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Rescue plan for Achilles: Therapeutics steering the fate and functions of stem cells in tendon wound healing

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ABSTRACT

Due to the increasing age of our society and a rise in engagement of young people in extreme and/or competitive sports, both tendinopathies and tendon ruptures present a clinical and financial challenge. Tendon has limited natural healing capacity and often responds poorly to treatments, hence it requires prolonged rehabilitation in most cases. Till today, none of the therapeutic options has provided successful long-term solutions, meaning that repaired tendons do not recover their complete strength and functionality. Our understanding of tendon biology and healing increases only slowly and the development of new treatment options is insufficient.

In this review, following discussion on tendon structure, healing and the clinical relevance of tendon injury, we aim to elucidate the role of stem cells in tendon healing and discuss new possibilities to enhance stem cell treatment of injured tendon. To date, studies mainly apply stem cells, often in combination with scaffolds or growth factors, to surgically created tendon defects. Deeper understanding of how stem cells and vasculature in the healing tendon react to growth factors, common drugs used to treat injured tendons and promising cellular boosters could help to develop new and more efficient ways to manage tendon injuries.

1. Introduction

In Greek mythology, Achilles, the demigod hero, is almost invulnerable except for his Achilles heel, whose injury resulted in his death. How could a tendon injury take such a prominent place in Greek mythology? This injury was obviously such a crucial and inexplicable event that it was extensively honored in the legendary Iliad of Homer. Presumably, the ancient Greeks have already wondered how it can happen that even in young powerful athletes the biggest tendon of man can suddenly break. Even today, we cannot explain or foresee when and why this greatest chord of man tears. Nor can we offer with great degree of certainty, especially in elderly individuals, complete reconstitution to normal strength and function of the tendon tissue once it has been ruptured.

As an integral part of the musculoskeletal system, tendons connect and transmit forces from muscle to bone. As a result of their composition and structure they are able to store elastic energy and withstand the high tensile forces that enable locomotion [1]. With an aging population and an increase in sports participation the risk for tendinopathy or tendon ruptures grows steadily. Approximately 45% of musculoskeletal injuries in the US are tendon or ligament injuries [2]. Tendon injuries are most common in the rotator cuff, the Achilles tendon and the patellar tendon [3] and the pathologies are often based on a degenerative process. Extensor and flexor tendons of the hand are also often subjected to direct lacerations in patients of all ages [2,3].

This review article aims to: (1) provide background information on tendon structure and the lengthy and insufficient healing process of tendon after injury including the clinical relevance; (2) highlight the influence of different types of mesenchymal stem cells on tendon healing; (3) summarize how different growth factors involved in tendon healing influence mesenchymal stem cells (MSCs) and their capability to

Abbreviations: ADMSC, Adipose tissue derived mesenchymal stem cells; bFGF, Basic fibroblast growth factor; Bgn, Biglycan; BM, Bone marrow; BMMSC, Bone marrow derived mesenchymal stem cells; BMP, Bone morphogenetic protein; Col, Collagen; COMP, Cartilage oligomeric matrix protein; CTGF, Connective tissue growth factor; Dcn, Decorin; ECM, Extracellular matrix; El, Elastin; ESFTT, Engineered scaffold free tendon tissue; ETM, Engineered tendon matrix; Fn, Fibronectin; Fmod, Fibromodulin; GDF, Growth and differentiation factors; GFP, Green fluorescent protein; GNT, Glyceryl trinitrate; HB-EGF, Heparin binding endothelial growth factor; IGF-1, Insulin-like growth factor-1; Il, Interleukin; INF, Interferon; Lum, Lumican; MMP, Matrix metalloproteinase; MRI, Magnetic resonance imaging; MSC, Mesenchymal stem cells; NO, Nitric oxide; PC, Perivascular cell; PDGF, Platelet-derived growth factor; PEG, Polyethylene glycol; PRP, Platelet rich plasma; Scx, Scleraxis; SFDLT, Superficial flexor digitorum longus tendon; SMA, Smooth muscle actin alpha; SMC, Smoot muscle cells; TGFβ, Transforming growth factor beta; TN-C, Tenascin C; TNF, Tumor necrosis factor; Tnmd, Tenomodulin; TSPC, Tendon stem/progenitor cells; VEGF, Vascular endothelial growth factor.

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enhance tendon healing; (4) clarify the effects of commonly used drugs on tendon-specific stem/progenitor cells and vasculature of the tendon and; (5) discuss the possibility of cellular boosters to amplify the positive effects of mesenchymal stem cells on tendon healing. In vivo and in vitro studies have been analyzed and summarized.

1.1. Tendon structure

Tendons are a hierarchically structured dense connective tissues (Fig. 1), designed to transmit forces between muscle and bone. They are mainly composed of collagen fibers and tenocytes that lie embedded in a well-ordered extra cellular matrix (ECM), containing high amounts of proteoglycans. The main purpose of the collagen fibers is to resist tension, while the proteoglycans provide the viscoelastic properties for the tendon. Cross-linked tropocollagen form insoluble collagen molecules, and aggregate into microfibrils. These microfibrils combined to form fibrils that group together into fibers aligning from end to end within the tendon. Fibers group together into a bundle that is ensheathed by a thin layer of loose connective tissue, known as the endotenon. In addition to binding the fibers together, the endotenon enables fiber groups to glide over each other and carries blood vessels, nerves and lymphatics to deeper portions of the tendon [1,4,5]. Fascicles are groups of fiber bundles ensheathed by endotenon. The epitenon, a dense fibrillary network of collagen, binds fascicles together to create the tendon [5]. This complex internal ultrastructure leads to high tensile force and resilience but also prevents damage and the separation of fibers under mechanical stress [6].

