Tendon mechanobiology in small animal experiments during post-transection healing

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Abstract: Tendon ruptures are common and costly, and no clinical consensus exists on the appropriate treatment and rehabilitation regimen to promote tendon healing and full recovery of tendon functionality. Although mechanobiology is known to play an important role in tendon regeneration, the understanding of how mechano-regulated processes affect tendon healing needs further clarification. A vast amount of small animal studies, particularly in rats and mice, have characterized the progression of healing in terms of geometrical, structural, compositional, mechanical, and cellular properties. Some of the properties are also studied under different mechanical loading regimens. The main focus of this review is to summarize and generalize the current status in the literature regarding spatial and temporal evolution of tendon properties during rodent tendon healing following full tendon-transection, as well as how this is affected by altered in vivo loading regimens.

Keywords: collagen – extracellular matrix – rupture – Achilles tendon – cells – unloading – immobilization – tissue differentiation – mechanical – heterogeneous

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List of abbreviations

ADAMT	A desintegrin and metalloproteinase with thrombospondin motifs
BMP	Bone morphogenic protein
ECM	Extracellular matrix
HIF	Hypoxia-inducible factor
MMP	Matrix metalloprotease
RUNX	Runt related transcription factor
SCX	Scleraxis
SLRP	Small leucine-rich proteoglycans
SMA	Smooth muscle actin
SOX	SRY-box
TIMP	Tissue inhibitors of matrix metalloprotease
TAZ	Transcriptional co-activator with PDZ binding motif
TGF	Tumor growth factor
VEGF	Vascular endothelial growth factor

Prospect of this review

Achilles tendon rupture can have severe long-term implications, such as loss-of-function, range-of-motion, pain, potential re-rupture, and thus can severely affect the quality of life. Yet, consensus on the optimal treatment for Achilles tendon rupture is lacking (Holm et al., 2015). Possibly due to knowledge gaps. During the last decade, an increasing amount of small animal studies, mostly rats and mice, have been performed to characterize the evolution of tendon properties throughout healing. This review aims to summarize recent data from rat and mouse studies on temporal evolution of tendon composition, organization, and mechanical properties post-transection. In addition, we strive to present a generalized overview on how different external loading protocols alters the temporal evolution of tendon properties (Fig. 1), and to identify trends and current gaps in knowledge. Additionally, we hope that it can inspire novel experimental and computational work. Particularly, computational studies of tendon mechanobiology during healing are still scarce (Richardson et al., 2018; Chen et al., 2018; Notermans et al., 2021). In this area, we can learn from other fields of musculoskeletal research that have developed a larger toolbox of adaptive computational models that can aid in identifying, exploring, and predicting important mechanobiological processes during tissue repair.

Introduction

Tendons play an important role in the biomechanical load-transfer of the limbs. Tendon is a load-bearing connective tissue that consists mainly of water (55-70% wet weight) and a highly aligned collagen type 1 matrix (60-85% dry weight) (Taye et al., 2020). The remaining 15-40% dry weight consists of other types of collagens and other ECM proteins, and enzymes (Taye et al., 2020). Intrinsic tendon fibroblasts are few, yet diverse (Kendall et al., 2020), display a low metabolic rate and have a low regenerative capacity (Galatz et al., 2015; Snedeker and Foolen, 2017; Stauber et al., 2019). Therefore, tendon healing depends heavily on extrinsically recruited factors, e.g. angiogenesis (Tempfer et al., 2015; 2018), immune cells, nerve system, and fibroblastic cells from the surrounding tissues (Snedeker and Foolen, 2017) (Fig. 1). Tendon healing displays classical wound healing

characteristic and starts with an initial inflammatory phase, which in rodents lasts for a few days, where the defect is filled with immune cells (Thomopoulos et al., 2014; Graham et al., 2018; Nichols et al., 2019). Subsequently, a fibroblastic/proliferative/reparative phase takes place that lasts for a few weeks in rodents. This second phase is characterized by significant infiltration and proliferation of fibroblasts, as well as extracellular matrix (ECM) production.

Tendon mechanobiology affects healing

Throughout healing, tendon cells in the defect (mainly fibroblasts) respond to mechanical loading by regulating matrix production (Muller et al., 2015). The effects of loading on tendon healing have been characterized previously in comprehensive review articles (Wang et al., 2006; Killian et al., 2012; Wang et al., 2012; Voleti et al., 2012; Muller et al., 2015; Freedman et al., 2014; Thomopoulos et al., 2014; Andarawaris-Puri et al., 2015; Nourissat et al., 2015; Hsu et al., 2016; Graham et al., 2018). This review employs quantitative analyses to generalize the findings of recent publications in order to get an overview of the temporal and spatial evolution of various tendon properties throughout Achilles tendon healing. We searched for all available literature and quantitative data on rat Achilles tendon healing after full transection with or without surgical repair. For mechanobiological analysis we subdivided studies in three different types of loading regimens: loaded - continuous free cage activity; mixed loading - multiple physical activity levels, e.g. 1-week cast immobilization followed by free cage activity; unloaded: continuous treatment that (supposedly) creates decreased physical loading of the Achilles tendon, e.g. intramuscular botox treatment, tail suspension, cast/boot immobilization. In areas where the data is scarce, qualitative findings from other tendons (e.g. patellar or flexor tendon) or other species (mice) were included. In those sections, the species and specific tendon that was investigated are clearly stated in the reported findings. This review is limited to findings from 124 publications on tendon healing (in the Achilles, flexor or patellar tendon) in rodents (rat and mouse).

