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MUSCLE & TENDON

Tendon healing: a concise review on cellular and molecular mechanisms with a particular focus on the Achilles tendon

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Tendon is a bradytrophic and hypovascular tissue, hence, healing remains a major challenge. The molecular key events involved in successful repair have to be unravelled to develop novel strategies that reduce the risk of unfavourable outcomes such as non-healing, adhesion formation, and scarring. This review will consider the diverse pathophysiological features of tendon-derived cells that lead to failed healing, including misrouted differentiation (e.g. de- or transdifferentiation) and premature cell senescence, as well as the loss of functional progenitors. Many of these features can be attributed to disturbed cell-extracellular matrix (ECM) or unbalanced soluble mediators involving not only resident tendon cells, but also the cross-talk with immigrating immune cell populations. Unrestrained post-traumatic inflammation could hinder successful healing. Pro-angiogenic mediators trigger hypervascularization and lead to persistence of an immature repair tissue, which does not provide sufficient mechano-competence. Tendon repair tissue needs to achieve an ECM composition, structure, strength, and stiffness that resembles the undamaged highly hierarchically ordered tendon ECM. Adequate mechano-sensation and -transduction by tendon cells orchestrate ECM synthesis, stabilization by cross-linking, and remodelling as a prerequisite for the adaptation to the increased mechanical challenges during healing. Lastly, this review will discuss, from the cell biological point of view, possible optimization strategies for augmenting Achilles tendon (AT) healing outcomes, including adapted mechanostimulation and novel approaches by restraining neoangiogenesis, modifying stem cell niche parameters, tissue engineering, the modulation of the inflammatory cells, and the application of stimulatory factors.

Cite this article: *Bone Joint Res* 2022;11(8):561–574.

Keywords: Achilles tendon, Tendon healing, Cell plasticity, Tendon-derived stem cells

Article focus

- Cell biological peculiarities and molecular events which influence the healing response in the Achilles tendon (AT).
- Open questions concerning factors which cause unsuccessful AT healing.
- Outlook on strategies to augment AT healing.

Key messages

- The AT shows a limited healing response.
- Unrestrained inflammation, hypervascularization, and changes in stem and progenitor cell niches might contribute to an inappropriate healing response.
- Individually adapted mechanostimulation is an important prerequisite for regenerative healing.

Strengths and limitations

- This review provides a cell biological point of view on novel aspects of the pathophysiology of AT healing.
- This overview on current literature remains narrative, and does not systematically evaluate the experimental works cited.

Tendon – structure and cells

Macroscopical structure. The Achilles tendon (AT) is the strongest tendon in the human body,[1,2] gaining its crucial importance with the upright gait of humans. It connects the triceps surae muscle, consisting of the medial and lateral heads of the gastrocnemius muscle and the soleus muscle forming the dorsal shape of the calf with the calcaneus

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doi: 10.1302/2046-3758.118.BJR-2021-0576.R1

Bone Joint Res 2022;11(8):561–574.

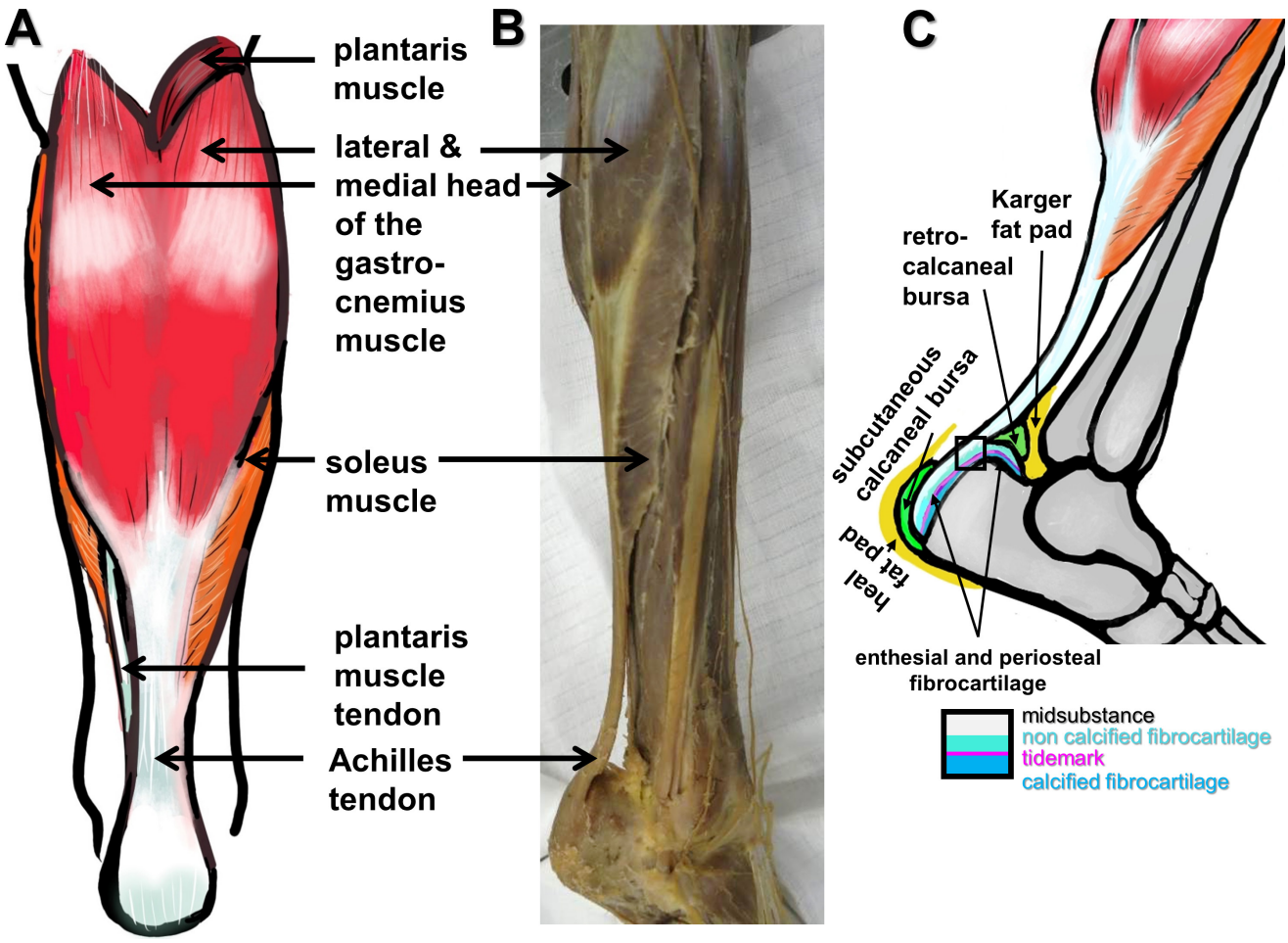


Fig. 1

Macroscopical anatomy of the Achilles tendon (AT). a) Scheme of a dorsal view. b) and c) Dorsolateral views: b) dissection photograph; and c) scheme of the AT, bursae, fat pads, and enthesis zones (inset). a) and c) The images were created by G. G. Schulze-Tanzil using Krita 4.1.7 (Krita Foundation, The Netherlands).

