Biochem 1996.

Aside from collagen type I, other collagen types, such as III, IV, V, and XII are distributed around type I fibril bundles or are associated with basement membranes and blood vessels.[61,71,76,83-85] Type III collagen forms longitudinal fibrils that are smaller in diameter than type I collagen fibrils or heterogeneous fibrils with type I collagen.[84,86] Type III collagen also

regulates type I collagen fibril size and strength during development.[87,88] The total collagen content and the proportions of small (40 nm diameter) and large (>200 nm diameter) collagen fibrils in a tendon determine its strength.[61,69,89] During post-natal development, tendons progress from having uniformly small collagen fibrils to a bimodal distribution of large and small fibrils, likely in response to load.[69] Large fibrils increase tendon resistance to deformation under load (stiffness) due to increased density of intra-fibrillary covalent crosslinks.[69] In contrast, small fibrils resist shear forces and slippage between fibrils by having increased total surface area for inter-fibrillary covalent crosslinks.[69] Small fibrils are therefore thought to contribute to the ability of the tendon to deform elastically (without permanent structural change) under load.[69] Elastin fibers compose 2-3% of total tendon content in the equine SDF and also contribute to tendon elasticity.[90]

The remainder of the tendon ECM is composed of non-collagenous ground substance, including proteoglycans, glycoproteins, and water.[51,69,70,91,92] Proteoglycans make up 1% of tendon dry weight and consist of a protein core with covalently-bound negatively-charged glycosaminoglycan (GAG) units.[91] The GAG units can be sulfated or non-sulfated, with dermatan sulfate (DS) composing 60% of GAG in adult energy-storing tendon.[51,69,70] The net negative charge of the bound GAG groups draws water into tendons, which lubricates fibrils during tendon elongation and maintains spacing between fibrils.[67,91,93] Proteoglycans in tendon consist of small leucine-rich proteoglycans (SLRP) and large aggregating (modular) proteoglycans.[91,94,95] The SLRP include decorin, biglycan, fibromodulin, and lumican.[96,97] These small proteoglycans modulate tendon strength by binding to and regulating type I collagen fibril diameter, shape, and organization during development.[51,91,92,95-101] While all small proteoglycans are needed for normal tendon development, the distribution and importance of

each proteoglycan varies by tendon in mice and people.[96,97,100,101] It is unknown whether the content and relative importance of each small proteoglycan varies between tendons in horses.

Glycoproteins are also crucial for developing and maintaining tendon structure and strength.[102-104] Cartilage oligomeric matrix protein (COMP) is a pentameric glycoprotein that binds collagen types I, II and IX in a zinc-dependent manner.[104-106] COMP is expressed in tendons, ligaments, and cartilage.[63,91,105,107] In energy-storing tendons, COMP regulates collagen fibrillogenesis during growth and development and thereby contributes to mature tendon strength.[63,68,104,106-108] COMP is expressed at the highest levels in the tensional region of energy-storing tendons and at low levels in positional tendons in young horses.[68,107,108] In the equine superficial digital flexor tendon, COMP levels increase during growth and in response to load, with levels peaking between 2-3 years of age.[63,68,107] The importance of COMP in guiding appropriate tendon development is underscored by the fact that mutations in the COMP gene in people cause multiple musculoskeletal disorders, including pseudoachondroplasia and multiple epiphyseal dysplasia.[104,109]

Tenomodulin is a type II transmembrane glycoprotein that is highly expressed in tendons and ligaments, but is also expressed in cartilage, muscles, eyes, and visceral organs.[102,103] Similar to decorin and biglycan, tenomodulin helps modulate collagen fibril size.[103] Tenomodulin also supports tenocyte proliferation in neonatal tendons and maintains tenocyte density in mature tendons.[103] Interestingly, tenomodulin inhibits new blood vessel formation (angiogenesis) by inhibiting the actions of vascular endothelial growth factor.[110] While the anti-angiogenic property of tenomodulin helps prevent vascular ingrowth and metastasis of tumors, it is unclear if or to what extent tenomodulin limits angiogenesis in tendons.[110]

Scleraxis and mohawk homeobox are transcription factors that regulate tendon development and homeostasis and are expressed in progenitor cells undergoing tenogenic differentiation.[40,111-115] Scleraxis is a basic helix-loop-helix transcription factor that is highly expressed in embryonic tendon and ligament progenitor cells and is transiently expressed in developing chondrocytes, cardiac valves, body wall and limb bud mesenchyme, and Sertoli cells.[116-121] Scleraxis is also expressed in the tensional region of mature load-bearing tendons and is expressed in response to load.[122,123] In mature tenocytes, scleraxis expression maintains cytoskeletal stiffness and promotes formation of adhesion proteins, both of which are required for mechanosensation.[124] Scleraxis also stimulates expression of type I collagen and tenomodulin.[125,126]

Mohawk homeobox belongs to the three loop amino acid loop extension of atypical homeobox genes.[127] Mohawk is expressed in developing tendons, muscles, cartilage, and urogenital tissues and in adult tendon.[127,128] In developing tendons, mohawk promotes type I collagen synthesis and increases collagen fibril size.[129,130,131] Mohawk also stimulates gene expression of scleraxis, decorin, and tenomodulin.[114,131] In mature tendon, mohawk regulates collagen fibril diameter and integrity, suppresses osteogenic gene expression, and maintains elongated tenocyte shape.[131,132]

Tendon cell morphology, arrangement, and functions

The primary cell type in tendons is the tenocyte.[133] Tenocytes are specialized, fibroblast-like cells that are responsible for synthesizing and organizing the ECM during development and in response to load and injury.[61,133] Tenocytes are variably elongated, spindle-shaped or stellate, and lie in rows between collagen fibers in the tensional region of tendon (Figure 2.4).[52,62,133]

Tenocytes connect to each other and to the surrounding ECM in a three-dimensional network of cytoplasmic projections.[52,134] Tendons also contain a smaller subset of progenitor cells that are multipotent, self-renewing, and can undergo tenogenic differentiation.[112,135,136]

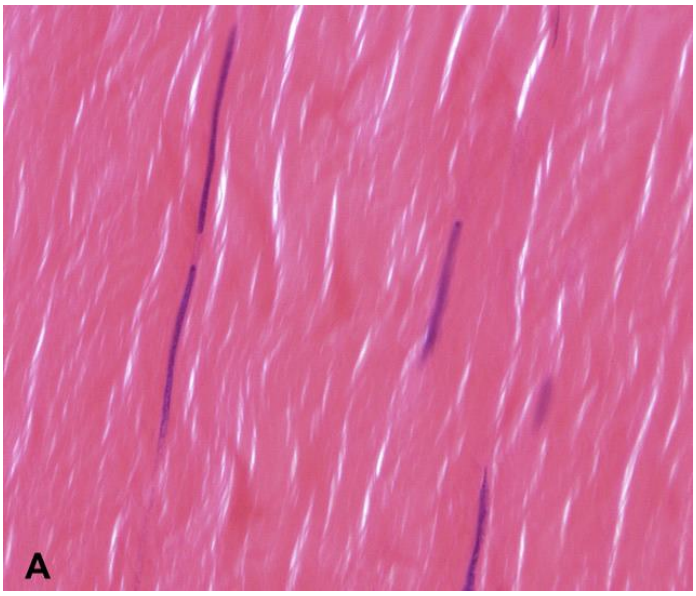

Figure 2.4 Hematoxylin and eosin-stained sections of equine digital flexor tendon (40x magnification). From Patterson-Kane JC et al J Comp Path 2012.

The specialized functions of tenocytes and tendon progenitor cells rely on their ability to sense and respond to load on the surrounding ECM (*mechanosensation*). Mechanosensation is driven by contact guidance between cells and the matrix.[137,138] The specialized cytoplasmic projections between tenocytes and the ECM enable tenocytes to detect strain on ECM via tension on the cytoskeleton and/or signaling through mechano- or osmotically-sensitive ion channels (Figure 2.5).[134,139-142] The connections between tenocytes contain gap junctions that allow intercellular communication and coordinated synthesis of ECM in response to strain.[134,137,143-145] Mechanosensation also stimulates mature tenocytes and progenitor cells to develop an elongated,

spindle, or stellate morphology and align along tension lines.[40,111,113,137,138,142,143] These morphologic changes require rearrangement of the actin cytoskeleton and may be mediated by intracellular calcium signaling.[138,139,141,146]

Detection of strain concurrently increases expression and enzymatic activity of matrix metalloproteinases (MMP).[40,111,113] MMP are a family of 23 zinc-dependent proteases that collectively digest all components of the ECM.[147-149] The collagenases (MMP-1, -8, and -13) target triple helix fibrillar collagen types I and III and are therefore important in tendon homeostasis.[147,150] MMP contribute to tendon remodeling in response to strain by removing collagen fibrils that are not aligned along tensional lines, thereby allowing synthesis of fibrils that are aligned along those lines.[55,150,151] In tendon homeostasis, MMP synthesis and activity are balanced by synthesis of tissue inhibitors of MMP (TIMP)-1, -2, -3, and -4, which bind to and inhibit all MMP.[148] The balance between MMP activity and inhibition thereof allows ECM degradation to be balanced with ECM synthesis, which preserves the structural integrity of the tendon.[147,150,151]

Adaptations to specific functions

The mechanoresponsive capabilities of tenocytes and tendon progenitor cells allow tendon composition and organization to be adapted to the specific forces applied.[54,67,68,71,80,108] The tensional region of the superficial digital flexor (SDF) tendon in adult horses has higher cellularity, GAG, and water content than the common digital extensor tendon (CDET).[54] These properties make the SDF matrix less stiff than the CDET, allowing it to elongate and store energy in the weight-bearing phase of the stride. In areas where tendons are compressed, such as where they pass over a high-motion joint such as the fetlock, collagen fibers are interwoven

and oriented perpendicular to the long axis of the tendon.[67] Tenocytes in compressed regions are round and arranged in columns, similar to chondrocytes.[67] Tendons in areas of compression contain higher levels of large aggregating proteoglycans, which maintain a greater water content than in the tensional region.[67,70] Compressed regions of tendon also have higher glycosaminoglycan and chondroitin sulfate contents than tensional regions.[70,80,92] These adaptations give the tendon a fibrocartilage-like character and enable it to resist compression.

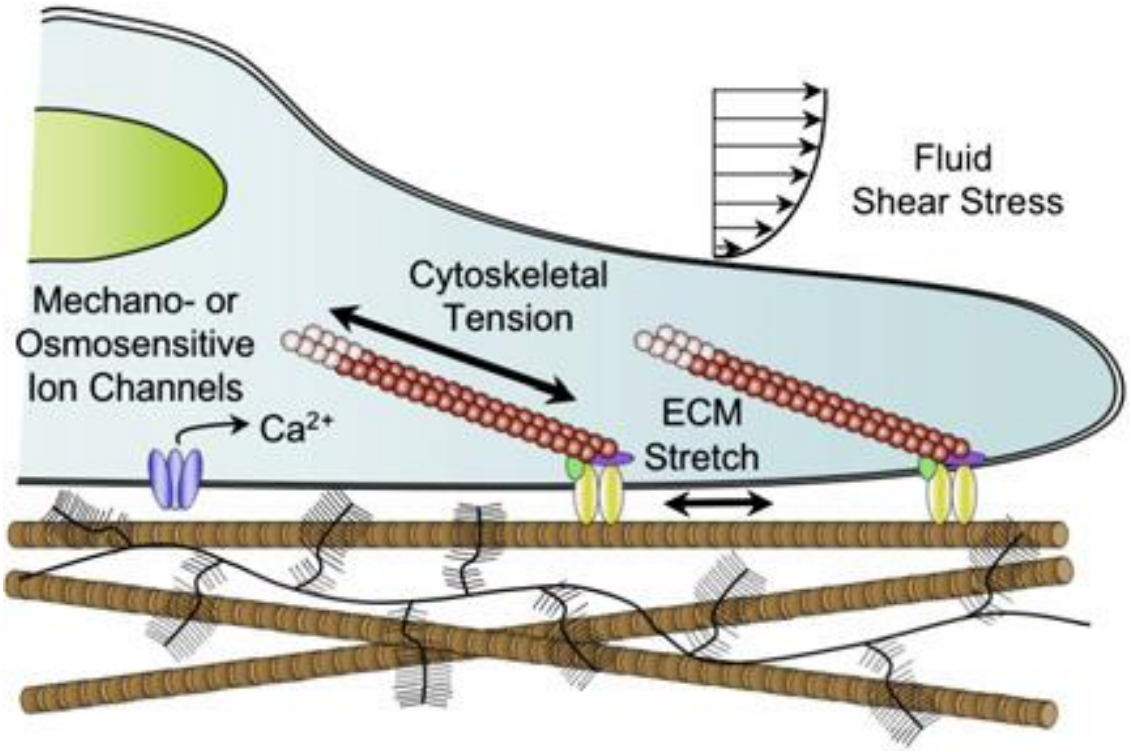

Figure 2.5 Cellular mechanisms of mechanosensation. From Guilak F et al Cell Stem Cell 2009.

While tendons adapt their ECM composition and organization to specific types of load, they also modify their ECM composition in response to repetitive exercise.[63,64,66,68,152] Cumulative exercise-induced changes in ECM composition over time lead to progressive loss of

ECM integrity and organization.[68,86,153-157] The accumulation of microscopic ECM degeneration, combined with excessive matrix degradation relative to matrix remodeling, eventually leads to gross tendon injury and clinical tendinopathy.