Collagen levels – genes and proteins

In this section we reviewed 25 studies investigating rat Achilles tendon healing after full tendon transection. Collagen (type 1 and type 3) gene expression are increased compared to intact levels throughout the first 4 weeks of healing (Fig. 2A-B). The increase of collagens appears to take place throughout the first week of healing with a peak sometime between 5-14 days, where collagen type 3 peaks earlier than collagen type 1 (Fig. 2C-F). The shift from predominantly collagen type 3 to type 1 occurs within 2 weeks of healing (Fig. 2G-H).

The temporal evolution of collagen protein content includes a wide range of observations (Fig. 2E-F). Several studies report more collagen type 3 content throughout the first 4 weeks of healing and a shift towards predominantly collagen type 1 content during later healing (Kueckelhaus et al., 2014; Dietrich et al., 2015; Genc et al., 2018). Albeit, histological studies on the temporal shift between collagen type 3 and collagen type 1 are somewhat inconsistent. Many studies show a decrease in collagen type 3 during the first 8 weeks of healing (Majewski et al., 2009; Majewski et al., 2012; Kueckelhaus et al., 2014; Guo et al., 2020), while some other studies show more constant levels of collagen type 3 (Carlsson et al., 2011) or collagen type 1/type 3 ratio during the first 8 weeks (Majewski et al., 2012). Histological findings on collagen type 1 have implied both increasing intensity (Guo et al., 2020; Kueckelhaus et al., 2014) and decreasing positively stained area (Da Silva et al., 2020) throughout 8 weeks of healing. Overall collagen content measured by hydroxyproline assay displayed a minor increase (10% dry weight) in collagen content between 2 and 8 weeks of healing (Kueckelhaus et al., 2014). Female rats displayed more collagen type 3 compared to male rats at the injury site at 6 weeks post-transection (Fryhofer et al., 2016). Histological analysis implied that re-suturing the paratenon increases early collagen formation after 1 week of healing (Muller et al., 2018).

Unloading through 2 weeks of cast immobilization has been shown to decrease the collagen type 3 content (-83%) compared to free cage activity loading (Schizas et al., 2010). However, the collagen type 1 vs. type 3 ratio was not systematically affected by different periods of cast immobilization in combination with and without surgical repair (Freedman et al., 2016; 2017a; 2017b). Unloading through intramuscular botox injection increased both collagen type 1 and type 3 gene expression at 8 days post-transection, yet collagen type 1 gene expression was thereafter lower in unloaded rats at 14 and 21 days post-transection, compared to rats undergoing free cage activity loading (Eliasson et al., 2009). Hammerman et al. (2018) showed how collagen (type 1 and 3) gene expression increases with increased continuous loading (botox+orthosis vs. botox vs. free cage activity) during early rat Achilles tendon healing. Additionally, needling-induced microtrauma also upregulated gene expression of collagen type 1 and 3 similar to loading-induced gene expression, displaying that loading-induced damage may play an important factor in governing matrix production during rat Achilles tendon healing (Hammerman et al., 2013).

Collagen structure and organization

In this section we considered 16 studies investigating rat Achilles tendon healing and 1 in mouse Achilles tendon healing after full tendon transection. Throughout the first four weeks, there was a temporal and spatial evolution of collagen fibril properties (D-spacing, fibril alignment, fibril adhesion, and packing), where most fibril properties did not recover to baseline intact values (Khayyeri et al., 2020). This study also observed a heterogeneous evolution of fibril properties, which implied a stronger collagen matrix maturation in the periphery of the defect. This heterogeneity emphasizes a need for spatial characterization of tendon properties throughout healing. In nonrepaired neonatal and adult mouse Achilles tendon healing, the intact collagen fibril diameter distribution measured by transmission electron microscopy was not recovered within 8 weeks of healing (injury: 30-80nm; intact: 30-230 nm) (Howell et al., 2017). In suture-repaired rats, half (~55nm) of intact average fibril diameter

(~90 nm) recovered after 2 and 4 weeks of healing (Cury et al., 2019). Furthermore, there appeared to be 2 families of fibrils, thicker and thinner. Thinner fibrils were located in the tendon core and thicker fibrils were found in the periphery of the defect after 2 weeks of healing. The average collagen fiber diameter increased between 2 weeks (~2μm) and 6 weeks (~4μm) of healing (Usman et al., 2015).

Studies have implied that loading potentially affects crosslinking. In terms of crosslinking in rat Achilles tendon healing, gene expression for lysyl oxidase increased after small changes in load, from complete unloading (botox injection + orthosis) to unloading by botox injection at 5 days post-transection (Hammerman et al., 2018). This contrasts findings in another study where the gene expression levels were higher in rats that were unloaded by botox injection compared to rats experiencing free cage activity at 8 days post-transection (Eliasson et al., 2009). Still, both these studies imply that loading potentially affects crosslinking and the formation of elastic fibers through loading-dependent expression of lysyl oxidase.