bone tuberosity (Figures 1a and 1b).[1,2] In 65% of cases the tendon of the plantaris muscle associates laterally with the AT (Figure 1a).[2] Thereby, the AT allows the transmission of forces generated by the triceps surae muscle, high enough to compensate a multitude of loading of the body weight[3] as main actor at the ankle joint mediating plantar flexion.[4] Hence, the tendon is highly stressed in sport disciplines associated with jumping, and sudden accelerations and stops, such as football, basketball, and running.[5] The AT is supported by two bursae (retrocalcaneal and subcutaneous calcaneal) protecting it from wear and tear and attached via a fibrocartilaginous enthesis at the calcaneal tuberosity (Figure 1c).[6] These structures are prone to overuse and the bursae and associated fatty tissues (Karger and heal fat pads, Figure 1c) might contribute to inflammatory responses and healing. Intra-articular and joint-associated fat pads including the Karger fat pad (Figure 1c) have multiple functions: they are paracrine-active, modulating inflammation, but also exert biomechanical functions by distribution of loading and protection of neighbouring structures.[7] In addition, they contain sensory nerve endings (mediating proprioception and sensation of pain), and hence can protect tissues from overload during the healing process.[8] Tendons including the AT[9] generally have a poor blood supply. The AT receives its blood supply mainly from the posterior tibial artery and to a lesser extent from the fibular (peroneal) artery.[10] Their branches run in the paratenon.[11] Three territories of vascular supply can be distinguished,[9] with the mid third being nourished by the fibular artery, and the proximal and distal thirds receiving nutrition from the posterior tibial artery. The mid third (around 2 to 6 cm above the calcaneal insertion) of the AT is more hypovascular than both other regions of the AT, and hence prone to injury.[10,12] In the AT there is a central area mostly devoid of blood vessels, and through twisting of the fibre bundles this area can be exposed to compressive forces.[13,14] Individual subtendon twisting patterns of the AT influence energy storage capacity and stiffness.[15] During gait, transverse rotatory forces are low compared to the tensile stretching forces of the AT but under load, the AT also becomes externally rotated

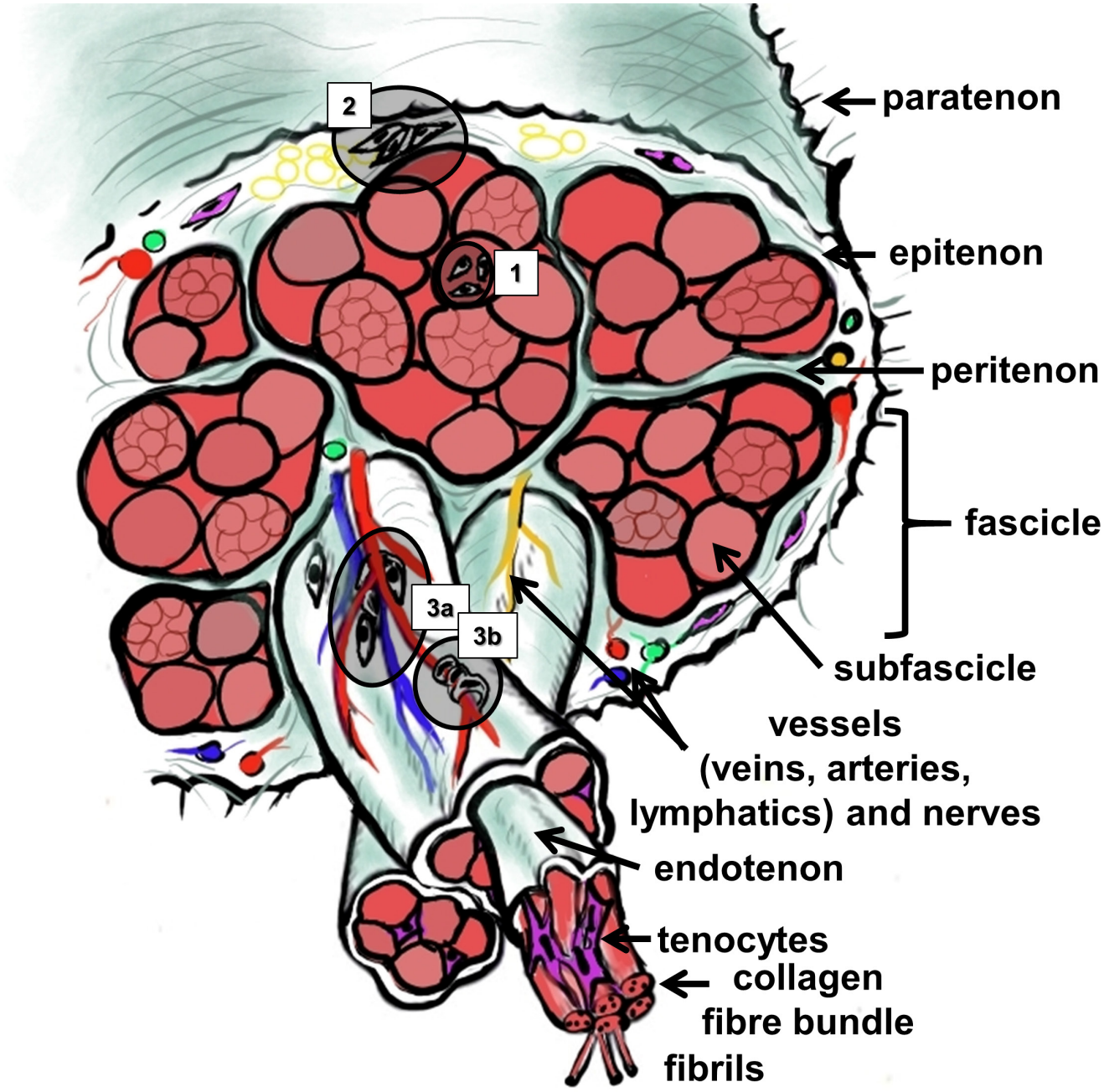


Fig. 2

Scheme of the microscopic anatomy of the Achilles tendon. The stem cell niches are numbered (1: within the tendon proper; 2: within the epi/paratenon; interfascicular niches comprise 3a: perivascular; and 3b: niches within the wall of small vessels, containing pericytes). The image was created by G. G. Schulze-Tanzil using Krita 4.1.7 (Krita Foundation, The Netherlands).

relative to its calcaneal insertion.[16] Despite the blood supply being increased during exercise in this region,[17] the AT is at highest risk of rupturing and failures in healing at its midsection. However, blood supply is further diminished with ageing.[12] Individuals who are characterized by poorer blood supply of the AT midsection have a higher risk of AT rupture.[10]

Microscopic structure. Tendon is a highly hierarchically composed tissue consisting of fascicles and subfascicles,[18] each surrounded by loose connective tissue sheathes (named peri- and endotenon) containing blood and lymphatic vessels and nerves, and the collagen fibre bundles (Figure 2). The entire AT is covered by a connective tissue sheath called epitenon and surrounded by a paratenon, the latter connecting it to the surrounding subcutaneous and adjacent tissue (Figure 2). With regard to force transmission, tendon fascicles with their interfascicular membrane belonging to the peritenon acted as independent units in the AT and lateral force transmission between fascicles was negligible.[19] Nevertheless,

Table I. Markers shown in cell types of Achilles tendon.