Tendinopathy

Tendinopathy is a clinical condition defined by tendon swelling, pain, and impaired function following tendon injury. While tendon injuries can result from an acute, single episode of internal or external trauma, they most often occur due to accumulated strain-induced degeneration of the extracellular matrix.[61,62,64,68,86,153,156] The increased incidence of tendon injuries with increased age and time in work supports progressive ECM degeneration as a primary cause of tendon injuries in athletic horses.[58,60,158,159] Cumulative ECM degeneration combined with a net imbalance in ECM metabolism, whereby ECM degradation outpaces ECM synthesis, results in net loss of ECM content and organization and contributes to ultimate structural failure.[160-164]

Epidemiology

Tendon injuries are common in horses and negatively impact quality of life, reduce athletic performance, and result in large economic losses.[58,60,64,159] Energy-storing tendons, such as the superficial digital flexor tendon, are most often affected.[58,60,165-167] Forelimb SDF tendon injury is one of the primary causes of lameness and loss-of-function in Thoroughbred racehorses, with a reported incidence of 11.1-65%.[58,60,159,167] SDF tendonitis was the reason for retirement in 14% of all racehorses at the Hong Kong Jockey Club[159] and was the most common site-specific reported injury at the National Hunt Club.[167] Recurrent SDF tendon injuries are common, with

reported incidences ranging from 16-66% in Thoroughbred racehorses.[58,165,166,168] The deep digital flexor (DDF) tendon is less commonly injured in racehorses; in a survey of equine musculoskeletal abnormalities in an abattoir, forelimb DDF injuries comprised only 1.4% of tendon/ligament abnormalities in Thoroughbred racehorses, compared to 11.8% of such abnormalities in other breeds.[59] DDF injuries are a common cause of lameness localized to the foot, comprising 59% of lesions diagnosed on magnetic resonance imaging in one study.[169] Longitudinal and/or marginal DDF tears are also a common cause of chronic digital flexor sheath tenosynovitis.[16,170,171]

Pathophysiology

Tendons demonstrate *viscoelasticity* within the physiologic range of strain: their mechanical properties vary with the rate of application of load.[172] The response of tendons to load is defined by a load (stress) versus deformation (strain) curve (Figure 2.6).[61,172,173] The first portion of the curve is the *toe region*, which represents the non-linear straightening of the fascicular crimp to absorb the initial load.[173] The linear region represents elastic deformation of tendon fibers, which occurs between 4-8% strain in the SDF.[172] The slope of the linear portion of the curve reflects the *elastic modulus* of the tendon, or ability of the tendon to resist permanent structural deformation.[89,172] Above the *yield point*, collagen fibrils and cross-links undergo irreversible, microscopic damage.[89] If ultimate tensile strain is applied, the tendon undergoes catastrophic failure.[89] While the equine SDF fails at 10-12% strain under *post-mortem* biomechanical testing, the equine SDF undergoes strains of up to 16% at the gallop in Thoroughbred racehorses.[89,172] The tendency of the SDF to operate at or above its failure limit during high-speed work may contribute to a high rate of clinical SDF injury in racehorses.[61,173]

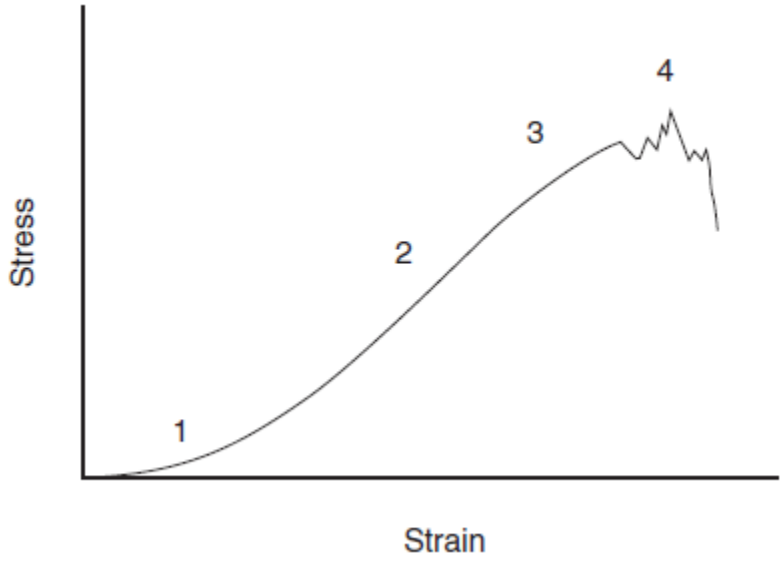

Figure 2.6 Stress-strain curve of the equine SDF tendon. 1: toe region; 2: linear deformation; 3: yield point; 4: failure point. From Ross MW and Dyson SJ, Diagnosis and Management of Lameness in the Horse 2011.

Exposure of load-bearing tendons to repetitive, high levels of strain not only physically damages the ECM but also alters tendon physiology in ways that contribute to ECM degeneration and tendinopathy. The elasticity of the SDF enables it to conserve kinetic energy when loaded but may also contribute to tendon pathology with repetitive exercise.[56,61,174] Load-bearing tendons in the horse lose approximately 5% energy as heat between loading and unloading, a process referred to as *hysteresis* (Figure 2.7).[89] A more elastic tendon, like the equine SDF, will have minimal hysteresis due to conservation of kinetic energy when loaded.[61] While minimizing hysteresis improves efficiency of locomotion, energy retention in the form of heat causes the tendon core to reach temperatures of 43-45°C during exercise.[156] Heat accumulation in the tendon core during repetitive work may therefore lead to thermal-induced

tenocyte damage and subsequent ECM degeneration.[156,174] Repetitive strain may contribute to relative tissue hypoxia during exercise and reperfusion injury following exercise, both of which may injure tenocytes and contribute to ECM degeneration.[157,175]

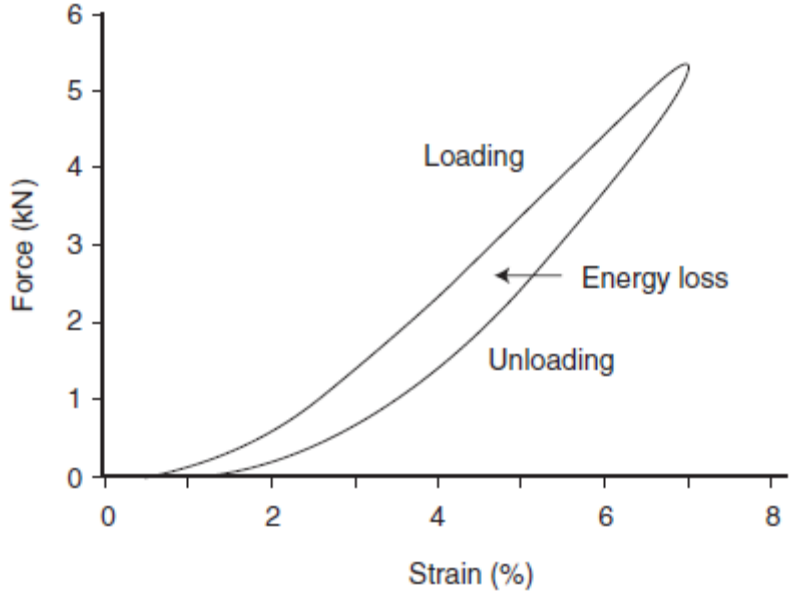

Figure 2.7 Hysteresis loop of the equine SDF tendon. The area of the loop represents the energy lost as heat between loading (top line of loop) and unloading (bottom line of loop). From Ross MW and Dyson SJ, Diagnosis and Management of Lameness in the Horse 2011.

Tendon ECM composition and organization adapt in response to exercise and age and these changes are thought to contribute to the development of tendinopathy.[64,66,86,153-155] In regularly exercised horses, the tensional region of the SDF has an increased proportion of small fibrils to large fibrils compared to tendons from non-exercised horses.[64,66] The increased proportion of small fibrils is thought to predispose the tendon to injury by decreasing tendon tensile strength.[66,69] Decreased fascicle integrity in the SDF core, and increased SDF stiffness with age and exercise, decrease the ability of the SDF to deform elastically when loaded and

increase the risk of injury.[57,153,154] While repetitive high-strain exercise leads to degenerative changes, moderate exercise during development is required for appropriate ECM development. Foals with free choice pasture exercise between 2-5 months of age showed faster increases in COMP levels in the SDF compared to foals with restricted exercise.[63] Foals unable to bear weight on a limb in the first year of life had significantly lower COMP levels in the unloaded versus the loaded SDF.[68] Foals receiving free choice pasture exercise from ages 1-5 months also had significantly greater load at failure in their SDF at 11 months.[65] Addition of controlled treadmill exercise to pasture turnout during the first year of life significantly increased SDF cross-sectional area compared to pasture turnout alone.[176] The increases in tendon COMP, mechanical strength and cross-sectional area of the SDF with regular, moderate exercise during development may protect against development of tendon injures in adult life.[63]

Response to injury

Tendons follow the same phases of wound healing as other tissues, but their healing process is slow.[133,177] The initial inflammatory phase lasts for 1-2 weeks and is characterized by hematoma formation at the site of fiber rupture, debridement of damaged ECM by macrophages, hypertrophy of the epitenon, and upregulation of growth factors and pro-inflammatory cytokines.[59,133,177,178] Matrix metalloproteinase (MMP) gene expression and enzymatic activity increase in response to pro-inflammatory cytokines to remove damaged matrix components.[150,160,162,164,178] The proliferative phase lasts for 6 weeks or more and is characterized by angiogenesis, migration and proliferation of tenocytes at the injury site, and deposition of small, disorganized, predominantly type III collagen fibrils.[133,158,177,178] The predominance of smaller diameter, disorganized type III collagen fibers in newly synthesized

ECM decreases the overall tensile strength of the tendon (Figure 2.8).[7,69,86,158,164,177] While tendons were traditionally thought to only heal by extrinsic methods, tendons seem to capable of extrinsic and intrinsic methods of repair.[133] The cells that proliferate at the injury site can be mature tendon fibroblasts or progenitor cells and may migrate to the injury site from the para-, epi-, and/or endotenon.[83,133,177,178] The vasculature in the endotenon is also a rich source of cells that can contribute to intrinsic tendon repair.[164] Active extrinsic and intrinsic healing during the proliferative phase is seen histologically as an increase in round, randomly oriented cells at the tendon injury site (Figure 2.8).[164,177]

Remodeling of newly synthesized tendon ECM lasts for up to a year.[177] During remodeling, newly synthesized collagen fibrils and tenocytes align along tensional lines and the relative proportion of type I collagen fibrils increases as type III collagen is replaced by type I collagen.[177] Even if healing proceeds uninterrupted, repaired tendon consists of variably organized fibrosis (scar tissue) that is stiffer and more prone to recurrent injury than normal tendon.[59,61,173,177] The balance between MMP synthesis and activity, and synthesis of their tissue inhibitors (TIMP), during healing also influence the integrity of the healed tendon.[55,160-164,179] Some MMP activity is required for tendon healing beyond the removal of damaged collagen fibrils and non-collagen ECM components in the acute inflammatory phase. In the remodeling phase, MMP remove initially synthesized disorganized fibrils, thereby allowing synthesis of fibrils that are organized along tensional lines.[150,151,164] Decreased MMP expression in the equine SDF compared to the common digital extensor tendon is actually thought to contribute to the high risk of SDF injury by decreasing ability of the SDF to remodel its matrix in response to strain.[55] However, gene expression and enzymatic activity of MMP, particularly MMP-13, are markedly increased in acute tendon injuries and are persistently increased in chronic tendon

injuries.[160-164,178] At the same time, expression of TIMP is downregulated in acute and chronic tendon injuries.[160,163,164] The increase in MMP expression and activity, along with the subsequent decrease in synthesis of its inhibitors, leads to net degradation ECM that further decreases tendon structural integrity and increases the risk of re-injury.[161,163,164,179]

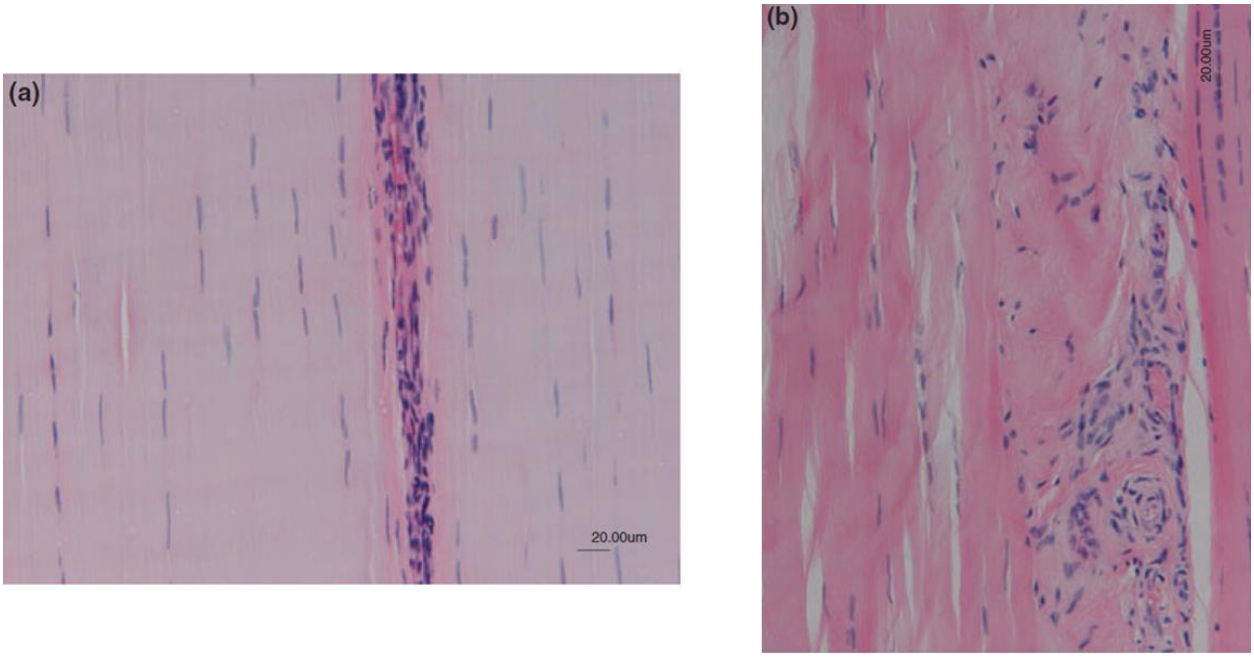

Figure 2.8 Hematoxylin and eosin-stained sections of equine SDF tendon. (a) Normal and (b) 8 weeks following injury. Scale bars = 20 µm. From Clegg PD et al Int J Cell Pathol 2007.

Diagnostic techniques

Diagnosis of tendinopathy in horses is based on a combination of clinical history, palpation and manipulation of the affected tendon, gait analysis, and diagnostic imaging.[1,180,181] Ultrasonography of the affected tendon, with evaluation of the contralateral tendon for comparison, is the primary method to diagnose tendinopathy.[181,182] Typical findings include increased tendon cross-sectional area, decreased margin definition, altered fiber density and organization, and tendon sheath effusion.[2,155,182,183] Ultrasonography is also used to monitor of

the progression of healing and thereby guide controlled exercise protocols.[2,3] Repeat ultrasonography is typically performed at 6-8 week intervals to allow adequate time for increased healing from the previous scan and to adjust the controlled exercise routine if evidence of re-injury is detected.[1,2]

While ultrasonography is readily available, non-invasive, relatively cost-effective, and can be performed in the standing horse, the ultrasonographic appearance of lesions may be difficult to correlate with the histologic appearance of lesions in the proliferative and remodeling phases.[184,185] Tendon structural integrity and progression of healing can be further assessed with sonoelastography.[186,187] Sonoelastography is an ultrasound-based technique that can used to calculate tendon elastic modulus by calculating strain on the tendon before and after tissue compression; a color-coded map is then superimposed on the gray-scale images that correlates with elastic modulus by region.[186,187] Magnetic resonance imaging (MRI) can be performed under standing sedation or general anesthesia and provides high anatomic detail, contrast between tissues, the ability to distinguish acute from chronic lesions, and allows visualization of structures within the hoof capsule.[16,171,188] MRI can therefore be a useful adjunct to ultrasound examination or, in the case of injuries within the hoof capsule, the imaging method of choice to evaluate tendon pathology.