Collagen dispersity decreases throughout healing, but does not return to intact levels of high alignment within the first months (Fig. 3A-B). The bulk of collagen matrix alignment happens within the first 4 weeks of healing (Burssens et al., 2005; Sasaki et al., 2012), but even after 4 months of healing the tendon displays more disorganized collagen alignment than intact baseline (Fig. 3B) (Hsieh et al., 2016; Santos da Silva et al., 2020). Khayyeri et al. (2020) found that collagen alignment measured from small-angle x-ray scattering (fibril scale level) and histology (tissue scale level) showed strong spatial variations throughout the first 4 weeks of healing regardless of loading (botox unloading vs. free cage activity). There is no strong evidence that mixed loading or constant unloading affect the collagen dispersity differently between 3 and 6 weeks post-transection (Fig. 3A-B). However, the collagen dispersity decreases with increasing dorsiflexed angle during cast immobilization (Hillin et al., 2019). There is a lack of experimental data regarding possible sex-dependent differences in collagen

alignment during tendon healing, only Fryhofer et al. (2016) found significantly increased collagen dispersity in male rats compared to female rats at 3 weeks post-transection. However, there was no differences detected after 6 weeks of healing.

Non-collagenous matrix components

In this section we considered 3 studies investigating rat Achilles tendon healing after full tendon transection. Gene expression of proteins that degrade collagen (MMPs) peak at 2-4 weeks of healing, while tissue inhibitors of MMPs peak at week 1-2. Some proteoglycans display increased gene expression throughout the first four weeks of healing, e.g. aggrecan, biglycan and versican, whereas others display decreased gene expression, e.g. decorin and fibromodulin (Sugg et al., 2014). Santos da Silva et al. (2020) used Alcain-blue staining to show that proteoglycan content peaked at 8 weeks in the healing tendon callus, but was significantly decreased at 17 weeks of healing. One study found higher protein levels of elastin in healing tendons compared to intact ones during the first 4 weeks of healing (Svärd et al., 2020).

Geometrical properties

In this section we considered 17 studies investigating rat Achilles tendon healing after full tendon transection. Throughout healing, the cross-sectional area of healing tendons is larger compared to intact tendon, irrespective of treatment (Fig. 4). In addition, the cross-sectional area increases with increased loading (Andersson et al., 2009), early return-to-activity (Freedman et al., 2016), and intactness of paratenon (Muller et al., 2018). There is no systematic difference in cross-sectional area between mixed loading and constant loading (Fig. 4). The cross-sectional area and gap distance increased when comparing rats subjected to free cage activity compared to rats that were unloaded by tail suspension, even when these unloaded rats had daily treadmill training (Andersson et al., 2009).

Female rats appear to display larger cross-sectional area of the healing tendon compared to male rats (Fig. 6A). Additionally, there is no strong evidence for a general effect of suture-repair on temporal changes in geometrical properties (Fig. 6B).

Mechanical properties

In this section we considered 38 studies investigating rat Achilles tendon healing after full tendon transection. Most structural mechanical properties (e.g. stiffness, peak force, and energy) evolve towards intact values within 2-4 weeks (Fig. 5A,C,E). On the other hand, material properties (such as Young's modulus and ultimate stress) do not return to intact baseline values during early healing (Fig. 5B,D,F). In addition, unloading (both mixed and constant unloading) rehabilitation regimens "slow down" the recovery of nearly all structural and material mechanical properties (stiffness, Young's modulus, peak force, peak stress, work and energy) (Fig. 5A-F). There is no strong evidence that mixed loading improves the final recovery of mechanical properties compared to constant unloading (Fig. 5A-F). Female rats display similar structural mechanical properties (stiffness and peak force) as male rats (Fig. 6C,E), but with a decreased Young's modulus and peak stress (Fig. 6G-I). The difference in material properties can be explained by the increased cross-sectional area (Fig. 6A). When comparing healing suture-repaired with nonrepaired tendons, there is no clear difference in evolution of mechanical properties (Fig. 6D,F,H,J). Yet, re-suturing the paratenon has been shown in one study to increase the recovery of mechanical properties (Muller et al., 2018).

Cell populations and distribution

In this section we considered 31 studies investigating Achilles, flexor and patellar tendon healing in rats and mice. It is explicitly mentioned when a study used a different model from the Achilles tendon in rats. There are many different cell types involved in tendon healing such as (myo)fibroblasts, tendon

stem/progenitor cells and immune cells, originating from various sources (tendon core, epitenon, paratenon, tendon sheath, lymphatic, blood, bone-marrow) (Nichols et al., 2019). Yet, thorough characterization of the spatial and temporal distribution and functional properties of these different cell populations during tendon healing is lacking. In general, cell proliferation and cell density peak at around 7-14 days of healing (Galatz et al., 2015) and decrease thereafter, but without returning to baseline levels within 4 or 8 weeks post-transection, respectively (Fig. 7A). There is no strong evidence whether different loading conditions affect cell density. Yet, the work of Palmes et al. (2002) suggested an increase in migration of inflammatory cells at 8 days post-transection for partially mobilized (allowing limited range-of-motion) mice compared to immobilized mice with fixated ankle joints.

