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Strategies for Improving Tenogenesis Directed by Equine Tendon Proper and Peritenon Cells

By

MONICA PECHANEC VOSS
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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of the

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ABSTRACT

Tendinopathies in equine athletes pose as a major musculoskeletal challenge due to the slow and incomplete repair process in tendons. Ongoing exploration into novel therapeutics to aid tendon repair into its pre-injury capacity spur doctors and researchers in multiple disciplines and across various species. Tendinopathies amount to one of the most common musculoskeletal injuries costing owners and breeders time and resources with no guarantee of return competition. As such, elucidation into the effects of current and novel therapeutics on tendon *in vitro*, in addition to, understanding the native tendon profile across age is crucial for improving tendon repair therapeutics.

Chapter one reviews the impact of tendinopathies, the molecular and cellular challenges causing incomplete tendon repair, and key components affecting tendon repair. Additionally, this chapter also covers the structure, assembly, maturation, and regulation of collagen in tendon. Regional cellular differences also exist within the tendon; the intrinsic (tendon proper; TP) and extrinsic (peritenon; PERI) cells play differing roles in tendon development and repair causing the response to injury must be assessed separately. Finally, aging also impacts the tendon repair outcome through changes in gene expression and epigenetics which ultimately may affect effective therapeutics for successful return to competition.

Chapter two investigates the *in vitro* effect of supplementation of biglycan and decorin, small leucine-rich repeat proteoglycans (SLRPs) that play critical roles in tendon development, growth, and maturation. Equine tendon proper and peritenon cell from the superficial digital flexor

tendon were seeded in three-dimensional constructs from five horses and supplemented with 5 nM or 25 nM of bovine biglycan or bovine decorin. Functionality and ultrastructural morphology using biomechanics, collagen content analysis, transmission electron microscopy (TEM), and gene expression assessed the effect of supplementation. The peritenon supplemented cells, compared to the tendon proper cells, produced constructs with better mechanical (or material) properties, in addition to, a tenogenic-like phenotype with decorin supplementation (5 nM decorin: increased *BGN* and *SCX* expression, ultimate tensile strength, Young's modulus, decreased *CSPG4* expression; 25 nM decorin: increased *BGN*, *COL1A1*, *FMOD*, and *SCX* expression, and increased collagen content, decreased *CSPG4* expression). Biglycan supplementation also produced positive results with trending improvements to biomechanics and increased gene expression of tenogenic and extracellular markers (*BGN, DCN, SCX* for 5 nM biglycan; *BGN, SCX, CSPG4* for 25 nM biglycan) but overall were not as effective as the decorin supplementation.

Chapter three explores the transcriptome and methylome of the superficial digital flexor tendon to assess the regional differences between the tendon proper and peritenon in adolescent (0-5 yrs), midlife (6-14 yrs), and geriatric (15-27 yrs) horses using RNA sequencing and DNA methylation techniques. RNA from 9 non-breed or sex specific horses were sequenced before RNASeq analysis to identify differentially expressed genes, gene ontology, and pathway analysis. DNA from 10 horses were sequenced by reduced representation bisulfate sequencing before methylation analysis for methyl calls, CpG island identification, and genomic annotation to identify differentially methylated regions from adolescent to geriatric horses. Across age, regional differences between the native tendon proper and peritenon cell populations persist in the transcriptome and methylome. Additionally, increased transcriptional activity is present in the

adolescent and geriatric groups most likely due to ongoing growth and maturation in the adolescent group with subsequent degradation of the tendon in the geriatric population.

Chapter four aims to understand the *in vitro* effect of co-culturing adipose-derived mesenchymal stem cells (ADMSCs), a common equine tendinopathy therapeutic, with tendon proper and peritenon cells. Regional differences between the intrinsic (tendon proper) and extrinsic (peritenon) cells exist and identifying the influence of ADMSCs on the two tendon cell populations is vital for gaining insight into their effect as therapeutic. Tendon proper and peritenon cells from 5 non-breed or sex specific horses were co-cultured with adipose-derived mesenchymal stem cells over 120 hrs and assessed by expression of tenogenic, perivascular, and extracellular assembly markers in addition to assaying cellular proliferation. ADMSCs co-cultured with either peritenon or tendon proper cells stimulates increased expression of a tenogenic phenotype by *LOX* and *SCX* and decreased *CSPG4* in the two tendon cell populations with further tenogenic specificity in the peritenon cells with increased *MKX* expression.

Lastly, chapter five covers the effect of decorin supplementation in tendon proper and peritenon cells in two-dimension, the challenges of adipose-derived mesenchymal stem cell transfection with the small leucine-rich repeat proteoglycan decorin, and the future trajectory of enhanced cell therapeutics in tackling tendinopathies.

Keywords: Tendon, SLRP, Equine, RNASeq, DNA methylation, ADMSC

Chapter One: Literature Review of Tendinopathies, Tendon Development and Organization, and Collagen in Repair and Aging

I.   **Impact of Tendinopathies**

Across numerous disciplines and species, tendinopathies in recreational and elite athletes represent an ongoing challenge due to inadequate therapeutics as a result of the poor healing capacity of tendons [1,2].   Specifically, musculoskeletal injuries in racehorses are the primary cause of death at about 2/1000 starts in Thoroughbreds and Quarter Horses and a majority of these injuries occur in the forelimb (80-90%) [3-9]. Catastrophic failure may not occur in all cases; factors such as field size, days since last race, and impact surface were identified as some of the race-start characteristics associated with catastrophic musculoskeletal injuries. Equine athletes of all disciplines are not exempt from these tendinopathies, so across breeds, there is a need for novel therapeutics.

Tendon repair is often slow and incomplete thereby resulting in increased reinjury rates and subsequent career-ending injuries [1,10,11]. The tendon healing process involves steps such as inflammation, proliferation, and remodeling; all of which take considerable time, partially due to the composition of the matrix and the hypocellular environment resulting in tendon that is still compromised relative to its pre-injury condition [12]. Numerous therapeutics have attempted to target increasing the healing capacity in tendons, but no treatment has been widely successful therefore resulting in ongoing clinical challenges [13]. Some gene therapies, such as bone morphogenic protein (BMP)-12 and platelet-derived growth factor (PDGF)-B, have succeeded in bolstering the extrinsic inflammatory phase of healing but not intrinsic tenocyte repair, thus leading to continued challenges. The immediate inflammatory response provides vascularity and

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scaffolding, in addition to attracting necessary cytokines and scavengers to clear the site [14]. This response only persists for a few days as the changes stimulate the proliferative phase to activate cells in the paratenon and synovial sheath to the injury site [15]. Subsequently, intrinsic tenocytes are also recruited to the site, though delayed, and both cell populations secrete increased levels of collagen III, extracellular matrix proteins, and glycosaminoglycans [16,17]. The neotendonous material remains until the remodeling phase in which increased levels of collagen I are produced. Collagen alignment in the direction of stress begins, and then subsequent decellularity occurs as the site is considered repaired, though not to pre-injury capabilities due in part by scarring [16,18,19].

The biomechanics of tendons also contribute to the challenges in tendon healing. Due to the constant recoil and elongation of the superficial digital flexor tendon (SDFT), it is particularly susceptible to injury and as a result is the most common tendon injury (75-93%) as compared to the deep digital flexor tendon or common digital extensor tendon [11,20-22]. The SDFT is an energy storing tendon that operates close to its maximal load in elite athletes thereby causing structural deformities such as microtraumas [23,24]. Currently, most tendinopathies are treated with both conservative and invasive strategies which span from systematic rehabilitation to surgical intervention. In some cases, stem cell therapies are utilized but this method has not produced an acceptable efficacy rate [25]. As of yet, an ideal therapeutic for tendinopathies has not been identified and in some part that may be due to the vast spectrum of injuries the tendon can sustain. This spectrum ranges from inflammation to microtrauma to partial or full ruptures. As a result, continued research into novel therapeutics that can be applied to a wide range of tendinopathies is of high interest.

In addition to other factors, proteoglycans play a key role in the regulation of tendon healing. Increases in small leucine-rich repeat proteoglycans (SLRPs) are seen after injury which is unsurprising as they play critical roles during development. Single-deficient and double-deficient mice in biglycan (BGN), decorin (DCN), lumican (LUM), and/or fibromodulin (FMOD) showed musculoskeletal alterations, including compromised tendons, due in part to their role in regulation of collagen fibrillogenesis, alignment, and maturation [14,26]. These alterations occurred in both mechanical and structural characteristics. SLRPs like decorin control fibril diameter and collagen fibril alignment resulting in abnormally large fibrils with decreased mechanical properties in failure load, force, and stiffness during decreased or absent levels of the SLRPs [27-30]. Similar changes to tendon are observed during age and injury. Most notably, decorin and biglycan expression mimic trends seen during development with biglycan expressing high levels acutely after injury while decorin expression is elevated later during healing. These findings imply that decorin and biglycan work in a step-wise manner resulting in a sequential order in response to injury [27]. Additionally, decorin can substitute for biglycan in biglycan-deficient mice but the reverse has not been observed to the same degree [31].

During aging, similar decreases in structural and mechanical properties occur with increasingly detrimental effects when decorin or biglycan are not expressed. Maintenance of collagen fibrils is done by SLRPs, in addition to, proteolysis protection of the fibrils by steric hinderance of collagenases to cleavage sites [32]. Decreases in cell morphology, cellularity, proliferation, mobility, and matrix turnover with maturation and aging may also contribute to tendon alteration with aging [33]. Some variation can be seen in these factors depending on the tendon type [33]. Nonenzymatic cross-linking formed by glycation in aging has been observed in all tendon types and it is relatively uncontrolled, contributing to the increasing distances between

collagen molecules leading to changes in molecular structure and accumulation of advanced glycation end products (AGEs) [34]. The accumulation of AGEs affects the water content present in tendons due to the dehydration that occurs with cross-linking [35-38].

II.   Tendon Collagen Structure, Assembly, Maturation, and Regulation

Tendons play a critical role in the musculoskeletal system; understanding the composition and function of tendons gives insight into their biomechanics at varying locations. Tendons connect muscle to bone and allow mechanical forces to be applied and transmitted through the system. The difference in origin and insertion, and size of the muscle to which the tendons are connected, changes the potential of the tendon. Additionally, composition and organization influence the tendons' ability to resist irregular displacement of bones and absorb applied loads. Overall, tendons are composed of varying amounts of water (50-60%), and by dry weight are predominantly collagens (70-80%) with the remaining components including proteoglycans (elastin, glycoproteins, and small-leucine rich proteoglycans) [39-41].

The overall structure of tendons also greatly influences their ability to disperse loads and withstand impacts. Tendons are comprised of fibrous collagen fibrils that are organized into bundles called fibers (**Fig. 1.1**). These fibers have numerous crosslinks between the fibrils and proteoglycans stabilizing the fibers. The fibers are further grouped together, with tendon cells called tenocytes, into fascicles with a loose connective tissue called the endotenon surrounding the fascicle. A collection of fascicles is surrounded by another loose connective tissue called the epitenon with the paratenon further surrounding it which ultimately comprises the whole tendon.

The hierarchical structure of tendons is critical for the function that these tissues play. Based on this structure and the cellular organization, tendons behave in a non-linear viscoelastic

manner under load. Visualization of the tensile properties of tendons can be done with a stress-strain curve defined as the deformation that a material can sustain at certain loads (stress; force per unit area) and the deformation a material exhibits at that load (strain) (**Fig. 1.2**). Tendons are relatively compliant at low loads. The first region of the stress-strain curve is the non-linear toe region which affects the crimps of the collagen fibers and any molecules interacting with collagens that have some viscoelastic properties to elongate at initial loads. With increasing tensile loads, these tissues become increasingly stiffer. Once all the collagen fibers are uncrimped, the stiffness of the tendons will follow a linear slope, termed the linear region. During this portion of the test, slippage occurs within the collagen fibrils, then between fibrils, and ultimately results in tearing of adjacent fibril molecules as the loads become too great resulting in tensile failure and the yield to failure region [42,43]. At this point, the tendons have reached their maximal load or point of ultimate tensile stress. As the load proceeds from initial strain to the point of failure, the area under the curve is considered the total energy absorbed.

Viscoelasticity, likely a result of the water, collagenous proteins, and interactions with proteoglycans, causes the mechanical behavior to be dependent on the rate of mechanical strain [44]. As a result of the individual differences between differing tendons, the rate of stress and stain is dependent on the time of displacement and load [45]. Viscoelastic materials are deformable at low strain rates but less so at high strain rates. Tendons at low strain rates can absorb more mechanical energy but conversely, become less effective at carrying loads from muscle to bone. As the tendon stiffness increases with increased loads, the viscoelastic property is overcome at higher strain rates. Additionally, three characteristics of tendon viscoelasticity (creep, stress relaxation, and hysteresis, the energy dissipated due to material viscosity) are now considered evident properties of tendons as these properties vary greatly across tendons. Changes to these

properties between high- and low-stressed tendons signal the potential for different material composition to compensate for higher fatigue in high-stressed tendons but much of the micro-structural origin of tendons is still unknown [46,47]. Recent models have shown that varying fibril lengths contribute to the viscoelastic nature of tendons. This model implies that fibrils and the matrix fundamentally have the same mechanical properties in a given tenon but differences between behaviors of differing fascicles is caused by varying distributions of the fibril lengths [47]. Though, this concept has yet to be combined with the dissipative theory of temporary interfibrillar bridges between collagenous and noncollagenous proteins in the matrix which would account for the fibril-matrix interaction as well as the fibril length distributions [48,49]. In conclusion, the relative stiffness and tensile strength of tendons is essential for maintaining force transmission and these properties change with loading rate.

Proper collagen fibril assembly is necessary for adequate force transduction to occur. The self-assembly process of fibril formation occurs both intracellularly and extracellularly, thereby allowing for greater regulation to occur at each step due to the compartmentalization of the process. Regulation includes stoichiometric control of gene products during synthesis, hydroxylation, glycosylation, folding and triple helix formation, packaging for secretion from the golgi apparatus, transport via secretion compartments at the cell surface, and formation of the extracellular compartments for matrix assembly [50]. The numerous regulatory steps involved in collagen assembly, in conjunction with interactions with processing enzymes, fibril associated molecules (proteoglycans and FACITs), and adhesive glycoproteins, allow for the specialization of various collagen types found throughout the body.

Collagen assembly begins with the synthesis of collagen mRNAs in the nucleus. These mRNAs are then transported out of the nucleus and translated into a preprocollagen where the

collagenous region is comprised of a repeating Gly-X-Y amino acid sequence. The X and Y amino acids in the sequence can be any amino acid though predominantly are the imino acids proline and hydroxyproline due to the helix formation and stability that these iminos provide. Glycine plays a major role in the sequence as it is the only amino acid that can adhere to the size constraints of the triple helix procollagen [51-53].

As preprocollagen is translated, a signaling peptide is attached to aid in the transport into the rough endoplasmic reticulum. Hydroxylation of the preprocollagen occurs within the rough endoplasmic reticulum after cleaving the signaling peptide and in the presence of vitamin C as a cofactor. Vitamin C plays a critical role in hydroxylation. Deficits in this vitamin can lead to diseases such as Scurvy, while supplementation with vitamin C enriched gelatin can increase collagen synthesis if taken before exercise [54,55]. Hydroxylation results in two unique amino acids, hydroxyproline and hydroxylysine, which play critical roles in downstream triple helix stability and glycosylation. At this point, a procollagen has been formed and the aggregation of the procollagen chains into triple helices will occur after glycosylation. Glycosylation occurs when some hydroxylysine residues receive O-linked sugar modifications by glycotransferases and galactotransferases of α-glucose and β-galactose [56]. The ultimate function of these glycosylation events is still not fully elucidated, but some working theories identify that the O-linked sugars provide slight destabilization of the collagen triple helices thereby enhancing the self-assembly or that these modifications affect lateral assembly of collagen fibrils [57-59]. Post-translational glycosylation impacts fibril assembly and structure in such that intermolecular center-to-center distances can change the collagen triple helical circumference and over glycosylation can lead to compromised fibrillar organization [60-63].

Assembled triple helices are then exocytosed from the Golgi body after traveling from the rough endoplasmic reticulum. Once outside the cell, the procollagen triple helix is processed into tropocollagens with peptidases removing globular N- and C- propeptides. Procollagen C-proteinase enhance-1 (PCPE-1) accelerates the proteolytic release of the C-propeptide from the fibrillar procollagens while members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family affect the N-proteinase activity [64,65]. Notably, ADAMTS defects lead to heterotopic ossification in the cell with ADAMTS7 and ADAMTS12 regulating tendon collagen fibril structure and inhibiting heterotopic ossification [66]. Finally, tropocollagen cross-linking occurs through lysyl oxidase (LOX) and results in covalent aldehyde links that aggregate tropocollagens into long linear subunits both linearly and laterally [67]. Fibromodulin (FMOD; member of the SLRP family) regulates LOX cross-link formation by inhibiting its access to the telopeptides, cross-linked N- and C- terminal ends. Deficient levels in FMOD cause increased C-telopeptide cross-linking ultimately leading to abnormal fibril formation and decreased mechanical strength of collagen fibers [68].

As the tropocollagens aggregate together, with their enzymatic and non-enzymatic chemical cross-linking, they are arranged into a quarter-staggered pattern in uniform and short D periodic protofibrils with diameters ranging between 20 – 40 nm, lengths of 4 – 12 μm, and with tapered ends resulting in the characteristic 67 nm D-banding pattern [51,69-72]. The newly assembled protofibrils are deposited and incorporated into the developing tendon extracellular matrix as small bundles of fibrils or immature fibers and stabilized via fibril-associated collagens with FACITs (fibril associated collagens with interrupted triple helices) and SLRPs. SLRPs, particularly decorin, interact with the collagen fibrils and regulate fibril growth by binding non-covalently at every D period (67 nm) to an intraperiod site on the surface of the collagen fibrils

[73]. As the tendons mature in linear and lateral fibril growth, associations, fusions, and end overlap are introduced to generate larger diameter fibrils resulting in cylindrical fibril structures. Inter- and intra-molecular cross-linking regulate structure turnover and stability allowing for greater mechanical stability depending on tendon location. Additionally, at this point a crimp pattern is introduced at the fibril and fascicle level by elastin thereby increasing complexity of the tendon.

Regulation of collagen fibrillogenesis is tissue-specific and involves interactions between different molecules such as processing enzymes, heterotypic fibril-forming collagens, FACITs, SLRPs, and other glycoproteins such as fibronectin and tenascin X [74]. During growth and development of tendon, significant increases in collagen fibril diameters occur resulting in direct affects to the mechanical properties of the tendon [75]. Organizers, nucleators, and regulators play key roles in aiding collagen fibril self-assembly and growth. Namely, collagen V and XI have been shown to nucleate collagen fibril formation and assembly of immature protofibrils [76-79]. The alpha-chain tissue-specific isoforms of collagen V and XI form heterotypic fibrils with collagens I and II regulating the collagen organization and fibril differences [78]. In the absence of collagen V, collagen I and II can still self-assemble but only after long lag phases indicating the need for collagen V to provide a fibril-forming site to allow for subsequent fibril intermediates and linear and lateral fusions [80,81].

Small leucine rich repeat proteoglycans (SLRPs) are crucial regulators of linear and lateral fibril growth with direct impacts on collagen fibril growth, maturation, and homeostasis [82]. Class I (biglycan and decorin) and class II (fibromodulin and lumican) SLRPs are tissue-specific and deficits in these molecules can result in affected functional characteristics (leucine-rich repeat ear or N- and C- terminus disulfide caps) with subsequent detrimental or fatal effects [6,26,27,82-

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85]. In addition to their functional characteristics, SLRPs sterically hinder collagenases access to cleavage sites therefore aide in collagen fibril proteolysis protection [82,86].

In tendons, linear and lateral fibril regulation is controlled largely by decorin and fibromodulin with modulations done by biglycan and lumican; therefore, any alterations to these molecules can result in changes to fibril growth and subsequent larger fibril diameters [6,26,87-91]. As a result, changes to the mechanical and structural properties of these affected tendons can occur, though, deficiencies of a single SLRP within its class can be compensated for by another in its class due to binding site similarity and molecular homology [92]. If deficiencies of two SLRPs in different classes occur, changes to tendon biomechanics, fibril diameters, and potential ectopic ossification within the tendon has been shown [26,89,93]. Unlike fibril-forming collagens, SLRPs regularly turnover allowing for changes in expression to affect fibrillogenesis and tendon structure throughout development, maturation, and injury.

III.   Regional Differences between Tendon Proper and Peritenon Tenocytes

The structure and function of the tendon throughout development, maturation, homeostasis, aging, and injury heavily depends on the roles of the tendon proper (TP) and peritenon (PERI) cell populations. As new evidence emerges, it has become apparent that these two cell populations located within (intrinsic) and around (extrinsic) the tendon respond, interact, and produce very different molecular and mechanical reactions but studies to elucidate the extent of their affects in injury and aging are still ongoing. The tenogenic transcription factor Scleraxis (SCX) has been identified as an early mesenchymal tendon lineage marker expressed during tendon differentiation in both progenitor and differentiated cells and constantly in tendon proper cells while only during injury in peritenon cells [94-97]. This discovery is of particular importance

because prior to this point, lineage tracing provided a significant challenge in developmental, healing, and maintenance models. Furthermore, SCX promotes tendon differentiation and maturation by activating genes such as *Col1a1* and tenomodulin, in addition to numerous other extracellular matrix genes [98-100].

Understanding the regional differences between the intrinsic and extrinsic cell populations becomes apparent as leukocytes and fibroblasts migrate to an injury site but the origin and contribution to repair of these cells remained unknown [97,101]. Further work identified that both the extrinsic peritenon and intrinsic tendon proper populations contributed to repair [93,102-104]. Progenitors of these populations expressed differing markers with tendon related markers present in the intrinsic population and vascular/pericyte related markers in the extrinsic population [97,104-108]. The intrinsic and extrinsic cell populations both respond to the lesion with cellular migration and proliferation, in addition to, secreting trophic factors, stimulating inflammatory cells, and secreting enzymes to break down and remodel [103,109-111]. The exact regional cellular response to injury indicates that both populations are capable of contributing collagen fibril-rich structures but tenogenic differentiation markers (SCX and tenomodulin;TNMD) are notably expressed by the intrinsic cells [102,106]. Peritenon cells migrate to the lesion early with evidence of releasing stimulatory factors bolstering expression of tenogenic differentiation markers and matrix assembly genes in tendon proper cells [102,103,106]

A large focus in the elucidation of the regional differences between the extrinsic and intrinsic cell populations of tendon has been towards developing novel strategies towards tendon healing and regeneration. The two populations unquestionably receive crosstalk in regard to repair and demonstrate as a potential target area for improving repair and regeneration of tendinopathies. Conversely, the changes between the two populations and their relative response to one another

has not been closely looked at. It is well established that aging affects tendons in a negative manner with increases in injury risk as a result of compositional changes, compromised mechanical properties, and decreased structural integrity [112-114]. As such, understanding the molecular and cellular changes that tendon proper and peritenon cell populations undergo during aging may further elucidate age-dependent alterations in tendon architecture and functionality.

IV.   Changes in Gene Expression and Epigenetics with Aging

Aging in tendons has long been hypothesized to play a role in compositional changes within the tendon matrix thereby resulting in alterations in the material properties of tendons leading to increased susceptibility to injury, especially when looking at the effects of age and exercise [115-128]. Although the effect of exercise on aged tendons has been well characterized, the effect of natural age-related changes of tendon genes, tendon fibril distribution, and the role of aging on tendinopathies is not well understood [129-132]. Aging has been proposed as a potential intrinsic factor involved in tendinopathies due to the gradual decrease in mechanical integrity of the aged tendons [133]. This is most likely due to the decrease in interfascicular sliding resulting in a stiffer interfascicular matrix and subsequent reduction of fibril size due to degenerated larger fibrils from tendons being loaded at an earlier point during tendon extension [119,130]. Additionally, decreased protein synthesis, cell functionality, ultrastructural changes, and viscoelasticity has been attributed to early degenerative changes in tendon resulting in microdamage and degraded collagens within the tendon [27,28,113,134-137]

Tenocytes and the surrounding tendon tissue undergo adaptation processes during tendon maturation, training, and aging in which the synthesis, degradation, and maintenance of the extracellular matrix is regulated by the tenocytes in response to the mechanical forces translated

to biochemical signals [137-140]. As a result of this, genes are most likely expressed differently throughout the life-stages of an individual potentially leading to the gradual decrease in the regenerative potential of the tendon [141-145]. As such, the molecular mechanisms that play a vital role in these age-related changes have yet to be elucidated but insight into this topic would aid in developing novel therapeutics and further preventative measures of tendinopathies.

Another consideration are DNA methylation changes that occur with age in tissues although the mechanisms and specificity are still poorly understood. As a result of these changes, chromatin modifications are associated with the regulation of transcriptional activity [146]. Methylation of DNA is the result of modification of cytosine to 5'methylcytosine by DNA methyltransferases most often found proximal to promoters of housekeeping genes and labeled as CpG islands (CGIs) [147-149]. Hypermethylation of CGIs is associated with transcriptional silencing and about 60 – 70 % of promoters are linked to CGIs in normally hypomethylated regions [150-152]. Gene body methylation, a feature of transcribed genes, and active transcription have been confirmed to have positive correlations resulting in tissue-specific intragenic methylated CGIs [153-157]. Gene body CGIs can be heavily methylated with no effect on transcription elongation despite chromatin features associated with repressed transcription at the transcription start site (TSS) [158]. Resultingly, methylation in the promoter is inversely correlated with expression and gene body methylation is positively correlated [159]. Exons, compared to introns, are more highly methylated with varying degrees of methylation at exon-intron boundaries suggesting methylation as a regulator of splicing [160]. These findings imply that gene body methylation will have functions outside the classic silencing of intragenic repetitive DNA sequences but further elucidation into this is necessary.

As new technologies emerge and the ability to analyze big datasets becomes increasingly possible, looking into differentially expressed genes through RNA sequencing (RNASeq) and differentially methylated regions by DNA methylation (RRBS; reduced representation bisulfite sequencing) become abundantly apparent. Although tendon specific genes, regulators, nucleators, and organizers play clear and vital roles during growth, maturation, and aging, the effects aging has on tendons is most likely on a larger scale, involving multiple pathways, and requires genome-wide analysis to elucidate key genes, pathways, and regulators responsible for age-related tendon changes.

V.   Summary of Studies

As previously mentioned, the repair and regeneration process in tendons is slow and incomplete. With aging, the risk of tendinopathies is even greater due to the mechanical, compositional, and molecular changes that the aging tendon undergoes. As such, the need for novel therapeutics and exploration into therapies resulting in stronger more complete repair is crucial and ongoing. Currently, in cases where exercise management and conservative treatments have yielded little to no results, treatments such as stem cell injections (either bone marrow-derived or adipose-derived mesenchymal stem cells) and platelet rich plasma (PRP) are utilized with the hope of better outcomes [161-165]. The trophic factors secreted by both the MSCs and PRPs provide many resources to the surrounding tendon cells, but they provide little direction in guiding the cells to stimulate repair, in addition to, a lack of understanding of the modes of action of the cells [166]. The concern with these therapeutics is also that each individual can respond differently to the treatment based on a number of factors such as time since injury, age of the animal, degree of severity of the injury, etc [164, 167-172].

The role of SLRPs in tendon development, homeostasis, and repair has been proven, thereby opening another avenue into potential therapeutic treatments. Biglycan and decorin are two candidate molecules due to their close incorporation with collagen fibrils and their role in fibril growth and homeostasis. Before these SLRPs can be utilized to treat tendinopathies in an *in vivo* study, understanding the changes to structure and gene expression with supplementation on an *in vitro* level must be done. Three-dimensional tendon constructs are used to more closely assess the role of biglycan and decorin on tendon collagen growth and maturation. Fibrin gel comprised of either tendon proper or peritenon cells and supplemented with a treatment (control, 5 nM biglycan, 25 nM biglycan, 5 nM decorin, or 25 nM decorin) loaded around anchor points allow the gel to contract and incorporate collagen fibrils longitudinally (**Fig. 1.3**). The constructs are then assessed for collagen content, collagen organization, and tenogenic or perivascular gene expression to understand the impact that supplementation has on the cells and the surrounding material. This study will pilot the use of SLRP supplementation for more directed tendon repair.

Tendinopathies occur in numerous equine disciplines and breeds but also varying ages. Tendon composition, morphology, and response to exercise from aging has been well established but these findings generally focus on a macro scale. To make progress in improving recovery from equine tendinopathies, understanding the changes that occur in the extrinsic and intrinsic cell populations throughout aging without the additional influence of injury is necessary to understand the baseline mechanisms at play. By understanding the effect of aging on a gene expression and epigenetic regulation level, certain therapeutics could be either utilized or ruled out based on the cells' potential response to the intended treatment at an individual's age. If certain tenogenic markers or pathways necessary for collagen homeostasis, development, or turnover are affected due to age, targeting a different therapeutic avenue as a response may be necessary. With the use

of RNA sequencing and DNA methylation, a more global approach to understanding the shifts in translation can shed light on the changes that occur in the tendon proper and peritenon cell populations throughout aging.

