TENDON REGENERATION: ROLES OF GROWTH FACTORS AND PHENOTYPIC DIVERSITY OF TENDON STEM CELLS

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ABSTRACT

Tendon injuries significantly impact quality of life and are often career ending. Mesenchymal stem cell (MSC) therapy is known to augment intrinsic tendon healing, however, little is known of the stem cells endogenous to tendon, the microenvironmental cues that induce tendon differentiation, and whether individual cells in an inflammatory milieu respond differently to these cues. To address these questions, a three-dimensional tenogenesis assay was developed as an efficient and reproducible metric of cellular capacity to differentiate toward tendon. In contrast to more complex assays of tenogenesis, this design incorporates a simple apparatus using commercially available plasticware for the application of uniaxial static strain in in a type I collagen cell-seeded hydrogel construct. Tendon-related gene expression, glycosaminoglycan levels, elongated cell morphologies and parallel cell alignments are enhanced with BMP-12 induction over ten days of culture. This dissertation provides novel insight to the roles of growth factors in MSC tenogenesis.

Tendon healing *in vivo* is dependent on endogenous tendon stem cells (TSC) that mediate the inflammatory response to injury and promote synthesis of collagen and matrix remodeling, among other extracellular processes. Recent evidence suggests that these cells exist on a spectrum of differentiation potencies, and may be differently committed to the tendon fate. Individual stem cells were isolated from the tendon, and their capacities for proliferation, tri-lineage differentiation and tenogenesis were evaluated. Three distinct TSC phenotypes were

revealed, and significant, positive correlations were found in quadra-differentiation potency (toward four lineages) and the expression of a strong, composite tendon phenotype.

These studies have important implications in the current standard-of-care in regenerative therapies for tendon. Our benchtop tenogenesis assay can be used to determine the therapeutic potential of allogeneic MSC lines and MSCs from novel sources for 'off-the-shelf' treatments. Our study of TSCs lends valuable insight to the diversity of cell phenotypes found in tendon, and the potential contributions of each phenotype to tendon healing and homeostasis. These results further strengthen the status of tendon as a superior source of stem cells for tendon repair.

GENERAL AUDIENCE ABSTRACT

Tendons are fibrous, elastic bands of collagen that connect muscles to bones and are essential to movement and proper functioning of the skeletal system. Weight-bearing tendons like the Achilles in humans and superficial digital flexor tendons in horses are particularly prone to damage and degeneration with overuse and/or aging. Bone marrow-derived stem cell treatments have shown promise in the reduction of pain and inflammation, and restoration of native tendon structure and function in cases of severe tendon injuries. However, the roles of stem cells in tendon healing, particularly their ability to transition to cell types native to tendon and integrate with an environment distinct from their own is unknown. Culturing of stem cells in three-dimensional (3D) environments has enabled us to identify and understand the biochemical and mechanical signals that trigger stem cell transitions to tendon cells in tendons, but currently available 3D culture systems are complex and inefficient. In this dissertation we have developed a cost-effective and high throughput 3D culture system to assay the potential of stem cells to form tendon cells and composite tendon-like tissues. Toward this, we have also optimized the effects of known tendon proteins on the tendon fate in 3D culture of stem cells.

Like most adult tissues, the tendon encompasses an in-house repository of stem cells. Tendon stem cells (TSCs) are primarily responsible for the inflammatory and reparative responses to tendon injury. Recent evidence suggests that TSCs are diverse in character, and differ from each other in their ability to form cells and tissues of fat, bone and cartilage. In this work, we provide evidence that TSCs are also differently committed to forming tendon tissue, and moreover that significant inter-relationships among gene expression patterns in these cells directly contribute to cultural diversity.

In sum, our results provide novel insight to the roles of stem cells in tendon healing, particularly their response to subtle changes in their biochemical environment, and the contributions of individual cells in a milieu to a holistic reparative response.

DEDICATION

To my parents, Mohamed Husein and Zarina Rajpar, for their unconditional love, support, and for summoning the courage to send their daughter to America. All I want is to make you proud.

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BCAR1	Breast cancer anti-estrogen resistance protein 1
bHLH	Basic helix-loop-helix
BMP	Bone morphogenetic protein
CD	Cluster of differentiation
COLI	Collagen type I
COX	Cyclooxygenase
CTGF	Connective tissue growth factor
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
Egr	Early growth response factor
ERK	Extracellular signal-regulated kinase
FABP4	Fatty acid binding protein 4
FGF	Fibroblast growth factor
FRS2α	FGF receptor substrate-2α
GAG	Glycosaminoglycan
GDF	Growth and differentiation factor
GDP	Guanosine diphosphate
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
IGF	Insulin-like growth factor
IL	Interleukin

IP_3	Inositol triphosphate
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
MRL	Murphy Roths large
MSC	Mesenchymal stem cell
Oct-4	Octamer 4
Pax1	Paired box 1
PDGF	Platelet-derived growth factor
Pea3	Polyoma enhancer activator 3
PGE_2	Prostaglandin E2
PI3K/Akt	Phosphoinositide 3-kinase/ Protein kinase B
PLC	Phospholipase C
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
Sca-1	Stem cells antigen-1
SDFT	Superficial digital flexor tendon
SMAD	Single Mothers Against Decapentaplegic
SHCA	Src Homology and Collagen A
SSEA	Stage specific embryonic antigen
SOS	Son of Sevenless
Sox9	SRY-Box-9
TAK-1	TGF-β-activated kinase-1

TC	Clonal tendon stem cell line
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TP	Parent tendon stem cell line
TRAF-6	TNF receptor-associated factor 6
TSC	Tendon stem cell
VEGF	Vascular endothelial growth factor
Wnt	Wingless/Integrated
2D	Two-dimensional
3D	Three-dimensional

OVERVIEW OF DISSERTATION

Chapter 1 is a review of introductory topics in tendon biology, including structure and function relationships, developmental paradigms and the mechanobiology of tendons. We have a general understanding of the etiology of tendinopathy, which is mainly characterized by the breakdown of collagen architecture and the dysregulation of integral functions involved in homeostasis. The goal of regenerative medicine is to promote scarless healing in adult tendons and thereby restore native structure and function.

Chapter 2 is an introduction to the principles and current knowledge of tendon growth factors, scaffolds and MSCs within the context of research described in this dissertation. A review of combined growth factors highlights recent, exciting results on synergistic effects in tendon differentiation and repair. Collagen hydrogels have successfully been used as 3D platforms for MSC characterization, and various parameters of hydrogel function have been reviewed with an emphasis on *in vitro* tendon models. The final section introduces MSCs and its current applications in tendon healing. The prevalence and importance of phenotypic diversity in MSC culture is described, concluding with a brief overview of current techniques in single cell analysis.

Chapter 3 describes original research based on the development of a tenogenesis assay for the rapid evaluation of MSC efficacy for tendon engineering and clinical applications. This benchtop assay was inspired by complex *in vitro* models and bioreactor systems to successfully accomplish a composite, tendon phenotype with a simple growth factor cocktail and standard,

tissue-culture plasticware. BMP-12 application over 10 days of culture was found to upregulate expression of scleraxis, augment MSC elongation, alignment and ECM GAG deposition, and balance contraction toward the generation of a mature, tendon-like construct. Additionally, significant results from the applications of novel growth factor combinations in this assay have been described, with direct comparisons to single factors in parallel experiments.

Chapter 4 is an original research study establishing the presence of distinct cell phenotypes in tendon, with an emphasis on TSC differentiation potencies *in vitro*. Regenerative tendon treatments can greatly benefit from a superior understanding of endogenous TSC function, specifically, the inter-relationships in gene and protein expression patterns that govern the intrinsic healing response to injury. Single-cell derived TSC lines were classified into three groups based on their ability to differentiate to 1) fat, bone, cartilage and tendon (AOCT), 2) bone, cartilage and tendon (OCT) or 3) fat, cartilage and tendon (ACT). The AOCT phenotype strongly differentiated to bone, fat and cartilage in standard tri-differentiation assays, exhibited several doublings in culture over three passages, and differentiated to a mature, tendon phenotype upon tenogenesis, by virtue of enhanced scleraxis and mohawk gene expressions, tendon-like tissue morphology and histology.

Chapter 5 describes major conclusions from chapters 3 and 4, potential future directions and proposed future experiments.

CHAPTER 1: INTRODUCTION TO TENDON BIOLOGY

Structure and function relationships

Tendons are fibrous connective tissues that transfer forces from muscles to bones. Tendons enable movement, and their health is essential to the quality of life. These tissues are primarily composed of a dense, collagenous matrix that consists mainly of collagen type I (65-80%) and elastin (1-2%)[4, 5].

Tropocollagen, the smallest modular unit of collagen, consists of three α-polypeptide chains (two of COLIα1 and one of COLIα2[6] woven together to form a triple-helix. Collagen microfibrils are the smallest microscopically detectable unit of collagen fibers, and composed of tropocollagen molecules, assembled in parallel but staggered arrays[7], and interconnected by lysyl oxidase-catalyzed crosslinks[8]. Microfibrils aggregate to form fibrils, which progressively assemble to form collagen fibers and fascicles. A connective tissue sheath termed the endotenon surrounds collagen fascicles or fiber bundles. The cross-sectional diameter and length of collagen fibrils are important markers of tendon health, type and their response to mechanical forces in development and disease. In addition to collagen type I, collagen types II, III, IV, V, IX, X, XI and XIV are also found in relatively smaller amounts[4]. Collagen type III is a fibrillar collagen and the second most abundant collagen found in tendon. It comprises 10% of the total collagen in healthy tendons[9].

Proteoglycans are important constituents of the tendon extracellular matrix (ECM), and play a role in the proper mechanical functioning of the tissue. They facilitate collagen fibrillogenesis[10] and "fiber gliding"[11, 12]. Decorin, biglycan, fibromodulin and lumican are small

leucine-rich proteoglycans. Of these, decorin constitutes 80% of the proteoglycans found in the load-bearing region of tendon[13, 14]. Production of these molecules decreases with age, rendering tendons susceptible to damage; however, moderate exercise may promote proteoglycan synthesis. Large proteoglycans like aggrecan and versican, are found in the fibrocartilaginous regions of tendons. They provide resistance against sudden compressive and tensile forces[15], by virtue of negatively charged sulfate groups on glycosaminoglycan (GAG) side chains that render the molecules hydrophilic. Proteoglycans attract and bind water molecules to provide resistance to external forces. Decorin has a single dermatan or chondroitin sulfate side chain[14]. GAG content is an important indicator of the physiological and pathophysiological state of tendon[16, 17]. GAGs may act as molecular cross-links between collagen fibrils, suggesting a role in the maintenance of normal tendon mechanics[12]. GAG-functionalized scaffolds exhibit superior mechanical properties. However, enzymatic removal of GAGs does not affect the elastic modulus of chick tendons in development[18].

The tendon ECM is interspersed with cells that are aligned in parallel to the tensional axis, and reside between adjacent collagen fibrils. These are generally spindle-shaped with a high nucleus to cytoplasmic ratio, but may differ morphologically[19]. Early investigations of tendons classified tendon cells into two types: the tenoblasts and tenocytes[20, 21]. Tenoblasts were undifferentiated, and usually found in the epitenon, whereas tenocytes were differentiated tendon cells and found in the tendon proper. Bi et al.'s investigations into the tendon stem cell niche have resulted in a paradigm shift in our understanding of tendon-derived cells[22]. It is now worth noting that most literary references to "tenocytes"m "tendon fibroblasts" and "tendon stem cells" encompass a mixture of tendon-derived cells at different stages of commitment to the tendon lineage. They may also express nuclear and surface markers of stemness including Oct-4 and

SSEA-4 and exhibit tri-lineage differentiation potency; hence the term "tendon stem cell"[23]. In addition to these cells, smaller amounts of chondrocytes, synovial cells and vascular cells are also found in tendon, particularly in regions subject to compressive forces such as the tendon-bone junctions[11].

Tendons have low metabolic activity and are preferential to anaerobic energy generation, due to which they can sustain loads for long periods[24]. The characteristic load-elongation curve of a tendon consists of three distinct regions: a toe region characterized by crimp straightening, a linear elastic region where increasing strain results in proportional increases in tendon length, and a yield region, where progressive accumulation of creep eventually results in tendon rupture[25, 26]. Supraphysiologic strains of more than 4% result in microdamage in the tissue, and strains beyond 8-10% result in intrafibrillar damage and macroscopic failure[24]. Mechanical testing is used to compute the mechanical properties of tendons[27].

Tendons transmit forces from muscles to bones by elongating their collagen fibrils (characterized by increases in the D-periods of adjacent collagen molecules)[11]. Tendon mechanics are sensitive to the orientation of collagen fibrils and the anatomical locations of tendons. Achilles tendons for example, are weight-bearing tendons and positioned to receive greater loads in the body compared to the anterior tibialis tendons. At low strains, they are less stiff and have a lower elastic modulus, however, they can adapt to high strains by acquiring greater stiffness and increasing their elastic modulus. Achilles tendons hence exhibit viscoelastic behavior, which enables them to withstand frequent, high strains of even more than 10% without rupturing. Since these tendons are usually subject to unidirectional forces along a longitudinal axis, their fibers are arranged in an orderly parallel alignment to the axis[4].

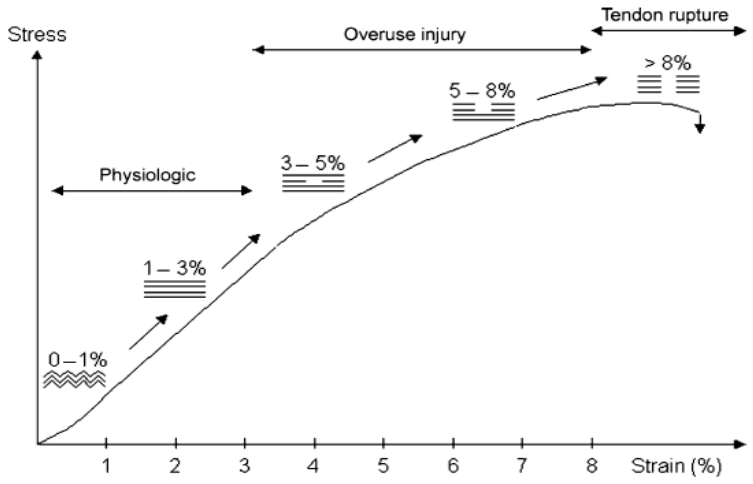


Fig 1.1. Classic load-elongation curve of a tendon under strain (*Adapted from Arnozcky et al. 2007*[2])

Origins and development

Tendons arise from two distinct domains of the developing embryo: the axial tendons, like those connecting the intercostal muscles to the ribs, arise from the somite, whereas appendicular tendons like the limb tendons arise from the lateral plate mesoderm. The discovery of scleraxis, a bHLH transcription factor and marker of early tendon progenitor cells, has greatly facilitated our understanding of early tendon development[28]. Scleraxis is expressed both in early tendon primordia as well as in late differentiated tendons.

In the somite of chick-quail embryos, expression of scleraxis is exclusive to a region distinct from the sclerotome, myotome and dermatome. This region is termed the syndetome, and it arises from the sclerotome. The two regions are morphologically similar; although, scleraxis expression does not overlap with Pax1, a sclerotome-specific marker[29]. Scleraxis transcripts are also evident in all 9-10 day embryonic chick limbs at muscle to bone attachment sites, the wing aponeurosis, and in muscle attachments in the trunk and neck. In the mouse limb bud, scleraxis is first induced as dorsal and ventral patches; however, by E12.5, an organized alignment of

scleraxis-expressing progenitor cells can be detected between the muscles and cartilage[30]. Scleraxis-null mutant mice embryos are viable, but may not develop long tendons in the body, and are functionally impaired.

The mechanisms underlying the induction of scleraxis and the tendon cell fate in development are not well understood. One hypothesis suggests that FGF-8-expressing cells, found in the central myotome, may be responsive to their receptors at the anterior and posterior edges of the myotome, and activation of FGF signaling in this region induces scleraxis expression in the adjoining sclerotome[29, 31]. However, ectopic expression of FGF-8 can also induce scleraxis[32], hence it is not clear how FGF signals co-localize with scleraxis-expressing cells in the somite. FGF may also induce scleraxis expression by activating the transcription factors Pea3 and Erm in cartilage progenitor cells of the sclerotome[33]. Cues from cell and tissue culture experiments further inform our knowledge on the specification of the tendon fate. For example, Wnt3a in concert with FGF-8 suppresses cartilage differentiation *in vitro* culture[34]. Scleraxis-expressing cells arise from cartilage progenitor cells in the somite[35], and hence this effect suggests a potential mechanism for FGF-8 to induce scleraxis expression in the developing embryo[36].

Members of the TGF-β superfamily have also been implicated in the regulation of scleraxis expression in the embryo[33, 37]. During early development, the interaction between FGF and TGF-β and resulting activation of the MAPK and SMAD2/3 pathways promote scleraxis expression in the embryo[38]. In one study, loss of TGF-β signals in TGF-β2-null and TGF-β3-null double mutant mouse embryos resulted in the loss of most tendons in the limbs, trunk, tail and head[39]. BMPs were initially considered as repressors of the tendon phenotype, and primarily transduce signals via the SMAD pathway. Application of Noggin (a BMP antagonist) to the

mouse limb mesenchyme results in a dramatic upregulation of scleraxis expression[28]. However, disruption of SMAD signaling does not result in a tendon-specific fate[40], suggesting that a different (non-SMAD) BMP pathway may be involved in tendon development.

In addition to scleraxis, several other transcription factors and matrix molecules are involved in tendon development, and their roles have evolved in the recent past. None of these markers are exclusive to, but highly expressed in embryonic tendon. Mohawk, an atypical homeodomain transcription factor, is essential for normal tendon morphogenesis, and strongly expressed in embryonic tendon[30]. Compared to scleraxis-null mutants, mohawk-null mutant mice exhibit less severe defects in postnatal development. Tendons in the latter are characterized mainly by hypoplastic, smaller and more homogenous collagen fibrils, but otherwise exhibit minimal functional deficits, typical of scleraxis-null mice[41, 42]. Egr1 and Egr2, belong to a family of zinc finger transcription factors. They are highly expressed in developing tendons, and modulate the induction of several other tendon-related genes, including scleraxis[41, 43]. Egr1 and Egr2 have different expression patterns in tendon development. Unlike transcription factors, the tendon glycoproteins, tenomodulin[44-46], tenascin[25] and thrombospondin[30] play minor roles in development. Their contributions may be restricted to tendon structural and functional properties in the postnatal stages, rather than cell fate specification. Nonetheless, they are highly relevant makers of the tendon phenotype.

Mechanobiology

Tendons frequently experience stress from external forces. The primary responders of these forces *in vivo* are the tenocytes, and their behavior depends on the magnitude, frequency, direction, duration and type of force transmitted[47]. The cellular response to external forces in turn

affects the material properties or "response" of the underlying substrate to cells. This feedback loop of behavioral responses in cells is termed the "inside-outside-in" pathway[11, 48].

Cells respond to changes in the stiffness and topography of the ECM[49] by inducing macromolecular changes in the cell membrane such as the formation of focal adhesions and stress fibers, the opening of stress-activated ion channels and changes in the cytoskeletal organization[50]. The resulting intracellular cascade in cells is driven by actin/myosin-dependent contractile forces[51] that enable physical interactions between cells and the ECM. Cells can thus alter their phenotype in response to changes in the ECM.

The integrins are heterodimeric transmembrane receptor proteins, comprising α and β subunits[52]. They mediate cytoskeletal attachments to the ECM by binding various matrix molecules including collagen, tenascin and fibronectin[50]. The binding of integrin receptors to matrix proteins induces the lateral movement and clustering of these receptors in the plasma membrane to form focal adhesions. Integrins thereby provide physical links between the ECM and the cytoskeleton, for anchorage, migration and signal transduction, and their functions are augmented by soluble TGF-β in the ECM[53]. In tendons, integrins are associated with the organization of collagen architecture. Evidence suggests that loss of the tenocyte-specific α2 and α11 integrin subunits in young tendons results in reduced collagen remodeling and matrix disorganization, typical of age-related degeneration[54]. In contrast, optimized loading protocols may elevate expression of this receptor in tenocytes[52].

Upon activation, integrins recruit stress fibers and signaling proteins like talin, an actin-integrin binding protein, into their cytoplasmic domains[55]. Stress fibers are composed of F-actin bundles held together by α-actinin-mediated crosslinks. They respond to small external forces by activating mechanically gated ion channels, such as the voltage-operated calcium channel in

tenocytes[20]. The opening of these stress-activated channels triggers an influx of calcium; the primary second messenger of signal transduction in cells[56, 57]. Integrin activation can elicit a variety of intracellular pathways downstream, but mainly involves the activation of Rho-GTPases. Activated focal adhesion kinases bind adaptor proteins like BCAR1 and guanine-nucleotide exchange factors that catalyze the conversion of Rho-GDP to Rho-GTP. Rho-GTPases are involved in several intra- and extracellular processes including the maintenance of cytoskeletal organization and the formation of specialized actin structures. Interactions between the Rho and ERK pathways have been implicated in the specification of MSC cell fate in response to substrate rigidity[50].

Calcium ions are in a constant flux between cells and the ECM. They are released from cells to the ECM through an IP_3-mediated pathway, but can also travel between cells through gap junctions[56]. Tenocytes respond to strain by increasing levels of intracellular calcium, and this response is dependent on the presence of a threshold level of extracellular calcium. Gap junctions enable cell-to-cell communication, and are composed of connexins; these are transmembrane proteins that assemble in the cell membrane to form a connexon or gap junction pore. Gap junctions are known to regulate DNA and collagen synthesis in tenocytes[58]. Cadherins are cell adhesion proteins that form cell-cell junctions in the cytokskeleton[57]. Like focal adhesions, cadherin-dependent adhesions are also mechanosensitive, but unlike focal adhesions, their mechanisms of signal transduction are not well understood.

Cilia are a different form of mechanical signal transducers; these are specialized organelles with slender projections extending from the cell surface into the ECM. Cilia respond to mechanical stimuli by shortening their lengths[59], presumably by the same actin-dependent contraction mechanisms involved in cytoskeletal homeostasis[60]. Conversely, lack of stimuli or

tissue laxity induces elongation of cilia. Tenocytes respond to induced tissue laxity by increasing their ciliary lengths over the first 24-hour period, followed by contraction of their cilia back to resting lengths over 7 days[59]. Cilia transduce mechanical signals into cells via numerous receptors on their surface like the tyrosine kinase receptors, that further relay these signals to the nucleus via the MAPK, P13K-Akt and the PLC pathways, among others[61]. Cilia are highly aligned to the tensional axis in load-bearing tendons[62], and may be involved in the anisotropic organization of collagenous matrices.

Pathophysiology and mechanisms of degeneration

Tendon injuries are classified into two types: acute and chronic. Acute tendon injuries occur due to a sudden and short-lived traumatic event. Chronic injuries or degeneration is characterized by the progressive accumulation of microdamage over time, usually in response to tendon overuse or aging. The term "tendinopathy" encompasses all tendon-related pathologies, whereas "tendonitis" or "tendinitis" fundamentally refers to the inflammatory component of tendon injury, though inflammation may not necessarily precede a degenerative response[63, 64]. Complete rupture of tendons maybe spontaneous; however, their pathophysiology is consistent with that of chronic tendinopathy.