For segments of tendon within a synovial sheath, tenoscopy allows direct evaluation of lesion presence and severity in the tendon and the surrounding sheath.[16,170,189] In addition to facilitating surgical treatment of these lesions, tenoscopy provides more accurate diagnostic and prognostic information about intrasynovial tendon and tendon sheath lesions than ultrasonography or MRI.[16,171,188] Tenoscopy is particularly helpful in diagnosing tears in the

digital flexor tendon margins, synovial masses, tears in the *manica flexoria,* and adhesions between the tendons, surrounding sheath, and the navicular bone and bursa.[15,16,170,171,183,189]

Current therapeutic options

The immediate objectives of treatment for tendon injuries are to reduce pain and inflammation and prevent continued damage to the tendon. Once the acute inflammatory response has subsided, the goal of tendon injury treatment is to encourage healing that restores the tendon's native structure, thereby restoring athletic function and preventing re-injury. Tendon injuries can be treated by medical and surgical therapies, and a combination of therapies is commonly used to achieve the best possible healing and return to soundness. The mainstays of treatment in the acute inflammatory phase are strict athletic rest, cold therapy, and non-steroidal anti-inflammatory medications to reduce pain, edema, and prevent continued damage to the tendon.[1,3] The horse should be confined to an area that is small, flat, and does not have deep footing to avoid excessive strain on the injured tendon.[2] The initiation and progression of a graduated exercise program are dictated by the lack of clinical signs of recurrent acute tendonitis and evidence of increased healing without of re-injury on serial ultrasound examinations.[1,2] Graduated exercise is important to encourage alignment of newly synthesized collagen fibers along the lines of tension without re-injuring the tendon.[1,173] Hoof trimming and shoeing can be adjusted to reduce stress on the affected tendon and prevent re-injury.[2,173] *In vivo* kinematics analyses suggest that heel elevation and shortening the toe decrease the strain on the DDF tendon by promoting flexion of the distal interphalangeal joint.[190,191] Conversely, maintaining a flat foot with no additional heel elevation or support is thought to decrease strain on the SDF tendon, particularly at high speeds, by limiting extension of the metacarpophalangeal joint.[190,192]

In the past 15-20 years, regenerative therapies to improve the quality of tendon healing have gained popularity. The intralesional injection of mesenchymal stem cells (MSC) for the treatment of tendon injuries has received the most attention clinically and in research studies.[4,166,193-195] MSC are progenitor cells that are capable of self-renewal, can differentiate into specific tissue types (multipotent), and are readily isolated from many adult tissues, including bone marrow and adipose tissue.[194,196-198] When exposed to mechanical strain, tendon-associated growth factors, and/or tendon ECM, MSC are also capable of undergoing tenogenic differentiation.[40,111-113,199-201] MSC are thought to promote healing by reducing fibrosis, promoting angiogenesis, recruiting endogenous stem cells to the site of injury, and promoting their division and differentiation.[139,202-205] In addition, MSC support physiologic healing by modulating the local immune response, including suppressing mononuclear cell proliferation and decreasing secretion of pro-inflammatory cytokines from resident leukocytes.[158,206-209] In naturally-occurring and experimental equine SDF tendon injuries, intralesional injection of autologous MSC improved histologic organization of healed tendon compared to controls.[4,5,193,194,210] Intralesional MSC injection into surgically-induced SDF lesions has also been demonstrated to reduce MMP-13 activity, water and GAG content and improve expression of tendon-associated genes.[194,210] The benefit of MSC treatment on collagen fibrillogenesis in tendon healing remains unclear; intralesional MSC injection did not restore fibril diameter or bimodal fibril distribution in surgically-induced equine SDF core lesions.[195] While data on the clinical response to MSC treatment in horses are sparse, MSC injection into clinical SDF lesions in Thoroughbred racehorses significantly reduced the re-injury rate compared to other non-surgical treatments in one study.[166] Overall rates of return to soundness and work following intralesional MSC injection range from 73-82% in Thoroughbred racehorses.[166,211]

Platelet-rich plasma (PRP) has also been investigated as a therapy to improve the quality of tendon healing. PRP is an autologous blood product in which the platelets have been concentrated in a small volume of plasma and can be injected intralesionally, either by itself or as a vehicle for MSC administration.[7,8,193,210,212] PRP provides a fibrin scaffold and multiple growth factors to support endogenous cell migration, proliferation, and tenogenic differentiation, including transforming growth factor (TGF)-β, platelet-derived growth factor-BB, and insulin-like growth factor-I.[6,7,213-217] Incubation of equine SDF explants with PRP increased gene expression of tendon matrix components without concurrently increasing MMP gene expression.[6,217] Similar to MSC, PRP injections into experimentally-induced SDF core lesions improved cellular and matrix organization of healed tendon compared to saline-injected controls.[7,216] PRP injection also improved load at failure and elastic modulus in surgically-induced SDF lesions in horses.[216] In retrospective clinical studies, the reported rate of soundness and return to work following intralesional PRP injection, with or without MSC, ranges from 50-93% across breeds, disciplines, and tendon injury sites.[8,218-220]

Surgical therapies for tendon injuries can also be pursued concurrent with or following non-surgical therapies to stimulate healing of tendon lesions and/or improve return to athletic use. Percutaneous tendon splitting is performed to stimulate healing and new vascular ingrowth in a chronic lesion or to decompress an acute core lesion.[11,221-224] In a collagenase-induced SDF tendonitis model, tendon splitting improved neovascularization, collagen orientation and crimp formation and the lesion site, and decreased lesion size faster than non-treated tendons.[223] Clinical outcomes for forelimb SDF splitting vary; 65-83.6% of horses raced post-operatively in retrospective studies of Thoroughbred racehorses and trotters of various breeds, although increased age at the time of surgery was negatively associated with return to racing.[11,221,222]

Transection (desmotomy) of the accessory ligament of the SDF tendon (ALSDFT, or "superior check ligament") has also been performed as a treatment for SDF tendonitis in racehorses. The ALSDFT originates from the caudodistal radius and inserts on the dorsal surface of the SDF, just distal to the musculotendinous junction.[1] The ALSDFT is thought to transfer load from the tendinous portion of the SDF to the distal radius when the tendon is loaded.[12] ALSDFT desmotomy is thought to reduce strain on the SDF and the risk of re-injury by lengthening the musculotendinous unit.[12] However, *ex vivo* documentation of increased strain on the SDF following ALSDFT desmotomy does not support this hypothesis.[225] In Thoroughbred racehorses, reported rates of return to racing following ALSDFT desmotomy range from 69-79%.[12,14] Similar to tendon-splitting, older horses ($\geq$ 5years) are less likely to return to racing post-operatively than younger horses.[14] The reported rates of return to racing are somewhat higher in Standardbred trotters, with 71-82% of horses racing post-operatively without recurrence of SDF tendonitis.[13,226]

For suspected tendon lesions within a synovial sheath and/or chronic tenosynovitis of unknown etiology, tenoscopy remains the gold standard for diagnostic exploration and treatment of those lesions.[170,183,227] Tenoscopy allows minimally invasive debridement of torn fibers, intrasynovial masses, adhesions, and hypertrophic synovium, and facilitates administration of local regenerative therapies.[15,16,170,171,183,189] Tenoscopy can also be used to guide desmotomy of the palmar/plantar annular ligament (PAL). The PAL overlies and maintains the position of the digital flexor tendons as they pass through the fetlock canal.[1] PAL desmotomy is indicated to alleviate the restriction on gliding movement of the digital flexor tendons that can occur due to PAL thickening or tenosynovitis-associated effusion.[13,15,183,189,227] Rates of return to soundness and work following tenoscopic treatment of deep digital flexor lesions, with or without PAL

desmotomy, range from 59-73%.[15,16,170,171,183,227] One retrospective analysis of tenoscopically-debrided *manica flexoria* tears in primarily ponies and cobs reported a 79% rate of return to pre-operative work; however, it is unclear whether similar outcomes would be achieved in other breeds and disciplines.[189]

Despite having several treatment options for tendon injuries in the horse, regaining the normal tendon architecture and preventing re-injury remain challenging for veterinarians. Reported SDF re-injury rates in Thoroughbred racehorses with conservative management alone are high, with 43-66% injuries recurring after prolonged rest and graduated exercise.[165,168,220] While the majority of horses are reported to return to racing following tendon splitting or ALSDFT desmotomy, increased age at the time of either surgery decreases the prognosis for racing post-operatively.[14,222] Furthermore, no improvement in return to racing was seen in Thoroughbred racehorses undergoing ALSDFT desmotomy versus those treated conservatively in one study.[168] Both tendon splitting and ALSDFT desmotomy also have potential complications which can be career-limiting. Exuberant granulation tissue formation and persistent lameness have been documented following SDF splitting.[10] In Standardbreds undergoing ALSDFT desmotomy, 13-18% of horses developed post-operative suspensory desmitis, which is thought to be due to hyperextension of the metacarpophalangeal joint following lengthening of the SDF musculotendinous unit.[13,225,226] For lesions requiring tenoscopic treatment, deep digital flexor tendon tears that are long and/or marginal, marked pre-operative tendon sheath effusion, increased duration of clinical signs, and extensive intrasynovial masses decrease the prognosis for athleticism post-operatively.[170,171,183,227]

Regenerative medicine therapies show promise in improving the quality of tendon healing and return to athletic use, but there are several limitations to their use. Autologous MSC

require 3-4 weeks to isolate in culture, which delays their use in treating tendon injuries.[9,139,166,210,212] While banked allogeneic MSC would be readily accessible, recent data suggest that allogeneic MSC are capable of inciting an antibody response in the recipient that results in MSC death.[228-231] MSC may also not be retained or survive within the lesion long enough to influence tendon healing. Only 24% of intralesionally-injected MSC were present in naturally-occurring SDF lesions at 24 hours after injection, with less retention reported for MSC administered via intravenous or intra-arterial regional limb perfusion.[232,233] Less than 5% of injected MSC were alive at 10 days after injection into surgically-induced SDF core lesions in another study.[234] The ability of MSC and PRP to guide healing also varies by donor age and the presence of systemic diseases.[215,235-239] Recent *in vitro* evidence in horses and people also suggests that the tenogenic capacity of MSC is compromised in an inflammatory environment.[131,136,240] The response of tendon injury to regenerative therapies may also vary by breed and discipline. Compared to conservative management, PRP injections into clinical SDF injuries increased return to soundness and work in Warmbloods and ponies but did not improve the rate of return to racing in National Hunt Thoroughbred racehorses.[8 220]

Although currently available tendon injury treatments can improve healing and soundness in work, there remains a crucial need for a treatment that can consistently restore tendon architecture and function to its physiologic state and thereby prevent re-injury. The ideal treatment would be safe, widely-available, cost-effective, minimally antigenic, and consistently effective at promoting physiologic healing across a wide range of individuals, athletic disciplines, and specific tendon injuries. Decellularized extracellular matrices (ECM) have the potential to fulfill these needs, as they are readily isolated from multiple tissues and have been successfully applied in a xenogeneic fashion to promote physiologic healing in several species

and tissues, including musculoskeletal injuries.[32,35,36,38,39,41,42] ECM therefore may be able to improve the quality of tendon healing and the prognosis for soundness and return to work without re-injury in equine athletes.

Extracellular Matrices in Tenogenesis

The use of decellularized extracellular matrix (ECM) scaffolds to promote connective tissue healing was first described in 1989 by Dr. Stephen Badylak at the McGowan Institute for Regenerative Medicine at the University of Pittsburgh.[241] In that report and those that immediately followed, porcine-derived small intestinal submucosa sheets were successfully utilized as experimental large-diameter vascular grafts in dogs.[241-243] Based on the evidence amassed since those initial reports, ECM scaffolds show substantial promise in guiding site-appropriate, physiologic tissue healing and remodeling.[32,36,38,39,42] ECM scaffolds have been isolated from several tissue sources and species, including porcine small intestinal submucosa, urinary bladder, and liver, bovine and human tendon, human and porcine dermis, and human pericardium.[18,25,27,36,40,41,244-247] All ECM scaffolds provide a three-dimensional scaffold of collagen and non-collagen proteins, including proteoglycans, glycosaminoglycans, adhesion molecules, and bioactive factors that stimulate endogenous cell migration, adhesion, proliferation, and tissue-appropriate differentiation.[20,24,36,40,245,248-250]

ECM isolation and mechanisms of action

ECM scaffolds are isolated in several specific steps. The initial isolation process requires physical separation from the scaffold from its parent tissue a process known as *delamination*.[20,250] Delamination is ideally performed in a manner that preserves the native

collagen fibril orientation of the scaffold.[250] The isolated scaffold is decellularized by freezing/thawing, agitation, sonication, or application of detergents to remove antigenic material responsible for precipitating an immune response following implantation.[24,250-252] Decellularization and the high degree of conservation of the primary ECM components across species facilitate xenogeneic (cross-species) application of ECM scaffolds.[33,34,251] The scaffold is then terminally sterilized by gamma or electron beam irradiation or exposure to ethylene oxide gas.[20] Following sterilization, the scaffold is either freeze-dried (lyophilized) or vacuum-dried to enable long-term storage and prevent potential leaching of soluble growth factors.[31,252]

ECM scaffolds guide physiologic healing and tissue remodeling by several mechanisms. The three-dimensional scaffold, along with it native adhesion molecules, provides surface area for endogenous cell migration, attachment, and proliferation.[20-22,36,40,244] Bioactive factors in the ECM, including vascular endothelial growth factor, TGF-β, and fibroblastic growth factor, encourage endogenous cell migration, proliferation, differentiation, and angiogenesis.[22,23,248,249,253] While all ECM scaffolds share the same basic components, the collagen fiber organization and biochemical composition of each ECM are specific and optimized to the functions of the tissue of origin.[23,36,40,244,245]

In order for bioactive factors to be released from the ECM, mononuclear cells must infiltrate and degrade the ECM scaffold.[25,245,254] Biodegradation of ECM by mononuclear cells has been documented histologically in ECM scaffolds derived from multiple tissues and takes approximately 60 days for complete degradation and replacement with new tissue.[25-27,35,245,250] ECM biodegradation also guides local macrophages to a regulatory (M2) phenotype, which alters the local milieu to be more anti-inflammatory and favors constructive tissue remodeling over inflammation and fibrosis.[19,29,30,255] The biodegradability of any ECM scaffold ultimately

determines its ability to guide macrophage polarization, support physiologic tissue healing, and prevent a chronic inflammatory reaction at the implantation site.[25-27] ECM scaffolds that are not readily biodegradable result in lack of site-appropriate tissue formation and/or chronic inflammation and fibrosis at the implantation site, the latter reflecting a foreign body response.[25,27,254]

Porcine urinary bladder matrix

Of all ECM types, porcine urinary bladder matrix (UBM) has received substantial attention for its ability to support physiologic healing in multiple tissues and its potential for use in treating musculoskeletal injuries.[32,42,44,48,49] UBM is isolated by separation of the mucosal basement membrane and *tunica propria* from the muscular layers and the mucosal epithelium of urinary bladders from pigs.[22,244,250] Following decellularization and terminal sterilization, UBM is lyophilized and processed into individual or stacked sheets for topical application to injuries.[21,42,256,257] UBM can also be pulverized and suspended in an aqueous solution, or chemically digested and incorporated into a hydrogel to inject into deep and/or irregularly-shaped lesions.[24,25,32,246,258,259] Both sheet (MatriStem™, ACell®, Columbia, Maryland) and lyophilized particulate forms (MatriStem™ MicroMatrix, ACell®) of UBM are commercially available.