As lameness remains the most common diagnoses in equine medicine, the need for improving regenerative medicine is ongoing [173,174]. The use of mesenchymal stem cells from differing origins, platelet rich plasma, autologous conditioned serum, and autologous protein solution have been used in regenerative medicine with varying success due to different cellular isolation practices, degree or induction of injury (microtrauma vs macrotrauma), and overall study design limited to small studies or individual cases with varying post-treatment regimens [164,166,175-179]. Notably, the interplay between the trophic factors of mesenchymal stem cells, such as growth factors, proteases, and cellular mediators, and their effect on the extrinsic and intrinsic native cells is still poorly understood [166,180-182]. As a result, further elucidation into the direct tenogenic impact that adipose-derived mesenchymal stem cells (ADMSC) have on tendon proper and peritenon cells *in vitro* on a gene expression level without injury as a confounding variable will further inform researchers and veterinarians the effect of ADMSCs on tendon.
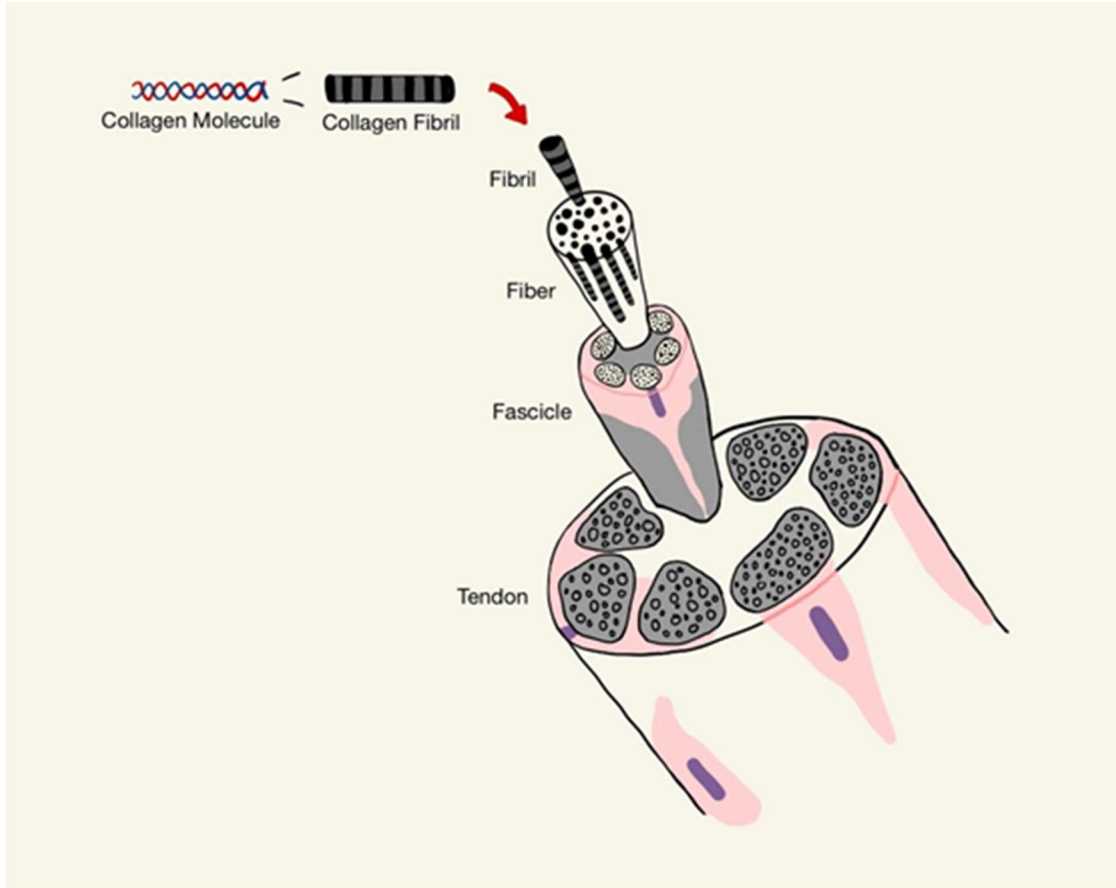
Figure 1.1. Diagram of the hierarchical structure of tendon. Tendons are hierarchal structures built together from triple helical collagens assembled into fibrils. Cross-linking of the fibrils allows for further structural assembly into fibers. Multiple fibers undergo linear and lateral elongation forming into a fascicle which is surrounded by tenocytes and loose connective tissue called the endotenon. Groups of fascicles are further bundled together ultimately forming a tendon that is surrounded by loose connective tissue, epitenon, and a synovial-like tissue, paratenon. The collection of the paratenon and epitenon layers comprise the peritenon while the core of the tendon is labeled as the tendon proper.
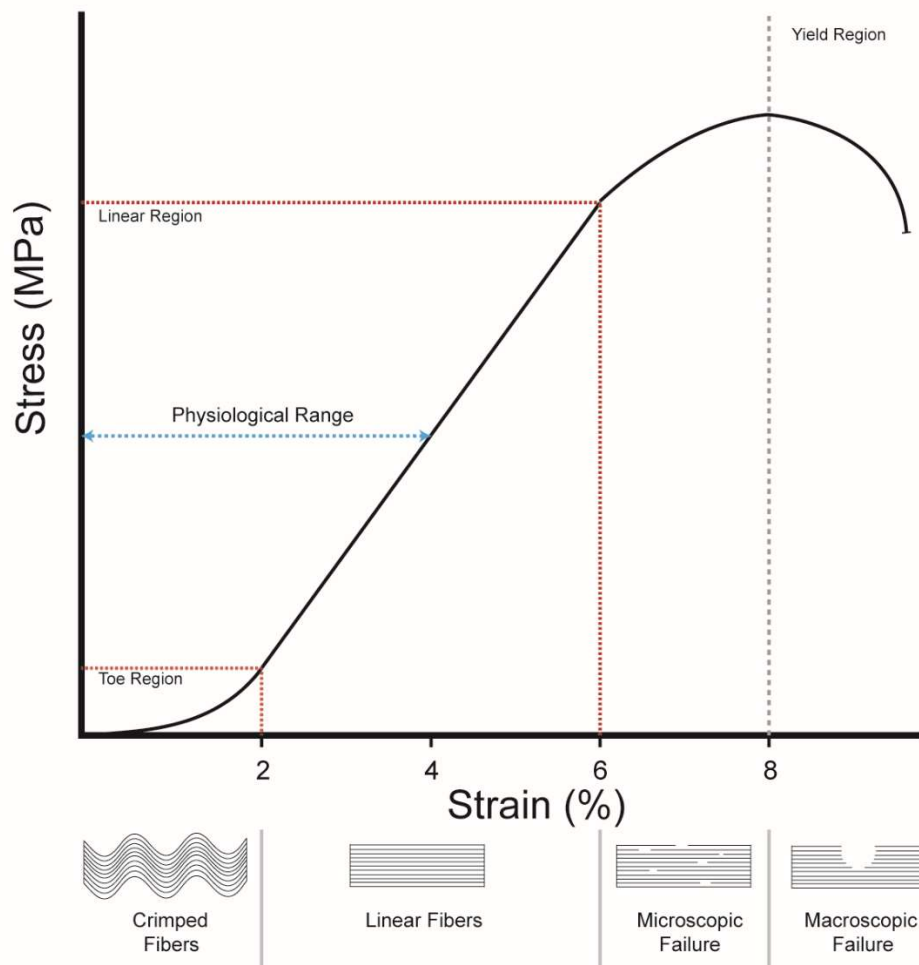
Figure 1.2. Load-elongation curve for tendons. The load-elongation curve, also known as a stress-strain curve, has three distinct regions defining tendon response to tensile loading. The toe region primarily affects the crimped collagen fibers and any viscoelastic properties from the molecules interacting with the collagens. This region is followed by a linear region where most physiological tensile loading occurs. In this region, as the tensile load increases the tendon elongates and increases in stiffness. Slippage between and within collagen fibrils occurs in this region. If the load applied to the tendon goes beyond the linear region, the yield region is breached as tearing of adjacent fibrils slip causing microscopic tears, macroscopic tears, or complete rupture.
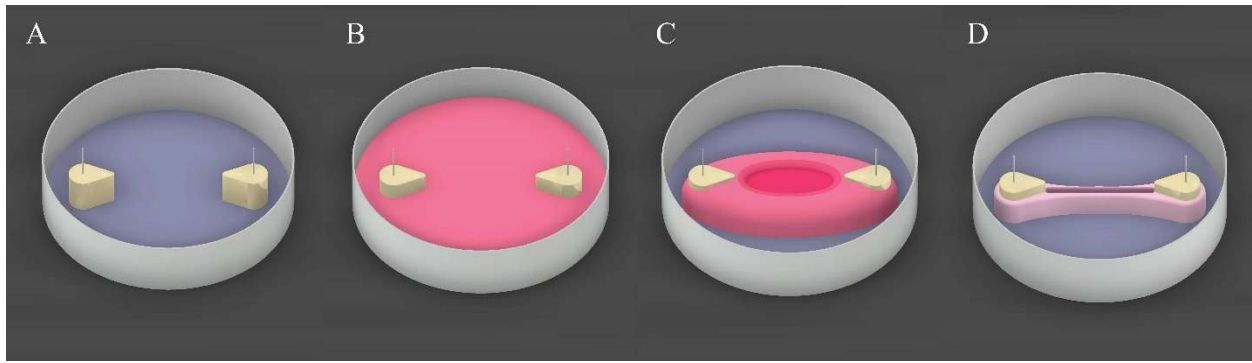
Figure 1.3. Three-dimensional rendering of the engineered constructs. (A) Six-well plates were coated with Sylguard silicone bottoms allowing brushite anchors to be placed 1 cm apart from one another. **(B)** The fibrillin gel with tendon proper or peritenon cells supplemented with a treatment was spread across the bottom of the plate. **(C)** The constructs were allowed to contract and form around the anchors over 14 days. **(D)** Final constructs were measured, weighed, and then processed for either collagen content, collagen organization, or gene expression analysis.

VII.   References

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Chapter Two: Adding Exogenous Biglycan or Decorin Improves Tendon Formation for Equine Peritenon and Tendon Proper Cells in Vitro

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I.   Abstract

Background: Tendon injuries amount to one of the leading causes of career-ending injuries in horses due to the inability for tendon to repair and the high reinjury potential. Tendon is highly acellularized which increases the difficulty for complete repair. As a result, novel therapeutics are necessary to improve repair with the goal of decreasing leg lameness and potential reinjury. Small leucine-rich repeat proteoglycans (SLRPs), a class of regulatory molecules responsible for collagen organization and maturation, may be one such therapeutic to improve tendon repair. Before SLRP supplementation could occur *in vivo*, proper evaluation of the effect of these molecules *in vitro* needs to be assessed. The objective of this study was to evaluate the effectiveness of purified bovine biglycan or decorin on tendon proper and peritenon cell populations in three-dimensional tendon constructs.

Methods: Equine tendon proper or peritenon cell seeded fibrin three-dimensional constructs were supplemented with biglycan or decorin at two concentrations (5 nM or 25 nM). The functionality and ultrastructural morphology of the constructs were assessed using biomechanics, collagen content analysis, transmission electron microscopy (TEM), and gene expression by real time – quantitative polymerase chain reaction (RT-qPCR).

Results: SLRP supplementation affected both tendon proper and peritenon cells-seeded constructs. With additional SLRPs, material and tensile properties of constructs strengthened, though ultrastructural analyses indicated production of similar-sized or smaller fibrils. Overall expression of tendon markers was bolstered more in peritenon cells supplemented with either SLRP, while supplementation of SLRPs to TP cell-derived constructs demonstrated fewer changes in tendon and extracellular matrix markers. Moreover, relative to non-supplemented tendon proper

cell-seeded constructs, SLRP supplementation of the peritenon cells showed increases in mechanical strength, material properties, and collagen content.

Conclusions: The SLRP-supplemented peritenon cells produced constructs with greater mechanical and material properties than tendon proper seeded constructs, as well as increased expression of matrix assembly molecules. These findings provide evidence that SLRPs should be further investigated for their potential to improve tendon formation in engineered grafts or post-injury.

II.   Background

Tendinopathies like those of the superficial digital flexor tendon (SDFT) result in major leg lameness and are debilitating for horses of all disciplines [1, 2]. For both acute and chronic tendinopathies, like those of the SDFT, a closer look at the pathology associated with tendon injury oftentimes implicates alterations in extracellular matrix (ECM) regulators of collagen fibrillogenesis and organization [3]. Alterations in the expression of ECM regulators lead to changes in biomechanical properties that impact the strength and stability of these energy storing tendons [4]. Due to this, novel therapeutics are necessary since complete repair is unlikely and further injury is a major concern [2, 3].

Small leucine-rich repeat proteoglycans (SLRPs) are a class of regulatory molecules that are essential for collagen organization in tendon development, maturation, and repair [5]. The contributions of SLRPs have been particularly well-characterized in tendons [6,7,8,9,10,11,12]. Besides directly affecting collagen fibrillogenesis, SLRPs like biglycan (BGN) and decorin (DCN) play roles in determining how tissue niche impacts cell biology, including: 1) the differentiation status of tendon progenitors in health and pathology [13]; 2) inflammatory regulation as Damage Associated Molecular Pattern proteins interacting with Toll-like receptors [14]; 3) recruitment of cells to sites of tissue repair or regeneration [15]; and 4) sequestration of growth factors essential for generation and maintenance of the tendon phenotype [13]. Previous work has demonstrated that the absence of these SLRPs dramatically affects tendon repair outcomes with BGN essential early in repair and DCN crucial later in tendon repair [7, 9]. Interestingly, expression of BGN and DCN in mature animals decreases after an injury and never recovers to the level seen during development and maturation [11], suggesting that low BGN and/or DCN may contribute to the impaired injury response.

After a mature tendon is injured, repair occurs as a result of extrinsic and intrinsic influences. Leukocytes and fibroblasts migrate into the lesion early in repair [15]. Post-injury, these fibroblasts originate from the extrinsic paratenon and have demonstrated distinct differences in marker expression and tenogenic potential as compared to the tendon proper fibroblast cell population [16,17,18,19,20]. Thus, when considering therapeutic interventions for tendon repair, both cell populations should be included since the role of each cell type remains unresolved.

Recognizing the value of BGN and DCN in tendon development and maturation and their subsequent decline at the time of repair, we hypothesize that addition of BGN or DCN to the tendon matrix would improve tendon formation. To test this hypothesis, equine tendon proper (TP) and peritenon (PERI) cells were seeded in an in vitro fibrin-based three-dimensional tendon construct model in which the gel contained two differing amounts of either exogenous bovine purified BGN or exogenous bovine purified DCN. The effects of the exogenous BGN or DCN on biomechanics, electron microscopic ultrastructure, collagen content, and gene expression were determined.

III.   Materials and Methods

2.3.1 Tendon Harvest and Cell Isolation.

Equine superficial digital flexor tendon (SDFT) cells were harvested from five horses of various breeds (ages 8–15 years) with approval from the University of California Davis Institutional Animal Care and Use Committee. All horses were property of the University of California Davis and were assessed as healthy with no known tendinopathies and were euthanized by intravenous injection of euthanasia solution (pentobarbital sodium and phenytoin sodium) for reasons unrelated to the study. After euthanasia, 2.5 cm of forelimb SDFT was harvested per horse

approximately 10–15 cm proximal of the forelimb fetlock. Tendons were transported to the lab in Dulbecco's Phosphate Buffer Solution (DPBS, Life Technologies, Benicia, CA, USA) containing 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B, Life Technologies). For each horse, tissue from the tendon proper and peritenon regions were isolated while submerged in DPBS containing 1% antibiotic/antimycotic. Under a dissecting microscope and sterile conditions, the peritenon region was isolated by removing the paratenon and 1 mm of the epitenon region using forceps and sterile scalpel blades. The tendon proper region of the tendon was isolated by removing a 2 mm square the length of the sample of the tendon core [5, 18, 20]. Separated regions were then used for digestion to isolate the different cell populations for each horse. Enzymatic digestion followed previous protocols using 0.3% type-I collagenase (CLS-1, Worthington, Lakewood, New Jersey, USA) and 0.4% Dispase II (Roche, Basel, CH) in Hanks Balanced Salt Solution (HBSS, Gibco, Benicia, CA, USA) with enzymatic inactivation after agitation in standard tenocyte media (alpha-MEM, 10% fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic/antimycotic) [20, 21]. Cells from each region for each horse were plated in T75 flasks and expanded in standard tenocyte media. Cells from each region for each horse were passaged before being cryopreserved in 10% dimethyl-sulfoxide (DMSO) solution in standard tenocyte culture media under liquid nitrogen after reaching P2.

To make constructs, frozen vials of peritenon and tendon-proper cells were thawed and seeded as P3 in T75 flasks at 6666 cells per cm2 and grown to 85% confluency in normal tenocyte media. Tear-drop shaped brushite anchors (100 mM citric acid and 3.5 M orthophosphoric acid added to dense β-tricalcium phosphate mixture (β-TCP; Plasma Biotal Limited, Derbyshire, UK) in a 1 mL per 1 g ratio) were pinned 1 cm apart in 35 mm tissue culture treated dishes cured with 3 mL of Sylgard (184 Silicone Elastomer Kit, Dow Corning, Midland, MI) [21,22,23]. For each

horse sample and treatment, a minimum of 3 constructs were used for biomechanics and subsequent collagen analysis, 1 for real time quantitative polymerase chain reaction (RT-qPCR), and 1 for transmission electron microscopy (TEM). Bovine biglycan (bBGN) (Sigma-Aldrich) or bovine decorin (bDCN) (Sigma-Aldrich) was supplemented into the fibrin gel mixture at high (25 nM) or low (5 nM) concentrations – doses that were previously investigated with cultured myotubules, cardiomyocytes, and type I collagen gels [24,25,26]. Therefore, at least 50 constructs were made for each horse in order to provide a minimum of 5 technical replicates for the control, high and low bBGN, and high and low bDCN conditions for both the peritenon and tendon proper cells. To make the tendons, cells were combined with the fibrinogen-thrombin matrix gel (681 μl cell suspension with supplementation or control media, 286 μl of 20 mg/mL fibrinogen, and 29 μl of 200 U/mL thrombin to get 998 μl total gel volume) at 300,000 cells per construct and seeded in a spread method around the anchors [21]. The suspension was allowed to gel for 15 min before adding tenocyte standard media supplemented with 200 uM ascorbic-2-phosphate into the wells [20]. Constructs were maintained at 37 °C in 5% CO2 for 14 days with media changes every 2–3 days.

2.3.2 Biomechanical Testing.

At day 14, length and width of a minimum of 3 constructs for each treatment was determined using digital calipers before being loaded into a horizontal uniaxial tensile testing machine within a saline bath [27,28,29]. Samples were tested to failure without preconditioning at a constant displacement rate of 0.4 mm/s [30]. LabVIEW (National Instruments, Austin, TX) software recorded the resulting force measurements and the load-deformation curve was used to determine the maximal tensile load (MTL) of the construct. The load and deformation values were normalized to the cross-sectional area (CSA) and initial construct length, respectively, to calculate

stress and strain. The ultimate tensile stress (UTS) was recorded as the highest stress value before failure, whereas Young's modulus was determined by calculating the slope of the linear portion of the stress-strain curve.

2.3.3 Collagen Content.

Following biomechanical testing, constructs were removed from anchors, patted dry, and placed on glass to dehydrate at 120 °C for 20 mins. Dried constructs were weighed and either stored in individual tubes until necessary or immediately processed for hydroxyproline analysis. Analysis followed a previously described protocol using 6 N hydrochloric acid at 120 °C for 2 h for hydrolysis, followed by 1.5 h to evaporate the hydrochloric acid. Hydroxyproline buffer (3.3% citric acid, 2.3% sodium hydroxide, 0.8% acetic acid in water, pH 6.0–6.5) was used to resuspend the pellets and resulting solution was stored in − 20 °C until further processing [27]. Stock samples were diluted to 9:1 or 4:1 hydroxyproline buffer:stock sample to allow for more accurate colorimetric detection. Chloramine-T (14.1 mg/mL) and aldehyde perchlorate solution were added in a step-wise fashion to each diluted sample before heating, cooling, and reading the samples and standards in a UV spectrophotometer at 550 nm [31,32,33,34].

2.3.4 Transmission Electron Microscopy.

At day 14, constructs were rinsed with phosphate buffer solution (PBS) and fixed at length by complete immersion in Karnovsky's fixative for 2 h at 4 °C then stored in transport solution for up to 1 week before embedding. Further processing of constructs for TEM followed previously described protocols [10, 18, 29,30,31, 35, 36]. Briefly, fresh epoxy resin was used to embed constructs cut in thirds cross-sectionally and polymerized for 12 h at 60 °C (EMBed – 812,

Electron Microscopy Sciences, Hatfield, PA, USA). Blocks sectioned at 70 nm by ultramicrotome were post-stained with 2% aqueous uranyl acetate and 1% phosphotungstic acid, pH 3.2 [20]. Images were taken at 80 kV using a FEI C120 transmission electron microscope (FEI Co, Hillsboro, OR) with a Gatan Orius CC Digital camera (Gatan Inc., Pleasanton, CA). All images used for fibril diameter analysis, fibril density, collagen organization, and structure were taken at 33,000x. Fibril diameter distribution was visualized using ImageJ software (National Institutes of Health, Bethesda, MD) and means were calculated from 5 images per testing group within each biological sample with no more than 100 fibrils per image counted for a total of 500 fibril diameters per biological sample. Fibril density and fibrils per area of extracellular matrix (ECM) per image were calculated using the same 5 images as the fibril diameters.

2.3.5 Total RNA Isolation and Real Time quantitative PCR (RT-qPCR).

At day 14, constructs were snap frozen in liquid nitrogen and stored at −80 °C until further processed. Homogenization of the tendon constructs was done using a BioSpec Tissue-Tearor and total RNA isolation was performed using the RNeasy Plus Micro Kit (QIAGEN, Valencia, CA) including a RNase-free DNase treatment (QIAGEN, Valencia, CA). Reverse transcription was performed on 500 ng total RNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Genes assessed included tenogenic differentiation (MKX, FMOD), ECM assembly (BGN, DCN, COL1A1, LOX), or perivascular (CSPG4) markers [7, 18, 20, 37, 38]. POLR2A was used as the housekeeping gene [19, 37, 39]. Taqman primers were designed from equine gene structure annotation (NCBI Equicab 3.0) using Primer3 or from predesigned primers (Life Technologies) (Table S-1) [40, 41]. For RT-qPCR analysis, 1 ul of cDNA template was combined with Taqman Master Mix (no UNG) (Life Technologies) and equine specific primers for a reaction

volume of 20 ul in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) [19]. Each sample of amplified cDNA was analyzed in duplicate for each gene with gene specific efficiencies calculated using LinRegPCR v 7.5 software [7, 18, 19]. The relative quantity ratios formula was used to calculate the relative quantity of mRNA for each gene [42, 43].

2.3.6 Statistics.

GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was used for all statistical analyses. Mean values for technical replicates within biological replicates were calculated before differences of the mean values were compared within testing categories to avoid pseudoreplicates. Statistical analyses were all performed using nonparametric Wilcoxon signed-rank tests in which each treatment (5 nM bBGN, 25 nM bBGN, 5 nM bDCN, 25 nM bDCN) was compared to its corresponding control with a one-sided test applied [44,45,46]. The H0 was that the addition of SLRPs to the constructs would lead to no improvement or a decline in tenogenic features; the Ha was that the addition of SLRPs would improve tenogenic properties, or promote tendon formation, as described in several studies: (1) increased UTS, Young's modulus, and MTL; (2) increased collagen content; (3) increases in collagen fibril numbers with increases in fibril diameters; (4) increased relative expression of tendon markers SCX and MKX, (5) increased expression of ECM assembly markers; and (6) decreased expression of perivascular markers [6,7,8,9,10, 12, 17,18,19,20,21,22, 27]. Significance level was set at $p \leq 0.05$. Given the limited "n," wherever analyses approached significance with $p = 0.0625$ (i.e., measurement ranks switched within one pair of the samples), it was noted in the results.

IV.   Results

2.4.1 Biomechanics and Collagen Content.

Constructs contracted successfully and were assessed at 14 days post seeding with cells aligned unilaterally and no qualitative differences seen between peritenon and tendon proper cells upon gross microscopic examination. The supplementation of SLRPs bBGN and bDCN improved biomechanics in many instances (**Fig. 2.1**). Peritenon cells supplemented with 25 nM bBGN showed significantly increases in UTS (p = 0.0313) and Young's modulus (p = 0.0313). Increases in UTS approached significance (p = 0.0625) when PERI cells were supplemented with 5 nM or 25 nM bDCN. Increases in Young's modulus approached significance (p = 0.0625) when PERI cells were supplemented with 5 nM bBGN, 5 nM bDCN, or 25 nM bDCN. For tendon proper cells, Young's modulus significantly increased for constructs supplemented with 25 nM bDCN (p = 0.0313). Otherwise, for TP cells, increases in Young's modulus approached significance (p = 0.0625) when supplemented with 5 nM bBGN or 5 nM bDCN. No significant increases in CSA were seen when TP or PERI cells were supplemented with bBGN or bDCN.

SLRP-treated PERI cell constructs were compared to TP cell control constructs in regard to biomechanics to discern if SLRP-supplemented PERI cells created biomechanically superior constructs (**Fig. S-2.1**). UTS was significantly greater for PERI constructs supplemented with 25 nM bBGN. Relative to TP cell control constructs, Young's modulus was significantly greater for PERI cells supplemented with 25 nM bBGN or 5 nM bDCN, and MTL was greater for PERI cell constructs receiving 5 nM bBGN or 25 nM bDCN.

Increases in collagen content approached significance (p = 0.0625) in TP and PERI constructs supplemented with 25 nM bDCN (**Fig. 2.2a**). For collagen content as a fraction of dry mass (%), neither tendon proper nor peritenon cells showed improvement with any

supplementation (**Fig. 2.2b**). Likewise, when comparing SLRP-treated PERI cell constructs with TP cell control constructs, supplementation with 25 nM bBGN or 5 nM bDCN increased collagen content (**Fig. S-2.2A**), yet not as a percentage of dry mass (**Fig. S-2.2B**).

2.4.2 Transmission Electron Microscopy.

TEM cross-sections were used to analyze collagen fibril diameters (**Fig. S-2.3**). Fibril analyses showed slight shifts towards smaller fibrils for PERI cells in constructs supplemented with 25 nM bBGN, 5 nM bDCN, and 25 nM bDCN, as well as for TP cells in constructs supplemented with 5 nM bBGN, 5 nM bDCN, and 25 nM bDCN (**Fig. 2.3**). A bimodal distribution can be seen for TP cell-derived constructs with 25 nM bBGN supplementation. When comparing mean fibril diameter (nm) of SLRP-supplemented constructs with their respective controls, though not statistically significant, mean diameters of PERI constructs were roughly the same size, and mean diameters of TP constructs were slightly smaller or the same size. For normalized fibrils per area of extracellular area, no significant differences were found (**Fig. 2.4**).

To understand how SLRP-treated PERI cell constructs compared to TP cell control constructs, fibril diameter distribution analyses demonstrated relative shift towards larger fibrils (**Fig. S-2.4**) and trends of more fibrils with more fibrils per area of ECM (**Fig. S-2.5**) when supplemented with bBGN and bDCN.

2.4.3 Gene expression.

Gene expression analyses informed how SLRP supplementation within the constructs affected TP and PERI cell tenogenic properties (**Fig. 2.5**). When PERI cells in constructs were supplemented with 5 nM bBGN, increased expression of *BGN*, *SCX*, and *COL1A1* approached

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significance (p = 0.0625); however, expression of CSPG4 was elevated. PERI cells supplemented with 25 nM bBGN had increased *BGN* and *SCX* expression (p = 0.0313). Supplementation of 5 nM bDCN led to increased expression (p = 0.0313) of *BGN* and *SCX* with an increase in *COL1A1* approaching significance (p = 0.0625). Moreover, expression decreased for *CSPG4* when PERI cells were supplemented with 5 nM *DCN* (p = 0.0313). PERI cells supplemented with 25 nM bDCN showed increased expression of *BGN*, *FMOD*, and *SCX* (p = 0.0313 for each), and decreased expression of *CSPG4* (p = 0.0313). Additionally, when PERI cell constructs were supplemented 25 nM bDCN, increases in expression of *MKX*, *DCN*, and *COL1A1* approached significance (p = 0.0625).

For the markers tested, overall fewer significant changes were seen in gene expression demonstrating improvements in tenogenesis for the tendon proper cell-derived constructs. TP cell constructs supplemented with 5 nM bBGN only demonstrated increased expression of *SCX* (p = 0.0313); supplementation of 25 nM bBGN led to an increase in *BGN* expression that only approached significance (p = 0.0625). No significant tenogenic improvements in expression were seen with supplementation of 5 nM bDCN or 25 nM bDCN.

When SLRP-treated PERI cell constructs were compared to TP cell control constructs, bBGN and bDCN PERI cell-derived constructs had similar matrix assembly marker expression levels as TP cell control constructs (**Fig. S-2.6**).

V. Discussion

Embedding small leucine-rich proteoglycans within a fibrin gel affected features of the engineered tendons. When considering gene expression, the supplementation of either exogenous biglycan or decorin had a greater effect on the tenogenic capacity of the equine peritenon cells than they did on tendon proper cells. Yet, biomechanical properties were bolstered by

supplementation of SLRPs for both cell types to varying degrees. PERI cells supplemented with bBGN or bDCN showed significant or approaching significant increased Young's modulus, and ultimate tensile strength in PERI cells increased with the addition of 25 nM bBGN. Moreover, tendon proper cell-seeded constructs had increased Young's modulus for and 25 nM bDCN, and nearly significant increases for 5 nM bBGN and 5 nM bDCN. These results suggest that SLRP supplementation can have positive tenogenic effects on extrinsic PERI cells and intrinsic TP cells.

Cells within a connective tissue can be affected by changes in their tissue niche. For example, if biglycan and decorin expression are absent during development the resulting changes affect the fibril structure with a shift toward larger diameters. In addition to alterations in mechanical properties, such as a failure at lower loads, decreased stiffness, and increase in percent relaxation, knocking out expression of biglycan and decorin affects collagen fiber realignment with a slower response to load [7, 9, 12]. Other knockout SLRP models, including biglycan, decorin, and double biglycan and fibromodulin, have varying degrees of apparent phenotypes including accelerated degeneration of articular cartilage, subchondral sclerosis, reduced growth rate of bone with decreased bone mass, and disruption of proper collagen fibrillogenesis [47,48,49,50]. Conversely, supplementation of SLRPs provides evidence for crucial roles in: signaling pathways, such as the TGF-β (transforming growth factor beta), WNT, TLR (toll-like receptor), EGFR (epithelial growth factor receptor) internalization, and Akt -dependant/−independent; collagenase shielding; collagen fibrillogenesis in the form of wound healing and scar mitigation; and proteoglycan regulation [26, 50,51,52,53,54,55,56,57,58,59].

When evaluating gene expression of the PERI supplemented cells, 5 nM bDCN showed significant increases in *BGN*, *FMOD*, and *Scleraxis* (*SCX*) and a significant decrease in *CSPG4*. The increase in tendon specific markers may be the result of regulation in the TGFβ pathway with

decreased activation of ERK1/2 resulting in increased expression of SCX and subsequently SLRPs like *BGN* by TGFβ [60, 61]. Although the cross-linking marker LOX tends to decrease, biomechanics (UTS and Young's Modulus) increase and the fibril distribution is shifted to smaller fibrils indicating that more collagen fibrils are being produced (supported by a trend towards increased *COL1A1* expression) but the fibrils are not maturing and cross-linking remains low (**Fig. 2.5, Fig. S-2.3**). Previous studies in DCN knockout mouse models identified an increase in fibril diameter with subsequent decrease in elastic and viscoelastic properties while alterations of the dermatan sulfate side chains had no effect on mechanical properties indicating that the decorin core protein itself is essential for the organization of collagen and its resulting tissue mechanics [12, 26, 47]. In contrast to *DCN*, the 5 nM bBGN supplementation produced increases in *CSPG4* expression, suggesting that although *BGN* and *DCN* have similar signaling pathways in collagen fibrillogenesis they are antagonistic in perivascularization. In breast carcinoma cells, *DCN* had an anti-angiogenic effect while BGN in bone fractures increased pro-angiogenic signals such as *VEGFA* (vascular epithelial growth factor A) showing that *BGN* and *DCN* have antagonistic effects, thus explaining the difference in *CSPG4* expression between bBGN and bDCN in PERI cells [62, 63]. This would indicate that during fibrillogenesis following an injury, *DCN* may play a vital role in mitigating scar formation by preventing vessel growth allowing improved tissue function [64, 65].

Tendon proper and peritenon cells responded differently to supplementation. Such variations could be due to the differences in niche composition within which these cells exist in vivo as well as differences in cell origins, both of which could affect the tenogenic capacity of these cell types [18,19,20, 66]. The tendon proper niche consists of a stiff, relatively acellular and hypoxic

environment with cells undergoing mechanical load and recoil activating characteristic signaling molecules such as *TGFβ* and *Egr1/2* through induction of *SCX* [67]. Other major regulators of tendon maturation and differentiation include *GDF5* (growth and differentiation factor 5) and mohawk (*MKX*) in addition to the SLRPs such as fibromodulin, and biglycan [13, 67, 68]. The peritenon niche is not as clearly defined but is comprised of cells which: (1) express perivascular markers such as endomucin (*EMCN*), *CD34*, and *CD45*; (2) secrete stimulatory factors during repair; (3) express matrix remodeling-related genes (matrix metalloprotease, *MMP1* and *MMP3*, and *COL3A1*); and (4) possess high cellular phenotypic heterogeneity [18,19,20, 66].

Peritenon cells had a more pronounced response to SLRP supplementation, particularly for DCN. This suggests that DCN may contribute to the peritenon cells transitioning into a tendon-like phenotype after the initial inflammatory response or that DCN aids in the collagen fibril assembly in the extracellular matrix. These functions could be instrumental for tendon repair since peritenon cells are a highly mobile cell type, reacting immediately in response to an injury. Additionally, from the expression and biomechanics data, DCN supplementation could improve peritenon cell utility in engineered tendon grafts. Though tenocytes (TP cells) might seem to be good tissue engineering candidates, it is interesting to compare the response of PERI cells to SLRP supplementation relative to the non-supplemented TP control. When PERI cell-seeded constructs supplemented with SLRPs were compared to TP control constructs – tenocytes that might be used in grafts. Increases in UTS, Young's modulus, and MTL were significant or approaching significance with all four doses of SLRPs used (**Fig. S-2.1**). Relative to TP control, the constructs with PERI cells had greater collagen content or increased levels approaching significance (**Fig. S-2.1**). Moreover, PERI cell-derived constructs had similar matrix assembly marker expression levels (**Fig. S-2.6**). Although there were no differences in fibril density or mean diameter, bBGN

and bDCN supplemented peritenon cells displayed a shift towards larger fibrils which partially explains the increased UTS, Young's modulus, and MTL (**Fig. S-2.1, S-2.4, S-2.5**). The expression of CSPG4 in bDCN supplemented peritenon constructs was similar control TP cell-derived construct levels, which supports a shift away from a perivascular-like phenotype. This suggests the utility of DCN, in particular for peritenon cells, as a phenotype influencing signaling molecule capable of affecting cells of both regions in the tendon. This finding could have implications during injury repair and cell selection for engineered grafts. Many findings in this study support the supplementation of SLRPs like BGN and DCN in therapeutic strategies. Further studies are required to discern the exact mechanisms by which supplemental SLRPs are affecting PERI cells.

This study has a number of important limitations. First, purified bovine proteins were used with the equine cells instead of equine-derived SLRPs. Second, cellular responses to SLRP supplementation are being described in an in vitro model where the active agent is continuously present in the matrix which would not be the case in vivo in a potentially pathological or inflammatory environment. Third, construct numbers were limited, and thus extensive histological analysis, analyses of other SLRPs, and the combinatorial effects of BGN ad DCN were omitted. Fourth, the tendon cells were isolated from horses with a range of ages and breeds. Fifth, the findings are based upon cells of the equine superficial digital flexor tendon. Therefore, the results might not translate to other tendons and ligaments. Finally, a limited n = 5 horses were used in to compare controls and treatments individually to determine if defined and hypothesized improvements in tendon formation were seen with SLRP supplementation. While this might limit the statistical power of the study, it allows for preliminary answers into the efficacy of SLRP supplementation for improving tendon formation. Moving forward, the evaluation of SLRPs in an in vivo injury model could provide further insight into this novel therapeutic intervention for injury

repair for equine athletes of all disciplines. Moreover, our findings lend support to further studies that include the incorporation of SLRPs in tendon engineering strategies.

