



Review

Using load to improve tendon/ligament tissue engineering and develop novel treatments for tendinopathy

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ABSTRACT

Tendon and ligament injuries are highly prevalent but heal poorly, even with proper care. Restoration of native tissue function is complicated by the fact that these tissues vary anatomically in terms of their mechanical properties, composition, and structure. These differences develop as adaptations to diverse mechanical demands; however, pathology may alter the loads placed on the tissue. Musculoskeletal loads can be generally categorized into tension, compression, and shear. Each of these regulate distinct molecular pathways that are involved in tissue remodeling, including many of the canonical tenogenic genes. In this review, we provide a perspective on the stage-specific regulation of mechanically sensitive pathways during development and maturation of tendon and ligament tissue, including scleraxis, mohawk, and others. Furthermore, we discuss structural features of healing and diseased tendon that may contribute to aberrant loading profiles, and how the associated disturbance in molecular signaling may contribute to incomplete healing or the formation of degenerative phenotypes. The perspectives provided here draw from studies spanning *in vitro*, animal, and human experiments of healthy and diseased tendon to propose a more targeted approach to advance rehabilitation, orthobiologics, and tissue engineering.

Introduction

Tendon and ligament injuries are prevalent worldwide, affecting individuals of all ages with long-term or permanent deficits in physical function and quality of life. Over a quarter-million full-time employees per year in the United States alone are afflicted with sprains, strains, or tears, with a median of 33 lost workdays per injury depending on the occupation, making it the most common cause of illness-related lost workdays[1]. In the US in 2016, musculoskeletal injuries to the neck and back alone resulted in the single greatest health care expenditure (~\$135 billion), with musculoskeletal disorders not related to the neck and back placing second (~\$130 billion). This means that the direct cost of musculoskeletal injury was more than diabetes (\$111 billion) and heart disease (\$89 billion) combined[2]. To make matters worse, movement deficits associated with these injuries may cause knock-on effects that increase risk of major metabolic diseases[3].

Despite the serious personal and socioeconomic impact, currently practiced treatments are limited. For example, surgical repairs of rotator

cuff tendons have failure rates of 16 to 39 % depending on the size of the tear[4,5]. Failure rates of Achilles tendon repairs are much lower, but even successful surgeries lead to persistent functional deficits[6]. In terms of conservative treatment, 60 % of Achilles tendinopathy patients still experience symptoms after completing an exercise-based rehabilitation program[7]. These data indicate that there is an urgent need to improve the way that tendon and ligament injuries are treated.

In recent years, major advances have been made in understanding tendon cell biology[8]. However, treatments have remained unchanged for centuries due to challenges in understanding the biomolecular origins of tendinopathy and stage-specific processes of tendon regeneration. In fact, the most common treatment for acutely injured tendons, immobilization, has changed very little in the last 4500 years[9]. While it is becoming better appreciated that tissue loading plays an indispensable role in healing, current clinically practiced methods of deploying load-based therapies do not result in full recovery. Importantly, there is a lack of mechanistic understanding of how loads regulate cell biology and how they should be used to regulate healing and

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prevent scar formation in tendons and ligaments. Therefore, translation of basic mechanistic findings to clinically relevant outcomes remains a crucial obstacle for the field.

Tendinopathy is a generic term that means tendon pain. Pain within a tendon is often, but not always, related to structural changes to the tissue that can be visualized using clinical imaging techniques[10]. The fact that pain does not always reflect structural deficits, at least in what is seen using clinical imaging, makes advances in basic tendon research more difficult to translate into effective interventions for patient populations. Even though painful tendons are not always associated with structural abnormalities, macro or microscopic changes in the structure of the matrix are a common feature of tendinopathic tissue[11,12]. This suggests that differences in tissue morphology underly differences between healthy and diseased tendons and ligaments.

Tendons and ligaments are tissues that primarily function to transmit tension. They therefore should exhibit material properties that enable them to perform this unique role. When force-transmitting structures are damaged or pathologically altered, function is compromised, and the tissue must undergo a process of repair. However, patients often present clinically only after the formation of a scar that, at a material level, lacks the native matrix composition and structural organization[12,13]. Therefore, a major clinical goal for treating tendon and ligament injuries is to regenerate, or in extreme cases, graft a replacement tissue that has the material properties of the native tissue.

Current methods of obtaining and using tissue grafts come with clinical complications. Autografts are associated with donor site morbidity, and allografts and xenografts are associated with immune rejection, pathogen transmission, and greater rates of re-rupture[14]. Engineered tissues provide a potential solution to this issue, as a small number of the patient's cells may be collected through minimally invasive procedures and used to grow a replacement tissue. However, tissue engineering of tendon and ligament as a field is still in its infancy and has yet to generate a functionally mature tissue: one with the structural and material properties of native adult tissue [8,15].

Strategies for tissue regeneration and tissue engineering may require similar processes since both have the goal of forming a healthy mature tissue. For regeneration, current strategies often involve inducing a sequence of developmental processes to recapitulate tissue formation. As will be discussed in later sections, current methods of engineering tendons and ligaments are limited to producing a developmentally arrested tissue that is similar to a scar. Therefore, understanding processes that drive the stage-specific development of native tissue *in vivo* can likely apply in parallel ways to tissue engineering. Afterall, the behavior of a cell in a remodeling tissue one way or another depends on its environmental inputs, whether in the body or in a Petri dish.

A variety of approaches to achieve tendon and ligament regeneration and understand pathology have been studied but results are equivocal [16]. Measurement of effective tissue regeneration is complicated by the inability to sample regenerating tissues in people, and the time-dependent transcriptional profile when using animal models[8, 17]. The fact that gene expression profiles naturally change throughout development within a single “healthy” tissue suggests context-specific contributions of these genetic signatures. However, how the factors that drive healthy development contribute to regeneration remains unclear. Furthermore, whether an observation in pathological tissue is a necessary component of healing, or a cause of disease, complicates data interpretation. Whether regenerating an injured native tissue or engineering mature tendons/ligaments by recapitulating development, the contribution of specific molecular factors to tissue formation and maturation needs to be better defined.

Comparisons of healthy and diseased states in tendon can provide paradoxical results that defy simple explanations. For example, healthy and scarred tendon are both characterized by an abundance of collagen [18]. Collagen is the most abundant protein in the body and type I collagen (COL1 or *Col1a1*) is recognized to be the material that is primarily responsible for the tensile strength of tendons and ligaments. As

such, greater amounts of collagen enable greater tensile strength. However, COL1 synthesis is greater in a region of scar than in healthy tendon[19]. Without context from other factors in parallel, the measurement of collagen synthesis alone can obscure distinctions between interventions that result in remodeling towards health versus pathology.

In healthy tendon, collagen is organized in hierarchical bundles of fibers that are crosslinked at their N and C termini[20] and aligned in parallel with the primary direction of load. By contrast, in scar collagen fibers are smaller, less crosslinked, and disorganized[21,22]. This means that beyond measuring collagen content/synthesis, the orientation, crosslinking, and size of the collagen fibrils should all be measured in pre-clinical studies since these can better distinguish healthy from pathological COL1 formation. However, for countless factors beyond COL1 that are suspected to influence health and disease, the contextual understanding of their expression, protein content, structure, and modification are poorly understood.

Like collagen, upstream and downstream regulators of collagen expression are also regulated in a complex manner. For example, transforming growth factor beta (TGF β) and early growth response factor 1 (EGR1) are each crucial for tendon formation[23,24], but are also thought to contribute to pathological scar formation in tissues across the body [23,25]. Matrix metalloproteinases (MMPs) are responsible for degradation of collagens but are also a natural component of the remodeling response to exercise and other anabolic stimuli [26]. Collagen III is often associated with degenerative scarring[18,27, 28], but is also crucial for successful formation of collagen fibrils during embryonic development and has been implicated in successful regeneration of amputated digit tips[29]. Although a general distinction can be made between scar that successfully heals into regenerated tissue and one that is degenerative, there is a complicated, poorly understood, and poorly defined overlap between the two at a structural and biomolecular level. While a scar that successfully heals is transient and serves as an intermediate state, for the purposes of this review a degenerative/pathological scar can be broadly considered as one that is chronically unresolving or diverges to an aberrant fate, often characterized by features that resemble fibrocartilage[30]. Together, these data suggest that the formation and maturation of tendon and ligament is molecularly complex and involves an undefined sequence of events, each of which may be beneficial or detrimental depending on the life history of the tissue and interaction with other molecular factors. By extension, development of treatments for tendinopathy, whether by regeneration or grafting engineered tissue, are not possible without better understanding the molecular mechanisms that are involved in the development of healthy and diseased tissues.

Molecular mechanisms that are involved in tendon and ligament development are potentially regulated by physical forces[27]. Forces within the musculoskeletal system can be generally classified into tension, compression, and shear. Despite having common cellular origins [31], during development tendons and ligaments across the body become transcriptionally and functionally (discussed in later sections) distinct[32]. The diversity in tendon phenotype is matched to the different physical role, and therefore different forces applied to the tissues, during movement (Fig. 1).