The basic ultrastructure of UBM is a complex, three-dimensional meshwork of collagens with intercalated non-collagenous proteins and growth factors (Figure 2.9).[17,247,259] Type I collagen predominates in UBM, with smaller amounts of collagen types III, IV, VII, and XIV present.[22,244,245,260] UBM contains multiple proteoglycans glycosaminoglycans, and proteins involved in cellular adhesion and migration[23,244,245,255] Multiple heat shock proteins important in

promoting cell survival through inhibition of apoptosis are also found in UBM.[22] Importantly, UBM contains bioactive factors that stimulate angiogenesis and chemotaxis, proliferation and tissue-appropriate differentiation of resident progenitor cells, including vascular endothelial growth factor, platelet-derived growth factor B, and keratinocyte growth factor.[23-25,30,42,256,261,262]

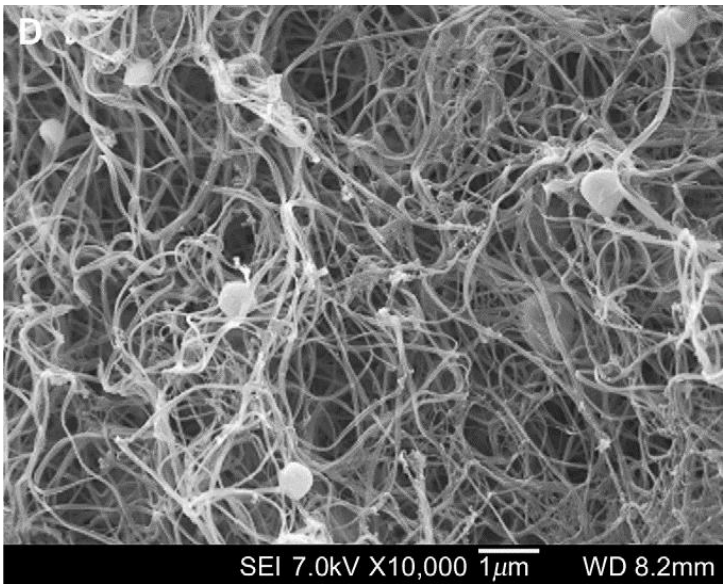

Figure 2.9 Ultrastructure of decellularized porcine urinary bladder matrix. Imaged obtained by scanning electron microscopy at 10,000x magnification. From Freytes DO et al Biomaterials 2008.

Similar to other ECM, UBM supports physiologic tissue via biodegradation, release of its bioactive factors, and promoting local macrophage polarization to a regulatory (M2) phenotype.[19,25,28,29,245,254,255,263] By encouraging M2 macrophage polarization, UBM has been demonstrated to promote a local anti-inflammatory environment by reducing secretion of pro-inflammatory mediators, including interleukin-1β, tumor necrosis factor-α, and inducible nitric oxide synthetase, and increasing secretion of the anti-inflammatory cytokine prostaglandin-

E_2.[19,28,255] However, macrophage polarization in response to UBM is not exclusively anti-inflammatory, and the concurrent presence of pro-inflammatory (M1) macrophages within the UBM scaffold is required for local angiogenesis.[19,26,28,30,245]

Evidence for ECM in guiding healing

ECM scaffolds from multiple tissue sources has been reported to promote site-appropriate tissue formation and thereby improve healing of musculoskeletal injuries. ECM from small intestinal submucosa (SIS) has been most extensively evaluated for the treatment of tendon injuries.[36-39] SIS application improved healing of experimental injuries of the rotator cuff in dogs and rats (Figure 2.10),[38,39] Achilles tendon in rabbits and dogs,[36,37] and the lateral collateral ligament of the metacarpophalangeal joints in a study of 10 horses.[35] While numerous reports of improved tendon healing with SIS application exist in animal models of tendon injury, the benefits of SIS treatment of human tendon injuries in clinical trials are less clear.[264,265] Tendon-derived ECM has also shown promise in healing musculoskeletal injuries.[40,41] Tendon ECM improved *in vitro* tenogenic differentiation of human adipose-derived cell-seeded collagen hydrogels and intralesional injection of MSC-seeded collagen hydrogels supplemented with tendon ECM improved the mechanical strength of healed experimental rotator cuff injuries in rats.[40,41]

Similar to SIS and tendon ECM, UBM has been demonstrated to support physiologic tissue. Topical UBM application improved corneal healing in cats, dogs, and horses.[49,50] UBM treatment has been reported to improve healing of complicated open wounds, urethral reconstruction, and rectopexies in people, experimental body wall hernias in pigs, and urinary

incontinence and partial cystectomies in dogs.[43-47,256,257] Recent data demonstrate the potential of UBM hydrogels in healing experimentally-induced stroke lesions in rats.[25,266]

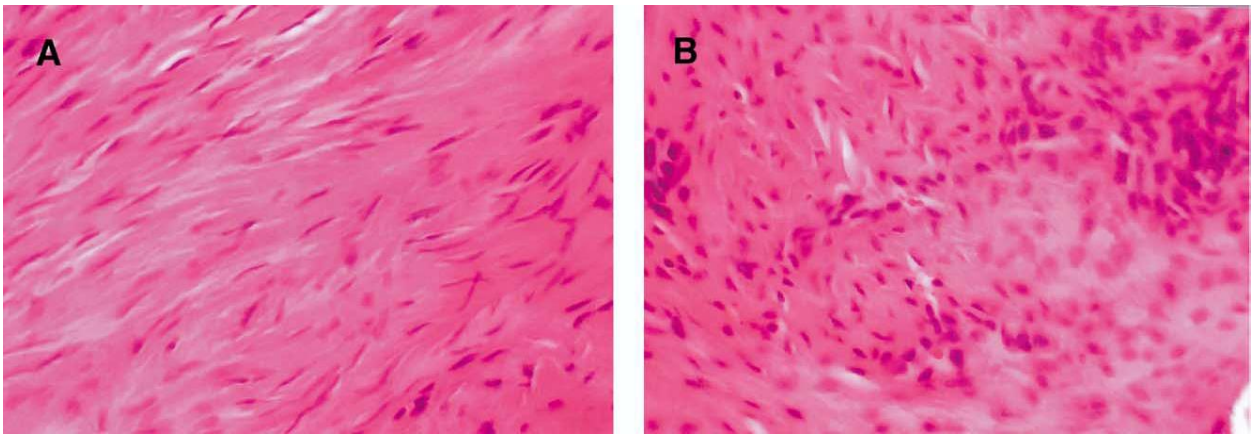

Figure 2.10 Hematoxylin and eosin-stained sections showing improved histologic healing following SIS application in experimental rotator cuff injuries in rats. Sections were obtained at obtained at 16 weeks after model induction. Note improved elongated cellular morphology and longitudinal organization in SIS-treated tendons (A) compared to control tendons (B). 40x magnification. Zalavras CG et al J Shoulder Elbow Surg 2006.

Like other forms of ECM, UBM also shows promise in healing musculoskeletal injuries, although data are sparser than for SIS.[32,42] Circumferential application of multilaminate UBM sheets to Achilles tenectomy sites in mice increased progenitor cell chemotaxis to the site by approximately 50% compared to no treatment or treatment with an autologous tendon graft.[42] Injection of aqueous, suspended ACell® powder into tendon and suspensory lesions in 107 horses (129 lesions) resulted in 85.9% soundness and return to work at 12 months after treatment.[32] Given that 75 of 129 proximal suspensory lesions were concurrently treated with fasciotomy, it remains unclear to what extent the UBM treatment versus the fasciotomy improved soundness in

those cases.[32] Injection of UBM suspension into experimental, collagenase-induced SDFT lesions in horses did not improve ultrasonographic or histologic healing over an 84-day treatment period.[258] It is unclear whether improved healing would have been seen with UBM treatment over a longer follow-up time, particularly given that lesion enlargement continued for up to 42 days after model induction and for 28 days following UBM treatment.[258]

In summary, UBM shows promise in promoting site-specific, physiologic healing in a variety of tissues. However, objective evidence to support the ability of UBM to induce tenogenesis and promote tendon healing is lacking. A better understanding of the cellular and biomechanical mechanisms by which UBM may guide tendon healing using a model of tenogenesis may help translate the application to the *in vivo* tendon environment.

Modeling tenogenesis in vitro

To improve success in treating tendon injuries, one approach is to develop a therapy that capitalizes on tenogenesis *in vivo* and thereby recapitulates the normal tendon structure and organization.[41,112,113] Tenogenesis is loosely defined as the generation of physiologic tendinous tissue.[40,111-113,138,200,267] While documenting a clinical response to novel tendon injury treatment is useful, understanding the cellular mechanisms by which that treatment heals tendon injuries requires its evaluation in an *in vitro* model of tenogenesis.

In vitro tenogenesis models have been used to study the cellular and biochemical processes involved in tenogenesis and to develop methods to optimize tenogenesis and thereby facilitate engineering of tendon tissue replacements.[40,111-113,199,200,268,269] Modeling tenogenesis *in vitro* is challenging because there are no established tendon-specific parameters to confirm the development of tendon tissue.[196] In contrast, other tissue types do have established tools, such as

detection of type II collagen and aggrecan for cartilage, calcification and alkaline phosphatase for bone, and fat droplets for adipose.[196,270-272] Because there are no universally accepted parameters, the criteria used to define tenogenesis vary between studies, but generally include cellular elongation and alignment along tensional lines, tendon matrix synthesis and organization, and expression of tendon-associated genes.[40,111,115,137,138,200,273]

Designing a model of tenogenesis

While tenogenesis can be modeled *in vitro* by many methods, three key design criteria must be considered: the type of cell used, the characteristics of the scaffold, and the protocol for applying strain to the model.[274] Most often, tendon fibroblasts or mesenchymal stem cells (MSC) derived from bone marrow or adipose tissue are used because they are mechanoresponsive, readily isolated, and in the case of MSC, are capable of tenogenic differentiation.[40,111,113,138,146,200,268,275-278] Some evidence suggests that tendon progenitor cells may be more ideal to model tenogenesis, as they upregulate tendon-associated gene expression more than bone marrow or adipose-derived MSC in the presence of mechanical strain and/or tendon ECM.[112,114,267,279]

The physical and biochemical characteristics of the scaffold used are also important to consider when modeling tenogenesis.[274] The scaffold should allow seeded cells to survive, proliferate, and integrate into the scaffold, and it should have the structural and biochemical properties required for the application.[40,112,200,274] Finally, the scaffold should be able to withstand the applied mechanical load.[274] Scaffolds can be constructed from synthetic or natural materials.[40,111-113,115,146,275,277,278] Synthetic polymer scaffolds can be readily synthesized with precise control over the scaffold topography.[115,200,268,278] However, the underlying fiber

alignment, scaffold stiffness, and the concurrent addition of tendon-derived ECM influence tenogenic differentiation of seeded progenitor cells.[115,200,201,268,280]

In contrast to synthetic scaffolds, natural ECM-derived scaffolds provide a more physiologic ultrastructural and biochemical environment for seeded cells and improve cell viability over synthetic scaffolds.[281,282] Natural ECM scaffolds can be digested and used to construct hydrogels, which are highly hydrated polymer networks that solidify at physiologic temperature and can be formed into elongated, three-dimensional structures suitable for modeling tenogenesis.[24,25,28,40,111,113,146,246,259,283,284] When constructing natural ECM-based hydrogels, the composition of the ECM scaffold and the method used to digest it will determine the ability of that ECM to guide tenogenesis *in vitro.* Purified type I collagen is commonly used for constructing hydrogels because it is the most abundant collagen in adult tendon, is readily isolated from multiple tissue sources, and is commercially available.[40,51,85,88,111,113,146] However, type I collagen-based hydrogel models lack other collagens and non-collagen proteins present in tendon ECM.[40,51] In contrast, native tendon-derived ECM provides non-collagen proteins that are crucial to collagen fibrillogenesis, cell-matrix adhesion, and expression of tendon-associated genes.[40,112,267] Rat tail tendon ECM, in particular, would be an ideal source of ECM to model the tendon niche, as it is readily isolated and retains the ECM composition and collagen fibril organization of native tendon.[51,260]

In addition to the ECM source, the specific technique used to digest the ECM scaffold prior to hydrogel construction is important to consider because it affects the structural and biochemical properties of the ECM.[85,115,252,285] Enzymatic digestion by pepsin is most commonly used;[24,25,28,85,115] however, pepsin cleaves the carboxy- and amino-terminal propeptides of the collagen triple helices, separating the helices and eliminating the capacity for fibril self-assembly

and alignment.[85,252,285] Acetic acid digestion preserves the propeptides and thereby allows fibril assembly and improved support for cell attachment, migration, and replication.[285] Pepsin digestion also decreases levels of active growth factors in the ECM compared to non-enzymatic methods of digestion.[115] Therefore, using a non-enzymatic digestion method such as acetic acid or urea may be ideal to preserve the ECM structure and biochemical profile and thereby the ability to the ECM to support tenogenesis in the model.[40,85,115,285]

Once the cell source, scaffold source and scaffold synthesis and processing protocol have been determined, the final consideration in modeling tenogenesis is the protocol for application of strain. Strain can be applied in a cyclic or static fashion, with amplitude ranging from 2-15%, frequency ranging from 0.017-1 MHz, and single versus repeated application of strain over the study period.[40,111-113,138,276-278] Consistent, controlled strain can be applied along the longitudinal axis of the model by linear motors or step motors as part of the bioreactor.[112,199,275,276] One commercial pneumatic actuator (Tissue Train® 3D Culture System, Flexcell® International, Burlington, NC) has been used to apply cyclic strain in many publications.[40,111,113,146] The Tissue Train® system creates strain by applying a vacuum to deform culture plates with flexible bottoms (Figure 2.8).[146] While the aforementioned systems precisely control the amplitude, duration, and frequency of strain, they are costly to purchase and maintain, and require adequate working knowledge to successfully use them. The Tissue Train® system also limits hydrogel construct volume to 200-300 µL, which can complicate sectioning for histology and results in low RNA yields for gene expression.[40,111,113]