The collagen network of the tendon matrix forms a regular sinusoidal pattern called “crimps”, which act as a buffer or a shock absorber within the tendon, permitting small longitudinal elongation of individual fibrils without permanent damage to the tissue [7]. It has been estimated that these crimps allow 1–3% stretching of the tendon tissue, and thus, provide a highly efficient “safety measure” for tendons to resist sudden, possibly hazardous tensile strains subjected on them by excessive contraction or elongation of the attached skeletal muscle [7]. 65–80% of the dry mass of tendon consists of collagen, with collagen I (Col I), accounting for up to 95% of the total collagen, while only 1–2% consist of elastin (El) [4,8–10]. Besides Col I, the tendon also contains collagen III (Col III), restricted to the tendon sheets in healthy tendons [6,11], as well as small amounts of Col V, VI, XII, XIV and XV [6,12].

Together with collagen crimps, elastic fibers made out of El and tenascin-C (TN-C) in the tendon provide extensibility and flexibility for the tissue and permit long-range deformability as well as passive recoil without energy input [13].

The ground substance in tendons is built up by proteoglycans like decorin (Dcn), biglycan (Bgn), fibromodulin (Fmod) and lumican (Lum), glycoproteins, El and inorganic molecules (copper, manganese and calcium) [14]. 60 to 80% of the total weight of the ground substance is water, leading to a hydrophilic, gel-like texture. The water binding capacity of the ground substance improves the elasticity of the tendon, making it more resistant against shear and compressive forces, and provides support to the collagen fibers [5].

Tenocytes and tenoblasts are approximately 90–95% of tendon cells. Tenocytes, being terminally differentiated, are spindle-shaped, with elongated nuclei and thin cytoplasmic protrusions anchoring the collagen fibers, while tenoblasts are more roundly shaped with a large, ovoid nucleus [15]. The discrimination between tenocytes and tenoblasts is based on cell shape appearance and it is still lacking precise molecular separation via marker gene expression. Therefore, the exact tendon cell differentiation process is not fully understood and lagging far behind the other musculoskeletal lineages. In recent years, several research papers based on knockout or reporter mouse models have

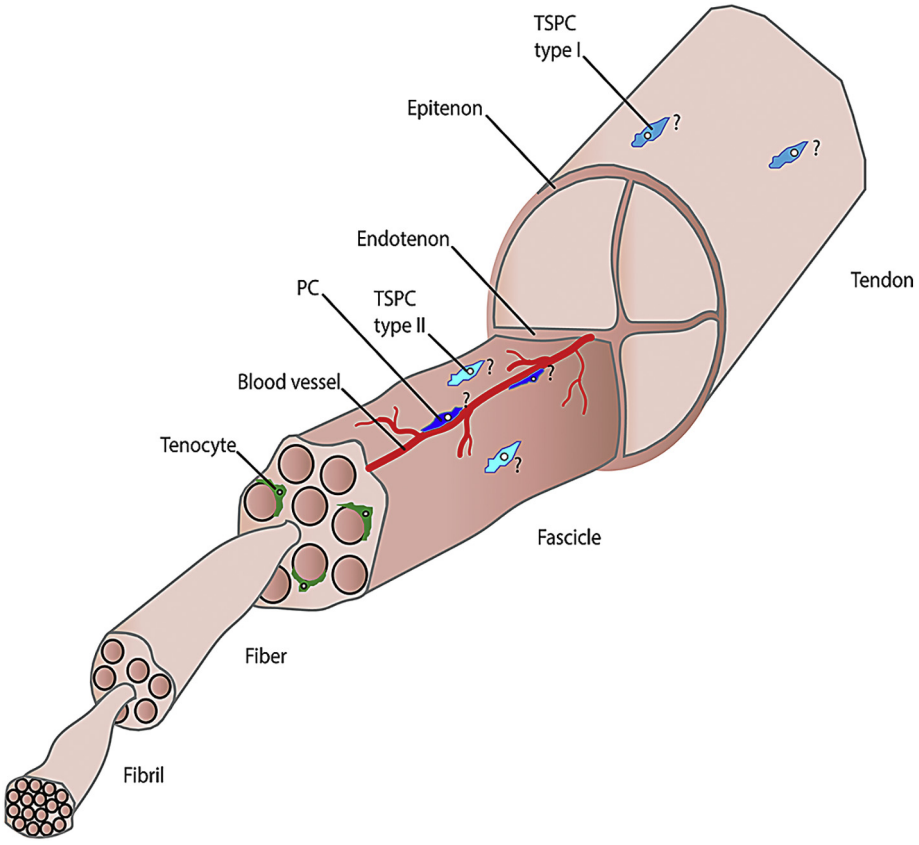

Fig. 1. A schematic drawing of tendon structure and the localization of tenocytes and TSPCs. The tendon is hierarchically structured in fascicles, fibers and fibrils, composed of collagen molecules. Despite tenocytes, tendons contain a pool of stem and progenitor cells. The exact location of these TSPCs is not clarified yet and therefore indicated with question mark. Locations discussed for different types of stem/progenitor cells in the tendon are the epitenon (TSPC type 1), the endotenon (TSPC type 2) and the perivascular region.

aimed to identify critical genes for tendon formation or maturation [16]. Despite some advancement in recapitulating the discrete molecular steps of the tenogenic differentiation cascade, the field is in need of identifying specific surface markers which will allow sorting of tendon cell populations and in turn will aid in better characterization of the tendon regional cell niches.

