TENDON MECHANOBIOLOGY IN SMALL-ANIMAL EXPERIMENTS DURING POST-TRANSECTION HEALING

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Abstract

Ruptures to tendons are common and costly, and no clinical consensus exists on the appropriate treatment and rehabilitation regimen to promote their healing as well as full recovery of 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. Many 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 focus of this review is to summarize and generalize the information in the literature regarding spatial and temporal differentiation 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

BMP	bone morphogenic protein
ECM	extracellular matrix
HIF	hypoxia-inducible factor
MMP	matrix metalloprotease
mTORC1	mTOR complex 1
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 DZ binding motif
TGF	tumor growth factor
VEGF	vascular endothelial growth factor

Prospect of this review

Achilles tendon ruptures 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 number of small-animal studies, mostly in rats and mice, have been performed to characterize the recovery of tendon properties throughout healing. This review aims to summarize recent data from rat and mouse studies on temporal healing of tendon composition, organization, and mechanical properties post-transection. In addition, the review strives to present a generalized overview on how different external loading protocols alters the temporal differentiation of tendon properties (Fig. 1) and to identify trends and current gaps in knowledge. Additionally, it is the authors' hope that this can inspire novel experimental and computational work. Particularly, computational studies of tendon mechanobiology during healing are still scarce (Chen *et al*., 2018; Notermans *et al*., 2021; Richardson *et al*., 2018). In this area, researchers can learn from other fields of musculoskeletal research that have developed a larger toolbox of adaptive computational models, which 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, ECM proteins, and enzymes (Taye *et al.*, 2020). Intrinsic tendon fibroblasts are few, yet diverse (Kendal *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 characteristics and starts with an initial inflammatory phase, which lasts for a few days in rodents, when the defect is filled with immune cells (Graham *et al.*, 2018; Nichols *et al.*, 2019; Thomopoulos *et al.*, 2015). Subsequently, a fibroblastic/proliferative/reparative phase takes place, which in rodents lasts for a few weeks. This second phase is characterized by significant infiltration and proliferation of fibroblasts, as well as ECM production.

Tendon mechanobiology affects healing

Throughout healing, tendon cells in the defect (mainly fibroblasts) respond to mechanical loading by regulating matrix production (Müller *et al.*, 2015).

The effects of loading on tendon healing have been characterized previously in several comprehensive review articles (Andarawaris-Puri *et al.*, 2015; Freedman *et al.*, 2014; Graham *et al.*, 2018; Hsu *et al.*, 2016; Killian *et al.*, 2012; Müller *et al.*, 2015; Nourissat *et al.*, 2015; Thomopoulos *et al.*, 2015; Voleti *et al.*, 2012; Wang, 2006; Wang *et al.*, 2012). The present review employed quantitative analysis to generalize the findings of recent publications to obtain an overview of the temporal and spatial evolution of various tendon properties throughout Achilles tendon healing. All available literature and quantitative data on rat Achilles tendon healing following full transection, with or without surgical repair, were analyzed. For mechanobiological analysis, studies were subdivided into 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 reduces physical loading of the Achilles tendon (*e.g.*, intramuscular Botox treatment, tail suspension, cast/boot immobilization). To allow the comparison between the different studies and to judge the recovery of specific properties, data from healing tendons were normalized to data from intact tendons, if these data were presented within the same study or a similar study from the same authors with an identical experimental set-up. In areas where the data were 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 were investigated are clearly stated in the reported findings. The present review is limited to findings from 124 publications on tendon healing (in the Achilles, flexor, or patellar tendon) in rodents (rat and mouse).
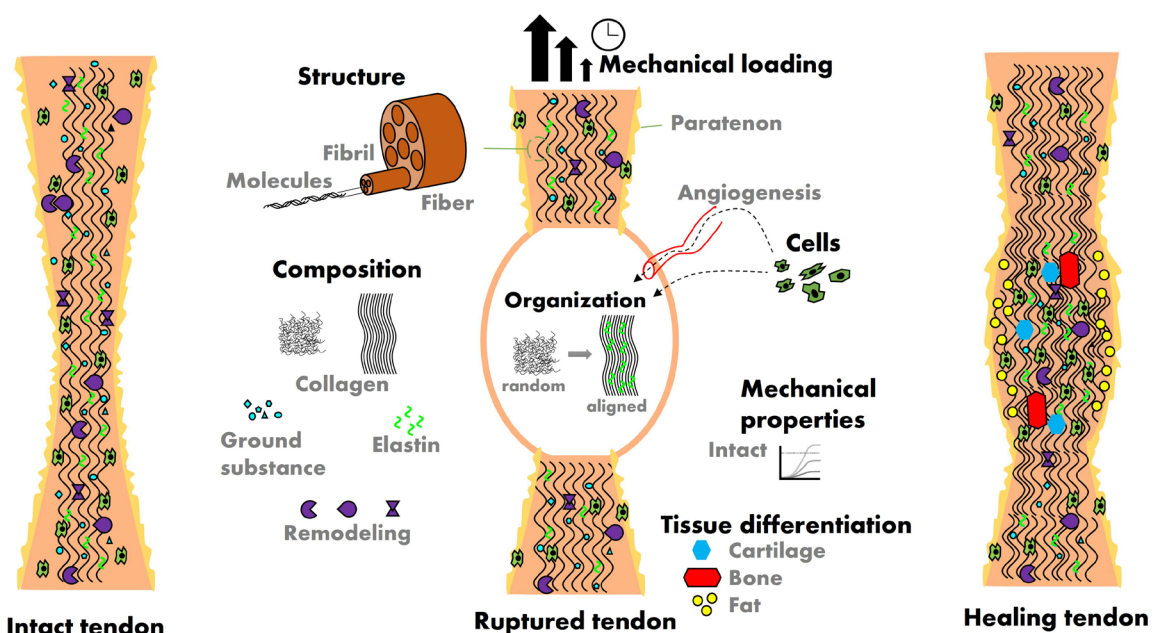

Fig. 1. Schematic overview of the main features involved in tendon healing that are discussed in the present review. The foremost focus is to identify how *in vivo* mechanical loading affects all these processes.