Evaluating tenogenesis in vitro

Once the model is designed, a combination of parameters must be selected that will allow the most complete evaluation of tenogenesis. Evaluation of cellular morphologic changes and matrix organization and remodeling are often evaluated through a combination of histologic stains and measurement of the change in construct width over time.[40,111,113,146] As for tendon cells *in vivo,* one of the hallmarks of tenogenesis *in vitro* is development of elongated cellular morphology with branching to each other and the ECM when placed under strain.[111-113,138,146,278] Cells may also migrate to the periphery of three-dimensional constructs (Figure 2.11).[40,146] It has been thought that the peripheral localization of cells on the constructs represents formation of an epitenon-like sheath, although the mechanism underlying this phenomenon remains unknown.[40,146] Development of cellular elongation and cytoplasmic branching may require at least 7 days of strain application, based on previous data from a tendon fibroblast-seeded collagen hydrogel model under cyclic strain.[146]

In three-dimensional hydrogel models, cells progressively remodel the ECM in which they are embedded in response to load.[40,111,113,146] Similar to *in vivo* tendon, remodeling occurs by MMP-mediated removal of disorganized collagen fibrils and synthesis of fibrils that are aligned along tensional lines.[40,111,113] Remodeling is grossly manifested by progressive narrowing of constructs over time (Figure 2.11) and increased construct stiffness and load at failure.[40,111-113,146,160]

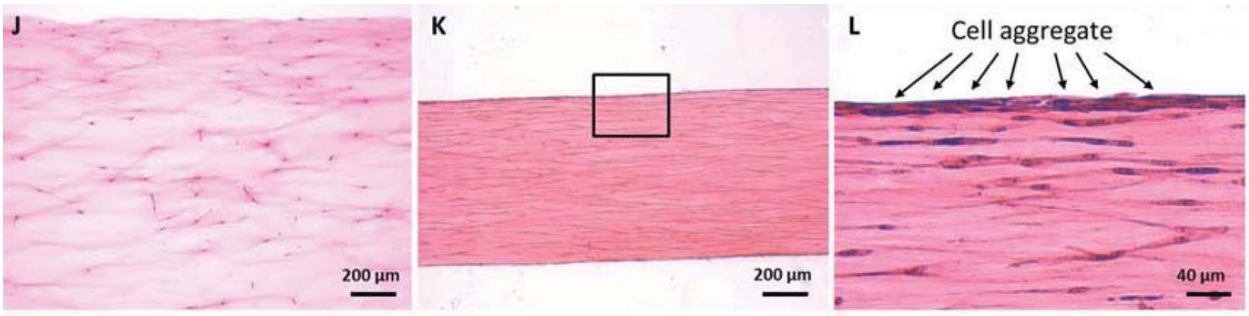

Figure 2.11 Changes in cell and matrix organization over time in cyclically-strained collagen hydrogels seeded with adipose-derived cells. Note the progression from wide constructs with disorganized matrix and round, randomly oriented cells on day 1 (J) to organized, compact matrix with elongated cells organized along tensional lines on day 7 (K and L). From Yang G et al Biomaterials 2013.

In addition to observation of cellular and matrix organization in response to strain, documentation of tenogenesis *in vitro* requires expression of tendon-associated genes, including tendon ECM components, MMP, and transcription factors important in tendon development and homeostasis.[40,111,113,115] Typical genes evaluated include those encoding for type I and type III collagen, small leucine-rich proteoglycans, ECM glycoproteins, MMP-1,-8-, and -13 (collagenases), and scleraxis.[40,146,199,278] Measurement of mohawk expression would also support evaluation of tenogenic differentiation, although the expression of mohawk in a three-dimensional tenogenesis model under strain has yet to be investigated.[114,115,128,131]

Several components of the tenogenesis model influence expression of these genes. Cyclic application of strain and the magnitude of strain are important factors in designing a model of tenogenesis because adequate mechanical strain is required to upregulate expression of tendon-associated genes.[111,113,122] Expression of scleraxis, a transcription factor important in tendon development and homeostasis, decreased over time under static strain but increased under

cyclic strain in MSC-seeded collagen hydrogels.[111] In another cell-seeded hydrogel model, scleraxis was expressed under both static and cyclic strain but was upregulated significantly more under cyclic strain.[122] Like scleraxis, MMP-13 expression increased 2.6-fold under 3 days of cyclic compared to static strain.[111] Expression of tenomodulin, an important glycoprotein in regulating collagen fibril development and tenocyte proliferation, was increased significantly in MSC-seeded hydrogels after 14 days of cyclic compared to static strain.[113] The relationship between cyclic strain and expression of other tendon-associated is less clear.[40,111-113,122] A relatively high amplitude of cyclic strain may also be required to upregulate expression of some tendon-associated genes; up to 10% cyclic strain was required to upregulate expression of type I collagen and scleraxis in one study.[122]

A final consideration in model design, and in evaluation of gene expression results, is the expected genetic variability of the subjects from which the cells used in the model are harvested. Cells isolated from the species of interest in tendon injury treatment (horses) may better model how tendon injuries in that species would respond to that treatment *in vivo* compared to cells from another species (mice). However, gene expression can vary widely between cells from genetically heterogeneous subjects.[111] Individual variation in gene expression, along with a small sample size, can result in a net lack of significant differences in gene expression over time.[111] Even if cells from genetically homogenous subjects are used and a wide panel of tendon-associated genes is evaluated, gene expression alone is inadequate to confirm tenogenic differentiation of those cells because none of the tendon-associated genes are exclusively expressed in tendon.[105,110,111,119,121] The gene expression results must therefore be considered concurrently with evidence of matrix synthesis and remodeling and histologic organization to confirm the presence of tenogenesis in the model.

Conclusion

The treatment of tendon injuries in horses remains a dilemma for veterinarians and horse owners, and a treatment that consistently promotes physiologic tendon healing and prevents re-injury is greatly needed. ECM scaffolds, including UBM, guide site-specific, constructive healing and remodeling and therefore have the potential to improve the quality of tendon healing and more closely approximate the normal tendon structure. The understanding of how UBM may guide tendon healing requires evaluation of its ability to guide tenogenesis in an *in vitro* model. While reported *in vitro* tenogenesis models have provided valuable insight into the various processes contributing to tendon healing, modification of some key features of these models might more accurately mimic the *in vivo* tendon environment. Specifically, the use of a tendon-derived ECM that is non-enzymatically digested to construct the model would more accurately manifest the *in vivo* tendon environment. The measurement of a wider panel of tendon-specific genes, including mohawk, will also facilitate assessment of tenogenesis *in vitro.* Improved *in vitro* modeling of tenogenesis will not only facilitate understanding of tenogenesis itself, but will further understanding of whether and how UBM may improve tendon healing.

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Chapter 3: Urinary Bladder Matrix Does Not Improve Tenogenesis in an *In Vitro* Equine Model

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ABSTRACT

Extracellular matrix (ECM) is responsible for tendon strength and elasticity. Healed tendon ECM lacks structural integrity, leading to reinjury. Porcine urinary bladder matrix (UBM) provides a scaffold and source of bioactive proteins to improve tissue healing, but has received limited attention for treating tendon injuries. The objective of this study was to evaluate the ability of UBM to induce matrix organization and tenogenesis using a novel in vitro model. We hypothesized that addition of UBM to tendon ECM hydrogels would improve matrix organization and cell differentiation. Hydrogels seeded with bone marrow cells (n = 6 adult horses) were cast using rat tail tendon ECM ± UBM, fixed under static tension and harvested at 7 and 21 days for construct contraction, cell viability, histology, biochemistry, and gene expression. By day 7, UBM constructs contracted significantly from baseline, whereas control

constructs did not. Both control and UBM constructs contracted significantly by day 21. In both groups, cells remained viable over time and changed from round and randomly oriented to elongated along lines of tension with visible compaction of the ECM. There were no differences over time or between treatments for nuclear aspect ratio, DNA, or glycosaminoglycan content. Decorin, matrix metalloproteinase 13, and scleraxis expression increased significantly over time, but not in response to UBM treatment. Mohawk expression was constant over time. Cartilage oligomeric matrix protein expression decreased over time in both groups. Using a novel ECM hydrogel model, substantial matrix organization and cell differentiation occurred; however, the addition of UBM failed to induce greater matrix organization than tendon ECM alone.

Keywords: urinary bladder matrix; extracellular matrix; tendon healing; tissue engineering; tendon hydrogel

Tendon injuries are common and are slow to heal, requiring extended periods of rest and rehabilitation. Healed tendon is at increased risk of reinjury because of the decreased elasticity and strength that occur as a result of scar tissue formation.[1] Conservative therapies for tendon injuries include cold therapy, anti-inflammatory medications, rest with controlled exercise, and physical therapy, including eccentric exercise.[2] While regenerative medicine therapies such as mesenchymal stem cells (MSC) and platelet-rich plasma show promise in treating tendon injuries,[3,4] evidence of their efficacy is limited. A critical need remains for development of therapies capable of promoting physiologic tendon healing and reducing the risk of reinjury.

Extracellular matrix (ECM) scaffolds from a variety of tissue sources, including small intestinal submucosa (SIS) and urinary bladder basement membrane (UBM), have been evaluated for their ability to guide physiologic healing and tissue remodeling in both preclinical animal studies and human clinical trials.[5-10] ECM scaffolds are a biological source of structural and functional elements in a complex three-dimensional arrangement of matrix (e.g., collagen, fibronectin, proteoglycans, and glycoproteins) and signaling molecules (e.g., growth factors and cytokines) that provide a favorable local environment for recruiting endogenous progenitor cells and facilitating matrix organization.[11,12] Additionally, the natural process of ECM degradation *in vivo* releases bioactive peptides that act as potent chemoattractants.[13-15]

Clinical and experimental evidence support the ability of ECM to promote tissue healing following musculoskeletal injury.[5-9] *In vitro*, addition of tendon-derived ECM to collagen hydrogels improved tenogenic differentiation of human adipose-derived stem cells compared to hydrogels without ECM.[16] *In vivo*, canine segmental Achilles tenectomy defects repaired with SIS regained the tensile strength of the contralateral tendon and healed with well-organized collagenous tissue compared to the disorganized control tissue.[7] Application of SIS membranes

and SIS-engineered tendon explants improved healing of experimental rotator cuff injuries in canine[5] and rodent[17] models. The potential benefits of SIS in repairing rotator cuff injuries based on human clinical trials are less clear.[18] In a mouse Achilles tendon transection model, circumferential application of stacked UBM sheets stimulated greater progenitor cell migration to the injury site compared to application of an autologous tendon graft.[14]

Porcine UBM, derived from decellularized, lyophilized urinary bladder basement membrane, stimulates progressive ECM organization *in vitro*.[11,12] The structural components of UBM are highly conserved across species[12] and decellularization further minimizes the antigenicity associated with the use of a xenogeneic product.[12] UBM is available off-the-shelf in two forms, without the need for tissue harvest or cell culture: (1) as a thin, translucent sheet for topical application (MatriStem™, ACell®, Columbia, Maryland); and (2) as a particulate form for injection into the injury site using a minimally invasively approach (MatriStem™ MicroMatrix, ACell®). Clinically, in people, UBM has been used successfully for urethral reconstruction,[19] rectopexy,[20] and to treat complex and non-healing wounds.[8,9,19,21] In veterinary medicine, UBM has been used for full-thickness corneal reconstruction in horses,[22] dogs and cats,[23] urinary incontinence in dogs,[10] and a variety of tendon and ligament injuries in horses.[24]

Despite the abundance of literature supporting the ability of ECM in general to improve tissue organization and healing of musculoskeletal tissues, there is a sizeable gap in the literature specifically related to the use of ECM products of any kind to treat tendon or ligament injuries. Anecdotal evidence suggests that particulate UBM therapy of tendon and ligament injuries is beneficial in horses.[24] Particulate tendon ECM improved tenogenic differentiation *in vitro*[16] and UBM sheets stimulated greater progenitor cell migration in an *in vivo* model of tendon healing.[14] Together, these reports support the use of the minimally invasive injection of particulate UBM as

an appealing biologic approach to treating tendon and ligament injuries. However, objective evidence for UBM to improve tendon or ligament healing is lacking. Therefore, the objective of this study was to evaluate the ability of UBM to induce matrix organization and tenogenesis using a novel hydrogel model *in vitro*. We hypothesized that addition of UBM to hydrogel constructs would improve cellular and matrix organization and markers of tenogenesis.

MATERIALS AND METHODS

Isolation of Equine Bone Marrow Cells

All procedures were approved by the Institutional Animal Care and Use Committee. Bone marrow cells were isolated by density gradient centrifugation from sternal bone marrow aspirates of 6 adult horses (3 females, 3 castrated males), aged 3-16 years. Horses were sedated and the sternum clipped, aseptically prepared, and locally anesthetized. Heparinized bone marrow aspirate was collected from the 4^{th} and 5^{th} sternebrae using an 11 gauge Jamshidi needle.[25] In a laminar flow hood, bone marrow aspirate was filtered, gently layered over density gradient medium (Ficoll-Paque™ Plus, GE Healthcare Life Sciences, Pittsburgh, PA), and centrifuged at 500 x g for 30 minutes at 4°C.[25] After supernatant removal, the mononuclear cells in Ficoll were aspirated, transferred to a sterile centrifuge tube, washed in Dulbecco's PBS (DPBS), centrifuged at 300 x g for 10 minutes at 4°C, the cell pellet resuspended in growth medium (Mesenchymal Stem Cell Medium with 5% fetal bovine serum [FBS]; ScienCell Research Laboratories, Carlsbad, CA) at 37°C, 5% CO_2, and 95% humidity, and passaged routinely using trypsin/EDTA prior to cells reaching 70% confluence. All reagents were purchased from Mediatech, Inc., Manassas, VA, unless otherwise noted. Bone marrow cells were cryopreserved at passage 2 (P2) in 90% FBS/10% DMSO and later thawed and expanded to

passage 5 (P5) in tendon growth medium (DMEM, 10% FBS, 300 µg/mL L-glutamine, 25 mM HEPES, 20 units/mL penicillin G, 20 µg/mL streptomycin sulfate, and 5 mg/mL ascorbic acid) for hydrogel seeding.

Hydrogel and UBM Preparation

Following euthanasia for reasons unrelated to this study, the tails of 3-month-old male Sprague-Dawley rats were collected, frozen at -20°C, and thawed in 70% ethanol for 15 minutes. Tendons were stripped from the tail, sterilized in 70% ethanol, digested with mixing in 0.1% acetic acid (100 mL/gram of tendon) at 4°C for 2 days. Tail tendon digests were centrifuged at 8,800 x g for 90 minutes at 4°C. The supernatant was lyophilized and stored at -20°C. Lyophilized tail tendon digest was reconstituted to 6 mg/mL in 1M acetic acid to make rat tail tendon ECM (rat tail ECM) for hydrogel formation. Prior to addition to hydrogels, UBM (MatriStem™ MicroMatrix, ACell®; particle size <500 µm) was hydrated in 0.1 % acetic acid (1.32 mg/mL) for 48 hours at 4°C and sonicated at 10 MHz for 10 seconds, repeated 3 times, to decrease the particle size. Following sonication, particle size was 10-100 µm as measured by stage micrometer.