The remaining 5–10% of cells in tendon is composed of chondrocytes, synovial cells of the tendon sheath, capillary endothelial cells and smooth muscle cells of arterioles.

Tendon cells engage in energy production via the Krebs' cycle, anaerobic glycolysis and the pentose phosphate shunt as well as in the biosynthesis of collagens and all additional components of the tendon matrix [1,10,17]. According to the most recent data, most of the tendon ECM is produced right after birth, thereafter very little ECM production takes place over the life span of an individual [18].With age, aerobic energy production and synthesis of ECM components decreases. The shift to anaerobic energy production leads to the ability to tolerate low oxygen levels, reducing the risk of ischemia and necrosis during extended periods of stress but also results in a poor and slow healing capacity [5].

1.2. Tendon healing

The exact mechanisms of tendon healing are still not completely understood, due to low number of detailed biochemical, histopathological and biomechanical studies as well as species-related differences in the healing process. Most insight has been gained from the analysis of animal models of experimentally induced tendon rupture [19,20], or by analyzing human ruptured tendons, but since these are models of acute injury only, they do not help in understanding the healing process in tendinopathy [21]. Currently, an optimal experimental model of tendinopathy is not available for two main reasons. Firstly, due to poor understanding of the pathogenesis of tendinopathy and especially of tendinosis and second, as there is no animal with exactly the same features of human tendons, no one species represents a gold standard [20]. From a translational point of view, non-human primates represent the most ideal species as they are the closes to humans in terms of tendon anatomy and physiology but their use is limited due to ethical considerations as well as high costs [20]. Small species such as rodents are the most common animal models. However, their tendons are better suited to withstand some types of stress such as exposing their extremities to excessive running or hill work (steep inclination or declination on treadmill), which leads to no significant structural changes in certain tendons [20]. When treated chemically, by injections of collagenase, corticosteroids or cytokines, the rodent tendons either do not induce pathology that replicates the human condition or over-respond by intense inflammation followed by progressive tendon reparation [20].

It is agreed that during the course of healing, a tendon passes through three main stages (Table 1 and graphical abstract), which may overlap, and that their duration depends on location and severity of the defect [14,22,23].

In the inflammation stage, starting directly after injury and lasting approximately for 3 days, the blood clot, created by the tear that also ruptures blood vessels, serves as a preliminary scaffold for invading cells. It then activates the release of chemoattractants from activated platelets, which initiate the migration of inflammatory cells, such as neutrophils and monocytes, from circulation to the injury site. Monocytes differentiate into macrophages, which digest necrotic material via phagocytosis and an increase of vasoactive and chemotactic factors results in the recruitment and activation of tenocytes [22,24,25]. During this stage, the formation of a vascular network by sprouting angiogenesis is initiated, which is essential for the survival of tenocytes engaged in the synthesis of the new fibrous tissue [26]. The newly formed tissue mainly consists of fibronectin and Col III, produced by tenocytes at the injury site [27].

The second proliferation stage lasts up to a few weeks. During this phase, macrophages release growth factors to direct cell recruitment and activity [25]. Thereafter, tendon fibroblasts from the epitenon and the synovial sheath and intrinsic tenocytes from the endotenon are recruited to the injury site to produce Col III, fibronectin and ECM components (e.g. proteoglycans) to create an initially unorganized ECM [28,29]. Then the production of Col III commences and it is replaced by substantially stronger Col I. Typical features of the proliferation stage are high cellularity and water absorption.

Following 6–8 weeks, the remodeling stage commences and takes about 1–2 years, depending upon age and condition of the patient. This stage can be subdivided into the consolidation stage and the maturation stage. The consolidation stage lasts up to 10 weeks and is characterized by tissue changes from a highly cellular to a more fibrous appearance. Metabolism of tenocytes is still high in this phase and the tenocytes and collagen fibers start to align in the direction of stress to restore tensile strength and tendon stiffness. Furthermore, the synthesis of Col III is replaced by the synthesis of Col I. Tendon fibroblasts transform to myofibroblast, which contract the large granulation tissue into substantially smaller, permanent scar tissue. The final maturation stage can take 1–2 years during which a change from fibrous tissue to a scar-like tendon tissue can be observed. During the course of this stage, tenocytes metabolism and tendon vascularity decrease [21]. Two different mechanisms, that are most likely acting conjointly, have been suggested for tendon healing. The extrinsic healing theory states that fibroblasts and inflammatory cells from the periphery and the blood vessels migrate to the injury site to proliferate and form adhesions. Extrinsic healing is believed to take place mostly in the early phases of healing. Then intrinsic healing takes over, meaning that cells

Table 1
Phases of tendon healing and growth factors involved.