VI.   References

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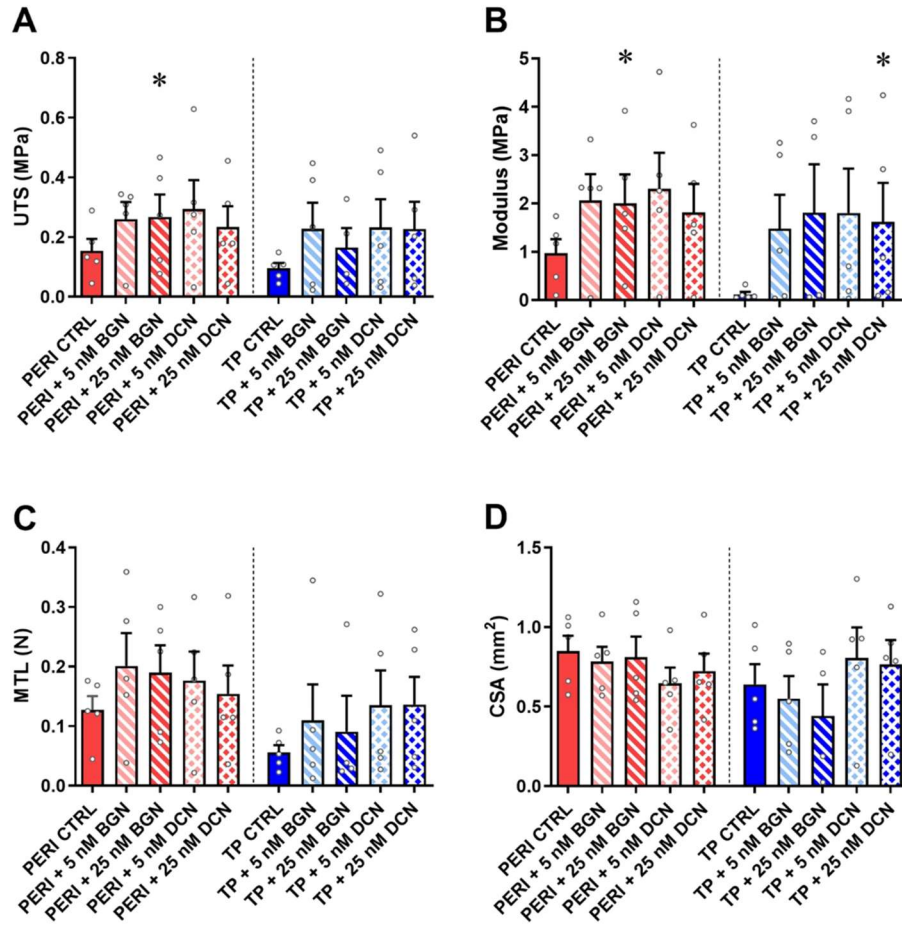
Figure 2.1. Biomechanical properties for 5 and 25 nM purified bBGN or bDCN supplementation. (A) Ultimate tensile strength (UTS), **(B)** Young's modulus, and **(C)** Maximum tensile load (MTL) were measured across five biological replicates and plotted as mean ± SEM. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation. n. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as $p \leq 0.05$, relative to the respective TP or PERI control; n = 5. Outliers detected by the Grubbs' test in technical replicates (UTS, 4; Young's modulus, 3; MTL, 2; CSA, 1; $p < 0.05$) were removed
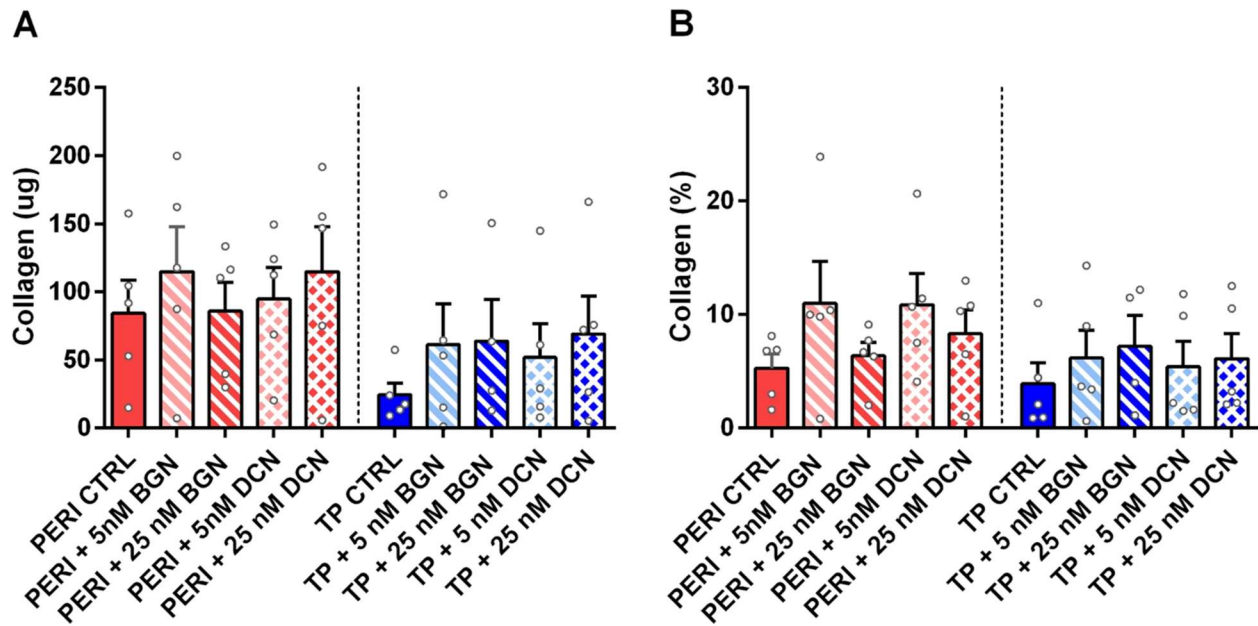
Figure 2.2. **Collagen content for 5 and 25 nM purified bBGN or bDCN supplementation.** Collagen analysis for **(A)** collagen content and **(B)** collagen fraction by dry mass in the tissues; plotted as mean ± SEM. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as p ≤ 0.05, relative to the respective TP or PERI control; n = 5.
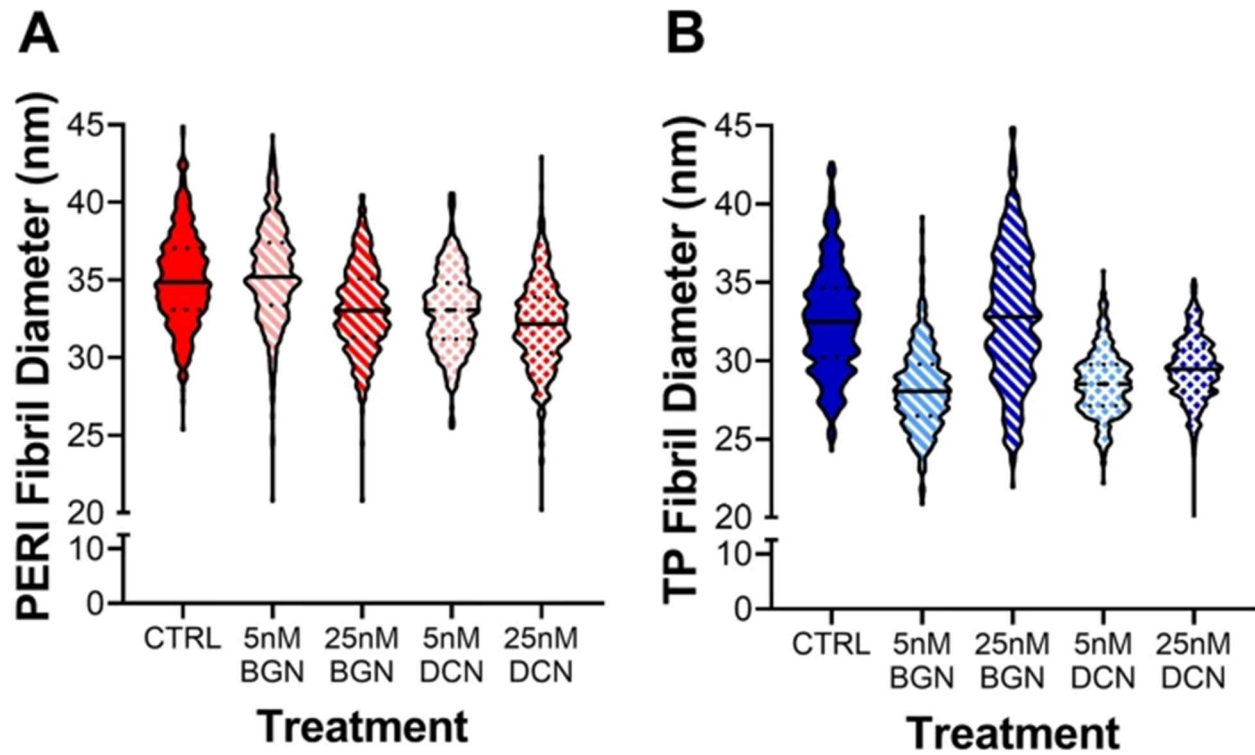
Figure 2.3. Fibril diameter analysis for samples supplemented with 5 or 25 nM bovine BGN or bovine DCN. Fibril diameter distributions are given as violin plots for constructs seeded with **(A)** peritenon (PERI) and **(B)** tendon proper (TP) cells, n = 5.
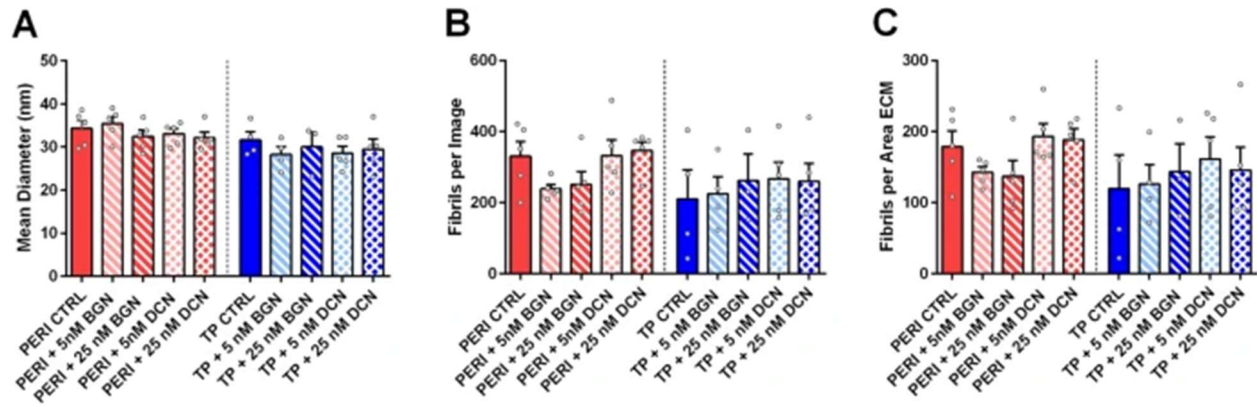
Figure 2.4. Fibril quantity analysis by mean diameter, density, and fibrils per area of extracellular matrix. (A) Mean fibril diameter, **(B)** Fibril number per image, and **(C)** fibrils per area of ECM per image were counted for all treatments; plotted as mean ± SEM. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation; ECM: extracellular matrix. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as $p \leq 0.05$, relative to the respective TP or PERI control; n = 5
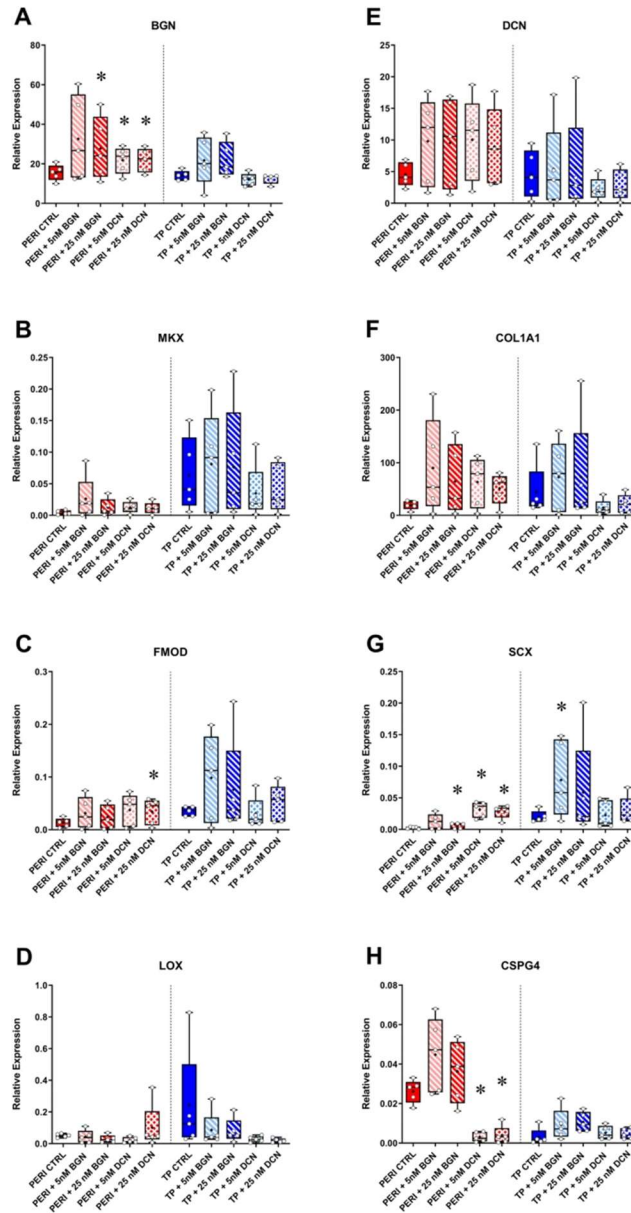
Figure 2.5. RT-qPCR analysis of Perivascular and Tenogenic markers. For each gene, the gene expression was plotted against the housekeeping gene *POLR2A* and plotted as mean relative expression ± SEM. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as $p \leq 0.05$, relative to the respective TP or PERI control; n = 5.

VIII.   Supplementary Information

	Fwd	Rev	Probe
POLR2A	CCAGGATGACCTGACTCACAAA	CGTCGAAGCTGATTGTTGATCT	TGGCGGACATTGTT
BGN	GGTGGGCGTCAACGACTT	GCCATTGTAGTAAGCCCGTTTG	CCCGTGGGTTTCG
DCN	TGCGAAAAGCGGTGTTCA	TGGGTTGGTGCCCAGTTCTA	ACTGAACCAGATGATAGTC
MKX	TCATGTTCCGAAGATGGAGAAA	ATTGTAGCCCCCTTCGTTCA	TCCTCCAAGAAACCAC
COL1A1	GGGCCGAGGGCAACA	GTGGTTTTGTATTCGATCACTGTCTT	CTTCACCTACAGCGTCAC
FMOD	AACCAAGGAGGCCAGACAGA	TGCATTTGTCTCTCTCAAGTTGAA	ACGTGGTCACTCTGAA
LOX	GCTTGGCCAGCTCAGCAT	TCTTAGCAGCACCCTGTGATCA	CAGGTCAGATGTCAGAGAT
CSPG4	CTCCTGGAGAGAGGTGGAACAG	TCAGTGTCTCGCTCCCATCA	AGCTGATCCGCTATGTG
SCX	ThermoFisher, cat no 4351372, Ec03818452_s1 -- Proprietary information		

Table S-2.1. Equine Taqman primer probe sets designed using Primer3 or predesigned for RT-qPCR. Forward, reverse, and probe sequences for *POLR2A, BGN, DCN, MKX, COL1A1, FMOD, LOX, CSPG4,* and *SCX* for TaqMan specific RT-qPCR. Primer probe sets underwent dimerization, hairpin formation, melting temperature and GC content scrutiny. All primer probe sets were validated in native equine tendon proper and peritenon tissue.

Figure S-2.1. Biomechanics comparing tendon proper (TP) control against peritenon cells supplemented with bBGN and bDCN. **(A)** Ultimate tensile strength, **(B)** Young's modulus, and **(C)** maximum tensile load for TP control cells were compared to peritenon treatment groups. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as $p \leq 0.05$, relative to the respective TP or PERI control; $n = 5$, plotted as mean + SEM. Measurements approaching significance (p=0.0625) included: **(A)** 5 nM bBGN, 5 nM bDCN, and 25 nM bDCN; **(B)** 5 nM bBGN and 25 nM bDCN; **(C)** 25 nM bBGN and 5 nM bDCN. Outliers detected by the Grubbs' test in technical replicates (UTS, 2; Young's modulus, 2; MTL, 1; $p < 0.05$) were removed.

Figure S-2.2. Collagen content and collagen fraction by dry mass comparing TP control and peritenon treatment groups. Tendon proper (TP) cells were plotted against peritenon treatment groups for (A) collagen content and (B) collagen fraction by dry mass. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as $p \leq 0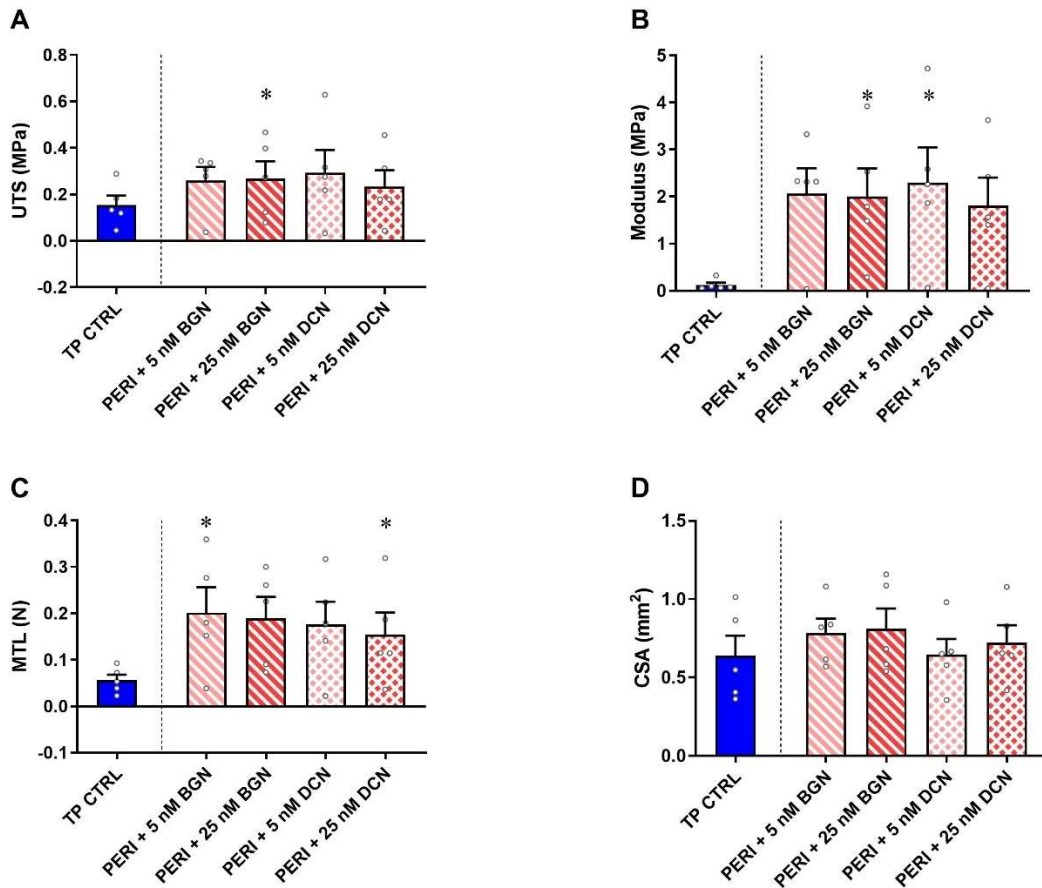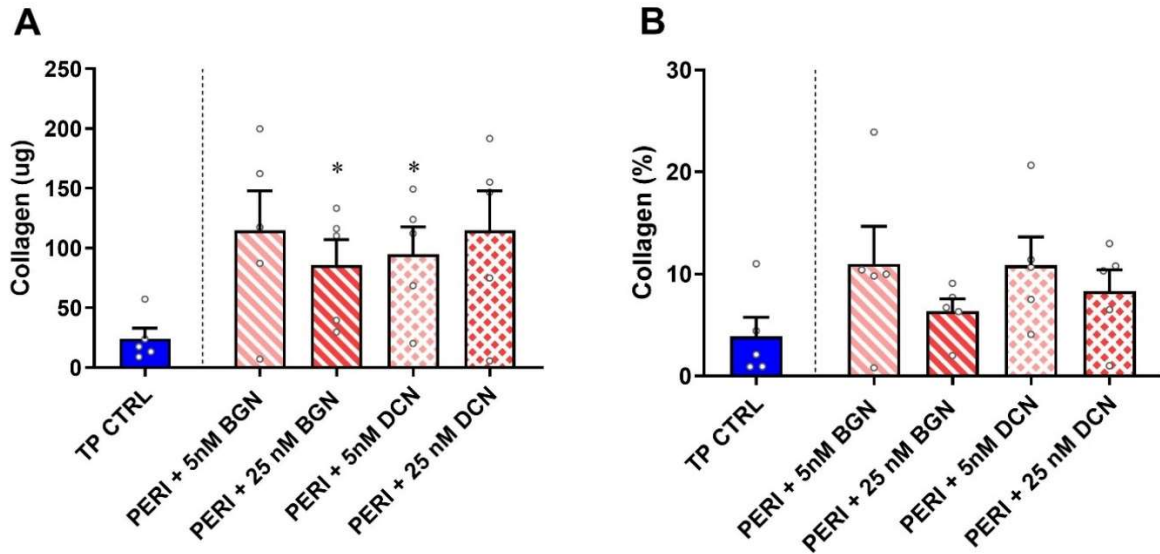.05$, relative to the respective TP or PERI control; $n = 5$, plotted as mean + SEM. Measurements approaching significance (p=0.0625) included: (A) 5 nM bBGN and 25 nM bDCN.
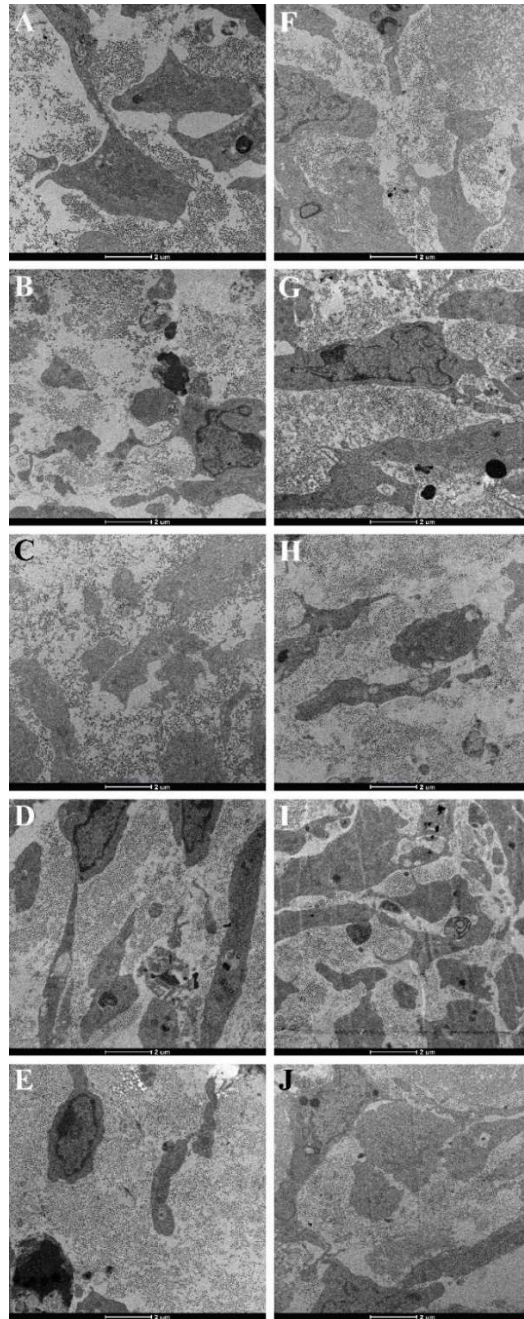
Figure S-2.3. **Qualitative representation of TEM cross-sections at 5300x magnification.** **(A)** PERI CTRL, **(B)** PERI + 5 nM bBGN, **(C)** PERI + 25 nM bBGN, **(D)** PERI + 5 nM bDCN, **(E)** PERI + 25 nM bDCN, **(F)** TP CTRL, **(G)** TP + 5 nM bBGN, **(H)** TP + 25 nM bBGN, **(I)** TP + 5 nM bDCN, and **(J)** TP + 25 nM bDCN are represented with low magnification TEM images.
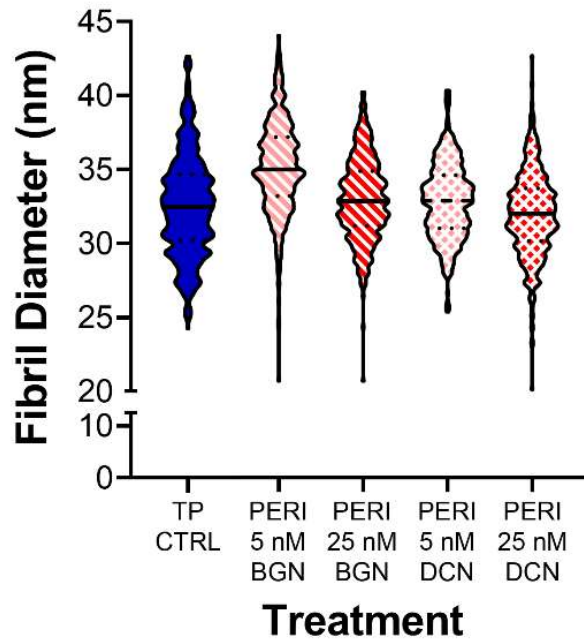
Figure S-2.4. Fibril diameter distribution analysis comparing tendon proper control against peritenon samples supplemented with 5 or 25 nM bBGN or bDCN. Fibril diameters were calculated and plotted as a violin plot for untreated TP constructs versus PERI +5 nM bBGN, PERI + 25 nM bBGN, PERI + 5 nM bDCN, and PERI + 25 nM, n = 5.
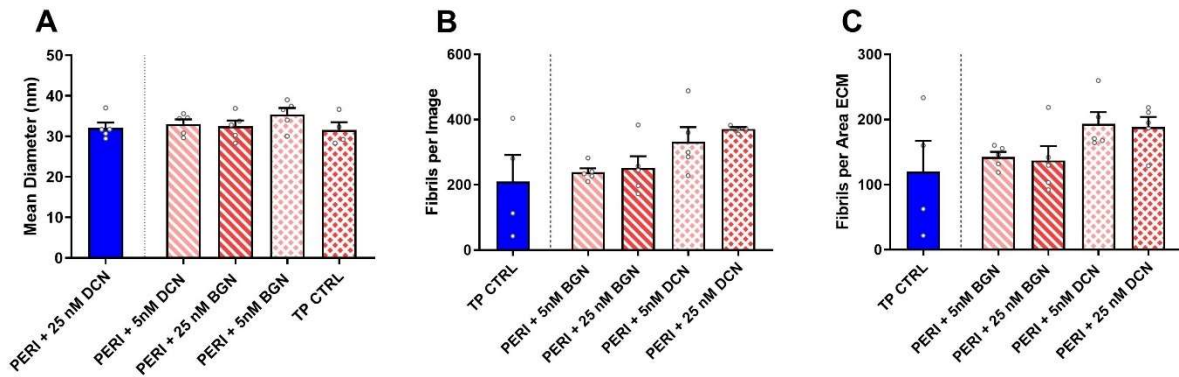
Figure S-2.5. Collagen fibril quantity analysis between TP and peritenon treatment groups. (A) Mean fibril diameter (nm), **(B)** fibril density, and **(C)** fibrils normalized per area of extracellular matrix were compared for TP control and peritenon samples treated with 5 or 25 nM bBGN or bDCN. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation; ECM: extracellular matrix. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as $p \leq 0.05$, relative to the respective TP or PERI control; n = 5.
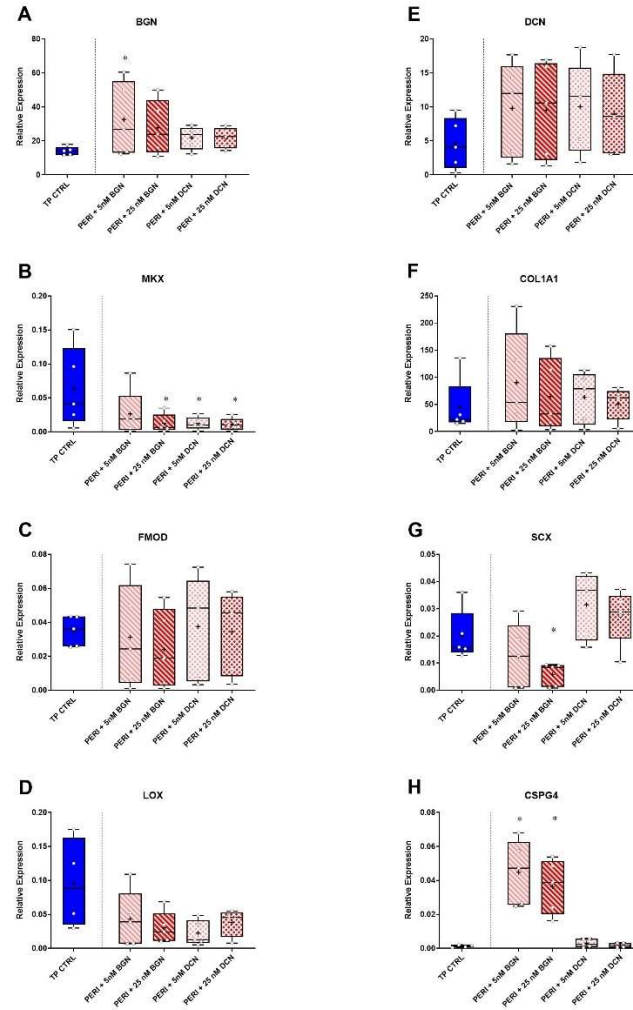
Figure S-2.4. RT-qPCR analysis for TP and peritenon treatment groups. Gene expression (*POLR2A* was used as the housekeeping gene) for TP control constructs were compared to peritenon constructs supplemented with 5 and 25 nM bBGN or bDCN and plotted as relative expression ± SEM. TP: tendon proper cells; PERI: peritenon cells; CTRL: no bBGN or bDCN supplementation. Expression is plotted in a box and whisker plot with "+" representing the mean, box representing first-third quartile, line representing median, and whisker representing range. Significance is based on one-sided nonparametric Wilcoxon signed-rank tests predicting improvement: *, significant as p ≤ 0.05, relative to the respective TP or PERI control; n = 5.

Chapter Three: Decoding the Transcriptomic Expression and Genomic Methylation Patterns in Resident Cell Populations in the Aging Horse Tendon

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Keywords: equine, tendon, peritenon, RNASeq, RRBS, bioinformatics

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I.   Abstract

Background: Tendinopathies in recreational and elite athletes remains as an ongoing challenge due to the poor healing capacity of tendons commonly resulting in high reinjury rates. Different cell populations, tendon proper and peritenon, contribute to the tendon matrix in differing capacities in times of inflammation, remodeling, and aging. As a result of the composition and mechanical action of energy-storing tendons, such as the superficial digital flexor tendon (SDFT), these tendons are susceptible to high injury rates. Furthermore, aged tendons have decreased repair potential but the underlying transcriptional and epigenetic changes that occur in the tendon proper and peritenon cells across age from adolescent through midlife and into geriatric ages is not well understood. The objective of this study is to assess the regional differences between the tendon proper and peritenon in adolescent (0-5 yrs), midlife (6-14 yrs), and geriatric (15-27 yrs) horses using RNA sequencing and DNA methylation techniques.

Methods: RNA and DNA from non-breed or sex specific horse samples were isolated for Illumina HiSeqv4000 RNA sequencing and Reduced-Representation Bisulfate Sequencing (RRBS). All samples were quality controlled for integrity before sequencing using RIN scores, UV spectrometry, or gel electrophoresis. Raw FASTQ sequenced files were quality controlled using FASTQC before undergoing adapter trimming and mapping to the EquCab3.0 genome. RNASeq analysis was conducted in RStudio using DESeq2 for identifying differentially expressed genes (DEGs), in addition to, gene ontology analysis through PANTHER, and pathway analysis by the Advaita iPathway Guide. DNA methylation analysis for methyl calls, CpG island, and genomic annotation was performed using the RStudio packages Methylkit and subsequently differentially methylated regions (DMRs) were annotated by Genomation.