The process of “ligamentization” of tendon autografts used for ACL reconstruction provide a fascinating example of the adaptive capabilities and similarities of tendon and ligaments in response to their environment. While the mature ACL is distinct from various tendons around the knee (e.g. patellar tendon, hamstring tendon) in terms of their biochemical properties (e.g. COL1/III ratio, glycosaminoglycan content, ratio of collagen crosslinking types), after a tendon is implanted as an autograft to reconstruct the ACL, both the mechanical and biochemical properties of the tissue converge towards those of the native ACL [33–35]. These data have two important implications. The first is that there is high potential for knowledge transfer in experiments between tendons and ligaments due to their behavioral similarities. This is especially important since there is a paucity of interventional studies on

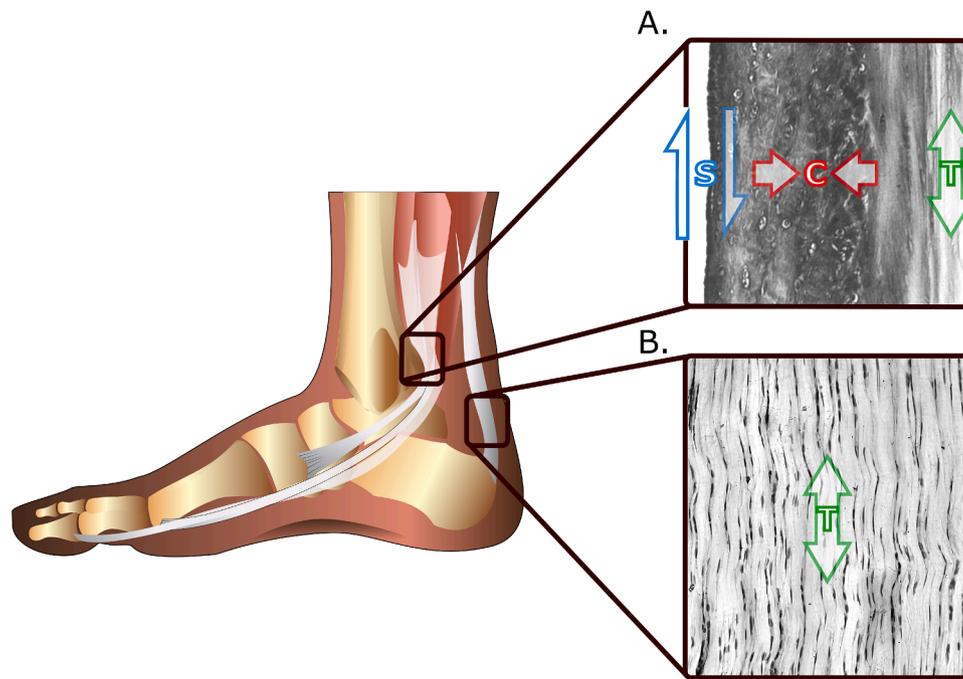


Fig. 1. Tendon structure varies by function. (A) The posterior tibialis tendon, which wraps around the medial malleolus like a pulley, experiences shear (S) and compression (C) on the anterior side. The anterior aspect exhibits rounded fibrocartilage cells along with higher proteoglycan content (dark stain). (B) The Achilles tendon, which experiences high amounts of tension (T) within the tendon proper, exhibits spindle-shaped fibroblasts along with highly aligned and densely packed collagen.

ligaments compared with those in tendons. Despite the lack of studies in ligaments, where data exist tendons and ligaments behave similarly in response to load stimuli. The second implication is that environmental factors can play pivotal roles in the development and deviation of tendons and ligaments, both in contexts of health and pathology.

The purpose of this review is to use normal anatomical variation in mechanical demands and morphological development as a platform to begin to understand the distinct roles of tension, compression and shear in the development, maturation, and pathology of tendons and ligaments. We will discuss how tendons vary in their physiological and material properties, and how development of tendons and ligaments depends on their mechanical environment. Our goal is to provide a perspective on how regenerative therapies and tissue engineering can be guided by the molecular response to physical forces during development, as well as how pathological states are likely caused by improper load across the tissue.

Tissue regeneration and recapitulation of development

Regeneration during adulthood is often achieved by recapitulating development. Genes that are normally only expressed in the developing tissue become re-expressed to regenerate the necessary cell mass and repair or replace damaged or lost tissue. This cell mass then differentiates to the specialized cells that establish and maintain tissue function. For example, in skeletal muscle[36], bone[37], and liver[38], stem/progenitor cells become activated, proliferate, and then differentiate into adult muscle, bone, and liver cells allowing the reestablishment of normal tissue size and function. In a murine model of digit tip regeneration after transection, regenerating tissues transiently upregulate embryonic transcriptomic profiles, whereas non-regenerating tissues at the same site lack this pattern[29]. However, injured tendons and ligaments tend to not regenerate in adults – they instead become degenerative and develop a distinct region where the cells resemble neither adult nor developing tendons[39].

Species with exceptional abilities to regenerate tissues may provide insight into strategies for achieving tendon and ligament regeneration in

humans. Axolotls for example, regenerate whole limbs, including the resident tendons and ligaments. Like humans, axolotl tissue regeneration begins by recapitulating developmental processes: cells revert to an embryonic-like state before differentiating into mature tissues[40,41]. In other instances, regeneration is possible only in young animals. For example, neonatal mice demonstrate the transient ability to regenerate transected portions of their heart[42]. They lose this regenerative ability within a few days after birth and the loss of regeneration is correlated with heart matrix stiffening. The same is true in tendons. Neonatal tendons demonstrate the ability to regenerate after injury[43], in contrast to their stiffer adult counterparts. Interestingly, in both cases regeneration can be rescued by application of β -aminopropionitrile, an agent that inhibits matrix stiffening[42,44]. Together, these data suggest that scar-free regeneration decreases as a function of the intrinsic stiffness of the tissue matrix.

Injured adult tendons become highly cellular and form a disorganized collagen matrix composed of small collagen fibrils[30]. Since the expression pattern within the scar is distinct from both adult and developing tendons[39], scars likely represent a failed attempt to use the developmental program to regenerate, or simply the inability of the tendon to regenerate due to the absence of an essential signal.

Observations from Murthy Roth's Large (MRL) Mice, a strain that has exceptional ability to heal and recover injured tendons, may offer some insights into how tendon and ligament healing can be achieved. At the beginning of their healing response, the transcriptome is dominated by genes that regulate the cell cycle[45]. In comparison, cells in wildtype mice remained relatively quiescent for these genes. Since neonatal tendon development in rats is characterized by high activity of the cell cycle[17], this suggests that the superior healing of MRL mice is initially mediated by improved ability to recapitulate development. Another study comparing MRL and wildtype mice healing found that while both undergo large increases in expression of *Snai1*, a gene required for embryonic development, the MRL mice do so to a 2–3-fold greater extent [46]. In the regenerating liver, too, *Snai1* is upregulated and its effects are required for successful regeneration to occur[47]. This suggests that while both MRL and wildtype mouse tendons respond to injury by

activating an embryonic signaling pathway, the superior healing of the MRL mice may be due to a more complete recapitulation of development. These data suggest that a conserved mechanism may be present for regenerating tendons and ligaments.

If developmental processes can be recapitulated in human tendon, as in the other parts of the body and regenerating species, then regeneration of injured tendon may be possible. These same processes may also enable the engineering of more mature tissues *in vitro*.

Anatomical variation in tendon and ligament morphology suggests that interventions for forming healthy mature tissue must be tailored with respect to the variety of potential outcomes. As such, it is crucial to understand the varied material properties of tendons and ligaments that underly their function.

Material properties reflect mechanical demands

Tension

Connective tissues must develop to meet the mechanical demands that are imposed upon them. However, mechanical demands vary depending on anatomical location. One of the clearest examples of this is seen when comparing “energy storing” tendons and “positional” tendons around the same joint. In humans, an example of this would be the Achilles (AT) and the tibialis anterior (TA) tendons, respectively. As their names suggest, energy storing tendons serve to store energy for efficient propulsion, whereas positional tendons serve to position joints to enable locomotion (in this case, dorsiflexing the foot to prevent the toes from dragging on the ground during walking/running). Energy storing tendons store the entire body weight (or more) and use the stored energy to propel the body forward, while positional tendons mostly function to lift a small segment of the body. For example, during running the AT is loaded with on average 4.15–7.71-times body weight[48] whereas the TA tendon is loaded using ~1 % of body weight (a ~400–700-fold difference). The difference in loads correspond with the AT having 5-fold greater cross-sectional area compared to the TA[49], suggesting a relationship with the magnitude of load that these tissues face. However, the anterior cruciate ligament experiences physiological loads that are an order of magnitude lower than the AT (hundreds versus thousands of newtons)[50,51], but have similar cross-sectional areas (~55 versus ~45mm[2])[52,53]. Considering such varied roles in these tissues, from being energy storing to positional, to joint stabilizing, it is evident that more than just tissue geometry distinguishes their specialized functions.