Collagen levels, genes, and proteins

25 studies investigating rat Achilles tendon healing after full-tendon transection were reviewed. Collagen (type 1 and type 3) gene expression is increased compared to intact levels throughout the first 4 weeks of healing (Fig. 2**a**,**b**). The increase in collagens appears to take place throughout the first week of healing, with a peak between 5 and 14 d, where collagen type 3 peaks earlier than collagen type 1 (Fig. 2**c**,**f**). The shift from predominantly collagen type 3 to type 1 occurs within 2 weeks of healing (Fig. 2**g**,**h**).

The temporal differentiation of collagen protein content includes a wide range of observations (Fig. 2**e**,**f**). Several studies report more collagen type 3 content throughout the first 4 weeks of healing and a shift towards a predominantly collagen type 1 content during later healing (Dietrich *et al.*, 2015; Genc *et al.*, 2018; Kueckelhaus *et al.*, 2014). However, 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 (Guo *et al.*, 2020; Kueckelhaus *et al.*, 2014; Majewski *et al.*, 2009; Majewski *et al.*,
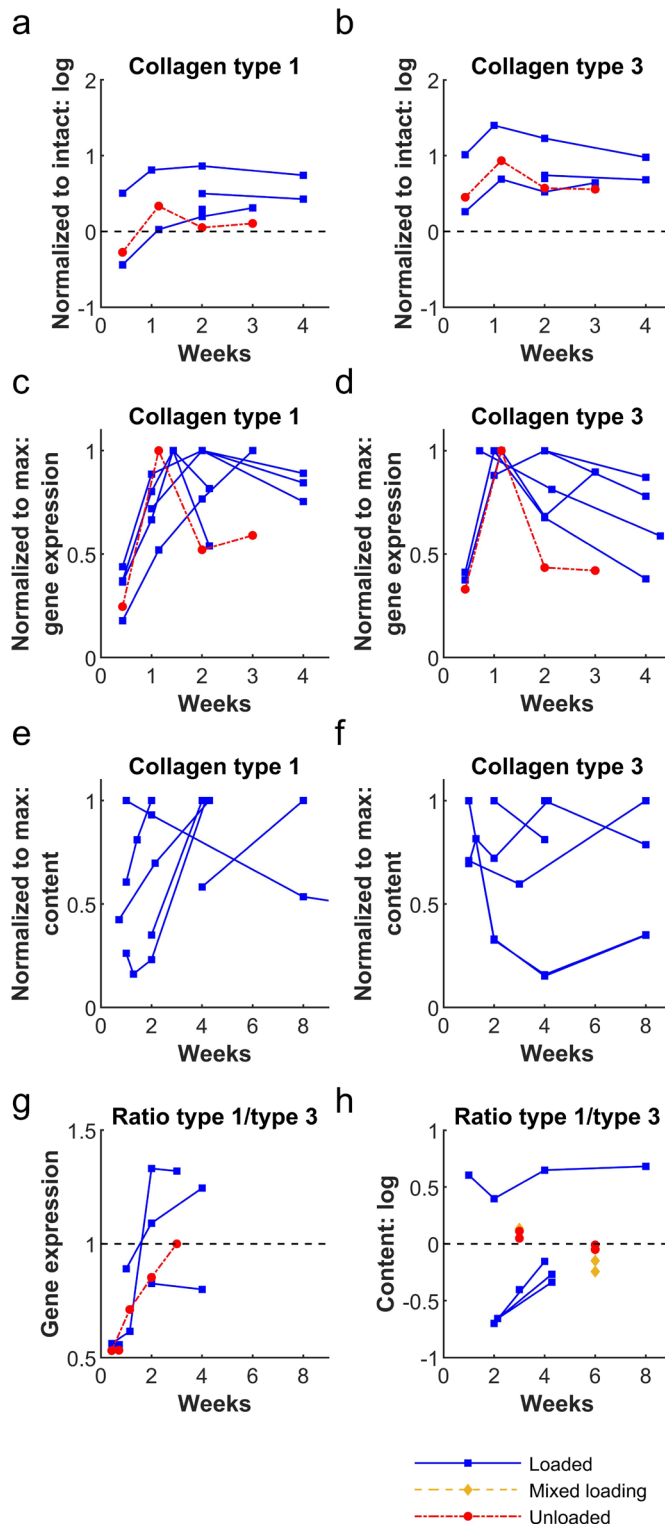

Fig. 2. Expression of collagen type 1 and 3 gene expression (a-d,g) and content (e,f,h) during early rat Achilles tendon healing. Gene expression (RT-qPCR) and protein content (histology, polarized light microscopy) is normalized to the (**a**,**b**) intact value or (**c**-**f**) peak value within every study. Ratio, defined as collagen type 1 divided by type 3, for (**g**) gene expression and (**h**) content. All features are compared between loaded (*i.e.*, free cage activity), mixed loading, or constant unloaded. Data are based on references for loaded (Ahmed *et al.*, 2012; Carlsson *et al.*, 2011; Chamberlain *et al.*, 2013; Dietrich *et al.*, 2015; Silva *et al.*, 2020; Eliasson *et al.*, 2009; Guo *et al.*, 2020; Jelinsky *et al.*, 2011; Kashiwagi *et al.*, 2004; Kaux *et al.*, 2012; Korntner *et al.*, 2017; Majewski *et al.*, 2009; Majewski *et al.*, 2012; Staresinic *et al.*, 2003; Sugg *et al.*, 2014), mixed loading (Freedman *et al.*, 2016, Freedman *et al.*, 2017a), and unloaded (Eliasson *et al.*, 2009; Freedman *et al.*, 2016; 2017a) rat Achilles tendons.

2012), while some others 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 suggest both an increasing intensity (Guo *et al.*, 2020; Kueckelhaus *et al.*, 2014) and a decreasing positively stained area (da Silva *et al.*, 2020) throughout 8 weeks of healing. Overall, collagen content measured by hydroxyproline assay displays a minor increase (10 % dry weight) between 2 and 8 weeks of healing (Kueckelhaus *et al.*, 2014). Compared to male rats, female rats display more collagen type 3 at the injury site at 6 weeks post-transection (Fryhofer *et al.*, 2016). Histological analysis suggests that re-suturing the paratenon increases early collagen formation after 1 week of healing (Müller *et al.*, 2018).