Resident cell type in AT	Marker expression	References
Tenoblast/tenocyte	Scleraxis (SCX), Mohawk, TNMD, thrombospondin 4, and Wnt family member, five collagen (COL) type I-expressing tenocyte cell populations: keratin-7/SCX-positive cells, pentraxin-related protein 3-positive cells, TPPP3/proteoglycan 4-positive chondrogenic cells, apolipoprotein D-positive fibro-adipogenic cells, and integrin α7-positive smooth muscle cells.	34,47
Fibrochondrocyte	COL types II, IX, XI, aggrecan, Sox9	25
Endothelial cells, pericytes	CD31, CD34	
SCs/TSPCs		
- interfascicular	CD146	33
- perivascular	Nestin, α-smooth muscle actin (αSMA), CD146*, CD90++*	44,47
- intrafascicular	CD90, stem cell antigen-1, CD44, SCX, tenascin-C, and TNMD	33,39,34
- epi-/paratenon	TPPP3, laminin, αSMA, platelet-derived growth factor receptor α, and osteocalcin	37,41,47

*High level.
AT, Achilles tendon; COL, collagen; SC, stem cell; SCX, scleraxis; Sox9, SRY box transcription factor 9; TNMD, tenomodulin; TPPP3, tubulin polymerization-promotion protein family member 3; TSPC, tendon stem/progenitor cell; αSMA, α smooth muscle actin.

the interfascicular ECM in the AT plays an important role in mediating isoelastic properties and, hence, biomechanics of this tendon.[20] Tendon is a hypocellular tissue consisting mainly of the ECM.[21] The ECM contains large amounts of collagen (mainly of type 1, 70% of dry weight),[22] elastic fibres, few proteoglycans (at least 1% of dry weight),[23] and various glycoproteins such as tenomodulin (TNMD) and tenascin C.[24] The composition of the ECM differs between the midportion and enthesis parts (the latter is mostly fibrocartilaginous) of the AT.[25] Types I, III, V, and VI collagens, decorin, biglycan, fibromodulin, and lumican, were found in both areas with partly differing distribution, whereas type II collagen and aggrecan were only found in the enthesis but versican exclusively in the midportion. This diverse distribution of ECM components correlates with differing mechanical cues.[25] The highly aligned type I collagen, as the main collagen in tendon, is mainly responsible for the mechanical characteristics of the tendon. The thinner type III collagen fibrils build a network with collagen type I, and their occurrence is associated with inferior tensile mechanical properties.[26] Further ECM proteins affect the mechanical properties, but also cross-linking of the proteins.[27,28] The interfascicular ECM provides sliding of tendon fascicles probably among other components supported by its content of lubricin and elastin.[29,30]

Tenocytes and progenitor cells. Tendon cells (Table I) are responsible for maintaining tendon homeostasis by sensation of mechanical signals, thereby allowing the ECM to adapt to mechanical challenges by a fine modulated remodelling process. The main cell population (90%) in tendon consists of tenocytes and tenoblasts.[3] Tenocytes, representing a highly specialized type of fibroblast, are arranged in rows, embracing collagen fibre bundles with their long communicating cytoplasmic extensions. Tenoblasts are less differentiated cells which are still able to divide, and hence can reconstitute micro-damages of the tissue. Recent data of single cell analysis indicate that tenocytes show some heterogeneity representing several subpopulations.[31] In addition to tenocyte populations, fibrochondrocytes, endothelial cells, pericytes, and stem cells (SCs) can be observed in tendon.[31,32] Fibrochondrocytes are localized at areas of the midsubstance of tendon exposed to compressive forces or the enthesis where the AT inserts into bone. Endothelial cells and pericytes are associated with the small capillaries. Pericytes are contractile cells surrounding the capillaries in an abluminal position, able to contribute to blood flow regulation. Moreover, SC capacities can be attributed to them in the AT (Figure 2).[31] Interfascicular SCs and pericytes are characterized by CD146 expression and recruited in AT injury.[33] SCs have been thoroughly described in tendons including the AT.[34,35] However, they seem not to represent a uniform population since their specific localization, with its unique niche characteristics as well as anatomical distribution in the AT,[36] and differentiation capacity, might substantially differ. There are three main regions where tendon stem/progenitor cells (TSPCs) have been localized, namely in the tendon fascicles (proper-derived TSPCs, Figure 2 (region 1)), in the epitenon (paratenon/epitenon-derived TSPCs, Figure 2 (region 2)), and in the vascularized region of the tendon (perivascular TSPCs, Figure 2 (regions 3a to 3b)) (reviewed by Walia and Huang[37] and Huang et al[38]). TSPCs localized in tendon fascicles did not only express the same cell surface markers as bone marrow mesenchymal stem cells (BMSCs), but also CD90, SC antigen (SCA)-1, CD44, scleraxis (SCX), tenascin-C, and TNMD as shown for the patellar tendon or the AT.[34,39] Furthermore, TSPCs confined in the paratenon/epitenon region are characterized by the expression of Tubulin polymerization-promotion protein family member 3 (Tppp3) as shown in the patellar tendon and AT,[40,41] laminin, α-smooth muscle actin (αSMA), platelet-derived growth factor receptor α (PDGFRα),[37,41] and osteocalcin.[41,42] Moreover, detailed transcriptome analysis identified *SCX*, Mohawk (*MKX*), thrombospondin (*Thbs)4*, and *Wnt10a* as genes to distinguish tendon proper progenitors from peritenon progenitors in AT.[43] Recently, using single-cell analysis of non-specified mouse tendons, nestin (Nes) was found to be a novel marker for a perivascular subpopulation of TSPCs with strong tenogenic potential.[44]

In addition, perivascular-TSPCs of the supraspinatus tendon expressed CD146, CD133, Endomucin (Emcn), Musashi1 (Msi1),[45] p75 neurotrophin receptor in patellar tendons,[46] and co-expressed αSMA;[37] the latter could also be shown in those of the porcine AT.[41] Moreover, recent publication based on multiomics single cell analysis of human tendons of different origin including AT identified a perivascular cell population that coexpressed high levels of CD90 and CD146, as well as five distinct collagen type I-expressing tenocyte populations in addition to endothelial cells, T-cells, and monocytes. These five collagen (COL)1A1/2-expressing tenocyte cell populations consisted of keratin-7 (KRT) KRT7/SCX-positive cells, pentraxin-related protein (PTX)3-positive cells, TPPP3/proteoglycan 4 (PRG4)-positive chondrogenic cells, apolipoprotein D (APOD)-positive fibro-adipogenic cells, and integrin α7 (ITGA7)-positive smooth muscle cells.[47] A further possible source of progenitor cells that might be involved in the healing of tendon tissue is the bursa, a friction-reducing tissue covering the joint capsule or underlying tendons or ligaments. Cells with a SC character were identified in the subacromial bursa of the glenohumeral joint.[48-50] Bursa tissue, namely the subcutaneous and retrocalcaneal bursae, is also associated with the AT (Figure 1), and it remains to be investigated whether both bursae could contribute to healing.[50]

Joint-associated fat pads such as the Kager fat pad might, as with the infrapatellar fat pad,[51] contain SCs which can directly contribute to healing by exerting trophic functions, engraftment into tissue defects, and lineage differentiation. The expression profile of SCs in the Kager fat pad and their contribution to AT healing have not been characterized, but their contribution to AT tendinopathy has been outlined.[7]