It is no surprise that with varied mechanical demands, material properties vary in parallel. In humans, the AT has a modulus of ~816 MPa[54] and can reach strains of up to ~11 %[55]. By contrast, the TA tendon has a modulus of ~1200 MPa and strains only ~2 %[56]. A similar trend is seen in the analogous superficial digital flexor tendon (SDFT) and common digital extensor tendon (CDET) of the horse. Screen et al. have reported that compared to the CDET, the SDFT has: higher failure strain, higher force at failure, higher cross-sectional area (CSA), and lower ultimate stress and elastic moduli[57]. Although potentially counterintuitive that in these examples the energy storing tendons have lower moduli (material stiffness) than the positional tendons, the energy storing tendons compensate by having a larger CSA resulting in greater maximal tensile load and higher stiffness index (N/strain)[58]. A potential explanation for this pattern is that it permits greater mechanical energy storage when combined with their ability to strain elastically to a greater extent (this helps create a greater area under the curve for the force-deformation plot of the elastic region of the energy storing tendon). At the level of collagen fascicles, the Screen lab has also shown that the SDFT behaves more elastically in terms of having lower hysteresis and also has a greater resistance to fatigue from cyclic load[59]. This is consistent with experiments at the level of individual collagen fibrils by a separate group[60,61]. Together, these data indicate that even for two tendons that act around the same joint, vastly different

mechanical and material properties develop because of the mechanical demands placed on them.

At the structural level, energy storing and positional tendons also differ. Upon fascicle strain, collagen fibers rotate in the SDFT, whereas they slide relative to one another in the CDET, suggesting that fascicles of the SDFT take on a more helical structure that potentially supports their ability to absorb greater loads, store and recoil more efficiently, and better endure repeated loading[59]. Interestingly, the average diameters of collagen fibrils in CDET are larger than those in SDFT[62]. Additionally, within SDFTs, larger average diameters are correlated with higher elastic modulus. Together, these data suggest that larger collagen fibril diameters are associated with higher moduli and that energy storage is more dependent on the macrostructural properties (helical v. parallel layout) of the fibrils.

At a compositional level, collagen comprises the majority of the dry weight for all tendons, reaching up to 80 %[63]. There are slight but significantly higher amounts in CDET compared to SDFT, again suggesting that the amount of collagen poorly reflects tissue function. There are more pronounced differences in collagen crosslinking type and density between the tendons: bovine SDFTs are characterized by stronger trivalent crosslinks whereas CDETs are characterized by divalent crosslinks[60,61]. Further, the SDFT has a higher density of thermally stable crosslinks compared to the CDET. There are also differences in non-collagenous components between tendon types. Sulfated glycosaminoglycans (GAGs) are higher in the equine SDFT compared to CDET [63]. Since GAGs are supposed to imbibe water due to their large negative charge, this may explain the higher water content of SDFT compared to CDET[64] (the evidence for the contribution of GAGs to the driving hysteretic properties however, are controversial[65]). Additionally, GAGs are a component of proteoglycans, some of which are recognized to play a crucial role in the regulation of collagen fibril morphology[66–69]. Therefore, differences in GAGs present a potential role in regulating the different collagen structures in the two tendons. Thus, there are several compositional differences that distinguish different tendon types and likely underly their mechanical differences.

Even between two tendons with more similar functions, local mechanical demands can vary resulting in different material properties. For example, the quadriceps and patellar tendons are arranged in-series and serve to transmit tension generated from the same quadriceps muscles into the same tibial tubercle, yet the quadriceps tendon has up to two-fold lower modulus than the patellar tendon[70,71]. The lower modulus reflects the greater cross-sectional area of the quadriceps tendon as the large quadriceps muscles converge on the patella. Even though these two tendons act in series, their mechanical environment is in fact quite different – the quadriceps tendon connects a compliant tissue to a stiff tissue (muscle to bone), whereas the patellar tendon connects a stiff tissue to a stiff tissue (bone to bone). Adapting the material and structural properties as a function of the tissues they connect helps to dissipate the stress concentrations that arise when applying a strain across tissues with dissimilar moduli. A similar pattern is seen across regions of the AT or TA tendon, where modulus increases from muscle to bone[72,73].

Compression

In addition to tension, compression is regularly experienced in a subset of tendons such as those that wrap around bones or under retinacula/pulleys[74] (Fig. 1A). For example, the peroneus brevis passes around the lateral malleolus on the outside of the human ankle, and the flexor digitorum profundus tendon (FDP) of the rabbit wraps around the talus. To determine the effect of *in vivo* compression on tendon function, the Vogel group surgically eliminated compressive stress from the rabbit FDP by translocating it in front of the talus[75]. At 4 weeks post-surgery, they found that releasing the compression on the FDP resulted in lower compressive stiffness compared to the sham surgery control. Therefore, like tendons under tension, tendons that experience compression

modulate their stiffness according to their mechanical environment.

In humans, regions of tendon that wrap around bone or under retinacula take on features of fibrocartilage[74](Fig. 1A). Cartilage and fibrocartilage are examples of tissues that specialize in resisting compressive forces using their high content of negatively charged large proteoglycans, which attract water, and type II collagen[76]. In the same study where the Vogel and her colleagues saw lower resistance to compression after eliminating physiological compression for 4 weeks, they also saw decreased amounts of large proteoglycans and type II collagen, and decreased thickness of the fibrocartilaginous region of the tendon[75]. Fibrocartilage in tendon is therefore a direct adaptation to local compression.

Shear

Shear forces are associated with the sliding of tendons, ligaments, muscles, and bones relative to one another, and around other structures. While the amount of shear between two tissues can increase as a function of the compression between two structures, there are examples where shear is present even with relatively little compression. One example is the human Achilles tendon close to the tibia where the tendon slides relative to the bone. A second example is the cortex of the canine flexor tendons, which are encased in a sheath. A third clear example is within the AT, where the deep portion displaces more than the superficial portion, resulting in sliding between fascicles[77]. The direction of this non-uniformity also varies as a function of knee angle [78].

The ability for adjacent structures to slide is an important clinical issue. For example, compared to young adults, middle aged adults were found to have less non-uniform AT displacement during passive dorsiflexion and this was associated with a lower range of motion[77]. Considering that during dorsiflexion the calcaneus changes orientation relative to the gastrocnemius, interfascicular sliding may be necessary to permit mobility. Perhaps more importantly, interfascicular sliding may optimize distribution of tensile stress concentrations across the cross-section of the tendon by appropriately adjusting the tautness of different fascicles according to joint position. This is consistent with the idea that AT ruptures are largely caused by high acute loads while in dorsiflexion[79]. Further support of this hypothesis comes from the fact that older individuals (who have less interfascicular sliding) have a 3-fold higher incidence of AT rupture[80] compared to young adults even though they exercise less[81]. Whether this relationship exists in rodents, whose tendons do not contain fascicles is unknown. Alternatively, sliding at the fiber and fibril level serves to attenuate strain that collagen fibers face from bulk tissue strain[82]. This idea is supported by findings that reduced sliding via experimental crosslinking increases tensile strain to the fibers[83]. Regardless of which is true, when regenerating or engineering functional tendons, adapting the ability to slide according to physiological demands is crucial. This can especially be true for those who participate in physical activities which demand high forces at end-ranges of motion.

The phenomenon that middle-aged adults have a higher incidence of AT injury despite exercising less may be explained by the same mechanism observed by Vogel previously[75], whereby compositional and functional adaptations develop in response to the loads (or lack thereof) that tendons habitually face. Therefore, the increased risk that the older population faces results from a lack of exercise-mediated shear stimuli and their associated tissue adaptations.

In both the human AT and canine flexor tendons, there is localization of the large proteoglycan lubricin at regions subject to shear. This includes the interface between tissues as well as the region between fascicles within a tissue[84,85]. Lubricin holds water at the interface to lubricate sliding[86,87]. While fibrocartilage is localized to the side of the tendon that is compressed by the pulley (Fig. 1A)[75], lubricin in canine flexor tendons is distributed evenly around the circumference of the tendon[85], indicating that it manifests independently of additional

compressive forces.

In terms of regenerating or engineering tendons and ligaments, anatomical variations in the mechanical demand necessitates specialized mechanical properties, and therefore, specialized composition and structure. By extension, the molecular response that allows tissues to adapt towards and achieve the desired mechanical properties needs to be understood within this context. It is no surprise then that the transcripts of tendons vary by their anatomic location, both between [32] and within[73] tendons. How this spatially varied genetic profile is achieved and coupled to mechanical demands is a crucial issue for both tissue regeneration and engineering.