Repair phase	Growth factors involved	Effects	References
Inflammatory	IGF-1	Invasion of inflammatory cells and fibroblasts	[253–258]
	PDGF	Chemo attraction, stimulation and proliferation of macrophages and fibroblasts, expression of growth factors	[259–262]
	TGF-β	Chemoattraction, cell migration and proliferation	
	VEGF	Angiogenesis, increase in capillary permeability	
	bFGF	Cell proliferation	[135,137,258,260,263,264] [235,265,266] [133,135,178]
Proliferative	IGF-1	Stimulation of migration, division and ECM expression of tenocytes	[267,268]
	PDGF	Stimulation of division, proliferation and ECM expression of tenocytes	
	TGF-β	Cell migration and ECM production	[260,268]
	GDF	Collagen and GAG production, Cell proliferation and realignment	[137,148,258] [94,263,269–272]
Remodeling	IGF-1	ECM remodeling	[258]
	TGF-β	Collagen synthesis, myofibroblasts, scar formation	[161,162,258,263,264]
	GDF	Collagen synthesis	[94,263,269–272]

from the endotenon are activated and migrate to the injury site, where they proliferate, produce and reorganize ECM and give support to the newly built vascular network [21,29]. There is only very little evidence on the origin of the participating cells. One study states that initially circulating cells (e.g. from bone marrow) invade the injury site and are then followed by activated local cells, which participate in the proliferative and remodeling phase, also confirming the biphasic model of tendon healing described above [30].

Various growth factors are involved in the activation and concertation of cellular processes during the different phases of tendon healing (Table 1 and graphical abstract) [21,29,31]. The release of growth factors is triggered first from the activated platelets straight after injury. Furthermore, all tissues contain growth factors in their inactive form and these inactive, "in storage" growth factors are activated in response to injury. Together, these growth factors initiate the inflammatory cascade and recruit more inflammatory cells to the site of injury within hours [24,25,31]. The inflammatory cells, in turn, secrete plenty of growth factors and amplify the inflammatory cascade [24,25,31]. The tendon cells located next to the injury site area are also activated and can produce growth factors, and mechanical loading placed on the injured tendon can further induce the production of growth factors [24,25,31]. The exact effect of these growth factors on stem cells in tendon healing will be discussed in Section 2 of this review.

The healing ability of tendons is limited and in almost all of the cases, the biomechanical properties of a healed tendon are not as good as that of an uninjured tendon [14,29]. Reduced tendon strength leads to thickening and an increased stiffness of the tendon, making it more prone to re-rupture [14,29].

1.3. Clinical relevance

Classification and terminology of different tendon overuse injuries are still not agreed upon completely. It is commonly accepted that while tendinitis is accompanied by the infiltration of inflammatory cells, the actual inflammatory tendinitis is almost non-existent in human tendon. However, acute, swollen, inflammatory reaction can be seen in epitenon as well as the loose connective tissue surrounding the tendon [15]. The pathological changes taking place within tendon itself in overuse injuries is tendinosis. Tendinosis (hypoxic, hyaline and mucoid degeneration, tendolipomatosis etc.) and acute tendon rupture are most likely caused by intratendinous degenerations without inflammation [32,33]. This view is supported by the fact that histopathologies of tendinopathy and acute tendon ruptures are identical; the degenerative pathology is just more severe in acute tendon rupture than in tendinopathy [34,35]. Furthermore, the current paradigm is that the onset of tendinosis is caused by hypoxia, (i.e. lack of oxygen) in the diseased tendon [32,33]. Angiogenesis is induced by the cells experiencing hypoxia and in the cells attempt to survive under hypoxia through secretion of soluble growth factors and cytokines, thus recruiting inflammatory cells [32,33]. Increased numbers of inflammatory cells were seen especially in hypervascular regions of tendinopathy. Due to neither inflammatory exudates nor accumulation of inflammatory cells in tendon bundles being detected, it was concluded that there is no inflammation in the tendinopathy. For example, Alfredson et al., [36] showed that no inflammatory mediators can be measured in the dialysate from chronic Achilles tendinopathy obtained by inserting a microdialysis catheter into the tendon. Furthermore, it should be considered that the great majority of the cells in tissue remodeling and repair are inflammatory cells (mainly macrophages) but their presence does not necessarily mean inflammation. A recent review by Millar et al., [37] challenges this dominating paradigm by suggesting that the lack of observation of an acute inflammatory infiltrate does not exclude a role for inflammation in the etiology of tendinopathy and that there might be the probability of preceding initial inflammation without clinical symptoms, finally leading to tendinopathy or spontaneous tendon rupture. Tendinopathy is a multifactorial condition and the precise role of inflammation in the tendinopathy process is still debatable and most likely cannot be viewed under the label of "one-size-fits-all". Therefore, future research has to carefully investigate inflammatory components in the various sub-types of tendinopathies.

The term tendinopathy is used throughout this article to describe overuse disorders affecting tendons, i.e. conditions that do not involve tendon rupture, but are accompanied by chronic pain.