Tendinopathy is a significant and unresolved clinical problem that affects millions of people in the United States annually. Sport-related injuries mainly involve the Achilles, patellar and/or tendons attached to the lateral epicondyle in the elbow. Achilles tendinopathy affects 1 out of every 10 people under the age of 45, with increased incidence in runners[65]. Patellar tendinopathies or "jumper's knees" account for 30% of all sport-related injuries[66]. Rotator cuff tears are common in the aging population, and incidence increases with age[67]. In the athletic

horse, tendons are involved in 46% of all limb injuries sustained on the racetrack, and mostly involve injuries of the superficial digital flexor tendon[68, 69]. Injuries to the SDFT are potentially career-ending; in one retrospective study of injured Thoroughbred racehorses, only 14.2% successfully returned to racing post-injury[70].

Tendinopathy is characterized by several histopathological changes in the tendon. Discoloration of tendons or "reddening" due to increased vascularization is one of the first signs of early onset degeneration in response to intensive exercise[1, 71]. Loading of tendons beyond this point results in the thinning, splitting, disintegration and angulation of collagen fibrils that are evident by macroscopic structural alterations in the ECM. Normal tendon is hypovascular and hypocellular[72], but the inflammatory response post-injury is characterized by damage to the blood vessels with hemorrhage and edema, followed by neovascularization and an influx of tenocytes to the injury site. While increased cellularity is expected, few studies have reported relatively acellular regions in macroscopically abnormal tendons[73]. A significant increase of sulfated GAGs is observed in degenerating tendons compared to normal, healthy tendons[66, 71] that may be attributed to corresponding increases in water and proteoglycans. A high collagen type III to type I ratio[74], and morphological changes in the tenocytes of tendinopathic tendons have also been reported.

Physiologic loading of tendons is essential to the maintenance of homeostasis[75]. Moderate exercise promotes an anabolic response in tendons, resulting in increased tenocyte proliferation, metabolism and tendon differentiation[76, 77]. In one study, low strain (4%) cyclic stretching of mouse tendon stem cells over 12 hours increased collagen type I and tenomodulin expression over unstimulated cells *in vitro*[1]. 4% stretching also decreases levels of pro-inflammatory COX-2 and MMP-1, and maintains normal tendon mechanics *in vitro*[75, 78].

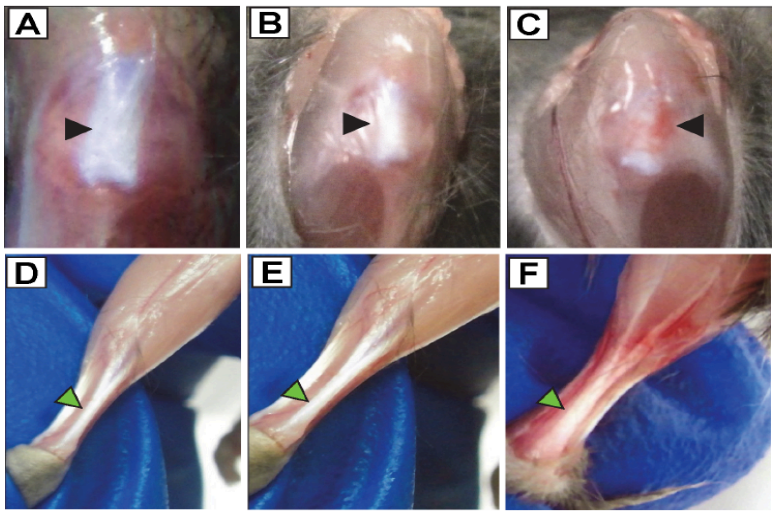


Fig 1.2. Gross appearance of mouse patellar (A-C) and Achilles (D-F) tendons: unstimulated control tendons (A,D), after moderate exercise (B-E), after intensive treadmill running (C-F) (Adapted from Zhang et al. 2013[1])

However, repetitive, supraphysiologic loading[2, 24] is associated with microdamage in the collagen architecture, increased expressions of pro-inflammatory PGE_2, IL-1β and MMPs, and perhaps even the induction of non-tenocytic phenotypes[1], typical of the early tendinopathic response. In fact, increased levels of collagenase mRNA in tendon may be induced simply by repetitive loading, and excessive loads beyond the physiologic range may actually exacerbate this effect, rather than induce it. Repetitive loading may overwhelm the intrinsic repair mechanisms in tendons, resulting in the progressive accumulation of microdamage[79], and altered cell-matrix interactions[2], which may serve as precursors to the degenerative response. This highlights the importance of optimizing loading protocols for tendon repair, also since optimal loads may vary, depending on the age, type, anatomical locations and regional variations in tendons[80].

The etiological mechanisms underlying the degenerative response in chronic tendinopathy are unclear. Increased production of pro-inflammatory cytokines[81], oxidative stress[82] and hyperthermia[83] are typical in tendons that have been subject to repetitive and prolonged durations of supraphysiologic loading. *In vivo* models of tendon overuse show

increased expressions of the pro-inflammatory mediators IL-11, IL-15, IL-6 and TNF-α[84]. Prostaglandins are involved in various physiological processes, but excessive levels of this cytokine in tendons during and post-exercise may have detrimental effects on the underlying collagen architecture[85]. IL-1β, a pro-inflammatory cytokine highly expressed in injured tendons[86], can induce PGE_2 production in a COX-2 dependent manner[87]. Interestingly, high levels of PGE_2 may also counteract the catabolic effects of IL-1β[88]. Fluoroquinolones may interact with PGE_2, leading to the enhanced production of catabolic MMPs like MMP-9 and MMP-13[89]. MMP-1 levels are significantly increased in tendinopathic tendons, whereas MMP-2 and 3 levels are reduced[90, 91]. These studies suggest that the imbalanced synthesis of prostaglandins and MMPs in favor of matrix catabolism may elicit the degenerative response in tendinopathy.

Reactive oxygen species (ROS) are produced during several cellular processes such as mitochondrial respiration, phagocytosis, dioxygenation and apoptosis[92]. Their production in tendons may be enhanced due to a traumatic event or overuse, resulting in significant cellular and tissue damage. ROS may be produced by tenocytes as well by adjoining tissues in the proximity of tendon[93]. High levels of peroxiredoxin 5, an antioxidant, are found in tendinopathic tendons, suggesting that ROS may contribute to the pathogenesis of degeneration[94]. Energy-storing tendons like the flexor tendons experience significant temperature increases during running, which can be attributed to energy loss during cyclic length changes (hysteresis)[83]. In fact, temperatures as high as 45°C have been recorded in the core of equine SDFTs during galloping[95]. This could explain the formation of degenerative lesions in runners with tendinopathy.

The horse as a model for tendinopathy

The SDFT is one of the largest tendons in the horse, and most susceptible to damage on the racetrack[96]. Subtle changes in SDFT structure can be detected as early as 1-2 days post- racing[97]. SDFT mechanics and structural organization is similar to human Achilles tendons; both operate close to their functional limits during intense exercise and are prone to injury and re-injury post-healing[98]. In the galloping horse, strains of up to 16% have been reported in the SDFT, which is a close figure to its strain limit at rupture of 21%[99]. Due to the similarities with Achilles tendons, SDFT injuries serve as good model systems for the development of translational treatment modalities. Equine tendons mature at 2 years[19], and the first signs of degeneration in equine SDFTs become apparent at about 3 years of age. The mid-metacarpal (core) region of the SDFT is particularly prone to degenerative lesions, perhaps due to the increased load sensitivity of collagen fibrils in this region[100]. In SDFT injury models, these core lesions provide natural and distinct enclosures for implantations and ultrasound-guided diagnosis.

Repair and regeneration

Tendons heal poorly, and surgical repair is often necessary to augment intrinsic healing after complete rupture or laceration[101]. In the first few days post-injury, tendons are characterized by hemorrhage, edema and an influx of inflammatory cells (mainly platelets and neutrophils) to the injury site. Platelets and leukocytes release growth factors and chemotactic factors that attract other inflammatory cells and tenocytes from the epitenon and endotenon, and induce neovascularization. The inflammatory phase is characterized by the initiation of collagen type III, GAG and fibronectin synthesis[102]. In the proliferative or regenerative phase, tenocytes rapidly proliferate and large amounts of collagen type III and GAGs are synthesized.

Inflammation reduces, and a matrix of disorganized collagen is present at the injury site. The final remodeling stage ensues in a few weeks. Cell proliferation and collagen type III synthesis is reduced, and collagen type I fibers are aligned to the tensional axis and cross-linked. The repair tissue matures at about ten weeks post-injury and results in the formation of a scar-like tissue in adult tendons[64].

Fibrous adhesions serve a dual purpose in the healed tendon; they contribute to tissue stiffness and mechanical strength, but they also restrict fiber gliding[103], which is essential to the elastic behavior of load-bearing tendons. Excessive scarring or formation of adhesions in the healed tendon tissue results in inferior mechanical and histological properties, and increases its likelihood for re-injury. The mechanisms underlying this fate in adult tendons are unclear. The reduced turnover of tendon components (the half-life of collagen type I is 200 years)[76], increased levels of TGF-β1, FGF-2 and CD44, improper loading or under-stimulation and hypoxia have been implicated in the poor healing response. In contrast, early fetal tendons heal without scar formation[65, 104, 105], perhaps due to the absence of an inflammatory response during early gestation and early remodeling of the collagen architecture[106]; however, the exact mechanisms are unknown. Genetically engineered PU-1 null MRL mice that lack functional macrophages and neutrophils can repair skin wounds in a scar-free manner[107]. In salamanders, skin wounds heal in the absence of neutrophils, but macrophages are indispensable to scar-free healing, and the dynamic control of pro- and anti-inflammatory cytokines in early healing appears to promote the regenerative response[108]. Hence it is uncertain whether modulation of the inflammatory response or its depletion will result in scarless wound healing in adult tendons. Further, the tendon ECM may also play a role in the intrinsic healing response. For example, in fetal skin wounds, higher levels of collagen type III, hyaluronic acid, fibromodulin and anabolic MMPs are present

compared to post-natal wounds, resulting in greater matrix organization without scarring[104]. While these findings lend insight to the potential mechanisms of fetal tendon healing, much investigation is needed to identify and develop translational approaches to augment adult tendon repair with fetal regenerative mechanisms *in vitro* and *in vivo*[109].

Regenerative healing is not a common feature of mammals. While certain tissues like the liver, bone marrow and blood can replenish their losses, most organs and body appendages cannot re-grow. The goal of regenerative medicine is to identify approaches to promote scarless healing in adult tendons towards the restoration of the native functional and structural properties of injured tendon tissue[65]. Tissue engineering is a branch of regenerative medicine that primarily combines cells, scaffolds and cues from tendon development and regeneration to generate tendon-like artificial graft material for the surgical repair of tendons *in vivo*. The early conceptual basis for tissue engineering as stated by Woo et al. is "the manipulation of cellular and biochemical mediators to regulate protein synthesis and remodeling"[110]. This *in vitro* approach also facilitates understanding of the cues that specify stem cell commitment to the tendon fate. A detailed review of the roles of growth factors, biomimetic scaffolds and mesenchymal stem cells in tendon is provided in the following chapter.

CHAPTER 2: GROWTH FACTORS, SCAFFOLDS AND MESENCHYMAL STEM CELLS FOR TENDON HEALING AND REPAIR

2.1 GROWTH FACTORS

Growth factors are signaling proteins involved in various complex cellular processes that are integral to tissue development, homeostasis, repair and regeneration. Tendon repair is orchestrated by endogenous growth factors that are released at the injury site by the surrounding cells[111]. They act in concert with each other and other signaling proteins, and exert their effects locally in an autocrine or paracrine fashion. The specific effects of growth factors on cells are largely dependent on the type of receptors they bind to and binding affinities for the receptors on the cell surface, the target cells themselves and their concentrations, and their temporospatial presence in the microenvironmental milieu. Tendon repair *in vivo* is characterized by three phases: the initial inflammatory phase, the proliferative phase and the remodeling phase[112] (each of these has been covered in detail in the preceding chapter). Growth factors are synthesized in high amounts during the pre-inflammatory phase immediately after injury and thereafter they are expressed in varying amounts throughout the healing period. Among tenogenic growth factors, the following are predominantly expressed during tendon repair and have been covered in detail in this section: the bone morphogenetic proteins (BMP), transforming growth factor-β (TGF- β), fibroblast growth factor (FGF) and insulin-like growth factor (IGF). Current knowledge of growth factors involved in tendon, their temporal, spatial and therapeutic effects *in vitro* and *in vivo* models have been described. Lastly, a review of combined growth factor applications is

presented with a focus on recent investigations, comparisons with single factors, and future directions.

Bone morphogenetic proteins

BMPs belong to a subclass of the TGF-β superfamily. Over 20 BMPs are encoded by the mammalian genome[113]. While most BMPs are primary inducers of the osteogenic phenotype, BMPs 12, 13 and 14[114] (human homologues of murine GDFs 7, 6 and 5 respectively) induce a strong tenogenic phenotype *in vitro* and *in vivo*[115-117], and are poor inducers of bone[117, 118].

BMP-12 is expressed in variable amounts throughout tendon healing, however expression peaks during the early phase, about 1 week post-injury[119], and is accompanied by an increase in the proliferation of tendon fibroblasts and procollagens type I and III[120]. Immunohistochemical staining of adult human patellar tendons showed that BMP-12 was expressed in highly organized, cellular and "active remodeling" sites of the tendon relative to the rest of the tissue, further providing evidence in its role as a master activator of tenogenesis[120]. Following the initial surge in expression, a gradual decline in BMP-12 levels is noted, returning to the baseline expression of healthy tendons at about 8 weeks post-injury[119].

BMP-12 is widely acknowledged as a tenogenic growth factor, though the cause of its functionally distinct behavior from osteogenic BMPs is not well understood. BMP-12 and BMP-2 (a FDA-approved bone-inducing agent[121]) signal cell differentiation by the same intracellular mechanisms[122, 123]. Nonetheless, application of BMP-12 to MSC culture augments tendon gene expression; most notably scleraxis expression[124, 125]. For example, in one study, bone marrow derived-MSCs treated with BMP-12 over a 24-hour period peaked expression of scleraxis at 12 hours post-treatment, followed by a steady decline in expression over the next 12 hours[126]. BMP-

12 also augments cell proliferation and collagen synthesis *in vitro* tendon fibroblasts[120]. Treatment of MSCs with BMP-12 prior to implantation induces the formation of tendon-like tissue *in vivo*[127, 128]. For example, implantation of BMP-12-treated MSCs in rat calcaneal tendon defects induces the formation of robust tendon-like tissues, accompanied by increased numbers of elongated cells, and increased cell alignment to the tensional axis, compared to untreated MSC implants. BMP-12 also improves the collagen organization[129] and biomechanics[130] of treated human patellar and Achilles tendons *in vivo* post-surgical repair; specifically, a higher load to failure and increased tissue stiffness compared to untreated controls were noted[131].

Evidence suggests that the tenogenic BMP proteins (BMP-12, 13 and 14) may functionally compensate for each other during intrinsic tendon healing. In one *in vivo* murine study, loss of BMP-12 did not result in significant deficits to the overall structure or functionality of the repaired tendon tissue[132], but a marked increase in the expressions of BMP-13 and BMP-14 was observed, compared to wild-type tendons. Gene-specific differences in BMP-tenogenesis, if any, are not well understood. BMPs 12, 13 and 14 share 82% sequence homology with each other[122] and their functional behavior *in vitro* maybe indistinguishable. One comparative study of BMP-12 versus BMP-14 application *in vitro* adipose stem cells did not reveal any significant differences in tenogenic outcomes[117].

Relative to the temporal effects of BMP-12, the concentration-specific effects are unclear. Liu et al. tested the effects of different BMP-12 concentrations (0, 1, 10, 50 and 100ng/ml) and culture durations (3, 7 and 14 days) on tendon stem cells grown in two-dimensional culture from rats, and found that 50ng/ml supplemented over 14 days was most effective for tendon differentiation[133]. Further evidence suggests that an early and low dose of

BMP-12 may be sufficient to induce and maintain the expression of scleraxis over extended culture, and higher BMP-12 doses may not necessarily augment this effect[117, 134].

Transforming growth factor-β

The mammalian TGF-β superfamily consists of over 30 growth and differentiation factors that are categorized into four subfamilies: TGF-β, BMP, activin and inhibin[135]. The TGF-β subfamily consists of three structurally-related isoforms, TGF-β 1, 2 and 3, that share at least 70% amino acid sequence similarity with each other[136]. Each of these isoforms is indispensable to tendon development and repair[39], and involved in a myriad of intra- and extracellular processes including but not limited to cell migration, proliferation, differentiation and collagen synthesis[137]. Predictably, TGF-β2/3 knockout mouse embryos fail to develop tendons and ligaments in the limbs, trunk, tails and head[39].

TGF-β1 is highly expressed in the initial inflammatory response to tendon injury[137], and TGF-β isoforms are variably expressed throughout tendon morphogenesis and post-injury. Evidence suggests that TGF-β1 is predominantly expressed during adult tendon healing, and comprises over 85% of the TGF-β found in adult wound fluid, whereas TGF-β2 and TGF-β3 predominate wound repair in fetuses[138, 139]. This may explain why adult tendons heal with a scar (one concerning predicament of TGF-β1 application), whereas fetal tendons regenerate. A previous study of the temporal expression of TGF-β1 in a rat Achilles tendon injury model revealed two distinct peaks over 8 weeks, one at week 1, followed by a steep decline in TGF-β1 levels at 2 weeks, and a dramatic increase at 8 weeks post-injury, compared to uninjured controls[119]. This suggests that TGF-β1 function is heavily involved in the initial inflammatory phase, and late remodeling phases of tendon healing[140].

Application of TGF-β *in vitro* is primarily associated with increased expression of tendon markers and collagen synthesis[141]. For example, addition of TGF-β3 to 3D cultures of equine embryonic stem cells significantly enhanced the expressions of scleraxis, collagen type I and cartilage oligomeric matrix protein in one study[142], and increased collagen synthesis in monolayer cultures of human bone marrow MSCs[143]. TGF-β application also augments tendon repair *in vivo*[144]. Specifically, the histological, biomechanical properties and remodeling of injured rat Achilles tendons were improved with TGF-β1 application in one study[131]. In a different study, the sustained delivery of TGF-β3 at healing tendon-to-bone insertions in rats resulted in accelerated healing and significant improvements to their structural and material properties at 28 and 56 days respectively compared to untreated controls[145].

Despite mounting evidence for TGF-β1 as a beneficial factor in tenogenesis, concerns surrounding its involvement in scar formation have not been alleviated and warrant investigation. Toward this end, Farhat et al. tested the effects of TGF-β1 on a 3D hydrogel seeded with human tenocytes and cultured under uniaxial static strain for 2 days[146]. Their results suggest that MMP16 (involved in the regulation of collagen fibril size) is downregulated in response to TGF-β1. This may explain the TGF-β1-mediated scarring in repaired tendon tissues by virtue of reduced matrix turnover.

A possible strategy to balance collagen synthesis with efficient collagen turnover in tendon may be to supplement MSC cultures with a competitive inhibitor of TGF-β1 binding such as mannose-6-phosphate[147], or use an isoform of TGF-β1. Like TGF-β1, TGF-β2 also induces scleraxis expression in bone marrow MSCs *in vitro,* but without any evident decreases in MMP16 expression [139]. However, simultaneous analysis of the three TGF-β isoforms is needed, to compare their potencies for tendon differentiation and to offset excessive fibrosis. Studies

comparing the three TGF-β isoforms to each other have achieved partial success[148, 149], and as a result, no definite conclusions can be drawn. For example, in one *in vivo* controlled study in rats, rat supraspinatus tendons were laterally transected and treated with 1) TGF-β1 or 2) TGF-β3 or 3) neutralizing antibodies to all three TGF-β isoforms over a span of 1-4 weeks. While loss of all three isoforms resulted in impaired repair, no significant improvement in either biomechanics or histology of repaired tendons was observed with TGF-β treatment in their study, and no significant differences between the function of TGF-β1 and TGF-β3 could be determined. An optimized cocktail of the three isoforms may be most effective and required to accelerate the intrinsic healing potential in tendons[138].

The dose-dependent effects of TGF-β1 on the expression of tendon markers in MSCs are unclear and contradictory evidence is presented in the literature[146, 148], suggesting that a moderate dose of 5-10ng/ml is best suited for *in vitro* differentiation.

Signal transduction by the TGF-β superfamily

Ligands of the TGF-β superfamily bind two transmembrane serine-threonine kinase receptors on the cell surface: the type I receptor and the type II receptor[150]. In mammals, at least 7 type I receptors and 5 type II receptors have been identified[151, 152]. Type I receptors incorporate a glycine-serine rich domain (the GS box), a key feature that distinguishes them from the type II receptors[153]. TGF-β ligands are distinct from BMP ligands in their preference of receptors[154]. Of the 7 type I receptors, BMP ligands bind 3 (ALK2, ALK3 and ALK6), and of the 5 type II receptors, they bind 3 (BMPR2, ACVR2A, ACVR2B)[113, 152]. TGF-β ligands bind 3 type I receptors (ALK1, ALK2 and ALK5) and the type II receptor TGF-βRII[155, 156].

Ligands first bind a type II or type I receptor, further incorporating the second receptor into the complex[157, 158]. Second receptors are activated by the phosphorylation of their serine residues by the kinase of the first receptor[159]. In the canonical TGF-β pathway, the phosphorylated receptor in a complex binds to the MH2 domain of a receptor-SMAD (R-SMAD), further phosphorylating it at the C-terminus and activating it. Phosphorylation induces a conformational change in the R-SMAD and binding of a co-mediator SMAD (SMAD-4). The R-SMAD/SMAD-4 complex can then translocate to the nucleus, where it cooperatively activates gene transcription. BMP ligands reportedly transduce signals to the nucleus via the SMAD1/5/8 complex, whereas TGF-β and its isoforms prefer the SMAD2/3 complex[154].

In addition to the SMAD-dependent canonical pathway, SMAD-independent pathways are also activated by TGF-β ligands. The activated TGF-β-receptor complex can recruit ubiquitin ligase (TRAF6) in the cytoplasm to activate TAK-1, leading to the activation of p38 MAPK and JNK signaling pathways. Further, the activated receptor can phosphorylate serine and tyrosine residues in the SHCA adaptor to recruit GRB2 and SOS which cooperatively activate the ERK pathway[113, 151, 160]. TGF-β ligands can also activate P13K independent of SMAD, leading to the phosphorylation and activation of AKT, and the Rho/ROCK pathway, via the small Rho-like GTPases RhoA, Rac and Cdc42[161].

Fibroblast growth factor

The basic-FGF or FGF-2 protein comprises 146 amino acids, and belongs to the heparin-binding family of growth factors[114]. In addition to FGF-2, FGF-4, -5 and -8 have been implicated in tendon development and repair[162, 163]; however, most investigations are based on FGF-2. Among its many roles in tendon development and repair, FGF-2 is involved in cell growth,

differentiation, induction of cell motility and angiogenesis, and production of structural proteins like vimentin and α-smooth muscle actin. FGF-2 is elevated early in the tendon healing process[164], followed by a gradual decline in expression[165] during the latter phase of healing in tendons.