Results: Across age, regional differences were present between the tendon proper (TP) and peritenon (PERI) population in the superficial digital flexor tendon (SDFT) as evidenced by RNASeq and DNA methylation. Cluster analysis of all TP and PERI samples indicate that regardless of age, distinctions between the regions exist with genes such as *DCN, COMP, FN1*, and *LOX* maintaining elevated expression in the TP while genes such as *GSN* and *AHNAK* are abundant in PERI. Gene ontology indicated that increased gene activity was present in adolescent and geriatric populations and decreased during midlife. Furthermore, looking at the changes of the TP and PERI cell populations across age, differences in abundance of differentially expressed genes (978, 110, 970; adolescent, midlife, geriatric) and differentially methylated regions were present (1587, 2319, 515; adolescent, midlife, geriatric). Notably, when evaluating all ages of TP against PERI, five genes (*HAND2, CHD9, RASL11B, ADGRD1*, and *COL14A1*) had regions of differential methylation as well as differential gene expression.

Conclusions: The tendon proper and peritenon cell populations in native tendon maintain regional differences regardless of age. Furthermore, increased activity in the adolescent and geriatric groups occur compared to midlife most likely due to the ongoing growth and maturation in adolescence and the degradation from aging in geriatric. In conclusion, applications of novel therapeutics to address tendinopathies would be most effective in the adolescent (0-5 yrs) and midlife (6-14 yr) age groups while the geriatric population is already undergoing a tendinopathy as a result of aging.

II. Background

Across numerous disciplines and species, tendinopathies in recreational and elite athletes are an ongoing challenge due to the inadequate healing capacity of tendons [1, 2]. Tendon repair is often slow and incomplete thus resulting in increased reinjury rates and subsequent career-ending injuries [1, 3, 4]. Specifically, musculoskeletal injuries in racehorses are the primary cause of death at about 2/1000 starts in Thoroughbreds and Quarter Horses, and a majority of these injuries occur in the forelimb (80-90%) [5-11]. Equine athletes of all disciplines are not exempt from these tendinopathies; as a result, there is a need for novel therapeutics. Additionally, aging also significantly affects the overall outcome of tendon homeostasis and repair due to decreased repair capacity and impaired structure at the extracellular, collagen, and gene expression level with increased age [12-18]. Due to the constant recoil and elongation of the superficial digital flexor tendon (SDFT), it is particularly susceptible to injury and subsequently is the most common tendon injury (75-93%) as compared to the deep digital flexor tendon or common digital extensor tendon [2, 19-21].

When energy-storing tendons are injured, there are several factors inhibiting adequate tendon repair. The overall structural complexity of tendons provides a gross mechanical challenge to healing and potential stress-shielded tissue [22]. Post injury, immunomodulatory hurdles must also be overcome as inflammatory cells release cytokines and growth factors to stimulate macrophage and tenogenic fibroblasts [23]. These factors affect the intrinsic tendon proper and extrinsic peritenon cell populations [13, 24, 25]. Furthermore, with aging, the challenges tendon healing must overcome are exacerbated by decreased tenocyte proliferation and increased matrix degeneration [15, 26]. Although it is well established that tendon healing decreases with age, as seen in mechanical and age-related injury model studies, the biological processes and molecular

functions underlying aging in equine tendon are not well understood [15, 18, 27]. Additionally, epigenetic changes associated with aging have emerged in recent years as another factor responsible for impaired tendon healing, but limited studies have considered the normal physiological process in aging tendon [28, 29]. Expressional and epigenetic assessments of tendon aging could provide essential biological context for predicting how novel therapeutics might work or fail when applied to injured equine tendons. The goals of this study are to elucidate gene expression markers defining the peritenon and tendon proper regions, to determine the gene transcripts associated with maturation and aging, and to determine associations of DNA methylation and changes in marker expression by age and location in the tendon. RNASeq and Reduced-Representation Bisulfite Sequencing (RRBS) were used to reach these goals.

III.   Materials and Methods

3.2.1 Tendon Harvest

Superficial digital flexor tendons were harvested from ten non-breed or gender specific horses from three age ranges (0-5 yrs adolescent, 6-14 yrs midlife, and 15-27 yrs geriatric) and two tendon regions (tendon proper and peritenon) to be used for RNA sequencing (RNASeq) and Reduced-Representation Bisulfate Sequencing (RRBS). Samples were collected from horses that were euthanized for reasons unrelated to this study; thus, they were exempt from approval of the University of California Davis Institute of Animal Care and Use Committee. Before collection and use of these tissues upon euthanasia, it was confirmed that these horses had no signs or known history of tendinopathies. For each sample, 2.5 cm of the superficial digital flexor tendon located 10-15 cm proximal to the forelimb fetlock were harvested. Immediately upon removal from the limb, samples were rinsed a minimum of three times in fresh Dulbecco's Phosphate Buffer

Solution (DPBS, Life Technologies, Benicia, CA, USA) containing 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10,000 ug/mL streptomycin, and 25 ug/mL amphotericin B, Life Technologies) before transport [24, 30]. Samples were either immediately snap frozen in liquid nitrogen as whole tendons or isolated into the tendon proper and peritenon regions under a dissecting microscope, snap frozen, and then stored at -80° C until further processed. Tendon proper tissue was isolated by excising a 2 – 2.5 mm diameter cylinder from the center core of the tendon. Peritenon tissue was isolated by harvesting some of the viscous paratenon in addition to 1 mm of the epitenon from the tendon. Samples were sectioned, if not already, on dry ice and powdered while frozen to allow for better RNA and DNA isolation [31].

3.2.2 RNA Isolation and Sequencing

Nine tendon proper and nine peritenon frozen samples (3 adolescent, 3 midlife, and 3 geriatric) were homogenized in QIAzol lysis reagent (up to 300 mg of tissue sample in 3 mL reagent) using a BioSpec Tissue-Tearor and total RNA isolation was performed with a QIAGEN RNeasy Plus Micro Kit following kit instructions (QIAGEN, Valencia, CA). Total RNA was RNase-free DNase treated (QIAGEN, Valencia, CA) during RNA isolation. RNA integrity was assessed via Experion Automated Electrophoresis Station (Bio-Rad) and UV spectrophotometer (Nanodrop) and reverse transcribed for Illumina next-generation sequencing using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) [24]. RNA integrity was deemed appropriate if samples scored an RNA integrity number > 8 and UV260:280 ratio > 1.9. The cDNA from each sample was submitted to the UC Davis Genome Center for stranded library preparation (200-300 bp inserts), barcoding, and sequencing done by Illumina HiSeqv4000 on two lanes to produce 150 bp pair-ended reads.

3.2.3 RNASeq analysis

RNASeq analysis was conducted using the Ubuntu 18.04.2 LTS with miniconda2 for package management and downstream analysis in RStudio v 1.4.463 [32-35]. Untrimmed FASTQ files were checked for corruption using respective md5sum files and then quality controlled using FastQC 0.11.8 [36]. All samples showed high quality and thus progressed to adapter trimming by Trimmomatic 0.39 (PE; Illuminaclip: TruSeq2-PE.fa:2:0:15; LEADING:15; TRAILING:15; SLIDINGWINDOW:10:20; MINLEN:25) and were reexamined for quality controlled using FastQC and MultiQC 1.7 [37,38]. Transcriptome mapping was accomplished using Salmon 0.14.1 and mapped to the horse genome (EquCab3.0) to produce quantification files to be further processed in RStudio using DESeq2 1.30.0 [39-44] (**Fig. 3.1**). Samples in DESeq2 were subset into age groups (Adolescent, Midlife, Geriatric) and contrasted for cell type (TP vs PERI); in addition, one analysis contained all samples with a cell type contrast using a Wald significance test. Differentially expressed genes (DEGs) were considered as genes that had a q value (false discovery rate; FDR) < 0.01 and a log 2-fold change (L2FC) < -1 and > 1. Additionally, a variance stabilizing transformation (vst) was performed for Principal Component Analysis (PCA) and the top 25 most variable genes for heatmapping which utilized clustering rows and columns by Pearson correlation and the method by Ward.D. Gene Ontology (GO) was accomplished using PANTHER from differentially expressed genes (DEGs) hits [45]. Pathway, upstream regulator, and downstream regulator analyses were conducted using the Advaita iPathway Guide using normalized counts and false discovery rate (FDR) p-value correction (padj) [46-48].

3.2.4 DNA Isolation and RRBS

DNA isolation for each sample (10 tendon proper and 10 peritenon; 3 adolescent, 4 midlife, and 3 geriatric) was done using the QIAamp Fast DNA Tissue Kit and DNA integrity was assessed by UV spectrophotometer (Nanodrop; UV260:280 > 1.8) and gel electrophoresis. The extra (10th) sample was added because there was space in the sequencing flow cells for DNA analysis. Samples were submitted to the UC Davis Genome Center for preparation. Genomic DNA underwent restriction enzyme digestion with *MspI* (5'-CCGG-3' target sequence) to generate libraries enriched for CpG island and CpG shore regions. Adapters were added, and then bisulfite conversion, barcoding, amplification, and purification were done. Samples were sequenced by an Illumina HiSeqv4000 to produce 100 bp single end reads. For 10x coverage, 10 samples per lane were loaded over 2 lanes for a coverage of 1.7 million CpGs [47]. Reduced-Representation Bisulfite Sequencing (RRBS) was used to assess CpG island regions throughout the genome [49].

3.2.5 DNA Methylation analysis

Output FASTQ files from the Illumina HiSeqv4000 sequencer were first quality controlled using FastQC before undergoing adapter trimming [36]. FASTQ files were adapter trimmed using Trim Galore! 0.6.5 and then processed using Bismark 0.20.0 with mapping to EquCab3.0 [40, 43,44,50,51]. Bismark was used for genome preparation, alignment, and methyl call extraction to generate coverage and BAM files to be used in further analysis. Methylkit 1.16.0 was used for generation of methyl calls, CpG islands, and genomic annotation [52] (**Fig. 3.1**). Samples were filtered by read coverage to prevent PCR bias and increase the power of the statistical tests by discarding bases with high (above 99.9th percentile) and low (below 10x) read coverage with each sequenced and filtered CpG site assigned a percentage methylation score. Systematic over-sampling bias potentially affecting statistical tests was reduced by normalizing coverage.

Hierarchical clustering used Canberra distances and subsequently Ward for the method. Annotation of differentially methylated regions was performed using the package Genomation 1.22.0 and referenced to EquCab3.0 with promoter regions designated at 2kb up and downstream of the transcription start site (TSS) [53].

IV. Results

3.3.1 RNASeq

The age groups that were used were adolescent (< 5 yrs), midlife (6-14 yrs), and geriatric (> 15 yrs). Of the nine samples used for RNA sequencing, after analysis, eight of the samples were used for further comparison between tendon proper and peritenon through age. One sample – the 10-day old foal tendon proper – proved to be an outlier as quantified using the Hubert Robust Principal Component Analysis Outlier (ROBPCA) computation [54, 55] **(Fig. S-3.1).** The paired TP and PERI samples for the 10-day old foal were removed from further analysis. As a result, two paired TP and PERI samples were used for the adolescent group, three for midlife, and three for geriatric.

Principal component analysis (PCA) of all samples showed a clear separation of the peritenon and tendon proper samples. Additionally, the tendon proper samples clustered more closely together compared to the peritenon (**Fig. 3.2A**). The differentially expressed genes (DEGs) for TP vs PERI were plotted using a Venn diagram for the three age groups. For these TP vs PERI comparisons, adolescent and geriatric age groups had far more (978; 970, respectively) DEGs compared to the midlife group (110) (**Fig. 3.2B**). The ratio of up and down regulated genes for the adolescent and midlife groups were about even but for the geriatric group, the percent of down regulated genes were over 10% greater compared to both adolescent and midlife (adolescent:

51.75% down regulated; midlife: 48.18%; geriatric: 62.58%). Contrasting the overall samples for

TP vs PERI groups, 446 DEGs were calculated and of these the top 25 of the most variable genes

were plotted as a heatmap (**Fig. 2C**). The most variable genes were calculated as the expression

strength of genes and their deviation from the gene's average across all samples. As such, genes

with low expression but great variance from the norm were parsed out from the data. The heatmap

further validated the distinction between tendon proper and peritenon cells, regardless of age.

Twenty-one of the twenty-five genes were upregulated in TP samples across all age groups **(Tab.

3.1)**. Four other genes (*GSN, AHNAK, COL1A2*, and *COL1A1*) were also considered as top variant

genes, and once variable abundance was combined, these genes were more abundant in PERI.

Further comparisons were made for tendon proper and peritenon cells across age groups

in consideration of gene ontology (GO) pathways using PANTHER for biological processes and

molecular function to further elucidate the overall systemic changes of the two cell populations

through aging. Considering biological processes, 15 GO terms were shared between adolescent,

midlife, geriatric, and all samples, while the molecular function had 8 similar GO terms. The total

number of genes for each comparison were plotted as bars with additional lines for the percent of

up or down gene hits against the total number of genes (circular point lines) and lines for the

percent of gene hits against the total number of process hits (triangle point lines). The additional

lines further inform the respective weight of the functional hit to better compare the results across

age groups as the raw number of DEGs between age groups vary. A general trend noted for

biological processes and molecular functions is that there are fewer expressional differences

between TP and PERI regions in midlife horses when compared to adolescent and geriatric as seen

by a 'V' in the line graph (**Fig. 3.3, 3.4**). Notably, across age, in biological processes from

adolescent through midlife to geriatric cellular component organization or biogenesis

(GO:0071840) decreases while response to stimulus (GO:0050896) and immune system process (GO:0002376) increases between TP and PERI cells (**Fig. 3.3A, 3.3F, 3.3O**). Additionally, biological regulation (GO: 0065007), cellular process (GO: 0009987), and metabolic process (GO: 0008152) had minimal change between groups but had a large proportion of function hits (triangle point lines) suggesting that regardless of age group these biological processes are critical for tendon proper cells though the overall genes comprising each group changes over age. In contrast, more consistent trends were seen in downregulated genes for biological processes across age contrasts with minimal percentage changes between adolescent, midlife, and geriatric indicating larger impacts from changes in up DEGs. Notably, biomineralization (GO:0110148) is only seen in aged samples, upregulated by the TP, and the DEGs persist when considering all samples.

Molecular function pathway analysis followed the same 'V' trend as for biological processes for both up and down regulated DEGs with some exclusions (**Fig. 3.4**). Varying trends were seen in molecular transducer activity (GO:0060089) and translation regulator activity (GO:0045182) with elevated molecular transduction in geriatric required for increased regulation and translation regulation in adolescent necessary during growth and development.

When assessing the expression differences in common tenogenic, extracellular matrix, and perivascular markers, there were significant differences in expression profiles between TP and PERI across age in some of these markers (**Tab. 3.2**). Midlife samples did not display any differences across these genes though adolescent and geriatric groups showed some changes for both PERI and TP. *BMP1, COL1A1*, and *COL5A1* had false discovery rates (FDRs), denoted as padj, less than 0.05 with negative log 2-fold changes. These changes either imply decreased expression in the tendon proper with maintained peritenon expression or maintained tendon proper expression and elevated peritenon expression. *CD44* indicated upregulation in the tendon proper

population compared to the peritenon most likely due to its anti-apoptotic pathway. Endomucin (EMCN) had two gene variants with significant expression though in differing directions. *EMCN* (XM_014738644) had normalized counts only in the peritenon samples while *EMCN* (XM_023637656) had normalized counts only registered in the tendon proper samples. Although *EMCN* was significantly detected in both cell populations, there may be variant specificity occurring depending on the location.

Alternatively, in the geriatric group comparing TP and PERI, numerous extracellular, organizational, and perivascular genes (*COMP, DCN, LOX, MKX*, and *CSPG4*) were significantly upregulated in the TP samples. Additionally, genes known to increase mineralization (*BMP2*) were also upregulated. *CD44* was downregulated in the geriatric group, further indicating the potential aging response that is occurring within the tendon between the TP and PERI.

Pathway analysis using Advaita iPathway Guide identified pathways between TP and PERI for Adolescent, Midlife, Geriatric, and all samples combined. For the adolescent group, dilated cardiomyopathy (14 genes; padj 0.011) was the only significant pathway with a padj < 0.05 (**Tab. 3.3**). Some of the genes that contributed to the identification of this pathway included *LAMA2*, *TGFB1, CACNA1S*, and *MYH6* for TP while *ITGB7* and *PRKACA* were identified in PERI. Midlife TP vs PERI samples did not identify any significant pathways. Conversely, the geriatric group had numerous significant pathways such as ECM-receptor interaction (25 genes; padj 0.002), focal adhesion (45 genes; padj 0.002), Rap1 signaling pathway (41 genes; padj 0.018), regulation of actin cytoskeleton (38 genes; 0.022), Hippo signaling pathway (27 genes padj 0.025), and proteoglycans in cancer (39 genes; padj 0.033) (**Tab. 3.3**). Notably, the ECM-receptor interaction pathway had 25 significant genes for this pathway and included genes such as fibronectin 1 (*FN1*), tenacin X (*TNXB*), *COL4A6*, *COL6A6*, and *CD44* were upregulated in TP for

this pathway. Thrombospondin 1 and cartilage oligomeric matrix protein (*COMP*) had log fold changes (LFC) indicating abundance in PERI relative to TP (**Tab. 3.3**).

The comparison of all samples of TP and PERI cell populations for pathway analysis indicate significant changes in the cAMP signaling pathway (21 genes; padj 0.016) and pathways associated with dilated cardiomyopathy (14 genes; 0.021) (**Tab. 3.3**). Specifically, within the cAMP signaling pathway, some genes of interest potentially affecting the TP cells include *ACOX1, CREBBP, PIK3CA, RHOA*, and *CREB5* while genes upregulated in the PERI cells in this pathway include *PDE3B, PPARA, F2R*, and *AKT3* (**Tab. 3.3**).

Using the Advaita iPathway Guide for identification of upstream activators or inhibitors, only the geriatric samples comparing TP vs PERI for upstream activation produced a significant result with a padj < 0.05 for the gene of interest (*CAST*; padj = 1.00E-06) and its activator status (*CAST*; padj = 0.029) (**Fig. 3.5**). The downstream genes that were inhibited by the activation of *CAST* were *TLN1, ITGB1, MMP2, PTK2*, and *PXN*.

3.3.2 DNA Methylation

Clustering of all samples for CpG methylation, showed grouping emerging with TP and PERI, regardless of age, separated with a third group of a geriatric sample clustering with itself (**Fig. 3.6**). The out clustering of this sample may indicate some underlying epigenetic factors present in the horse that could not be seen on a gross inspection or in gene expression.

Further investigation of the specific genomic regions between TP and PERI populations shows differentially methylated regions (DMRs) present in all age groups. DMRs were annotated to the EquCab3.0 genome for distances to transcription start sites (TSS), promoter, exon, intron, and intergenic regions, and association to gene features. Total DMRs ranged from 2319 in midlife

to 447 in all samples (515 geriatric; 1587 adolescent) (**Tab. 3.3**). Specifically, the highest percent of DMRs in the promoter region are in the geriatric age group (11.26%) and the lowest in all combined samples (7.83%; 9.64% adolescent; 9.83% midlife) (**Tab. 3.3**). DMRs in the exon region ranged between 2.52% to 4.7% (4.16% adolescent; 4.7% midlife; 2.52% geriatric; 2.68% all samples) (**Tab. 3.3**). Intergenic differential methylation for all groups remained around 47% and the greatest percent of DMRs for the intron region was in all samples contrasting TP and PERI (**Tab. 3.3**). The methylation changes between the two cell populations in all samples contributed to the TP presenting a greater proportion of hypomethylation across all chromosomes with cutoff values of qvalue < 0.01 and percent methylation difference > 25% (**Fig. 3.7**). The peritenon samples were denoted as the 'control' and the tendon proper as 'treatment' for comparing differential methylation across chromosomes. The tendon proper samples had more abundant hypomethylation compared to the peritenon. Chromosomes 4, 9, 13, 17, 21, 25 – 27, and 29 – 32 had no regions of hypermethylation compared to the peritenon. Chromosomes 14 and 23 had slightly higher regions of hypermethylation in the tendon proper as compared to other chromosomes (**Fig. 3.7**).

When comparing the DMRs to DEGs that were identified in RNASeq between TP and PERI for all samples, 8 targets were identified across 5 genes (*ADGRD1, CH9, COL14A1, HAND2*, and *RASL11B*) (**Tab. 3.5**). Two genes (*ADGRD1* and *COL14A1*) had two differential methylation targets associated contributing to the additional targets. The shared gene targets were mainly located within the intron region although *HAND2* and *RASL11B* were in the intergenic region. All target genes were hypomethylated in the tendon proper samples indicating that these genes are hypermethylated in the peritenon cell population.

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V.   Discussion

The intrinsic tendon proper and extrinsic peritenon populations play differing roles in tendon development, maturation, and healing.   Secondarily, with age, tendons undergo compositional changes in the tendon matrix causing alternations to material properties [56-61]. As such, elucidation of the gene expression and epigenetic changes of the intrinsic and extrinsic cell populations of tendons across age will give insight into the potential effectiveness of a therapeutic at a certain age.

In both gene expression and methylation, the intrinsic tendon proper population and extrinsic peritenon population of the tendon remain as two distinct regions. This has been well established in numerous study models; however, even with age, in normal tendon without tendinopathies the populations maintain regional physiological genomic differences as seen with differential gene expression (DEG) and differentially methylated regions (DMR) [24, 25, 62-68].   Although there are nuances to the gene profiles within the population, the regions maintain separation. Furthermore, regardless of age, common tenogenic genes such as *DCN, COMP*, and *LOX* maintain elevated expression in the tendon proper while genes such as *GSN*, a cell migration, proliferation, and inflammatory gene, and *AHNAK*, a cell proliferation and differentiation gene, are more abundant in the peritenon [69].   The genes abundant in the peritenon may in part attest to their role of early migration and proliferation in tendinopathies as well as the inflammatory response that accompanies injury.

When evaluating DMRs and DEGs across all ages to compare between TP and PERI, five genes are identified. Three genes express abundance in the PERI (*CHD9, ADGRD1*, and *COL14A1*) and two genes in TP (*HAND2* and *RASL11B*). The adhesion G-protein-coupled receptor (GPCR) family (*ADGRD1*) has been indicated to play pivotal roles in the

musculoskeletal system and G protein cascades, though their role in tendon has yet to be elucidated. Additionally, *CHD9* has recently been implicated in upregulating *RUNX2* which may shed light on ectopic ossification that may occur after injury [70]. *HAND2* plays a major role in limb development, crucial for the establishment of the anterior-posterior axis but is also required for vascular development and regulation of angiogenesis. Potentially, the vascularization that occurs as a result from the peritenon may be undergoing regulation from the tendon proper cell population through the expression of *HAND2* [71-73]. *RASL11B*, which is more abundant in TP, plays a role in maturation of primary macrophages with indication that it affects TGF-B1-mediated developmental processes and pathophysiology related to inflammation. It also contributes to tendon differentiation. As such, some elucidation into the regulation of the peritenon by the tendon proper may be concluded as it is known that the peritenon plays distinct roles in pro-angiogenesis and pro-inflammatory responses.

Notably, the greatest gene variation occurs in the adolescent, as tendon is maturing and the skeletal system is becoming fully developed, and in the geriatric when tendon is mechanically and compositionally compromised therefore needing repair (**Fig. 3.2B, 3.3, 3.4, Tab. 3.2**). Furthermore, there is also a shift in the expression profile between the two age groups. In adolescence, the DEGs between TP and PERI are similar in amount, while in geriatric, a shift towards TP downregulation with potential higher expression in the PERI region is occurring. Evidence of a lack of tendon proper cellular capacity is seen in the geriatric injury model which may be resulting in a peritenon compensation to maintain tendon integrity [74-76]. A similar response is potentially occurring in uninjured aging tendon due to the structural and compositional changes [77]. Additionally, evaluation of tendon genes of interest showed increased expression of *BMP2* in TP with pathway evidence of biomineralization. Potentially

in response, increases in ECM matrix assembly (*DCN, LOX, MKX*), vascular (*CSPG4*), and tendon growth (*COMP*) markers were seen with decreased *CD44* further supporting that even in uninjured tendon, at a geriatric age, the tendon is already undergoing a repair process as a result of compositional changes [78-80]. The ECM-receptor interaction pathway, responsible for its role in adhesion, proliferation, apoptosis, degradation, and mechanoreceptors, further indicates that the geriatric cellular population is undergoing compositional changes and attempting to address the compromised tissue structure and function (**Fig. S-3.2**). As the collagen is degraded through age, the intrinsic tendon proper population aims to maintain tendon homeostasis but fails as evidence in biomechanics, structural, and functional shortcomings indicating that any additional injury to the tendon may be overtaxing the system [18].

Contrastingly, during midlife a notable lack of significant expression from both the tendon proper and peritenon is seen. Overall DEGs, genes of interest, gene ontology, and pathway analysis all indicate that although a regional difference is maintained (as indicated by clustering in both RNA expression and methylation) there a level of homeostasis that is occurring in the tendon during midlife. During adolescence, growth, development, and maturation are occurring; in the geriatric age, there is a response to a degenerating structure. During aging, increases in methylation have been observed in other tissues such as skeletal muscle, blood, liver, and epidermis supporting evidence of age-related changes in DNA methylation by increased promoter methylation in the aging geriatric tendon (**Tab. 3.4**) [81-86]. The differentially methylated regions in midlife are much greater compared to the other groups (2319 vs 1587 and 515; adolescent and geriatric respectively). Although, percent methylation in the promoter, exon, intron, and intergenic regions remains comparable to the other ages with

far fewer significant genes detected in differential gene expression (110 vs 978 and 970; adolescent and geriatric respectively). With a majority of the DMRs being hypomethylation, access to the genome is present but transcription is not proceeding due to factors potentially outside of histone modifications, DNA methylation, and chromatin remodeling (**Fig. S-3.3**). As such, without an overabundance of genes is already being expressed in the tendon, when injury occurs, a preexisting profile of genes may not be interfering with the healing process and therefore a potential for targeted therapeutics may exist.

From this study we can conclude that regional differences in expression persist for many genes across age between the intrinsic (tendon proper) and extrinsic (peritenon) tendon populations. Expression differences seem greatest in earlier (adolescent) and later (geriatric) ages of the tendon (possibly due to growth, development, and maturation; degeneration and aging). Additionally, in DNA methylation of the geriatric individuals, a striking difference of hypermethylation develops in the promoter region. During midlife, although abundant expressional differences were not present, expressional changes could be stimulated due to the drastic levels of DNA hypomethylation in this age group. These findings are valuable in providing insight for future therapeutics by understanding the transcriptome and methylome across age. With this, identification of novel therapeutics can be age tailored as changes in the methylome and transcriptome may impact future tendon repair outcomes.

VI. References

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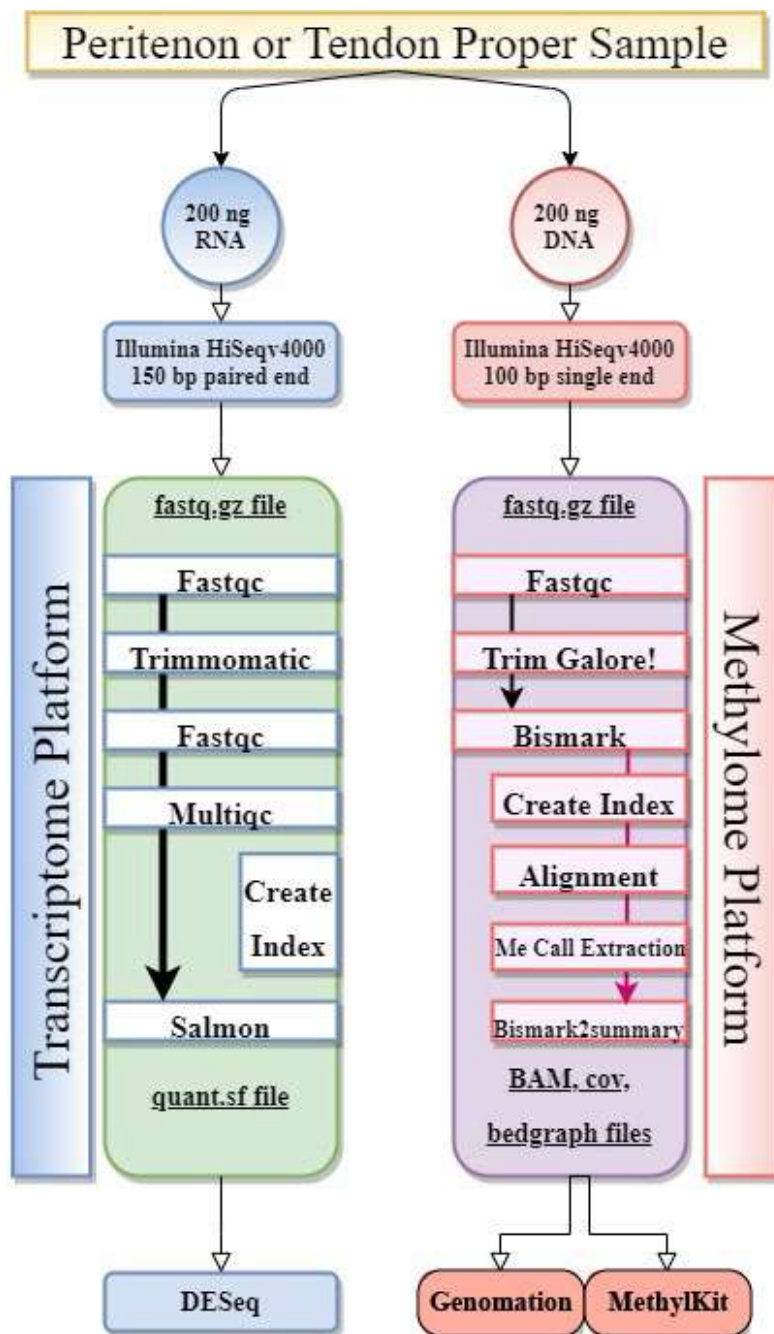
Figure 3.1. The workflow for sample preparation and analysis. Samples were taken through either the transcriptome or methylome workflow platform with alignment to EquCab3.0 before further downstream analysis.
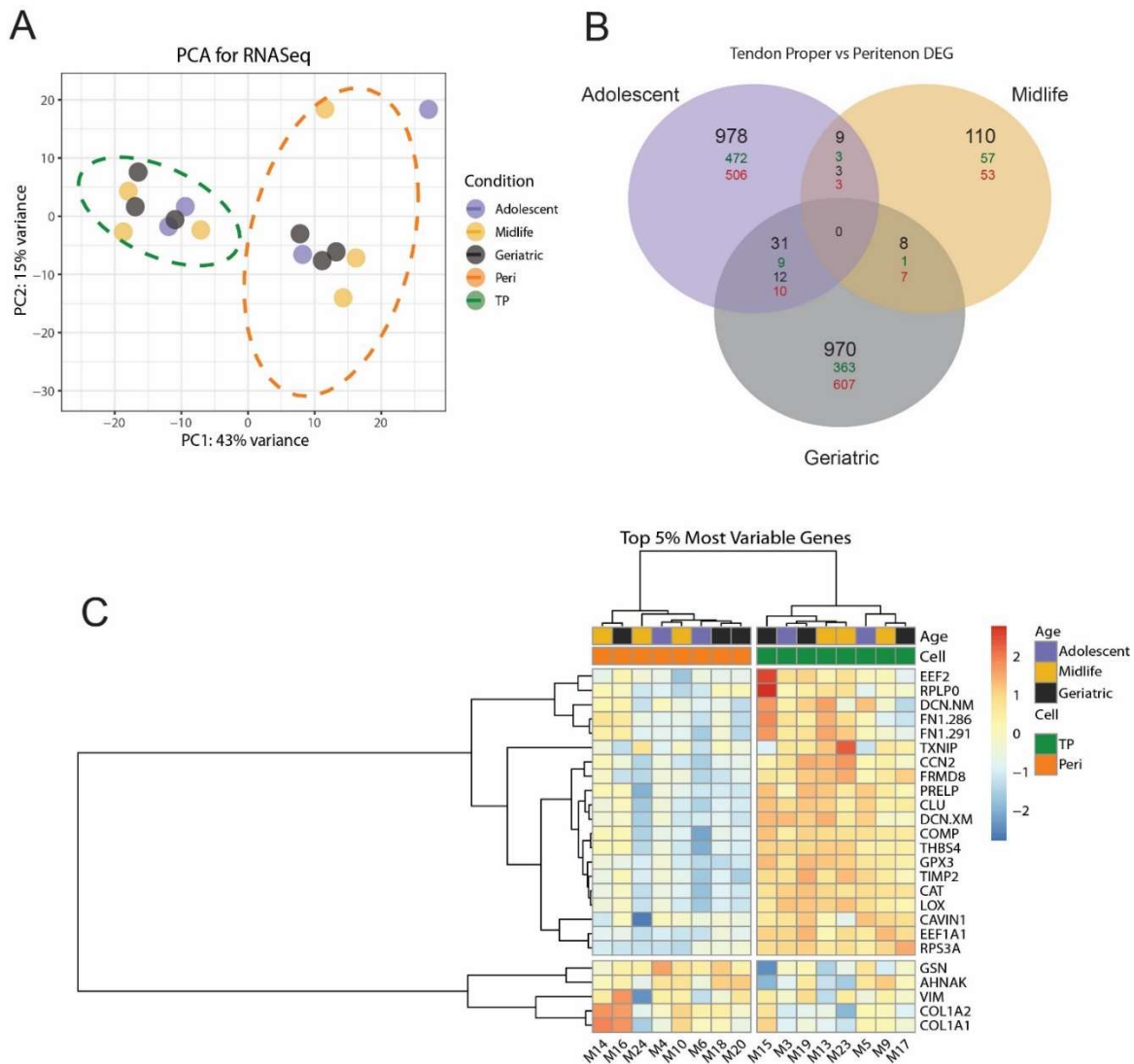
Figure 3.2. RNASeq analysis identifying sample relatedness. (**A**) The principal component analysis of all samples shows clear separation of TP and PERI cell populations with limited separation of samples by age. (**B**) A Venn diagram of samples by age when comparing TP vs PERI presented more DEGs in adolescent and geriatric ages. Black numbers signify the total DEGs, green as more abundant in TP compared to PERI, red as PERI more abundant than in TP, and blue as dependent on the sample the gene was either increased in TP or PERI. (**C**) A heatmap of the top 25 most variable genes further supported a separation between TP and PERI cell populations in genes closely tied to either region.