Healthy tendon has a poor natural healing ability due to hypocellularity and hypovascularity [38] and very low, almost non-existent metabolic rate [18], but as long as the ruptured parts remain in contact to each other and the epitenon is intact, healing without surgical intervention is possible [21]. Therefore, there is an ongoing debate as to whether to treat ruptured tendons surgically or conservatively, as these treatments provide almost the same outcome in randomized controlled trials in some tendons such as the Achilles tendon [39,40]. In Achilles tendon ruptures, surgical intervention reduces the risk of re-rupturing but on the other hand increases the risk of other complications such as surgical wound infections [41,42]. With respect to surgical treatment of rotator cuff, tears does not lead to a better functional outcome, but reduces pain and disabilities [43]. Moreover, the outcome also depends on patient's age, degree of tendon degeneration and extent of laceration [44]. Rupture of the patellar tendon leads to abolition of knee extension [45]. To restore the extensor apparatus of the knee, surgical treatment is inevitable [46] Sutures are the most common approach to re-establish tendon alignment, whereas bone anchors are needed when there is an avulsion/rupture of tendon from bone. Numerous techniques have been established, each especially adapted to the specific tendon. In some cases, tendon autografts may be necessary to recreate tendon structure, especially in the cases of tendon retraction or loss. These autografts have to be taken from donor sites, resulting in the risk of donor site morbidity [25]. Recently, use of allografts to bridge defects has increased [47,48]. The use of allografts avoids the problem of donor site morbidity but comes with the concern of immune rejection and disease transmission [49]. Overall, surgical repair of ruptured tendons is often unsuccessful and many become chronic tendinopathies [2].

However, not only age or overuse can cause tendon disorders, also several intrinsic factors, including body weight, vascular perfusion, anatomical variants, systemic disease, nutrition and even blood group may be the causative factors [6,50]. More recently, genetic polymorphisms associated with an increased risk for Achilles tendinopathy have been discovered. In the gene encoding for matrix metalloproteinase MMP 13, three different variants have been shown to be associated with Achilles tendinopathy in a south African Caucasian population [51], while two variants of the COL5A1 gene also increase the risk for this disease [52,53].

Acute tendon ruptures and chronic tendinopathies affect a growing number of people, restricting their quality of life and their capability to work. Additionally, they are the cause for the enormous budget spent each year by the worldwide healthcare system.

Hence, it is of great interest to develop new effective therapeutic treatments, like stem cell-based tissue engineering, growth factor cocktails or other drugs, for curing tendon diseases and augmenting tendon repair.

2. Stem cells in tendon

Stem cells are cells with the ability to differentiate into a multitude of cell types. Due to their potential to differentiate into tenocytes, a high proliferative and synthetic activity, the secretion of paracrine factors and the ability to exhibit immunomodulatory effects to promote tendon regeneration, the use of stem cells in tissue engineering for tendon repair is of great promise. Stem cells of different origins have been analyzed for their effect on tendon healing in vitro and in vivo (Table 2).

Table 2
Effect of different stem cell types in tendon repair.

Cell type	Origin of cells	Study model	Outcome	References
Tendon stem/progenitor cells (TPSC)	Rat	Rat, patellar tendon, surgical window defect, 1 mm in width, transplantation of TPSCs in fibrin glue, analysis at 1, 2 and 4 weeks	Accelerated healing, increased Col production, increased ultimate stress and Young's modulus at week 4.	[79]
	Rat	Rat, patellar tendon, surgical window defect, 1 mm in width, TPSC-fibrin construct transplantation (with our without CTGF and ascorbic acid treatment), analysis at 2, 4 and 8 weeks.	Accelerated and enhanced tendon repair by treated TPSC up to week 8 and 16 compared to untreated TPSC and controls. Shown by histology, ultrasound imaging and biomechanical testing.	[83]
		Rat; patellar tendon; surgical window defect, 1 mm in width, transplantation of mock-TDSCs in fibrin glue, Scx-TPSCs in fibrin glue or scaffold only, analysis at 2, 4 and 8 weeks.	Better tendon healing in Scx-TPSC group compared to Mock-TPSC and scaffold only. Shown by histology, viva CT, biomechanical testing, immunohistochemistry.	
		Rat, patellar tendon, surgical window defect, 1 mm in width, implantation of TPSC cell sheet, analysis 2, 4 and 8 weeks after surgery.	Improved healing, increased cellularity, increased ECM production, no differences in Col expression, higher ultimate stress and Young's modulus. Shown by histology, immunohistochemistry and biomechanical testing.	
	Rat		More ingrowth of fibroelastic cells, less infiltration of lymphocytes, denser ECM, more physiological native tendon structure, higher Col I and Col III production, increased biomechanical properties. Shown by histology, immunohistochemistry and biomechanical testing.	[80]
		Rabbit, rotator cuff tendon, surgical defect, 10 × 5 mm, implantation of TPSC seeded silk-collagen scaffold, analysis 4, 8 and 12 weeks after injury.	Improved tendon healing in ETM group, thicker and more organized Col fibrils. Shown by histology.	
	Rat			[84]
		Rat, patellar tendon, surgical defect, 2 mm diameter, implantation of TPSCs with or without ETM gel, analysis 8 weeks after surgery.		
	Rabbit			[72]
	Human			[85]
Bone marrow mesenchymal stem cells (BMMSCs)	Rat	Rat, Achilles tendon, surgical window defect, Injection of BMMSCs, Analysis after 1, 2 and 4 weeks	Increased tissue repair, higher ultimate failure load, increased Col production. Shown by histology, biomechanical testing and RT qPCR	[100]
	Human	Rat, Achilles tendon, Collagenase induced injury; implantation of BMMSCs; analysis 2, 4 and 6 weeks after injury.	Accelerated healing, increased Col production and better organization, no differences in biomechanical properties. Shown by histology, immunohistochemistry and biomechanical testing	[101]
			Improved tendon healing, increased ECM production and better organization. Shown by histology and immunohistochemistry	
		Rat, Achilles tendon, surgical defect, implantation of BMMSC-loaded mesh, analysis 6 and 14 days after injury.	Increased load to failure ratio after 2, but not after 4 weeks, no difference in stiffness, no difference in tissue organization and Col synthesis. Shown by Histology, immunohistochemistry and biomechanical testing.	