FGF ligands initiate signal transduction by binding a pair of tyrosine kinase receptors on the cell surface. The mammalian genome encodes 4 FGF receptors (FGFR1-4)[166]. Ligand binding induces receptor dimerization and activation. Activated FGF receptors in turn phosphorylate intracellular substrates; chief among these are FRS2α and PLCγ1[167]. Further downstream, activated FRS2α conveys messages into cells via the P13K-AKT or the MAPK pathway. Among these, the MAPK pathway is predominantly activated by FGF ligands. Increased levels of phosphorylated-ERK (a major effector of the MAPK pathway) can be detected in cells following FGF-2 transfection[168].

Like other tenogenic growth factors, *in vitro* applications of FGF-2 are associated with enhanced gene expressions of tendon markers and collagen synthesis[169, 170], but with a greater emphasis on tenocyte proliferation[171, 172]. This is not surprising, since FGF-2 is well established as an agent of cell proliferation[173]. Adenoviral transfection of FGF-2 in human bone marrow-derived MSCs results in consistently elevated expressions of a panel of tendon marker genes over a 14-day culture period, suggesting that in addition to activating differentiation, FGF-2 also maintains the tendon phenotype *in vitro*[168]. One study suggests that higher doses of FGF-2 (more than 10ng/ml) may suppress collagen synthesis in a dose-dependent manner[174], lending support to its role in the modulation of collagen synthesis and possibly, maintenance of the collagen type I to type III ratio in tendons. The *in vitro* tenogenic effects of FGF-2 are also translated *in vivo.* Moreover, significant improvements in tendon biomechanics have been observed with FGF-2

injection[175]. In a rat model of rotator cuff tendon repair, FGF-2 injection enhanced ultimate load to failure and tissue stiffness at 6 weeks, and tissue vascularity and collagen fiber orientation of repaired tendons at 8 weeks compared to untreated controls[176].

In comparison to FGF-2, FGF-4 and FGF-5 are the less investigated FGFs in tendon repair. During early embryonic development, FGF-4 is increasingly expressed in muscle fibers adjacent to developing tendons, a region which later matures into the myotendinous junction. This suggests that FGF-4 may play a role in early tendon development. Consequently, loss of FGF-4 results in the downregulation of scleraxis and tenascin in embryonic chick limbs[177], whereas exogenous implantation of this growth factor restores expression of these tendon markers[32]. However, exogenous application of FGF-4 downregulates scleraxis and tenomodulin expression *in vitro*[139, 162], suggesting that a different FGF, such as FGF-2 may predominantly mediate repair in adult tendons. Ectopic expression of FGF-5 promotes expression of tenascin-C in the hind limbs of chick embryos, lending support to its role as a tendon differentiation factor, and this effect was reproduced with equine adipose-derived MSCs *in vitro*[163].

Insulin-like growth factor

IGF-1 is a single chain polypeptide that belongs to the insulin-related family of peptides. It was named after its structural resemblance to proinsulin[178]. Temporal expression of IGF-1 peaks during the early proliferative and remodeling phases of healing. In healing equine flexor tendons, a 40% reduction in IGF-1 levels is observed at 2 weeks post-injury, followed by a surge in IGF-1 activity at 4-8 weeks, compared to untreated tendons[8, 179]. This suggests that IGF-1 is predominantly involved in the latter end of the healing cascade during tendon repair. However,

high amounts of IGF-1 are found in the inflammatory milieu of injured soft tissues immediately post-injury[137].

IGF-1 binds two tyrosine kinase receptors for signal transduction; it binds the IGF1 receptor with high affinity and the insulin receptor with low affinity[180]. Both receptors can also from heterodimers to bind IGF ligands. Receptor activation results in the binding of adaptor molecules such as the insulin-receptor substrates and Shc[181]. Downstream of the ligand-receptor complex, activated substrates transduce signals via the P13-AKT and MAPK pathways.

IGF-1 primarily induces cell proliferation and collagen synthesis in tendon repair[182], and these effects are recapitulated *in vitro*[183-185]. In addition, IGF-1 is associated with the induction and maintenance of the tenocytic phenotype *in vitro*. Like BMP-12, IGF-1 can induce expression of scleraxis in MSCs. It is worth noting that expression of scleraxis over 28 days of MSC culture was unaffected by IGF-1 concentration in one study (three doses tested: 1, 10 and 100ng/ml)[134], and scleraxis levels were highest at the 28-day time point. IGF-1-mediated scleraxis expression is also detected in 3D culture of human tenocytes, accompanied by increases in tenomodulin[184]. Interestingly, in this study, scleraxis and tenomodulin were upregulated at different time points; scleraxis levels peaked at day 28, whereas tenomodulin levels peaked at day 14. This observation lends support to the role of IGF-1 in the modulation of MSC differentiation.

In an equine model of flexor tendinitis, intratendinous injection of IGF-1 resulted in a significant reduction in lesion size, 4 weeks post-treatment, accompanied by increased stiffness and reduced swelling in the tendons compared to saline-treated controls[186]; however, these results were not replicated when pure IGF-1 injection was replaced with IGF-1-transduced MSCs. In the latter case, the authors did not observe any differences in the mechanics of their controlled versus IGF-1-treated tendons[187]. Different methods of growth factor delivery are

known to affect study outcomes, and hence this result is not surprising. In a different *in vivo* study using a rat model of Achilles tendon healing, IGF-1 injection resulted in a relatively shorter time to functional recovery compared to untreated tendons, but no significant differences in biomechanics or histology were noted[188]. Overall, these studies suggest that the therapeutic roles of IGF-1 in tendon repair are not well understood; it may boost intrinsic healing by reducing pain and inflammation[188] in injured tendons, thereby aiding faster recovery, but further studies are needed to understand IGF-1-mediated effects at the tissue level in tendons.

Other growth factors implicated in tendon healing

Platelet-derived growth factor is a 30kDa protein and expressed as three isoforms: PDGF-AA, PDGF-BB and PDGF-AB. Of these, PDGF-BB has been most investigated and predominantly involved in tendon[189, 190]. PDGF is expressed throughout the tendon healing period, and its receptor is highly expressed even 6 months post-injury[190]. In addition to its role as an agent of MSC proliferation, PDGF stimulates chemotaxis, neovascularization, is involved in the modulation of matrix protein synthesis, and promotes the biomechanics of rat Achilles tendons *in vivo*[131]. Vascular endothelial growth factor was originally described as an endothelial cell-specific mitogen[191], but is now known as a potent inducer of angiogenesis in tendon healing. VEGF expression increases about 10 days post-injury and at the end of the inflammatory phase[192], and expression is sustained throughout the proliferative and remodeling phases of tendon healing[137]. VEGF is highly expressed in ruptured human Achilles tendons, compared to healthy tendons[193]. Exogenous application of VEGF increases the tensile strength of surgically lacerated tendons in rats[192], compared to untreated tendons. Additionally, it promotes nerve innervation and neovascularization in damaged tendons[194]. Connective tissue growth factor is an

extracellular matrix protein, and works in concert with TGF-β to mediate matrix remodeling in development[195]. It is highly expressed throughout the tendon healing period[164, 165]. Reports suggest that pre-treatment of MSCs with CTGF promotes tendon repair *in vivo*[133, 196], compared to untreated MSC implants.

Combined growth factor applications

The studies described above lend ample support to the use of tenogenic growth factors as agents of tendon differentiation or healing. Evidence also suggests that modulation of growth factor concentrations or the duration of their treatments can optimize their efficacy. However, endogenous growth factors always work in concert with each other and other signaling proteins, and tendon repair is the cumulative result of the complex interplay in these proteins[197]. In the recent past, tendon biologists have attempted to identify and replicate the synergistic effects of two or more tendon growth factors in culture systems and *in vivo* models of repair. Several challenges are underway: for example, discrepancies in the published literature making it difficult and impractical to translate results, limited numbers of comprehensive studies that investigate different parameters of growth factor efficacy, and the general lack of consensus on their mechanisms and functional outcomes, to name a few. Nonetheless, in addition to potentiating the roles of growth factors and combined growth factors in therapy, these studies also lend insight to the complex mechanisms of endogenous growth factor repair, specifically the interdependent relationships in them and potential antagonism at different stages of healing. This section reviews some of the recent studies on combined growth factors and their potential benefits in tendon healing.

Application of a single factor to injured tendons may induce the endogenous synthesis of a different factor. For example, one report showed that VEGF application to injured rat Achilles tendons stimulated endogenous TGF-β expression 4 days post-surgery[192], suggesting a concerted effect of the two factors on tendon healing which was not elucidated in this study. Both VEGF and TGF-β are also highly expressed during the initial inflammatory response and subsequent inflammatory phase of injury[137, 192], and have varied functions in tendon repair. This suggests that the early combined application of VEGF and TGF-β may elicit a synergistic effect *in vitro*. To test this hypothesis, Hou et al. transduced bone marrow MSCs with adenoviruses encoding genes for each factor alone, or in combination[198]. Transduced MSCs were implanted in injured rabbit Achilles tendons for 8 weeks. While no synergistic effects were observed, results from this study showed that VEGF-mediated angiogenesis was reduced at 4 weeks in the combined factor group compared to the VEGF-only group. This suggests that TGF-β1 may have a role in the modulation of angiogenesis during tendon repair.

Combined growth factors may stimulate the expressions of several tenogenic markers in comparison to single factors that may only induce a few. However, this effect may be accompanied by a decrease in the efficacies of the individual factors involved. For example, Caliari et al. hypothesized that the combined application of BMP-14 and IGF-1 would result in increased levels of tendon marker genes (BMP-14), and enhance tenocyte proliferation (IGF-1) in their 3D-cultured equine tenocytes[199]. Their hypothesis was successfully proven, though, fewer tenogenic genes were upregulated at 1 week of culture in comparison to BMP-14 application alone.

Different members of the TGF-β superfamily are involved in tendon repair; however, their functionally distinct roles *in vivo* warrant investigation on their combined effects. A recent

study attempted to identify the potential synergism in BMPs (BMP-12 or BMP-14) and TGF-β3 for *in vitro* tendon differentiation in rabbit MSCs[200]. Application of this growth factor pair resulted in increased expressions of collagen type III and tenomodulin over a week of culture and compared to singly supplemented BMP groups. However, TGF-β3 was not independently investigated in this study, and hence it is difficult to conclude whether BMPs and TGF-β3 work in synergy *in vitro*. Yin et al. did not observe any synergistic effects of BMP-12 and TGF-β1 on the tenogenesis of *in vitro* bone marrow MSCs in their study, rather the pattern of increased tendon gene expressions in the combined group closely mimicked that of the solo TGF-β1 group[141].

PDGF, IGF-1 and FGF-2 are potent inducers of MSC proliferation and matrix synthesis in tendon repair. PDGF stimulates the production of IGF-1 and its receptors during the proliferative phase of tendon healing[194], and one report suggests that IGF-1 and PDGF may work in concert to augment tendon fibroblast proliferation *in vitro*[201]. Recent studies also suggest that cell proliferation is most benefited when IGF-1 and PDGF are combined with FGF-2, and applied to *in vitro* culture[202, 203]. These results are promising, and suggest that combined growth factor applications may be particularly beneficial when a single outcome of tenogenesis, such as cell proliferation, is of most importance.

The aforementioned studies suggest that combined growth factor applications may be a pragmatic approach to: 1) modulate the effects of single factors, 2) elicit multiple tenogenic outcomes toward a composite tendon phenotype, 3) investigate the concerted effects of factors that are phylogenetically-related and, 4) augment specific outcomes of tenogenesis such as cell proliferation. However, this approach is limited by our understanding of the complexities of growth factor biochemistry and *in vivo* mechanisms. MSCs may respond to growth factors in a

time and concentration-dependent manner, and it is important to note that small variations in culture parameters may not recapitulate previously described combined factor effects. Further, a truly combined effect can only be discerned in the absence of endogenous forms of the growth factor. While optimizing growth factor concentrations in MSC cultures may account for the effects of intracellular feedback loops, it may be beneficial to quantify the endogenous levels of growth factors in the pertinent system for accurate representations of combined effects. Parallel experiments with optimized single and combined growth factors are needed to successfully translate their effects to a variety of culture systems and *in vivo* models.

2.2 BIOMIMETIC SCAFFOLDS

Scaffolds have long been used as delivery vehicles for cells and regenerative agents, or independently to augment tissue mechanical strength and remodel tissue architecture *in vivo*. Scaffold-enhanced repair of tendons can augment function, reduce wastage and reduce overall healthcare costs. They are usually sourced from either natural tissues or synthetic polymers.

The ideal scaffold material should closely mimic the natural ECM environment (hence the term *biomimetic*), specifically the mechanical and biochemical properties of the repair tissue, and should be slow-degrading, non-toxic and non-immunogenic[204]. In tendon repair, factors such as wound size, anatomical location, regional variations, age, species and underlying pathologies of tendon must also be considered in deciding the optimal scaffold material. Tendons are primarily mechanical tissues, and may be subject to up to 40% of their peak *in vivo* forces during periods of intense activity on a daily basis[205]. Hence, the ideal scaffold must be able to withstand these repetitive, intense forces and even exceed peak forces without failing. Mechanical pre-conditioning of scaffolds may be necessary prior to implantation in tendon defects, so that their strength and stiffness may more closely match normal tendon parameters[206, 207].

In addition to their therapeutic applications, scaffolds are also used as platforms for studies on MSC characterization and differentiation *in vitro*. A 3D scaffold permits unrestricted cell migration and the assembly of matrix macromolecules in a third dimension, namely, across its depth, as opposed to 2D culture systems that only permit lateral and longitudinal movement and organization. 3D scaffolds can thus, accurately capture the complexities of the native tendon environment. Of these, hydrogels are water-based, soluble formulations of natural, synthetic or composite polymers that are cross-linked to form a viscous gel. Their porous structures are

permeable to nutrients, oxygen and other therapeutic agents. Further, their material properties can be easily manipulated, making them very adaptable to a variety of applications including tissue engineering[208]. Among natural hydrogel polymers, collagen, fibrin, alginate, chitosan and hyaluronic acid are frequently used[209]. Of these, collagen hydrogels are most prevalent in tendon-related applications, since collagen type I comprises 65-80% of the native tendon ECM collagen[27]. Like tendons, collagen hydrogels demonstrate nonlinear viscoelastic behavior under strain[210], and can be remodeled by natural cell-driven contractile mechanisms[211].

Several intrinsic and extrinsic factors influence tendon hydrogel designs, making comparisons between published studies difficult. Intrinsic factors may include the collagen source and concentration, cell source and concentration, and constituents of the diluent, to name a few[212]. Extrinsic factors such as the construct apparatus design, anchorage, and strain protocols may also affect the physiochemical characteristics of tendon hydrogels.

Among the main sources of collagen type I, rat tendons are most commonly used, whereas porcine tendons, dermis and bovine dermis are less frequently used. Collagen hydrogel is obtained by the dissolution of rat tail tendons in a dilute (1:25,000) preparation of acetic acid; a process termed as 'acid solubilization[213]. While low temperatures and pH maintain the viscous gel-like consistency of hydrogels, higher temperatures (>20°C) and neutralized solutions (pH of 7.4-8.4) induce fibril assembly and collagen polymerization. At 37°C, polymerization is most optimal, and the collagen structure is permeable to cells and nutrient macromolecules[212]. A majority of studies suggest that a concentration of 1-3mg/ml collagen is sufficient for the preparation of tendon hydrogels. Higher collagen concentrations may result in a dense fiber structure, decreased diffusion rates, decreased matrix organization and restricted cell division and migration.

Tenocytes in a collagen hydrogel bind to and exert tractional forces on the surrounding fibrils, resulting in gel contraction and condensation, and this process simulates the wound healing mechanism of fibroblasts and myofibroblasts *in vivo*[214, 215]. Predictably, tenocytes and myofibroblasts share similar phenotypic characteristics, including the presence of actin stress fibers aligned in parallel to the axis of tension. Isotropic contraction of "floating" cell-seeded hydrogels results in randomly oriented, low-strength fibrils and rounded cells[216]. To engineer tendon hydrogels, rectangular molds, troughs or vessels are generally used to provide instructional cues and fitted with anchors to provide length-wise constraints to contraction. Cell-seeded hydrogel suspensions are poured into these molds and allowed to gel. In this design, uniaxial tension provided by anchors and combined with cell-mediated tension forces induces the anisotropic contraction of hydrogels. After few days of culture at 37°C, this process results in the formation of a compact, stiff tendon-like structure, with highly aligned cells and collagen fibrils.

Matrix stiffness is known to affect cell fate, and induces a tendon-like phenotype in MSC-seeded 3D hydrogels[217]. However, it is unclear if static loading is sufficient or dynamic loading is necessary for MSC tenogenesis. Dynamic loading protocols may be necessary to enhance the mechanical strength and stiffness of tendon scaffolds for *in vivo* repair[207, 218, 219]. However, matrix stiffness is not the only parameter of MSC tenogenesis, and more analyses including tendon gene and protein expression, histology and contraction are needed to determine optimal loading protocols. Further, Feng et al. showed that the ultimate stress and material modulus of their static fibroblast-seeded hydrogels was greater than dynamic-loaded hydrogels[220]. In two other studies, dynamic loading did not affect the linear stiffness and modulus of tendon and bone marrow-derived MSC-seeded hydrogels when compared to static controls [221, 222]. Matrix protein synthesis has also been investigated in this regard. Mechanical

stimulation of 3D bone marrow MSC cultures over 21 days increased collagen type I, collagen type III and tenascin expression over static controls in one study[218]. Kuo et Tuan's study was the first to investigate changes in the expressions of a panel of tendon markers in response to static and dynamic-loaded bone marrow MSC-seeded hydrogels[223]. Very few differences were noted, mainly pertaining to scleraxis gene expression. Scleraxis was upregulated on day 1 in response to both loading protocols, but dynamic loading maintained the upregulated scleraxis expression over time, whereas static loading did not. To summarize, further studies are needed to determine whether static or dynamic loading can induce a holistic MSC response to tenogenesis in 3D culture. Static loading may be more suitable in short-term MSC characterization studies involving small molecules such as growth factors, to reduce or eliminate the influence of macromolecular structural changes associated with dynamic loads. Synergistic effects of growth factors and dynamic strain on MSC tenogenesis, specifically increases in scleraxis expression and MSC alignment have also been observed[124].

Gel contraction is a parameter of the MSC response to a number of external stimuli including growth factors[224], strain[225] and oxygen tension[143], and may be influenced by several intrinsic factors, most notably cell-seeding densities and collagen concentration. Three phases of gel contraction exist: the lag phase (no contraction), a rapid contraction phase and a slow contraction phase. Higher seeding densities of 4-8 million cells result in faster contraction rates[226], and more oriented and elongated cell nuclei, compared to lower densities in all three phases of contraction[227]. However, this effect may not be observed beyond a threshold number of seeded cells or a certain duration in culture[228]. An inverse relationship is observed between contraction rates and collagen concentrations[228]. Some studies suggest that the initial cell to collagen ratio in hydrogels is more important than the individual cell seeding densities and

collagen concentrations, to achieve optimal contractility and hydrogel mechanics[229, 230]. Growth factors such as TGF-β1 may increase tenocyte-seeded hydrogel contraction in a dose-dependent manner[146], in tune with their *in vivo* roles in scar formation. Differences in uniaxial strain amplitudes may also affect hydrogel contraction; in one study, 10% strain significantly increased the contraction of bone marrow MSC-seeded hydrogels compared to 12% strain over 14 days[225].

While studies on gel contraction lend insight to a variety of cell behaviors and cell-ECM interactions involved in tendon repair, from a technical standpoint, the control of gel contraction *in vitro* culture and *in vivo* applications may prove burdensome. Excessive contraction may hinder long-term culture *in vitro,* which may be necessary for tendon differentiation. On the other hand, insufficient contraction may result in poor matrix organization and the formation of macroscopic pores, which may compromise hydrogel integrity. Excessive or insufficient contraction may also hinder wound healing *in vivo*[231]. Culture supplements such as hydroxyapatite[232], cytochalasin D[227] or lactoferrin[233] may be used to regulate gel contraction without affecting cell viability or hydrogel stability, but safety and efficacy studies are needed to potentiate their benefits *in vivo* applications.

2.3 MESENCHYMAL STEM CELLS

The concept of MSCs first arose from studies on *in vivo* engraftments of bone marrow tissue in "diffusion chambers"[234]. The donor bone marrow differentiated to de novo bone tissue in the host system, suggesting that bone marrow had osteogenic potential, and specifically, a subset of cells with osteogenic potential. Subsequent culturing of bone marrow on tissue culture plastic revealed the presence of an adherent stromal fraction of cells. These cells are now commonly referred to as mesenchymal stromal or stem cells, due to the naïve stem-like properties of these cells. Several phenotypic surface markers of MSCs such as STRO-1, SSEA-4 and CD146 were identified at the time; however, their applicability was limited due to the general paucity of knowledge on bone marrow anatomy and immunological techniques[235]. Even today, the use of these markers is controversial. Instead, a more reliable approach for MSC selection and enrichment may be based on the simultaneous expression of a panel of surface markers. In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed a set of minimal criteria to define MSCs[236]; namely, 1) they must be plastic adherent, 2) they must express the surface markers CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR, and 3) they must be able to undergo tri-lineage differentiation[237]. While these criteria define a universal framework to encompass all cells of mesengenic origin, their applicability is limited to *in vitro* use and may not extend to a functional MSC *in vivo*.

Most adult tissues encompass a stem cell niche; an anatomical and functional microenvironment for "housing" stem cells and maintaining them in a naïve, undifferentiated state[238, 239]. In mammals, the perivascular MSC niche lining the blood vessels in the bone marrow

has been best described[240]. In tendons, two distinct stem/progenitor cell niches may exist. Perivascular cells are found in the peritenon, whereas non-vascular tendon cells are found in the tendon proper, aligned in parallel to each other and adjacent collagen fibrils. Cells from both the peritenon and tendon proper express surface markers of stemness, tendon markers, and also exhibit tri-lineage differentiation potential *in vitro*[241]. Niches govern all cell functions and cell-cell interactions involved in the maintenance of tissue homeostasis[22]. They also regulate tissue expansion, renewal and repair in response to sudden losses or chronic degenerative changes. MSCs respond to niche-activated signals by either actively replicating their DNA (proliferation), exiting the cell cycle to undergo a phenotypic shift (differentiation), or entering a quiescent G0 phase. Thus, the main role of a functional niche is to maintain a balance in these various MSC fates[242].

MSCs are multipotent; specifically their differentiation ability is restricted to cells of a few lineages. In contrast, embryonic stem cells are pluripotent, and have the ability to form whole tissues and body organs with distinct structures and functions that can integrate into the whole organism. The term "plasticity" refers to the ability of MSCs to transition to phenotypes distinct from their tissue of origin[243]. This transition may include both the trans- and dedifferentiation of MSCs to alternative mesodermal lineages, and to non-mesodermal germ layers[244]. MSC differentiation is a multi-step, sequential process that is regulated by bioactive factors and several other regulatory elements *in vivo*. MSCs can differentiate to all cells of the skeletal tissues including cartilage, bone, tendon, ligament, and bone marrow, and also cells of other connective tissues including blood, adipose, and those found in the heart and brain[235, 245]. A single MSC can divide extensively to form a clonal progeny of cells, and was hence first

identified as a colony-forming-unit fibroblast[234]. When transplanted *in vivo,* this colony can generate chimeric replicas of organs or organoids.