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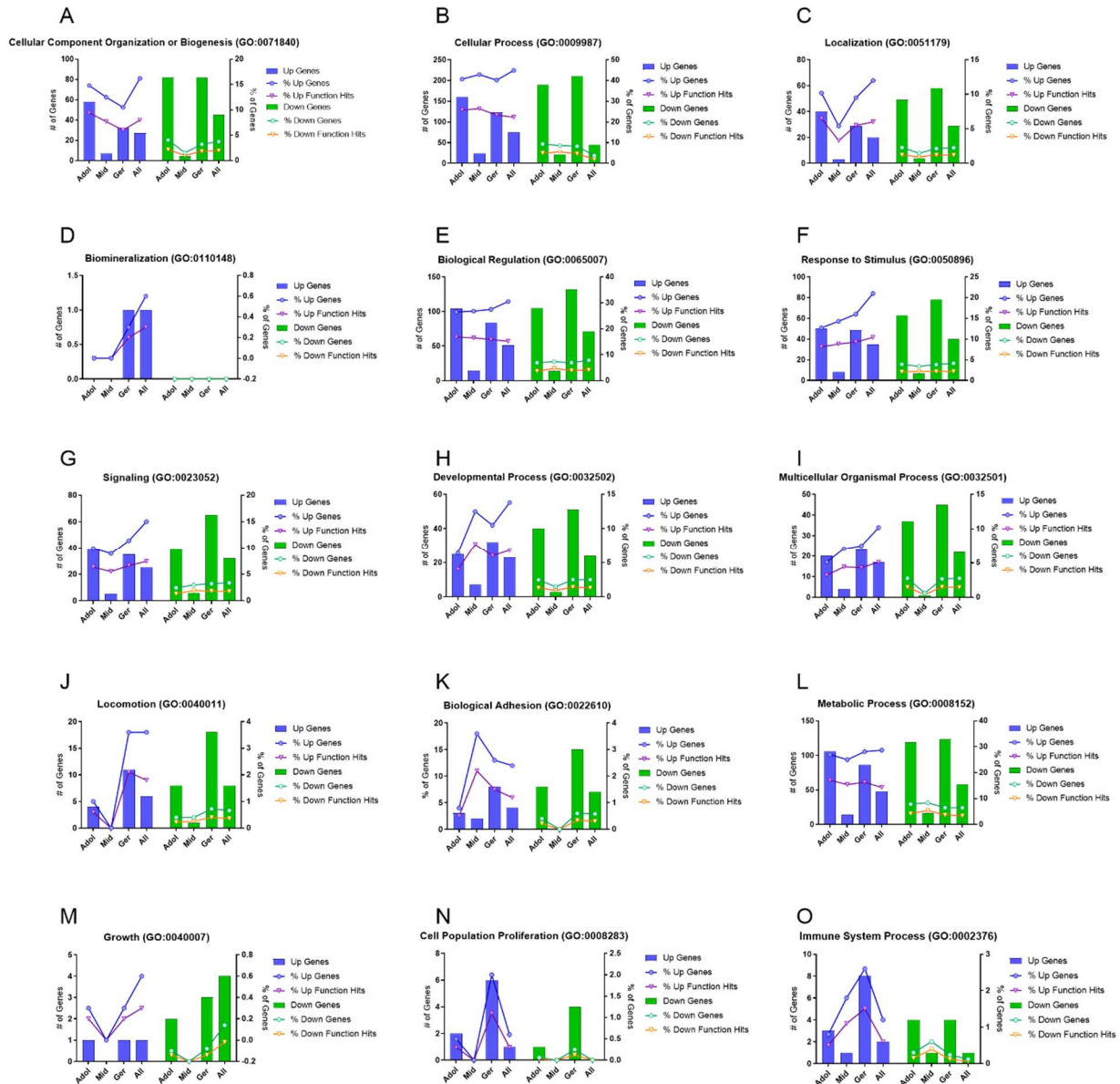
Figure 3.3. GO analysis for biological processes using PANTHER. Gene Ontology (GO) analysis for age groups between TP and PERI samples with at least two groups sharing a common GO term. Up regulated genes are in blue histogram bars while down regulated genes are in green. Further clarification for comparison was made with line graphs showing the percent of genes hit compared to the total genes (circle point line) and the percent of genes hit compared to the individual process (triangle point line).
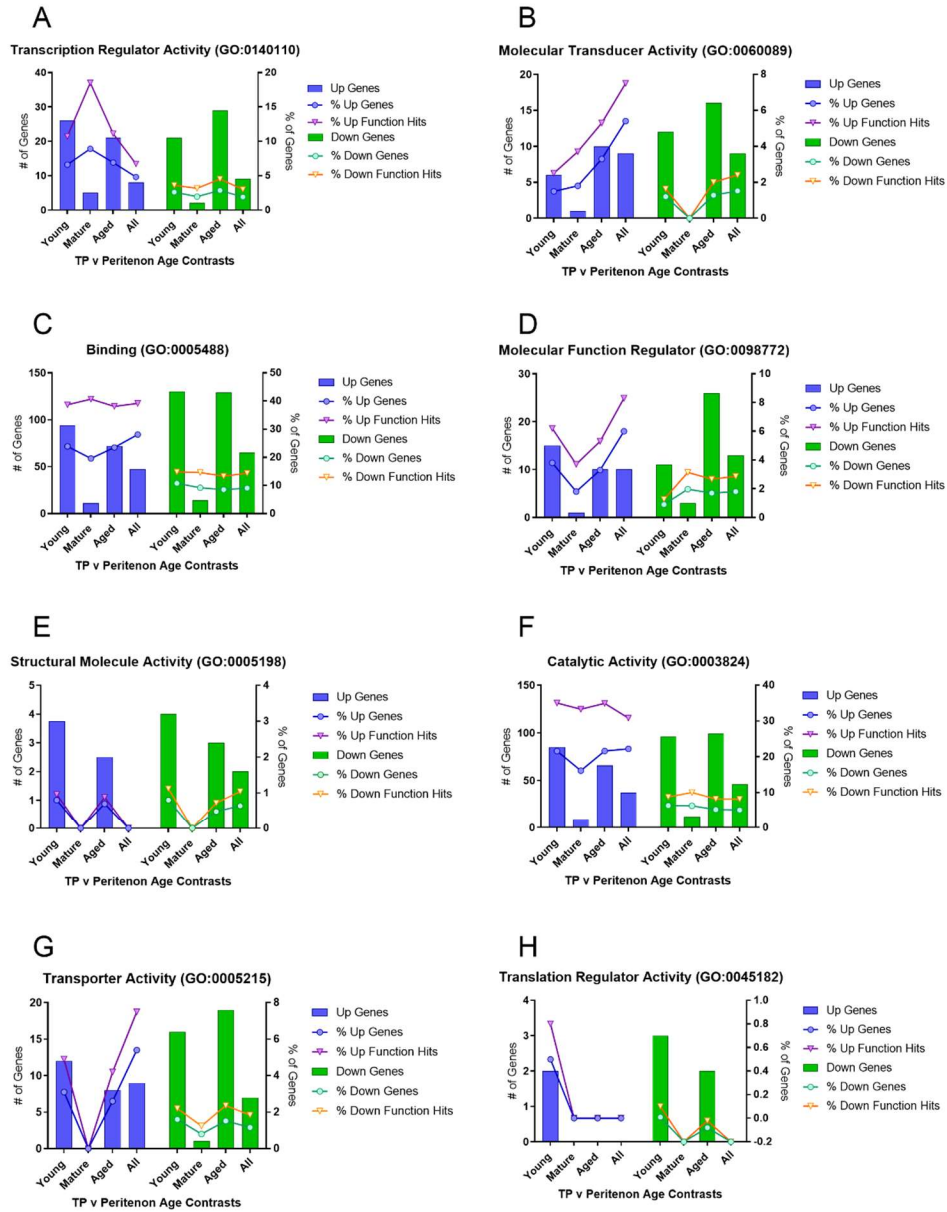
Figure 3.4. GO analysis for molecular function using PANTHER. Gene Ontology (GO) analysis for age groups between TP and PERI samples with at least two groups sharing a common GO term. Upregulated genes are plotted as blue histogram bars and downregulated genes in green. Line graphs were used to further clarify comparisons with circle point lines showing the percent of genes hit to the total genes and the triangle point lines indicating the percent of genes hit for an individual function.
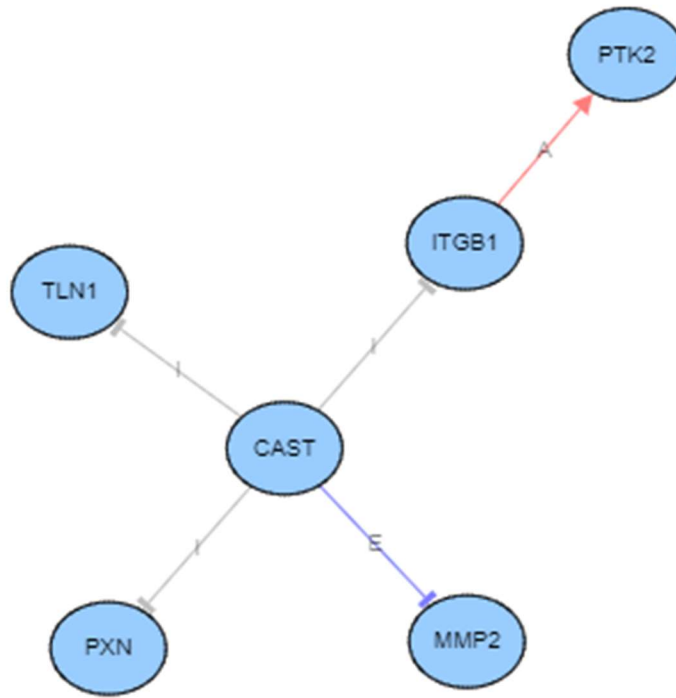
(c) Advaita Corporation 2021

Gene	padj	LFC	Measured DE Targets	padj Activator
CAST	1.00E-06	10	5/8	0.029
TLN1	0.000001	-10		
ITGB1	0.000001	-1.341		
MMP2	0.006795	-1.300		
PTK2	0.000029	-0.992		
PXN	0.015553	-0.890		

Figure 3.5. Upstream Activation Regulator for Geriatric TP vs PERI. The upstream regulator predicted as an activator for TP vs PERI in geriatric samples and its downstream genes are diagramed with the interaction effect (I: Inhibition; A: Activation; E: Expression). Padj and LFC related to the expression profiles for TP vs PERI are included in the table accompanying the figure.
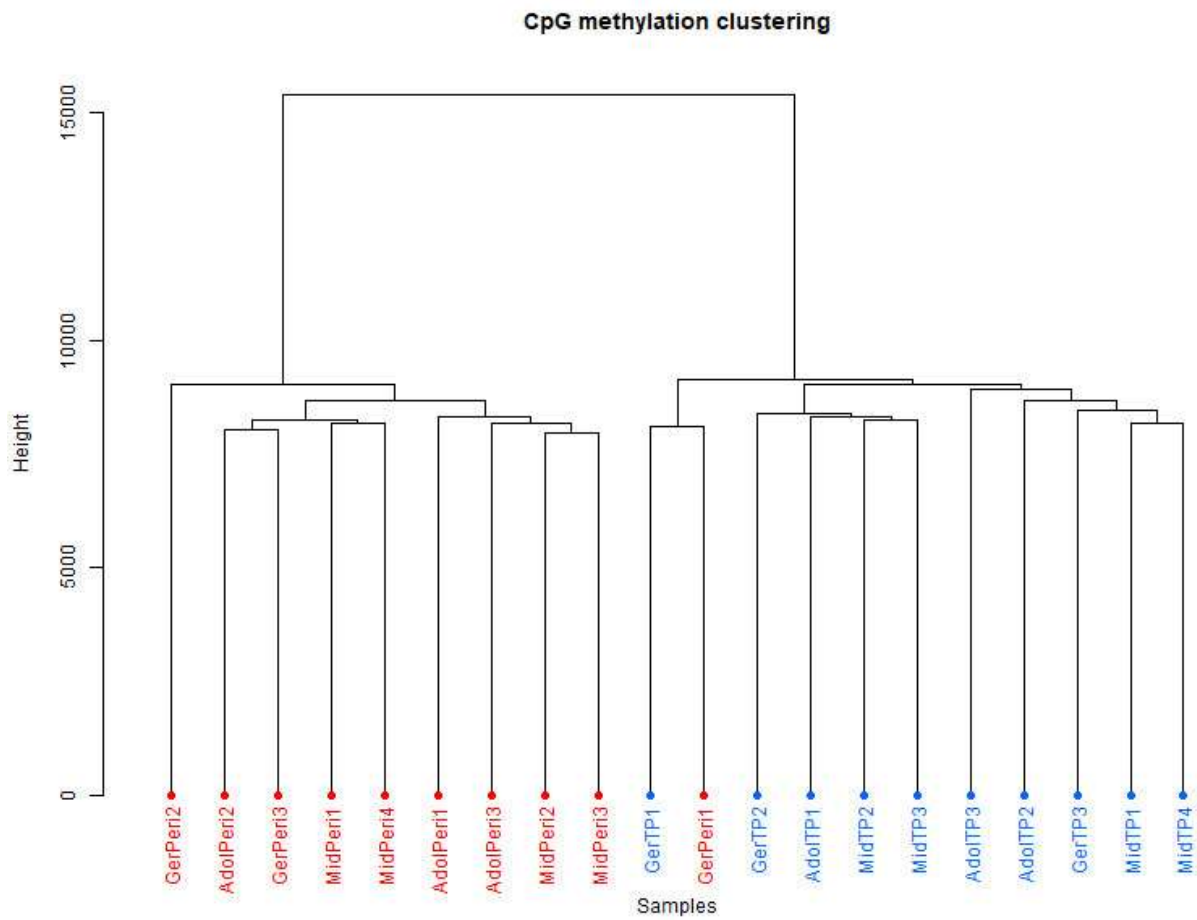
Figure 3.6. CpG methylation clustering for all samples across all age groups. Hierarchical clustering of all samples showed separation of TP and PERI cell populations. A geriatric sample clustered with itself, indicating a potential underlying epigenetic alteration.
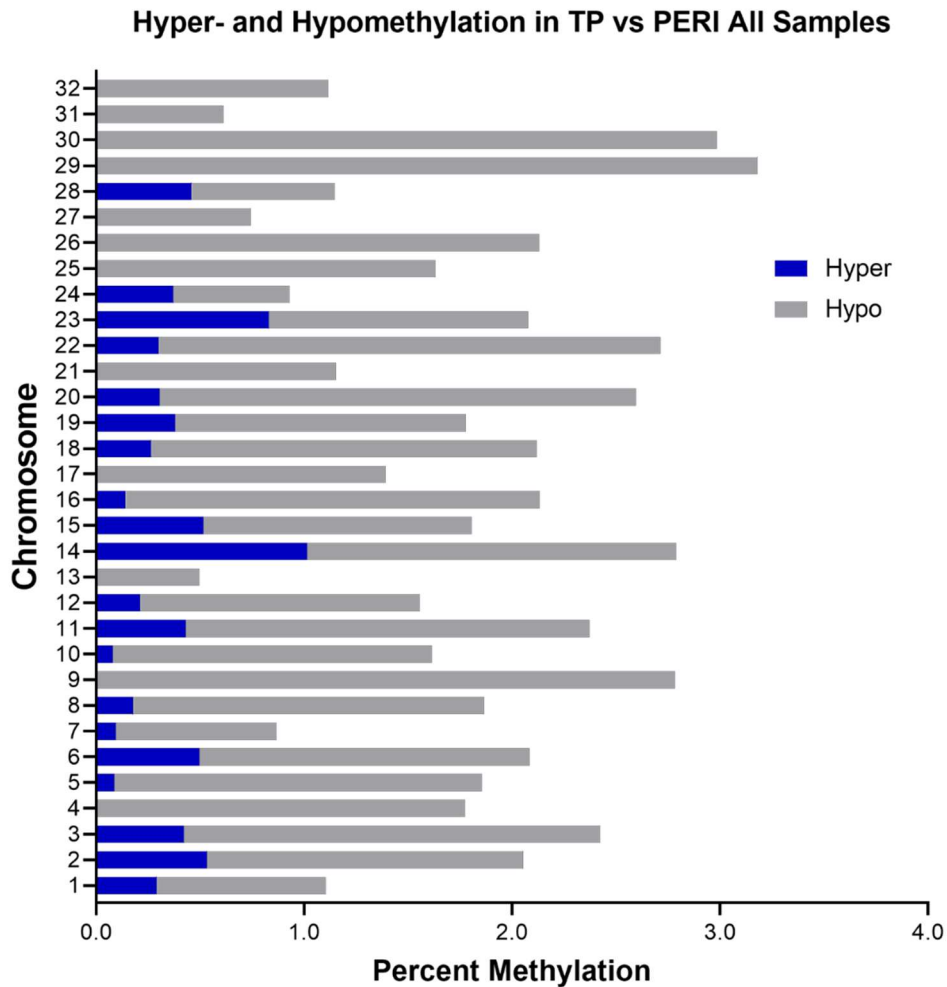
Figure 3.7. Hyper- and hypomethylated percent differences across all equine chromosomes when comparing TP against PERI for all samples. When looking at hyper- and hypomethylation, the tendon proper expresses more regions of hypomethylation across all chromosomes compared to the peritenon. The peritenon is treated as a 'control' and the tendon proper methylation calls are designated as 'treatment'. Although some increased regions of hypermethylation also occur, such as on Chr 14 and 23, substantially more chromosomes are hypomethylated in the tendon proper compared to the peritenon. Differentially methylated regions were considered hits with a qvalue < 0.01 and percent differential methylation > 25% between groups.

Gene	RefSeq	Gene Name	Cell Type Adundance	Variable Abundance
EEF2	XM_001915097	Eukaryotic translation elongation factor 2	TP	0.105
RPLP0	NM_001252576	Ribosomal protein lateral stalk subunit P0	TP	0.138
DCN.NM	NM_001081925	Decorin	TP	0.513
FN1.286	XM_023642286	Fibronectin 1	TP	0.595
FN1.291	XM_023642291	Fibronectin 1	TP	0.934
TXNIP	XM_023641298	Thioredoxin interacting protein	TP	0.259
CCN2	XM_023651101	Cellular communication network factor 2	TP	1.212
FRMD8	XM_023654435	FERM domain containing 8	TP	0.638
PRELP	XM_001915274	Proline and Arginine rich end leucine rich repeat protein	TP	1.134
CLU	NM_001081944	Clusterin	TP	1.180
DCN.XM	XM_005606467	Decorin	TP	1.008
COMP	NM_001081856	Cartilage oligomeric matrix protein	TP	2.182
THBS4	XM_023618094	Thrombospondin 4	TP	1.622
GPX3	NM_001115158	Glutathione peroxidase 3	TP	1.251
TIMP2	XM_023651899	TIMP metallopeptidase inhibitor 2	TP	0.723
CAT	XM_001914718	Catalase	TP	1.935
LOX	XM_023617820	Lysyl oxidase	TP	1.625
CAVIN1	XM_001494473	Caveolae associated protein 1	TP	0.359
EEF1A1	NM_001081781	Eukaryotic translation elongation factor 1 alpha 1	TP	0.192
RPS3A	XM_001501474	Ribosomal protein S3A	TP	0.255
GSN	XM_023628604	Gelsolin	PERI	-0.420
AHNAK	XM_023654210	AHNAK nucleoprotein	PERI	-0.132
VIM	NM_001243145	Vimentin	TP	0.006
COL1A2	XM_001492939	Collagen type I alpha 2	PERI	-0.666
COL1A1	XM_023652710	Collagen type I alpha I	PERI	-0.335

Table 3.1. Top 25 most variably expressed genes. *EEF2, RPLP0*, two transcript variants of *DCN*, two transcript variants of *FN1, TXNIP, CCN2, FRMD8, PRELP, CLU, COMP, THBS4*, *GPX3, TIMP2, CAT, LOX, CAVIN1, EEF1A1*, and *RPS3A* were all upregulated in the TP samples compared to PERI. Four genes (*GSN, AHNAK, COL1A2*, and *COL1A1*) were upregulated in PERI though when looking at individual variable abundance across all the samples, the genes had more variability between TP and PERI. *VIM* was very slightly more abundant in TP.

Gene Symbol	RefSeq	Adolescent TP v PERI		Midlife TP v PERI		Geriatric TP v PERI		All TP v PERI	
		padj	L2FC	padj	L2FC	padj	L2FC	padj	L2FC
BGN	NM_001081839	0.8510	-0.5706	0.99995	-1.3885	0.7059	1.0439	0.9998	0.2716
BMP1	XM_005607660	**0.000002**	**-1.7199**	0.99995	-0.7324	0.9796	-0.1753	0.9998	-0.0160
BMP1	XM_005607662	0.9999	-0.1805	0.99995	-0.9983	0.9166	0.8803	**0.0041**	**8.0417**
BMP2	XM_023626136	0.9999	-0.0311	0.99995	0.8360	**0.0002**	**1.9449**	**0.0064**	**1.9852**
CD44	XM_005598023	**0.0012**	**1.0756**	0.99995	-0.1368	**0.0399**	**-0.8104**	0.9998	-0.3052
COL1A1	XM_023652710	**0.0275**	**-1.1807**	0.99995	-0.6733	0.8201	-0.7596	0.9998	0.1383
COL1A2	XM_001492939	0.3906	-0.9936	0.99995	-0.6628	0.5029	-1.1818	0.9998	-0.5151
COL5A1	XM_023629284	**0.0133**	**-0.7507**	0.99995	-1.0524	0.9741	-0.1735	0.9998	-0.1165
COL14A1	NM_001163870	0.9755	0.4472	0.99995	-0.8647	**0.00090**	**-1.8458**	**0.0077**	**-1.9667**
COMP	NM_001081856	0.9918	-0.7957	0.99995	0.1852	**0.0018**	**3.4633**	**0.0162**	**4.5950**
CSPG4	XM_005602901	0.5524	-1.3342	0.99995	-0.2909	**0.0219**	**1.6050**	0.2564	1.9340
DCN	NM_001081925	0.9853	-0.3141	0.99995	-1.0096	0.3940	1.3663	0.6764	1.2716
DCN	XM_005606467	0.9875	0.4134	0.99995	-0.6091	**0.0120**	**1.9728**	**0.0426**	**2.2465**
EGR1	XM_001502553	0.1714	1.0839	0.99995	-0.0547	0.8385	0.3928	0.9471	-0.4547
EMCN	XM_014738644	**0.00007**	**-8.6756**	0.89027	7.3201	1.0000	-2.8830	0.9175	-0.4980
EMCN	XM_023637656	**0.0034**	**7.7222**	1.00000	-4.0162	0.9897	-0.2128	0.9998	0.6460
FMOD	NM_001081777	0.7095	-1.1449	0.99995	-1.7627	0.3612	1.6307	0.6198	1.7823
FMOD	XM_005609562	0.7049	-1.2166	0.99995	-1.5459	*0.0857*	*2.1297*	0.4892	1.9520
LOX	XM_023617820	0.9866	0.8119	0.99995	0.6449	**0.0064**	**2.6260**	**0.0017**	**3.6832**
LOX	XM_023617821	0.9999	-0.4132	0.99995	0.1023	**0.0155**	**2.9197**	**0.0007**	**4.7060**
MKX	XM_023632372	0.9999	0.2827	0.99995	0.9987	**0.0244**	**2.3604**	**0.0416**	**2.5332**
SCX	NM_001105150	0.4276	-2.3190	0.99995	0.4775	*0.0626*	*2.8441*	0.6767	2.1033
TIMP1	XM_023633181	0.9999	0.0389	0.99995	-0.6297	0.9972	0.0180	0.9998	0.0881
TIMP2	XM_023651899	0.9997	0.1975	0.99995	0.6356	0.1015	1.3328	0.0644	1.5320

Table 3.2. RNASeq differential expression for tendon related genes of interest. Tendon genes of interest were parsed out from their respective tendon proper and peritenon contrasts in adolescent, midlife, and geriatric groups, in addition to all samples. Gene contrasts with a false discovery rate (padj) < 0.01 are in bold with the accompanying log 2-fold change (L2FC) while gene contrasts between 0.1 and 0.05 padj are in italics. All genes have their accompanying RefSeq number specifying the gene variant. Significant gene contrasts that have a positive L2FC, indicating higher expression in tendon proper or a contrastingly lower expression in peritenon, are highlighted in green while genes that have a negative L2FC, indicating higher expression in peritenon or contrastingly lower expression in tendon proper, are highlighted in orange.

Adolescent TP vs PERI				Midlife TP vs PERI			
Pathway name	DE Genes	padj	LFC	Pathway name	DE Genes	padj	LFC
Dilated Cardiomyopathy	**14**	**0.011**		**None**			
	LAMA2	0.037	7.86				
	TGFB1	0.016	7.317				
	CACNA1S	0.659	0.005				
	MYH6	2.69E-04	2.478				
	ITGB7	0.016	-0.701				
	PRKACA	0.029	-6.678				
Geriatric TP vs PERI							
Pathway name	DE Genes	padj	LFC	Pathway name	DE Genes	padj	LFC
ECM-Receptor Interaction	**25**	**0.002**		**Focal Adhesion**	**45**	**0.002**	
	FN1	1.00E-06	10		*DOCK1*	1.00E-06	10
	TNXB	1.00E-06	10		*FN1*	1.00E-06	10
	COL4A6	0.043	8.717		*TNXB*	1.00E-06	10
	COL6A6	0.002	5.337		*TLN1*	1.00E-06	1.341
	CD44	0.04	0.81		*PDGFRB*	0.006	-1.988
	THBS1	0.004	-2.462		*ITGB10*	0.00000181	-3.642
	COMP	0.002	-3.463		*MYLK*	1.00E-06	-10
Rap1 Signaling Pathway	**41**	**0.018**		**Regulation of Actin Cytoskeleton**	**38**	**0.022**	
	TLN1	1.00E-06	10		*DOCK1*	1.00E-06	10
	LPAR1	1.00E-06	9.694		*FN1*	1.00E-06	10
	PLCE1	1.00E-06	9.368		*SSH2*	0.008	9.961
	ITGB3	1.00E-06	2.062		*MYLK*	1.00E-06	-10
	FGF9	0.0000068	-3.508	**Hippo Signaling Pathway**	**27**	**0.025**	
	PARD3	0.0000466	-8.437		*CTNNA3*	1.00E-06	10
	RALGDS	0.042	-8.635		*FRMD6*	1.00E-06	8.074
Proteoglycans in Cancer	**39**	**0.033**			*WNT4*	1.00E-06	7.935
	FN1	1.00E-06	10		*BMP7*	1.00E-06	6.991
	PLCE1	1.00E-06	9.368		*SMAD2*	0.039	6.366
	WNT4	1.00E-06	7.935		*WNT2*	0.0000059	6.008
	MAPK3	0.002	-0.807		*TGFBR2*	1.00E-06	2.885
	DCN	0.012	-1.973		*SMAD3*	0.026	-1.457
	FZD8	1.00E-06	-2.683		*BMP2*	0.0001715	-1.945
	PLCG1	1.36E-04	-3.031		*PARD3*	0.0000466	-8.437
All TP vs PERI							
Pathway name	DE Genes	padj	LFC	Pathway name	DE Genes	padj	LFC
cAMP Signaling Pathway	**21**	**0.016**		**Dilated Cardiomyopathy**	**14**	**0.021**	
	ACOX1	0.001	10		*ITGA7*	9.13E-04	10
	CREBBP	1.84E-05	10		*TPM1*	0.009	10
	PIK3CA	3.36E-04	10		*DES*	0.007	3.03
	RHOA	1.00E-06	10		*ITGA10*	8.23E-04	-8.929
	CREB5	1.00E-06	10		*CACNA1C*	0.003	-10
	PDE3B	0.04	-7.713				
	PPARA	0.006	-9.22				
	F2R	1.33E-05	-10				
	AKT3	1.00E-06	-10				

Table 3.3. Advaita iPathway Guide Analysis. Pathway analysis for age groups comparing TP vs PERI with a padj < 0.05 was performed. The number of DE genes for the pathway and the padj are listed next to the pathway. Some genes of interest and their padj and LFC were included below the corresponding pathway.

Differential Methylation Annotation					
Contrast TP vs PERI	Promoter (%)	Exon (%)	Intron (%)	Intergenic (%)	Total DMR
Adolescent	9.64	4.16	37.3	48.9	1587
Midlife	9.83	4.7	38.55	46.92	2319
Geriatric	11.26	2.52	38.64	47.57	515
All Samples	7.83	2.68	43.4	46.09	447

Table 3.4. The differentially methylated regions (DMRs) of all ages for TP and PERI samples. When comparing the DMRs for TP and PERI samples across ages, much of the methylation occurs in the intergenic and intron regions but methylation also occurs in the promotor and exon regions. Genomic regions were significant targets if the differential methylation was a qvalue < 0.01 and differentially methylated > 25%. Annotation of gene regions was done to EquCab3.0 and promoter boundary flanking regions were > or < 2kb of the TSS.