The acute inflammatory stage during the first days of healing is characterized by an extensive influx of immune cells (macrophages, neutrophils, mast cells, monocytes, B-cells, and T-cells) that peak throughout the first week of healing and subsequently decrease rapidly in density. However, the number of inflammatory cells does not appear to return to baseline levels within 4 weeks of healing (Fig. 7C). Fibroblast or tendon-like cells (expressing scleraxis, tenomodulin, S100a4, or mohawk) peak around 7-14 days of healing and contribute to matrix production (Sugg et al., 2014), also in mouse flexor tendon (Ackermann et al., 2019a) (Fig. 7A-B). Different rat and mouse tendon healing studies observe strong recruitment and proliferation of extrinsic cells (Snedeker and Foolen, 2017; Dyment et al., 2013, 2014; Best et al., 2019a; 2019b; 2020a; 2020b). In addition, multiple studies show a strong cell-presence at the stump-defect interface. Best et al. (1993) found round cells throughout the defect at 3 days, and more longitudinally aligned fibroblast cells on the interface with the intact stumps at 9 days post-transection in a suture-repaired rat Achilles tendon model. A series of studies investigating repaired mouse flexor tendon healing identified strong presence of macrophages (F4/80), myofibroblasts (aSMA+) and fibroblasts (SCX+, s100a4+) at the tendon stump-interface throughout 4 weeks of healing.

Still, the exact distribution, function and evolution of the different cell populations found during tendon healing is unclear (Nichols et al., 2019).

One well-studied cell population in tendon healing is cells expressing scleraxis. For example, Sakabe et al. (2018) showed in a partial-width injury that mouse Achilles tendon does not heal when the scleraxis (SCX) gene is knocked out, whereas Best et al. (2019a) found that deletion of SCX-lineage cells improved mouse flexor tendon healing (Best et al., 2019a). In addition, Howell et al. (2017) observed that the intrinsic SCX+ cells in neonatal tendon healing displayed high proliferative capacity, whereas intrinsic SCX+ cells remained quiescent in adult mice at day 3 of healing. Consequently, they also showed that the defect was deprived of SCX+ cells at 14 days post-surgery for adult mice whereas neonatal mice had a strong presence of SCX+ cells. In a repaired mouse flexor tendon model, extrinsically recruited SCX+ fibroblasts arrive at the periphery at 8 days post-transection, and migrate into the defect. By 14 days the whole defect is filled with SCX+ and S100a4+ fibroblasts (Best et al., 2019a; 2020a). In a nonrepaired longitudinal injury model in the mouse patellar tendon, SCX+ paratenon cells proliferated after injury and at 14 days post-transection these cells had formed a bridge of cells and newly produced matrix in the periphery of the defect (Dyment et al., 2013). Also, SCX+ and SCX-lineage cells have been reported to contribute to chondroid (cartilage-like) and endosteal (bone-like) cells and tissue regions of trauma-induced heterotopic ossifications in Achilles tendons for rats (Howell et al., 2017) and mice (Agarwal et al., 2017).

Moreover, a significant population of aSMA+ contractile fibroblasts (myofibroblasts) have been identified throughout tendon healing (Howell et al., 2017). Myofibroblasts are thought to contribute to restoring tension in the ECM matrix, and stump-to-stump bridging by enforce wound closure, but they can also contribute to scarring/persistent fibrotic tissue formation, as suggested in a study on mice (Howell et al., 2017) (Nichols et al., 2019). In neonatal (scarless) mice, myofibroblasts contributed to early

(day 3) Achilles tendon healing. Oppositely, in adult mice, myofibroblasts appeared later (day 14) throughout the defect and around blood vessels (Howell et al., 2017). Gene expression of aSMA in adult rats peaked at 7 days (Sugg et al. 2014).

Stem cells have been observed during tendon healing. In a window defect model in rat patellar tendon, tendon stem cells found in tendon periphery migrated, proliferated and activated tenogenic markers in the defect (Tan et al., 2013). In addition, a stem cell-marker (nucleostemin) revealed presence of stem cell-like cells throughout 17 weeks of rat Achilles tendon healing, peaking at 2 weeks of healing (Runesson et al., 2015). Tendon stem/progenitor cells appear to play a role during tendon healing by regulating inflammation during early healing in mouse patellar tendon (Tarafder et al., 2017). Furthermore, tenomodulin in stem cells has been described to regulate fat accumulation and scar formation during early healing (Lin et al., 2017). Tendon stem/progenitor cells have been found to be mechanosensitive through tenomodulin signaling (Dex et al., 2017). Also, platelet-derived growth factor signalling has been described to be critical in tendon stem cell populations for regulating regeneration and fibrosis in mouse patellar tendon (Harvey et al., 2019). The stem cell niche was identified early by Bi et al. (2007) and found to be highly dependent on biglycan and fibromodulin. Restoring this niche may be key for tissue regeneration. Further characterization of the cellular contribution to healing for the different cell populations may be key to reduce scar formation and induce more regenerative tendon healing.

Tissue differentiation

In this section we considered 16 studies investigating Achilles, flexor and patellar tendon healing in rats and mice. It is explicitly mentioned when a study used a different model from the Achilles tendon in rats. Many tendon healing studies have recently identified fat-, cartilage- and bone-related gene markers (Lin et al., 2010; Sugg et al., 2014; Omachi et al., 2015 rat PT; Korntner et al., 2017), cells (Lin et

al., 2010; Howell et al., 2017 mouse AT, Khayyeri et al., 2020; Santos da Silva et al., 2020), and tissue formation (Lin et al., 2010; Hsieh et al., 2016; Howell et al., 2017 mouse AT; Korntner et al., 2017; Misir et al., 2019; Huegel et al., 2019) during tendon healing.