In addition to their roles in proliferation and differentiation, MSCs also secrete a variety of bioactive factors, such as growth, chemotactic and anti-fibrotic factors. Further, they facilitate tissue repair by modulating the host immune response, specifically by suppressing T-cell, B-cell and interferon-γ synthesis, and homing to sites of injury (chemotaxis)[246]. Secreted factors may influence both, functions of MSCs that secrete them, as well as functions of other MSCs. Growth factors may be directly involved in the immunomodulatory roles of MSCs[247], maintenance of the microenvironmental milieu that supports MSC expansion and differentiation, stimulation of angiogenesis and wound healing[248].

Applications in tendon healing

MSC therapy has shown promise in the treatment of adult soft tissue injuries, with an increased propensity toward regenerative "scar-free" healing and increased quality and functionality of the repair tissue. The bone marrow is a preferred source of MSCs for tendon repairs, owing to its easy accessibility both *in vivo,* and through culture expansion *in vitro*. Alternative sources of MSCs such as adipose tissue, tendon and the umbilical cord have also been identified[249], and they display marked variations in their *in vitro* characteristics[250]. Like bone marrow MSCs, adipose-derived MSCs are widely available and well characterized; however, their applications in tendon repair have not been well investigated[251].

MSC therapy has been shown to augment intrinsic tendon repair, specifically by improving the mechanical properties of repair tissue, resulting in increased functional recovery. These effects have been observed in both injured Achilles and patellar tendons[23]. In surgically

created lesions in rabbit patellar tendons, MSC therapy increased the maximum stress and modulus of repaired tissues compared to non-MSC controls[252]. However, no significant structural improvements were observed, other than the increased crimping of collagen fibers and tenocyte numbers in a few treated tendons. MSC therapy during the early phases of healing may be most beneficial; in one study, improved tendon mechanics of MSC-treated injured rabbit Achilles tendons were observed at 3 weeks, but not at 6 weeks post-injury[253].

MSC therapy is a particularly attractive treatment option for equine SDFT injuries, which occur with great morbidity and poor functional recovery, resulting in wastage and severe economic losses. No adverse effects of MSC implantations in SDFT lesions have been reported; rather MSCs may prevent the progression of degenerative lesions[254]. Further, decreased numbers of inflammatory cells and small improvements in collagen organization and alignment[255] have been observed in treated lesions compared to untreated. MSC therapy may also improve function and reduce re-injury rates in injured horses[256]. In one study of 71 National Hunt racehorses with SDFT core lesions, only 13% horses re-injured post-MSC treatment and a 48-week rehabilitation program[68]. Autologous MSCs are usually preferred over allogeneic transplants, to offset the likelihood of graft versus host disease. However, allogeneic MSC transplants may not adversely affect healthy equine tendons as shown in one study[257]. Allogeneic transplants offer several advantages including a reduced time to treatment compared to autologous MSCs and opportunities for characterization and the selective enrichment of MSCs.

Studies suggest that the inflammatory stimulus in tendon injury may elicit a reparative and anti-inflammatory response in MSCs. Several underlying mechanisms may be involved, for example, the MSC-mediated reprogramming of macrophage phenotypes[258]. Pre-conditioning or "priming" of MSCs with exposure to TNF-α has been shown to reduce numbers of pro-

inflammatory M1 macrophages and IL-12 synthesis in rat Achilles tendon defects, accompanied by increased collagen synthesis and enhanced functional recovery of the treated tendons[259].

While MSC therapy has proven *in vivo* benefits, its mechanisms in tendon repair are not well understood. Several questions remain unanswered. For example, are MSCs actively present in the inflammatory milieu or do they exert paracrine effects from a distance? Do MSCs differentiate to tenocytes and integrate in the host tendon or are removed after a short-lived response by the host's immune system? Further, pre-clinical trials with bone marrow MSCs have reported inconsistent results, which may be attributed to donor-specific differences, different culture and isolation protocols and heterogeneity in MSC populations. Future investigations are warranted to fully understand and harness the potential of MSCs for superior clinical outcomes and truly regenerative treatments for tendon.

Heterogeneity

MSC populations are intrinsically heterogeneous. The early onset of heterogeneity may simply be associated with the removal of MSCs from their *in vivo* environment (the stem cell niche)[260]. MSCs derived from a single population may exhibit marked variations from each other *in vitro* culture, including differences in MSC morphologies, proliferative capacities, differentiation potentials and the expression of surface markers. Clonal colonies derived from parent bone marrow, adipose, umbilical cord, dental pulp, tendon and blood tissues have reported differences in their *in vitro* characteristics[261-264]. Intra-population heterogeneity refers to donor-specific differences between MSC populations. Inter-clonal heterogeneity refers to differences in single cells derived from a parent colony.

The heterogeneous character of MSC populations presents a significant problem. It may affect outcomes of *in vitro* culture and translational research, and impact efforts to discover and develop functional MSC markers or tests of potency. Attempts to identify and isolate potent MSC populations from stromal fractions using phenotypic surface markers like CD271, CD146, SSEA-3, SSEA-4 or STRO-1 have only been partially successful, since these markers are not uniformly expressed in MSCs from different sources, may select for undesirable traits and/or select for a few rather than all preferred traits[265-268]. For example in one study, isolation of a CD271+ fraction of bone marrow MSCs from a mixed population of plastic-adherent cells resulted in a greater proportion of highly proliferative cells in the CD271+ fraction, but both populations exhibited similar differentiation potencies which was unexpected[261]. Another study suggests that superior MSC performance in a panel of *in vitro* tests rather than a single test may be an accurate predictor of therapeutic efficacy[269]. However, their approach did not select for highly potent MSC lines, rather, all cell lines that were successful *in vitro* culture were selected. Lee et al. suggested that highly potent MSCs represent a subset of the population bearing small cell diameters, low cell stiffness and high nuclear membrane fluctuations, since these MSCs also represented a fraction of highly proliferative and multipotent cells *in vitro* and *in vivo*[270]. All MSC characteristics are heterogeneous *in vitro* culture and hence, methods to enrich MSC populations for these traits have not yielded consistent or optimal results. The implications of MSC heterogeneity *in vitro* and *in vivo*, and the molecular basis of heterogeneity is unknown[244].

Sources of heterogeneity

Morphological distinctions in MSC populations are the most obvious indicators of heterogeneity, and first evoked investigations to the source and implications of these differences

in vitro cultures. A fraction of plastic adherent-bone marrow MSCs for example, may consist of osteoblasts, adipocytes, fibroblasts and smooth muscle cells that differ morphologically from each other. Early studies reported two distinct cell morphologies in cultures of bone marrow MSCs plated at low densities: large, flat cells and spindle shaped cells[271]. Colter et al. identified a third cell type; these were rounded cells that were very small in size, divided rapidly, and exhibited robust differentiation potentials compared to large, flat cells. Proteomic analysis further identified differences in the protein expression patterns of rounded versus flat cells[272]. Recent studies have attempted to understand the significance of these morphological differences. One proposed model of MSC growth suggests that cell size is a function of asymmetrical cell division[239, 273]. In this model, all cells grow larger over time until division, which produces two equally sized daughter cells. A finite probability exists that one daughter cell then continues to grow in size and does not undergo further divisions, whereas the second daughter cell continues to divide and remains the same size. Thus, at any given point, MSC cultures may encompass unequal numbers of differently sized cells. However, cell size may not be an accurate predictor of potency or age. Larger cells may be senescent, or may have stopped dividing and entered a quiescent phase. A negative correlation in cell diameter and potency was found in one study, but was not strong[270].

Aging adversely affects MSC potency. Early evidence suggests that low bone mass in the aged population may be associated with the decreased potencies of MSCs in the bone marrow[245]. As expected, MSCs from younger donors display more potent characteristics compared to adult donors, including greater self-renewal and differentiation capacity[274]. MSC populations encompass differently aged MSCs that have aged at different rates, and the control of aging may be a potential mechanism to reduce heterogeneity and increase potency. MSC aging may be

classified as a decrease in regenerative capacity, deterioration of intercellular regulatory networks, shortening of chromosome telomeres, and increased expression of β-galactosidase (a marker of senescence)[275, 276]. *In vitro*, extensive periods of culture or serial culture on plastic may contribute to MSC aging. Extensive culturing has contributed to changes in MSC surface marker and global gene expression profiles, decreased homing capacity and decreased multipotency in MSC populations[244]. Aging may exacerbate the underlying heterogeneity in MSC populations, by virtue of a greater number of senescent MSCs in the aged population. MSC age is a particularly important consideration *in vivo* applications. Younger MSC populations that have not been exposed to extensive or serial culturing may yield greater therapeutic benefits like increased proliferation and migration to injury sites resulting in enhanced repair outcomes.

Culture conditions may also affect cellular heterogeneity. MSCs at different stages of the cell cycle or lineage commitment may selectively respond to culture stresses including variations in temperature, oxygen tension, pH of the medium, supplemented nutrients, strain and in the underlying substrate. A few scenarios can be envisioned: 1) only a fraction of MSCs in a population may respond to a stressful condition, 2) all MSCs in a population may respond to the same condition, but to different degrees and 3) individual MSC responses may affect non-responsive MSCs in the same population. Each of these scenarios can significantly affect MSC fate, by inducing fluctuations in inter- and intra-cellular protein levels or gene expression patterns, which may contribute to heterogeneity in the population.

Proliferative rates of single MSCs in a population are inconsistent. Early studies have noted that cells at the periphery of clonal colonies proliferate faster than cells at the center[277]. Clones with higher proliferative potentials can divide extensively in culture over several

passages, and give rise to secondary and tertiary colonies upon replating, compared to lowly proliferative clones that cease division after one or two passages[278].

Several studies have demonstrated variations in the differentiation potentials of MSCs derived from a single population. In one proposed model of hierarchical lineage commitment in bone marrow MSCs, decreasing proliferative potential is associated with a sequential loss of differentiation potentials in MSCs. At the top of this hierarchy, MSCs display potent tri-differentiation and proliferative potentials[263, 279]. In one study, 14 bone marrow-derived MSC clones were analyzed, of which 5 were found to be highly proliferative and tripotent, 3 were bipotent, and 4 were unipotent and lowly proliferative[280]. The proportion of highly proliferative and multipotent bone marrow MSCs in a population may be variable; in one study, only 7% of the clones analyzed met these criteria, and additionally gave rise to secondary colonies upon replating[278], whereas in a different study more than 50% of the clones analyzed fulfilled this criteria[3]. Among the three lineage-specific differentiation potentials (adipo-, osteo- and chondrogenic) adipogenic potential may be most sensitive to MSC aging. Muraglia et al. showed that adipogenic potential was progressively lost with increasing cell doublings in culture, followed by chondrogenic potential. 60-80% of their tested clones displayed osteo-chondrogenic potential, whereas osteo-adipogenic, chondro-adipogenic, purely adipogenic or purely chondrogenic clonal phenotypes were not observed in their study[281]. However, this pattern of lineage divergence with age may only be observed in bone marrow MSCs that are inherently committed to osteogenesis. Further, Russell et al. were able to identify and isolate bone marrow MSC clones in all eight categories of tri-lineage potential (Fig. 2.1), including all clonal phenotypes that were not previously identified by Muraglia et al.[3]. This suggests that hierarchical relationships in lineage commitment are complex, and possibly that a sequential pattern of

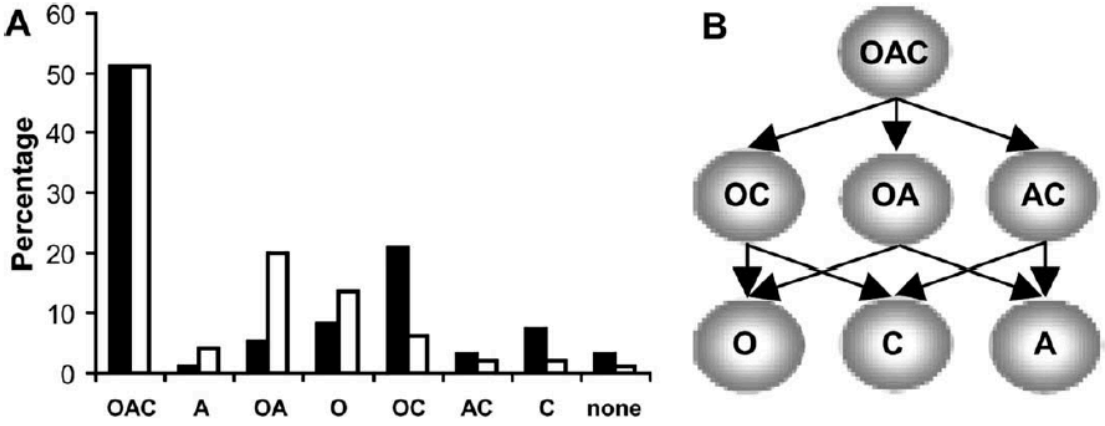


Fig. 2.1. Analysis of clonal bone marrow MSCs: Differentiation potencies of clones derived from two mouse donors (A), and predicted model of hierarchical lineage commitment (B). A-adipogenic, O-osteogenic and C-chondrogenic clone. (Adapted from Russell et al. 2010[3])

lineage commitment with progressive loss of potential as described previously may not exist. Sarugaser et al.'s model clarified this complexity to some extent. They found that highly clonogenic MSCs were capable of differentiating to more than three lineages (for example, myogenic and fibroblastic), and that a fibroblastic phenotype represents a state of complete restriction at the lowest level of the hierarchy[282]. Their data suggests that this subset of highly potent MSCs is also capable of multilineage repair *in vivo*.

Significant relationships in MSC immunomodulatory effects and differentiation potency have been investigated previously[261, 283]. For example, James et al. found a strong negative correlation in differentiation potency and pro-inflammatory IL-7 expression in clonal bone marrow MSCs. Weak correlations have been found in surface marker expressions and differentiation potency. For example, MSC flow-sorting with a combination of CD271, W8B2 and CD56 markers differentiated adipogenic clones from chondrogenic clones, but did not distinguish tri-, bi- and unipotent clones from each other[284]. A similar study found that CD200

was selectively expressed in clones with osteogenic potential and chondro-osteogenic potential, whereas SSEA-4 and CD140A marked adipogenic clones with very low osteogenicity[285]. Biophysical MSC traits such as cell stiffness may be related to reduced potency and increased osteogenicity[270].

Significance of heterogeneity

The biological significance of heterogeneity in MSC populations is not well understood. It may be a mechanism for MSCs to transition between two or more metastable cell states, while remaining inherently non-committal or naïve in character. For example, cells can undergo differentiation while maintaining their self-renewal potential[286]. At the molecular level, heterogeneity may be characterized as a constant state of change in the lineage-specific gene and protein expressions in MSCs resulting in "non-genetic individualities". These molecular fluctuations or "noise" in MSCs may be required to maintain a cultural equilibrium that is conducive to self-renewal and may be reduced in response to culture stresses like high oxygen tension, resulting in a switch in cell states or differentiation[287]. Conversely, decreasing oxygen levels may restore molecular heterogeneity in MSCs.

As discussed earlier, at a given point in culture a population of MSCs may encompass cells that are predisposed to distinct fates. A typical example of this scenario is observed in hematopoietic stem cell cultures. Sca-1 is a surface marker of the hematopoietic stem cell line, and heterogeneously expressed in a broad, bell-shaped pattern *in vitro* culture. However, culturing either Sca-1+ or Sca-1- fractions of a myeloid progenitor cell line were found to restore the original heterogeneous Sca-1 expression pattern in each population, without external intervention. Cells at the extremes of this spectrum exhibited distinct potentials; Sca-1+ cells

were biased toward myeloid differentiation, whereas Sca-1- cells were biased toward erythroid differentiation[288]. This suggests that a mixture of hematopoietic phenotypes is required to provide a conducive environment for the sustenance and homeostasis of a cell population *in vitro* culture.

Clonal analysis

Several molecular biology techniques are used to isolate and analyze single MSCs. These include limiting dilution plating, flow cytometry, fluorescence-activated cell sorting, fluorescence microscopy and the use of microfluidic devices[289]. Clonal analysis can be classified into two types: 1) physical isolation of single MSCs (prospective analysis), and 2) tracking of single MSCs *in situ* (retrospective analysis), which retains their native microenvironment[290]. Physical isolation and expansion of single MSCs may affect their growth and viability, especially over serial culture. Researchers have attempted to alleviate this issue by modifying intercellular signaling pathways or providing "feeder layers" for cell growth and conditioning. However, modified culture conditions may not be conducive to differentiation, or studies of alternative MSC responses. Single cell tracking *in situ* can be accomplished with the genetic recombination of retroviral sequences in the cell genome, which provides a unique molecular signature to each cell by "retroviral marking"[291]. In retrospective analysis, single cells are randomly selected, and information on the behavior of their progeny is retrospectively deduced after the culture has been analyzed *in vitro* or *in vivo*. Clonal tracking methods *in vivo* have been most investigated in the hematopoietic system. Lastly, a thorough understanding of stem cell heterogeneity requires the simultaneous analysis of both, molecular and functional characteristics of single cells. However, the current knowledge on inter-relationships in stem cell characteristics is based on correlated

data from different clonal cells, and is hence limited by the assumption of these cells being homogeneous[291].

CHAPTER 3: OPTIMIZING GROWTH FACTOR INDUCTION OF TENOGENESIS IN THREE-DIMENSIONAL CULTURE OF MESENCHYMAL STEM CELLS

ABSTRACT

Adult tissue stem cells have shown promise for the treatment of debilitating tendon injuries. However, few comparisons of stem cells from different tissue sources have been made to determine the optimum stem cell source for treating tendon. Moreover, it is likely that the addition of tenogenic growth factors will improve tendon stem cell treatments further, and a comprehensive comparison of a number of growth factors is needed. Thus far, different types of stem cells cannot be evaluated in a high-throughput manner. To this end, we have developed an approach to culture MSCs isolated from the bone marrow in collagen type I hydrogels with tenogenic growth factors using economical, commercially available supplies. To optimize growth factors for this assay, FGF-2, TGF-β1, IGF-1, and or BMP-12 were tested singly and in novel combinations of: 1) BMP-12 and IGF-1, 2) TGF-β1 and IGF-1, and or 3) BMP-12 and FGF-2 over 10 days. Our data suggests that BMP-12 supplementation alone results in the strongest expression of tendon marker genes, a controlled contractility of constructs, a higher degree of cell alignment and tendon-like tissue morphology. This easy-to-use bench-top assay can be used to screen novel sources of stem cells and cell lines for tissue engineering and tendon healing applications.

INTRODUCTION

Tendon injury is a significant clinical problem, and involved in over half the musculoskeletal injuries sustained each year[67]. Human sporting activities frequently involve overuse or acute injuries of the Achilles tendon[292]. Tendon injuries of the rotator cuff are prevalent in over 20% of the adult population, and incidence increases with age[293]. The poor healing response in mature tendons results in a disorganized scar with inferior functional outcomes and a high incidence of reinjury[294]. MSCs have shown promise for the treatment of tendon injuries, with a reduced re-injury rate of treated SDFTs in horses[256], and improved collagen organization and mechanical properties of Achilles tendons in humans[253]. A major impediment to the development of MSC therapies for tendon healing is a lack of screening assays for optimal stem cells. Benchtop assays of tri-lineage differentiation, which are currently used to assess MSC potency, are poor indicators of tenogenesis[295]. In the absence of a facile tenogenesis assay, novel sources of MSCs and different donor cell lines cannot be evaluated in an efficient and high-throughput manner.

Collagen type I comprises approximately 95% of the total collagen found in tendon[296]. As a hydrogel, it is readily accessible and customizable *in vitro*. MSCs, by virtue of interacting actin and myosin filaments[233], can remodel an immobilized collagen hydrogel into a tendon-like form. Previous attempts to engineer tendon constructs *in vitro* have utilized silicone molds[146, 220], synthetic sutures[207], vacuum pressure systems[223] and a variety of artificial biophysical agents. For example, Feng *et al*. designed rectangular silicone molds affixed with T-shaped silver wires at the mold edges for gel anchorage[220]. Butler *et al*. customized glass petri dishes to incorporate small, rectangular troughs with tensioned synthetic sutures to aid gel attachment and contraction[207]. Each of these systems rely on some or all of the following: careful manipulation and handling, expensive vessels or bioreactors that are impractical for high numbers of replicates, and custom manufacture of the apparatus.

Growth factor supplementation of MSCs facilitates tenogenesis, but there is incomplete understanding of which growth factors are sufficient and optimal. FGF-2 stimulates angiogenesis[137] *in vivo*. TGF-β1 improves tendon biomechanical properties in rat patellar tendons[141]. IGF-1 is an anti-inflammatory agent[188], and promotes MSC chemotaxis at sites of injury[137] *in vivo*. BMP-12 is primarily a tendon differentiation factor for MSCs[133], and BMP-12 tenogenesis has been previously reported via adenoviral gene transfer and protein supplementation *in vitro*[116, 128], and BMP-12-releasing sutures *in vivo*[129]. All of the aforementioned growth factors stimulate tendon-specific gene expression and matrix protein synthesis *in vitro* MSCs[134, 163, 184].

While single tenogenic growth factors have been well investigated, their synergistic effects in tenogenesis are poorly understood. It is likely that growth factors work in concert during tendon repair and for the maintenance of tendon homeostasis *in vivo.* Combined effects of a few growth factors have been reported *in vitro* studies[199, 200]. For example, IGF-1 in concert with FGF-2 was shown to increase cell survival over single factor supplemented scaffolds in one study[203]. However, whether combined factors are truly more efficacious than single factors, and if yes, the optimal growth factor combination for tenogenesis is unknown. Further investigation is warranted to address these questions, and to understand the complex mechanisms of growth factor-mediated regeneration.

The goals of this study were to 1) develop a high-throughput, facile tenogenesis assay using commercially available materials and reagents, 2) determine from known tenogenic factors, which growth factors are sufficient and/or optimal for the tenogenic induction of bone marrow MSCs in our 3D assay. The aforementioned growth factors (FGF-2, TGF-β1, IGF-1 and BMP-12) were evaluated individually, and in combination, and compared to unsupplemented 3D

controls. BMP-12 was combined with either FGF-2 or IGF-1. In addition, TGF-β1 combined with IGF-1 was also evaluated as a third dual factor group[297]. Tenogenesis was evaluated by collagen gel contraction, cell morphology and longitudinal alignment, gene expression of tendon markers and GAG content on day 10 of culture. We hypothesized that 3D culture of MSCs with BMP-12 and IGF-1, maintained under contraction-induced uniaxial strain would result in a facile and practical tenogenesis assay. We further hypothesized that synergistic growth factors would augment stress-induced tenogenesis over individual factors.