Cellularized Hydrogel Constructs

Cylindrical hydrogels were cast using ethanol-sterilized, 3 mm internal diameter plastic drinking straws cut to 7 cm lengths. Straws were rinsed in DPBS, placed upright in polymerized 4% agarose to create a sealing plug at the end of the straw and enable hydrogel loading, and a hole was created 5 mm above the agarose plug using a 25 gauge hypodermic needle to allow air escape during filling. Visible air bubbles were released after filling. Bone marrow cells at P5

were trypsinized, resuspended at $7x10^5$ cells/mL in 10% v/v FBS followed by ice cold neutralized rat tail ECM (pH 7.4-7.6; consisting of 70% v/v rat tail tendon solution (6 mg/mL), 18% v/v buffered 5x DMEM (pH 9.0), and 2% v/v HEPES buffer). For hydrogels containing UBM, 10% v/v of rat tail ECM was replaced with 10% v/v of UBM solution. An aliquot of $1x10^6$ P5 bone marrow cells was used as the day 0 control for gene expression. Cylindrical hydrogels were created by removing a straw from the agarose and holding it at 45° with sterile forceps while the rat tail ECM/cell solution (500 µL) was pipetted into the straw. Straws were then replaced in agarose to polymerize at 37°C for 45 minutes in a vertical position (Fig. 1). Once polymerized, hydrogels were released into tissue culture dishes by removing the agarose plug from the bottom of the straw. Hydrogels were fixed under static tension using needles embedded in ethanol-sterilized rubber stoppers adhered to the bottom of 60 mm culture dishes with cyanoacrylate. Maximum tension that immobilized constructs without tearing was applied. Fresh, untensioned hydrogels were rinsed with DPBS and set aside for baseline viability staining. Hydrogel constructs were maintained in tendon growth medium for 7 or 21 days and duplicate constructs collected for viability, histology, biochemistry, and gene expression. The tensioned portion of each constructs was harvested by sharp transection at the base of the tethers.

Contraction of Hydrogel Constructs

Constructs (6 per treatment group, time point, and horse) were digitally photographed (iPhone 6S, Apple Inc., Cupertino, CA) at a fixed focal distance with a ruler for scale for measurement of the construct width in triplicate at the midpoint (Fig. 3.1; ImageJ, National Institutes of Health, Bethesda, MD). Mean width was used for statistical analysis. Construct

contraction at days 7 and 21 was normalized to the day 0 width (% of baseline width), using the change in width between time points for statistical analysis.

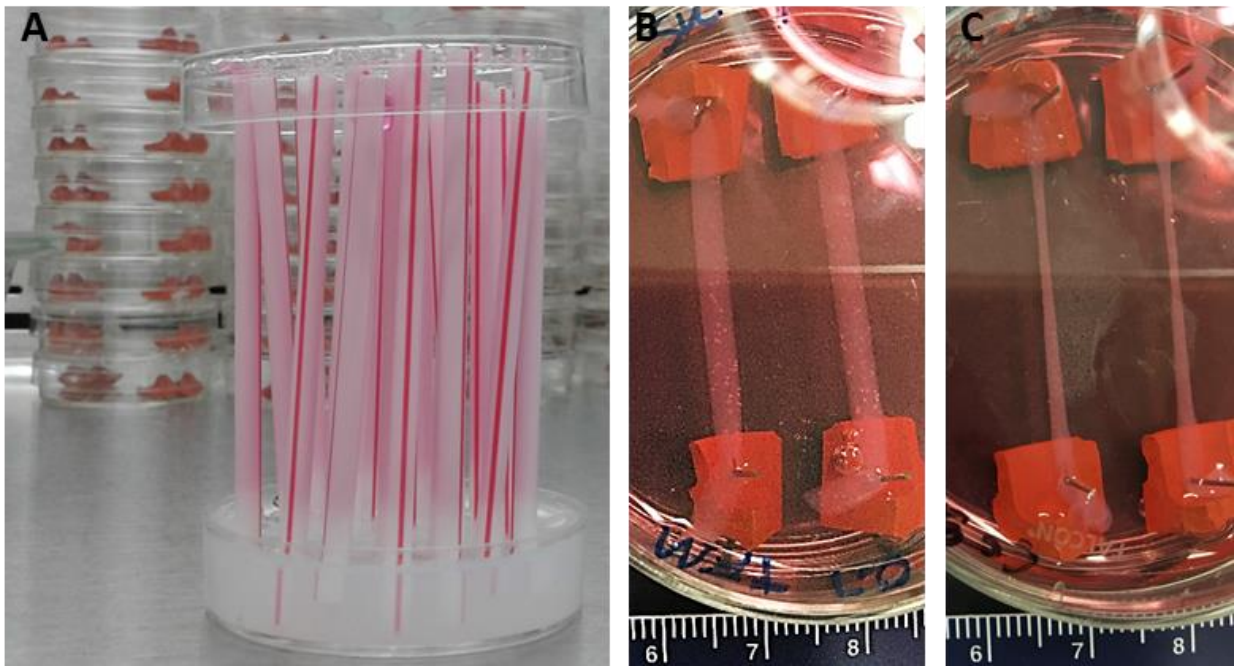

Figure 3.1 (A) Cylindrical hydrogels were created by casting cell-seeded rat tail extracellular matrix ± urinary bladder matrix in ethanol-sterilized straws standing vertically in sterile agarose, and allowed to polymerize at room temperature. (B) Following removal from the straws hydrogels were tensioned between rubber stoppers using needles and cultured in tendon growth medium for up to 21 days (C).

Phase Contrast Microscopy, Cell Viability, and Histology

Cell viability was determined in duplicate constructs at days 0, 7, and 21 using calcein AM-ethidium homodimer staining (Thermo-Fischer Scientific, Waltham, MA). Constructs were photographed using phase contrast microscopy (CKX41, Olympus Corporation, Center Valley,

PA) and standard imaging software (INFINITY CAPTURE©3, Lumen*era* Corporation, Ottawa, Ontario, Canada) to assess cell shape, orientation, and matrix organization prior to viability staining. Constructs were then rinsed with DPBS *in situ* and stained with calcein AM and ethidium homodimer at room temperature for 20 minutes in the dark. Stained constructs were protected from light, immediately viewed under fluorescence microscopy (CKX41, Olympus Corporation), and photographed using commercial software (INFINITY CAPTURE©1, Lumen*era* Corporation).

At days 7 and 21, duplicate constructs were fixed *in situ* in 10% neutral buffered formalin for 24 hours at 4°C, rinsed with DPBS, processed, paraffin embedded, and sectioned at 10 μm. Sections were stained with hematoxylin and eosin (H&E) for cell morphology and orientation, and extracellular matrix organization; 4′,6-diamidino-2-phenylindole (DAPI) for quantification of nuclear aspect ratio; and picrosirius red for collagen orientation and cross-linking. H&E-stained sections were imaged using light microscopy and digital photography (Eclipse C*i* Microscope, DS-Fi1 Camera with DS-U3 Controller, and NIS Elements Imaging Software version 3.22.00, Nikon Instruments, Inc., Melville, NY). DAPI-stained sections were photographed using fluorescence microscopy as described for viability staining and nuclear aspect ratio was calculated (CellProfiler™, Broad Institute, Cambridge, MA). Polarized light microscopy was used to image picrosirius red-stained sections (LMC-2000, Laxco, Inc., Bothell, WA; 18.0MP USB3.0 Digital Camera, OMAX microscope, Gyeonggi-do, Korea).

Biochemical Analysis

Duplicate constructs harvested at days 7 and 21 were lyophilized, digested in 0.5 mg/mL papain for 4 hours at 65°C, and glycosaminoglycan (GAG) content quantified by the

dimethylmethylene blue assay, with the dye titrated to pH 1.5.[26] Samples were digested for an additional 16 hours at 65°C for DNA quantification using the bisbenzamide fluorometric assay.[26] GAG content was analyzed with and without normalization to DNA content. Values are reported per construct.

Gene Expression

Total RNA was isolated from day 0 bone marrow cells and duplicate constructs by guanidinium isothiocyanate-phenol-chloroform extraction (TRIzol®, Thermo Fisher Scientific, Waltham, MA) and column purification (Direct-zol™ RNA Miniprep Kit, Zymo Research, Irvine, CA), quantified by spectrophotometry (NanoDrop, NanoDrop Products, Wilmington, DE), and reverse transcribed to cDNA (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA).[26,27] Complementary DNA was amplified by fluorescent real time PCR (StepOne Plus™, Applied Biosystems). Primers and TaqMan® probes for tendon-related genes were designed using equine specific sequences (Primer Express v 3.0, Applied Biosystems; Table 3.1) or commercial primer-probe sets (Scleraxis, assay #Ec03818452_s1; tenomodulin, assay #Ec03467883, Applied Biosystems).[26] Relative gene expression was calculated using the comparative threshold cycle method (ΔΔCt) with GAPDH expression as the endogenous control, and normalized to day 0 expression levels.[26,27]

Statistical Analysis

Normal probability plots were used to assess whether data followed a normal distribution. Data were summarized as mean ± SD when normally distributed or median [range] when skewed. Skewed outcomes (nuclear aspect ratio and gene expression for collagen type I,

Table 3.1 Equine-specific primer/probe sequences used for gene expression analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
Collagen Type I	GCCAAGAAGAAGGCCAAGAAGAA	TGAGGCCGTCCTGTATGC	ACATCCCAGCAGTCACCT
Collagen Type III	CTGCTTCATCCCACTCTTATTCTG	ATCCGCATAGGACTGACCAAGAT	AACAGGAAGTTGCTGAAGG
COMP	GAGATCGTGCAAACAATGAACAG	GACCGTATTCACGTGGAACGT	CTGGCTGTGGGTTACA
Decorin	AAGTTGATGCAGCTAGCCTGAGA	GGCCAGAGAGCCATTGTCAGAA	ATTTGGCTAAATTGGGACTG
MMP-13	AAGCCACTTTGTGCTTCCTGAT	GGATCGCATTTGTCTGGTGTT	TCTCTATGGTCCAGGAGAT
Mohawk homeobox	TGAATTTGAGGAAGAATTGGTGTCT	TTCCAGAGTGTCTGTGCGATAGA	CCTCGTCATCAGAAAC
GAPDH	CAAGTTCCATGGCACAGTCAAG	GGCCTTTCCGTTGATGACAA	CCGAGCACGGGAAG

COMP, cartilage oligomeric matrix protein; MMP-13, matrix metalloproteinase-13; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

cartilage oligomeric matrix protein (COMP), matrix metalloproteinase (MMP)-13, and Mohawk homeobox) were log transformed (base *e*) before assessing the treatment effects. Effect of treatment, time, and the interaction between time and treatment on each outcome were assessed using mixed model ANOVA (SAS version 9.4, SAS Institute Inc, Cary, NC). The linear model specified horse identification as the random effect. The interaction between time and treatment was further analyzed (sliced) to compare treatments at each time point and to compare time points within each treatment. Where appropriate, *p*-values were adjusted for multiple comparisons using Tukey's procedure. A value of n=6 horses was used for all analyses. Significance was set at $p \leq 0.05$.

RESULTS

Contraction of Hydrogel Constructs

Constructs contracted significantly over time ($p < 0.001$), regardless of treatment group (Fig. 3.2). Contraction of constructs varied widely between horses, as well as within a single horse and treatment group at a given time point. At day 7, UBM constructs had contracted significantly from baseline ($p = 0.039$), whereas control constructs had not ($p = 0.077$). Both control and UBM constructs contracted significantly compared to day 0 by day 21 ($p < 0.001$ for both). There were no significant differences between treatment groups at any time point. Overall, constructs that did show progressive contraction were narrowest at the center and subjectively appeared taut on their tethers based on decreased motion when transported.

Phase Contrast Microscopy, Cell Viability, and Histology

Day 0 phase contrast and fluorescence microscopy revealed circular bone marrow cells with high viability, evenly distributed throughout amorphous and homogeneous hydrogel constructs (Fig. 3). Between days 0 and 7, and 7 and 21, constructs in both groups maintained good cell viability and showed progressive cell elongation and organization along lines of tension. By 21 days, constructs were substantially narrower with more densely packed and longitudinally oriented matrix (Fig. 3.3). The amount of matrix compaction and organization varied between constructs cast with cells from the same horse and between constructs cast with cells from different horses. No differences were noted between treatment groups at any time points.

Nuclear aspect ratio did not change significantly over time ($p = 0.146$) or between treatment groups ($p = 0.402$). On day 21, the nuclear aspect ratio was significantly greater compared to day 7 in the control group ($p = 0.027$), but not in the UBM-treated group ($p = 0.784$). At day 21, the nuclear aspect ratio in the control group was higher than in the UBM-treated group; however, this difference failed to reach statistical significance ($p = 0.067$; Fig. 3.4). Day 7 constructs stained with H&E showed amorphous eosinophilic background staining with sparsely distributed cells that varied in shape from elongated to more stellate (Fig. 3.5). Regardless of cell shape, there was a consensus of cell orientation along lines of tension, consistent with the mean nuclear aspect ratio of ~1.50 (cells were longer than they were wide).

Although constructs were not collected for nuclear staining on day 0, the round cell shape seen on phase contrast is consistent with a nuclear aspect ratio of ~1.0. At day 7, there was frequent evidence in both treatment groups of cell migration to the periphery of the constructs,

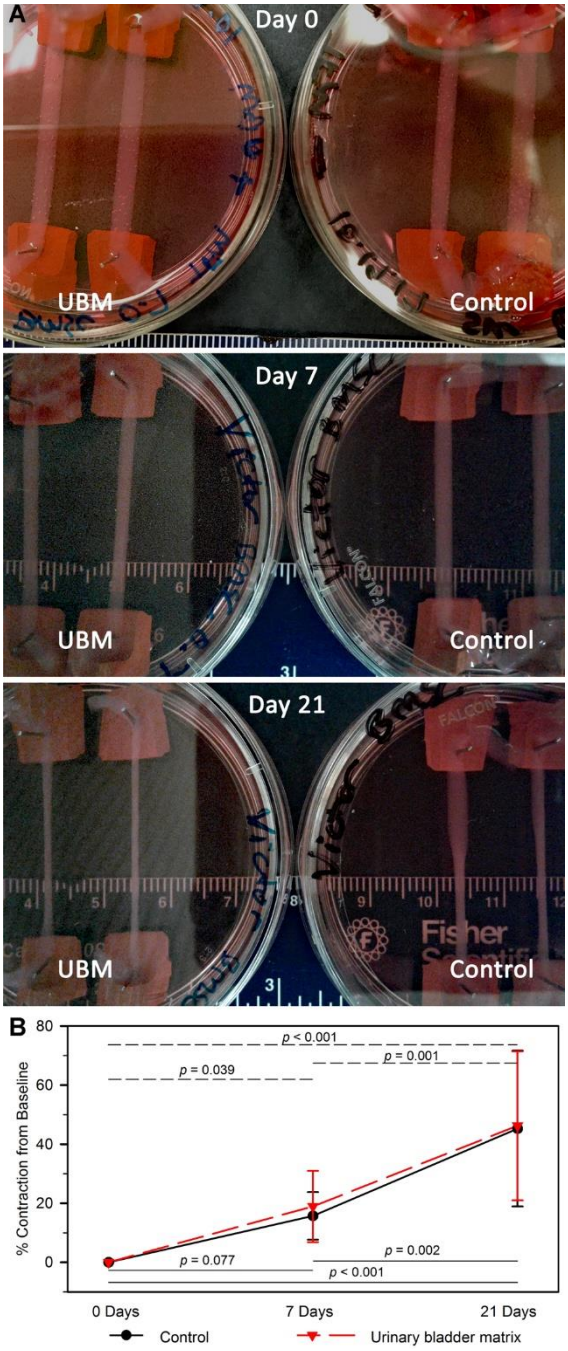

Figure 3.2 (A) Representative images of urinary bladder matrix (UBM; left) and control (right) cell-seeded hydrogel constructs after 0, 7, and 21 days showing reduced construct width (contraction) over time, as well as the variability between constructs of the same horse (Day 21). (B) Construct contraction over time.