Tendon healing

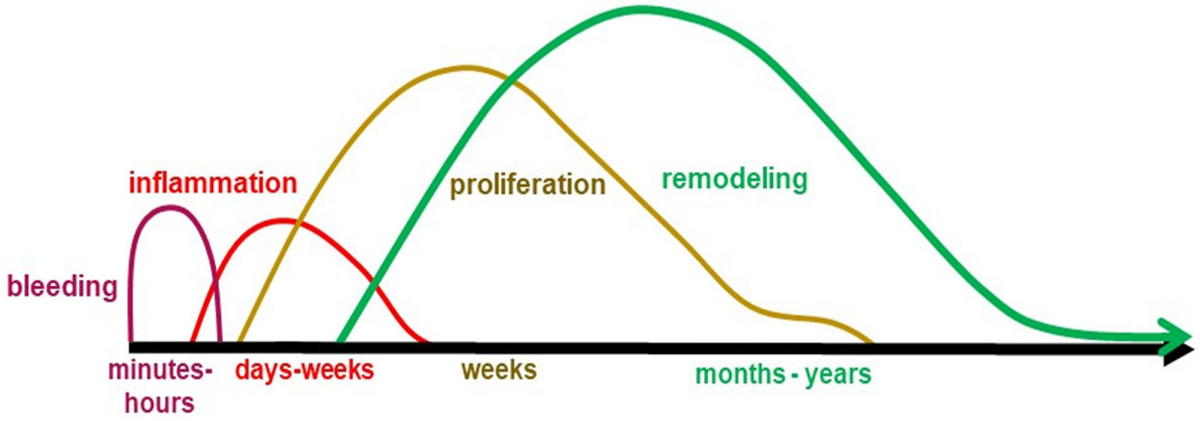
The AT is one of the most frequently ruptured tendons and its rupture represents nearly 20% of all injuries in large tendons, with increasing rates of occurrence.[5,10] It is more common in males and affects men most often at an age between 40 and 50 years.[5] AT rupture often leads to the end of a professional sports career. The AT midsection, characterized by weakest vascularization, is the region of most frequent rupture, 2 to 6 cm proximal of its insertion.[5] Usually, a degeneration of the AT precedes rupture. Eriksen et al[52] found an increased amount of type III collagen at the site of rupture, possibly due to preceding microtraumata and healing associated with lower tensile strength. These unfavourable tissue conditions might limit the subsequent healing process.

Healing phases. The phases of AT healing can be divided into three main phases: inflammation, proliferation, and remodelling (Table II). With the rupture of the tendon, vessels also rupture and a haematoma forms. The haematoma contains immune cells, such as platelets, neutrophils, monocytes, and macrophages. They secrete proinflammatory factors necessary to initiate the repair process (Figure 3). Tenocytes at the rupture undergo apoptosis, while progenitor cells and tenocytes from adjacent tissue invade the rupture zone, proliferate, and differentiate. In the remodelling phase, collagen type III of the initial ECM of the repair callus is replaced by collagen type I. During all three phases, various pro- and anti-inflammatory cytokines, inflammation-resolving factors, and growth factors are expressed, and the timely balanced abundance of the factors is important for the healing progress (for review see Molloy et al[53] and Thomopoulos et al[54]).

Healing outcome. AT healing could bear diverse drawbacks with functional deficits. Unsatisfying outcomes observed in AT repair can comprise an increased length of the AT combined with reduced calf muscle volume and persisting deficits in plantar flexion strength after surgically assisted repair of ruptured AT. Loss in strength and muscle volume of the triceps muscle is partly compensated by hypertrophy of the flexor hallucis longus muscle acting as a synergist, but 11% to 13% deficits in triceps surae muscle volumes and 12% to 18% loss in the strength of plantar flexion persist even after long-term observation.[59] Moreover, shear wave elastography (SWE) revealed lower shear wave velocity (SWV) in unilaterally ruptured AT compared to contralateral non-injured and healthy AT, even two years after rupture.[60] SWV is proportional to Young's modulus and therefore quantifies AT stiffness and material elastic properties.[61] The calculation of tissue stiffness using SWE is based on the principle that shear waves travel faster through stiffer (less elastic material) than through softer tissue. Moreover, stiffness is directly proportional to Young's modulus; hence, lower SWV is indicative for lower modulus and softer tissue.[62] Interestingly, a pilot study using the same technique, but focusing on acute AT ruptures, showed an elevated bisigmoidal SWV over time.[63] A significant increase in tendon elastic properties was observed between the third and the sixth week after AT rupture and a second less substantial one after the ninth week, displaying a time correlation of biomechanical properties with the biological healing stages of tendon tissue.[63] Similarly, Zhang et al[64] showed that repaired tendons gradually became stiffer postoperatively and that tendon functional outcome positively correlated with the elasticity of the repaired AT. While the majority of rehabilitation protocols allow increased weightbearing over time, there is still an enormous variability in rehabilitation protocols for treating AT ruptures (operative or nonoperative, weightbearing, range of motion, physiotherapy, and choice of orthosis), underpinning the necessity for further research of AT response to biomechanical challenges to improve AT healing and avoid degeneration.[65,66]

Degeneration of AT: impact on healing. Degenerative tendon diseases are a common cause of chronic disorders and pain, and eventually promote tendon rupture. In this way, degeneration and ageing affect multiple aspects of the anatomy and physiology of ATs. They lower the metabolic activity of the tendon, as shown for the patellar tendon,[67] and tissue regenerative potential. Tendinopathy due to degeneration therefore describes a

Table II. Tissue changes in the healing Achilles tendon.



Crucial features in healing AT	Effects	Factors	References
Bleeding (hours)	Bleeding and coagulation, cell apoptosis, necrosis		[52-58]
Inflammatory phase (few days)	Emigration of leucocytes from vessels into tissue (leukodiapedesis), particularly macrophages, phagocytosis: removal of cell and ECM debris, release of diverse proinflammatory mediators	TNFα, IL-6, IL-1β, IL-12, IL-17, and growth factors such as IGF-1, TGFβ, PDGF, bFGF, VEGF	
Proliferation phase (weeks)	Intrinsic and extrinsic (from the epitenon) cell activation, cell migration, proliferation, myofibroblast formation, angiogenesis, precursor cell commitment and differentiation. Release of anti-inflammatory mediators and growth factors Comprises the so-called granulation phase: formation of a cell-rich vascularized repair tissue Resolution of inflammation	Anti-inflammatory and inflammation-modulating mediators such as IL-10, PGE_2 and growth factors such as IGF-1, TGFβ, VEGF, PDGF, FGF	
Remodelling phase (months-years) "callus maturation"	Myofibroblast contraction, neo-ECM synthesis (collagen type III > I, later: type I > III) and degradation to allow ECM reorganization, cell and ECM alignment, neo-ECM stabilization by cross-linking	Collagen type III > I, later: type I > III, increase in elastin ECM degrading enzymes, e.g. matrix metalloproteinases (MMPs)	

AT, Achilles tendon; (b)FGF, basic fibroblast growth factor; ECM, extracellular matrix; IGF, insulin-like growth factor; IL, interleukin; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PGE_2, Prostaglandin E_2; TGF, transforming growth factor; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

complex multifaceted pathology of the tendon, clinically characterized by activity-related tendon pain, decline in function, restricted mobility, and disability. In the course of the disease, morphological changes occur within the tendon tissue. Since the tensile strength of the tendon decreases during the degeneration, it can lead to spontaneous tendon rupture, which means ruptures without an appropriate trauma.