MATERIALS AND METHODS

Experimental design

Previously isolated and cryopreserved bone marrow MSC lines derived from equine sternal bone marrow aspirate with Animal Care and Use Committee approval were used in this study (n=3). Constructs were prepared by seeding collagen gel with bone marrow MSCs suspended in tenogenic growth media on day 0. On day 1 of culture, constructs were supplemented with one of four single growth factor cocktails, namely 1) FGF-2, 2) TGF-β1, 3) IGF-1 or 4) BMP-12, or combined growth factor cocktails, namely 1) BMP-12 and IGF-1, 2) TGF-β1 and IGF-1 or 3) BMP-12 and FGF-2. Recombinant human growth factors were purchased from commercial vendors. FGF-2 and IGF-1 were from BioVision (San Francisco, CA), TGF-β1 was from R&D Systems (Minneapolis, MA), and BMP-12 was purchased from Sigma-Aldrich (St. Louis, MI). The concentrations of each growth factor in media are described in Table 1. Each growth factor concentration was selected based on results of previous studies inducing tenogenesis[116, 148, 203]. Each test group was plated in triplicate (n=3). For all outcome measures, growth factor groups

were compared to 3D, unsupplemented controls. Additionally, gene expression data was computed using an equine juvenile tendon reference control.

Table 3.1. Growth factor treatment groups and concentrations of each growth factor

Growth Factor Concentration	**Treatment Groups**						
	FGF-2	TGF-β1	IGF-1	BMP-12	BMP-12 IGF-1	TGF-β1 IGF-1	BMP-12 FGF-2
FGF-2	5 ng/ml						5 ng/ml
TGF-β1		5 ng/ml				5 ng/ml	
IGF-1			10 ng/ml		10 ng/ml	10 ng/ml	
BMP-12				50 ng/ml	50 ng/ml		50 ng/ml

Tenogenesis apparatus

Commercially available 4-welled, non-adherent, Nunc rectangular dishes (12.8 x 8.6 cm, Thermo Scientific, Waltham, MA) were purchased for this study. Each well in the plate was affixed with two sterile cloning cylinders (0.8 x 0.8 cm, Corning Inc, Christiansburg, VA) set three centimeters apart from each other along the longitudinal midline of the well, held in place by sterile silicone (Fig. 3.1).

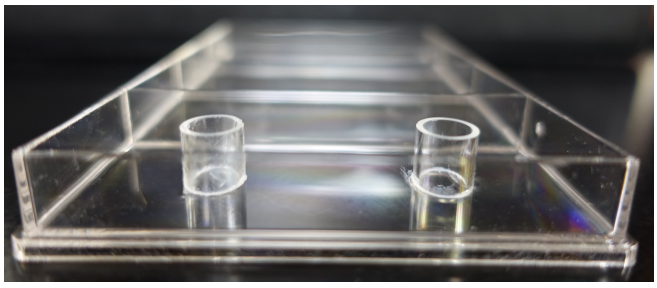


Fig. 3.1 Tenogenesis apparatus.

Mesenchymal stem cell culture and derivation of 3D constructs

MSC lines were previously determined to be positive for the expression of CD90, CD105, Oct-4, and undergo tri-lineage differentiation. Cells were expanded in culture for one passage. Upon confluence, cells were trypsinized and 1 million cells per gel were suspended in 5 milliliters of tenogenic growth media comprising high glucose DMEM (Thermo Scientific), 10% Cellect^{TM} Silver fetal bovine serum (MP Biomedicals, Santa Ana, CA), 37.5 μg/ml L-ascorbic acid (Sigma-Aldrich), 1% penicillin G (Sigma-Aldrich) and 0.8 mg/ml rat tail collagen I (Corning Life Sciences, Tewksbury, MA). Cell suspensions were plated immediately following preparation on day 0 and allowed to solidify in culture incubators maintained at 37°C, 5% CO_2 and 90% humidity. After 1 hour of gelation, a sterile spatula was used to release the gels from the well walls to facilitate contraction. On day 1 of culture, test groups were supplemented with their designated media. All constructs were fed on alternate days during a 10-day culture period.

Cell morphology

Longitudinal sections of each construct were stained with a commercial two-color fluorescent assay (LIVE/DEAD Viability Kit, Thermo Scientific) for qualitative analysis. Construct images are representative of the average of 6 replicates, and were acquired using a fluorescence microscope (EVOS^{TM} FL Imaging System, Thermo Scientific). Independent samples were fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS and submitted for histology (Laudier Histology, New York, NY). Samples were embedded in an acrylic resin, sectioned into 6 micron thick longitudinal slices and stained with Masson's trichrome stain. Images were acquired using a phase-contrast microscope (Olympus Corp, Center Valley, PA).

Quantification of cell alignment

Two histological sections per representative sample were used to quantify cell alignment using built-in ImageJ software analytical tools[298]. Fifty cellular angles per section were measured relative to the construct longitudinal axis. Parallel alignment to the construct longitudinal axis was assigned 0º, and angles of each cell relative to 0º (0º-90º) were used to construct histograms. The resulting histograms for each sample were averaged to draw group-wise comparisons.

Gel contraction analysis

On day 0, 1, 3, 5, 7 and 10, digital images of all constructs were taken to assess percentage area and determine contraction of the constructs. Images were analyzed using built-in ImageJ software analytical tools. The contracted area at each time point was calculated as a percentage of the initial area of the gel (% contraction).

Gene expression analysis

A section of each construct was used for gene expression analysis. Samples were homogenized in TRIzol reagent (Thermo Scientific) for RNA isolation according to the manufacturer's protocol. Genomic DNA contamination of RNA pellets was removed using RNeasy spin columns and on-column DNase treatment (QIAGEN Inc., Germantown, MD) and purified RNA was quantified using a Nanodrop spectrophotometer. First-strand complimentary DNA (cDNA) synthesis was performed using a high-capacity reverse transcriptase kit (High-Capacity RNA-to-cDNA kit, Thermo Scientific). Real-time quantitative PCR (7500 Real-Time PCR System, Thermo Scientific) was performed using custom TaqMan-MGB probes and sequence detection primers (Thermo Scientific) designed with Primer Express^{TM} software (Version 3.0, Thermo Scientific). The comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) was employed for relative quantification of gene expression[299]. Data was normalized to GAPDH, which was validated for stability in our study using qbase^{+} software (Biogazelle, Zwijnaarde, Belgium). Tenogenic

marker expression is reported as fold change with respect to a tendon reference control. A list of primers and probes is included in Table 2.

Table 3.2. Custom designed equine primer and probe sequences

	Forward primer	Reverse primer	Probe
GAPDH	CAAGTTCCATGGCACAGTCAAG	GGCCTTTCCGTTGATGACAA	CCGAGCACGGGAAG
Scleraxis	CGCCCAGCCCAAACAG	TTGCTCAACTTTCTCTGGTTGCT	TCTGCACCTTCTGCC
Collagen type I	GCCAAGAAGAAGGCCAAGAA	TGAGGCCGTCCTGTATGC	ACATCCCAGCAGTCACCT
Collagen type III	CTGCTTCATCCCACTCTTAT	ATCCGCATAGGACTGACCA	AACAGGAAGTTGCTGAAGG
Decorin	AAGTTGATGCAGCTAGCCTG	GGCCAGAGAGCCATTGTCA	ATTTGGCTAAATTGGGACTG
Biglycan	TGGACCTGCAGAACAATGAGAT	AGAGATGCTGGAGGCCTTTG	TCTGAGCTCCGAAAGG

Glycosaminoglycan quantification

Representative constructs from each replicate were homogenized overnight in a papain-containing digest buffer (Sigma) at 60°C. Cumulative GAG content within the construct was quantified by the 1,9 dimethylmethylene blue dye (Sigma, USA) colorimetric assay[300], using chondroitin sulfate (Sigma) as a reference standard. Data was normalized to total DNA content as determined by a Nanodrop spectrophotometer. Data is reported as μg GAG/ μg DNA.

Statistical analysis

Data was tested for normality using the Shapiro-Wilk test. Non-normal data was log transformed and achieved normality prior to analysis. For gel contraction, a one-way, multivariate analysis of variance with a repeated-measures design and Student's t-tests were used to assess differences between groups over time and at each time point. For gene expression and GAG data, a one-way analysis of variance was performed followed by post hoc Tukey's tests for pairwise comparisons

between groups. Cell alignment was assessed using Student's t-tests. Quantitative data is reported as mean ± standard error. Distinct letters denote significant differences on graphical data for cell alignment, gene expression and GAG content. For gel contraction, groups with significant between group differences at each time point are denoted with an asterisk, and detailed statistical data are presented as supplemental tables. A *p*-value of less than 0.05 was considered significant. Computation was performed in JMP Pro 15 (SAS Institute, Cary, NC) and MS Excel 11 (Microsoft, Redmond, WA).

RESULTS

Growth factors modulate cell alignment and anisotropic contraction

In all groups, the MSCs uniformly integrated into the 3D gel constructs and progressively aligned with the longitudinal axis of tension (Fig. 3.2). In all groups, >99% of MSCs exhibited elongated cell morphology and were viable. On day 10, MSCs in BMP-12 constructs were significantly better aligned when compared with control, FGF-2, BMP-12/FGF-2 and TGF-β1/IGF-1 constructs (Fig. 3.3).

Gel contraction (contracted gel area) was significantly improved in each group over time, and significant differences were seen in between group comparisons. From day 0 to day 3, all groups contracted rapidly to 20-50% of the initial area, whereupon the rate of contraction decreased (Fig. 3.4B). The BMP-12 group was significantly more contracted than TGF-β1 and BMP-12/FGF-2 on days 5, 7 and 10. No significant differences were noted between BMP-12 and BMP-12/IGF-1. TGF-β1/IGF-1 contracted the most of any group and significantly more than TGF-β1 and BMP-12/FGF-2 at most time points (days 3, 5, 7 and 10). BMP-12/FGF-2 contracted the least of any group and was significantly less contracted than the control, FGF-2,

BMP-12, BMP-12/IGF-1 and TGF-β1/IGF-1 groups at most time points (days 3, 5, 7, and 10). The remainder of the results is presented in Supplemental Tables A1-A5.

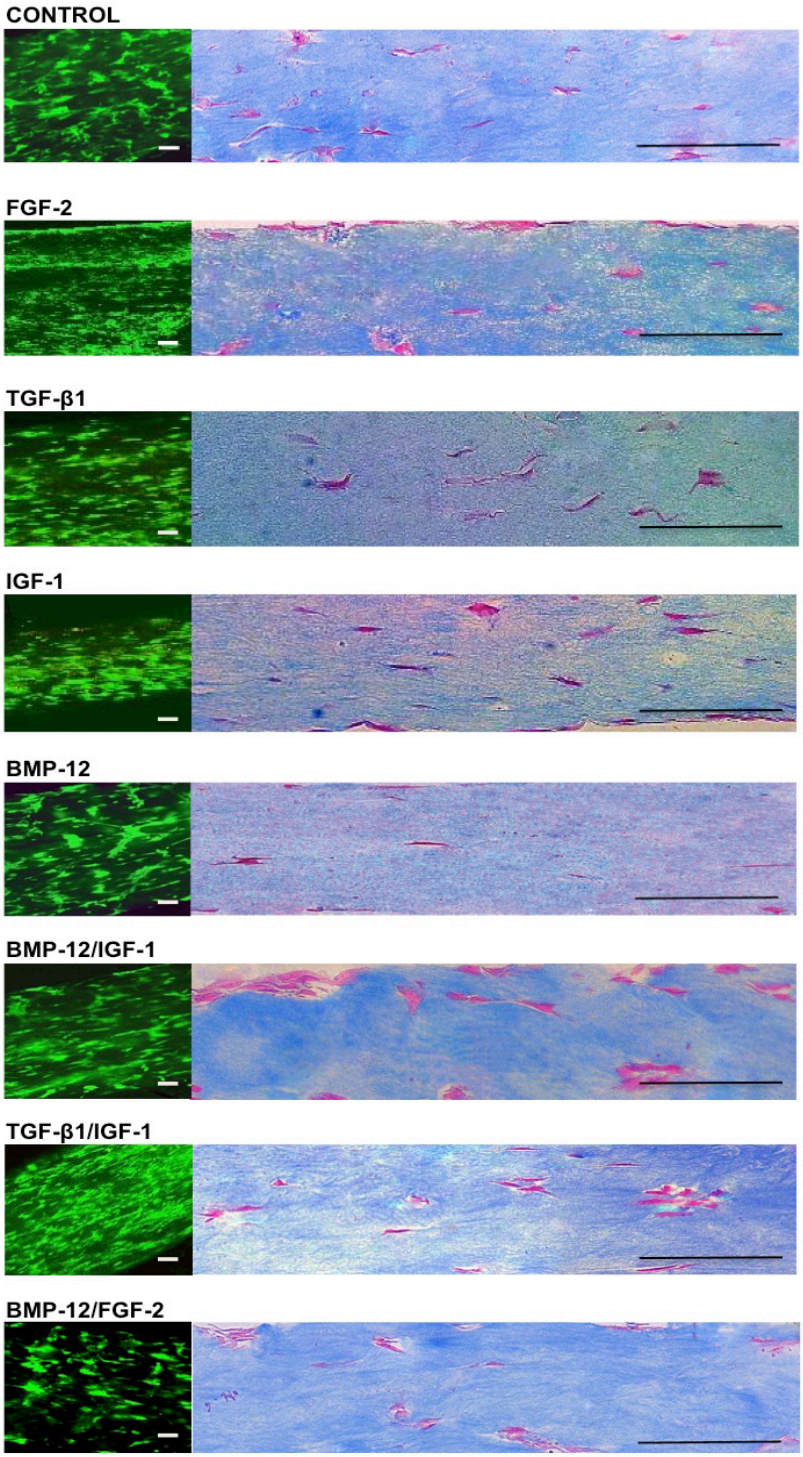


Fig. 3.2. Fluorescence microscopy (left), and Masson's trichrome histology images (right) of day 10 gels. All constructs exhibited uniform integration of MSCs in three dimensions accompanied by progressive alignment along the axis of tension. Scale bars represent 125 μm.

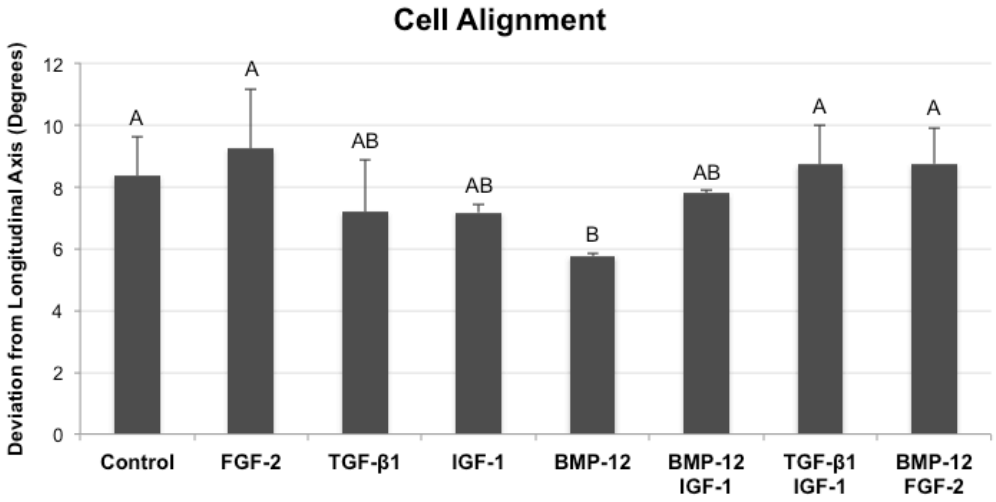


Fig. 3.3. MSC angle of deviation from the longitudinal construct axis in day 10 gels with perfect longitudinal alignment = 0º. BMP-12 enhanced parallel cell alignment significantly more than control, FGF-2, TGF-β1/IGF-1, and BMP-12/FGF-2. Data points that do not share a letter are significantly different.

Tendon gene expression in 3D culture is augmented by BMP-12

Control constructs expressed all tendon-related genes evaluated in this study on day 10, and the addition of BMP-12 and BMP-12/IGF-1 augmented gene expression toward a tendon phenotype (Fig. 3.5). BMP-12 induced more than a 3-fold increase of scleraxis over controls ($p = 0.0338$). BMP-12-mediated matrix remodeling was evident by more than a 5-fold increase of collagen type III expression over TGF-β1 ($p = 0.0116$) and TGF-β1/IGF-1 ($p = 0.0172$). BMP-12/IGF-1 significantly increased collagen type III expression over all groups except BMP-12, BMP-

12/FGF-2 and control. Decorin expression was the greatest in BMP-12 and BMP-12/IGF-1, significantly greater than the control, FGF-2, TGF-β1 and TGF-β1/IGF-1 groups. Biglycan expression remained unchanged in response to growth factor treatment. Interestingly, combining FGF-2 with BMP-12 significantly decreased BMP-12-induced scleraxis expression ($p = 0.0215$). Consistent with this, BMP-12/FGF-2 expression of collagen type I was significantly lower than IGF-1 ($p = 0.0186$), although the level was not significantly reduced from other groups.

Growth factors in combination increase cumulative GAG content

Single growth factors in this study failed to increase cumulative GAG content within the constructs compared to control (Fig. 3.6). BMP-12 combined with IGF-1, resulted in a 2-fold increase in GAG composition over control ($p = 0.0427$) and all single growth factor groups. TGF-β1/IGF-1 and BMP-12/FGF-2 also increased GAG compared with control and single growth factor groups. Interestingly, FGF-2 had significantly lower GAG compared with control ($p = 0.0483$). No other significant differences were detected.

DISCUSSION

The overarching goal of this study was to develop an easy-to-use tenogenesis assay for the efficient and relatively rapid evaluation of candidate stem cells for tendon engineering and treatment of tendon injuries. Toward this end, we designed and validated a simple apparatus using commercially available plasticware for the application of uniaxial static strain. We determined that BMP-12 protein supplementation of bone marrow derived stem cells in this tenogenesis apparatus induces a composite tendon phenotype over 10 days.

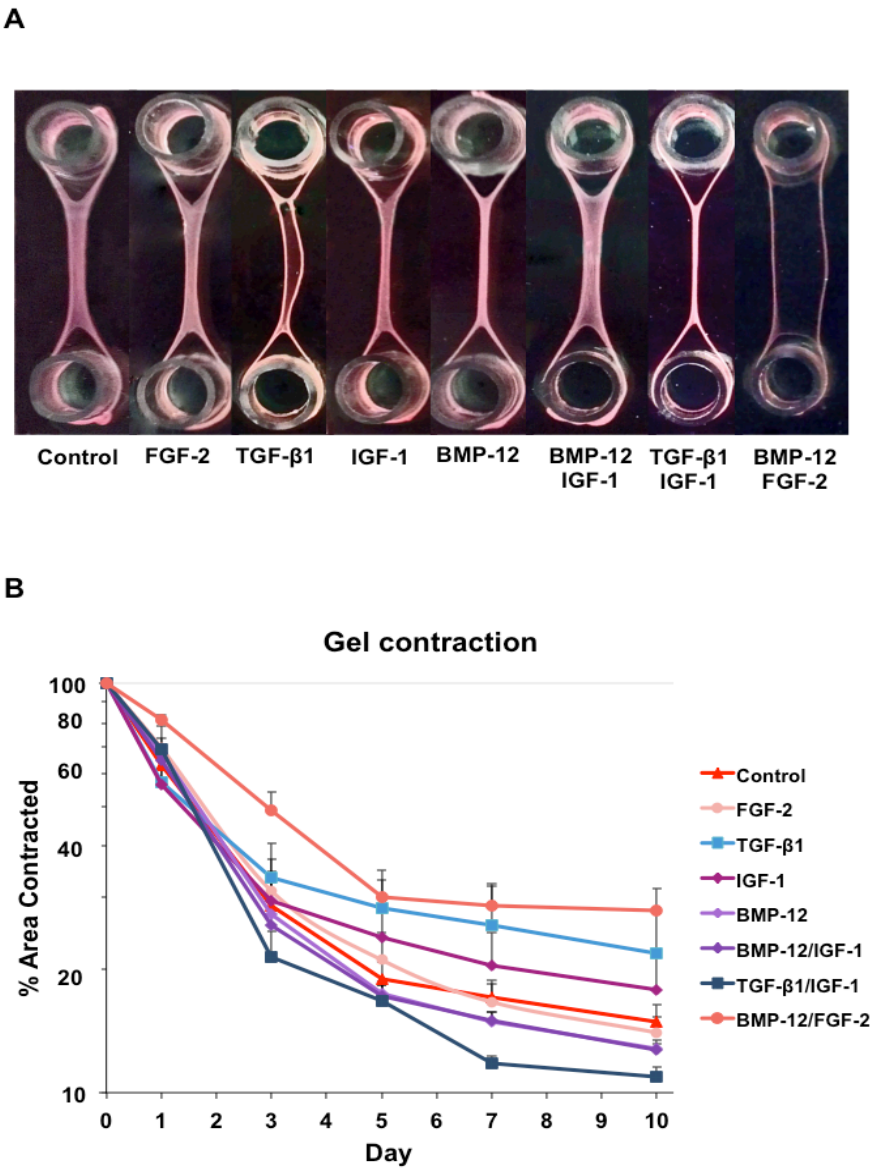
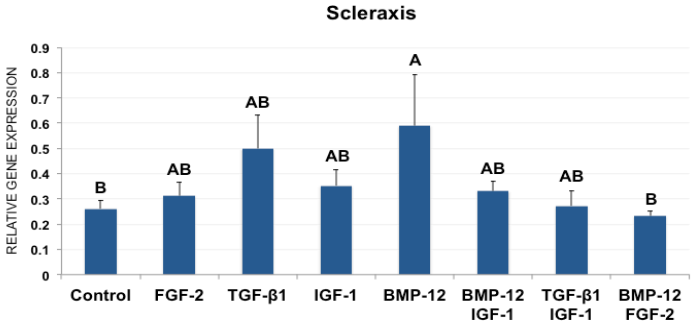
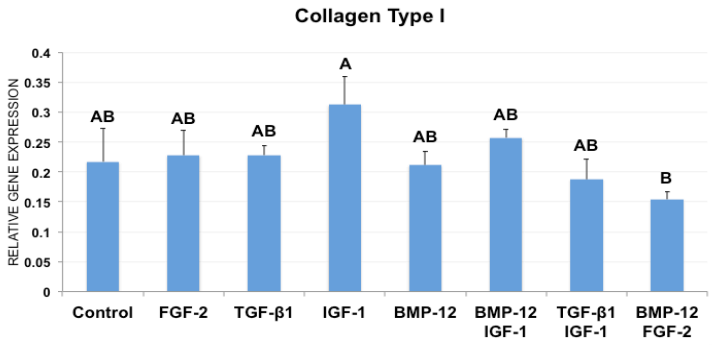


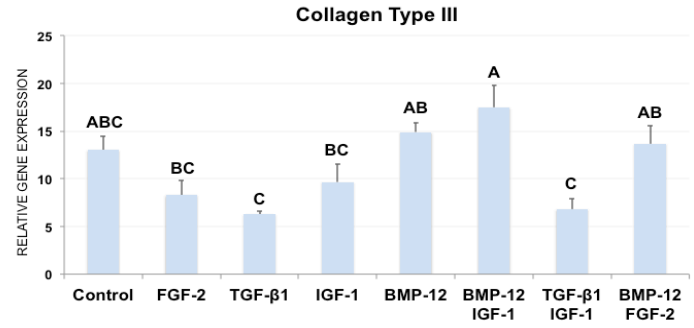
Fig. 3.4. Digital images of constructs at harvest (A) and graphical representation in logarithmic scale of percentage contraction of constructs over 10 days of culture (B). BMP12/FGF2 and TGF-β1 constructs had contracted the least at culture endpoint, significantly less than BMP-12, BMP-12/IGF-1 and TGF-β1/IGF-1 constructs.