Specific forces drive specific molecular programs

In the previous section we discussed the gradient in modulus that is present in the tendon from muscle to bone[72,73]. Importantly, when immobilized by denervation, this gradient is lost and instead the whole length of the tendon becomes as stiff as the bone-adjacent region[72]. This suggests that physiological forces are required for the regional variation in tendon mechanics. The effect of the mechanical environment on cell behavior is well recognized. An intervention as simple as varying *in vitro* cell culture substrate stiffness is sufficient to regulate the differentiation of stem cells towards different fates, including nerve, muscle, and bone[88]. Therefore, it is no hyperbole to say that cell fate and tissue composition are critically regulated by the mechanical environment. Of course, biochemical and hormonal signaling play a significant role in modifying these outcomes. But as we will see, tendons and ligaments are primarily influenced by the mechanical environment. In fact, chromatin accessibility in regions related to matrix remodeling is altered by the amount of resting tension in a tendon[89], possibly due to tensile deformation of the nucleus[90]. This suggests that the contributions of at least some mechanical and biochemical stimuli towards physiological function are interdependent. However, data in this area is currently limited. With tissue regeneration or engineering in mind, it is therefore crucial to recreate the mechanical environment that best recapitulates the developmental molecular program achieved physiologically, while suppressing alternative and potentially pathological fates.

Killian and Thomopoulos have provided an excellent review of the mechanisms by which tendons sense (mechanotransduction) and respond (adaptation) to physical forces and how they have been implicated in injury and healing[27]. However, there is still a crucial lack of understanding of the context-specific role of the mechanical signals in the stage-dependent development and maturation of tendons and ligaments. It is also still unclear how mechanotransduction regulates healing from injury and how this can be used to improve tissue engineering. The goal of this section is to: (1) provide a perspective on the stage-specific roles of various mechanosensitive signals in tendon development; and to (2) demonstrate that the same mechanotransduction pathways that drive development of tendon in early life also are involved in the formation of pathological tissue during adult tendon healing.

Tension

One hallmark of healthy and mature tendons and ligaments is their array of aligned collagen fibrils with a bimodal distribution of small and large diameters (~35 nm and ~150 nm, respectively)[91,92]. In this brief example (which we will elaborate on later), manipulation of the tensile environment of tendons by way of treadmill exercise[93], shielding tensile load[94], or mutating a tension-activated mechanosensor[95], has direct impact on the formation of healthy fibril diameters and other functional aspects of their phenotype. However, there is a poorly understood balance between tensile loads that are beneficial versus detrimental[27]. To understand these properties, their biomolecular foundation needs to be better understood.

The earliest known marker of tendon development is scleraxis (Scx), which is expressed in all tendon and ligament cells during embryonic

development and is critical for their embryonic growth[96,97]. Embryonic increases in COL1 depend on the binding of *Scx* to the *Col1a1* promoter[98]. While *Scx* is initially expressed in a cell autonomous fashion, continued expression during embryonic development requires muscle activity[24]. Similarly, in the injured rat patellar tendon, *Scx* expression is increased in response to acute loading[39].

In mice, *Scx* expression decreases after birth while *Col1a1* expression is unchanged[99]. This suggests that as the tendon matures, other molecular factors become more important for tendon homeostasis. While *Scx* does participate in the bulk tissue growth response to exercise in the AT[99], the Asahara group observed that exercise-induced increases in collagen fibril diameter can occur without an increase in *Scx*[93]. A variety of tenogenic genes did increase although *Scx* expression was unchanged. These genes included *Col1a1*, *Tnmd*, *Fmod*, and *Mkx*. These data suggest that during adulthood, tendon adaptations to tensile load can occur independent of *Scx*.

Embryonic (early) stages of tendon development are characterized by an increase in collagen fibril number while fibril diameter is relatively unchanged. By contrast, post-natal development is characterized by enlargement of fibrils to produce the bimodal distribution of fibrils discussed earlier[92,100] (Fig. 2A). These distinct types of growth suggest that tendon and ligament development can be understood by conceptually compartmentalizing development into an early stage where the number of nascent fibrils increase and a late stage where these fibrils mature by enlarging and potentially creating other macromolecular structures like fascicles. As such, distinct biological factors may be responsible for driving each developmental stage and this may explain why postnatal tendon remodeling and expression of the associated genes can occur without a change in *Scx* activity.

Interestingly, knockout of *Scx* affects tendons by reducing the number of collagen fibrils formed during embryonic stages[8]. This indicates an early developmental role of *Scx*. To study the role of *Scx* in tendon formation, a mouse model of adult neotendon formation in the plantaris tendon via synergist ablation of the AT has been used. In this model, *Scx* is required to form the neotendon, which forms at the periphery of the preexisting tendon[101]. Following ablation of the AT, proteomic analysis revealed that *Scx* knockout reduced collagens III, V, XI, and XII in the plantaris neotendon[101], all of which are involved in initial stages of fibril formation[102–105]. Furthermore, the same analysis showed that despite impaired neotendon formation in the *Scx* knockout group at both 7 and 14 days after synergist ablation, there was no

difference in COL1 (a collagen that characterizes mature fibrils) levels between *Scx* knockout and wildtype mice at these same timepoints, even with continued growth in the wildtype group. These data suggest that while *Scx* plays a crucial role in the initial formation of neotendon, it may do so through synthesis of a collection of proteins other than COL1. In another study using the same synergist ablation model, the same investigators showed that while *Scx* expression drastically increases in plantaris tendons after the surgery, *Scx*⁺ cells localize peripherally in the neotendon tissue, in contrast to its relative absence in the preexisting tendon tissue[106]. These data support the hypothesis that *Scx* serves to support tendon formation by generating new collagen fibrils, rather than altering existing collagen fibrils.

Perhaps more strikingly, when *Scx* is conditionally knocked out during adulthood, fibril diameters increase[101,107]. This suggests not only that *Scx* is not required for fibril diameter growth, but also that increased fibril diameter potentially compensates for the inability to generate new fibrils. This is consistent with the finding that, when *ColV* and *ColXI* are knocked out (which were decreased in synergist ablated *Scx* knockout mice[101]), there are not only fewer collagen fibrils but also larger fibril diameters[105], supporting the idea that *Scx* drives tendon development by increasing fibril number and has limited role in fibril growth. Together, these data suggest that another factor is responsible for driving enlargement of fibril diameter during tendon maturation and adaptation to tensional load.

Although knockout of *Scx* does not limit fibril diameter, knockout of mohawk (*Mkx*), a load-dependent homeobox transcription factor[108], severely impairs fibril diameter enlargement during postnatal maturation[109]. The growth response of collagen fibrils to exercise training during adulthood is also completely lost in *Mkx* knockout mice[93]. Consistent with the idea that *Scx* and *Mkx* have distinct roles, the effects of *Mkx* knockout on embryonic fibril morphology is minimal[109]. Therefore, it seems that the increase in new fibrils that characterizes early development is driven by *Scx*, whereas later increases collagen fibril diameter may be driven by *Mkx* during both postnatal development and in response to exercise training. The idea that *Scx* and *Mkx* perform distinct and sequentially related roles is further supported by the fact their expression is largely mutually exclusive at the single cell level, as indicated by single cell RNA sequencing of the periodontal ligament[110].

Mkx activity may play a role as a “state switch” from a nascent and multipotent tendon progenitor towards a more mature and

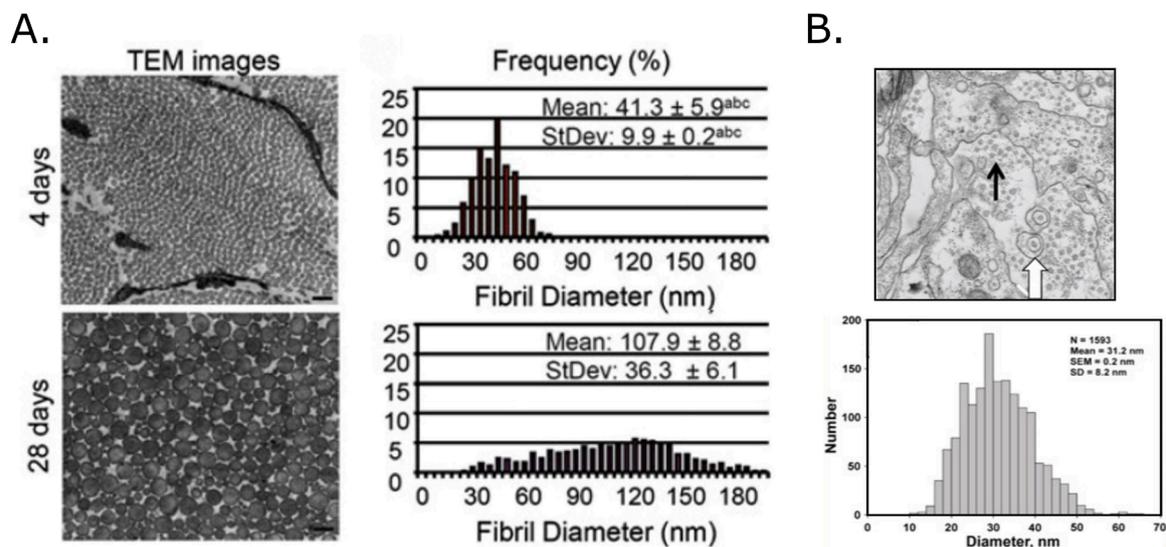


Fig. 2. The microstructure of engineered tendons/ligaments are limited to an immature phenotype. (A) Collagen fibril diameters of mouse Achilles tendons increase and become bimodal during postnatal development. (B) Engineered tendons formed from adult tendon cells seeded in fibrin hydrogel produce a uniform array of collagen fibrils (~35 nm) that resemble immature, neonatal tendon. Fibripositors are also seen, which are another feature of embryonic development. Adapted from [79,93].

differentiated tensional tendon. *Mkx* knockout increases *Scx*, *Bgn*, *Col3a1*, and *Tnc*, and decreases *Dcn*[111]. This transcriptional profile is opposite to that of normal maturation[68,100,112], suggesting an impaired ability for tensional tendons to mature in the absence of *Mkx*.