Inappropriate and insufficient healing of microdamages can lead to AT degeneration. Degeneration of tendons post injury could be associated with an increase in vascularization with randomly arranged blood vessels and a disordered collagen fibre arrangement with inhomogeneous distribution of collagen, irregular crimp formation of fibre bundles, and an elevated fraction of collagen type III compared to type I.[52] Many studies have shown that degenerative tendon diseases, e.g. in the rotator cuff, preferentially develop in hypovascular regions.[68] Degeneration reduces the mechanical properties of tendons and predisposes them to re-injury due to disorganization of collagen fibrils and neovascularization in the context of inflammation. Studies on tendinopathy have revealed that vascularization is critical in tendon healing, demonstrating direct impairing effects of neoangiogenesis on biomechanical properties as shown for degenerative patellar tendons.[69] Age-related structural changes such as decrease in collagen content may also impair tendon healing after degeneration and injury. The prominent histological and molecular features of degeneration include tendon thickening, disorganization of collagen fibrils, an increase in the microvasculature and sensory nerve innervation, dysregulated ECM homeostasis, increased immune cells and inflammatory mediators, and enhanced cellular apoptosis.[70,71] The key features of tendon degeneration are summarized in Table III.

Inflammation in AT healing. Inflammation is decisive for the outcome of AT healing and bears the risk of AT

Table III. Key tissue changes in the degenerated Achilles tendon.

Key features in degenerated AT	Effects	References
Increase in collagen type III / decrease in type I	Biomechanical stability decreases	52,52,72
Fibre arrangement: tendon thickening, disorganization of collagen fibrils	Change in biomechanical properties (> stiffness, < elasticity)	70,71,73
Increased angiogenesis, irregular arrangement	Impaired biomechanical properties, VEGF↑, MMP-3↑	69,72
Increased nerve ingrowth	Pain	74
Misrouted SC differentiation	Ossification, adipogenesis	75,76,77
Transdifferentiation, e.g. myofibroblast transition	Contraction, scarring	78,79

AT, Achilles tendon; MMP, matrix metalloproteinase; SC, stem cell; VEGF, vascular endothelial growth factor.

degeneration. Like in every tissue of the body, immune cells can also be found in AT. Tendon pain, oedema, and inflammation represent the immediate response to injuries associated with emigration of leucocytes from vessels into tissue (leukodiapedesis) and an early healing response.[80] In a very elaborative study, Ribitsch et al[81] compared fetal and adult tendon healing and saw regeneration in the fetal tendons, while the adult tendons formed scar tissue. In contrast to fetal tissue, adult tissue was characterized by fewer macrophages but increased neutrophils associated with an increased detection of neutrophil-specific proteins. In human ATs, rupture affects the expression of inflammatory cytokines with decreased expression of IL-33, but increased expression of IL-6, IL-10, CD68, and macrophage inhibitory factor-1 (MIF-1) compared to an intact tendon.[82] Dakin et al[83] summarized in a review that not only inflammation, a natural process during healing, but the resolution of inflammation is also important for tendon healing. Inflammation activates a highly regulated process with specialized pro-resolving mediators allowing healing. The group furthermore showed that pro-resolving mediators, such as lipoxin and maresin, modulate the inflammatory status of stromal cells from AT ruptures.[84] IL-1β stimulated cells co-incubated with the pro-resolving mediators showed reduced levels of inflammation initiating factors and increased levels of pro-resolving mediators. To improve healing, exosomes derived from tendon SCs were injected, and notably reduced inflammation (by modulating macrophage polarization and cytokine expression) and apoptosis were seen in healing rat ATs.[85] Characterization of the effect of exosomes revealed a dosage-dependent stimulation of tenocytes' proliferation and migration.[85] The influence of inflammation on the performance of tendon SCs and tendon healing is summarized by Vinhas et al.[86]

Tenocyte cross-talk with immune cells in AT healing. The exchange of exosomes and soluble mediators might also play a role in tenocyte communication with leucocytes. Tenocytes are well known to interact with blood-derived leucocytes via soluble mediators. The presence of peripheral blood mononuclear cells (PBMCs) induced tenocyte proinflammatory gene expression (IL-1β, tumour necrosis factor (TNF)-α, IL-6) in an autologous Achilles tenocyte/PBMC indirect co-culture system; this suggested that the interplay between both cell types was mediated by the exchange of soluble factors.[87] Tenogenesis of equine adipose tissue-derived SCs, e.g. shown by SCX expression and proliferation, was compromised in the presence of leucocytes.[88] Macrophage tenocyte interactions in particular occur in tendon injury and healing processes. An abnormal macrophage number and profile accompanied by hypervascularization, as well as erroneous ECM deposition, was identified in the early phase of tendon repair following surgically induced AT injury in mice deficient of TNMD (a mature gene marker for tendons).[89] Macrophages are required to remove damaged cells and ECM fragments. However, it was observed that M1-polarized macrophages mediate tendon inflammation and degeneration.[90] In contrast, anti-inflammatory M2 macrophages supported AT healing.[91] They could be generated by exposing macrophages to MSC-derived exosomes.[91] An improvement in AT healing could be achieved by reducing M1-polarized macrophages and generating a shift to the M2 phenotype to stimulate regenerative processes.[91] The direct but also indirect interaction (via soluble mediators) of macrophages with co-cultured tenocytes derived in this case from the supraspinatus tendon led to an increase in CD80, but reduced HLA-DR expression on macrophages, indicating their mixed type of polarization, and evoked an increased release of IL-6, IL-8, and monocyte chemoattractant protein-1, predominantly after inflammatory pre-stimulation of tenocytes.[92] In general, proinflammatory cytokines, as well as MMPs and chemokines, are part of the senescence-associated phenotype shift to a senescence-associated secretory phenotype (SASP, Figure 3), which affects paracrine senescence, immune cell invasion, and chronic inflammation.[93]

Pathophysiology of tendon tissue and cells affecting AT repair

De- and transdifferentiation in tendon healing. Tenocytes are known to undergo phenotypic shifts under pathophysiological conditions and during the first passages of in vitro culture, as observed in tenocytes isolated from ruptured AT.[94] In cultured chick embryonic tenocytes, a maximum of four passages was recommended to avoid phenotypic shift and senescence.[95] The transition from terminally differentiated cell to a less-differentiated state within the same lineage is known as dedifferentiation.[96] In tendon biology, this effect has been described with tenocytes reverting to tenoblasts during healing.[97] Tan et al[98] showed recently, using a tissue-specific conditional TGFβ type II receptor-knockout model (Tgfbr2; ScxCre),

that upon the loss of TGFβ signalling most of the mutant tendon cells not only lost SCX, COL type I α 1 chain (*Col1a1*), and TNMD gene expression, but also acquired some stem/progenitor features such as SCA-1, CD44, and CD34 expression. However, it was also stated that loss of TGFβ might prevent these cells from gaining the total spectrum of stemness or plasticity.[98]

The term 'transdifferentiation' refers to the cell regression to a point from which a switch to another lineage can occur.[96] Unsuccessful tendon healing associated with aberrant cell differentiation towards myofibroblasts can result in erroneous ECM deposition and fibrosis[78,79] and trauma-induced heterotopic ossification (HO) via chronic inflammation[99] or endochondral ossification (Figure 3).[100] Interestingly, loss of TNMD in mice could lead to misrouted differentiation towards adipocytes in the early repair phase,[89] and osteocytes via endochondral ossification in the late repair phase.[101] The presence of adipocytes might weaken the compact tendon structure, structural integrity, and biomechanical properties, thereby increasing the risk of rupture.[102] In addition, these cells are known to be paracrine-active,[8] hence it can be hypothesized that paracrine signals from adipose tissue-derived cells might also interfere with AT homeostasis. It has been shown that the inflammatory status of adjacent tissue, such as Kager's fat pad, is increased in patients suffering from tendinopathy.[7] Maintenance of the tissue-specific differentiated phenotype is guaranteed by epigenetic regulators.[103] Changes of epigenetics including the de-/methylation of DNA and modifications of histones, which organize DNA packing and accessibility for transcription as well as non-coding RNAs, might alter SC lineage differentiation by influencing gene activities. Ageing can lead to the inability of cells to maintain their differentiated phenotype and then ultimately to cell senescence,[103] and might therefore reduce the healing capacity of the cells.