Gene Information				Differential Methylation (DMR)								RNASeq (DEG)	
Chr	Chr.num	gene.name	feature.name	dist.to.feat	feat.strand	prom	exon	intron	intergenic	qvalue	meth.diff	padj	L2FC
NC_009145	2	*HAND2*	XM_001915555	3321	+	0	0	0	1	1.79E-57	-33.362	0.0018	3.396
NC_009146	3	*CHD9*	XM_023636982	15124	+	0	0	1	0	8.91E-11	-32.128	0.0092	-19.925
NC_009146	3	*RASL11B*	XM_001493834	-23724	-	0	0	0	1	6.86E-25	-32.073	0.0091	3.071
NC_009151	8	*ADGRD1*	XM_001494181	92393	+	0	0	1	0	9.02E-23	-42.390	0.0002	-2.583
NC_009151	8	*ADGRD1*	XM_001494181	92478	+	0	0	1	0	1.00E-10	-25.466	0.0002	-2.583
NC_009152	9	*COL14A1*	NM_001163870	34739	+	0	0	1	0	2.62E-16	-39.619	0.0077	-1.967
NC_009152	9	*COL14A1*	NM_001163870	34833	+	0	0	1	0	2.20E-22	-45.921	0.0077	-1.967

Table 3.5. DMR and DEG targeted genes for TP and PERI for all samples. Comparing the DMR and DEG hits for TP and PERI for all samples identified 5 genes across 4 chromosomes. These targets are hypomethylated in tendon proper samples and present in either the intron or intergenic regions. Two genes (*ADGRD1* and *COL14A1*) had two significantly differentially methylated regions associated with the gene target.
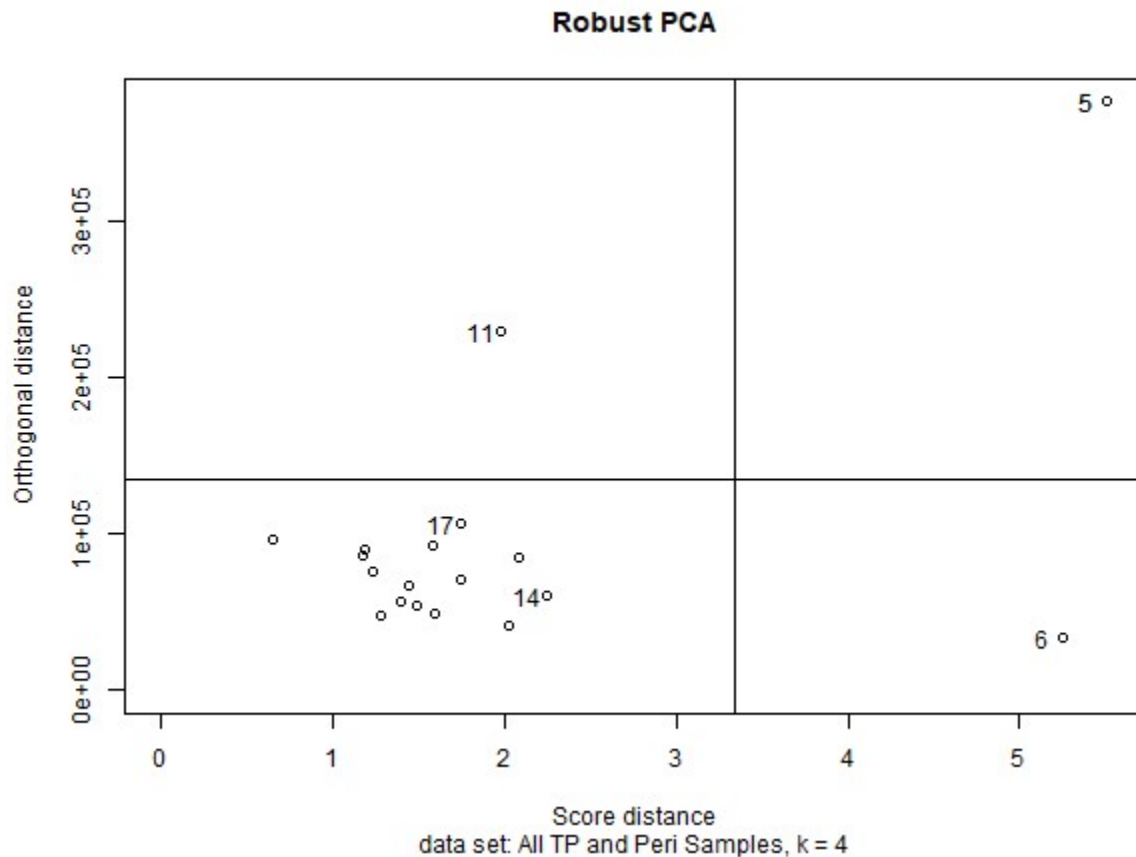
Figure S-3.1. Robust PCA for Outlier Detection. A robust variation of outlier detection using the Hubert ROBPCA was done using normalized counts. Based on the screeplot generated for the normalized data of all the samples, k = 4 was determined as the number of principle components to retain. The 10-day old TP sample (denoted as 5 on the plot) was outside the Wilson-Hilferty transformation for a χ^2 distribution (orthogonal distance; y-axis) and the 97.5% quartile of χ^2_k distribution (score distance; x-axis). Samples 6 (PERI 10-day old) and 11 (TP geriatric) were outside one of the two criteria and therefore were not immediately excluded. Sample 5, 10-day old TP was excluded with its PERI counterpart. Plotting for the outlier test was conducted using the PcaHubert command within the rrcov package.
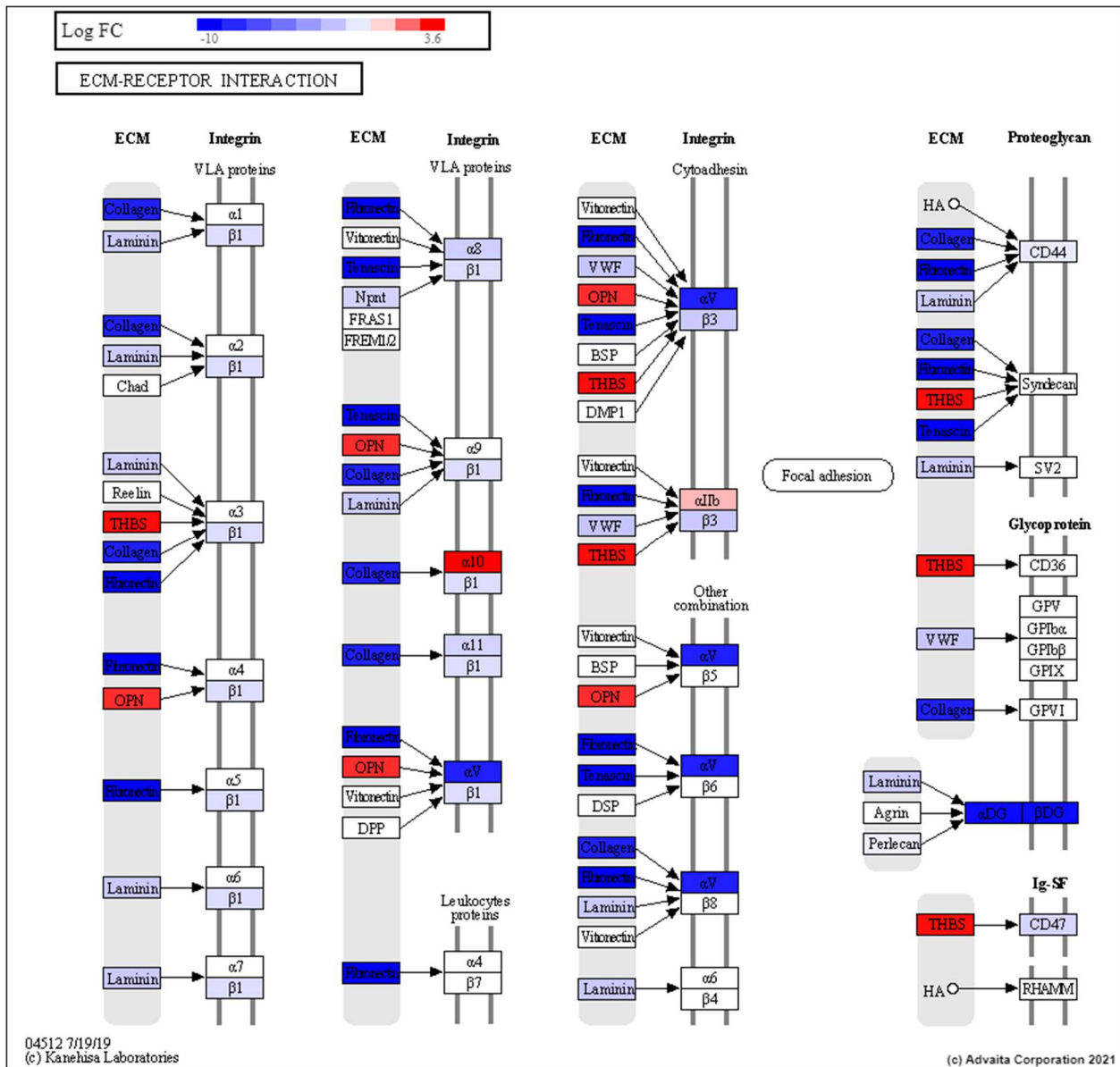
Figure S-3.2. ECM-Receptor Interactions for Advaita iPathway Analysis for Geriatric TP vs PERI. The blue boxed genes are overrepresented LFC genes for TP and red boxed genes for PERI in the ECM-receptor interaction pathway.
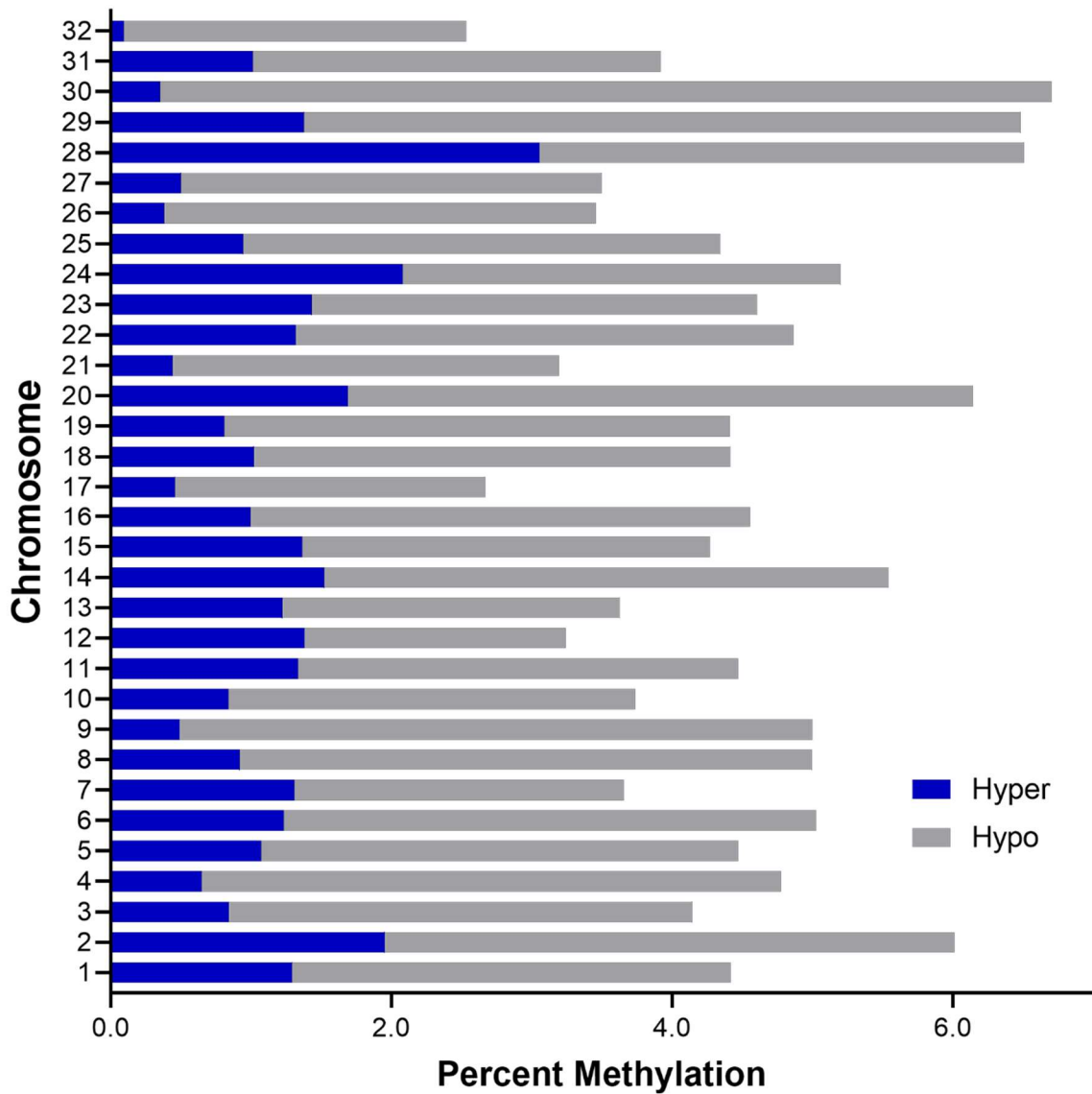
Figure S-3.3. Hyper- and hypomethylation percent differences for midlife samples when comparing TP vs PERI. The tendon proper expresses more regions of hypomethylation than hypermethylation compared to the peritenon when assessing all midlife samples.

Chapter Four: The Effect of Co-Culturing Equine Adipose-Derived Mesenchymal Stem Cells with Tendon Proper and Peritenon Cells In Vitro

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Key words: equine, tendon, peritenon, ADMSC, RT-qPCR

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I.   Abstract

Background: Tendinopathies remain the leading contributor to career-ending injuries in horses because of the complexity of tendon repair. As such, novel therapeutics to address the slow and incomplete repair process have gained traction in recent years. Notably, cell-based therapies like injections of adipose-derived mesenchymal stem cells (ADMSCs) into tendons are becoming increasingly popular but their long-term efficacy on a molecular and wholistic level remains contentious due to the variability in cellular isolation, dosage, and usage. As such, we examined the base effect that ADMSCs have on intrinsic (tendon proper) and extrinsic (peritenon) equine tendon cell populations.

Methods: Equine tendon proper or peritenon cells were co-cultured with equine ADMSCs over 120 hrs. Gene expression for common tenogenic, perivascular, and differentiation markers were quantified at 48 and 120 hrs after co-culture. Additionally, cellular metabolism of proliferation was examined by MTT assay every 24 hrs for peritenon and tendon proper cells co-cultured with ADMSCs.

Results: ADMSCs co-cultured with tendon proper cells expressed trending or increased expression of Chondroitin sulfate proteoglycan 4 (*CSPG4*) and decorin (*DCN*) indicating alterations to the ADMSC profile. Peritenon cells co-cultured with ADMSCs had a trending and significant decrease in biglycan (*BGN*) and CSPG4 at 48 hrs and 120 hrs but overall significant increases in lysyl oxidase *(LOX)*, mohawk (*MKX*), and scleraxis (*SCX*) within 48 hrs. Tendon proper cells co-cultured with ADMSCs also exhibited a decrease in *CSPG4* but increases in *LOX* and *SCX* all at 48 hrs. An overall increase in proliferation in tendon proper cells co-cultured with ADMSCs was seen at 72 hrs with minimal changes in differences of cellular metabolism in peritenon cells.

Conclusions: Adipose-derived mesenchymal cells (ADMSCs) co-cultured with either peritenon or tendon proper cells stimulates increased expression of a tenogenic phenotype by *LOX* and *SCX* and decrease *CSPG4* with further tenogenic specificity in peritenon cells with increased *MKX* expression.

II.   Background

Lameness in equine athletes remains the primary cause for time out of work and death across all equine disciplines [1-8]. Furthermore, tendon and ligament injuries remain one of the most common often resulting in early retirement due to slow and incomplete repair with an increased risk of reinjury [9-14]. Specifically, tendinopathies of the superficial digital flexor tendon (SDFT) are the most prevalent, both acutely and chronically, often with alterations to the extracellular matrix (ECM) regulators of collagen fibrillogenesis and organization resulting in compromised biomechanical properties and potentially catastrophic injury [12,14-18].

Due to these challenges, a variety of therapeutics have emerged to aid the healing capacity of tendons. These include the use of mesenchymal stem cells, platelet rich plasma, and autologous conditioned serum, among many. All of these therapeutics have had varying success in regeneration most likely due to different cellular isolation practices, degree or induction of injury (collagenous vs microtrauma), and overall study design limited to small studies or individual cases with varying post-treatment regimens [19-24]. Additionally, evidence shows that the intrinsic cellular population of tendon (tendon proper; TP) and the extrinsic (peritenon; PERI), play differing roles in development, maturation, aging, and injury of tendon [25-29]. Specifically, during injury, both regions secrete trophic factors, stimulate inflammatory cells, and secrete enzymes to break down and remodel the tendon, but the peritenon cells migrate to the site of injury initially with evidence of expressing stimulatory factors bolstering expression of tenogenic differentiation markers and matrix assembly genes in tendon proper cells [27,30-33]. Although the crosstalk of a tenogenic nature is occurring between the populations, slow and fibrotic scarring repair remains a problem [34].

The use of mesenchymal stem cells (MSCs) as a therapeutic has been utilized, among other cell-based therapies, due to its substantial secretion of trophic factors promoting tenogenesis to positively influence tendon regeneration and stimulate the surrounding cell populations [34,35]. Some evidence of an antiapoptotic and anti-inflammatory effect, stimulation of vascularization, recruitment of local cells, and release of growth factors could also be contributing to the repair though the exact role of MSCs in tendon is unknown [35-40]. Adipose-derived mesenchymal stem cells (ADMSCs) and bone marrow-derived mesenchymal stem cells (BMMSCs) are two established MSC sources often used in regenerative medicine with major phenotypic similarities though harvest and establishment of ADMSCs is less invasive further contributing to its selection for use in tendinopathy treatment [39,41,42].

Although ADMSC lesion injection is one of the most common cell-based therapies, the interplay between the trophic factors of the adipose-derived mesenchymal stem cells, such as growth factors, proteinases, and cellular mediators, and their effect on the extrinsic and intrinsic native cells is still poorly understood [43-46]. In this study, we hypothesized that equine ADMSCs would promote proliferation and a tenogenic expression profile for tendon proper and peritenon cells when co-cultured with these populations in vitro. To assess this, we co-cultured ADMSCs and tendon proper or peritenon cells on insert wells to allow trophic factor interaction but prevent cell migration. The effect of the co-culture was evaluated by the expression of tenogenic, perivascular, and extracellular assembly markers in addition to assaying cellular proliferation.

III.   Materials and Methods

4.2.1 Adipose Harvest and Adipose-Derived Mesenchymal Stem Cell Generation

Adipose tissue from the tail base located above the dorsal gluteal muscle was harvested from three mares (1-10 years; Thoroughbred or Quarter Horse) at the University of California Davis Horse Barn following the approved IACUC protocol 20901. The horses received a dose of 1.1 mg/kg IV flunixin meglumine and then were briefly sedated with 1.0 mg/kg xylazine IV. Their tail base was washed with soap and water to remove grossly observable dirt and debris. Then the tail head region was trimmed with #40 blade trimmers and surgically prepared with three each of chlorhexidine scrubbed and saline rinses. A local 2 % lidocaine chloride anesthetic was applied as an inverted L-block A 5 cm incision was made to visualize the adipose tissue. About 5 g of adipose tissue was harvested and placed into ice cold sterile phosphate-buffered saline (PBS) with antibiotics and antimycotics. The skin was sutured with 2/0 prolene using simple continuous and simple interrupted patterns. Post-operatively the next day each mare received another dose of 1.1 mg/kg IV flunixin meglumine. The adipose tissue was rinsed in sterile PBS with antibiotics and antimycotics. Cells were isolated based upon a protocol adapted from previous studies [47-49]. To begin the cell isolation, the tissue was mechanically separated with a no. 15 scalpel blade in a sterile PBS solution containing 0.1 % type I collagenase (Type I; Worthington Biochemical) and 1% bovine serum albumin with incubation at 37° C and continuously shaking at 90 rpm for 50 minutes. The sample was centrifuged at 260x g for 5 minutes. 5. Stromal cell separation continued by briefly and vigorously agitating the pellet. After discarding the supernatant containing oil, primary adipocytes, and collagenase solution, the stromal–vascular fraction pellet containing the nucleated cell portion of the adipose tissue harvest is again centrifuged at 260× g for 5 minutes. Cells were rinsed in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1 % antibiotic/antimycotic before being spun down and seeded at 5,000 cells per cm2 and incubated in DMEM (high glucose), 10 % FBS, and 1 % antibiotic/antimycotic in 5 % CO_2

with passages occurring at 70% confluency and media changed every 2-3 days [50,51]. Gene expression and tri-lineage assays were performed for MSC marker and differentiation capacity validation.

4.2.2 RT-qPCR Validation of MSCs

To confirm that the isolated cells were expressing mesenchymal stromal cell markers, total RNA was isolated from cells at each passage using a RNeasy Micro kit (QIAGEN) according to manufacturer instructions and including the optional RNase-free DNase treatment step. The mRNA (500 ng) was reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/ThermoFisher). RT-qPCR was performed for MSC-distinguishing markers *CD29, CD34, CD44, CD90*, and *MHCII* with *POLR2A* as the normalizing gene (**Tab S-4.1**). RT-qPCR was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems/ThermoFisher).

4.2.3 ADMSC Tri-lineage Assay

To confirm that the cells belong to the mesenchymal stem cell lineage, a tri-lineage assay was applied to the cells. Thus, the cells were differentiated with adipogenic, osteogenic, and chondrogenic media. To assess adipogenesis, 35,000 cells were seeded per well in a twelve-well plate and then basal media (DMEM, 10% FBS, 1x antibiotic/antimycotic) with 1 µM dexamethasone, 10 µg/mL insulin, 100 µM indomethacin, and 0.5 mM isobutylmethylxanthine for 21 days with media changed three times weekly [52,53]. Adipogenesis was evaluated after Oil Red O staining using 0.18% Oil Red O in 60% isopropanol (Thermo-Fisher) [33]. To assess osteogenesis, 17,500 cells were seeded per well in a six-well plate; StemPro™ Osteogenesis

Differentiation media was applied to the cells for 21 days according to manufacturer instructions with media changed three times weekly. Osteogenesis was assessed with Alizarin Red S staining using 2% Alizarin Red, S., pH 4.1 (Sigma) [33]. To assess chondrogenesis, 250,000 cells were seeded as a pellet at the bottom of a 15 mL centrifuge tube; StemPro™ Chondrogenesis Differentiation media was applied to the cells for 21 days according to manufacturer instructions with media changed three times weekly. Chondrogenic pellets were inspected by microscope. Trilineage differentiated cells were compared to cell grown in culture with basal media.

4.2.4 Tendon Harvest and Cell Isolation

Superficial digital flexor tendon (SDFT) samples were harvested from five non-breed or sex specific horses (ages 8 – 15 years) that were euthanized for reasons outside of this study; as such, they were exempt from approval of the University of California Davis Institute of Animal Care and Use Committee. No known history of tendinopathy was present in these horses and visual assessment of lameness was also conducted, and deemed negative, before selecting a horse for tendon harvest. Immediately upon euthanasia, sterile instruments were used to harvest 2.5 cm of the superficial digital flexor tendon located 10-15 cm proximal to the forelimb fetlock. Upon removal of the tendon, it was rinsed three times in Dulbecco's Phosphate Buffered Solution (DPBS, Life Technologies, Benicia, CA, USA) containing 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10,000 ug/mL streptomycin, and 25 ug/mL amphotericin B, Life Technologies) before transport for further isolation [33,54]. Tendon samples were isolated into peritenon (PERI) and tendon proper (TP) regions sterilely under a dissecting microscope where 2 – 2.5 mm of the central tendon core was used for the tendon proper and the peritenon consisted of the viscous paratenon in addition to 1 mm of the epitenon. Separated tendon proper and peritenon

regions were enzymatically digested following previous protocols using 0.3% type-I collagenase (CLS-1, Worthington, Lakewood, New Jersey, USA) and 0.4% Dispase II (Roche, Basel, CH) in Hanks Balanced Salt Solution (HBSS, Gibco, Benicia, CA, USA) and inactivated following agitation in standard tenocyte media (alpha-MEM, 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 1 % antibiotic/antimycotic) [27,55]. Cells were expanded in T75 tissue culture treated flasks and passaged to P2 before cryopreservation in 10% dimethyl-sulfoxide (DMSO) in standard tenocyte media and maintained in liquid nitrogen until further processing. All horse samples were maintained separately to ensure independent biological replicates.

4.2.5 Co-Culture Setup and Harvest

Cryopreserved vials of the peritenon and tendon proper for the five horses were thawed and seeded in T75 flasks at 6,666 cells per cm^2 and grown to 85 % confluency to ensure enough cells for the co-culture setup. Concurrently, ADMSC cells were thawed and seeded at 5,333 cells per cm^2 and allowed to expand until 75% confluency. ADMSC, TP, and PERI cells were expanded in P3 in standard tenocyte media (α-MEM, 10% FBS, 1x antibiotics/antimycotics, 1x L-Glutamine). The co-culture setup used 6-well cell culture transwell 0.4 μm polycester membrane inserts for the RT-qPCR analysis and 12-well cell culture transwell 0.4 μm polycarbonate membrane inserts for the MTT assay analysis. The cell culture inserts allowed trophic factors and cell secretions to travel between cell groups, but migration of cells was not possible. The TP and PERI tenocytes were seeded in the bottom well at 5,263 cells per cm^2 for TP and PERI in the 6-well plates. ADMSCs were seeded at 5,263 cells per cm^2 in the 6-well culture inserts. At this point, cells were fed a tenocyte media containing 1% FBS to halt cell proliferation. Samples were harvested at 48 hrs and 120 hrs for further RT-qPCR analysis. Media was changed at 48 hrs for

the 120 hr samples. Cellular groups that were harvested included ADMSC control (MSC), TP control (TP), PERI control (PERI), ADMSC co-cultured with TP (MSC:TP), ADMSC co-cultured with PERI (MSC:PERI), TP co-cultured with ADMSC (TP:MSC), and PERI co-cultured with ADMSC (PERI:MSC). For the MTT assay co-culture, tissue co-culture inserts for 12-well plates were used with TP and PERI cells seeded at 6,579 per cm^2 on the bottom well and ADMSC cells were seeded at 6,579 per cm^2 in the 12-well culture insert. PERI control, TP control, PERI:MSC, and TP:MSC samples were processed for the MTT analysis at 24, 48, 72, 96, and 120 hrs. Media was changed at 48 hrs for all samples to be harvested after 48 hrs. Cells were fed with phenol red-free alpha-MEM media supplemented with 1% FBS, 2 mM L-glutamine, and 1 % antibiotic/antimycotic for the MTT co-culture.

4.2.6 Total RNA Isolation and Real Time quantitative PCR (RT-qPCR)

At 48 and 120 hrs, cell layers were processed for RNA isolation, after rinsing in PBS, using the RNeasy Plus Micro Kit (QIAGEN, Valencia, CA) RLT lysis solution with 1% beta mercaptoethanol (BME) and stored at -80° C until further processing. All samples underwent RNA isolation following manufacturer protocol and treated with RNase-free DNase (QIAGEN, Valencia, CA). Reverse transcription was performed on 500 ng RNA using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). To identify the effect of the co-culture, tenogenic differentiation genes (*MKX, FMOD, SCX*), ECM assembly (*BGN*, *DCN*, *COL1A1*, *LOX*), perivascular (*CSPG4*), and cell proliferation (*MKI67*) markers were assessed with *POLR2A* as the housekeeping gene [27,33,56-60]. Taqman primers were designed using the equine gene structure annotation EquCab3 or from predesigned primers (**Tab S-4.2**) (Life Technologies) [61,62]. RT-qPCR analysis followed previously described protocols and each sample of amplified

cDNA was run in duplicate for each gene [33,54,56]. Gene specific efficiencies were calculated using LinRegPCR v 7.5 software and the relative quantity ratio formula calculated the relative quantity of mRNA for each gene and plotted in Log10 [63,64].

4.2.7 MTT Assay

Samples were rinsed in PBS before processing the MTT assay (CyQUANT MTT Cell Proliferation Assay Kit, Invitrogen). Reagents for the assay were prepared following manufacturer protocol. To begin, 100 uL fresh culture media was added to each sample with 10uL of 12nM MTT stock solution and allowed to incubate at 37° C for 4 hrs. Following incubation, each sample received 100 uL of SDS-HCl solution and incubated for 12 hrs at 37° C in a humidified chamber. Samples were read in triplicate on a 96-well flat bottom plate at 570 nm absorbance.

4.2.8 Statistics

Statistical analysis performed using GraphPad Prism (Graphpad Software, Inc. San Diego, CA) with significance levels set at $p \leq 0.05$ and trending at $p > 0.05$ and ≤ 0.1. For RT-qPCR, mean values for technical replicates were calculated for each biological replicate and graphed in Log10. Reported p-values were calculated by a mixed-effect full model with Geisser-Greenhouse correction and corrected for multiple comparisons by the false discovery rate (95 % confidence interval).

IV.   Results

4.3.1 Adipose-Derived Mesenchymal Stem Cell RT-qPCR and Tri-lineage Assay Validation

The primary (P0), P1, and P2 passages of isolated and expanded cells were *CD29+ CD34- CD44+ CD90+ MHCII-* by RT-qPCR which is characteristic of ADMSCs (**Fig. 1**). Induction of adipogenesis, osteogenesis, and chondrogenesis was possible with the AD-MSCs (**Fig. 2**).

4.3.2 Gene Expression

Adipose-derived mesenchymal stem cells were seeded on transwell inserts with either peritenon or tendon proper seeded below and harvested at 48 and 120 hrs. When comparing ADMSCs co-cultured with PERI or TP cells, after 120 hrs (AD)MSC:TP had a significant increase in decorin (*DCN*) and trending significance in *CSPG4* for (AD)MSC:TP at 48 hrs post co-culture (**Fig 4.3C, Fig 4.3D**). ADMSCs without co-culture were included in graphing to provide relevance but removed from statistical analysis due to lack of power.

Peritenon cells co-cultured with ADMSCs were compared to control peritenon tenocyte resulting in a trending decrease in PERI:(AD)MSC biglycan (*BGN*) at 48 hrs and a significant decrease in *CSPG4* for PERI:(AD)MSC at 120 hrs (**Fig 4.4A, Fig 4.4C**). Alternatively, there were significant increases in expression for PERI:(AD)MSC at 48 hrs for *LOX, MKX*, and *SCX* (**Fig 4.4F, Fig 4.4H, Fig 4.4I**).

Tendon proper cells co-cultured with ADMSCs were contrasted to control tendon proper tenocytes with resulting significant decreases in expression of *CSPG4* in tenocytes co-cultured with ADMSCs at 48 hrs (**Fig 5C**). Expression of *LOX* and *SCX* increased at 48 hrs of co-culturing tendon proper cells with ADMSCs (**Fig 4.5F, Fig 4.5I**).

4.3.3 Cellular Metabolic Activity

A cell proliferation assay was done to assess the cellular metabolic activity of the peritenon and tendon proper cells that were co-cultured with ADMSCs. The percent viability was tracked over 120 hrs with harvests every 24 hrs (**Fig 4.6**). Over this point, the proliferative capacity of the tendon proper cells co-cultured with ADMSCs were significantly greater compared to peritenon cells at 72 hrs. Peritenon cells maintained a relatively steady percent viability while the tendon proper cells increased until 72 hrs, followed by a dip at 96 hrs, and then increase again at 120 hrs (**Fig 4.6**).

V.   Discussion

Tendinopathies remain a major concern for horse owners and breeders of all breeds and disciplines due to the inability for tendons to properly heal to pre-injury states along with the exorbitant time and cost to achieve a marginal level of success. Unfortunately, even if horses are able to return to their event after injury, the risk of re-injury is still present. As such, ongoing research into novel ways for better, more complete, tendon repair is of great importance for both the financial perspective and quality of life of the horses. One therapeutic that has been utilized for numerous years is the use of mesenchymal stem cells, but results have not been conclusive, due to variations in cell isolation, dosage, usage, and other factors, and their use at times is anecdotal. As a result, identification of the effect of mesenchymal stem cells, specifically adipose-derived due to their ease of isolation for future therapeutics, on the native tendon cells will provide vital information. Common tendon related genes required during maturation, development, and repair, along with the effect on proliferation, will provide insight into the changes, if any, that tendon cells experience when exposed to mesenchymal stem cells *in vitro*.

ADMSCs release numerous trophic factors which ultimately affect the surrounding cells. Although ADMSCs can differentiate into tendon cells, as these cells are multipotent, their nature when injected into an injury site does not lead to full incorporation into the tissue matrix [65,66]. As such, understanding their influence on tendon cells *in vitro* further identifies the base mechanism by which ADMSCs can benefit tendon repair. Alterations in tendon cell gene expression was observed with ADMSC co-culture but ADMSCs were also affected by the tendon proper or peritenon cells to which they were exposed. Of the genes observed, the perivascular marker *CSPG4* and the small leucine rich-repeat proteoglycan decorin tended to increase in the ADMSCs exposed to tendon proper cells. Specifically, an increase in decorin at 120 hrs further suggests that ADMSCs present in the tissue at the timepoint after injection could be contributing important collagen fibril building and signaling molecules in addition to other trophic factors. Since decorin is not increased in tendon proper or peritenon cells, such ADMSC-derived resources could be critical for proper collagen alignment and maturation as long as ADMSCs are still present at the site of injection five days post-injection.

Further shift towards a tenogenic phenotype were observed within the peritenon cell population and the tendon proper when co-cultured with ADMSCs. A decrease in the perivascular marker *CSPG4* indicates a shift away from pericyte activity towards more of a tenogenic tendency [67,68]. Notably, in both the peritenon and tendon proper cells co-cultured with ADMSCs expression of lysyl oxidase (*LOX*), a collagen cross-linking molecule, and scleraxis (*SCX*), a tendon differentiation marker, were upregulated within 48 hrs. This implicates that the ADMSCs, regardless of region, further stimulate collagen fibril organization and tendon differentiation, which is beneficial for repair. Also compelling was that the peritenon cells were further stimulated towards tenogenic influence as evidenced by an increase in mohawk (MKX). Mohawk regulates

extracellular matrix deposition in addition to activating transcription of *COL1A1*, *COL1A2*, *tenomodulin*, and *decorin* by the complexation of SMAD2/3 thereby stimulating collagen production, assembly, and maturation [69-73].

Co-culture of ADMSCs with tendon proper cells stimulates proliferation, an increase in cellular metabolism, after 72 hrs with gradual increases prior to this point. Peritenon cells have minimal differences between PERI and PERI:MSC most likely due to the increase in numerous transcription markers shifting priorities away from replication [74-76].