On the cellular level, very limited spatial, temporal and mechanobiological observations have been made concerning differentiation. Throughout healing, many non-tenogenic cell populations are also found, in particular, adipocytes, chondrocyte-like and bone-like cells (Lin et al., 2010; Sugg et al., 2014; Omachi et al., 2015 rat PT; Howell et al., 2017 mouse AT; Korntner et al., 2017; Khayyeri et al., 2020; Santos da Silva et al., 2020). Khayyeri et al. (2020,) saw adipocytes and chondrocyte-like cells throughout the first 4 weeks of rat Achilles tendon healing. Adipocytes inside the newly formed tendon tissue appeared more in rats exposed to loading (free cage activity) compared to unloading (by botox). For the unloaded tendons, adipocytes were located more at the periphery around the healing tendon tissue. Chondrocytes were located closer to the stumps for loaded and unloaded tendons, increasing in numbers towards 4 weeks.

Cartilage and bone formation have been identified through histology (Lin et al., 2010; Hsieh et al., 2016; Korntner et al., 2017; Misir et al., 2019; Santos da Silva et al., 2020) and x-ray tomographic imaging (Lin et al., 2010; Hsieh et al., 2016; Howell et al., 2017 mouse AT; Huegel et al., 2019). In these studies, practically all rats develop cartilage/bone-like tissues of substantial size (~4mm^3 after 6 weeks of healing (Huegel et al., 2019); ~ 7 mm^2 after 16 weeks (Hsieh et al., 2016)). One explanation for this is that pluripotent or tenogenic cells (trans) differentiate into cartilage and/or bone forming cells under the influence of skeletal growth factors (TGF-β1,2,3, HIF-1α, VEGF, BMP-2,4,7, SOX9, RUNX2) (Lin et al., 2010; Nichols et al. 2019). Also, Asai et al. (2014 mouse AT) already showed the potential for tendon progenitor cells to start displaying cartilage-like properties during healing. Lin et al. (2010) identified a potential large role for hypoxia induced factor-1a to induce chondrogenesis. Galatz et al.

(2015) hypothesized that the appropriate (spatio-temporal) signaling to induce tenogenic differentiation in mesenchymal stem cells is missing, rather than an active transdifferentiating process. A study on mouse Achilles tendon, identified a potential role for scleraxis in regulating cartilage formation and ectopic ossification (Sakabe et al., 2018). Interestingly, Howell et al. (2017) found no bone formation in neonatal mouse Achilles tendon. Yet, work on adult mouse Achilles tendon showed that progressive heterotopic ossification affected biomechanical properties (Zhang et al., 2016).

Interestingly, there are some reports that mechanical loading may affect cartilage, fat or bone formation during healing. In a combined burn and tenotomy model in mice, joint immobilization led to no mineralization after 9 weeks of healing, compared to mice subjected to free cage activity, treadmill (1hr/day) or passive range-of-motion exercise (Huber et al., 2020). They found that mobilization increased collagen alignment, cell spreading and TAZ signaling and ectopic bone formation. Oppositely, joint immobilized mice displayed decreased collagen alignment, cell spreading, TAZ signaling, and increased adipocyte differentiation. Another study on suture-repaired mice observed less fibrocartilage formation in mice that were subjected to limited range of motion compared to full joint immobilization, after 16 weeks of healing (Palmes et al., 2002). Similarly, Chen et al. (2017) found that mild joint immobilization led to a decrease in bone volume after 6 weeks of healing compared to full joint immobilization. However, rats allowed free cage activity displayed the largest bone volumes. This study also identified the mTORC1 pathway to regulate mechanically induced heterotopic ossification.

Many questions on cartilage and bone formation during tendon healing remain unanswered. Why/how does the evolution of cartilage or bone regions arise and how do these regions affect tendon function? Do they increase the risk for tendon (re)rupture?

Discussion

In this work, we have summarized and generalized the current literature on spatial and temporal evolution of tendon properties during rodent tendon healing following transection, and how this is affected by in vivo loading regimens. In particular, we have focused on collagen levels, structure, and organization, noncollagenous matrix components, geometrical and mechanical properties, cellular distribution and tissue differentiation. We identify a few distinct gaps in knowledge.

Need for extensive characterization of tendon properties

Continuous loading by free cage activity predominantly has a positive effect on early recovery of mechanical properties during rat Achilles tendon healing. Particularly, considering mechanical properties (e.g. stiffness, Young's modulus, peak force/stress, and energy), all loading scenarios that impose less than free cage activity loading impede the temporal evolution of mechanical properties. However, a generalized understanding of the effect of external loading on the temporal evolution of viscoelastic properties (e.g. stress-relaxation, creep, and hysteresis) and fatigue properties (e.g. cycles to failure and dynamic modulus) is lacking. Besides mechanical characterization, there is a whole spectrum of tendon properties that needs to be investigated to fully evaluate the evolution of tendon function throughout healing, and effects of mechanical loading on tendon healing. To address this, the tendon community has developed elaborate protocols to investigate mechanical, histological, compositional, structural and ambulatory analysis of healing tendons. However, this is an emerging field of research and only a small selection of studies have actually elaborately analyzed the effect of different loading regimens, as well as compared the effect of surgical and nonsurgical repair, on rat Achilles tendon healing.