Tendon tissue ageing, cell senescence, and premature cell death. In AT, the natural ageing process contributes directly to and correlates with the occurrence of ruptures, and alters both, the cellular and ECM compartments.[5] Some of the best-described features associated with the ageing process at a cellular and molecular level are cell cycle arrest,[104] the decrease of cell proliferation capacity, and increased cellular senescence.[76,105] Senescence describes 'cell ageing', and it is known to be triggered by epigenetic alterations such as DNA methylation and histone modifications in MSCs,[106,107] as well as by DNA damage accumulation caused via reactive oxygen species[108] and by telomere erosion, leading to the activation of the $p14^{ARF}$ and $p16^{INK4A}$ tumour-suppressor pathways in SCs.[109]

A detailed comparison of the cellular and molecular characteristics of human TSPCs revealed increased senescence in the cells derived from aged/degenerative AT, namely increased number of β-galactosidase (β–gal) positive cells and enhanced expression of $p16^{INK}$.[76] Similarly, a recent study demonstrated that a higher number of senescent cells can be isolated from aged rat ATs.[110] Senescence was associated in aged tenocytes with the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Figure 3). Inhibition of this pathway reduced cellular senescence and SASP, as shown by reduced expression of inflammatory and catabolic mediators including IL-6, IL-1β, MMP-3, MMP-9, and CXCL12, thereby restoring the cell function. Moreover, it was demonstrated that the incubation of young tenocytes with proinflammatory IFN-γ promoted senescence.[110] Interestingly, Yan et al[111] demonstrated that aged TSPCs are also less competent at forming 3D tendon organoids in vitro, suggesting that such cells will fail in building tendon tissue during repair. Among other features, aged tendons present compositional, structural, and biomechanical modifications that slow down repair. Studies have shown that ECM gene expression of collagen types I, III, and IV, elastin, and lubricin is decreased due to ageing.[112,113] Conversely, the activation of genes related to ECM remodelling such as MMP-2, MMP-8, and MMP-9 is strongly increased with age.[114,115] Analysis of collagen fibril diameter in human and murine ATs from three- and 18-month-old animals revealed increased presence of small fibrils in the neonatal period versus increased content of larger and higher variability of fibril diameters in adults.[73,112] The diminished and inferior viscoelastic properties in aged tendons are associated with the enhanced cross-linking of collagen,[116] leading to an increased cross-sectional area of the AT as well as AT stiffening.[117] Further intensive research is needed to unravel the role of ageing in AT repair.

Loss of instructive cell niches. Regarding cellular niches in tendon, inflammation might influence the performance of tendon SCs and niche characteristics.[86] Also, changes in the tendon ECM properties can affect cell behaviour. For example, Bi et al[39] demonstrated a crucial role of two ECM components – biglycan and fibromodulin. Depletion of these proteoglycans greatly altered TSPC proliferation and differentiation due to alterations in the bone morphogenetic protein signalling pathway.[39]

A novel strategy to achieve cell rejuvenation is supplying cells with instructive niches and providing proper surface topography (e.g. by surface roughness), the appropriate micro-nanoscale organization (e.g. fibril size, distribution and alignment), as well as stiffness (Young's modulus). For example, when provided with a 3D self-assembling nanofibre hydrogel, aged human TSPCs showed a comparable cell survival and proliferation to young TSPCs as well as restored cell morphology and cytoskeletal architecture.[118] Jiang et al[119] could demonstrate that decellularized ECM from young TSPCs enhanced cell proliferation and differentiation potential of aged TSPCs, and reduced cell senescence. Hence, the identification of novel regulatory biochemical, topographical, and biomechanical factors in the tendon cellular niches during tissue homeostasis, and the ageing and healing processes, would be of great relevance for the tendon field. Expanding such knowledge