Table 2 (*continued*)

Cell type	Origin of cells	Study model	Outcome	References
	Human	Rat, Achilles tendon, 2.4 mm punch injury, injection of BMMSCs, analysis 2 and 4 weeks after injury.	Faster closure of gap in Achilles tendon, increased ultimate failure load (hypoxic higher than normoxic), increased numbers of mature tenocytes, reduced fibrosis, increased Col I production. Shown by biomechanical testing, histology and immunohistochemistry.	[102]
			No differences in load to failure and stiffness, no differences in Col content and organization. Shown by biomechanical testing and histology.	
	Rat	Rat, Achilles tendon, complete incision, injection of normoxic or hypoxic MSCs, analysis 2 and 4 weeks after incision.	Better ultrasound images, return to full exposure, reduced risk of re-rupture. Shown by ultrasound and follow up analysis.	[105]
	Rat	Rat, Achilles tendon, 2.4 mm punch, injection of MSC-eGFP or MSC-bFGF, analysis 12 weeks after surgery.		[103]
		Horse, superficial flexor digitorum longus tendon, spontaneous lesion, injection of MSCs, follow up till 2 years after treatment.		
	Rat			[104]
	Horse			[107]
Adipose-tissue derived mesenchymal stem cells (ADMSCs)	Horse	Horse, superficial flexor digitorum longus tendon, spontaneous lesion, injection of ADMSCs, analysis 9 to 24 weeks after injection.	Sonographic improvement of defect, reduced lameness. Shown by MRT.	[122]
	Rabbit	Rabbit, Achilles tendon, surgical incision, covered with PRP gel or PRP-ADMSC gel, analysis 4 weeks after surgery.	Increased tensile strength, more longitudinally arranged Col fibers, increased Col I production, increased FGF and VEGF synthesis, decreased TGF-β synthesis. Shown by biomechanical testing, histology and immunohistochemistry.	[119]
			No differences in biomechanical properties, no differences in Col production. Shown by biomechanical testing and histology.	
		Rat, supraspinatus tendon, surgical detachment, implantation of ASDCs in collagen carrier, analysis after 24 h, 1, 2 and 4 weeks.	Increased structural organization. Shown by histology.	
	Rat	Rabbit, Achilles tendon, cross section, transplantation of ADMSCs, analysis after 14 and 28 days.	No difference in lesion area, increases linearity of Col fibers, no differences in Col I and III expression and synthesis. Shown by ultrasound, histology, immunohistochemistry and qRT-PCR.	[121]
		Horse, superficial flexor digitorum longus tendon, collagenase induced lesion, injection of MSCs 2 weeks after lesion induction, Ultrasound every second week till 16 weeks after injection, tendon biopsy 16 weeks after injection.	Lower levels of degenerative changes, higher density of collagen fibers, decreased Col III/Col I ratio. Shown by histology, immunohistochemistry and RT-PCR.	
	Rabbit	Rat, Achilles tendon, collagenase induced lesion, injection of MSCs 1 week after lesion induction, analysis 4 or 12 weeks		[118]

(*continued on next page*)

Table 2 (*continued*)

Cell type	Origin of cells	Study model	Outcome	References
		after injection.		
	Horse			[273]
	Rat			[120]
Stem cells of other sources	human (umbilical vein)	Rabbit, Rotator cuff, 5 mm punch, injection of MSCs, analysis 4 weeks after surgery.	Reduced tendon tear size, growth of fibroblastic bundles, increased Col I production. Shown by histology and immunohistochemistry.	[274]
		Mouse, subcutaneous implantation of alginate microspheres loaded with TGF-β3 and PDLSCs, GMSCs or BMMSCs, analysis 8 weeks after implantation.	Neoformation of tendon-like structures in all groups, more organized ECM and Col in PDLSC group. PDLSCs show best capability for form tendon-like tissue. Shown by histology and immunohistochemistry.	[275]
	Human (periodontal ligament, gingivial tissue)		Better repair with denser connective tissue and increased ECM production in iPSC-NCSC group, higher failure load and Young's modulus. Shown by histology and biomechanical testing.	
		Rat, patellar tendon, surgical window defect 1 × 4 mm, implantation of iPSC-NCSCs in fibrin glue or fibrin glue alone, analysis 4 weeks after surgery.	Loaded tissue constructs form thicker neotendinous tissue, faster Col maturation, increased expression of Scx, Tnmd and TNC. Shown by histology and immunohistochemistry.	
	Human (induced pluripotent, neural crest)	Mouse, subcutaneous implantation of DSPC-PGA scaffolds with our without mechanical loading, analysis 8 and 14 weeks after surgery.		[276]
	Human (dental pulp stem cells = DSPCs)			[277]
Perivascular cells	Rat	Rat, patellar tendon, window defect 1 × 4 mm, peritenon sutured or peritenon scratched, analysis 1 and 4 weeks after surgery.	Faster tendon healing in peritenon sutured group. Shown by macroscopic observation.	[131]
		Rat, patellar tendon defect, transplantation of CTGF pretreated or untreated CD146+ TSPCs, analysis after 2 weeks	Improved tendon healing only in CTGF-TSPC group, reorganized collagen orientation. Shown by histology.	
	Rat			[132]