The fact that baseline levels of *Scx* and *Col3a1* are higher in response to *Mkx* knockout suggests that tendons are compensating for the fact that they are unable to increase the diameter of existing fibrils and generate more fibrils instead[111]. Alternatively, it is plausible that this phenotype is not a functional compensation, but rather a symptom of being developmentally arrested due to a lack of *Mkx* activity. In support of either of these hypotheses, the frequency distribution of fibril diameters in adult *Mkx* knockout mice closely resembles the more homogenous and small diameter fibrils seen in neonates, compared with the more bimodal distribution of larger diameter fibrils seen in adults [93,100]. Although *Scx* is a foundational transcription factor in the development of tendons, it seems to be essential in only the early stages of tendon formation or regeneration when nascent fibril generation is important, whereas *Mkx* is necessary for later-stage maturation and growth of these fibrils. Considering that *Scx*, but not *Mkx*, is increased after patellar tendon injury[39], it appears that tendon reflects at least some aspects of early development following injury.

Scar is similar to embryonic tendon in that there is an abundance of small diameter collagen fibers. This may correspond with the increased *Scx* that is seen in healing tendon. Scar and embryonic tendon might be similar in that they form the foundational nascent fibrils that have the potential to mature into larger diameter fibers given the right signals. The data above suggest that both embryonic and postnatal tendon require tension but that the intracellular mediators are distinct. This may also be the case for healthy healing following injury (as discussed earlier, although embryonic *Scx* is initially expressed independently of load, its expression is sustained by load). Some aspects of development are reflected in healing tissue, but the matrix is unable to mature. This suggests that load is not passing through the tissue in the way it does as the tissue matures during development.

Stage-dependent developmental processes driven by tension appear to be implicated in tissue engineering of tendons and ligaments too. Investigators have only been able to generate a homogenous array of small-diameter collagen fibrils, even if the cells in the engineered tissue were derived from adult tendons[113–115] (Fig. 2B). It is therefore likely that the signals necessary to develop the bimodal array of small and large diameter fibrils are missing. The small diameter collagen fibrils indicate that engineered tissues resemble embryonic or scar tissue. Like scar or embryonic tendons, engineered tissues have a higher density of cells, display developmental isoforms of collagen, and have cellular protrusions that typically characterize embryonic development: fibroblasts[116]. What is lacking from the tissue engineering environment may simply be postnatal-like dynamic and distractional tensile strain. Indeed, most tendons and ligaments are engineered and held at a uniform length, resembling an immobilized state[117]. When engineered tendons were implanted *in vivo*, collagen fibril diameter increased[115], indicating that some aspect of the physiological environment, likely tensile load, is essential for fibril maturation.

Compression

The enthesis is subject to compressive forces[118] and develops regions of fibrocartilage that are dependent on muscle activity[119]. The fact that these chondrogenic features, including expression of *Col2a1* and aggrecan (*Acan*; a large proteoglycan), arise postnatally in tendons specifically where they wrap around anatomic pulleys suggests that compressive forces during movement guide differentiation[120,121]. Interestingly, cartilage-like tissue can be ectopically induced in a tendon midsubstance if it is subjected to compression. Experimentally, the role of compression in driving chondrogenic fates was first demonstrated by Vogel and colleagues when they observed increased *Acan* expression and synthesis of large proteoglycans within an otherwise healthy bovine

tendon that was compressed *ex vivo*[122].

As mentioned above, tendons share the same embryological origins as cartilage (and bone)[31]. During embryonic development, there is diffuse expression of not only *Scx*[97], but also the cartilage transcription factor *Sox9* in tendons and ligaments[123]. At the cellular level, many tendon progenitors express both *Scx* and *Sox9*[124–127]. These *Scx*⁺/*Sox9*⁺ progenitors later separate into distinct *Scx* and *Sox9* domains, where the *Scx* cells localize to the tendon midsubstance and the *Sox9* cells localize to the enthesis, consistent with spatial transcriptomic data from adult tendons showing that expression of *Col1a1* and *Col2a1* are spatially distinct and are expressed in the midsubstance and enthesis, respectively[128]. It appears that this distribution is achieved by *Scx*⁺/*Sox9*⁺ populations downregulating either *Sox9* or *Scx* depending on the location within the tissue[124,125]. These multipotent progenitors appear to be implicated in healing too, as a recent paper has shown that the initial healing response to injury in the AT midsubstance requires a massive infiltration of a population of stem-like cells (*Axin2*⁺) which have multi-lineage potential, including the ability to express *Sox9* [129]. However, controlled experiments that measure *Scx* and *Sox9* expression in precursor cells following tension or compression have not been reported. In addition, how *Sox9* is activated and its role in developing the fibrocartilaginous regions of tendon pulleys or degenerative tendon scar is not known[130].

Interestingly, deletion of *Sox9* in *Scx*-expressing cells leads to disruption in only the enthesis[124,125]. Despite originally being expressed in the progenitors of the midsubstance, *Sox9* does not appear to play any role in the development of this region. Instead, tendon and ligament progenitors appear to be multipotent during development and undergo a “state switch” towards divergent fates depending on the loading environment. This same mechanism may be implicated in the multipotent *Axin2*⁺ cells that drive the initial healing response of the tendon midsubstance.

Importantly, *Mkx*, which is activated in the rat AT by tensional load [93], can suppress expression of cartilage genes, including *Sox9*[108]. When *Mkx* is deficient in mice, ectopic mineralized fibrocartilage forms in the tendon midsubstance[108]. Furthermore, this ectopic tissue forms in the same region where *Scx* disappears[131]. Interestingly, the ectopic mineralized fibrocartilage that appears in the quadriceps tendon, patellar tendon, and cruciate ligaments of mice when the proteoglycans biglycan (*Bgn*) and fibromodulin (*Fmod*) are knocked out, can be reduced by moderate exercise[132]. As discussed above, exercise increases *Mkx* suggesting that *Mkx* plays a direct role in the tendon/fibrocartilage switch. In support of this hypothesis, the cellular response to tensile strain in wildtype tenocytes increases tenogenic genes, but in *Mkx* knock-out tenocytes cartilage genes increase instead[108]. Together, these data suggest that tendon cells have an innate capacity for expressing fibrocartilage genes, which is prevented by *Mkx* downstream of tensional load.

Shear

Shear is a much less studied force on the remodeling of tendons and ligaments, but no less important. Clear indications of roles of shear in tissue remodeling come from studies in passive mobilization of sheath-encased flexor tendons. In a canine model of flexor tendon healing after surgical repair, early passive mobilization resulted in greater interphalangeal joint range of motion and tendon excursion in response to a given load[133].

When discussing shear, lubricin is a protein of interest, as *in vivo* models show that it localizes to areas where there is shear to facilitate sliding between tissue surfaces[84,85]. Furthermore, lubricin-knockout mice have greater tendon gliding resistance in their deep digital flexor tendon compared to wildtype and heterozygous controls[134]. While there are currently no indications whether shear can induce lubricin expression in engineered tendons and ligaments, there is good evidence for this in both engineered and native cartilage[135]. Considering the

clinical complications caused by scar adhesions in poorly repaired tissues such as flexor tendons and herniated intervertebral discs, there is a valuable opportunity to study whether physical forces can effectively modulate lubricin expression in tendons and ligaments.

Hyaluronic acid (HA) is another large proteoglycan that promotes tendon gliding, and it has been suggested as a potential therapeutic for tendinopathy[136]. However, the exogenous application of HA is invasive and clinically difficult. Therefore, it would be valuable to better understand the mechanism underlying the regulation of HA synthesis by hyaluronic acid synthase (HAS). As with lubricin, there are no studies investigating the localization and activation of HAS in response to shear in tendon. Considering their critical role in lubrication, the effect of shear on HA and lubricin has the potential to impact clinical care.

At a smaller scale, shear mechanosensing and the resultant adaptations may be distinct from the adaptations discussed above. Piezo1, a shear-sensitive calcium ion channel, promotes lysyl oxidase (a cross-linking enzyme) activity in rat tail tendon fascicles[137]. In isolated fascicles of rat tail tendon, physiological strains are sufficient to induce piezo1 channel activity. This is potentially counterintuitive since physiological shear is likely also responsible for promoting adaptations that facilitate sliding between adjacent structures. However, the Asahara group has shown that piezo1 also promotes lubricin gene expression [95]. It is currently not clear how piezo1 simultaneously regulates these shear-stabilizing and -promoting adaptations.