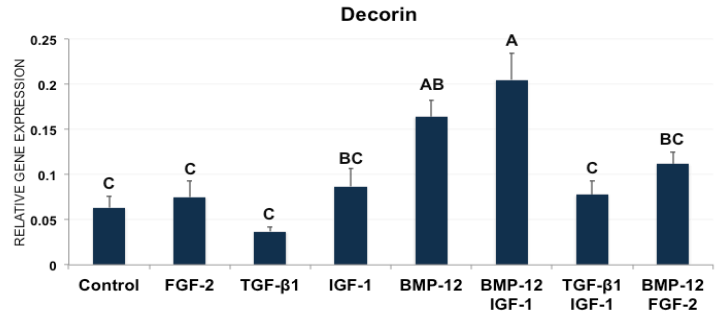
Native tendon tissue is characterized by a highly organized array of cells and collagen fibrils, aligned in parallel to the longitudinal axis of tension[301]. Progressive cellular alignment in response to tissue anisotropy is a hallmark of tenogenesis[302], and was observed in all constructs over the culture period. Further, to the best of our knowledge, this study is the first to perform quantitative comparisons of MSC orientation in response to exogenous growth factors during differentiation. The increased cell alignment in response to BMP-12 in this study further strengthens the hypothesis that BMP-12 augments strain-mediated tendon tissue morphology *in vitro*[126], however this effect was reduced by the BMP-12/FGF-2 group.

BMP-12 and BMP-12/IGF-1 stimulated the greatest overall increases in tendon gene expression compared to other growth factor groups. The increased expression of scleraxis (a bHLH transcription factor)[28] in response to BMP-12 was expected, since BMP-12 is well documented in the literature as a tenogenic growth factor[117, 200]. BMP-12 has been shown to augment strain-induced scleraxis expression in MSCs, establishing a combined effect of uniaxial strain and BMP-12[124], which was also observed in this study. Decorin and biglycan are small leucine-rich proteoglycans (SLRP) abundantly found in the tendon matrix[303]. Expression of decorin in response to BMP-12 in MSCs has previously reported contradicting results; some studies report significant increases[304], while others report unaffected expression[125]. In this study, the strong upregulation of decorin expression in BMP-12 and BMP-12/IGF-1 constructs may explain the lack of induction of biglycan observed in the same groups. Decorin and biglycan may functionally compensate for each other, evident by the increasing expression of biglycan in response to loss of decorin *in vivo*[14].


Scleraxis
RELATIVE GENE EXPRESSION
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
B
AB
AB
AB
A
AB
AB
B
Control
FGF-2
TGF-β1
IGF-1
BMP-12
BMP-12 IGF-1
TGF-β1 IGF-1
BMP-12 FGF-2


Collagen Type I
RELATIVE GENE EXPRESSION
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
AB
AB
AB
A
AB
AB
AB
B
Control
FGF-2
TGF-β1
IGF-1
BMP-12
BMP-12 IGF-1
TGF-β1 IGF-1
BMP-12 FGF-2


Collagen Type III
RELATIVE GENE EXPRESSION
25
20
15
10
5
0
ABC
BC
C
BC
AB
A
C
AB
Control
FGF-2
TGF-β1
IGF-1
BMP-12
BMP-12 IGF-1
TGF-β1 IGF-1
BMP-12 FGF-2


Decorin
RELATIVE GENE EXPRESSION
0.25
0.2
0.15
0.1
0.05
0
C
C
C
BC
AB
A
C
BC
Control
FGF-2
TGF-β1
IGF-1
BMP-12
BMP-12 IGF-1
TGF-β1 IGF-1
BMP-12 FGF-2

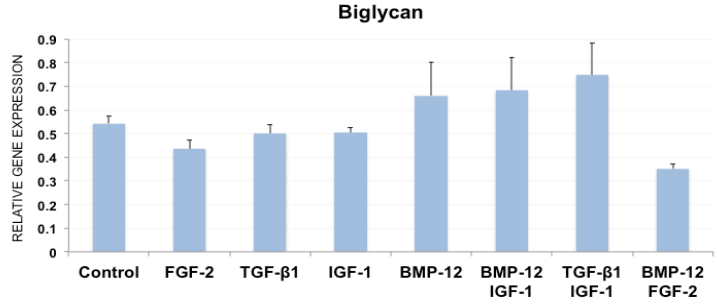

Biglycan
RELATIVE GENE EXPRESSION
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
FGF-2
TGF-β1
IGF-1
BMP-12
BMP-12 IGF-1
TGF-β1 IGF-1
BMP-12 FGF-2

Fig 3.5. Gene expression profiles of tendon marker genes scleraxis, collagen type I, collagen type III, decorin and biglycan. BMP-12 and BMP-12/IGF-1 constructs consistently increased expression of all markers on day 10. Data is reported as fold change with respect to tendon reference control. Data points that do not share a letter are significantly different.

The *in vitro* process of collagen gel contraction by tenocytes recapitulates their physiological behavior to remodel a collagenous matrix in response to an external stimulus, such as strain[305] or inflammation[75]. A ratio of 1 million cells to 4mg collagen in this study resulted in a reduction of collagen gel area to 29% of the initial area by day 3, and to 18% by day 7, consistent with previous results[306]. TGF-β1 has been shown to enhance the contraction of tenocyte-seeded matrices under strain[146]. In this study, TGF-β1 independently did not increase contraction over controls, however, the synergistic effects of TGF-β1 and IGF-1 augmented tissue contractility over both individual factors. Wound healing in rabbit patellar tendons is enhanced with the combined application of TGF-β1 and IGF-1[297], which may be attributed to enhanced tenocyte contraction and GAG synthesis. BMP-12 (singly and combined with IGF-1) construct contraction was not significantly different from TGF-β1/IGF-1, in support of BMP-12's role as a tendon healing agent *in vivo*[127]. When FGF-2 was combined with BMP-12, contraction was significantly reduced compared to either factor alone.

GAGs are the side chains of tendon proteoglycans[12]. The cumulative GAG content in control constructs (1.2 μg GAG/μg DNA) is consistent with the physiologic levels of equine SDFT (0.2 to 0.8 μg GAG/ μg DNA)[307]. An increased accumulation of GAGs, resulting from a synergistic effect of BMP-12 and IGF-1 in this study, is expected during tendon neogenesis[179] (2.1 μg GAG/μg DNA compared to 1.2 μg GAG/μg DNA of control constructs). Further, this

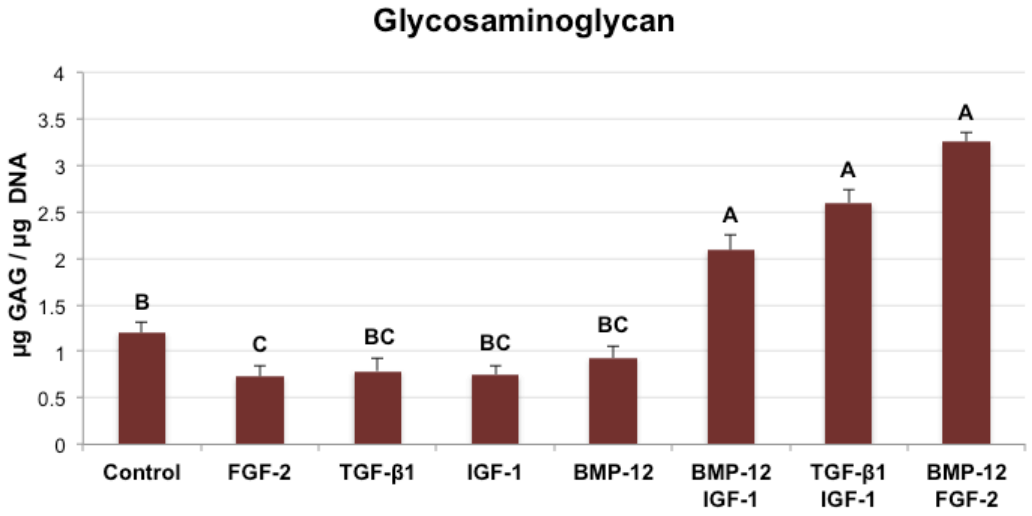


Fig 3.6. Glycosaminoglycan levels of day 10 constructs were assessed using the 1,9 dimethylmethylene blue dye assay. Synergistic effects of dual factor groups were detected for GAG. BMP-12 combined with IGF-1, synergistically increased GAG levels. Endpoint GAG content in the BMP-12/FGF-2 constructs was the highest at harvest, significantly greater than all single factor groups and control. Data points that do not share a letter are significantly different.

observation may be correlated to a similar increase in decorin expression in the same dual factor group. BMP-12 independently did not influence GAG levels, contrary to one previous report[132].

Results from this study support the claim that BMP-12 is primarily a tendon differentiation factor, whereas FGF-2, TGF-β1 and IGF-1 may be better described as inducers of matrix synthesis and/or cell proliferation[111, 137, 308]. However, our study is not without limitations. Bioactive levels of growth factors were selected in this study [116, 133, 134, 148, 199, 203], and a single concentration of each growth factor was evaluated. Further, outcomes from this study represent a single time point in the culture period. It may be beneficial to test outcomes from different

concentrations of growth factors at several time points using this assay, to understand their spatiotemporal control of tenogenesis.

Our investigation of synergistic growth factors in this study is comprehensive and accurate. In this study, 1) all single growth factor groups were incorporated, and 2) single factor groups were incorporated in parallel rather than separate experiments with combined factor groups, so that accurate assessments of growth factor synergism could be made. In addition to the synergistic effects described above and in this study, an inhibitory effect of FGF-2 on BMP-12 was observed, which has not been previously reported. Specifically, BMP-12-induced MSC alignment, contraction and induction of scleraxis gene expression were inhibited with the addition of FGF-2. Adenoviral transfer of BMP-12 did not affect the endogenous production of FGF-2 in a previous study of rat Achilles tendons[119]. In contrast, results from this study suggest that the simultaneous overexpression of FGF-2 and BMP-12 may downregulate tenogenic differentiation, further suggesting cross talk in these two growth factors. Addition of IGF-1 to BMP-12 constructs did not augment tenogenesis over BMP-12 alone, except for an increase in GAG content. Hence, for the purpose of this assay, BMP-12 is sufficient to induce tenogenic differentiation. The effects of BMP-12 were manifested by the stronger expression of tenogenic genes, controlled contractility of constructs, a substantial degree of cell alignment and tendon-like tissue morphology.

This novel bench-top assay does not require sophisticated materials or machinery. Uniaxial static tension combined with BMP-12 is sufficient to induce tenogenic differentiation within a 10-day culture period. This assay can be used to assess large numbers of donor MSC cell lines for the optimum stem cells for allogeneic treatments. This would enable off-the-shelf treatment of tendon injury.

ACKNOWLEDGEMENTS

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CHAPTER 4: HETEROGENEOUS DIFFERENTIATION AND QUANTIFICATION OF LINEAGE-SPECIFIC MARKERS IN TENDON STEM CELLS

ABSTRACT

Tendon injury is a significant clinical problem. Mature tendons heal poorly, and the development of tendon treatments is hindered by our limited understanding of endogenous tendon stem cells. Recent evidence suggests that TSCs may exist on a spectrum of differentiation potencies, and are not homogeneous in character. However, a detailed analysis of single-cell generated lines is required to understand the molecular basis of each TSC phenotype. Toward this end, we have developed a tenogenesis assay of tenogenic potential, so that in addition to standard tri-lineage differentiation assays, a comprehensive assessment of differentiation potency towards four lineages could be made. *We hypothesized that TSCs varied in their differentiation capacity towards tendon, and to the bone, cartilage and fat lineages.*

Thirty clonal TSC lines (TCs) were isolated and expanded in culture for one passage (N=3). To quantify tenogenic potential, TCs were seeded in 3D collagen hydrogels and supplemented with BMP-12 and IGF-1 over 10 days. Tri-lineage differentiation potency was determined over 21 days of culture in lineage-specific differentiation media. Outcomes of differentiation were quantified by the gene expression analysis of lineage-specific markers, and tenogenesis was additionally evaluated by histology and contraction analysis. Statistical significance was determined using ANOVA, with post hoc Tukey's tests and Pearson's correlation analysis. A p-value of ≤ 0.05 was considered significant.

15 TCs (N=3) were successfully analyzed for quadra-differentiation potency and classified into three groups based on differentiation phenotype (AOCT, OCT and ACT). TCs of

the AOCT phenotype strongly differentiated to bone, fat and cartilage, and produced a mature, tendon-like construct over 10 days of induction in our tenogenesis assay, manifested by the strongest expressions of scleraxis and mohawk, controlled contractility, elongated cell morphologies and superior cell alignment.

This study has important implications in the current standard-of-care in regenerative therapies for tendon. Our results suggest that while TSCs of the AOCT phenotype are strongly tenogenic, those of the OCT phenotype may offer select advantages in tendon healing, including a decreased proclivity to ectopic bone formation. Future studies may be targeted to understanding the functional contributions of each phenotype *in vivo*.

INTRODUCTION

Tendon injuries are debilitating, and significantly impact quality of life. Over 32 million cases of musculoskeletal injuries involving the tendon are reported every year, and the incidence of tendon injuries is increasing, especially in the elderly population[207]. Acute and chronic injuries of the Achilles, patellar and rotator cuff tendons are most prevalent in the adult population. Acute injuries are common in athletes, whereas chronic injuries usually arise from tendon overuse or aging[292, 293]. Tendons heal poorly[309], and surgical intervention is often necessary to augment healing. However, the incidence of re-injury following surgical repair can be as high as 20-60%[310]. In recent times, regenerative therapies, specifically MSCs have shown promise in the restoration of native tendon structural and functional properties. Among these, bone marrow MSCs are generally preferred; they are easily accessible, well characterized and have been efficacious *in vivo* models[256]. However, they are also predisposed to bone, may not differentiate to tenocytes and may require pre-transplantation conditioning in bioreactors or with growth

factors to facilitate their roles in tendon repair. ***This suggests that an alternate MSC source such as tendon may be better suited for regenerative tendon healing***[241].

Endogenous TSCs are found in the tendon epitenon and endotenon, and arise from the tendon progenitor niche that is predominantly a collagen-rich ECM[22]. Evidence suggests that these cells are the principal mediators of the regenerative processes that occur during tendon healing, including the control of the inflammatory response and the synthesis and remodeling of collagen[311, 312]. Like bone marrow MSCs, TSCs exhibit surface markers of stemness, have extensive proliferative potentials and can differentiate to non-tendon lineages[242] *in vitro*. Transplantation of these cells can also augment tendon repair *in vivo*[313]. However, recent studies indicate that TSCs from different tendon regions such as the inter- and intra-fascicular matrix, the peritenon and the tendon core exhibit differences in their morphologies and tendon healing potentials[312, 314]. For example, one study showed that while TSCs from both the tendon core and peritenon are multipotent, those from the core express higher levels of scleraxis and tenomodulin, whereas those from the peritenon express elevated levels of vascular and pericyte markers[241]. ***This suggests that TSCs are not homogeneous in character, and their phenotypes may not even be restricted to two categories as previously described.*** Bi et al. further clarified this dilemma in their study of human hamstring and mouse Achilles tendons. In their study, they identified several TSC phenotypes based on differentiation potencies and proliferative potentials[22]. However, a comprehensive analysis of single TSC lines is required to understand the molecular basis of these differences, and their contribution to tendon healing and the maintenance of homeostasis *in vivo.*

The presence of distinct TSC phenotypes in tendon is not surprising. MSCs derived from the bone marrow, adipose, umbilical cord, dental pulp and blood have reported differences in

their *in vitro* characteristics[261-264]. The presence of phenotypic variations in MSCs challenges the current paradigm of one phenotype that encompasses all MSCs, and hence, Dominici *et al.*'s proposed critieria may not strictly characterize all MSCs[236, 273]. The source of these phenotypic variations is also not well understood, and may be attributed to asymmetrical cell division[239], which results in daughter cells committed to one of three fates: 1) quiescent and inactive, 2) partially differentiated progenitors or 3) actively proliferating with a constrained differentiation fate[315]. The advent of single-cell or clonal analysis has further enriched our understanding of MSC heterogeneity. Consequently, several studies have attempted to isolate and propagate clonal MSC lines, and shown that in reality MSCs exist on a spectrum of stem-like potency. For example, in a mixed population of bone marrow MSCs, cells from all eight categories of tri-lineage differentiation potential can be found[3]. ***However, benchtop assays of tri-lineage differentiation are poor indicators of tenogenic potential, and it is unknown whether individual TSCs are differently committed to the tendon fate from each other.***

Our goal in this study was to compare the differentiation potentials of single TSCs to each other and toward four lineages: bone, fat, cartilage and tendon and to determine the significance of differences, if any, in their *in vitro* potencies. Toward this end, we have incorporated a previously developed 3D tenogenesis assay using collagen type I gel and tenogenic growth factors to assess the TSC's ability to form their tissue of origin. In addition we have determined tri-lineage differentiation potency (AOC) using standard benchtop assays and population doubling assays to assess proliferative capacities. We hypothesized that TSCs would exhibit heterogeneous differentiation *in vitro* culture, and that quadra-potent TSCs would successfully generate a mature, composite, tendon-like phenotype upon tenogenesis.

MATERIALS AND METHODS

Experimental design

30 clonal tendon stem cell lines (TCs) were isolated and archived from equine TPs (parent tendon stem cell lines) (IACUC approved)(N=3; horses 211 and 961 were 1-3 months and 755 was a year old at euthanasia). All TPs were confirmed for the expressions of markers of stemness (CD90, CD105, GNL3 and Oct-4) using gene expression analysis and an adult equine muscle reference control. Archived TCs were previously isolated by the method of limiting dilution cloning[316]. Briefly, 1000 nucleated cells from SDFT stromal vascular fractions were plated in 100x17mm polystyrene dishes (Nunc™, Thermo Scientific, Waltham, MA), and confluent colonies were isolated with 10mm cloning cylinders (0.8 x 0.8 cm, Corning Inc, Christiansburg, VA)[317]. Recovered TCs were expanded in culture for one passage before plating for differentiation assays and population doublings. 15 TCs were analyzed for the gene expression of Oct-4 in monolayer culture relative to the respective day 0 TP cultures.

Assay of proliferative potential

10,000 cells per cell line were plated in individual wells of 24-well tissue culture dishes (Nunc™, Thermo Scientific), trypsinized at 70-80% confluence, and counted with an automated cell counter (Beckman Coulter, Brea, CA). Population doubling numbers (DN) and doubling time (DT) for each passage, and cumulative doubling numbers (CDN) over 3 successive passages were calculated using the following formulae:

$DN = \log_2(\text{cell number at confluence/cell number at seed})$

DT (days) = culture duration*log(2)/log(cell number at confluence/cell number at seed)

CDN = sum of DNs from each passage

Tenogenesis assay

A million cells were suspended in 5 milliliters of tenogenic growth media comprising high glucose DMEM (Thermo Scientific), 10% Cellect^{TM} Silver fetal bovine serum (MP Biomedicals, Santa Ana, CA), 37.5 μg/ml L-ascorbic acid (Sigma-Aldrich, St. Louis, MI), 1% penicillin G (Sigma-Aldrich) and 0.8 mg/ml rat tail collagen I (Corning Life Sciences, Tewksbury, MA). Gels were plated immediately following preparation in 4-welled, rectangular dishes (Nunc™,12.8 x 8.6 cm, Thermo Scientific) affixed with two sterile cloning cylinders (0.8 x 0.8 cm, Corning Inc.) set 3 centimeters apart from each other along the longitudinal midline of the well and held in place by sterile silicone on day 0. Gels were maintained at 37°C, with 5% CO2 and 90% humidity. On day 1, gels were supplemented with 50ng/ml BMP-12 (recombinant human, Sigma Aldrich) and 10ng/ml IGF-1 (recombinant human, BioVision, San Francisco, CA). Gels were fed on alternate days over a 10-day period.

Histology and analysis of cell alignment

Longitudinal sections of each tenogenic construct were fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS and submitted for histology (Laudier Histology, New York, NY). Two, 6 micron thick longitudinal slices per sample section were stained with Masson's trichrome stain. Images were acquired using a phase-contrast microscope (Olympus Corp, Center Valley, PA). Cell alignment was quantified using ImageJ software analytical tools[298]. Fifty cellular angles per histological section were measured relative to the construct longitudinal axis. Parallel alignment to the construct longitudinal axis was assigned 0º, and angles of each cell relative to 0º (0º-90º) were averaged for each sample to draw comparisons between samples.

Gel contraction analysis

Digital images of all constructs were taken on days 1, 3, 5, 7 and 10 to determine the percentage of contracted area at each time point relative to day 0. Images were analyzed using built-in ImageJ software analytical tools.

Tri-lineage differentiation assays

Tri-lineage differentiation potential was assessed using standard benchtop assays of adipogenesis, osteogenesis and chondrogenesis. For adipogenesis and osteogenesis, cells were plated at high (21,000 cells/cm^2) and low densities (4000 cells/cm^2) respectively in tissue culture dishes (Nunc™, Thermo Scientific), and cultured in growth media comprising high-glucose DMEM and 10% fetal bovine serum (Thermo Scientific). At 70-80% culture confluence, media was replaced with differentiation media and maintained for 21 days. For chondrogenesis, 200,000 cells per cell line were aliquoted and centrifuged at 800g for 10 minutes to obtain a pellet. Pellet cultures were maintained in growth media comprising high glucose DMEM and 1% insulin-transferrin-selenium mix (Gibco^{TM}, Thermo Scientific) for 2 days prior to differentiation for 21 days. Differentiation media comprised the following: for adipogenesis, StemPro^{TM} adipogenesis differentiation medium (Thermo Scientific), for osteogenesis, growth media supplemented with 10mM beta-glycerophosphate, 50µg/ml ascorbate 2-phosphate and 100nM dexamethasone (all Sigma-Aldrich), and for chondrogenesis, growth media supplemented with 37.5µg/ml ascorbate 2-phosphate, 100nM dexamethasone (both Sigma Aldrich) and 10ng/ml TGF-β3 (recombinant human, R&D Systems, Minneapolis, MA). On day 21, cultures were either fixed with 10% formalin (Sigma Aldrich) or frozen for gene expression analysis. Results from gene expression analysis were confirmed with Oil Red O (adipogenesis) and Alizarin Red S (osteogenesis) staining(both Sigma Aldrich), and images were acquired using an inverted microscope (Olympus Corp, Center Valley, PA).