Although this study was conducted in a 2-dimensional monolayer, the control that this model creates provides insight into the underlying effect that ADMSCs have on tendon cells. The equine model resembles the individuality of human cells more closely lending it to be valuable when significant differences are identified. Understanding that ADMSCs have cellular diversity due to isolation, dosage, and application is pertinent for future therapeutics. Some limitations of this study include the use of one ADMSC candidate for further co-culture experiments, five equine candidates within a mature age group and not adolescent or geriatric, and the longevity of the experiment since injected ADMSCs may provide trophic factors longer than 120 hrs [76-79]. Finally, as this was a co-culture experiment with two cell types, these experiments did not include other cells one might find in tendon repair provisional matrix or scar tissue.

In conclusion, we did not see a change in overall proliferation capacity for tendon proper and peritenon cells treated with ADMSCs. Instead, we did find that ADMSCs stimulated the tendon cells toward expression profiles of a more tenogenic phenotype, which is particularly important for peritenon cells that might be directing repair in adult tendon tissue.

VI.   **References**

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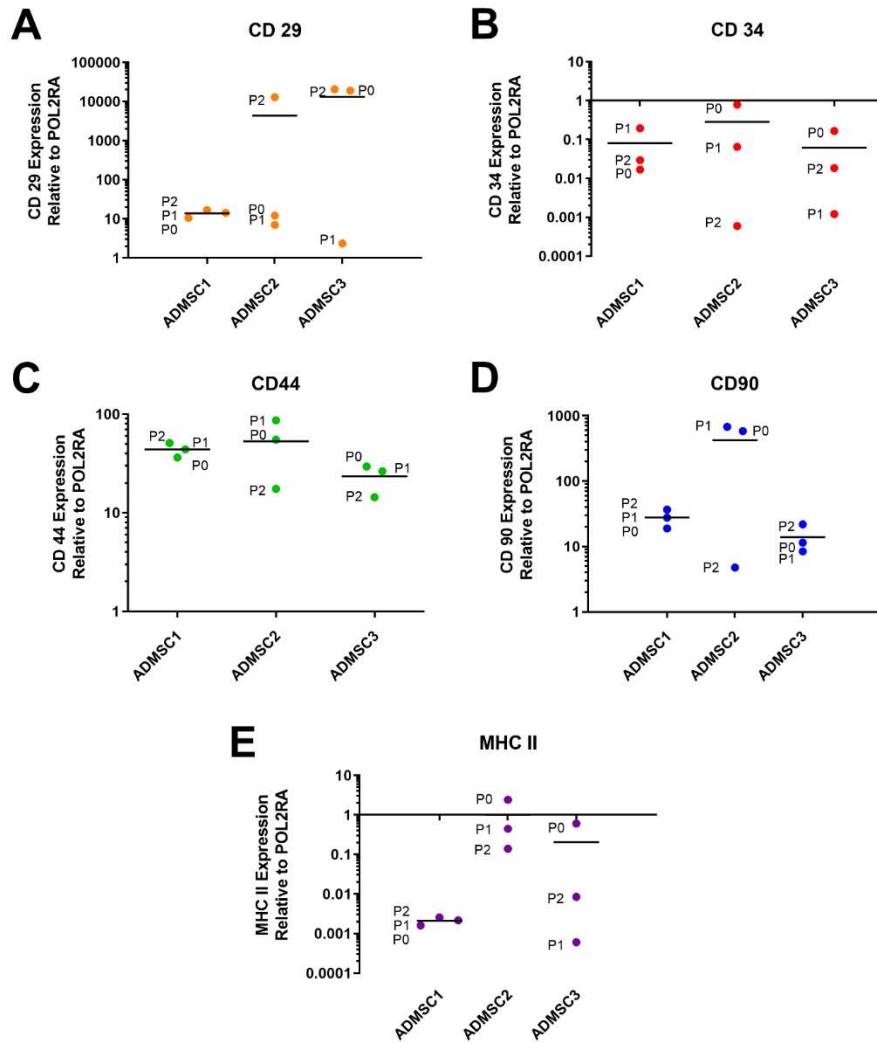
Figure 4.1. RT-qPCR gene expression for MSC markers. Isolated adipose-derived cells from

Horses 1, 2, and 3 were passaged twice. Cells at every passaged were checked for expression of

(A) *CD29*, (B) *CD34*, (C) *CD44*, (D) *CD90*, and (E) *MHCII*, relative to *POL2RA* abundance.

Relative expression is graphed in Log10. P0, primary cells; P1, first passage; P2, second passage.
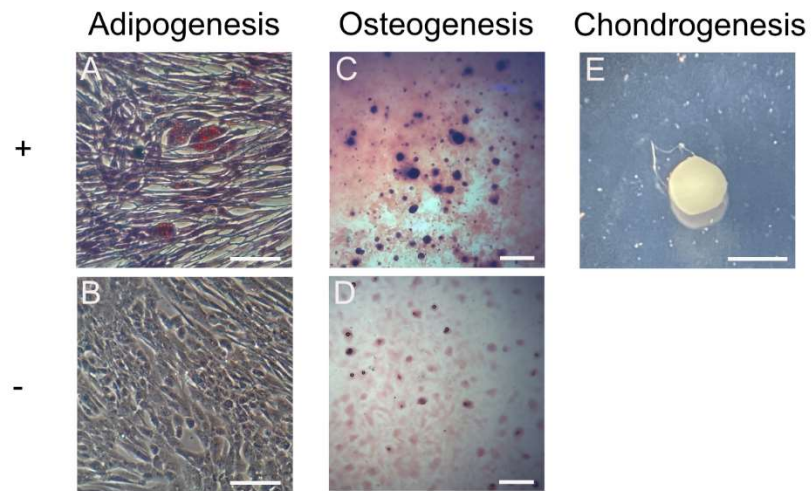
Figure 4.2. ADMSCs Differentiated by Trilineage Assay. These images are representative of the ADMSCs from the three horses. They are capable of adipogenesis (red oil droplets from Oil Red O stain), osteogenesis (dark red staining of calcium deposits by Alizarin Red), and chondrogenesis (cartilage pellet). Scale bars included are 100 μ (**A, B**), 2 mm (**C, D**), and 1 mm (**E**).
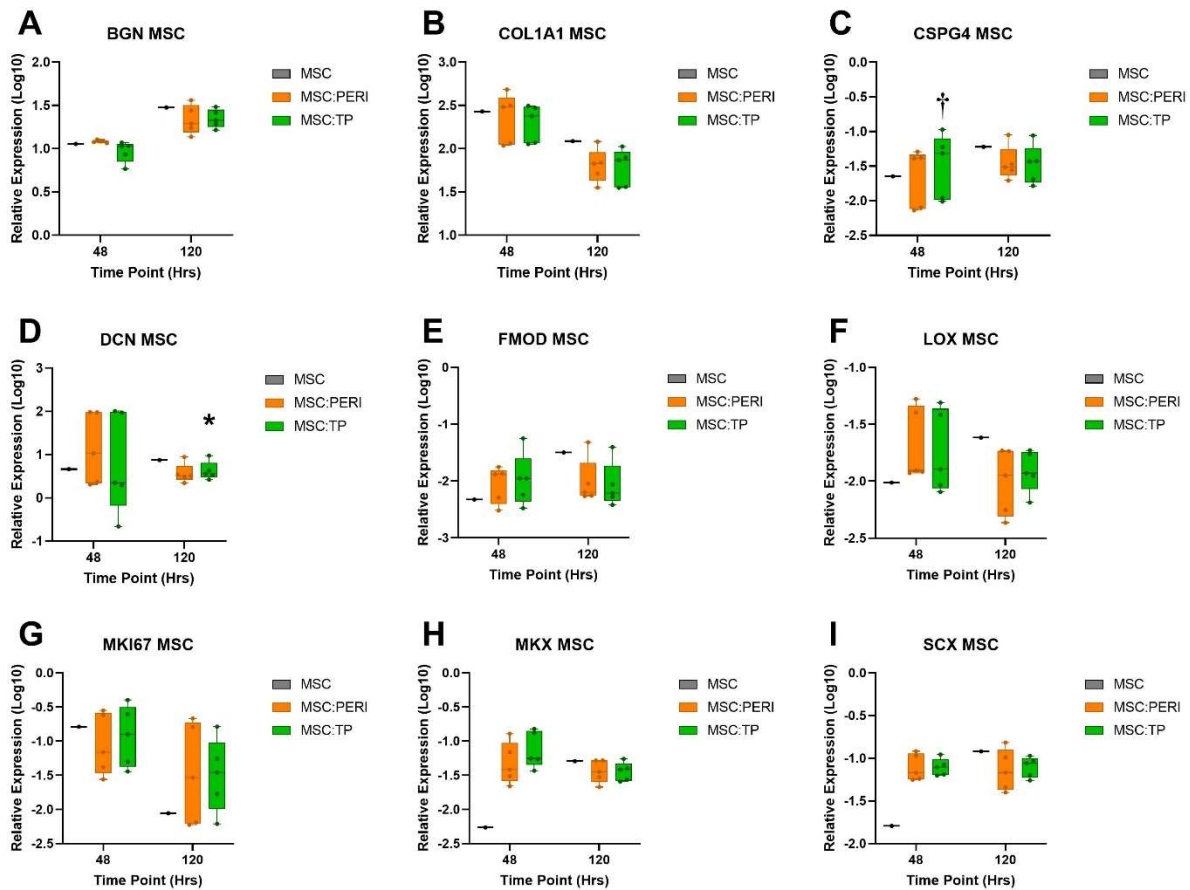
Figure 4.3. RT-qPCR gene expression for ADMSCs co-cultured with PERI or TP. After 48 or 120 hrs, ADMSCs co-cultured in transwells with PERI or TP cells were harvested and gene expression quantified. (AD)MSC:PERI and (AD)MSC:TP expression levels were used to calculate the difference in PERI and TP co-culture. (AD)MSC levels were included to provide relevance but were not included in statistical calculations. Relative expression is graphed in Log10 and significance was set at $p \leq 0.05$ (*) and trending at $p > 0.05$ and ≤ 0.1 (†).

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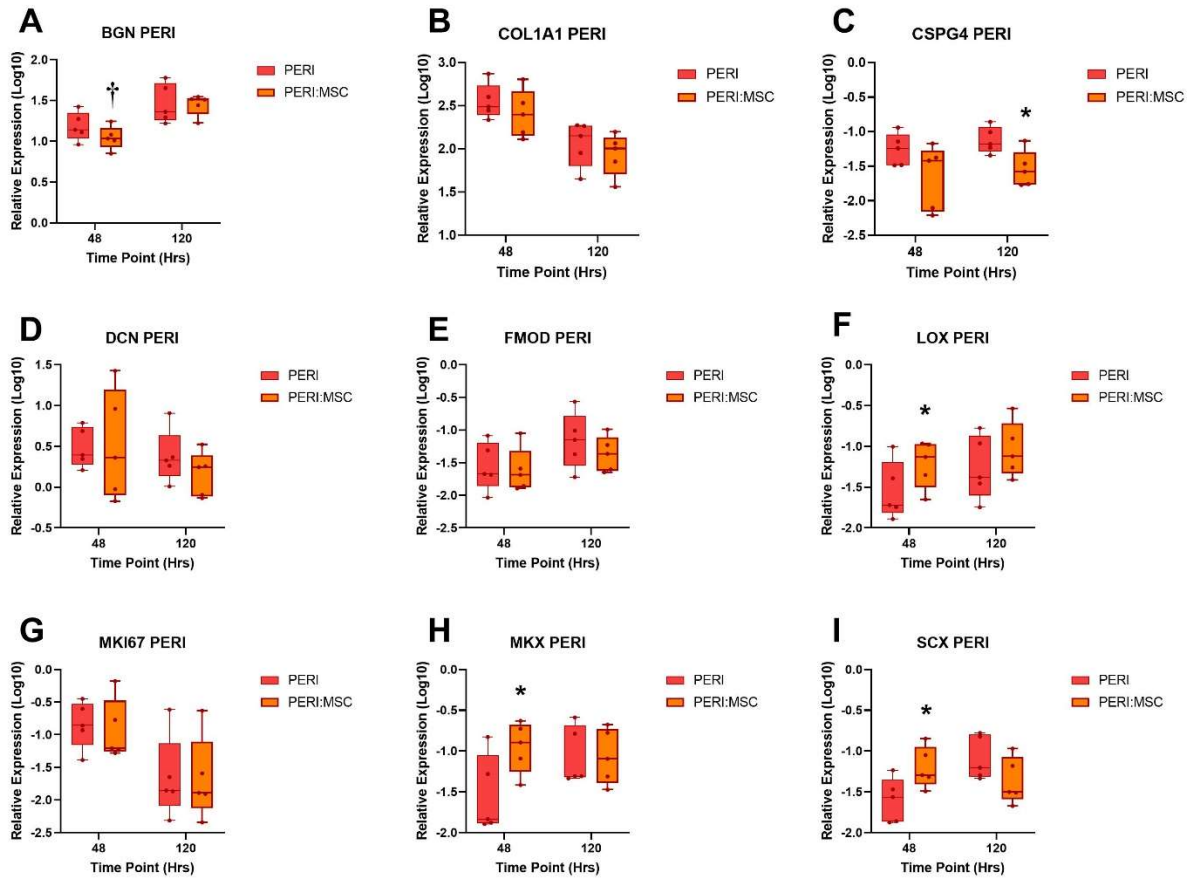
Figure 4.4. RT-qPCR gene expression for PERI and PERI cells co-cultured with ADMSCs.

After 48 or 120 hrs, PERI and PERI cells co-cultured on transwells with ADMSCs were harvested and gene expression quantified. Relative expression is graphed in Log10 and significance set at p ≤ 0.05 (*) and trending at p > 0.05 and ≤ 0.1(†).
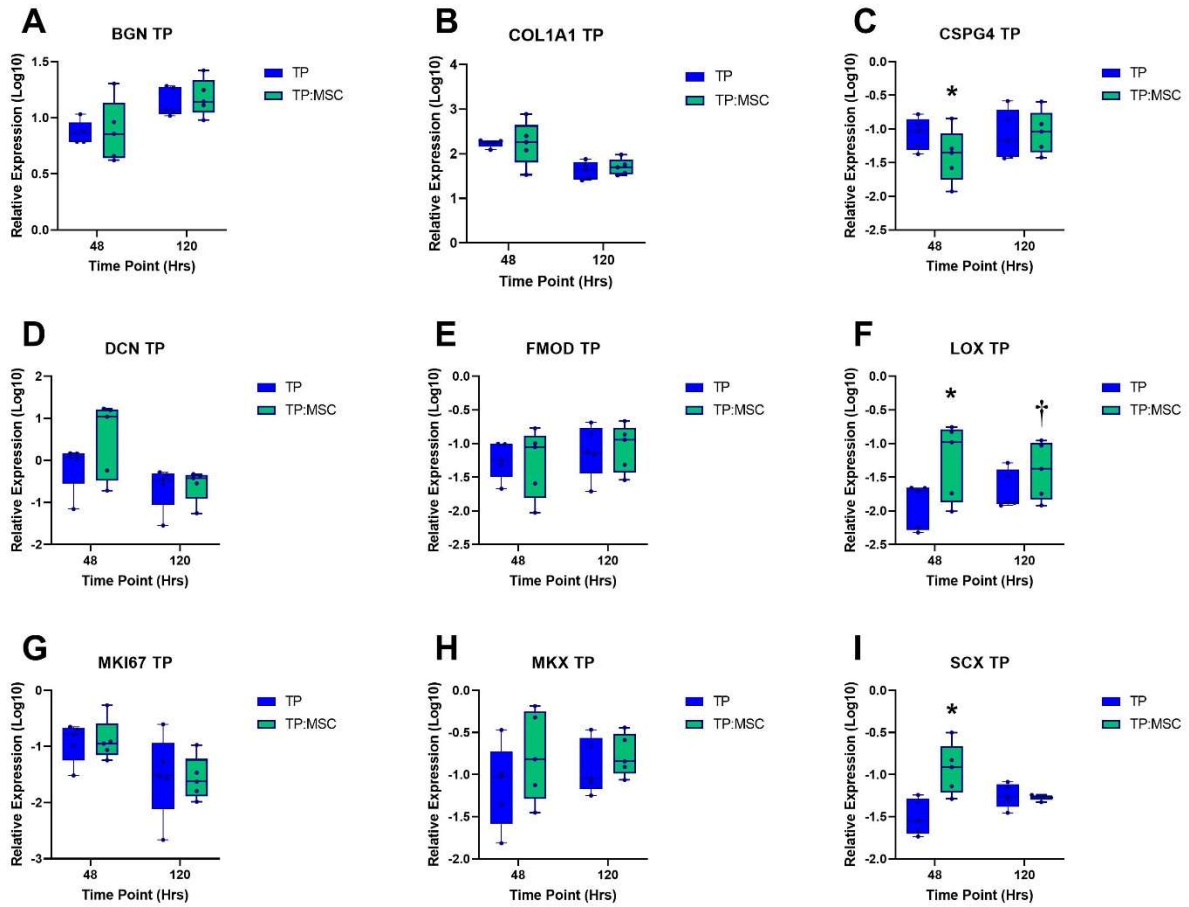
Figure 4.5. RT-qPCR gene expression for TP and TP cells co-cultured with ADMSCs. After 48 or 120 hrs, TP and TP cells co-cultured with ADMSCs were harvested and gene expression quantified. Relative expression is graphed in Log10 and significance was set at p ≤ 0.05 (*) and trending at p > 0.05 and ≤ 0.1(†).
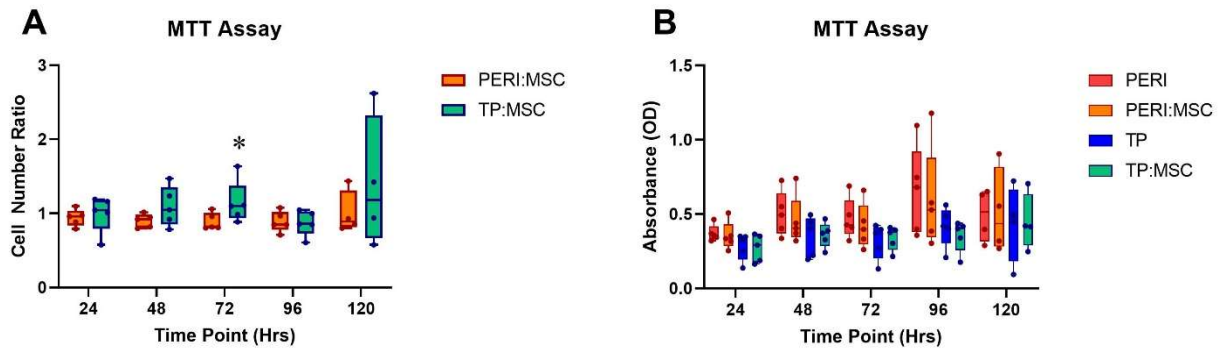
Figure 4.6. MTT Cellular Metabolic Assay. The MTT assay was used to calculate cell viability by cellular metabolism over a 120 hr period with harvests every 24 hrs. (**A**) The cell number ratio using control and treatment cells was calculated for normalization to compare peritenon against tendon proper cells co-cultured with ADMSC. (**B**) The optical density absorbance was graphed to indicate that although peritenon cells were overall more abundant compared to tendon proper, the tendon proper cells had more proliferation when supplemented with ADMSCs compared to peritenon. Significance was set at $p \leq 0.05$ (*).

Supplemental Tables

	Fwd	Rev	Exons
POLR2A	CCAGGATGACCTGACTCACAAA	CGTCGAAGCTGATTGTTGATCT	5-6
CD29	CCTCAGCCAGGCCAGGTT	CAGCAGTCATCCACATCCTTCTC	14-15
CD34	GGCCAGGCCACATCCA	ATCAGCCACCACGGGTTGT	7-8
CD44	CATAGAAGGGCACGTGGTCAT	TTGGCGGCACAGATGGA	1-2
CD90	GGCAGACCAGAGCCTTCGT	ATGGGTGTGGCGGTGGTAT	1-2
MHCII	CAGAGCGAGTGCGGTACTTG	GCTGTCGAAGCGCACGTA	2

Table S-4.1. Primers for Distinguishing MSCs. Forward and reverse primers were designed using the EquCab3.0 annotation in Primer3 SYBR green RT-qPCR.

	Fwd	Rev	Probe
POLR2A	CCAGGATGACCTGACTCACAAA	CGTCGAAGCTGATTGTTGATCT	TGGCGGACATTGTT
BGN	GGTGGGCGTCAACGACTT	GCCATTGTAGTAAGCCCGTTTG	CCCGTGGGTTTCG
DCN	TGCGAAAAGCGGTGTTCA	TGGGTTGGTGCCCAGTTCTA	ACTGAACCAGATGATAGTC
MKX	TCATGTTCCGAAGATGGAGAAA	ATTGTAGCCCCCTTCGTTCA	TCCTCCAAGAAACCAC
COL1A1	GGGCCGAGGGCAACA	GTGGTTTTGTATTCGATCACTGTCTT	CTTCACCTACAGCGTCAC
FMOD	AACCAAGGAGGCCAGACAGA	TGCATTTTGTCTCTCTCAAGTTGAA	ACGTGGTCACTCTGAA
LOX	GCTTGGCCAGCTCAGCAT	TCTTAGCAGCACCCTGTGATCA	CAGGTCAGATGTCAGAGAT
CSPG4	CTCCTGGAGAGAGGTGGAACAG	TCAGTGTCTCGCTCCCATCA	AGCTGATCCGCTATGTG
MKI67	ThermoFisher, cat no 4351372, Ec07039069_g1 -- Proprietary information		
SCX	ThermoFisher, cat no 4351372, Ec03818452_s1 -- Proprietary information		

Table S-4.2. Taqman Primers for Equine Tenogenic, ECM Assembly, Perivascular, and Proliferation Markers. Forward, reverse, and probe sequences for *POLR2A, BGN, DCN, MKX, COL1A1, FMOD, LOX, CSPG4, MKI67,* and *SCX* designed using the EquCab3.0 annotation in Primer3 or predesigned primer probe sets for Taqman specific RT-qPCR. All primer probe sets underwent quality scrutiny, in addition to, validation in native equine tendon proper and peritenon tissue.

Chapter Five: Decorin Secreting Adipose-derived Mesenchymal Stem Cell and the Future

Trajectory of Enhanced Cell Therapeutics in Tackling Tendinopathies

I.   **Effect of Biglycan and Decorin Supplementation in 2-dimentional Culture**

Numerous studies have assessed the effect of decorin, a small leucine rich-repeat proteoglycan, *in vivo* and in three-dimensional tendon constructs, but the effect of decorin *in vitro* in a two-dimensional system have yet to be explored. This is of particular interest because supplementation of decorin on a two-dimensional level will identify the direct gene expression changes without extracellular matrix crosstalk. Specifically, expression of genes of interest were quantified including the extracellular matrix, perivascular, and tenogenic markers for both the intrinsic and extrinsic tendon population.

Assessing the decorin supplementation on the peritenon (PERI) and tendon proper (TP) over time will aid in understand the role that decorin has in enhancing or decreasing gene expression of various genes of interest. For this, we used SDFT cells from 5 horses for both TP and PERI and supplemented with either 5 nM or 25 nM of decorin (DCN) or biglycan (BGN) and cell harvests were done 48 or 120 hrs after initial supplementation. Cell isolations were done following well established protocols [1,2] and SLRP supplementation was done with bovine decorin and bovine biglycan (Sigma-Aldrich). Both cell populations were thawed after cryopreservation and seeded at 6,666 cells per cm^2 in normal tenogenic media (alpha-MEM, 10 % fetal bovine serum, 2 mM L-glutamine, and 1 % antibiotic/antimycotic) to allow for cellular expansion. Once enough cells were prepared, cells were seeded at P4 in 6-well plates at 150,000 for TP and 37,500 for PERI per well with either control media, 5 nM supplemented media, or 25 nM supplemented media with either bDCN or bBGN in duplicate for all 5 horses. The same set up was performed for both harvests at 48hrs after initial supplementation and 120 hr

supplementation with supplemented media replaced at 48 hrs. Duplicate samples were pooled during harvest to increase RNA yield.

RNA isolation was performed on all samples using the QIAGEN RNeasy Plus Micro Kit with a RNase-free DNase treatment (QIAGEN, Valencia, CA). After RNA concentration was measured using a Nanodrop, reverse transcription was performed on 500 ng total RNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Genes assessed included tenogenic differentiation (*MKX, FMOD, SCX*), ECM assembly (*BGN, DCN, COL1A1, LOX*), or perivascular (*CSPG4*) markers with *POLR2A* as the housekeeping gene [1-9]. Taqman primer probe sets utilized in previous studies were used and combined with 1 ul of the cDNA template and Taqman Master Mix (no UNG) (Life Technologies) for a 20 uL (10 ng/ul) reaction volume ran in duplicate in the StepOnePlus Real-Time PCR System [2]. To calculate relative expression, gene specific efficiencies calculated using the LinRegPCR v 7.5 software into the relative quantity ratios formula calculated the relative quantity of mRNA for each gene [3,4,8,10,11]. Significance was established as p-value < 0.05.

Supplementation of bDCN and bBGN after 48 hrs across all genes of interest indicated that there was no effect with either low (5 nM) or high (25 nM) dosage for TP or PERI (**Fig 5.1**). On the other hand, supplementation after 120 hrs showed changes in expression levels. Notably, 5 nM BGN increased expression in PERI *BGN*, TP *COL1A1*, TP *CSPG4*, TP *DCN*, TP *FMOD*, TP *LOX*, and a decrease in TP *BGN* expression. The supplementation of 25 nM BGN increased PERI *BGN*, TP *COL1A1*, PERI *CSPG4*, TP and PERI *DCN*, PERI *FMOD*, TP *MKX*, and PERI *MKX*. For 5 nM bDCN supplementation, an increase was seen in PERI *BGN*, TP *COL1A1*, PERI *CSPG4*, TP and PERI *DCN*, TP and PERI *FMOD*, TP *LOX*, TP *MKX*, PERI *SCX*, and a decrease in PERI *LOX* expression. Finally, for 25 nM bDCN, increases in PERI *BGN*, TP *COL1A1*, TP *CSPG4*, TP

DCN, TP *FMOD*, TP *LOX,* TP and PERI *MKX*, PERI *SCX,* and decreases in PERI *LOX* and TP *SCX* expression were seen.

From this information, changes in gene expression for tenogenic, perivascular, and extracellular matrix markers were seen after supplementation of bDCN and bBGN. For a low dosage of bBGN, the effect was mostly seen in the tendon proper cellular population except for PERI *BGN* which increased across all supplementation groups. 5 nM bBGN was also the only dosage that affected the expression of TP *BGN* with it causing a decrease in expression potentially indicating that the dosage was low enough to decrease expression in a feedback mechanism. The same effect was not seen with 25 nM BGN but the variability by sample was much greater indicating individual responses to the supplementation ultimately resulting in a reaction to increase expression of other 'injury related' reorganizing genes such as TP *COL1A1*, PERI *CSPG4*, TP and PERI *DCN* and *MKX*, and PERI *FMOD*. Supplementation of bDCN was slightly more uniform between 5 nM and 25 nM for TP, except for 25 nM *SCX* and *CSPG4*, with an overall push towards tenogenic differentiation for the PERI cells as indicated by the increase in *SCX*, *MKX*, and *FMOD*. Overall, the effect of supplementation of 5 nM bDCN showed the greatest shift for both TP and PERI to a tenogenic phenotype after 120 hrs. Additionally, the supplementation indicates that the effect is not immediate and there is a delay in changes to gene expression that can be identified at 120 hrs.

Paired together with the results of 3D construct bDCN supplementation and the potential changes to the PERI population towards a tenogenic phenotype from bDCN supplementation, this SLRP is a strong candidate for transfection with mesenchymal stem cells (MSC). After injury, utilization of stem cell therapy has, to date, provided the best tendon repair success compared to other therapeutics but it still has numerous factors affecting the overall outcome with actual low

success rates [12-19]. As such, enhancing the beneficial components of stem cell therapy with a guide to appropriate gene expression for tenogenic repair of the tendon proper and peritenon could be achieved with mesenchymal stem cell decorin transfection.

II.   Isolating and Sequencing Decorin cDNA

For transfection of a decorin plasmid into mesenchymal stem cells, it was necessary to reverse-transcribe and amplify the full-length cDNA of equine decorin from *DCN* mRNA for proper downstream expression, transcription, and translation. Reverse transcription and amplification of the equine mRNA was done using equine tendon SDFT mRNA from Horse 16 (tendon proper, 5 year-old Thoroughbred mare) with the SuperScript™ IV One-Step RT-PCR System (Invitrogen, ThermoFisher Scientific cat no 12594025). Each RT-PCR reaction contains the following components: 1 μg total RNA, 25 μL 2x Platinum™ SuperFi™ RT-PCR Reaction Mix, 2.5 μL 10-μM forward primer, 2.5 μL 10-μM reverse primer, 0.5 μL SuperScript™ IV RT Mix, and nuclease-free water to get to a 50 μL reaction volume. The thermal cycler program for reverse transcription was: (1) reverse transcription, 50°C for 10 minutes; and (2) RT inactivation/initial denaturation, 98°C for 2 min. The thermal cycler program for the amplification step was: (1) 98°C for 10s, (2) 60° for 10s, (3) 72°C for 60s, all repeated 40 times; and (4) a final extension for 5 minutes at 72°C, followed by a hold step at 4°C. Three sets of equine specific decorin primers were used for the reactions **(Tab 5.1)**. Decorin-specific primers were created using the full length decorin sequence from the EquCab3.0 annotation and Primer3 software. RT-PCR products were visualized via gel electrophoresis for individual bands per reaction and gene length, and bands were cleaned up with the PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen/ThermoFisher Scientific) and then amplified with PCR using Elongase Enzyme

Mix (Invitrogen/ThermoFisher Scientific). The PCR products were also purified with the PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen/ThermoFisher Scientific) **(Fig. 5.2)**. The full-length cDNAs underwent Sanger sequencing at the Veterinary Genetics Laboratory located on campus at the University of California Davis. The 1257 bp "DCN 2" cDNA was chosen for downstream cloning **(Fig. 5.3)**.

III.   Cloning Equine Decorin

After sequence validation, the full-length cDNA was introduced into a pcDNA™3.1/V5-His TOPO™ plasmid from the pcDNA™3.1/V5-His TOPO™ TA Expression Kit (Invitrogen/ThermoFisher Scientific, cat no K4800-01). The *DCN* cDNA PCR product was incubated in salt solution with 1 μL of TOPO vector for 8 minutes at room temperature. Then the TOPO reaction was placed on ice. Two microliters of the TOPO cloning reaction were added to a vial of One Shot® TOP10 Chemically Competent *E. coli*; the vial was mixed gently and then incubated for 15 minutes on ice. *E. coli* cells were then heat-shocked for 30 s at 42°C, and then the cells were immediately transferred to ice. Afterward, 250 μL room temperature SOC medium was added, and the vial was capped and shook horizontally (200 rpm) at 37°C for 1 hour. The transformation media (25 μL and 100 μL) was then spread on pre-warmed 50 μg/ml ampicillin LB agar plates plates (Sigma-Aldrich, cat no SIAL-L0168-10EA) and were incubated overnight at 37°C. Ten clones were picked from the plates and then cultured overnight in 3 mL LB medium with 50 μg/ml ampicillin. Plasmid DNA was purified with a PureLink™ HiPure MiniPrep Kit (ThermoFisher Scientific). Plasmid DNA was analyzed by PCR for correct orientation using T7, BGH, and DCN specific primers using Promega GoTaq® Green Master Mix. Two clones ("8" and "9") showed the best amplification in both directions, so they were selected for further growth and

stored in frozen glycerol stocks. Equine *DCN* TOPO expression vectors ("8" and "9") were then grown in large-scale along with a TOPO control *LacZ* vector in 400 mL of LB medium with 50 μg/ml ampicillin. Large-scale growth of plasmids was purified using a Purelink™ Expi Endotoxin-Free Maxi Plasmid Purification Kit (ThermoFisher Scientific).

IV.   **Transfection Efficiency and Efficacy of Equine Adipose-Derived Mesenchymal Stem Cells**

Numerous sources can be used for stem cell therapy, such as adipose-derived, bone-marrow derived, and embryonic. For this study and for future ease of treatment, adipose-derived mesenchymal stem cells were selected due to their less invasive harvest compared to bone-marrow derived while still maintaining their ability to secrete trophic factors. Before decorin transfection can occur, establishing a protocol for the best transfection efficiency was necessary. Two different kits were used for transfection, Lipofectamine 3000 and Lipofectamine STEM (Life Technologies), with varying amounts of lipofectamine and cDNA following manufacturer guidelines to establish a transfection efficiency for equine adipose-derived mesenchymal stem cells (ADMSC) in 6-well plates. To visualize and quantify the efficiency, transfection with a pcDNA™DNA3.1/V5-His-TOPO/*lacZ* control vector (Invitrogen, ThermoFisher Scientific) was used, and efficiency was predicted using a β-gal staining kit (X-gal; Life Technologies).