Mechanobiology: Working towards rehabilitation-like regimens in rat Achilles tendon healing

As stated in Hillin et al. (2019), current AAOS guidelines for rehabilitation therapy in humans describe incremental increase in loading during tendon repair. In rat Achilles tendon healing, several studies have implemented such a rehabilitation regimen which starts with different types of cast immobilization, followed by a period of free-cage activity, treadmill training, and more extensive treadmill exercise (Hillin et al., 2019; Freedman et al., 2016; 2017a; 2017b).

Freedman et al. (2016; 2017a; 2017b), found that surgical repair increased the cross-sectional area. Additional effects of surgical repair varied with (im)mobilization regimen or were minor or absent. For example, surgical repair decreased the number of cycles to failure during fatigue testing in shortly immobilized tendons (1 or 3 weeks immobilization, followed by 5 or 3 weeks of loading), but not in long-term immobilized tendons (6 weeks immobilization) (Freedman et al., 2017a). These findings together with earlier data (Fig. 6B, D, F, H, J), results in a lack of consensus on whether to surgically repair the Achilles tendon or not.

Prolonged duration of cast immobilization has been found to decrease geometrical properties (cross-sectional area), mechanical properties (e.g. strength, cycles to failure) and ambulatory properties (e.g. range-of-motion) (Freedman et al., 2016; 2017a; 2017b). However, long-term evaluation of early cast immobilization (1 or 3 weeks) showed no effect of the immobilization in mechanical, histological, muscle fiber-type and locomotion properties in a long-term follow up at 16 weeks (Freedman et al., 2017b).

An interesting finding described by Hillin et al. (2019) was that an incremental change in ankle immobilization angle, followed by continued immobilization may inflict damage and hinder tendon

healing. Furthermore, more dorsiflexed immobilization angles improved functional tendon properties. However, significant (and unwanted) tendon lengthening, and decreased push-off strength was also observed with this regimen. Therefore, a moderately plantarflexed immobilization angle and early return-to-activity was identified as more successful rehabilitation regimen for non-surgically repaired healing tendons.

Another series of studies (Andersson et al., 2009; Eliasson et al., 2011; 2012) investigated how short periods of treadmill running can affect tendon healing compared to immobilization (through tail suspension) or free cage activity. In general, treadmill running during immobilization increased mechanical properties (e.g. stiffness, peak force), but not to the level of properties in rats with free cage activity (Andersson et al., 2009, Eliasson et al., 2011; 2012). On the other hand, free cage activity led to tendon elongation, which was not observed after short term treadmill running. Furthermore, once a threshold duration of treadmill running was completed (15 minutes/day) the mechanical properties did not increase further. In addition, a single episode of treadmill running only affected gene expression up to 24 hours after running, emphasizing the need for daily mechanical stimulation to enhance healing (Eliasson et al., 2012). Another experiment showed that botox unloading led to improved material properties while more loading mainly resulted in a larger cross-sectional area and thereby increased mechanical strength but not necessarily improved material properties (Andersson et al., 2012).

Studies investigating rehabilitation regimen have not extensively characterized long-term effects of degree of loading on evolution of tendon properties. Altered loading may affect properties throughout early healing but the effect may diminish throughout the remodeling phase of healing. For example, the difference in tendon properties between free cage activity and botox unloaded tendons was minimal after 4 weeks of healing (Khayyeri et al., 2020). On the other hand, 1 or 3 week cast

immobilization had significant effects on tendon healing after 3 (Freedman et al., 2016) and 6 (Freedman et al., 2017a; Hillin et al., 2019) weeks, but had very minimal effects after 16 weeks (Freedman et al., 2017b).

Spatio-temporal heterogeneity of healing

Spatial variation in tendon properties throughout healing has been identified, however it has scarcely been characterized how different rehabilitation regimens affect the heterogeneous distribution in the callus. Sasaki et al. (2012) showed that production of collagen fibers during early rat Achilles tendon healing occurs in a spatio-temporal manner. On a fibrillar collagen level, this heterogeneity was also shown, identifying increased collagen production and/or maturation in the periphery of the defect compared to the tendon core (Cury et al. 2019; Khayyeri et al., 2020). On a cell-level, several studies of mice have started to characterize heterogeneity in tendon healing by analyzing the spatio-temporal distribution of different cell populations in flexor tendons (Ackermann et al. 2017; 2019; Best et al., 2019a; 2019b; 2020a), patellar tendons (Dyment et al., 2013; 2014) studies and Achilles tendons (Howell et al., 2017). These studies describe how intrinsically and extrinsically recruited cells contribute to healing. There is very limited data available on the spatio-temporal evolution of different cell populations, and how mechanical loading may affect this, during rat Achilles tendon healing. Future investigations of this could be essential in identifying and resolving limiting factors in tendon healing. One hypothesis is that throughout healing, mechanical overloading and/or metabolic insufficiency of the tendon core may recruit cells from the extrinsic compartment (Snedeker and Foolen, 2017), potentially stimulating matrix production from the periphery inwards towards the core. In agreement with this idea, a disruption in the external compartment through removal of the paratenon after tenotomy surgery had a detrimental effect on recovery of mechanical properties in healing rat Achilles tendons (Muller et al., 2018). During healing, the paratenon has early appearance of leukocytes, blood vessels and proliferative cells (Chbinou et al., 2004) that can aid early healing. For example, the