Unloading through 2 weeks of cast immobilization decreases collagen type 3 content (− 83 %) compared to free cage activity loading (Schizas *et al.*, 2010). However, collagen type 1 to type 3 ratio is 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 increases both collagen type 1 and type 3 gene expression at 8 d post-transection, yet collagen type 1 gene expression is thereafter lower in unloaded rats at 14 and 21 d 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 upregulates gene expression of collagen type 1 and 3 similar to loading-induced gene expression, displaying that loading-induced

damage may play an important role in governing matrix production during rat Achilles tendon healing (Hammerman *et al.*, 2013).

Collagen structure and organization

This section examined 16 studies investigating Achilles tendon healing in rat and 1 in mouse, following full-tendon transection. Throughout the first 4 weeks, there is a temporal and spatial differentiation of collagen fibril properties (D-spacing, fibril alignment, fibril adhesion, and packing), where most fibril properties do not fully recover to baseline intact values (Khayyeri *et al.*, 2020). The authors also observed a heterogeneous differentiation of fibril properties, which implies 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 non-repaired neonatal and adult mouse Achilles tendon healing, the intact collagen fibril diameter distribution as measured using transmission electron microscopy is not recovered within 8 weeks of healing (injury: 30-80 nm; intact: 30-230 nm) (Howell *et al.*, 2017). In suture-repaired rats, half (~ 55 nm) of intact average fibril diameter (~ 90 nm) is recovered after 2 to 4 weeks of healing (Cury *et al.*, 2019). Furthermore, there appeared to be 2 families of fibrils, one thicker and the other thinner. Thinner fibrils are located in the tendon core and thicker fibrils are found in the periphery of the defect following 2 weeks of healing. The average collagen fiber diameter increases between 2 weeks (~ 2 μm) and 6 weeks (~ 4 μm) 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 of lysyl oxidase increases after small changes in loading, from
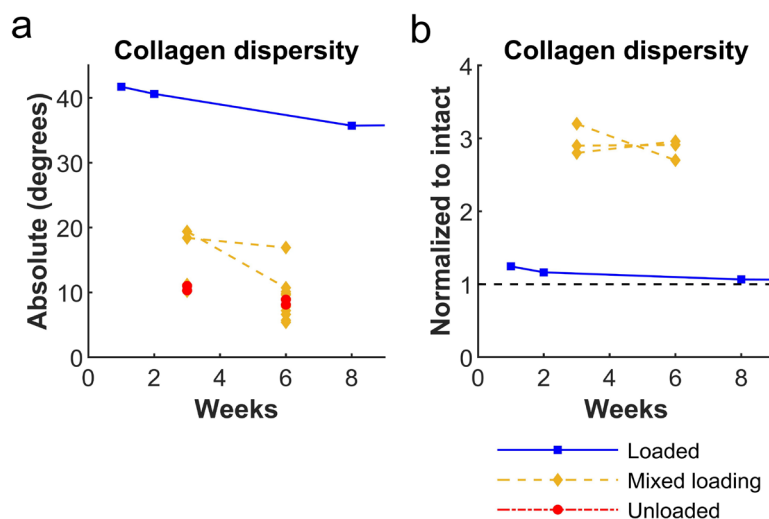

Fig. 3. (a) Absolute and (b) intact-normalized collagen dispersity during rat Achilles tendon healing. Three loading levels: free cage activity (loaded), unloading followed by loading (mixed loading), and unloaded. Data are based on references for loaded (da Silva *et al.*, 2020), mixed loading (Cheema *et al.*, 2019; Freedman *et al.*, 2016; Freedman *et al.*, 2017a; Fryhofer *et al.*, 2016; Hillin *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) based on high-frequency ultrasound. da Silva *et al.* (2020) used picrosirius red staining and circular deviation using fast Fourier transformation.

complete unloading (Botox injection + orthosis) to unloading by Botox injection at 5 d post-transection (Hammerman *et al.*, 2018). This contradicts findings of another study where gene expression levels were higher in rats that were unloaded by Botox injection compared to rats experiencing free cage activity at 8 d post-transection (Eliasson *et al.*, 2009). Nevertheless, 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 the intact-tendon levels of high alignment within the first months (Fig. 3**a**,**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 a more disorganized collagen alignment than intact-tendon baseline levels (Fig. 3**b**) (da Silva *et al.*, 2020; Hsieh *et al.*, 2016). Khayyeri *et al.* (2020) found that collagen alignment measured using small-angle X-ray scattering (fibril scale level) and histology (tissue scale level) shows 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. 3**a**,**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, no difference was detected after 6 weeks of healing.

Non-collagenous matrix components
3 studies investigating rat Achilles tendon healing after full-tendon transection were examined. Gene expression of proteins that degrade collagen (MMPs) peaks at 2-4 weeks of healing, while expression of tissue inhibitors of MMPs peaks at week 1-2. Some proteoglycans display increased gene expression throughout the first 4 weeks of healing, *e.g.*, aggrecan, biglycan, and versican, whereas others display decreased gene expression, *e.g.*, decorin and fibromodulin (Sugg *et al.*, 2014). da Silva *et al.* (2020) used alcian blue staining to show that proteoglycan content peaks at 8 weeks in the healing tendon callus but is significantly decreased at 17 weeks of healing. Svärd *et al.* (2020) found higher protein levels of elastin in healing tendons compared to intact ones during the first 4 weeks of healing.

Geometrical properties

17 studies investigating rat Achilles tendon healing following full-tendon transection were examined.

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 (Müller *et al.*, 2018). There is no clear difference in cross-sectional area between mixed loading and constant loading (Fig. 4). The cross-sectional area and gap distance increases when comparing rats subjected to free cage activity compared to rats that are unloaded by tail suspension, even when these unloaded rats have 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. 6**a**). Additionally, there is no strong evidence for a general effect of suture repair on temporal changes in geometrical properties (Fig. 6**b**).