From the β-gal staining, transfection efficiency was best for the Lipofectamine 3000 with 1000 ng plasmid and 4 ul of lipofectamine reagent and for Lipofectamine STEM with 2500 ng plasmid with 3.75 ul of lipofectamine reagent (**Fig 5.4**). Transfection efficiencies were slightly lower than the expected 20-40 % seen in previous studies [20,21]. With this, validation that the transfection efficiency is within a working range has been established for equine ADMSCs.

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V. Decorin Plasmid Transfection into Adipose-Derived Mesenchymal Stem Cells

Incorporation of the decorin plasmid into equine ADMSC followed manufacturer guidelines for Lipofectamine 3000 and Lipofectamine STEM at the previously mentioned concentrations. The study setup followed the transfection efficiency setup utilizing 6-well plates. Assessment of decorin production in the transfected ADMSCs was done by ELISA, Western Blot, and RT-qPCR in addition to a Pierce BCA Protein Assay to assess total protein.

The Pierce BCA Protein Assay quantified the total protein produced by the transfected cells; it showed that there was a trend in decreased total protein compared to control untransfected cells except for Lipofectamine STEM where there was an increase though not significant (Fig 5.5). This implies that cellularity between all groups is not significantly different. Although total protein is present, it was necessary to determine the amount of decorin produced by the cells to identify if transfection of the equine *DCN* plasmid was successful and productive. An equine decorin specific ELISA kit (MyBioSource, cat no MBS281010) was used with specificity up to 0.31 pg/ml. Additionally, according to manufacturer specification, no significant cross-reactivity or interference between horse decorin and analogues was observed. Sample preparation and reagent preparation was performed according to manufacturer guidelines and samples were run in triplicate with standards provided by the manufacturer with downstream wavelength correction. Across all treatments, there was a general decrease in decorin compared to control and it was trending ($p > 0.05$ and ≤ 0.1) in significance for Lipofectamine 3000 at 24 hrs and 48 hrs. A Western Blot to detect decorin was performed using the iBlot system following manufacturer protocol. Cell lysis was performed using a protein lysis buffer to prepare samples for further processing. Protein was

heated to 70°C within Bolt™ Reducing Agent and Bolt™ LDS Sample Buffer (ThermoFisher Scientific) and loaded into a a 8-12% Bolt™ Bis-Tris Plus MiniGel. Samples underwent denaturing protein gel electrophoresis with MES running buffer for 22 minutes at 200 V constant according to manufacturer instructions. Proteins were then transferred onto nitrocellulose using the iBlot™ 2 Dry Blotting System. Rabbit anti-decorin primary antibodies (Aviva Biosystems ARP48199) were applied and goat anti-rabbit secondary HRP antibodies (Invitrogen/ThermoFisher Scientific, 32460) to the blot. SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) was applied. Imaging was done on a UVP ChemiDoc-It^{TS2} (UVP, LLC) with exposure set at 30 min due to the sensitivity and lack of decorin protein in the samples. Detection of the decorin protein in the Western Blot showed no detectable level of the protein in all samples using Lipofectamine 3000. Lipofectamine STEM was more successful in producing the decorin protein at 24 hrs but overall, the control ADMSC cells had the highest abundance of the decorin protein by Western Blot **(Fig. 5.6)**. Additionally, no detectable levels of decorin were seen in samples at 48 hrs post transfection. Combined with the ELISA data, it can be inferred that the transfection of the decorin plasmid and transfection in general may be causing additional stress to the cells thereby decreasing auxiliary protein production and instead addressing the stressors.

To establish whether if transfection may be causing additional stress on the cells, RT-qPCR was done for genes enhancing apoptosis (*BAX2*), anti-apoptosis (*BCL-2*), cell proliferation (*MKI67*), metabolic stress (*PERK*), and decorin (*DCN*) with *POLR2A* as the housekeeping gene. To calculate relative expression, technical replicates within biological replicates were averaged and the relative quantity ratio was used as previously described in Chapter 2 section III.2.3.5. Statistical calculations were done in Graphpad Prism (Graphpad Software, Inc. San Diego, CA)

with significance set at $p \leq 0.05$ (*) and trending at $p > 0.05$ and ≤ 0.1 (†) using a nonparametric Kruskal-Wallis ANOVA for multiple comparisons controlled with false discovery rate. After 24 hrs post transfection, the only gene that was increased was decorin. Lipofectamine STEM transfection had a trending increase in *DCN* while Lipofectamine 3000 had a significant increase in expression compared to control ADMSC cells. There were no significant changes in apoptosis, anti-apoptosis, metabolic stress, or proliferation after 24 hrs while for 48 hrs post transfection Lipofectamine 3000 showed trending increases in *BAX* and *BCL2* and a significant increase in decorin after 48 hrs **(Fig. 5.7)**. *BAX* and *BCL2* are antagonistic associated mechanisms and the ratio of *BAX* to *BCL2* determines survival or death of a cell. With this, Lipofectamine STEM and 3000 both show trending significance towards cell death compared to control ADMSCs.

In conclusion, decorin plasmid transfection in equine ADMSCs does not show adequate transfection and secretion of decorin by way of ELISA, Western Blot, and RT-qPCR analysis after 24 and 48 hrs post transfection. Lipofectamine 3000 at 48 hrs was the only treatment that showed a significant increase in decorin expression expected after transfection but subsequent protein production of the SLRP was not quantified. Conversely, the ELISA for this treatment had trending significance for decreased abundance of decorin and the western blot also did not bind any decorin for visualization. As such, further development of the protocol for decorin transfection of ADMSCs is necessary and more extensive cell treatment would be necessary to apply this procedure as a tendinopathy therapeutic.

VI. **Limitations and Changes Necessary for Decorin Secreting Adipose-Derived Mesenchymal Stem Cells**

As mentioned in the previous section, more extensive elucidation into establishing a decorin secreting ADMSC as a therapeutic option is necessary. Some limitations and considerations that need to be made for future work include the use of a non-viral DNA vector for incorporation of the DNA (minicircle vectors) or mini-intronic plasmid containing bacterial elements, increasing transfection time or proliferation of successful decorin transfected cells, and increasing the selection options of adipose donor tissue to enhance transfection [21-25]. Cytomegaloviral (CMV) DNA vectors have shown varying degrees of transfection success with some evidence of cellular stress leading to decreased transfection rates in MSCs. As such, alternatives including lentiviral vectors or non-viral vectors may increase transfection efficiency and with non-viral vectors addressing the cellular stress occurring in the cells. Additionally, priming cells with glucocorticoids, such as dexamethasone, can further improve transfection in MSCs [21,26,27]. Another consideration is the effect that individual donor tissue has on the transfection efficiency and expression of the target gene. If the consideration of autologous cell therapeutics drives the appeal of this method, understanding that individual differences in the efficacy of the therapeutic may exist. To combat this concern, identification of a prime ADMSC candidate that has high transcription of the gene of interest is necessary. The therapeutic would then be allogeneic but would produce a fast reproducible result. Elucidation of cryopreservation on transfected ADMSCs would be necessary to make this a viable option.

Finally, increased transfection time, longer proliferation periods, and cell sorting of transduced cells would further increase the number of successfully transfected cells. Establishment of this could be done with green fluorescent protein (GFP) labelling and subsequent

harvests of transfected cells after 3 days, 1 week, and 3 weeks to establish short- and long-term success. RT-qPCR can be used for quantification of transcription levels and enzyme-linked immunoassay (ELISA) or Western Blot assays will quantify downstream protein levels. Identification of the minimum time necessary for ideal or sufficient enhanced protein production is instrumental for establishment of this procedure as a therapeutic.

VII.   Future Trajectory of Enhanced Cell Therapeutics for Tendinopathies

Tendinopathies are one of the most common career-ending injuries in horses of all breeds and disciplines. Specifically, injuries to the superficial digital flexor tendon, a load-bearing tendon, accounts for 46 – 89 % of tissue injuries depending on the discipline [28-31]. Additionally, tendon repair after injury is slow and incomplete with a high reinjury rate. Due to this major concern in equine medicine, therapeutics to aid in the repair process have been an ongoing endeavor. Some examples include mechanical stimulation like shockwave therapy, cell-based injections such as mesenchymal stem cells or other stem cells, and platelet-rich plasma therapy [32,33]. Across all therapeutics, the stimulation of extracellular matrix proteins, growth factors, and differentiation factors is key to a better repair response. There have been varying degrees of success due to the variation in harvest, isolation, and development into an injection [32]. Additionally, therapeutic success is also dependent on the severity and time from initial injury. Injection with MSCs, more prominent with bone marrow-derived, has noted greater success across varying tendinopathies than other therapeutics but even still a reinjury rate of 27% is observed in racehorses [34]. As such, to enhance the specificity of the expressed trophic factors and guide the surrounding tissue for regeneration, incorporation of additional extracellular matrix proteins that

are responsible for collagen maturation, ECM scaffolding, and collagen cross-linking could increase the repair in tendon.

Enhanced therapeutics via genetic alterations may cause further variations due to autologous individuality from MSC isolation or induction, in addition, to donor variation causing loss of finances and loss of time to training due to the increased variables. Although a single component capable of long-term protein of interest and trophic factor secretion would be far preferential, the nuances of this cause concern for actual application. Studies involving supplementation of certain SLRPs, growth factors, or collagen co-factors have all shown increases in collagen production, incorporation, or strengthened tissues shedding light on their importance [35-38]. Understanding *in vitro* supplementation usually involves specific concentrations in a specific model system that is not *in vivo*, typically in a 2D or 3D system without interference from external factors. Due to this, basic understanding of a tenogenic effect can be elucidated but the reaction in native tendon may be different including interfering, enhancing, or suppressing mechanisms. Furthermore, in mature tendon, collagen turnover and remodeling are limited from epigenetic and transcriptional limitations during this stage of development [39].

At this point in tendon regenerative research, using a two- or three-pronged approach in an injection would potentially enhance tendon repair decreasing reinjury and strengthening native tendon. In this approach, a combination of MSCs, SLRPs, and co-factors would ideally lead to a pre-injury tendon structure. Although not as effective as bone marrow-derived mesenchymal stem cells, adipose-derived mesenchymal stem cells still secrete numerous necessary trophic factors and are far less invasive in their harvest contributing to their appeal. Utilizing ADMSCs for their growth factor secretion, while supplementing an injectable dosage with SLRPs such as decorin and vitamin C would create a multipronged approach to repair. The appeal of this would include

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utilizing an autologous cellular source with a researched dosage for the additional components. With this, the issue of individual transfection efficiencies and subsequent secretions would be addressed and components such as co-factors and non-protein sources can be added to the injections for better, more direct repair.

VIII.   References

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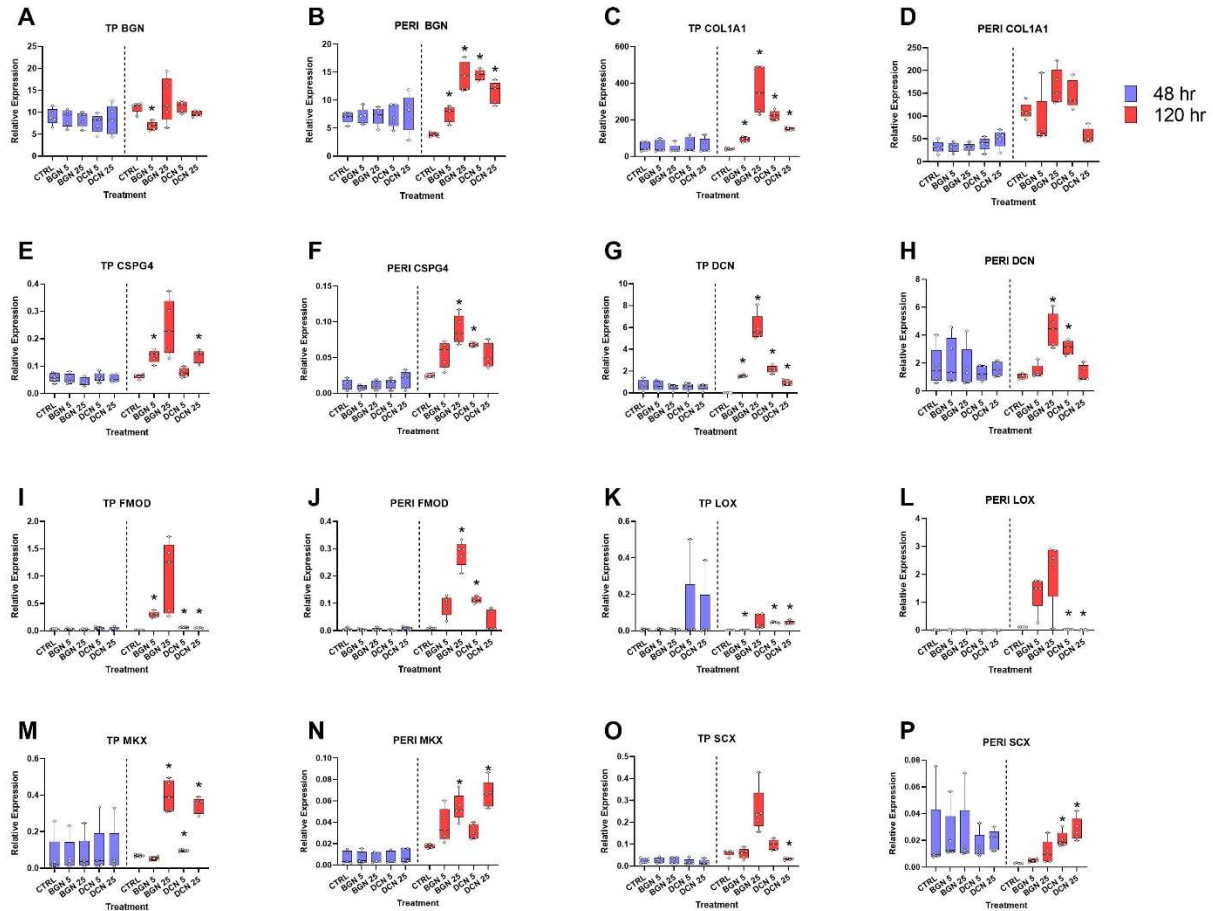
Figure 5.1. Gene expression of decorin supplemented tendon proper and peritenon cells in 2D. Tendon proper and peritenon cells from 5 horses were supplemented biglycan or decorin at 5 nM or 25 nM and harvested at 48 hr and 120 hr. Statistical significance denoted as $p \leq 0.05$ (*).

Figure 5.2. Validation of isolated decorin cDNA genomic size before gene sequencing. Three

different primers designs were utilized for decorin cDNA isolation based on the available sequence

in EquCab3.0. The gel confirmed cDNA isolation of decorin in the expected range.

DCN amplification #2, 1257 bp
Primers: DCN F2, DCN R1
8 exons

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0     AGAAGCAGGAGGTTTTCAACCTAGTGACAGTCACAGAGCAGCACCTACCCCCTCCTCCTTTCCACACCTG  70
71    CAAACTCTTTTGCTTGCGCTGAAAATTTAGTGTAATTACATCTCAGCTTTGAGGGCTCCTGTGGCAAATC  140
141   CCCGGATTAAAAGGTTCCTTGGTTGTGAAAATACATGAGATAAATCATGAAGGCAACTATCATTTTCCTT  210
211   CTGCTTGCACAAGTTTCCTGGGCTGGACCATTTCAACAGAGAGGCTTATTTGACTTCATGCTAGAAGATG  280
281   AGGCTTCTGGGATTGGCCCAGAAGATCGCATTCATGAAGTTCTAGACTTAGAGCCTCTGGGACCAGTGTG  350
351   TCCTTTCCGCTGTCAGTGCCATCTTCGAGTTGTCCAATGTTCTGATTTGGGTCTGGACAAAGTGCCCAAA  420
421   GATCTTCCCCCTGACACCACGCTGCTGGACCTGCAAAACAACAAAATAACCGAAATCAAAGATGGAGACT  490
491   TTAAGAACCTGAAGAATCTTCATGCGTTGATTCTTGTCAACAACAAAATTAGCAAAATCAGCCCTGGAGC  560
561   ATTTACACCTTTGGTGAAACTGGAACGACTTTATCTGTCCAAGAATCATTTGAAGGAATTGCCAGAAAAA  630
631   ATGCCCAAAACTCTTCAGGAGCTGCGTGTCCATGAGAACGAGATCACCAAAGTGCGAAAAGCGGTGTTCA  700
701   ATGGACTGAACCAGATGATAGTCGTAGAACTGGGCACCAACCCACTGAAGAGCTCAGGAATTGAAATGG  770
771   AGCCTTCCAGGGGATGAAGAAGCTGTCCTACATCCGCATTGCTGACACCAACATAACCACCATCCCTCCA  840
851   GGTCTTCCTCCTTCCCTTACTGAATTACATCTTGATGGCAACAAAATCACCAAAGTTGATGCAGCTAGCC  910
921   TGAGAGGACTGAATAATTTGGCTAAATTGGGACTGAGTTTCAACAGCATCTCTGCTGTTGACAATGGCTC  980
991   TCTGGCCAACACTCCTCATTTGAGGGAACTTCACTTGGACAACAACAAGCTTATCAAAGTGCCTGGTGGG  1050
1061  CTGGCGGATCATAAGTACATCCAGGTTGTCTACCTTCATAACAACAATATCTCTGCAGTTGGATCTAATG  1120
1131  ACTTCTGCCCACCTGGATACAACACCAAAAAGGCTTCTTATTCGGGTGTGAGCCTTTTCAGCAACCCAGT  1190
1191  CCAGTACTGGGAGATCCAGCCATCCACCTTCCGATGTGTCTATGTGCGCTCTGCCATTCAGCTCGGAAAC  1260
1261  TACAAGTAACTCCCAGGAAAGCCCTCATCTTTATAAGCTGGCAACATCCCGTTAATGTCATTGCTC  1326
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Fig. 5.3. Equine DCN cDNA amplification selected for cloning. The second amplification was used for cloning. The *DCN* gene has 8 exons. These eight exons are demarcated by different colors of letters. Italicized font represents untranslated regions; underlined font is where the amplifying primers were located within the sequence.
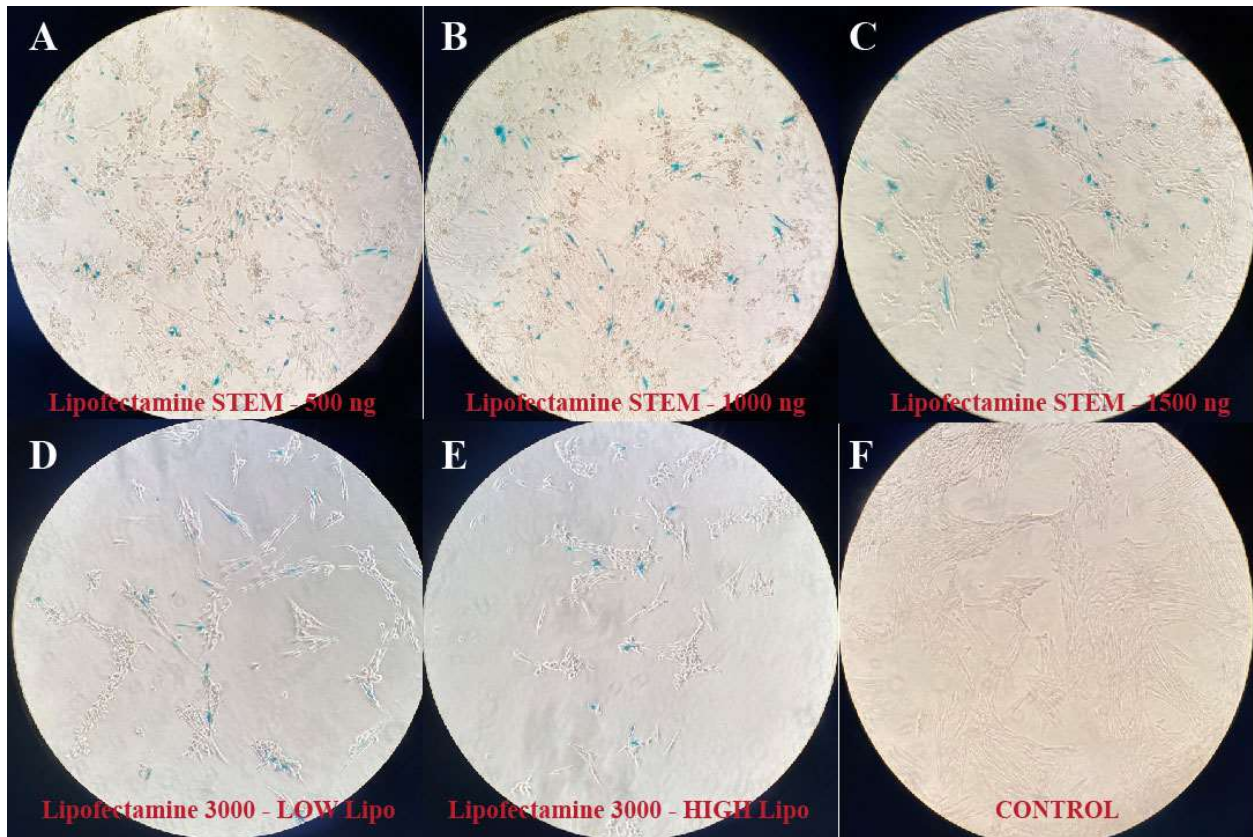
Figure 5.4. X-Gal staining to validate transfection efficiency of equine adipose-derived mesenchymal stem cells. The β-galactosidase reporter transfection was used to validate the transfection efficiency of the ADMSCs either using Lipofectamine STEM (at three different DNA concentration: (**A**) Lipofectamine STEM – 500 ng, (**B**) Lipofectamine STEM – 1000 ng, (**C**) Lipofectamine STEM – 1500 ng) or Lipofectamine 3000 (at two lipofectamine concentrations: (**D**) Lipofectamine 3000 – LOW Lipo, (**E**) Lipofectamine 3000 – HIGH Lipo) and an ADMSC control (**F**).
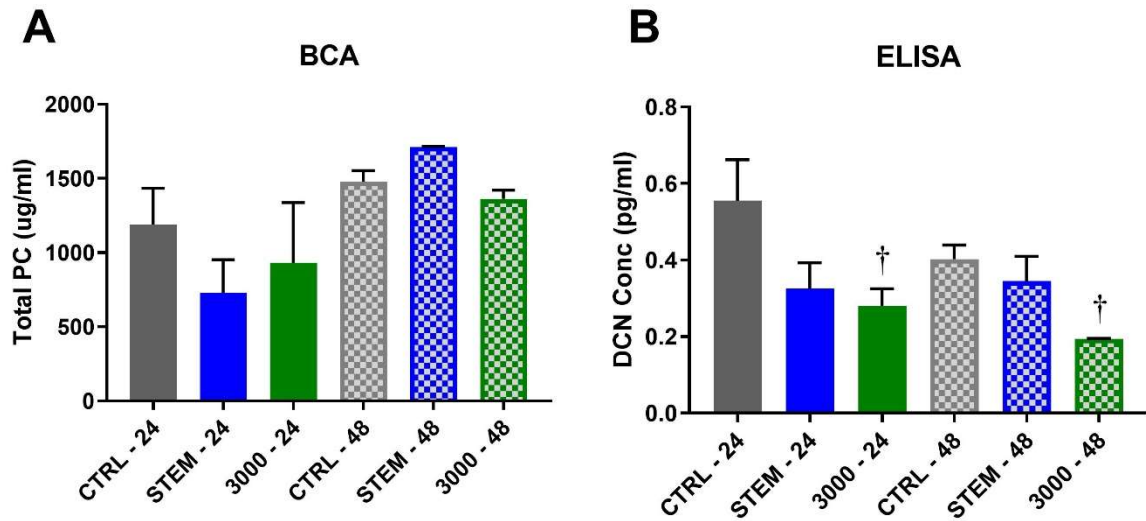
Figure 5.5. Total Protein and Decorin Concentration in Decorin Plasmid Transfected ADMSCs. (**A**) Pierce BCA Protein Assay for decorin plasmid transfected ADMSCs with Lipofectamine STEM and Lipofectamine 3000 at 24 and 48 hrs post transfection. (**B**) Decorin specific ELISA assay for decorin plasmid transfection. Statistical significance denoted as $p \leq 0.05$ (*) and trending at $p > 0.05$ and ≤ 0.1 (†).
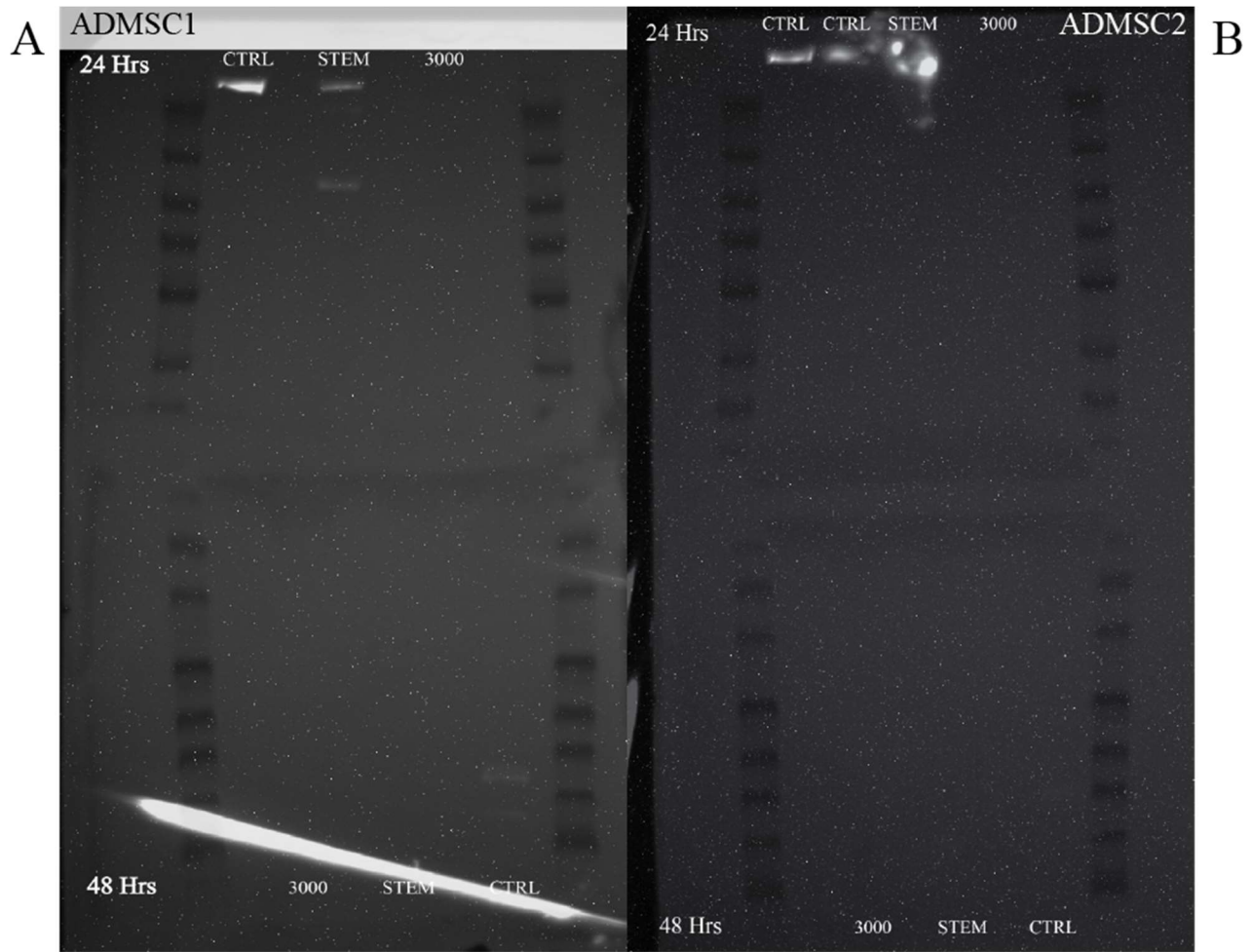
Figure 5.6. Western Blot images for Decorin Transfected MSCs. A western blot was done to validate the presence of decorin in transfected ADMSC cells. **(A)** ADMSC1 transfected cells using Lipofectamine STEM or Lipofectamine 3000 with a decorin plasmid for 24 hrs or 48 hrs post transfection. **(B)** ADMSC2 decorin transfected cells using Lipofectamine STEM or Lipofectamine 3000 for protein detection after 24 or 48 hrs post transfection.

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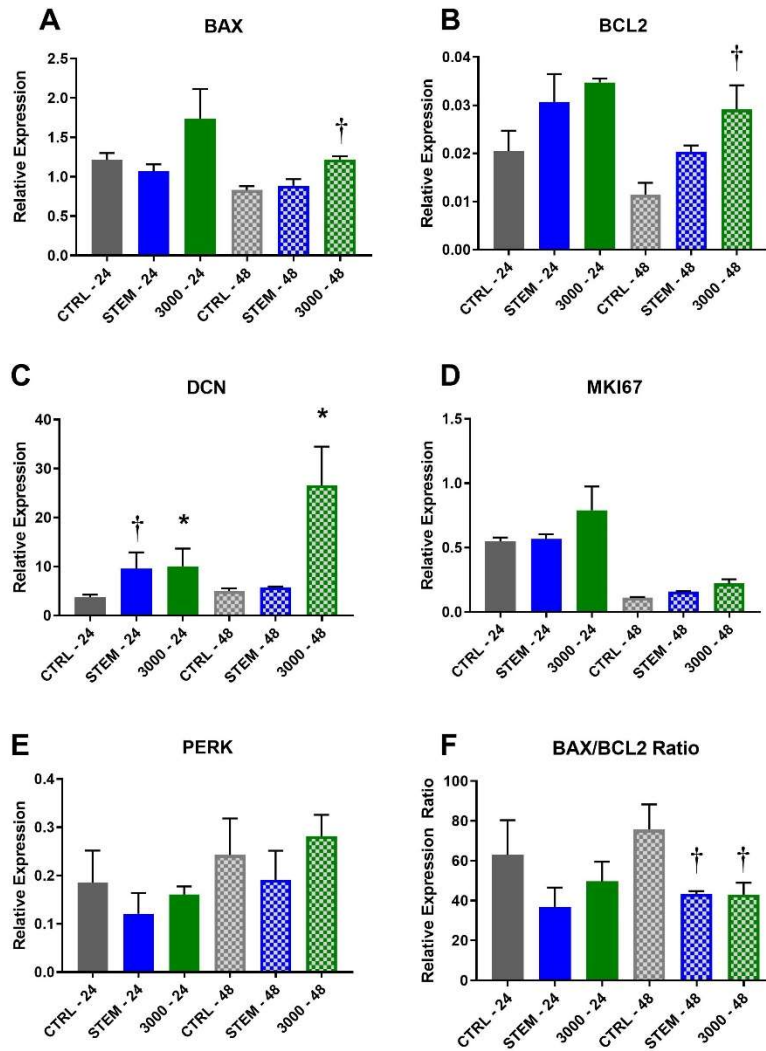
Figure 5.7. RT-qPCR of decorin transfected ADMSC. Gene expression at 24 and 48 hrs post transfection of genes of interest included pro- and anti- apoptotic markers (BAX; BLC2), decorin (DCN), cell proliferation (MKI67), and metabolic stress (PERK) to better elucidate the stress of transfected ADMSCs. Statistical significance denoted as $p \leq 0.05$ (*) and trending at $p > 0.05$ and ≤ 0.1 (†).

X. **Tables:**

RT ID (Length)	Forward Primer	Reverse Primer
DCN 1 (1166 bp)	F1: 5' GCAAATCCCCGGATTAAAAG-3'	R1: 5'-AGCTTATAAAGATGAGGGCTTTCC-3'
DCN 2 (1257 bp)	F2: 5'-ACCTACCCCCTCCTCCTTTC-3'	R1: 5'-AGCTTATAAAGATGAGGGCTTTCC-3'
DCN 3 (1255 bp)	F3: 5'CTACCCCCTCCTCCTTTCC-3'	R1: 5'-AGCTTATAAAGATGAGGGCTTTCC-3'

Table 5.1. Equine-specific DCN primers for RT-PCR of full-length cDNA. Using Primer3software and the Equcab3.0 annotation, three primer designs were generated to for PCR amplification for the equine decorin sequence.

Kit Type	DNA Conc (ng)	Reagent Amt (ul)
Lipofectamine 3000	500	2
Lipofectamine 3000	1000	4
Lipofectamine 3000	1500	6
Lipofectamine STEM	2500	1.875
Lipofectamine STEM	2500	3.75

Table 5.2. Lipofectamine Transfection Concentration Setup. Based on manufacturer guidelines, the above concentrations for DNA and volume for lipofectamine reagent were selected to test the optimal transfection efficiency for equine ADMSCs.