Gene expression analysis

RNA isolation was performed using the TRIzol™ method (Thermo Scientific). RNA pellets were subjected to RNeasy spin columns for removal of genomic DNA contamination (QIAGEN Inc., Germantown, MD) and purified RNA was quantified using a NanoDrop 2000c spectrophotometer. CDNA was synthesized using a commercial kit (High-Capacity RNA-to-cDNA kit, Thermo Scientific). Real-time qPCR (7500 Real-Time PCR System, Thermo Scientific) was performed using custom TaqMan-MGB probes and primers (Thermo Scientific) included in Table 4.1. Predesigned Runx2 (Assay ID: Ec03469741_m1) and Sox9 (Assay ID: Ec03469763_s1) assays were obtained from Thermo Scientific. The comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) was employed for the relative quantification of gene expression[299]. Data was normalized to GAPDH. Tenogenic marker expression is reported as fold change with respect to an equine juvenile tendon reference control. Tri-lineage marker expression is reported as fold change relative to monolayers cultured in growth media on day 21.

Statistical analysis

Non-normal data was log transformed prior to analysis. Sample data for analysis of quadra-differentiation potency was classified into three groups (AOCT, OCT, ACT). Group means were compared to each other using a one way ANOVA and post hoc Tukey's tests for gene expression, population doublings (number and time) and cell alignment. Significant differences in contraction were assessed using a one way, MANOVA with a repeated measures design and Tukey's tests. Pearson's correlation analysis was used to assess significant relationships between outcomes. A *p*-value of less than 0.05 was considered significant. Computation was performed in JMP Pro 15 (SAS Institute, Cary, NC) and MS Excel 11 (Microsoft, Redmond, WA).

Table 4.1. Custom designed equine primer and probe sequences

	FORWARD	REVERSE	PROBE
GAPDH	CAAGTTCCATGGCACAGTCAAG	GGCCTTTCCGTTGATGACAA	CCGAGCACGGGAAG
Scleraxis	CGCCCAGCCCAAACAG	TTGCTCAACTTTCTCTGGTTGCT	TCTGCACCTTCTGCC
Collagen I	GCCAAGAAGAAGGCCAAGAA	TGAGGCCGTCCTGTATGC	ACATCCCAGCAGTCACCT
Collagen III	CTGCTTCATCCCACTCTTAT	ATCCGCATAGGACTGACCA	AACAGGAAGTTGCTGAAGG
Decorin	AAGTTGATGCAGCTAGCCTG	GGCCAGAGAGCCATTGTCA	ATTTGGCTAAATTGGGACTG
Biglycan	TGGACCTGCAGAACAATGAGAT	AGAGATGCTGGAGGCCTTTG	TCTGAGCTCCGAAAGG
FABP4	AAAATCCCAGAAACCTCACAAAAT	TCACTGGCGACAAGTTTCCA	TGTGATGCATTTGTAGGCA
Aggrecan	GACCACTTTACTCTTGGCGTTTG	GTCAGGGTCTGAAACGTCTACTGA	ACTCTGAGGGTCATCAC
GNL3	TTCGGGAAGCTGAGCTAAGG	CTGTCAAGCTTCTGCTGCTGTT	AACAGCGGCTTGAAG
CD90	GGCAGACCAGAGCCTTCGT	ATGGGTGTGGCGGTGGTAT	TGGACTGCCGCCATG
CD105	TCCACATCCTCTTCCTGGAGTT	GGACCTTTGGATAGTCAGCTTCA	CCAAGGGATGTGTCAGAG
Oct-4	CAGCTCGGGCTCGAGAAG	TTCTGGCGACGGTTGCA	ACGTGGTACGAGTGTGG
Mohawk	CCCACCAAGACGGAGAAGATACT	CACCTGCACTAGCGTCATCTG	TTGGCGCTCGGCTC
Tenascin C	GTTGGACTCCTGTACCCATTCC	GGCCCGAGGTCGTGTCT	TCCCAAGCGATGCTG

RESULTS

Single-cell derived clonal cell lines from the tendon are successful in vitro culture

All TPs used in this study expressed the stem cell markers CD90, GNL3, CD105 and Oct-4 in monolayer culture at day 0 and at confluence, and a general trend of increased expression relative to control was observed with CD90, GNL3 and Oct-4 (Fig. 4.1 (top)). 30 TCs successfully recovered from cryopreservation and were expanded for this study. Table 4.2. depicts five categories of TCs seeded for varied numbers of tests based on cell yield at passage. 26 out of 30 TCs yielded a million cells or more at confluence, and were cultured in 3D hydrogels for tenogenesis. Of these, 18 TCs were additionally plated for population doubling tests and all three tri-differentiation assays (AOC). 15 out of 18 TCs adhered to plastic and

collagen, divided successfully in uninduced monolayer and 3D cultures, and expressed stemness markers in monolayer culture (Fig.4.1 (bottom)). 15 TCs were hence, further analyzed for quadra-differentiation potency.

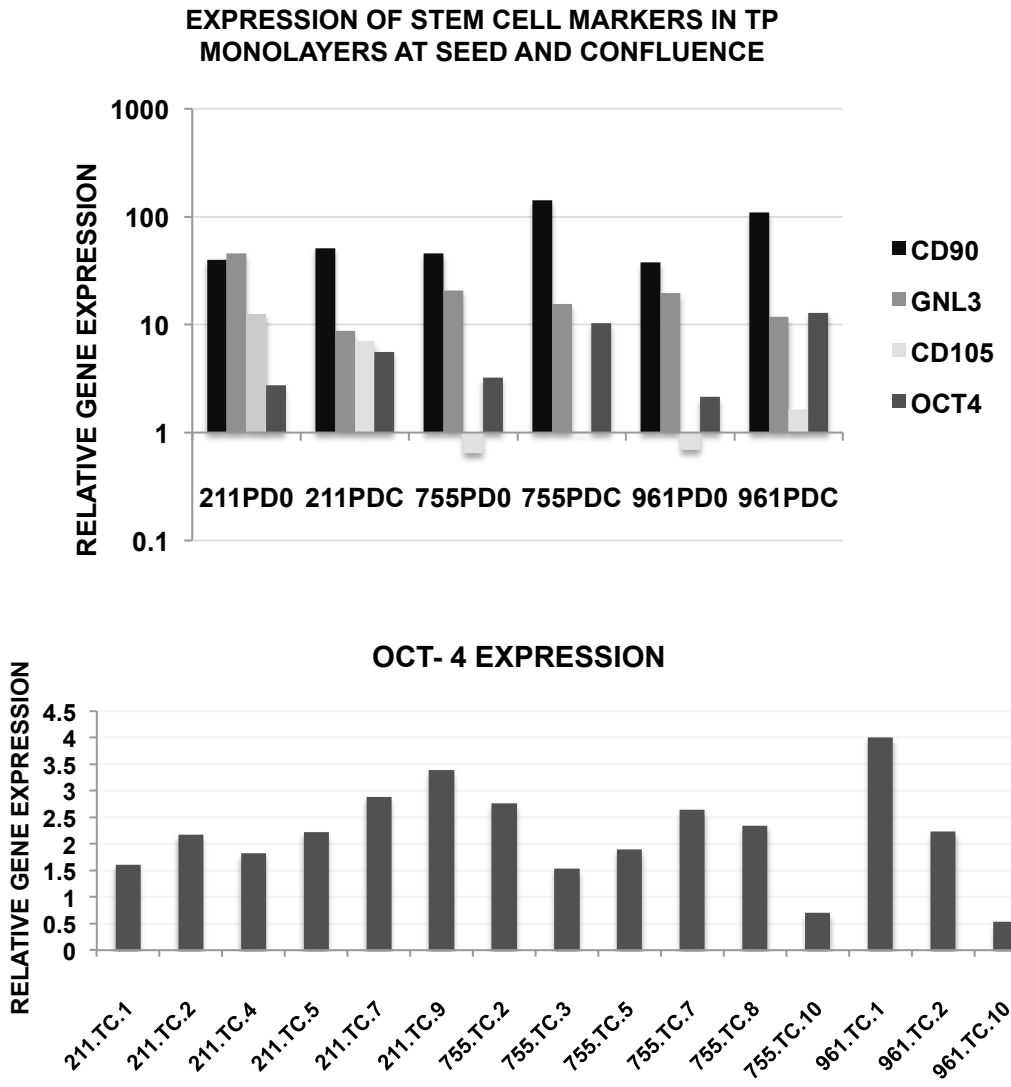


Fig. 4.1. Gene expression of markers of stemness in TPs (top) and TCs analyzed for quadra-potency (bottom). 211, 755 and 961 represent horse IDs, D0 = day 0 cells, DC = cells at confluence. CD90, GNL3 and Oct-4 were increased in all TPs on day 0 and at confluence.

Table 4.2. Cell yield of TCs versus tests seeded. A tick mark indicates that cells were seeded for that test.

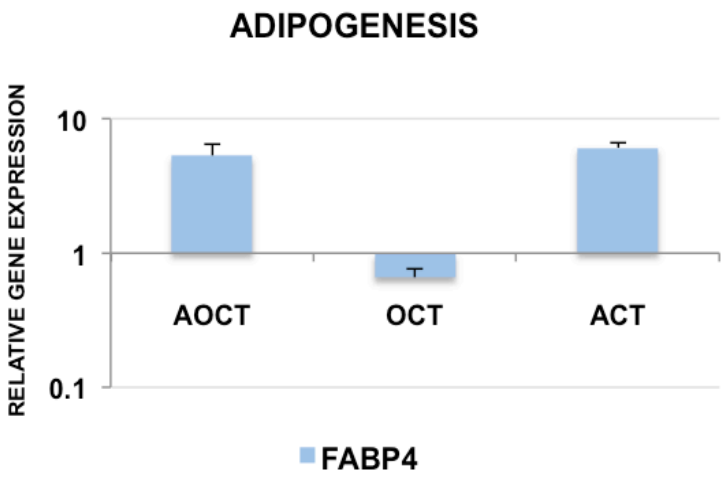
TC YIELD (MILLIONS)		ASSAY TYPE					CLONES SEEDED	CLONES SUCCESSFUL
		POPULATION DOUBLINGS	ADIPO -GENESIS	OSTEO -GENESIS	CHONDRO -GENESIS	TENO -GENESIS		
1	1.4 - 4	✔	✔	✔	✔	✔	18	15
2	1.2	✔	✔	✔		✔	5	5
3	1 - 1.6					✔	3	3
4	0.8	✔	✔	✔	✔		1	1
5	0.4 - 0.5	✔	✔	✔			3	2

Three distinct tendon stem cell phenotypes can be found in tendon

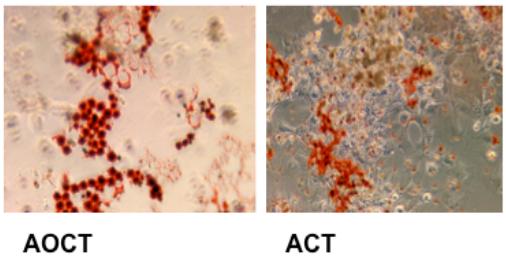
Of the 15 TCs analyzed for quadra-differentiation potency, all were positive for chondrogenic and tenogenic potential, and expressed all markers of the respective lineages upon differentiation. 8 out of 15 TCs differentiated to all four lineages evaluated in this study (AOCT phenotype), 5 did not under adipogenic differentiation and did not express FABP4 (OCT phenotype) (Fig.4.2.A), and 2 did not undergo osteogenic differentiation and did not express Runx2 (ACT phenotype) (Fig.4.2.C). Oil Red O staining confirmed the presence of intracellular oily droplets in the AOCT and ACT groups (Fig.4.2.B). Likewise, osteogenic differentiation was confirmed with the formation and staining of calcium nodules with Alizarin Red S in the AOCT and OCT groups (Fig. 4.2.D). Among equine donors, most TCs derived from the 211.TP belonged to the OCT phenotype, whereas most TCs derived from 755.TP and 961.TP belonged to the AOCT phenotype.

Chondrogenic potential significantly differed among the three groups (Fig.4.3). Expression of Sox9 was significantly increased in the AOCT group compared to ACT and OCT (p = 0.0167 and 0.0017), whereas aggrecan expression was the greatest in the OCT group, and significantly greater then AOCT (p = 0.029).

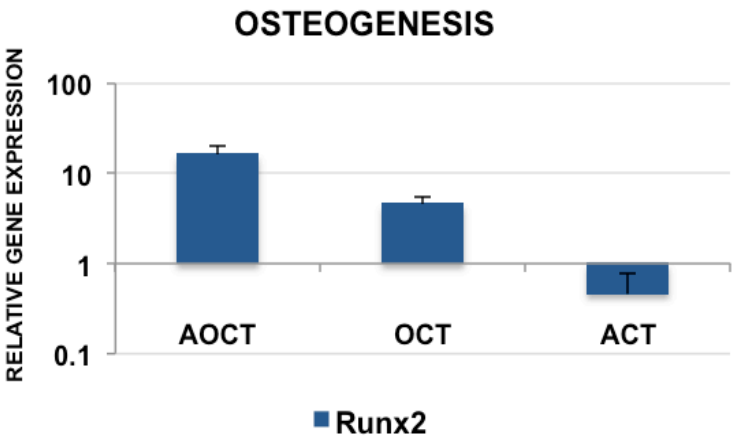
4.2.A



4.2.B



4.2.C



4.2.D

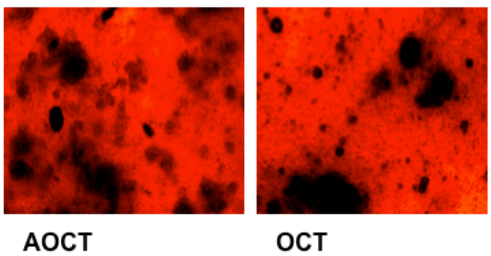


Fig.4.2 Adipogenesis assay (4.2.A,B) and osteogenesis assay (4.2.C,D) results. Gene expression of FABP4 relative to uninduced monolayer cells on day 21 (4.2.A), visualization of oily droplets in representative AOCT and ACT clones by Oil Red O staining of day 21 adipogenic cultures (4.2.B), gene expression of Runx2 relative to uninduced monolayer cells on day 21 (4.2.C) and visualization of calcium nodules in representative AOCT and OCT clones by Alizarin Red S staining of day 21 osteogenic cultures (4.2.D). Images are at a 10X magnification.

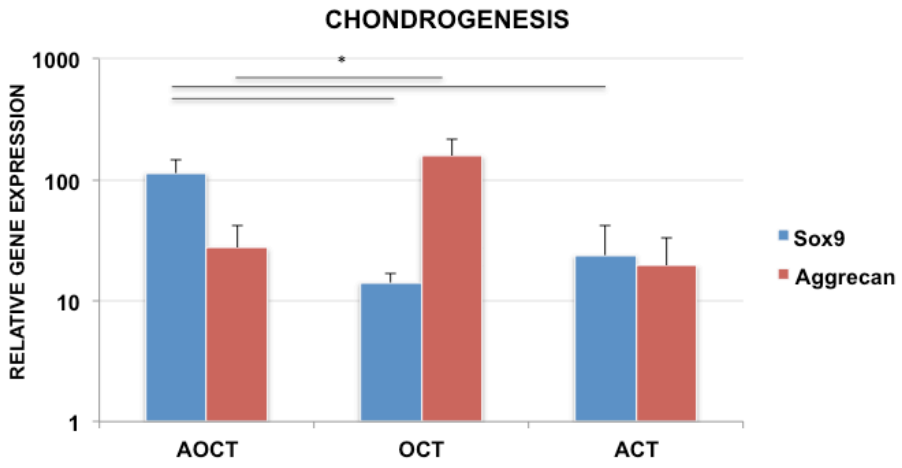


Fig.4.3 Chondrogenesis assay results. Gene expression of Sox9 and aggrecan relative to uninduced monolayer cells on day 21. Lines and asterisk indicate significant differences between groups. Sox9 expression was the highest in the AOCT group, whereas aggrecan expression was the highest in the OCT group.

Differences were also detected in the population doubling numbers of TCs over three passages of culture (Fig.4.4). In the AOCT group, a significant decrease in doubling numbers was observed

in passages 4 and 5 relative to passage 3, and a corresponding increase in doubling times was also detected from passage 3 to 4, but was not significant. In contrast to the AOCT group, TCs of the OCT and ACT groups did not significantly reduce their doubling numbers over 3 passages. The OCT group exhibited a significantly greater number of cumulative doublings compared to the AOCT and ACT groups (p = 0.0105 and 0.0392).

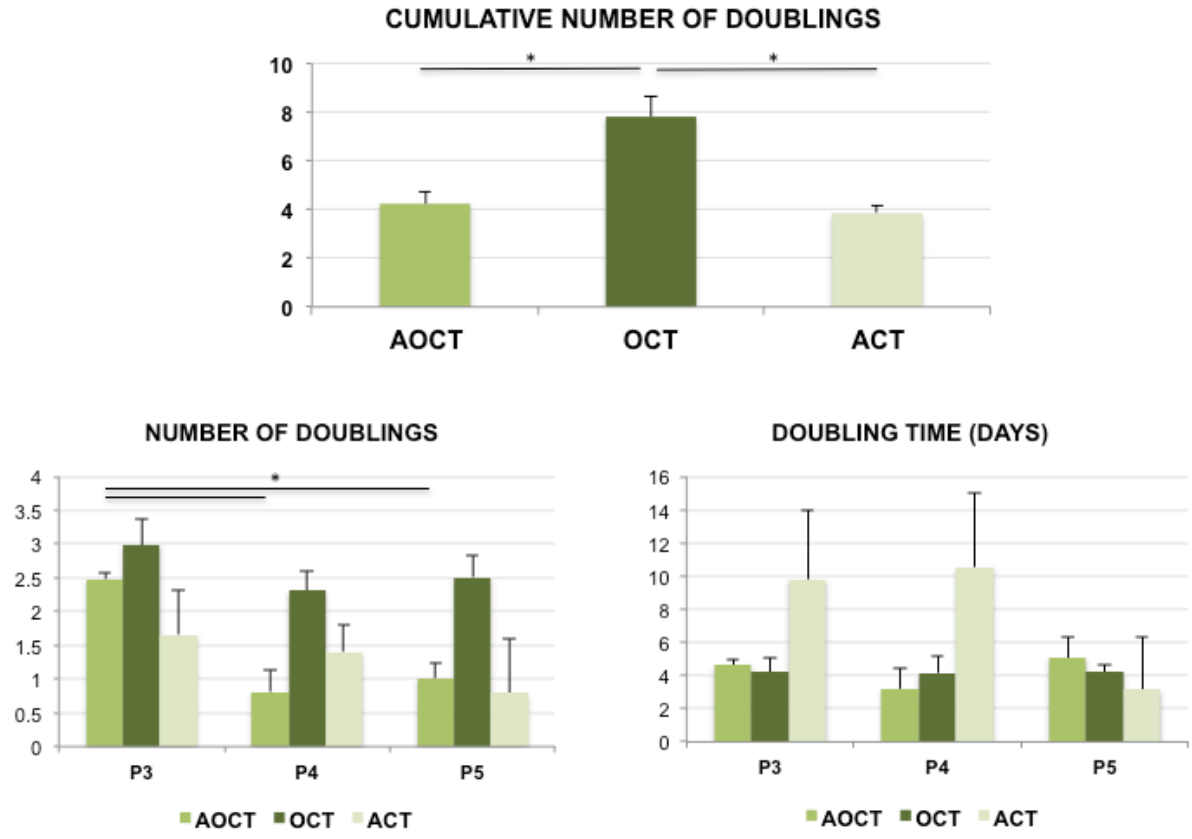


Fig.4.4 Assay of proliferative potential: cumulative doubling numbers (top), number of doublings (bottom left) and doubling time (bottom right) over 3 successive passages. Cumulative doubling numbers were the greatest in the OCT group. Lines and asterisks indicate significant differences between groups.

Quadra-potent TCs strongly differentiate to a composite tendon phenotype

All TCs expressed all gene markers of the tendon phenotype analyzed in this study, and significant differences were detected between the AOCT and OCT groups (Fig.4.5). Specifically, expressions of the tendon transcription factors scleraxis and mohawk were atleast 3-fold increased in the AOCT group compared to the OCT group, and this difference was significant (p = 0.0454 for scleraxis and 0.0431 for mohawk). In contrast, a corresponding increase in collagen type I expression in the AOCT group was not observed in this study, and message levels in the AOCT and OCT groups were comparable on day 10. Expression of collagen type III, a marker of the regenerating tendon phenotype, was significantly elevated in the OCT group compared to AOCT (p = 0.0315). All three groups consistently expressed high levels of the tendon matrix glycoproteins tenascin, decorin and biglycan. Decorin levels were 2-fold increased in the AOCT compared to the OCT group, however this difference did not reach significance.

TCs in all three phenotypic groups contracted collagen matrix in three dimensions albeit to different degrees (Fig.4.6.B). Placement of cloning cylinders in culture dishes resulted in length-wise constraints to hydrogel contraction and the generation of tendon-like constructs on day 10. Significant differences were noted in contracted gel area over time and at two time points between groups (Fig.4.6.A). The ACT group was significantly less contracted than the AOCT and OCT groups on days 5 (p = 0.0004 and 0.0008) and 7 (p = 0.0031 and 0.0026).

TCs in all three groups were uniformly interspersed in the 3D matrix and aligned in parallel to the axis of tension on day 10 (Fig.4.7.A). Histology revealed a greater proportion (>90%) of cells with an elongated morphology in all constructs relative to a small number of spherical cells (<5%). No significant differences in cell alignment were observed between the three groups (Fig.4.7.B).

DISCUSSION

The goal of this study was to determine whether individual TSCs are differently committed to four connective tissue lineages from each other, and the molecular basis or contribution of these differences *in vitro*. All lineage-specific markers were heterogeneously expressed among TCs, and the most significant differences were based on adipogenic potential. TCs of the AOCT phenotype strongly differentiated to bone, fat and cartilage, exhibited several doublings in culture, and strongly differentiated to a native tendon-like phenotype when induced with tenogenic stimuli in our 3D benchtop assay.