recruitment of blood vessels, characterized through gene expression of HIF-1a and angiogenesis marker VEGF, are highly expressed after 2 weeks of healing (Sugg et al., 2014) and subsequently decrease gradually towards 10 weeks after injury (Lin et al., 2010). Interestingly, a recent partial-width transection study showed that modulation of the blood vessel density and size (through an injection of anti-VEGF antibody) showed temporal effects on both mechanical properties and collagen alignment throughout the first 4 weeks of healing (Riggin et al., 2019).

There are no experimental studies quantifying the magnitude, rate, duration or frequency of loading that the Achilles tendon is subjected to during healing. Additionally, there are no spatial and/or temporal experimental characterizations of tissue-level or cell-level deformation or strain throughout healing. Yet, this data could help identifying how certain rehabilitation regimens are related to impaired healing through local mechanical over- or unloading. In particular, the identification of loading-induced damage or microtrauma may help identifying appropriate levels of stimulation throughout healing. Hammerman et al. (2018) showed that free cage activity in healing rats causes microtrauma throughout the first week of healing, which triggers additional matrix production, but also prolongs the inflammatory response. Additionally, early loading may inflict damage and loss of tension in a premature matrix, causing decreased mechanosensing of cells.

Inducing regenerative healing

A main limitation when interpreting experimental work on Achilles tendon healing in rodents is a lack of definitions, understanding and evidence of what 'optimal', 'scarless' and 'regenerative' healing means (Andarawaris-Puri et al., 2015; Galatz et al., 2015). Interestingly, a new 'superhealer' mouse model (MRL/MpJ) has shown improved healing outcome (superior mechanical properties, decreased inflammation, enhanced cell migration), which may allow for identifying key aspects of regenerative healing (George et al., 2020).

In general, there is a lack of long-term studies to determine whether tendon properties (composition, structure, mechanical properties) eventually return to intact/healthy properties and if not, which properties are disrupted the most. Subsequently, the clinical question remains on how to utilize treatments (e.g. (non)surgical interventions, rehabilitation regimen, biomaterials, injections of growth factors) to induce the best possible long-term healing. In this discussion, it also becomes apparent that it is of importance to know how Achilles tendon healing in animal models differs from humans, to judge the clinical relevance of the small animal studies.

Outlook

In this review, a generalized overview of the temporal and spatial evolution of various tendon properties throughout Achilles tendon healing in rats and mice was established. However, more work is needed to characterize temporal and spatial evolution of compositional, structural, mechanical, functional and cellular properties throughout healing. In particular, these studies should investigate the effect of different levels and timing of mechanical loading, on both early and long-term tendon healing. Multiscale characterization of the extracellular (collagen) matrix may be vital to assess tendon regeneration. Additionally, the contribution, spatio-temporal distribution and mechano-sensitivity of different cell populations present during Achilles tendon healing has not been established, which may be key to understand and prevent excessive scar formation.

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Fig. 1. Schematic overview of the main features involved in tendon healing that are discussed in this review. The foremost focus is to identify how in vivo mechanical loading affects all of these processes.

Fig. 2. Temporal evolution for collagen type 1 and 3 gene expression (A-D, G) and content (E,F,H) during early rat Achilles tendon healing. The gene expression (RT-qPCR) and protein content (histology, polarized light microscopy) is normalized to the intact value (A-B) or peak value (C-F) within every study. We also present a ratio, defined as collagen type 1 divided by type 3, for gene expression (G) and content (H). All features are compared between loaded (i.e. free cage activity), mixed loading, or constant unloaded. The data in this figure is based on the following references for loaded (Staresinic et al., 2003; Kashiwagi et al., 2004; Eliasson et al., 2009; Majewski et al., 2009; Carlsson et al., 2011; Jelinsky et al., 2011; Ahmed et al., 2012 Kaux et al., 2012 Majewski et al., 2012; Chamberlain et al., 2013; Sugg et al., 2014; Dietrich et al., 2015; Korntner et al., 2017; Guo et la., 2020; Santos Da Silva et al., 2020), mixed loading (Freedman et al., 2016, Freedman et al., 2017a) and unloaded (Eliasson et al., 2009; Freedman et al., 2016; 2017a) rat Achilles tendons.

Fig. 3. Temporal evolution of absolute (A) and intact-normalized (B) collagen dispersity during rat Achilles tendon healing. Three loading levels: Free cage activity (loaded), unloading followed by loading (mixed loading), and unloaded. The data in this figure is based on the following references for loaded (Santos Da Silva et al., 2020), mixed loading (Freedman et al., 2016; Freedman et al., 2017a; Fryhofer et al., 2016; Hillin et al., 2018; Cheema et al., 2019; Huegel et al., 2019) and unloaded (Freedman et al., 2016; 2017a) rat Achilles tendons. Most studies calculated collagen dispersion

(circular standard deviation) from High-Frequency ultrasound. Santos da Silva et al., 2020, used picrosirius red histology, circular deviation using fast fourier transformation.