2.1. Tendon stem/progenitor cells

In 2007, Bi et al., first demonstrated the existence of stem cells in tendon tissue. They showed that human and mouse tendons contain a minor cell population which possess clonogenic capability, a distinct mRNA expression profile, multipotent and have a high proliferation capability [54]. The existence of stem/progenitor cells (TSPCs) could be confirmed in various tendons and ligaments from different species [31,54–64]. They exhibit classical criteria of adult mesenchymal stem cells, like typical surface antigens, self-renewal, clonogenicity and three-lineage differentiation (adipogenic, osteogenic and chondrogenic) [31,54–64]. In contrast to MSCs of other origins, they express the tendon-related genes scleraxis (Scx), tenomodulin (Tnmd) [16], cartilage oligomeric matrix protein (Comp) and TN-C [54–59]. Various studies have focused on the isolation, characterization and the finding of specific markers of TSPCs (reviewed in [60,61]). TSPCs are positive for some common stem cell markers, which can also be found on the surface of other mesenchymal stem cells (MSCs). They express Sca-1, CD44, CD90, CD90.1, CD105, CD146, Stro-1, nucleostemin, Oct-4 and SSEA-1 but not CD18, CD31, CD34, CD45, CD106, CD117, CD144 and

Flk-1 [61]. Since there are no molecular markers that allow discrimination between TSPCs, tenoblasts and tenocytes, it is impossible to isolate pure subsets of cell populations from these differentiation stages (Fig. 2). The exact role of TSPCs in tendon maintenance and healing is not completely understood till now. Hence, there is a great need for in vitro and in vivo studies demonstrating their role and location.

Recently it was shown that cells simultaneously expressing tendon and pericyte-associated marker genes are localized in the perivascular space of tendon tissues, suggesting that the perivascular niche might be a source of another type of local stem/progenitor cells [55]. Furthermore, it was proposed that there is a regional distribution of different stem/progenitor cells within tendon, namely in the outer tendon sheet (TSPC type I) and within the tendon proper (TSPC type II) (Fig. 1) [31, 65]. Comparison between these subpopulations revealed that the peritenon-derived cells have increased vascular and pericyte markers, while the tendon proper-derived cells are more proliferative and exhibit higher levels of Scx and Tnmd [65]. The study of Bi et al., shows that TSPCs reside in a niche which consists mostly of ECM. Two ECM molecules, namely fibronectin (Fn) and Bgn seem to play an essential role in the control of TSPC function. Tendons of double-knockout mice for Fn and Bgn showed higher cellularity and decreased fibril thickness. TSPCs of this knockout strain exhibited increased colonogenicity and proliferation, while the expression of Scx and Tnmd were reduced, leading the authors to hypothesize that tendon ECM influences TSPC self-renewal and differentiation and that alteration in ECM composition could lead to tendon malformation and ossification. Conforming data for this hypothesis was also presented in this study, as TSPCs from the Fn/Bgn knockout mice were more responsive to BMP signaling, which leads to increased differentiation towards the osteogenic lineage.

Mesenchymal stem cells (MSCs) from different tissue origins display common stem cell properties, but still might have tissue specific characteristics and therefore different functions [66]. Since TSPCs show higher clonogenicity, proliferation and multi-lineage differentiation potential compared with bone marrow-derived MSCs (BMMSCs) in vitro [54,60, 67] and also express higher levels of bone morphogenetic protein (BMP) receptor IA, IB and II [68], TSPCs are most likely a distinct cell type from BMMSCs. Furthermore, when implanted subcutaneously, TSPCs formed tendon- and enthesis-like structures, while BMMSC implantation led to the formation of bone and bone marrow-like structures [54].

As mentioned above, the transplantation of tissues or cells faces some challenging problems. Transplantation of allogenic cells may lead to an immune reaction. This problem could be overcome by using autologous cells, but the retrieval of such cells can cause donor site morbidity. Another problem that needs to be resolved is that tendon derived cells may undergo phenotypic drift during in vitro expansion. Over time, cell shape and expression patterns of Col I, Col III and Dcn change in human tenocyte culture, if cells are cultivated in monolayer [69–71]. One possibility to avoid cell phenotype lost is to mimicking the natural niche of tendon-derived cells in vitro. In the tendon, tenocytes lay embedded in a dense three-dimensional (3-D) network of collagens, other ECM components and cells. AAs a result, Schulze-Tanzil et al., [72]suggested to cultivate tenocytes in high-density culture, where they form 3-D pellets. This technique led to stable cell morphology and expression patterns of Scx, Col I and Col II over 14 days, indicating that tenocytes retained their phenotypic identity in 3-D culture [72]. An alternative of the 3-D high density culture is a self-assembly model with tendon-derived cells resulting in 3-D tendon cell sheet structure that can be subjected to static [289] or even dynamic axial load. Further proof for the usability of tendon cell sheets formed by TSPCs was provided by a study showing that the implantation of tendon cell sheets in a rat Achilles tendon defect improved the overall tendon healing and reduced the defect size, and resulted in better organized collagen fibers with elongated spindle shaped cells and higher ultimate load, four weeks after surgery [73].