Mechanical properties

38 studies investigating rat Achilles tendon healing following full-tendon transection were examined. Most structural mechanical properties (*e.g.*, stiffness, peak force, and energy) evolve towards intact values within 2-4 weeks (Fig. 5**a**,**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. 5**b**,**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. 5**a**-**f**). There is no strong evidence that mixed loading improves the final recovery of mechanical properties compared to constant unloading (Fig. 5**a**-**f**). Female rats display similar structural mechanical properties (stiffness and peak force) as male rats (Fig. 6**c**,**e**), but with a decreased Young's modulus and peak stress (Fig. 6**g**-**i**). The difference in material properties can be explained by the increased cross-sectional area (Fig. 6**a**). When comparing healing of suture-repaired and non-repaired tendons, there is no clear difference in recovery of mechanical properties (Fig. 6**d**,**f**,**h**,**j**). Yet, re-suturing the paratenon has been shown to increase the recovery of mechanical properties (Müller *et al.*, 2018).

Cell populations and distribution

31 studies investigating Achilles, flexor, and patellar tendon healing in rats and mice were examined. 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 system, 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 d of healing (Galatz *et al.*, 2015) and decrease thereafter but without returning to baseline levels within 4- or 8-weeks post-transection, respectively (Fig. 7**a**). There is no strong evidence as to 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 d 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. 7**c**). Fibroblast or tendon-like cells (expressing SCX, tenomodulin, S100a4, or mohawk) peak around 7-14 d of healing and contribute to matrix production (Sugg *et al.*, 2014), also in mouse flexor tendon (Ackermann *et al.*, 2019) (Fig. 7**a**,**b**). Different rat and mouse tendon healing studies have observed

strong recruitment and proliferation of extrinsic cells (Best *et al.*, 2019a; 2019b; 2021; Dyment *et al.*, 2013; 2014; Snedeker and Foolen, 2017). In addition, multiple studies have shown a strong cell presence at the stump-defect interface. Best *et al.* (1993) found round cells throughout the defect at 3 d and more longitudinally aligned fibroblast cells on the interface with the intact stumps at 9 d post-transection in a suture-repaired rat Achilles tendon model. A series of studies investigating repaired mouse flexor tendon healing identified a strong presence of macrophages (F4/80), myofibroblasts (aSMA$^+$), and fibroblasts (SCX$^+$, s100a4$^+$) at the tendon stump interface throughout 4 weeks of healing. Nevertheless, the exact distribution, function, and differentiation of the different cell populations found during tendon healing is unclear (Nichols *et al.*, 2019).

One well-studied cell population found in tendon healing is cells expressing SCX. For example, Sakabe *et al.* (2018) showed in a partial-width injury that mouse Achilles tendon does not heal when *SCX* is knocked out, whereas Best *et al.* (2019a) found that deletion of SCX-lineage cells improves mouse flexor tendon healing (Best *et al.*, 2019a). In addition, Howell *et al.* (2017) observed that intrinsic SCX$^+$ cells in neonatal tendon healing display high proliferative capacity, whereas intrinsic SCX$^+$ cells remain quiescent in adult mice at day 3 of healing. They also showed that the defect is deprived of SCX$^+$ cells at 14 d post-surgery in adult mice whereas neonatal mice have a strong presence of SCX$^+$ cells. In
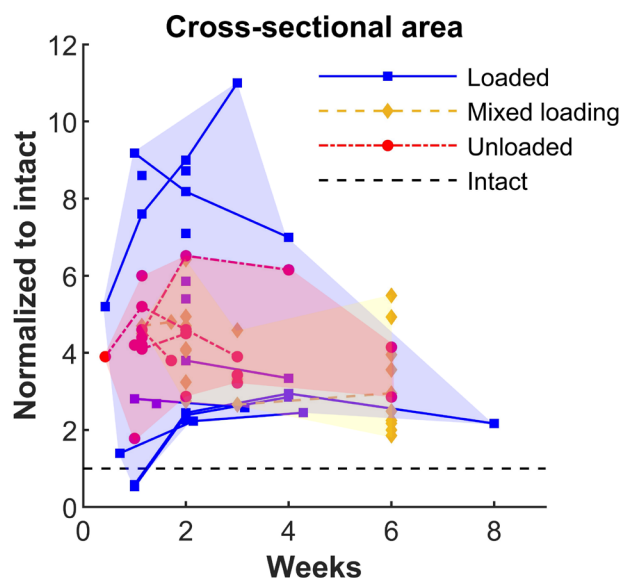