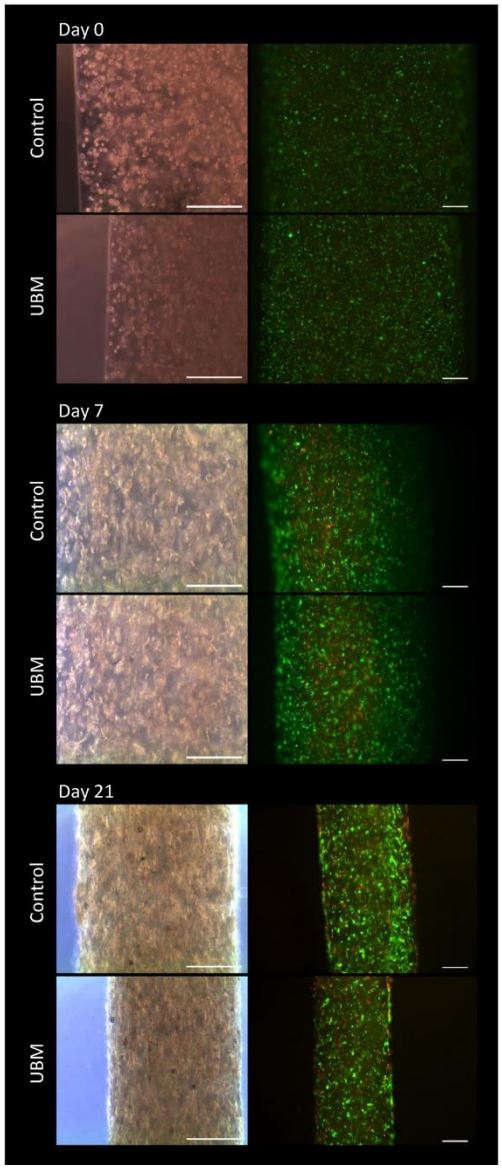

Figure 3.3 Representative phase contrast (left column) and calcein AM (green)-ethidium homodimer (red) images (right column) of cell-seeded rat tail hydrogel constructs ± urinary bladder matrix (UBM) from one horse at 0, 7, and 21 days showing progression of cell and matrix organization over time. No clear differences were noted between control and UBM-treated constructs. From Day 0 to 7, cell morphology and orientation changed from round and randomly oriented to elongated and oriented along lines of tension. Constructs became progressively narrower over time, with increased matrix organization seen in day 7 and 21 images. Images were taken at the middle of each construct. Scale bars = 400 μm.

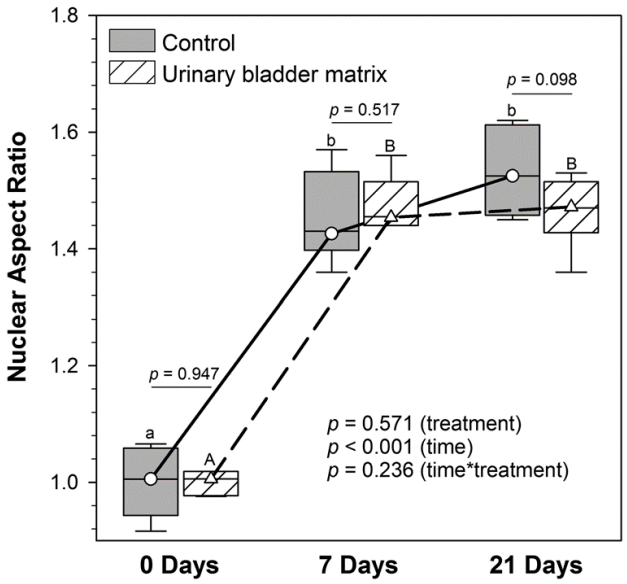

Figure 3.4 Nuclear aspect ratio of bone marrow cells seeded in rat tail hydrogel constructs ± urinary bladder matrix at days 0, 7, and 21. On day 0, cells were round, with a nuclear aspect ratio of 1.0. The horizontal line in each box indicates the median; boxes represent the 25th and 75th percentiles; whiskers represent the 10th and 90th percentiles. Line plots show relationship between median values over time within each treatment. Superscript letters indicate statistically significant differences over time within the same group (lower case for control and upper case for UBM-treated; $p \leq 0.05$). *P*-values for comparisons between treatment groups at individual time points are shown above each time point.

resulting in a series of several elongated cells (Fig. 3.5). No differences in matrix organization or cell morphology were noted between control and UBM-treated constructs. Between days 7 and 21, control and UBM-treated constructs were visibly contracted, with increased eosinophilia and longitudinal matrix organization (Fig. 3.5). Areas of increased matrix density were evident immediately surrounding many of the cells and were subjectively more intensely eosinophilic

(more prominent) in UBM-treated constructs. The majority of cells remained elongated with variable numbers of visible cytoplasmic extensions into the surrounding matrix in both groups. Subjectively, there were more frequent cytoplasmic extensions into the adjacent matrix observed in UBM-treated constructs. Additionally, at day 21, there was increasing organization of cells along the edges of the constructs in both groups (Fig. 3.5). Overall, minimal evidence of collagen crosslinking was observed on picrosirius red-stained sections under polarized light in either group. Birefringence varied between horses and was most evident along the edges of constructs at both 7 and 21 days in both treatment groups (Fig. 3.6).

Biochemical Analysis

There were no significant differences in DNA or GAG content (with or without normalization to DNA) over time or between treatments (Table 3.2).

Gene Expression

There were no significant effects of time or treatment on expression of collagen types I or III (Fig. 3.7). Decorin and MMP-13 expression increased significantly over time ($p < 0.001$ for both); however, there was no effect of treatment (Fig. 3.7). Expression of COMP decreased significantly from day 0 to days 7 and 21 ($p < 0.001$), but was not significantly affected by UBM treatment (Fig. 3.7). Tenomodulin was not expressed in either treatment group at any time point. Expression for Scleraxis increased over time in both control and UBM-treated constructs ($p = 0.016$), primarily in the first 7 days. There was no difference in Scleraxis expression between control and UBM-treated constructs ($p = 0.793$; Fig. 3.7). Mohawk was expressed consistently over time ($p = 0.953$) without difference between treatments ($p = 0.837$; Fig. 3.7).

Table 3.2 DNA and glycosaminoglycan (GAG) content of cell-seeded extracellular matrix hydrogel constructs ± urinary bladder matrix (UBM). Both total GAG content (μg/mL) and GAG content normalized by DNA content of the same construct (GAG/DNA) are reported. Mean ± SD.

	DNA (μg/mL)		**GAG (μg/mL)**		**GAG/DNA**	
	Control	**UBM**	**Control**	**UBM**	**Control**	**UBM**
Day 7	109.5 ± 50.0	129.3 ± 50.0	84.5 ± 44.8	106.9 ± 45.9	0.75 ± 0.14	0.81 ± 0.13
Day 21	127.4 ± 48.7	117.9 ± 68.9	98.1 ± 19.7	98.4 ± 36.6	0.86 ± 0.32	0.92 ± 0.24
	p = 0.776 (treatment) p = 0.860 (time) p = 0.426 (time*treatment)		p = 0.419 (treatment) p = 0.853 (time) p = 0.431 (time*treatment)		p = 0.522 (treatment) p = 0.232 (time) p = 0.970 (time*treatment)	

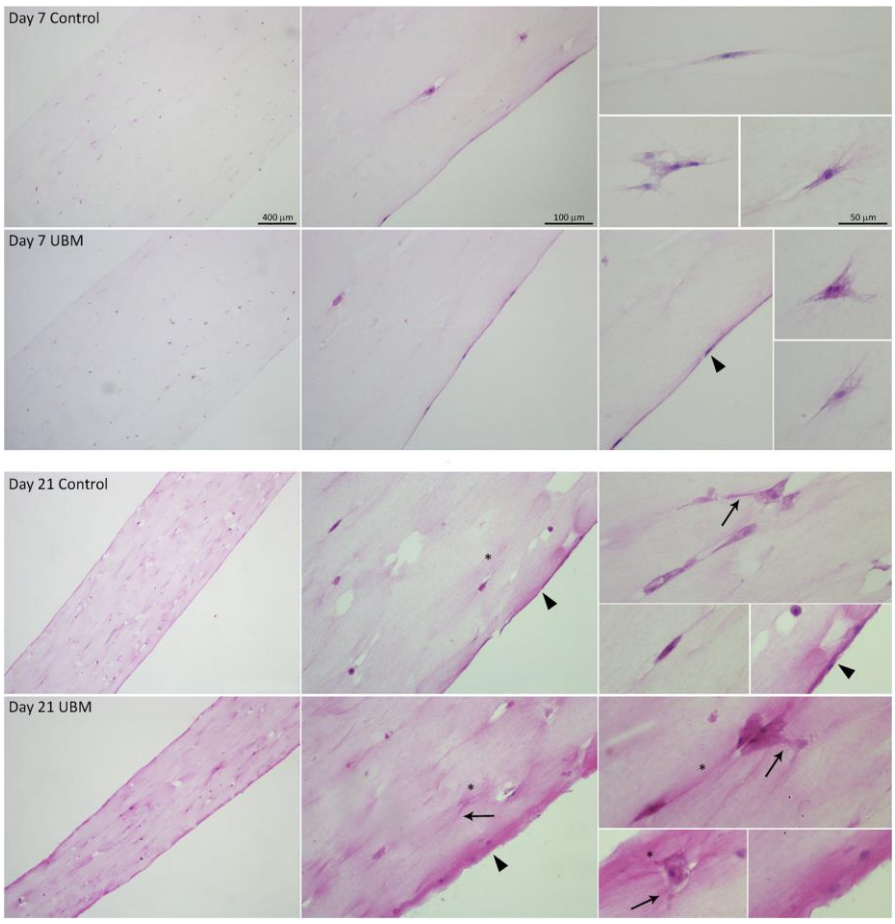

Figure 3.5 Representative H & E-stained sections of cell-seeded rat tail hydrogel constructs at 7 and 21 days at increasing magnification (see scale bars). Constructs progressed from being wide with lightly stained, amorphous matrix on day 7 to narrower with more densely eosinophilic and longitudinally organized matrix on day 21. Cells became increasingly oriented along lines of tension and more uniformly elongated over time. Matrix density was increased immediately surrounding cells (*), and a proportion of cells exhibited multiple cytoplasmic extensions projecting toward the matrix (black arrows). Both of these observations were subjectively more prominently in UBM-treated constructs compared to controls. Progressive cell migration and linear organization along the periphery of constructs (arrowheads) was observed in both treatment groups from day 7 to 21. There were no differences noted between control and UBM-treated constructs.

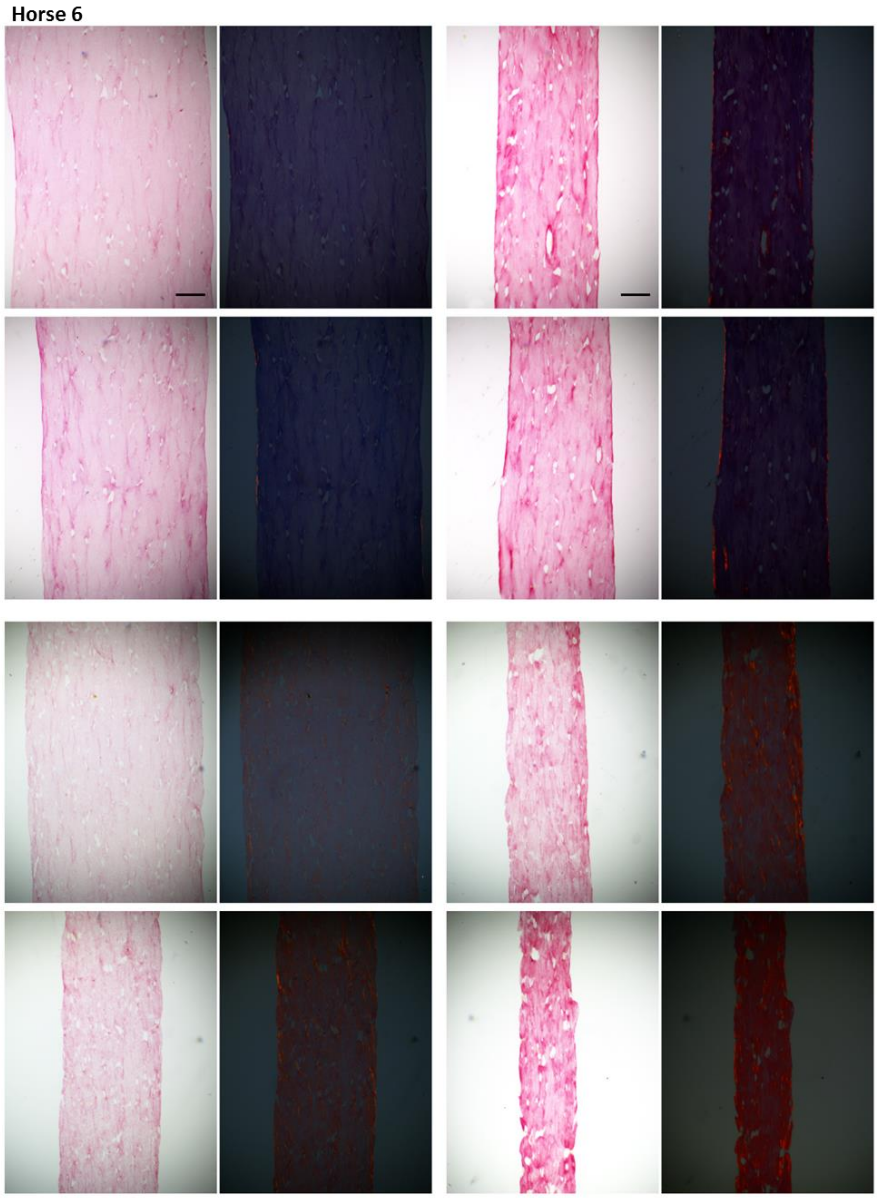

Figure 3.6 Representative images picrosirius red-stained cell-seeded rat tail hydrogel constructs ± urinary bladder matrix (UBM) from two horses at 7 and 21 days (direct illumination, left; polarized, right). Birefringence varied between horses and was most evident along the edges of constructs. Scale bars = 200 μm.