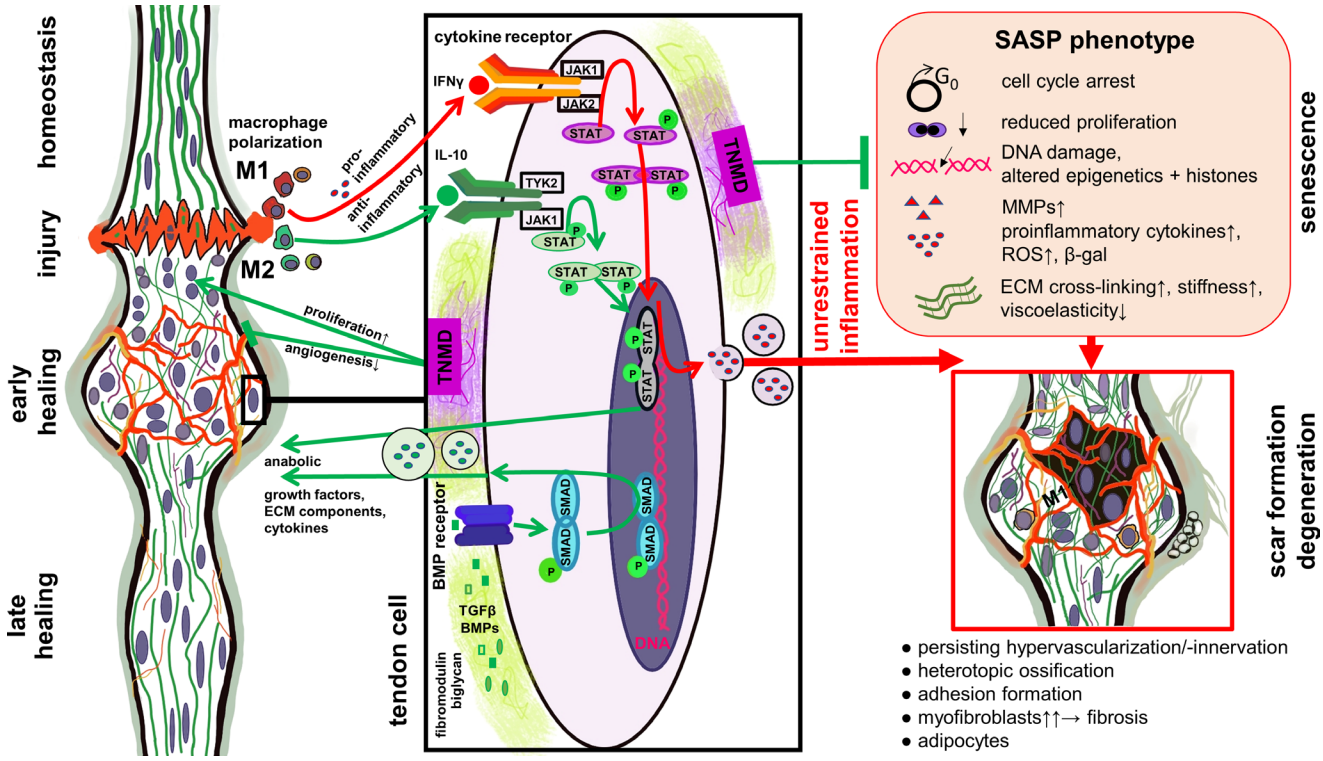


Fig. 3

Role of Janus kinase signal transducer and activator of transcription (JAK/STAT) and bone morphogenetic protein (BMP) pathways in tendon healing and degeneration. Simplified scheme of selected signalling pathways involved in tendon healing. The balance of M1 and M2 macrophage polarization plays a central role in resolving the inflammatory phase and affects the outcome of tendon healing. Proinflammatory cytokines released during dominant and prolonged M1 macrophage polarization stimulate molecular factors released in response of the JAK/STAT pathway activation, which can trigger the senescence-associated secretory phenotype (SASP) shift of tendon cells. SASP is associated with degenerative features in healing tendons. Mediators released during M2 macrophage polarization, including anti-inflammatory cytokines such as interleukin (IL)-10, stimulate other parts of the JAK/STAT pathway. Proteoglycans such as biglycan and fibromodulin can bind and stabilize growth factors activating the BMP pathway. Tenomodulin produced by the tendon cells can exert pro-proliferative effect as well as protective roles against cellular senescence and unrestrained angiogenesis. The image was created by G. G. Schulze-Tanzil using Krita 4.1.7 (Krita Foundation, The Netherlands). β-gal, β-galactosidase; ECM, extracellular matrix; IFNγ, interferon γ; MMP, matrix metalloproteinase; ROS, reactive oxygen species; TGFβ, transforming growth factor β; TNMD, tenomodulin.

could contribute to the design of smart strategies to steer progenitor/SCs towards desired behaviour and better healing outcomes.

Key mediators and processes during AT healing

Tenomodulin as molecular factor influencing the repair process. A multitude of molecular factors contribute to AT healing depending on the healing phase (Table I). A molecular factor not listed in Table I but known to be important during AT repair is TNMD. This gene encodes a type II transmembrane glycoprotein with a cleavable C-terminal cysteine-rich domain secreted in the tendon ECM, and has a dual function, namely as a pro-proliferative agent in tendon cells[120] and anti-angiogenic factor inhibiting vascular cell migration.[121] AT of *TNMD*-deficient mice exhibits a substantially reduced cell density and proliferation in vivo, paralleled with pathological thickening of collagen fibrils,[120] which is a phenotypical modification associated with premature tissue ageing.[122] In order to uncover *TNMD* role during healing, Lin et al[89] surgically induced a complete AT injury in the midsubstance in *TNMD*-deficient mice. Detailed analysis at day 8 post injury revealed a profound difference in scar organization, with greatly reduced cell proliferation and increased cell apoptosis, senescence, inflammation, and adipocyte and blood vessel accumulation.[89] Interestingly, when assessed at later timepoints of the healing process, *TNMD*-knockout tendons exhibited markedly increased heterotopic ossification (HO) paralleled by diminished biomechanical and functional properties of the ATs.[101]

Neovascularization/angiogenesis/innervation. Unbridled inflammation during healing is associated with an abundance of diverse mediators such as cytokines and chemokines inducing hypervascularization, which is in contrast to the mature healthy AT known as a hypovascular and hypoinnervated tissue.[9,123] Recent research showed that impairing neoangiogenesis in ruptured tendons promotes healing in the AT.[124] Sprouting of sensory nerve endings during healing AT is also guided by inflammatory mediators such as chemokines.[125] Nerve ingrowth into the tendon fascicles, along with time-dependently emerging sensory, autonomic, and glutamatergic mediators, amplifies and modulates tendon inflammation and

healing.[74] Nerve branches follow newly formed blood vessels into the healing area of the tendon, but relocate later during the remodelling and reorganization process to the surrounding AT, particularly the paratenon.[74,125] However, painful degenerated tendons remaining after unsuccessful healing might reflect an uncoupled reinnervation process.[74]

ECM remodelling. The composition and organization of the ECM determines the mechanical properties of the tendon, and clear histological alterations can be seen after rupture. On a histological level, Maffulli et al[72] showed alterations in AT fibre structure and arrangement, nuclei morphology, and increased cellularity, vascularity, and glycosaminoglycan content, but decreased collagen stainability. As mentioned earlier, the main ECM protein of healthy tendon is collagen. In intact tendons, collagen has a very low turnover rate and it is hypothesized that formation occurs only in the first 17 years of life.[126] Using double quantum filtered nuclear magnetic resonance (NMR) spectroscopy, the maturation of collagen fibres in regenerating rabbit AT was monitored by Ikoma et al.[127] The authors found an ongoing reorientation of the collagen fibres with an increasing alignment of the collagen over time. The data from the non-invasive NMR spectroscopy were supported by the histology. The main collagen subtype in tendon is collagen type I, with lower amounts of types II, III, V, and VI.[25] During human AT healing the amount of collagen type III is increased, which might lead to thinner collagen fibres and decreased mechanical properties.[52] There was also a substantial increase in elastin (from 2% to 4%) content observed in healing AT, influencing mechanical properties.[58] Although tendons have a low ECM metabolism, remodelling takes place in healthy and injured tendons mainly due to the activity and balance of MMPs and their inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs). Analyzing the expression in ruptured human ATs, collagen types I and III, as well as MMP-1, MMP-2, MMP-13, and TIMP-1 increased with time after rupture while MMP-3 and MMP-10 decreased.[57] Improved ECM maturation and superior biomechanical properties after rupture were seen in a rabbit AT model with TGF-β1 gene transfer in comparison to the controls without gene transfer, according to a study by Hou et al.[128] These authors also concluded that there was an improved cross-linking of the collagen, as synthesis of the cross-linking enzyme lysyl oxidase (LOX) can be upregulated by TGF-β1. Cross-linking between collagen and elastin fibres is catalyzed by LOX enzymes resulting in insoluble ECM complexes with modified mechanical characteristics, and this is an important process in intact as well as in healing tissue.[129]

Mechanoresponse. ECM remodelling is strongly influenced by mechanical stimuli (Figure 4), since tenocytes are mechanosensitive cells.[130]

It is well known that immobilization of a healing tendon is detrimental to the healing process. Disturbed mechanoresponse and mechanotransduction in tendon cells can lead to failure in healing. Runesson et al[36] demonstrated that exercise elevated in vivo cell proliferation in rapidly dividing cells, whereas the stem/progenitor population did not change in number. Moreover, exercise has also been shown to influence the immune response in tendons.[66] Eliasson et al[131] performed several in vivo studies to investigate the effect of load on rat AT healing. In a study from 2009, they investigated the effect of mechanical unloading (by botox injection into the gastrocnemius muscle) on gene expression during healing. Unloading resulted in a reduced transverse tendon area and reduced mechanical properties. On the gene expression level, an increased expression of TNF-α, TGF-β1, LOX, and procollagens types I and III was seen in the unloaded healing tendons at the early timepoints. At the later timepoints, most investigated markers had increased expression in loaded tendons. In a subsequent study, the AT of tail-suspended rats was transected and gene expression after one episode of loading was analyzed.[132] Strong upregulation of 86 genes and downregulation of 64 genes were seen three hours after loading, while only a few genes were regulated after 48 hours. Upregulated genes were associated with inflammation and coagulation, while proteoglycans and collagens were downregulated. Loading also resulted in an increased peak force at day 7. Applying different loading regimens, Hammerman et al[133] were able to show that mild load (around 10 Newtons in animal experiments) increased the mechanical properties without affecting the expression of inflammatory genes, which were activated after strong loading.