Fig. 4. Temporal differentiation of intact-normalized cross-sectional area of the healing rat Achilles tendon callus. Three loading levels: free cage activity (loaded), unloading followed by loading (mixed loading), and unloaded. Data are based on references for loaded (Ahmed *et al.*, 2012; Andersson *et al.*, 2009; Andersson *et al.*, 2012; Black *et al.*, 2012; Eliasson *et al.*, 2009; Kaux *et al.*, 2012; Khayyeri *et al.*, 2020; Majewski *et al.*, 2018; Müller *et al.*, 2016; Murrell *et al.*, 1997; Murrell *et al.*, 2008; Schizas *et al.*, 2010), mixed loading (Andersson *et al.*, 2009; Eliasson *et al.*, 2011; Eliasson *et al.*, 2012; Freedman *et al.*, 2017a; Hillin *et al.*, 2019; Huegel *et al.*, 2019), and unloaded (Eliasson *et al.*, 2009 Eliasson *et al.*, 2011; Eliasson *et al.*, 2012; Freedman *et al.*, 2016; Freedman *et al.*, 2017a; Hammerman *et al.*, 2014; Huegel *et al.*, 2019; Khayyeri *et al.*, 2020; Schizas *et al.*, 2010) rat Achilles tendons.
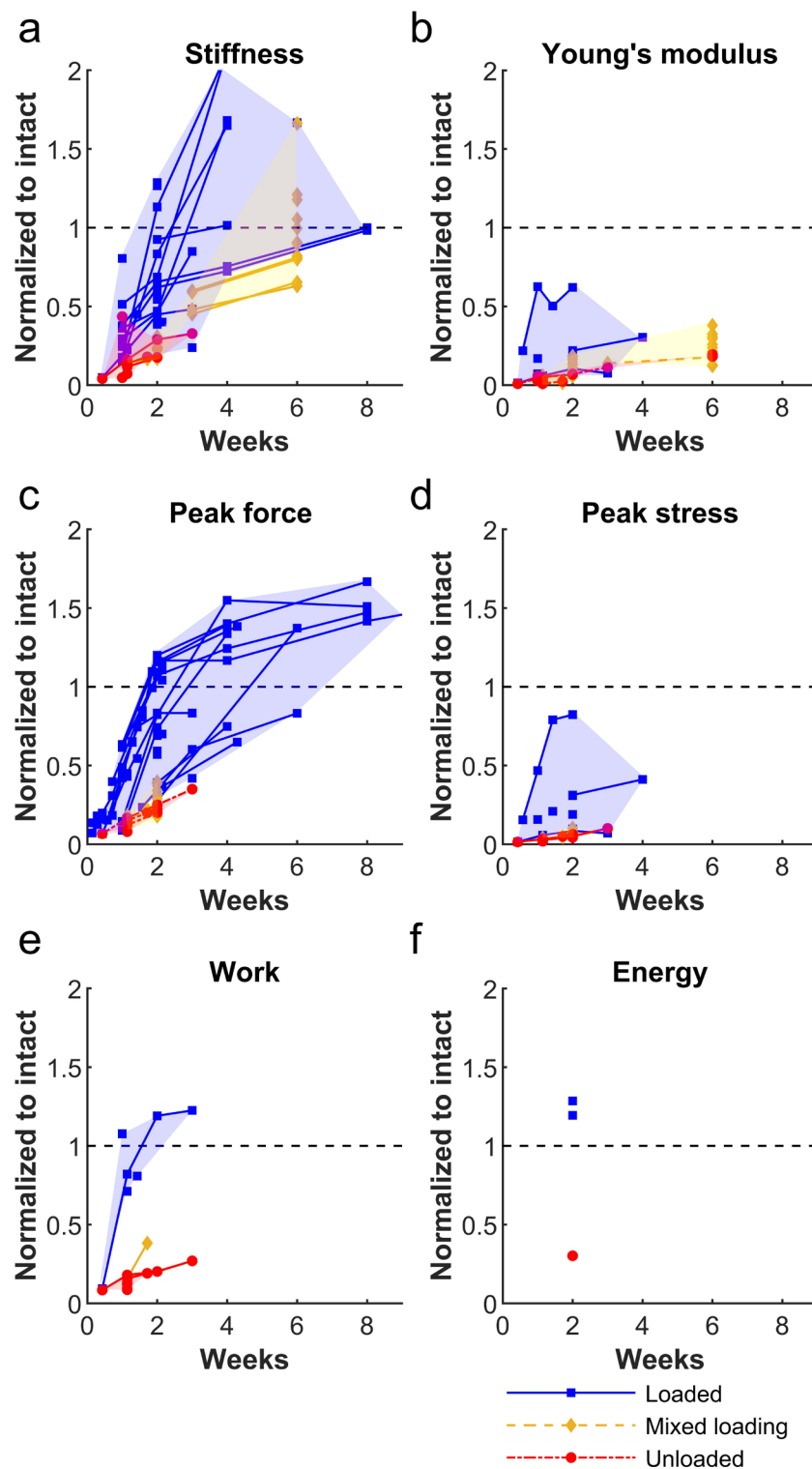
Fig. 5. Temporal differentiation of intact-normalized structural (a: stiffness; c: peak force; e: work) and material (b: Young's modulus; d: peak stress; f: energy) mechanical properties in rats during early Achilles tendon healing. Three loading levels: free cage activity (loaded), unloading followed by loading (mixed loading), and unloaded. Data are based on references for loaded (Ahmed *et al.*, 2012; Andersson *et al.*, 2009; Best *et al.*, 1993; Black *et al.*, 2012; Bolt *et al.*, 2007; Devana *et al.*, 2018; Eliasson *et al.*, 2009; Genc *et al.*, 2018; Kaux *et al.*, 2012; Khayyeri *et al.*, 2020; Komatsu *et al.*, 2016; Korntner *et al.*, 2017; Kurt *et al.*, 1999; Majewski *et al.*, 2008; Majewski *et al.*, 2012; Majewski *et al.*, 2018; Misir *et al.*, 2019; Müller *et al.*, 2016; Müller *et al.*, 2018; Murrell *et al.*, 1997; Murrell *et al.*, 2008; Schizas *et al.*, 2010; Staresinic *et al.*, 2003; Usman *et al.*, 2015; Weng *et al.*, 2020; Wieloch *et al.*, 2004) mixed loading (Andersson *et al.*, 2009; Eliasson *et al.*, 2011; Eliasson *et al.*, 2012; Freedman *et al.*, 2016; Freedman *et al.*, 2017a; Fryhofer *et al.*, 2016; Hillin *et al.*, 2019; Huegel *et al.*, 2019), and unloaded (Andersson *et al.*, 2012; Eliasson *et al.*, 2009; Eliasson *et al.*, 2011; Eliasson *et al.*, 2012; Freedman *et al.*, 2017a; Hammerman *et al.*, 2014; Huegel *et al.*, 2019; Khayyeri *et al.*, 2020; Schizas *et al.*, 2010;) rat Achilles tendons.
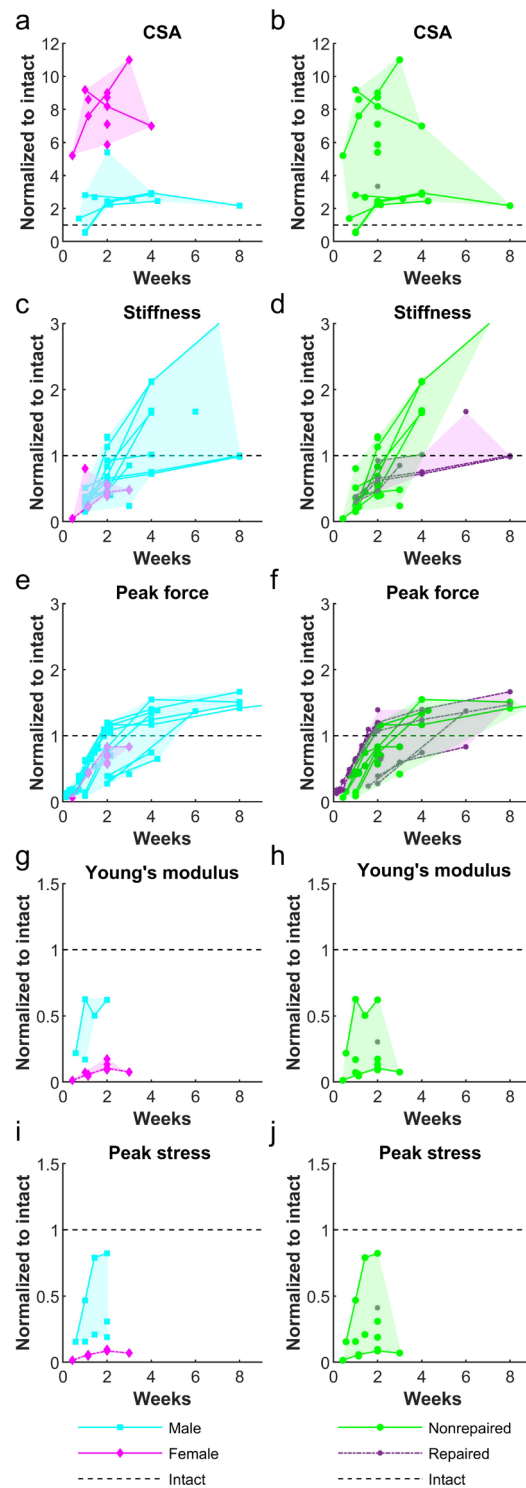
Fig. 6. Temporal differentiation of intact-normalized properties (a,b: cross-sectional area; c,d: stiffness; e,f: peak force; g,h: Young's modulus; i,j: peak stress) in rats allowed free cage activity during early rat Achilles tendon healing. (**a**,**c**,**e**,**g**,**i**) male *vs.* female rats; (**b**,**d**,**f**,**h**,**j**) non-repaired *vs.* suture-repaired rats. Data are based on references for male (Ahmed *et al.*, 2012; Best *et al.*, 1993; Devana *et al.*, 2018; Genc *et al.*, 2018; Kaux *et al.*, 2012; Komatsu *et al.*, 2016; Kurtz *et al.*, 1999; Majewski *et al.*, 2008; Majewski *et al.*, 2012; Majewski *et al.*, 2018; Misir *et al.*, 2019; Müller *et al.*, 2016; Müller *et al.*, 2018; Murrell *et al.*, 1997; Murrell *et al.*, 2008; Schizas *et al.*, 2010; Staresinic *et al.*, 2003; Usman *et al.*, 2015; Wieloch *et al.*, 2004) and female (Andersson *et al.*, 2009; Andersson *et al.*, 2012; Eliasson *et al.*, 2009; Khayyeri *et al.*, 2020; Korntner *et al.*, 2017;), as well as for suture-repaired (Best *et al.*, 1993; Black *et al.*, 2012; Bolt *et al.*, 2007; Genc *et al.*, 2018; Komatsu *et al.*, 2016; Majewski *et al.*, 2008; Majewski *et al.*, 2012; Misir *et al.*, 2019; Usman *et al.*, 2015; Weng *et al.*, 2020) and non-repaired (Ahmed *et al.*, 2012; Andersson *et al.*, 2009; Andersson *et al.*, 2012; Devana *et al.*, 2018; Eliasson *et al.*, 2009; Kaux *et al.*, 2012; Khayyeri *et al.*, 2020; Korntner *et al.*, 2017; Kurtz *et al.*, 1999; Majewski *et al.*, 2018; Müller *et al.*, 2016; Murrell *et al.*, 1997; Murrell *et al.*, 2008; Schizas *et al.*, 2010; Staresinic *et al.*, 2003; Wieloch *et al.*, 2004) rat Achilles tendons.