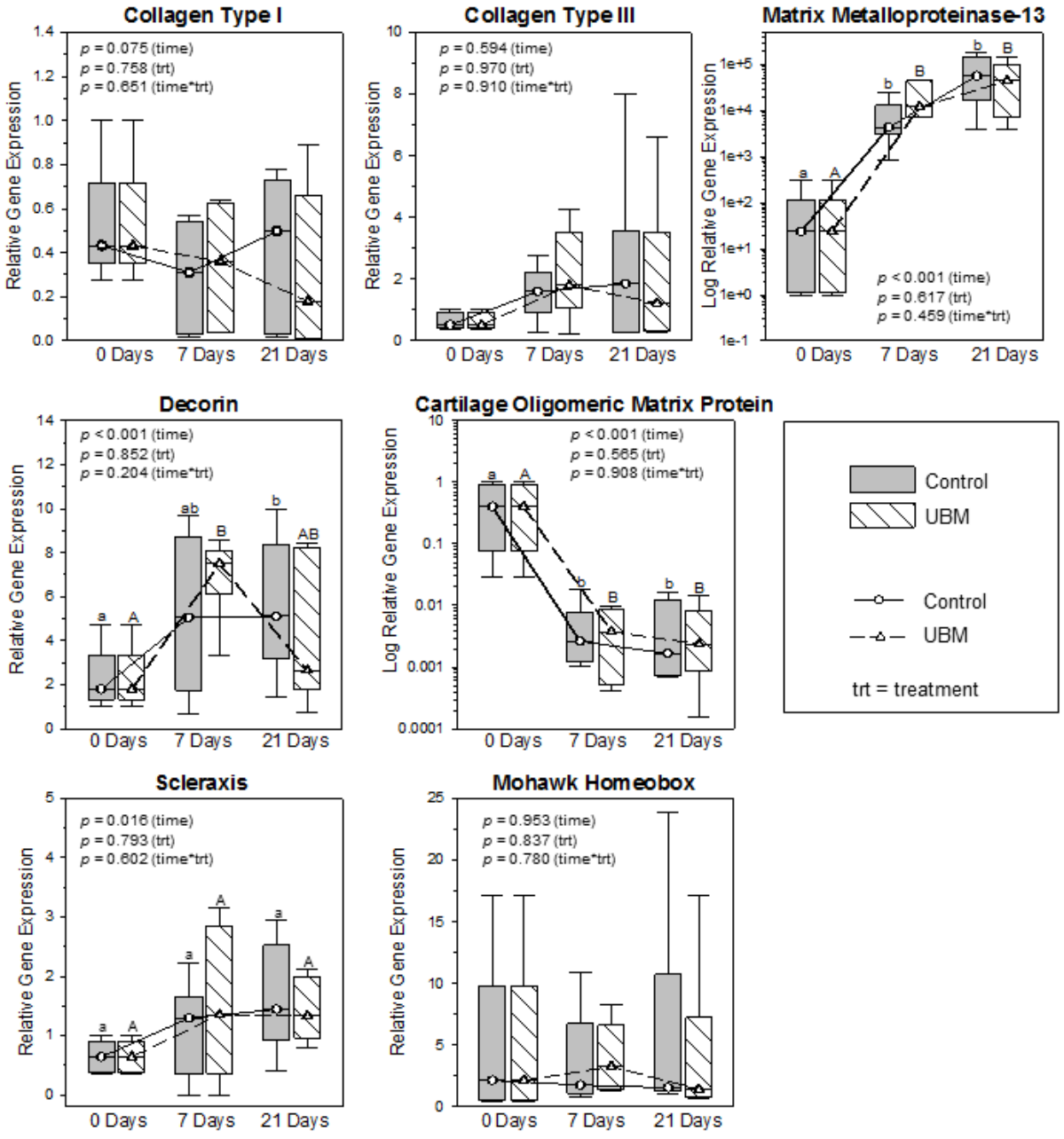

Figure 3.7 Relative gene expression over time for bone marrow cells seeded in rat tail tendon extracellular matrix hydrogels ± urinary bladder matrix (UBM). The horizontal line in each box indicates the median; boxes represent the 25th and 75th percentiles; whiskers represent the 10th and 90th percentiles. Line plots show relationship between median values over time within each treatment. Superscript letters indicate statistically significant differences within the same group (lower case for control and upper case for UBM-treated; $p \leq 0.05$).

DISCUSSION

In our novel model of cellularized rat tail tendon hydrogel constructs maintained under static tension, we observed changes in cell morphology, ECM organization, and gene expression over time. Cells elongated and aligned progressively, and the ECM contracted and organized along lines of tension in both control and UBM-treated constructs. Scleraxis and Mohawk, regulatory genes important in tendon development and structural maintenance, were either increased (Scleraxis) or unchanged (Mohawk) over time in both groups. Tendon-related ECM genes were unchanged (collagen types I and III) or increased with time (decorin) except for COMP, which decreased. Based on these comprehensive findings, our static tension model was effective in stimulating matrix organization and early tenogenic differentiation. However, in our model, the addition of UBM to rat tail tendon hydrogel constructs did not induce changes beyond those appreciated in control constructs, suggesting that rat tail ECM alone supports tenogenesis in equine bone marrow cells.

Determination of tenogenesis can be challenging, based on the lack of strictly tendon-specific genes. A panel of tendon-related genes, similar to those used in other studies,[16,28-30] and that could be interpreted collectively, was selected to assess tenogenic differentiation in our study. Scleraxis and Mohawk homeobox are transcription factors expressed during tendon development and in response to physiologic load in adult tendon,[26,30-34] and that regulate expression of genes encoding tendon ECM proteins.[34-36] In our 21-day model of static strain, Scleraxis expression significantly increased over time. This finding differs from a previous *in vitro* hydrogel study in which scleraxis expression decreased under static tension in collagen hydrogels seeded with human bone marrow MSC.[28] Another *in vitro* hydrogel study using a

MSC cell line (C3H10T1/2) in collagen hydrogels reported similar results to ours, where Scleraxis increased from 0 to 6 days under static load, but remained constant between 14 and 21 days.[37] In both studies, Scleraxis expression increased with addition of cyclic strain.[28,37] The observed lack of changes in collagen types I and III expression is consistent with one previous report,[37] and different from another that reported increased expression under static strain.[28] Increased Scleraxis expression, sustained expression of Mohawk and collagen types I and III, and increased decorin expression in both groups are supportive of a developing tenogenic phenotype.[16,38] Decreased COMP expression may be the result of inadequate mechanical load in our static-tensioned model.[38] COMP expression was increased on stiffer collagen gels.[39] The substantial and progressive increase in MMP-13 expression in both groups corresponds with active matrix remodeling, evidenced by construct contraction and histological changes, and is consistent with the literature.[16,40,41] In addition to gene expression changes over time, we observed histological changes consistent with tenogenic differentiation, including cell elongation and matrix organization along lines of tension, and cell migration and linear organization around the periphery of the hydrogel constructs.[16,28,29]

The *in vitro* hydrogel model used in this study has several unique features, including use of acid-digested rat tail tendon for hydrogels and creation of large cylindrical hydrogels. *In vitro* models commonly use purified bovine type I collagen for hydrogels.[16,28-30] Although type I collagen accounts for the majority of tendon dry weight, other matrix molecules (e.g., other collagen types, glycoproteins, proteoglycans, and growth factors) are critically important in the function of tendon and in providing the appropriate niche for progenitor cell differentiation.[12,42,43] Complementary components in ECM hydrogels provide a more natural niche for embedded cells than purified collagen when the goal is tenogenesis. Commercial

collagen is frequently purified by enzymatic digestion in pepsin, and therefore lacks fiber alignment and self-assembly because pepsin digestion cleaves the non-helical telopeptides, leaving only the collagen triple helix.[12,44] Rat tail tendons in our study were digested in acetic acid to preserve the carboxy- and amino- terminal propeptides and better support collagen fiber assembly, cell-matrix attachments, and cell migration.[44] ECM extracted from various sources understandably varies in its composition and organization, with the ECM derived from tendon being highly aligned compared to other sources.[12] The tendon-derived extract used in our model was therefore selected to provide a native, physiological complex of aligned collagenous and non-collagenous proteins for supporting tenogenesis. Increased Scleraxis expression despite only static tension in our model could be a function of the use of a native tendon ECM hydrogel in place of collagen.

The rat tail tendon ECM used in our study supported excellent cell viability and progression toward a tenogenic phenotype. However, the hypothesized differences between control and UBM-treated constructs were not observed. Two possible explanations exist for the lack of improved matrix organization and tenogenesis in the UBM-treated group. Assuming cell response and matrix remodeling would be proportionate to the source of ECM, the effect of the 10% (v/v) UBM may not have been substantial enough to detect. Had we used purified collagen as the base for hydrogels and then added the same 10% v/v UBM, the relative effect of the changes due to UBM may have been relatively larger and therefore detectable, as previously reported.[16] In other words, the rat tail tendon ECM provided the necessary niche for supporting tenogenesis, making the additional UBM redundant.

Inclusion of a single cell type in our model provides a second possible explanation for the lack of a UBM-specific effect. Bioactive breakdown products of ECM are chemotactic for

progenitor cells, initiate angiogenesis, and stimulate cell division.[13,15] In addition, growth factors are liberated as the ECM is degraded. *In vivo*, the degradation process occurs rapidly and ECM is replaced with native neotissue within ~60 days.[45] ECM degradation and remodeling *in vivo* is primarily a result of mononuclear cell infiltration.[12,45] Modulation of infiltrating mononuclear cells to the regulatory, M2 macrophage phenotype is one of the most important functions of ECM, including UBM, in orchestrating effective tissue remodeling.[46] *In vitro*, it is challenging to reproduce the complexity of the host response to ECM. We chose bone marrow progenitor cells for our studies because they are mechanoresponsive,[47,48] capable of tenogenesis,[28,38] readily available, contribute to tendon healing *in vivo*, and have promising therapeutic potential.[49] Inclusion of mononuclear progenitor cells, such as those isolated from bone marrow aspirate, along with mesenchymal progenitors, would better mimic the *in vivo* environment and may have resulted in the UBM breakdown required to induce superior matrix organization and tenogenesis.

We used a unique method to prepare hydrogels in an effort to produce large cylindrical constructs and avoid the need for expensive equipment. Previously reported methods for creating *in vitro* tenogenesis models include a vacuum-based tissue culture system capable of uniaxial static or cyclic strain.[16,28-30] Other models seed cells on decellularized tendon scaffold or electrospun synthetic meshes that are then connected to a bioreactor to generate cyclic strain.[38,49] These models, while advantageous in many ways, require acquisition, maintenance, and a working knowledge of specialized equipment and are prone to their own imperfections. The small construct size generated by the vacuum-based system can require pooling of numerous constructs for gene expression analysis and can be challenging to section for histology. Our model is relatively simple and cost-effective to set up, and generated consistent, large constructs, increasing RNA yields for gene expression and providing constructs that were relatively easy to

section for histology. Based on the good cell viability throughout our constructs, diffusion of medium was not a problem.

Finally, an important factor to consider in interpreting our results is the use of static rather than cyclic strain. As expected, cellular remodeling of constructs did increase construct tension over time, consistent with other static models.[16,28,29] Cyclic strain may be required to generate improved tenogenesis and more substantial matrix remodeling *in vitro*.[28,30] In fact, relatively high strain levels may be required for tenogenesis.[37] An objective means of standardizing day 0 tension across all constructs in our model would reduce variability between individual constructs; however, variability between individual horses would remain high. Variability in response between cells from individual horses is a common challenge and is consistent amongst species with higher genetic variation than that found in inbred strains of rats and mice or cell lines.

Using our model, both control and UBM-treated constructs demonstrated gross and histologic evidence of matrix organization over time, as well as gene expression changes consistent with development of a tenogenic phenotype. Lack of fundamental differences between control and UBM-treated constructs may have been due to the use of tail tendon ECM rather than collagen alone as the basis for our hydrogels and/or the use of static tension rather than cyclic strain. Future studies incorporating a combination of cell types, including MSC and macrophage progenitors, would better model the *in vivo* breakdown of UBM and potentially substantiate its ability to improve matrix organization and tenogenesis.

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Chapter 4: Final Comments

This thesis investigated the ability of UBM to induce matrix organization and tenogenesis in a novel rat tail ECM hydrogel model seeded with equine BMSC. We specifically assessed tenogenesis by evaluating histologic organization, matrix contraction, GAG synthesis, cellular viability and density, and expression of tendon-related genes over time. The greater intent was to understand the mechanisms by which UBM may improve healing of tendon injuries in a controlled, *in vitro* environment.

We used rat-tail tendon ECM in our hydrogels to mimic the *in vivo* tendon environment more closely than purified type I collagen alone. We used equine BMSC in our model because they are readily available, mechanoresponsive, capable of tenogenic differentiation, and contribute to tendon healing in the horse. Finally, our static strain model generated large hydrogels to facilitate measurement of gene expression and sectioning for histology and did not require expensive equipment to construct. Our model successfully modeled tenogenesis. Most tendon-associated genes had sustained or increased expression, cell viability was maintained, and cells acquired a tenocyte-like morphology and progressively organized the ECM over time. However, the advantageous features of this model may have contributed to the lack of effect of UBM treatment on our parameters. The relative contribution of UBM to the entire hydrogel volume was small, such that any effect of UBM on promoting tenogenesis was overwhelmed by the effect of a larger volume of rat tail tendon ECM. Mononuclear cells infiltrate and degrade UBM *in vivo*, releasing factors that stimulate local progenitor cell chemotaxis, proliferation, differentiation, and angiogenesis. UBM also encourages macrophage polarization toward an anti-inflammatory phenotype. Inclusion of bone marrow mononuclear cells, which contain

mononuclear and mesenchymal progenitors, or co-culture of BMSC and macrophage progenitors in our model may more accurately reflect the native *in vivo* response to UBM. Application of standardized, cyclic strain to our model may have increased expression of tendon-associated genes and matrix organization over time or with UBM treatment. Finally, BMSC from individual horses in this study varied widely in their ability to undergo tenogenic differentiation. The differences in behavior of these cells did not seem to be related to horse age at the time of cell collection. High variation between individual horses, combined with a small sample size, may have masked any effect that UBM had on tenogenesis.

Our results do not support the use of UBM to treat tendon injuries. Given the limitations of our model and *in vivo* evidence supporting treatment of injuries in equine tendon and other tissues with UBM, further *in vitro* investigation is necessary to determine how UBM may guide tissue healing. The ability of rat tail tendon ECM to support tenogenesis in our model is promising and emphasizes that ECM in general encourages formation of physiologically appropriate tissue and can be a valuable tool in tendinopathy treatment.