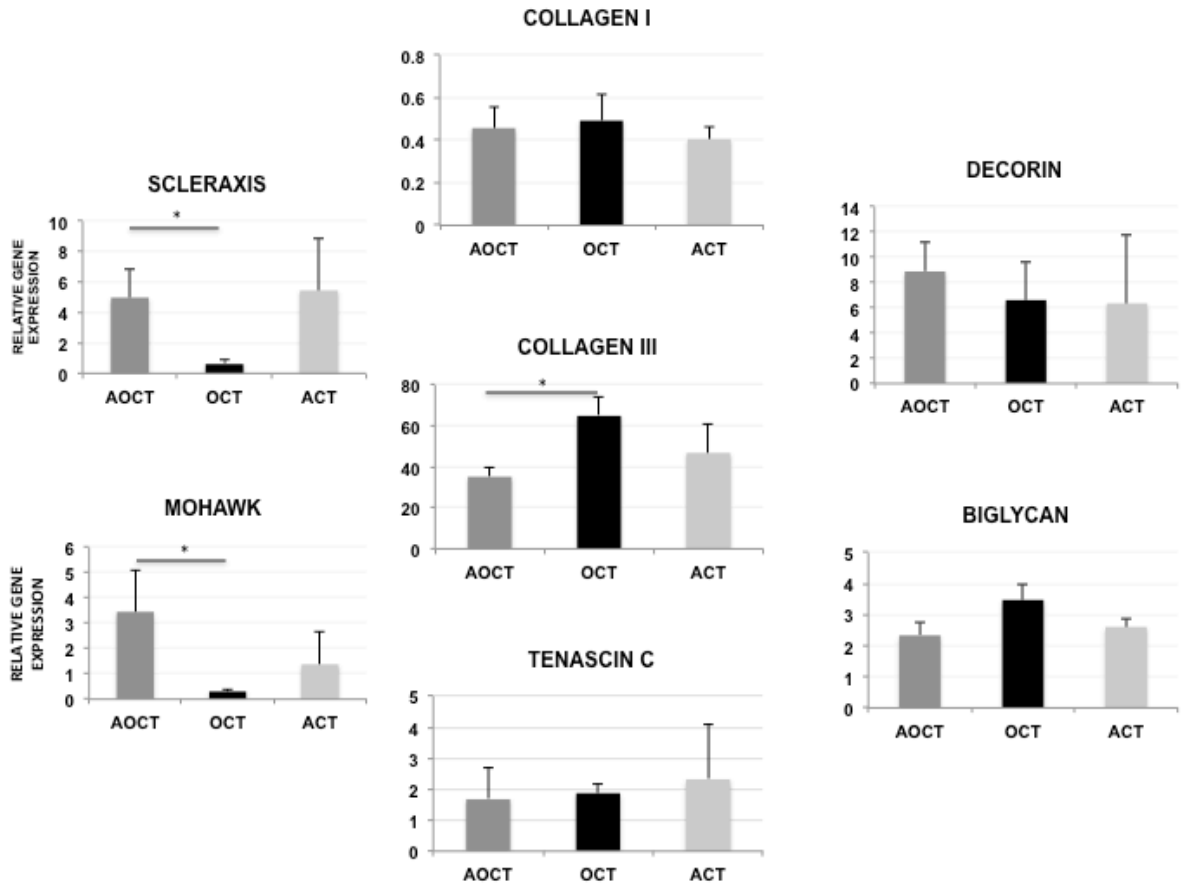


Fig.4.5 Tenogenesis assay: gene expression of tendon-related genes in 3D constructs on day 10. Scleraxis and mohawk expression was significantly greater in the AOCT group compared to OCT. Lines and asterisks indicate significant differences.

This study is the first to evaluate the tenogenic potentials of individual TSCs derived from the equine tendon. In the absence of tendon-specific markers, we evaluated a range of markers that collectively identify the tendon phenotype[242, 318]. Scleraxis is a bHLH transcription factor[28], and was increasingly expressed in the AOCT group compared to the OCT group, suggestive of tendon neogenesis[319]. Further, the increased expression of scleraxis in this group coincided with increased chondrogenic potential by virtue of Sox9 expression, typical of the overlapping expression patterns of these factors in early tendon development[36, 320]. Mohawk is a member of the TALE superclass of atypical homeobox genes[321]. Interestingly, the strong expression pattern of scleraxis in the AOCT group coincided with a similar, strong expression pattern of mohawk in the same group. Scleraxis is essential to tendon fate specifications in the early embryo, but also expressed in mature, differentiated tendons[29], whereas mohawk plays a critical role in the maturation of embryonic tendons[42]. This could explain the overlapping expression patterns of these transcription factors observed in this study. Further, both scleraxis and mohawk promote collagen type I synthesis in tendons[322], which was confirmed by these results. A high collagen type III to type I ratio was observed in the OCT group (compared to AOCT), is typical of tendinopathic and calcified regions in tendons, and is manifested *in vivo* by poor matrix organization and small diameter collagen fibrils[74, 323]. This may also suggest that constructs of the OCT group were actively undergoing matrix remodeling as opposed to the mature, organized matrices of the AOCT group at harvest. Opposing trends in the expressions of tendon

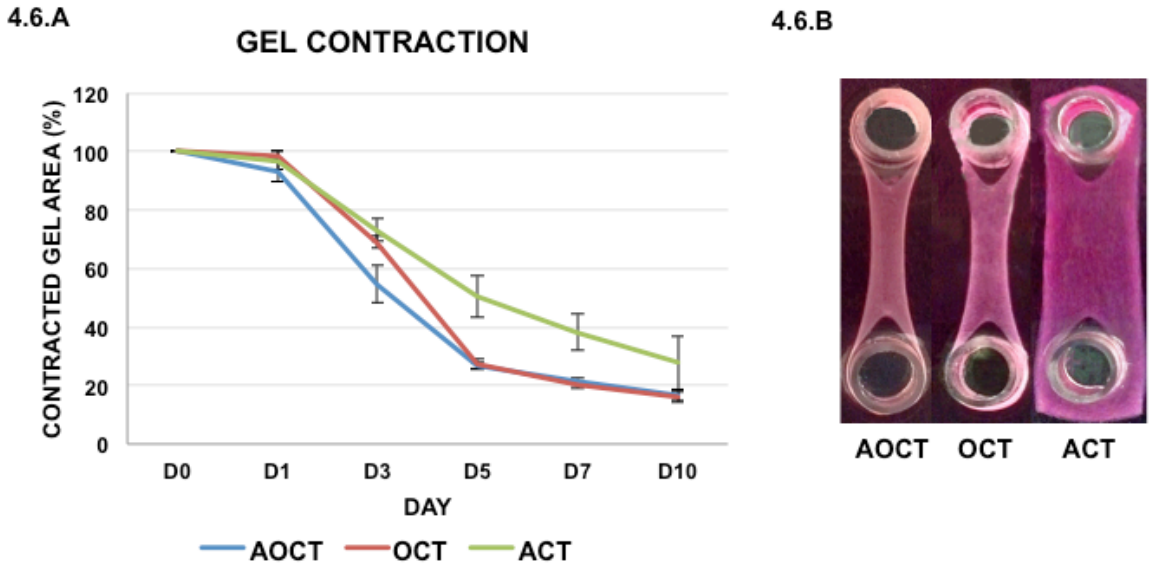


Fig.4.6 Contraction of tenogenic constructs: Percentage contracted gel area over 10 days of culture (4.6.A), and macroscopic images of constructs at harvest (4.6.B). The ACT group had contracted the least at culture endpoint.

proteoglycans were observed in the AOCT and OCT groups, but were not significant. Decorin and biglycan are like-minded entities and function by compensation *in vivo* tendons[14].

TSCs seeded in a collagenous matrix exert tractional forces on the surrounding collagen fibrils, resulting in matrix contraction *in vivo*[324-326]. In the presence of unidirectional strain along a longitudinal axis, contraction is anisotropic, and coupled with the progressive alignment of cells parallel to the tensional axis, typical of tendons[223]. Our tenogenesis assay design successfully recapitulated the physiological behavior of TSCs *in vitro,* and consistently generated tendon-like constructs on day 10 of culture. Further, significant differences in contraction were observed in the ACT group compared to the AOCT and OCT group. Wound healing *in vivo* is driven by a balanced rate of contraction by adjoining collagen fibrils[215]. The reduced contraction of ACT constructs observed in this study suggests that the complete loss of osteogenic potential

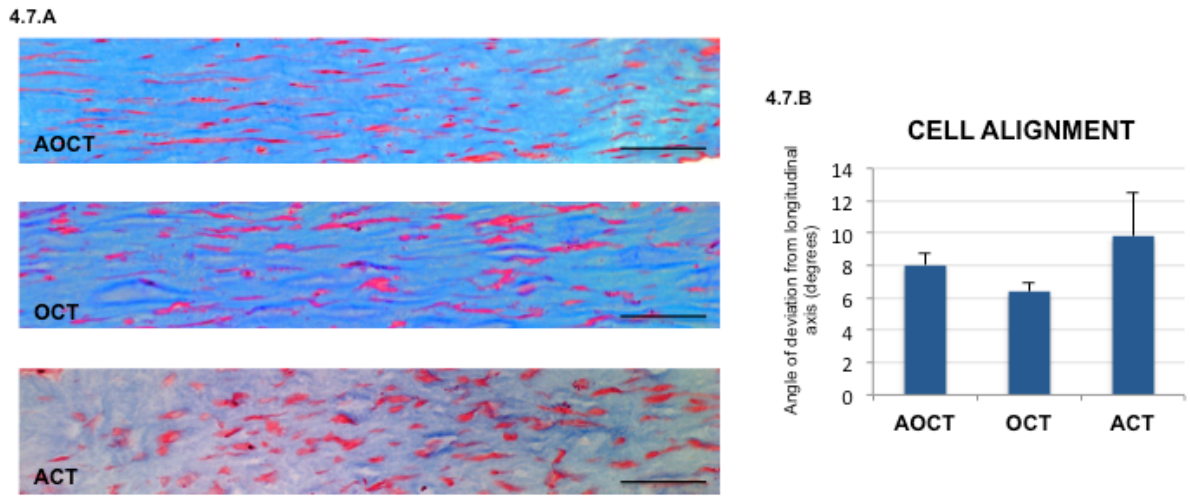


Fig.4.7 Histology of day 10 tenogenic constructs: Masson's trichrome staining of 6 micron thick sections of representative constructs from each group (4.7.A), and quantification of cell alignment in histological sections (4.7.B). All groups exhibited hypercellularity and superior alignment in parallel to the tensional axis on day 10. A greater proportion of elongated cells were observed in all constructs. For cell alignment, an angle of 0° demarcates perfect parallel alignment. Scale bar on images represents 250 microns.

in TSCs may impede tendon repair *in vivo.* Runx2 overexpression in MSCs is known to augment tendon-to-bone healing, by inducing site-specific bone formation, and inhibiting adipogenesis and heterotopic ossification[327].

Our results confirm the hypothesis that only a subset of TSCs in a population is capable of tri-lineage differentiation to the fat, bone and cartilage lineages and can undergo several doublings in culture over successive passages. This subset was represented by the AOCT phenotype in this study[22] and constituted more than 50% of the total TC's analyzed for quadra-potency, in partial agreement with a previous result with human hamstring-derived TCs[22] and two previous studies with bone marrow MSC clones[3, 280]. The proportion of quadra-potent TSCs

in a population can lend insight to a variety of population characteristics including age, purity and therapeutic efficacy that can be used to optimize tendon treatments.

Sox9, a key transcription factor in early chondrogenesis[328-330], can also induce the expression of adipogenic transcription factors such as C/EBPβ in TSCs[331]. In this study, a significant, positive correlation in Sox9 and FABP4 expression was found in quadra-potent TCs ($r = 0.5398$, $p = 0.0378$). Further, Sox9 enhances the promoter activity of aggrecan[332], a major structural proteoglycan found in cartilage. However in this study, the patterns of chondrogenic gene expression in quadra-potent TCs did not coincide, which may be attributed to the temporal regulation of these genes in the progression of chondrogenesis by virtue of intracellular feedback loops.

Our study is not without limitations. Expansion of single-cell derived colonies is notoriously difficult *in vitro*, and TC yield limited our analysis of quadra-differentiation potency to 18 TCs. Of these, 3 TCs failed to adhere to plastic, or adhered to plastic and failed to expand to confluence for some differentiation assays. Feeder layers or alternative methods to supplement cell growth such as FGF-2 application were not used in this study to avoid their potential effects on study outcomes. However, sufficient cell volumes were acquired from 15 TCs to enable gene expression analysis as a superior outcome of differentiation in comparison to stain absorbance and microscopy. Recent studies have emphasized the need to develop superior techniques such as flow sorting to isolate single cells for truly representative clonal colonies. While we acknowledge that our methods of single cell isolation may not have been optimal, we were able to provide strong preliminary evidence of TSC heterogeneity to serve as a foundational basis for future studies.

In bone marrow MSCs, high proliferative potential is correlated with multi-lineage differentiation potential[3]. In this hierarchical model, adipogenic potential is lost early with successive cell doublings and aging[281], resulting in an almost complete loss of doubling capacity in purely osteogenic clones[279]. In tendon, our results suggest that TSC doubling capacity is reduced in quadra-potent TSCs. Significantly greater numbers of cumulative doublings were detected in the OCT group compared to the AOCT group. This suggests that 1) adipogenic potential may not be a strong indicator of MSC multi-lineage differentiation or proliferative potential in tendons, and 2) the AOCT phenotype represents a group of TSCs that are strongly tenogenic, and when induced with tenogenic stimuli can actively differentiate to tenocytes and form mature tendon-like tissues. In contrast, the OCT phenotype represents a group of weakly tenogenic and non-committed TSCs that may partially differentiate to tendon but fail to produce a mature, composite tendon phenotype. It is possible that TSCs of the OCT phenotype may acquire adipogenic potential with age and transition to the AOCT phenotype, by virtue of their inherent plasticity[333, 334].

This study is the first to analyze the tenogenic potentials of single TSCs at a clonal level in tendons. Of the three TSC phenotypes revealed in this study, those of the AOCT phenotype exhibited the strongest expressions of scleraxis, mohawk and produced a mature tendon-like construct with progressive contraction, increased cellularity, tenocyte-like cell morphologies and superior cell alignment over 10 days. TSCs of the OCT phenotype exhibited a trend of reduced osteogenic potentials compared to AOCT, warranting future investigation to their therapeutic efficacies *in vivo*.

ACKNOWLEDGEMENTS

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY OF RESULTS

Bone marrow MSCs are the current standard in MSC therapies for tendon treatments. More recently, novel sources of stem cells such as adipose, tendon and the umbilical cord have been investigated, with the goal of developing superior alternatives to aid a faster recovery. However, attempts to harness the tenogenic potential of MSCs have not been successful, in part due to our limited understanding of the microenvironmental cues that trigger MSC differentiation to tendon and the inefficiency of available systems to quantify tenogenic potency. The discovery of TSCs has opened new avenues to understanding MSC behavior in adult and fetal tendons, and in tendon-like collagenous matrices by tissue engineering. TSCs may be predisposed to tendon differentiation, but recent studies suggest that this characteristic of TSCs may not be homogeneous in a population or in TSCs from different regions of the tendon. In this dissertation we have attempted to address these concerns. We have introduced a novel 3D assay to quantify the tenogenic potential of MSCs, and to enable comparisons in differently sourced MSC lines.

In phase 1, we have developed this assay with commercially available, polystyrene, rectangular wells, which serve as culture vessels for MSC-seeded collagen hydrogels. Length-wise anchorage was provided in each well to encourage anisotropic contraction and matrix organization. Our preliminary results suggested that this standalone system was sufficient to elicit a mild tenogenic response by virtue of tendon collagen and glycoprotein expression, but growth factor induction was necessary to generate a composite tendon phenotype and to translate these results to the clinic. To optimize growth factors for this assay, FGF-2, TGF-β1, IGF-1, and

or BMP-12 were tested singly and in various combinations. For phase 1, we hypothesized that the synergistic effects of BMP-12 and IGF-1 would augment stress-induced tenogenesis over BMP-12 or IGF-1 application alone. While a synergistic increase in ECM GAG, a marker of the regenerating tendon phenotype, was observed in the BMP-12/IGF-1 group, our data suggested that solo BMP-12 supplementation was sufficient to induce the strongest expressions of scleraxis, collagen type III and decorin, the balanced contraction of constructs, a higher degree of cell alignment and tendon-like tissue morphology. Uniaxial static strain combined with 50ng/ml BMP-12 consistently generated a robust tendon-like phenotype over 10 days in this assay.

In phase II, we incorporated this assay to quantify differences in TSC commitment to the tendon fate (T). In addition, standard assays of tri-differentiation toward fat (A), bone (O) and cartilage (C) were used to comprehensively assess TSC differentiation potencies. Clonal TSC lines were derived from parent TSC populations and classified into three groups based on differentiation potencies in the aforementioned assays (AOCT, OCT and ACT). In experiment 2, we hypothesized that the AOCT phenotype would exhibit superior tenogenic potential among the three groups and our hypothesis was successfully proven. The AOCT phenotype of TSCs exhibited the strongest expressions of scleraxis and mohawk, balanced contractility, and elongated cell morphologies with superior cell alignment. This group consistently generated mature, native tendon-like constructs over 10 days of culture.

These studies have important implications in the current standard-of-care in regenerative therapies for tendon. Benchtop assays of tenogenesis are appealing alternatives to their cumbersome counterparts, and can enable ‘off-the-shelf’ treatments of tendon injury. Our study of TSCs lends valuable insight to the diversity of cell phenotypes found in tendon, and will foster research on the therapeutic capabilities of TSCs *in vivo*.

FUTURE DIRECTIONS

Gel contraction in our tenogenesis assay is dependent on the ability of MSCs to uniformly integrate in three dimensions on day 0, and sustain growth to permit the acquisition of a tendon phenotype over 10 days. Rectangular wells were specifically selected over tendon-shaped molds or troughs so that macroscopic changes in contraction could be monitored over time. While all parameters of this assay were optimized, a greater number of cells are required for seeding this assay in comparison to some published methods, and which may not be available for every application. One possible solution to this problem may be to scale down the well and cloning ring dimensions, and test a proportionally reduced seed number. Alternatively, coating wells with cell adhesion enhancers such as fibronectin may encourage an early and sustained log phase of cell growth, with lower seed numbers.

While cell and collagen concentration were optimized to control contraction, culture supplements such as hydroxyapatite or lactoferrin may also facilitate control to sustain culture over extended periods, to augment the maturation of gels, expression of tendon markers, and perhaps mechanical strength. This would enable mechanical testing as an additional parameter of tenogenesis. Static, uniaxial strain combined with growth factor induction proved sufficient to achieve tenogenesis in our experiments. Future experiments may investigate the effects of varied strain amplitudes and multiaxial strains in a bioreactor with or without growth factors, on hydrogel mechanics and tendon differentiation.

Tests of *in vitro* potency in phase II were limited to differentiation to four lineages and proliferative capacity. It may be interesting to analyze various other characteristics of single TSCs including the expression of surface markers (other than Oct-4), morphology and age.

Analysis of the proteomic and transcriptomic profiles of single-cell derived colonies *in vitro* will further our understanding of the cellular diversity found in tendons, and the regulatory pathways involved in its sustenance.

APPENDIX A: SUPPLEMENTARY TABLES FOR CHAPTER 3

TABLE A1. Group-wise differences (*p*-values) in contraction on day 1

Day 1	Control	FGF-2	TGF-β1	IGF-1	BMP-12	BMP-12 IGF-1	TGF-β1 IGF-1	BMP-12 FGF-2
Control	-	0.662	0.295	0.307	0.59	0.797	0.505	0.078
FGF-2	0.662	-	0.142	0.148	0.918	0.857	0.817	0.178
TGF-β1	0.295	0.142	-	0.979	0.117	0.195	0.091	0.006
IGF-1	0.307	0.148	0.979	-	0.123	0.203	0.096	0.007
BMP-12	0.59	0.918	0.117	0.123	-	0.777	0.897	0.212
BMP-12/IGF-1	0.797	0.857	0.195	0.203	0.777	-	0.681	0.129
TGF-β1/IGF-1	0.505	0.817	0.091	0.096	0.897	0.681	-	0.262
BMP-12/FGF-2	0.078	0.178	0.006	0.007	0.212	0.129	0.262	-

TABLE A2. Group-wise differences (*p*-values) in contraction on day 3

Day 3	Control	FGF-2	TGF-β1	IGF-1	BMP-12	BMP-12 IGF-1	TGF-β1 IGF-1	BMP-12 FGF-2
Control	-	0.783	0.543	0.953	0.753	0.68	0.157	0.005
FGF-2	0.783	-	0.738	0.829	0.556	0.493	0.093	0.011
TGF-β1	0.543	0.738	-	0.583	0.358	0.31	0.046	0.025
IGF-1	0.953	0.829	0.583	-	0.709	0.68	0.141	0.006
BMP-12	0.753	0.556	0.358	0.709	-	0.921	0.266	0.002
BMP-12/IGF-1	0.68	0.493	0.31	0.68	0.921	-	0.31	0.001
TGF-β1/IGF-1	0.157	0.093	0.046	0.141	0.266	0.31	-	<0.001
BMP-12/FGF-2	0.005	0.011	0.025	0.006	0.002	0.001	<0.001	-

TABLE A3. Group-wise differences (*p*-values) in contraction on day 5

Day 5	Control	FGF-2	TGF-β1	IGF-1	BMP-12	BMP-12 IGF-1	TGF-β1 IGF-1	BMP-12 FGF-2
Control	-	0.702	0.093	0.314	0.669	0.605	0.5	0.011
FGF-2	0.702	-	0.189	0.529	0.419	0.37	0.293	0.027
TGF-β1	0.093	0.189	-	0.486	0.037	0.03	0.021	0.347
IGF-1	0.314	0.529	0.486	-	0.155	0.131	0.097	0.106
BMP-12	0.669	0.419	0.037	0.155	-	0.928	0.804	0.003
BMP-12/IGF-1	0.605	0.37	0.03	0.131	0.928	-	0.874	0.002
TGF-β1/IGF-1	0.5	0.293	0.021	0.097	0.804	0.874	-	0.001
BMP-12/FGF-2	0.011	0.027	0.347	0.106	0.003	0.002	0.001	-

TABLE A4. Group-wise differences (*p*-values) in contraction on day 7

Day 7	Control	FGF-2	TGF-β1	IGF-1	BMP-12	BMP-12 IGF-1	TGF-β1 IGF-1	BMP-12 FGF-2
Control	-	0.827	0.091	0.469	0.44	0.454	0.03	0.002
FGF-2	0.827	-	0.058	0.347	0.578	0.595	0.0502	0.001
TGF-β1	0.091	0.058	-	0.324	0.016	0.017	0.0003	0.145
IGF-1	0.469	0.347	0.324	-	0.139	0.145	0.005	0.017
BMP-12	0.44	0.578	0.016	0.139	-	0.145	0.151	0.0003
BMP-12/IGF-1	0.454	0.595	0.017	0.145	0.145	-	0.145	0.0003
TGF-β1/IGF-1	0.03	0.0502	0.0003	0.005	0.151	0.145	-	<0.0001
BMP-12/FGF-2	0.002	0.001	0.145	0.017	0.0003	0.0003	<0.0001	-

TABLE A5. Group-wise differences (*p*-values) in contraction on day 10

Day 10	Control	FGF-2	TGF-β1	IGF-1	BMP-12	BMP-12 IGF-1	TGF-β1 IGF-1	BMP-12 FGF-2
Control	-	0.722	0.147	0.45	0.422	0.383	0.077	0.0006
FGF-2	0.722	-	0.074	0.269	0.653	0.602	0.153	0.0002
TGF-β1	0.147	0.074	-	0.478	0.027	0.023	0.002	0.027
IGF-1	0.45	0.269	0.478	-	0.123	0.108	0.014	0.004
BMP-12	0.422	0.653	0.027	0.123	-	0.943	0.322	<0.0001
BMP-12/IGF-1	0.383	0.602	0.023	0.108	0.943	-	0.358	<0.0001
TGF-β1/IGF-1	0.077	0.153	0.002	0.014	0.322	0.358	-	<0.0001
BMP-12/FGF-2	0.0006	0.0002	0.027	0.004	<0.0001	<0.0001	<0.0001	-

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