a repaired mouse flexor tendon model, extrinsically recruited SCX[+] fibroblasts arrive at the periphery at 8 d post-transection and migrate into the defect. By 14 d the whole defect is filled with SCX[+] and S100a4[+] fibroblasts (Best *et al.*, 2019a; 2021). In a non-repaired longitudinal injury model in the mouse patellar tendon, SCX^{+} paratenon cells proliferated after injury and at 14 d 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 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 enforcing wound closure, but they can also contribute to scarring/persistent fibrotic tissue formation, as suggested in a study performed in mice (Howell *et al.*, 2017; Nichols *et al.*, 2019). In neonatal (scarless) mice, myofibroblasts contributed to early (day 3) Achilles tendon healing. Conversely, 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 d (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 the tendon periphery migrated, proliferated, and activated tenogenic markers in the defect (Tan *et al.*, 2013). In addition, a stem cell-marker (nucleostemin) revealed the presence of stem-cell-like cells throughout 17 weeks of rat Achilles tendon healing, peaking at 2 weeks (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 tendons (Tarafder *et al.*, 2017). Furthermore, tenomodulin in stem cells has been described to regulate fat accumulation and scar tissue 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 signaling has been described to be critical in tendon stem cell populations for regulating regeneration and fibrosis in mouse patellar tendons (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 tissue formation and induce more regenerative tendon healing.

Tissue differentiation

16 studies investigating Achilles, flexor, and patellar tendon healing in rats and mice were examined. It is explicitly mentioned when a study used a different model from the rat Achilles tendon. Many tendon healing studies have recently identified the role of fat-, cartilage- and bone-related gene markers (Korntner *et al.*, 2017; Lin *et al.*, 2010; Omachi *et al.*, 2015, rat patellar tendon; Sugg *et al.*, 2014), cells (da Silva *et al.*, 2020; Howell *et al.*, 2017, mouse Achilles tendon; Khayyeri *et al.*, 2020; Lin *et al.*, 2010), and tissue formation (Howell *et al.*, 2017, mouse Achilles tendon; Hsieh *et al.*, 2016; Huegel *et al.*, 2019; Korntner *et al.*, 2017; Lin *et al.*, 2010; Misir *et al.*, 2019) during tendon healing.