Future perspectives

Tendon ruptures are common injuries, and the AT is affected in 20% of cases. The tendon heals by the formation of an inferior scar tissue, and the function is often impaired. Epidemiological investigations have shown that the incidence is increasing in both athletic and occupational populations. The incidence of AT ruptures ranges between six and 37 per 100,000 inhabitants in cohorts from the European Union and Canada.[134-136] The overall incidence has been rising in recent decades, based on an increasing incidence of this condition in the older population due to degeneration,[136] and elastic properties of a healed AT are inferior even a long time after rupture.[60] To optimize and coordinate the AT healing process as early as possible, a mechanostimulation protocol adapted to the individual patient's healing progress would be highly advantageous. Studies have revealed that in patients with chronic tendinopathy, eccentric training and local administration of a sclerosing agent, polidocanol, improved the sport activity level and pain by reducing the number of neovessels.[137] Eccentric training describes the muscle work when a muscle continuously elongates during load-bearing, for example by slowly releasing a carried load to the floor. Preclinical results with non-invasive electromagnetic

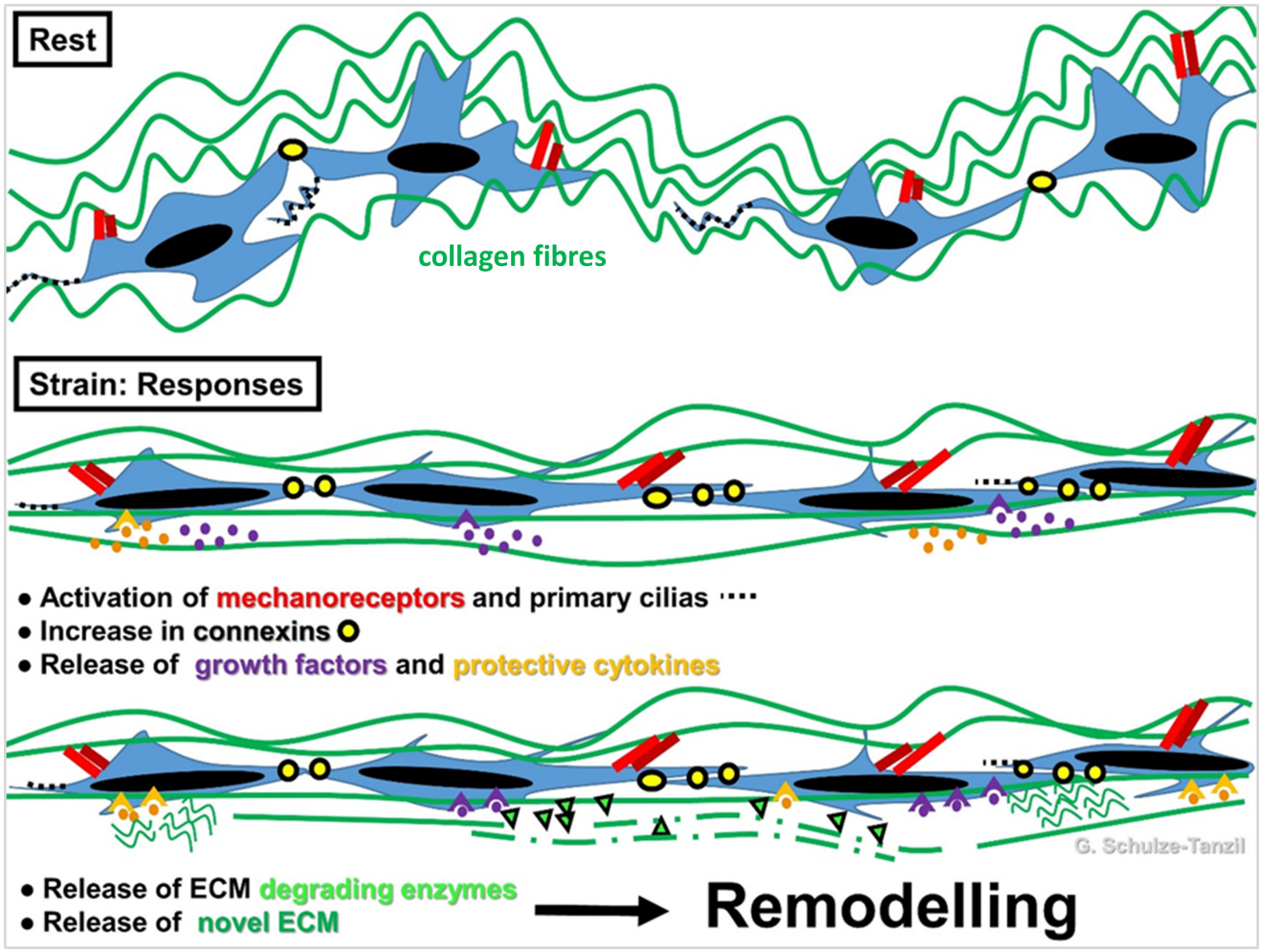


Fig. 4

Scheme of tenocyte mechanoresponse. Stretching at a physiological level leads to activation of mechanoreceptor and primary cilias. Crimping of the collagen fibre bundles disappears (in biomechanical measurement: toe region of a stress-stain curve). Connexin expression is elevated and hence, cell–cell signalling via gap junctions is also elevated. Protective cytokines and anabolic growth factors are released followed by de novo extracellular matrix (ECM) synthesis and ECM-degrading enzyme release, which mediate reorganization of ECM by a remodelling process to adapt the ECM biomechanics according to the stretch direction. The image was created by G. G. Schulze-Tanzil using Krita 4.1.7 (Krita Foundation, The Netherlands).

stimulation of AT during healing of tendinopathy are promising,[138] and could be beneficial for clinical treatment. These clinical findings are in line with the results showing increased neovascularization and VEGF synthesis in affected tendons, and previous observations where vascularization was directly correlated to biomechanical properties of the tendon.[139] Considering the pivotal role that the successful resolution of inflammation plays during AT healing, an anti-inflammatory treatment might present a future approach to reduce unrestrained neovascularization and overflowing innervation associated with pain.[124] In vivo monitoring, as well as controlling and modulating vascularization during tendon regeneration, might be a promising treatment approach in the future.[69]

MSC-derived exosomes could present a strategy to resolving overflowing inflammation in healing AT.[91] Understanding how different cell populations engage and are regulated by the discrete niches they reside in would also be very beneficial in identifying novel strategies to navigate cell behaviour during repair. Modifying niche parameters could further contribute to more satisfactory healing outcomes. Taking into account that in cases of critical AT defects healing might not be successful at all, and that suitable autografts are limited, tissue engineering could provide an approach to bridge these defects, e.g. based on allogenic or xenogenic AT ECMs or synthetic biomaterials.[140,141] Biomaterial-free cell sheets made from SCs could also be of advantage to improving biomechanical properties of healing ATs.[142] Recently, it was found that tenocytes differentiated from induced progenitor SCs could successfully be used to augment AT healing and restore function.[143] All in all, based on the complexity of the AT, as well as due to demographic changes and rising incidences of tendon pathologies, a multifactorial

approach combining different imaging, biochemical, cellular, and biomechanical aspects is necessary to improve AT repair and achieve major breakthroughs in tendon injury management.

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Funding statement:
- The authors received no financial or material support for the research, authorship, and/or publication of this article.