Fig. 4. Temporal evolution of intact-normalized cross-sectional area of the healing rat Achilles tendon callus. Three loading levels: Free cage activity (loaded), unloading followed by loading (mixed loading), and unloaded. The data in this figure is based on the following references for loaded (Murrell et al., 1997; Murrell et al., 2008; Andersson et al., 2009; Eliasson et al., 2009; Schizas et al., 2010; Ahmed et al., 2012; Andersson et al., 2012; Black et al., 2012; Kauxet al., 2012; Muller et al., 2016; Majewski et al., 2018; Khayyeri et al., 2020), mixed loading (Andersson et al., 2009; Eliasson et al., 2011; Eliasson et al., 2012; Freedman et al., 2017a; Huegel et al., 2019; Hillin et al., 2019) and unloaded (Eliasson et al., 2009 Schizas et al., 2010; Eliasson et al., 2011; Eliasson et al., 2012; Hammerman et al., 2014; Freedman et al., 2016; Freedman et al., 2017a; Huegel et al., 2019; Khayyeri et al., 2020) rat Achilles tendons.

Fig. 5. Temporal evolution of intact-normalized structural (stiffness; A, peak force; C, work; E) and material (Young's modulus; B, peak stress; D, energy; F) mechanical properties in rats during early Achilles tendon healing. Three loading levels: Free cage activity (loaded), unloading followed by loading (mixed loading), and unloaded. The data in this figure is based on the following references for loaded (Best et al., 1993; Murrell et al., 1997; Kurt et al., 1999; Staresinic et al., 2003; Wieloch et al., 2004; Bol et al., 2007; Majewski et al., 2008; Murrell et al., 2008; Andersson et al., 2009; Eliasson et al., 2009; Schizas et al., 2010 Ahmed et al., 2012; Black et al., 2012; Kaux et al., 2012; Majewski et al., 2012; Muller et al., 2016; Komatsu et al., 2016; Korntner et al., 2017; Usman et al., 2015; Majewski et al., 2018; Muller et al., 2018; Devana et al., 2018; Genc et al., 2018; Misir et al., 2019; Khayyeri et al., 2020; Weng et al., 2020) mixed loading (Andersson et al., 2009; Eliasson et al., 2011; Eliasson et al., 2012; Freedman et al., 2016; Fryhofer et al., 2016; Freedman et al., 2017a; Hillin et al., 2019; Huegel et al., 2019), unloaded (Eliasson et al., 2009; Schizas et al. 2010; Eliasson et al., 2011; Andersson et al., 2012; Eliasson et al.,

2012; Hammerman et al. 2014; Freedman et al., 2017a; Huegel et al., 2019; Khayyeri et al., 2020) rat Achilles tendons.

Fig. 6. Temporal evolution of intact-normalized properties (cross-sectional area; A-B, stiffness; C-D, peak force; E-F, Young's modulus; G-H, Peak stress; I-J) in rats allowed free cage activity during early rat Achilles tendon healing. Comparison between male and female rats (A, C, E, G, I), and non-repaired compared to suture-repaired rats (B, D, F, H, J). The data in this figure is based on the following references for male (Best et al., 1993; Murrell et al., 1997; Kurtz et al., 1999; Staresinic et al., 2003; Wieloch et al., 2004; Majewski et al., 2008; Murrel et al., 2008; Schizas et al., 2010; Ahmed et al., 2012; Kaux et al. 2012; Majewski et al., 2012; Usman et al., 2015; Komatsu et al., 2016; Muller et al., 2016; Devana et al. 2018; Genc et al., 2018; Majewski et al., 2018; Misir et al., 2019; Muller et al., 2018) and female (Andersson et al., 2009; Andersson et al., 2012; Eliasson et al., 2009; Kornter et al., 2017; Khayyeri et al., 2020), as well as for suture-repaired (Best et al., 1993; Bolt et al., 2007; Majewski et al., 2008; Black et al., 2012; Majewski et al., 2012; Usman et al., 2015; Komatsu et al., 2016; Genc et al., 2018; Misir et al., 2019; Weng et al., 2020) and non-repaired (Murrell et al., 1997; Kurtz et al., 1999; Staresinic et al., 2003; Wieloch et al., 2004; Murrell et al., 2008; Andersson et al., 2009; Eliasson et al., 2009; Schizas et al., 2010; Ahmed et al., 2012; Andersson et al., 2012; Kaux et al., 2012; Muller et al., 2016; Kornter et al., 2017; Devana et al., 2018; Majewski et al., 2018; Khayyeri et al., 2020) rat Achilles tendons.

Fig. 7. Temporal evolution of cell densities (#cells/area) for various cell populations (all cells, proliferating cells, tendon-like cells, myofibroblasts, inflammatory cells, stem cell-like cells) measured during early rat Achilles tendon healing. All values are normalized to intact reference values and plotted on a logarithmic scale. All rats experienced free cage activity loading. The data in this figure is based on the following references: Chamberlain et al., 2013; Runneson et al., 2015; Kornter et al., 2017; Hsieh et al., 2016.

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