At the cellular level, very limited spatial, temporal and mechanobiological observations have been made concerning differentiation. Throughout healing,
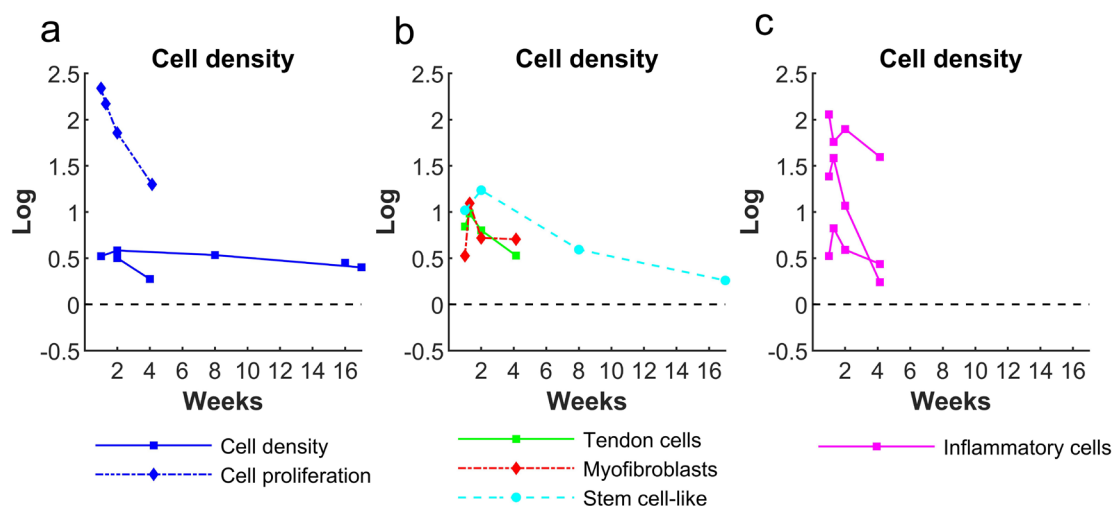

Fig. 7. Temporal differentiation of cell densities ([#] cells/area) for various cell populations (all cells, proliferating cells, tendon-like cells, myofibroblasts, inflammatory cells, stem-cell-like cells) measured during early rat Achilles tendon healing. All values are normalized to intact reference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

many non-tenogenic cell populations are also found, in particular, adipocytes, chondrocyte-like, and bone-like cells (da Silva *et al.*, 2020; Howell *et al.*, 2017, mouse Achilles tendon; Khayyeri *et al.*, 2020; Korntner *et al.*, 2017; Lin *et al.*, 2010; Omachi *et al.*, 2015, rat patellar tendon; Sugg *et al.*, 2014). Khayyeri *et al.* (2020) observed adipocytes and chondrocyte-like cells throughout the first 4 weeks of rat Achilles tendon healing. Adipocytes inside the newly formed tendon tissue appeared to be 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, becoming more numerous towards week 4.

Cartilage and bone formation within tendons has been identified through histology (da Silva *et al.*, 2020; Hsieh *et al.*, 2016; Korntner *et al.*, 2017; Lin *et al.*, 2010; Misir *et al.*, 2019) and X-ray tomographic imaging (Howell *et al.*, 2017, mouse Achilles tendon; Hsieh *et al.*, 2016; Huegel *et al.*, 2019; Lin *et al.*, 2010). In these studies, all animals developed cartilage/bone-like tissues of substantial size [~ 4 mm^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 Achilles tendon) showed the potential for tendon progenitor cells to start displaying cartilage-like properties during healing. Lin *et al.* (2010) identified a potentially significant role for HIF-1α 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 it being an active transdifferentiating process. A study on mouse Achilles tendon identified a potential role for SCS 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 its 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 (1 h/d) or passive range-of-motion exercise (Huber *et al.*, 2020). The authors found that mobilization increased collagen alignment, cell spreading, TAZ signaling, and ectopic bone formation. Conversely, joint-immobilized mice displayed decreased collagen alignment, cell spreading, and TAZ signaling as

well as increased adipocyte differentiation (Huber *et al.*, 2020). Another study on suture-repaired mice showed less fibrocartilage formation, after 16 weeks of healing, in mice that were subjected to a limited range of motion when compared to full-joint immobilization (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 mTORC1 pathway to regulate mechanically induced heterotopic ossification.

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

Discussion

The present review summarized and generalized the information in the literature on spatial and temporal differentiation of tendon properties during rodent tendon healing following transection, and how this is affected by *in vivo* loading regimens. In particular, focus was placed on collagen levels, structure, and organization, non-collagenous matrix components, geometrical and mechanical properties, cellular distribution, and tissue differentiation. A few distinct gaps in knowledge were identified.

The need for extensive characterization of tendon properties

Continuous loading by free cage activity has a predominantly 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 recovery of mechanical properties. However, a generalized understanding of the effect of external loading on the temporal recovery 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 recovery of tendon functions throughout healing and the effects of mechanical loading upon 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 elaborately analyzed the effect of different loading

regimens, as well as compared the effect of surgical and nonsurgical repair, upon rat Achilles tendon healing.

Mechanobiology: working towards rehabilitation-like regimens in rat Achilles tendon healing
American Academy of Orthopaedic Surgeons guidelines for rehabilitation therapy in humans describe an incremental increase in loading during tendon repair (Hillin *et al.*, 2019). 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 (Freedman *et al.*, 2016; 2017a; 2017b; Hillin *et al.*, 2019).