Clues can be gained from the Snedeker group's comparison of the effect of a gain-of-function mutation in piezo1 on the mechanical properties of individual tail tendon fascicles versus whole plantaris tendon[137]. Interestingly, an increase in maximum tensile load was seen only in the whole plantaris tendon, whereas an increase in stiffness was seen in both samples. The relative effect was 2-fold larger in the whole plantaris tendon compared to the isolated fascicles. The different observations between individual fascicles and whole tendon suggests that increases in bulk tendon mechanical properties result from adaptations beyond just individual units of collagen fibrils, including, between fibrils and fascicles. This would have implications for the shear mechanisms discussed earlier whereby stress concentrations could potentially be dissipated by sliding, thereby allowing a greater number of collagen fibers to be engaged simultaneously. In fact, the Asahara group showed that piezo1 gain-of-function mutations in mice result in greater strain to failure and energy stored to failure in the AT[95]. Studies that investigate how shear stimuli at different scales influence spatial distribution of crosslinking, lubricating proteins, shear mechanisms, mechanosensing, and resultant mechanical behavior would clarify this issue. With such influence on tendon (and ligament) function, these are crucial factors to consider for both tissue regeneration and engineering.

Spatial arrangement

As a tissue whose function is determined by mechanical and material properties, the spatial arrangement of proteins within tendon and ligament is critical. Spatial remodeling is also regulated by physical forces. Fibrilpositors, which align in parallel with cells, are responsible for the deposition of aligned collagen fibrils during embryonic development [114,138]. Therefore, fibrilpositor and cell alignment are essential for generating an organized collagen matrix. Fibrilpositors form in concert with the tension-sensitive actin cytoskeleton[116]. Uniaxial tension can trigger cell alignment in an initially disorganized state, and this change corresponds with the production of a collagen matrix that corresponds with cell alignment[139,140]. Releasing the tension from engineered tendons results in the loss of fibrilpositors and collagen fibril alignment [114]. Together, these data suggest a crucial role for tension-guided cell alignment in the development of the parallel collagen matrix that forms the backbone of healthy tendons and ligaments.

Non-cellular roles of mechanical stimulation on collagen matrix remodeling

While this review focuses on cell-based mechanisms of matrix remodeling in response to mechanical stimulation, it is crucial to also consider effects that exist independent of cells. When collagen gels devoid of cells with randomly oriented fibers experience tensile strain, collagen alignment and density increases, and these features were maintained even after the tissue was returned to resting length[141]. Notably, this adaptation resembles characteristics of a healthy, mature tendon or ligament. This raises the question of how much of collagen remodeling *in vivo* is cell-mediated versus passive remodeling.

Interestingly, if these collagen gels were crosslinked before loading, the effects on collagen alignment and density were reversed when they returned to resting length. This suggests that biochemical alterations to the matrix, such as crosslinking, determines the degree of passive remodeling in response to load. Practically, this means that when crosslinks are high in tendons and ligaments, such as in diabetics and older individuals, passive changes to tendon architecture may not be as lasting or as inducible as in young healthy individuals. In contrast, since enzymatic crosslinking enzymes like lysyl oxidase are increased in response to loading[137], enzymatic crosslinking could be a mechanism to stabilize passive load-induced changes in collagen architecture. This could facilitate permanence in the highly crosslinked, dense, and aligned collagen fibrils that we know characterize mature tendon and ligament tissue. These data suggest a mechanism by which passive loading cooperates with biochemical activity to give rise to physiological adaptations.

Tensile strain also increases the resistance of collagen matrices to collagenases, whether bacteria-derived or endogenous MMPs (reviewed by Saini et al. [142]). Therefore, the passive remodeling of collagen architecture by tensile loading prevents its breakdown, resulting in a more stable matrix.

Even though mechanical stimulation affects collagen remodeling in ways that are independent of cellular intervention, the relative contribution of these passive effects and biochemical factors remains unknown. For example, treadmill exercise induces Mx-dependent collagen fibril enlargement; however, whether the biochemical cascade downstream of Mx alone is sufficient to induce collagen fibril adaptation remains unknown. It is likely that both passive collagen remodeling and biochemical signals like Mx are needed for the full collagen fibril growth response. However, studies that test the synergy of passive mechanics and biochemical factors are missing from the literature.

Absent and aberrant forces play a role in tendon and ligament degeneration

Clinically, healing tendon presents traits of an immature matrix. Similar to development, injured tendons have: 1) higher Scx expression [39,126]; 2) higher expression of fibrillogenic collagens (III[102,143], V [105,144], XI[105,145]); 3) uniformly small diameter collagen fibrils [100,146,147]; 4) greater vascularization[148]; 5) greater mitochondrial gene expression[17,149]; 6) higher cell density[150]; 7) rounded cells[151,152]; and 8) Scx⁺/Sox9⁺ cells[126]. These traits also resemble embryonic tissues prior to loading (see above). Therefore, cells in an injured tendon may scar because they lack the native mechanical signals needed to mature.

During fetal development, paralysis reduces Scx expression by decreasing transforming growth factor (TGF) β signaling[24]. This mechanism has implications for tendon regeneration, as Scx-mediated tendon healing in neonatal mice requires TGF β [153]. Early growth response (*Egr*)1 is another mechanically-induced transcription factor that is essential for tendon formation[23]. In injured ATs, application of botox to the gastrocnemius muscle results in a reduction of *Egr*1 and other tendon genes[154]. Importantly, tendon gene expression can be rescued by overexpression of *Egr*1. If *Egr*1 is expressed in embryonic

tissues beyond tendon, ectopic expression of tendon genes is also induced[155]. Interestingly, *Egr1* simultaneously suppresses *Col2a1* expression, and silencing *Egr1* has the opposite effect on *Col2a1*, providing another molecular switch that could drive tendon and inhibit cartilage gene expression. It is therefore likely that tensile load is required to upregulate *Egr1* and direct tendon/ligament regeneration and maturation. As discussed above, when tendon that normally does not experience compression is compressed, *Acan* expression increases [122]. Together, these data suggest a direct effect of aberrant/absent loading on tendon and ligament degeneration. To achieve tissue regeneration, it is necessary to not only have the appropriate forces, but also minimize pathological forces.

These data raise the question of how degenerative states originate in tendon: does abnormal loading initiate abnormal morphology, or vice versa? Considering the ability of load to generate abnormal morphology, it is likely that biomechanical, biochemical, and cellular changes originate simultaneously and positively regulate each other. The subsequent section on stress-shielding will focus on the biomechanical aspects of this cycle.

In tendon healing, collagen III (COLIII) is often associated with scar [18,27,28]. It is recognized as mechanically inferior due to its disorganized orientation and small diameter[156]. However, neonatal mice, which are known to regenerate tendons scar-free, have greater *Col3a1* expression following injury[43]. Furthermore, “super-healer” MRL mice, which have an extraordinary ability to heal adult tendons with minimal scarring as discussed earlier, have an even greater increase in COLIII protein 1-week after injury than wildtype mice[143]. Importantly, by 6 weeks COLIII is lower in MRL mice than wildtype mice. Together, these data indicate that prolonged COLIII abundance is likely a symptom, and not the cause, of failed repair.

Importantly, loading regulates COLIII and COLI gene expression. Eliasson, Andersson, and Aspenberg found that after AT transection, unloading by botox injections results in increased COLIII gene expression acutely after injury (3 and 8 days), and at later timepoints COLI expression is lower compared to loaded controls (14 and 21 days)[157]. The lower COLI was associated with an impaired return to native biomechanical properties in another study by the same group[158]. These data suggest a time-dependent, context-dependent, and functional relationship between COLIII and COLI, especially in response to load-mediated healing.

Consistent with the hypothesis that COLIII is not causing degenerative scars, during development COLIII precedes the formation of mature collagen fibrils. In fact, COLIII deficiency results in an impaired ability to form fibrillar collagen and is fatal[103]. COLIII is present around small diameter fibrils during early development, but is lost when the fibrils increase in diameter during maturation[159]. Furthermore, in the skin of mice, the ratio of COLI to COLIII increases from birth up until it plateaus at 9 weeks old[160]. The increase in this ratio corresponds with increases in fibril diameter and the acquisition of the bimodal distribution of fibril diameters. These data suggest that COLIII aides in the formation of a developmental matrix and decreases naturally with maturation. Consistent with this hypothesis, *Scx*, which shows a similar developmental pattern (see above), promotes *Col3a1* expression, along with other collagens that are essential for fibrillogenesis such as collagens V and XI[101].

Our lab has recently shown that in healthy adult rat patellar tendons, *Col3a1* is expressed only in a population of fibroblasts within the paratenon that is distinct from the main population of fibroblasts within the midsubstance of the tendon where *Col1a1* is expressed[128]. Again, since the increased *Scx* expression that results from growth-inducing exercise is localized to the tendon periphery[99], this is consistent with the idea that *Scx* plays a role in initializing connective tissue formation through fibrillogenic collagens such as COLIII. Furthermore, since cells from the paratenon are important for repair[161], the increase in *Col3a1* expression following injury could indicate that cells from the paratenon have entered the tissue to help initiate repair or

neotendon growth in a *Scx*-dependent manner, before other, more mature, mechanical signals such *Egr1/Mkx* take over.

Even small forces are sufficient to elicit remodeling activity. The Aspenberg group elicited three levels of unloading after rat AT injury by either injecting the gastrocnemius with botox, suspending their hindlimbs, or both[158]. Surprisingly, they found that the combination had even greater detriments to recovering mechanical properties than either intervention alone, suggesting that passive motion from contractions without an external load are sufficient to improve mechanics relative to complete unloading. A similar pattern was seen in the mechanical properties of rat supraspinatus repair in which the tissues were treated with either botox, casting, or both[162]. In another study, even 1 % strain applied *ex vivo* was sufficient to elicit a 20-fold decrease in MMP13 gene expression in rat tail tendon[163]. These studies demonstrate the high sensitivity of tendon and ligament cells to their mechanical environment.

Stress shielding in tendons and ligaments

As discussed above, healing tendons and ligaments resemble developmental tendons in many ways. However, one clear difference between healthy and healing tendons and ligaments is that the disorganized collagen fibrils in the scar are surrounded by a stiff native matrix. Considering that tendon cells need tension to align, and that fibroblasts deposit collagen parallel to their alignment[114,138–140], impaired stress-mediated cell alignment may underlie the disorganized matrix that characterizes scar. Further, in the absence of tension, *Mkx* and *Egr1* would remain low and *Sox9* and *Col2a1* would rise, inhibiting matrix maturation and potentially making the matrix prone to fibrocartilage development as seen in scar.

Biomechanically, a compliant structure positioned in parallel to a stiff structure will be stress-shielded (Fig. 3). Stress-shielding is well-recognized in bone, where native bone can be shielded from physiological stresses by stiffer materials that act in parallel, resulting in a state of disuse[164]. This is best appreciated with internal or external fixation using metal implants. For example, in hip replacement surgery a metal (often titanium, stainless steel, or other high-strength alloy) prosthetic covered in ceramic material is inserted into the medulla of the femur. Because of the superior stiffness of the prosthetic, during the normal strain of movement there is less stress on the bone that acts in parallel with the implant. As a result, the native bone atrophies over time. Stress shielding is prevented by using materials that are more similar in stiffness to native bone, allowing the adjacent bone to sense stress and providing a stimulus to the resident cells to adapt.

By analogy, a scar within an adult tendon has a lower stiffness than the native tissue[165]. A compliant scar acting in parallel with the stiffer healthy portion of a tendon may therefore be stress-shielded (Fig. 3). Three lines of evidence point to an important role for stress-shielding in scar formation. First, when the Hayashi group inserted a stainless-steel wire between the patella and tibial tubercle (in parallel to the healthy patellar tendon), the tensile strength, maximum load, and strain to failure of the native patellar tendon decreased in as little as 1 week and continued to decrease over time[166]. Further, they found that by simply stress-shielding a healthy patellar tendon with a wire, cellularity increased five-fold, median fibril diameter decreased ~58 %, and the orientation of the matrix decreased[94]. These data indicate that shielding a healthy tendon from stress results in what histologically and biomechanically would be defined as a scar. The second line of evidence implicating stress-shielding comes from injured horse tendons. Here, injury-induced scar formation is eliminated by injection of β -aminopropionitrile (BAPN), an inhibitor of the primary collagen crosslinking enzyme lysyl oxidase. BAPN injection into the healthy part of the tendon decreased overall tendon stiffness and when the BAPN treated horses were exercised, the collagen disorganization, hypercellularity, and the ratio of type I to III collagen all returned towards healthy levels[44], suggesting that decreasing stress-shielding by weakening the native

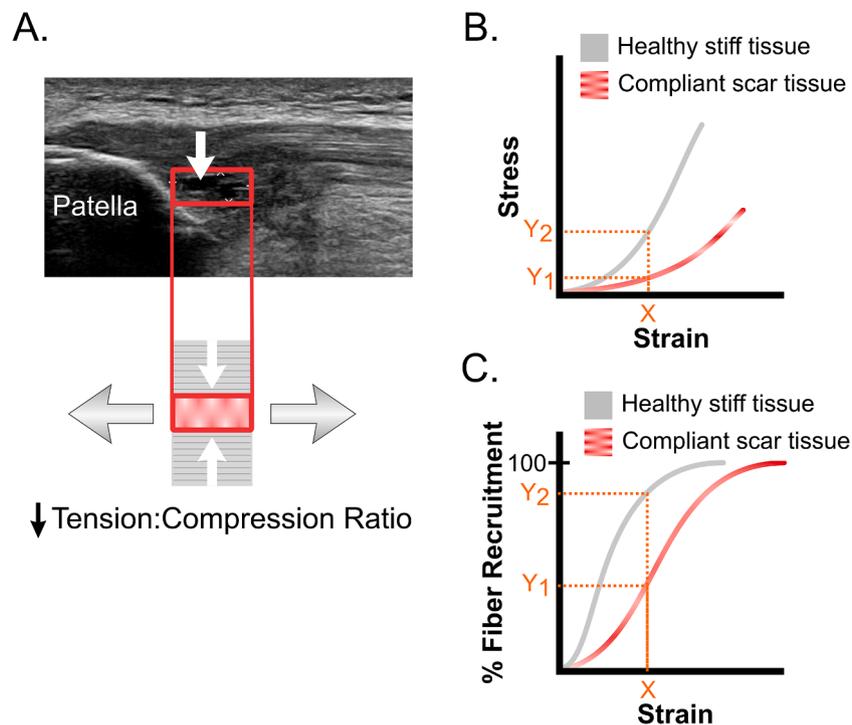


Fig. 3. Stress-shielding in an injured tendon. (A) Ultrasound of patellar tendon in an individual with Jumper's knee. Note the hypoechoic region (white arrow flanked by + and x at the edges) parallel with the tissue with normal signal. This suggests that the injured tissue acts in parallel with relatively healthy tissue, which presumably is stiff in comparison. (B) The stress-strain curves of stiff and compliant viscoelastic materials. Note that at any given strain the stiff material would experience more stress (Y_2) than the compliant material (Y_1) in parallel, thereby stress-shielding the scar. This may lead to deprived mechanosensing that is necessary for healing. (C) Representative fiber recruitment-strain curves for tendon. Due to a higher degree of collagen fiber organization in the healthy tissue, at any given strain the healthy tissue will have engaged a higher percentage of collagen fibers. Therefore, for physiological loads, the scar tissue will be at a deficit in terms of the number of resident cells that receive tensile/shear stimulation.

tissue is sufficient to regenerate the tissue. The third piece of evidence that stress-shielding plays an important role in scar formation comes from a series of experiments carried out in sheep[150,151]. In these experiments, members of the Soslowky lab studied the progression of the exact same injury in fetal and adult sheep tendons. Where the adult tendon formed a scar, the fetal tendon healed in a scar-free manner [151]. Transplanting the fetal tendon to the adult environment had no negative effect on regeneration, suggesting that an environmental factor was not important in scar-free healing[150]. The factor that most likely explained the scar-free healing in the fetal tendon was the fact that it had only ~2% of the stiffness of the adult, potentially allowing a more equal match in mechanical properties between the native and healing tissue.

As a crimped fibrous tissue such as tendon or ligament undergoes strain, the stress-strain response is composed of microscopic material behaviors that determine the mechanical signals that are imparted onto the resident cells. The "toe region" of the stress-strain curve is believed to be caused by the gradual uncrimping, and realignment of individual collagen fibers, whereas in the linear region, all collagen fibers are taut [167,168].

Compared to uninjured tendons, injured tendons with a disorganized ECM exhibit greater ECM realignment across a range of strains[165]. Importantly, computational modeling suggests that compared to native tissue, healing tissues require greater bulk strain before the collagen fibers within the matrix align with the tensile load and contribute to force (Fig. 3C)[165]. Additionally, this may correspond with the scar tissue having a lower percentage of fibers engaged in tension for any given bulk tissue strain seen physiologically. This can result in fewer cells receiving tensile or shear stimuli. At the level of the cell, these effects correspond with lower changes in nuclear aspect ratio (a measure of tensile stress on resident cells) in response to the same strains in post-injury tendons compared to uninjured tendons[165]. If a scar is in parallel with a healthy tendon matrix, at any given strain the scar would

experience less collagen fiber alignment, fiber stress, and cellular stress compared with the healthy tissue (Fig. 3C). In other words, the cells within the scar would be shielded from the stress required for the molecular signals described above to drive maturation.

These mechanisms may also manifest in more pernicious way. Sub-rupture fatigue loading can elicit microscopic levels of damage to collagen fibrils whereby isolated microscopic regions would become more crimped[61,169]. In more extreme cases, microscopic rupture at the level of individual fibers can occur[170]. This likely reflects a local slackening or detensioning of the collagen matrix. Experimental detensioning of tendons has been shown to result in degenerative states, whereby a decrease in mechanical properties is seen, alongside increases in the gene expression of collagen-degrading proteins[89]. Likewise, the detensioning could decrease the substrate stiffness perceived by residing cells, which also would impact the activity of signaling pathways involved in tendon formation, including ERK1/2[171]. Furthermore, a decreased stiffness[165] can result in increased stemness of residing cells, potentially increasing their potential to differentiate towards aberrant fates[171]. Therefore, localized stress-shielding may affect small fractions of cells initiating degeneration, decreasing the overall function of the tissue, and becoming a self-perpetuating cycle that can lead to a more significant injury or rupture.