Freedman *et al.* (2016; 2017a; 2017b) found that surgical repair increases the tendon cross-sectional area. Additional effects of surgical repair vary with (im)mobilization regimen or are minor or absent. For example, surgical repair decreases 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. 6**b**,**d**,**f**,**h**,**j**), result 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 immobilization on mechanical, histological, muscle fiber-type, or 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 immobilization angle, followed by continued immobilization may inflict damage and hinder tendon healing. Furthermore, more dorsiflexed immobilization angles improve functional tendon properties. However, significant (and unwanted) tendon lengthening and decreased push-off strength is also observed with this regimen. Therefore, a moderately plantarflexed immobilization angle and early return to activity was identified as being a more successful rehabilitation regimen for non-surgically repaired healing tendons.

Andersson *et al.* (2009) and 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 increases mechanical properties (*e.g.,* stiffness, peak force) but not to the level observed in rats allowed free cage activity (Andersson *et al.*, 2009; Eliasson *et al.*, 2011; 2012). On the other hand,

free cage activity leads to tendon elongation, which is not observed after short-term treadmill running. Furthermore, once a threshold duration of treadmill running is completed (15 min/d), the mechanical properties do not improve further. In addition, a single episode of treadmill running only affects gene expression up to 24 h after running, emphasizing the need for daily mechanical stimulation to enhance healing (Eliasson *et al.*, 2012). Another experiment showed that Botox unloading leads to improved material properties while more loading mainly results in a larger cross-sectional area and thereby increased mechanical strength but not necessarily improved material properties (Andersson *et al.*, 2012).

Studies investigating rehabilitation regimens have not extensively characterized long-term effects of degree of loading on differentiation of tendon properties. Altered loading may affect properties throughout early healing but the effect may diminish throughout the remodeling phase. For example, the difference in tendon properties between free cage activity and Botox unloaded tendons is minimal after 4 weeks of healing (Khayyeri *et al.*, 2020). On the other hand, 1 or 3 week cast immobilization has significant effects on tendon healing after 3 (Freedman *et al.*, 2016) and 6 (Freedman *et al.*, 2017a; Hillin *et al.*, 2019) weeks but 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. At the 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). At the cellular level, several studies in mice have started to characterize the heterogeneity of tendon healing by analyzing the spatio-temporal distribution of different cell populations in flexor (Ackerman *et al.*, 2017; 2019; Best *et al.*, 2019a; 2019b; 2021), patellar (Dyment *et al.*, 2013; 2014), 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 differentiation of different cell populations and how mechanical loading may affect this, during rat Achilles tendon healing. Future investigations 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 to the external compartment through removal of the paratenon after tenotomy surgery has a detrimental effect on recovery of mechanical properties in healing rat Achilles tendons (Müller *et al.*, 2018). During healing, the paratenon presents early appearance of leukocytes, blood vessels, and proliferative cells (Chbinou *et al.*, 2004) that can aid early healing. For example, regarding the recruitment of blood vessels, *Hif1a* and the angiogenic marker *Vegfa* are highly expressed after 2 weeks of healing (Sugg *et al.*, 2014) and decrease gradually towards 10 weeks following injury (Lin *et al.*, 2010). Interestingly, a recent partial-width transection study showed that modulation of blood vessel density and size (through an injection of anti-VEGF antibody) showed temporal effects upon 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, these data could help identifying how certain rehabilitation regimens are related to impaired healing through local mechanical overloading or unloading. In particular, the identification of loading-induced damage or microtrauma may help to identify 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 cellular mechanosensing.

Inducing regenerative healing

The main limitations when interpreting experimental work on Achilles tendon healing in rodents are a lack of definitions, understanding, and evidence as to what optimal, scarless, and regenerative healings mean (Andarawaris-Puri *et al.*, 2015; Galatz *et al.*, 2015). Interestingly, a new 'superhealer' mouse model (MRL/MpJ) has shown improved healing outcomes (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, and mechanical) eventually return to an intact/healthy level and, if not, which properties are disrupted the most. Subsequently, the clinical question remains as to how treatments are utilized [*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 important to know how Achilles tendon healing in animal models differs from humans, in order to judge the clinical relevance of the small-animal studies.

Outlook

In the present review, a generalized overview of the temporal and spatial differentiation of various tendon properties throughout Achilles tendon healing in rats and mice was established. However, more work is needed to characterize temporal and spatial differentiation 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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Discussion with Reviewer

Andreas Traweger: How comparable are the results from the various studies included in this review? What would the authors suggest to the tendon research community to make results more comparable?

Authors: This is of course a very important question. If you consider variability between studies and experimental setups, one of the first things to consider is the variability in the animal model. Although many studies utilize the Sprague-Dawley rat, it is clear that gender, age/maturity, and weight will affect the healing. Also, the exact protocol for the surgery to induce a complete rupture, *i.e.*, scalpel transection, location of the transection, re-suturing of the tendon, re-suturing of the skin, cutting of the plantaris tendon, can all have their effects on healing. Yet, given all these differences, it is fascinating to

see how low the variance is in some experimental measurements. For example, the recovery of the peak force (or ultimate strength) of the tissue (Fig. 5**c**) is quite similar in most studies. This is also a result of the fact that the measurement of the peak force is more straightforward than other mechanical properties (stiffness, fatigue, or viscoelastic properties), and mechanical testing is way more established than, for example, measuring collagen alignment and organization or cell densities (of different populations). All in all, it remains difficult to compare the results between different experiments with completely different experimental protocols. In terms of consistency, one major advantage of the small (yet rapidly expanding) tendon research field is that most experimental work is done within a few laboratories that have been extremely consistent with the protocols for the animal experiments and measurements for all different properties. In addition, many laboratories investigate tendon healing from many different angles, by measuring many different properties *i.e.*, structure, organization, geometry, mechanical

properties, cells, and ambulatory properties within large studies. Also, some studies have investigated both male and female animals from the same species to compare the sex-dependent response in many different properties. In conclusion, there are rather few laboratories that have been building a vast amount of experimental data using consistent protocols, while addressing different hypotheses; however, it remains unclear whether the existing or new research groups should converge towards a single, widely used, animal experiment. Note that the tendon community within the Orthopaedic Research Society (ORS) is in the process of writing and publishing guidelines on streamlining experimental protocols. The authors highly recommend taking these guidelines into account when designing new experiments, particularly for new emerging research groups.

Editor's note: The Scientific Editor responsible for this paper was Denitsa Docheva.