Other than mechanical fatigue, biochemical alterations may also alter how loads are sensed across the tissue. Since non-enzymatic crosslinks such as AGEs can alter shear stresses randomly throughout the tissue, stress transfer may be impaired in the absence of a direct injury. Aberrant crosslinking could result in cells being shielded from strain while others sense abnormally high forces. Furthermore, these pathological crosslinks may simply prevent shear stress, as they could prevent the sliding of fibers or fascicles relative to one another. Likewise, alterations in lubricating proteins such as lubricin or hyaluronic acid could have similar effects resulting in adhesions that cause impaired

force transfer. Other compositional changes such as increased GAG and water content, fatty infiltration, or calcification may also affect biomechanical behavior, although the effects of those are less well-studied.

In addition to a dampened ability to sense bulk tensile stress, reductions in transverse CSA that result from tension[172,173] can pose further issues. These reductions in CSA may translate to radial compression of cells within the scar. This can be especially true if the injury occurs in the core of the tendon, as is a common feature in the patellar tendon with what is known as jumper's knee[12]. In response to radial compression and the absence of tensile strain (Fig. 3A), the cells would sense compression more than tension, *Mkx* would decrease and chondrogenic genes would increase. As the divergence of tenogenic and chondrogenic differentiation may be a result of the balance between tensile and compressive forces (discussed above), cells within a scar in the middle of a tendon may be uniquely positioned to receive aberrant mechanical signals and shift from a tendon to fibrocartilage phenotype (Fig. 3A).

If the absence of physiological mechanical signals and the presence of aberrant ones drive tendon degeneration, tendons and ligaments should recover scar-free if tensile loads reach the cells within the scar (Fig. 4). Since tendons are viscoelastic tissues, consideration of their time-dependent properties is crucial in this pursuit[174]. Stress-relaxation and creep are inherent to tendons. Stress-relaxation is the decrease in stress as a function of time in response to the same amount of strain, whereas creep is the slow deformation of a material subjected to a constant mechanical stress. As discussed above, a scarred matrix requires greater bulk tissue strain before the collagen fibers within the matrix align with the tensile load. This suggests that loading that permits creep may be better for healing scar than dynamic loads. In support of this hypothesis, loading an injured rat patellar tendon with isometric contractions increased tenogenic genes, whereas a time-under-tension-matched dynamic protocol increased *Col2a1* a chondrogenic gene[39]. Here, it is likely that the isometric contractions resulted in creep within the healthy part of the tissue sufficient to impart tension to the cells within the scar, circumventing stress-shielding and activating the tenogenic program. Over time, this type of isometric loading program has been shown to reverse tendinopathy and return normal tissue structure and function[175].

Physical loading for clinical therapies and tissue engineering

Tendons and ligaments in humans were classically thought of as inert tissues that have little to no capacity for remodeling due to poor

vascularity. However, tendons demonstrate high metabolic activity that varies as a function of loading, suggesting they can adapt to changing demands[176]. Furthermore, protein turnover rates of human tendon and ligament tissue are no different from muscle[177] and protein synthesis following exercise increases more in connective tissue protein than in muscle[178].

Tendon size and stiffness in humans increases as an adaptation to loading[179]. In terms of post-injury healing in rodents and horses, an exercise therapy program helps to recover tissue structure[44,180]. These data indicate a crucial and indispensable role of loading for clinical applications, whether to facilitate native healing or to facilitate progression and prevent degeneration from a variety of surgical interventions. However, this is a highly complex topic since the effect of loading on tissue remodeling varies as a function of the exercise parameters, timing of exercise, and the type of tendon[27,180,181].

In contrast, when human legs are immobilized as a result of spinal cord injury, patellar tendons were found to be more than two-fold less stiff and had nearly a 20 % smaller cross-sectional area compared to healthy controls[182]. This deterioration of tendon mechanical properties associated with unloading in humans can be reduced with exercise training[183]. This effect is independent of systemic confounders, as indicated by increased mechanical function post-tendon repair with passive mobilization of the rabbit TA and canine flexor tendon[133, 184]. Therefore, tendons are highly dynamic tissues that respond to mechanical stimuli with clinically relevant structural and mechanical adaptations. Although immobilization in the form of bracing in the early stages of healing after injury can be necessary to form an initial tissue bridge and improve clinical outcomes[185], targeted and careful mobilization during this stage may accelerate early stages of healing.

Conclusion and future directions

Loading is crucial for the formation and homeostasis of tendon and ligament tissue. This is true for periods of development, adulthood, and in the response to injury. The development and maturation of engineered tissues may similarly be affected by load, as these tissues currently demonstrate limited maturation. In this review, we provide a perspective on potential mediators of load-induced tissue maturation including: *Scx*; *Mkx*; *Egr1*; and *Col3a1*. Each of these factors appear to contribute to tissue formation in a stage-dependent manner and are coordinated by mechanical loading. Likewise, the expression of these factors, as well as many others not discussed, may be detrimentally modified in scar formation due to inappropriate, aberrant, or absent

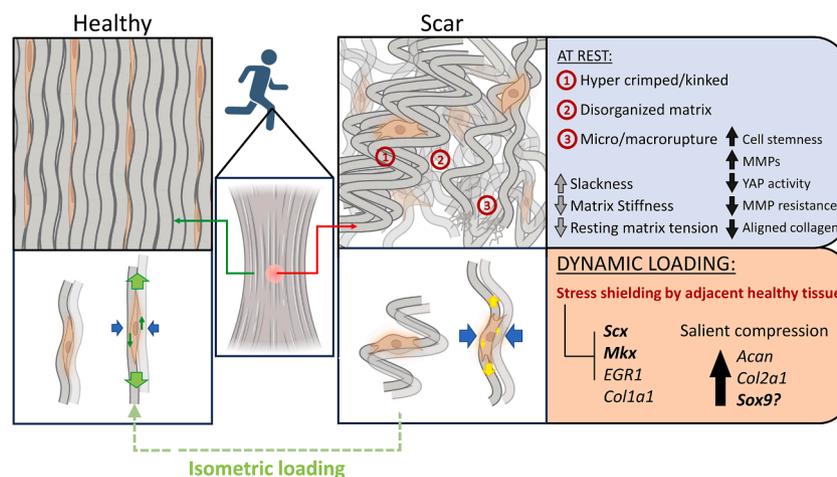


Fig. 4. Structural features of scar may pathologically alter matrix remodeling. The mechanical environment is altered during both rest and dynamic loading, posing mutually exclusive mechanisms that can be targeted for therapies. These pathological mechanisms may be bypassed by strategies to impart physiological load or load-mediated signals (e.g. isometric loading or orthobiologics) onto the tissue's residing cells to restore the activity of mechanically driven developmental signaling pathways and suppress aberrant ones.

forces. Tissue formation, whether through regenerative therapies or tissue engineering, requires a finely tuned profile of mechanically induced signals to facilitate development and maturation.

Tissue engineered models may allow translational obstacles to be circumvented. As tissues whose function is defined by their mechanical properties and based in their structure, it is important that both aspects of these tissues are measured. Unlike traditional *in vitro* models, tissue engineered models allow measurement of tissue remodeling and mechanical adaptation in response to activity or pharmacological stimuli. Unlike *in vivo* models, the mechanical environment is completely controlled and unlike *ex vivo* models, a consistent and specific population of cells within a native matrix can be tested. Therefore, tissue engineered models are well-positioned to elucidate the molecular mechanisms and translate these findings to the clinic. The fact that engineered tendons and ligaments are developmentally arrested means that they are perfectly positioned to discover the loading environment needed for maturation.

Since stress shielding is a likely culprit for the development of tendinopathy, *in vivo* studies to determine whether stress shielding occurs using microstructural analyses including cellular strain and spatial determination of mechanosensing are desperately needed.

Finally, since tendon and ligament repair often manifest as a sequence of processes that fail to completely recapitulate development, targeted exercise and therapies may be developed based on the gene expression of stress-shielded cells. Interventions that shift expression within the scar to resemble that seen during development should have the capacity to promote tendon regeneration and improve clinical outcomes.

With these advances, maybe we can finally move away from the 4500-year-old tradition of immobilizing tendon and ligament injuries [9]. In place of these ancient therapies, targeted early loading using creep and novel pharmaceutical interventions timed based on the stage of repair may reverse tendinopathy and improve quality of life for the hundreds of millions of people suffering from musculoskeletal pain.

Declaration of competing interest

KT has no conflict of interest to declare. KB has received has received grants, consulting fees, speaking honoraria, and donations from nutritional companies such as PepsiCo, Bergstrom Nutrition, and GelTor to study the effect of dietary supplements on matrix synthesis. He is also a founder of SinewUS, a company that is developing tendon loading devices.

Data availability

No data was used for the research described in the article.

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