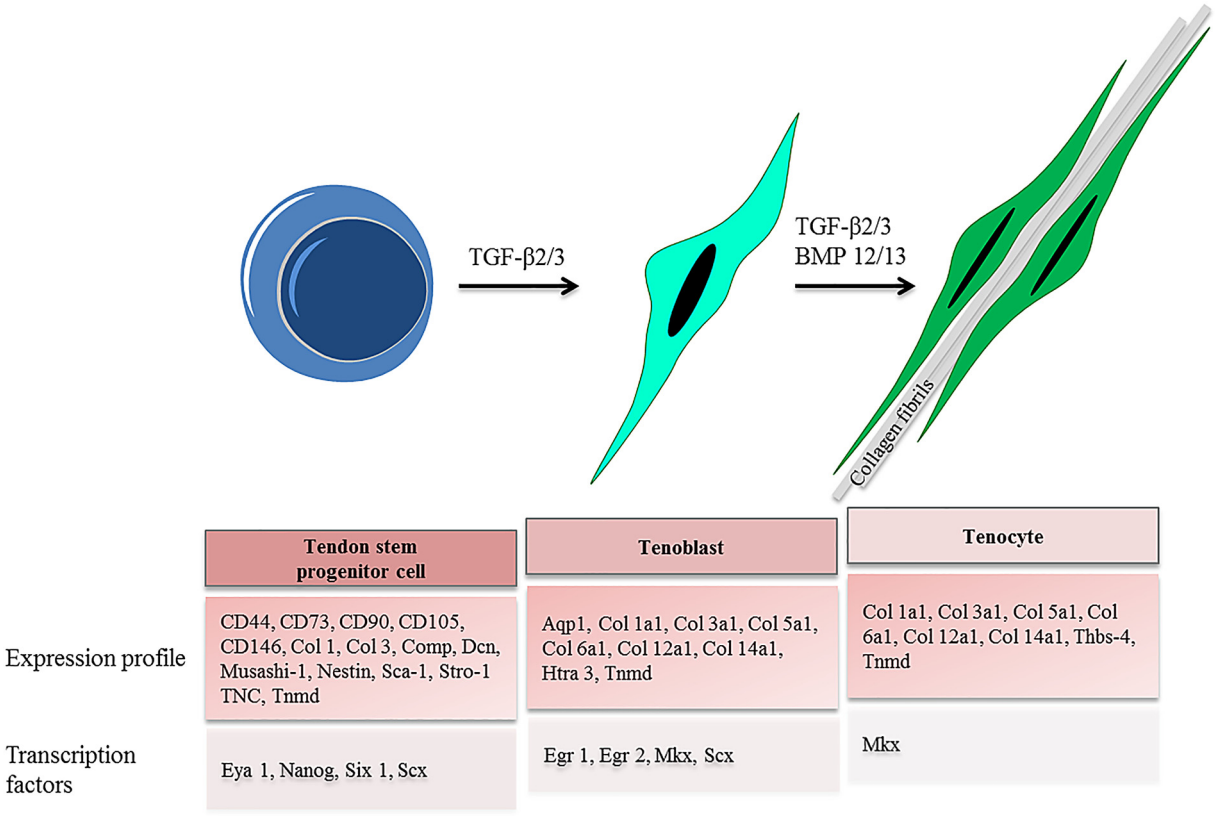

Fig. 2. Differentiation of a TSPC to a tenocyte. The expression profile of a TSPC changes during differentiation to a tenocyte. Tenogenic differentiation is mostly driven by TGF-β2/3 and BMP 12/13.

Other relevant ways to sustain stable cell phenotype is TSPC cultivation on adequate 3-D scaffolds that are similar to native tendon ECM molecular composition, organization, topography and overall biomechanical properties [31,74–76]. TSPC is also a mechanosensitive cell population [77], hence subjecting the cells to mechanical stimulation in vitro could provide a relatively simple option to stabilize their characteristics during prolonged cell culture periods.

The number of studies using TSPCs in tendon defect models has only slowly increased. One of the first studies by Zhang et al., used human TSPCs with or without engineered tendon matrix (ETM) in a rat patellar tendon window defect. They could show that the implantation, especially in combination with the ETM, led to increased tendon healing with the production of thicker and more organized collagen fibrils. In addition, they also reported that cultivation in ETM stimulates proliferation and preserves stemness of TSPCs in vitro, further highlighting the importance of the ECM niche for TSPCs [78]. Most studies analyzing the effect of TSPC implantation on tendon healing use the rat as a model organism. By transplanting GFP-TSPCs in fibrin glue into a rat patellar tendon window defect, Ni et al., showed that TSPCs significantly enhanced tendon healing and were observable in the tendon for two weeks after transplantation. TSPC transplanted tendons exhibited significantly increased tendon healing with increased collagen production and fiber alignment, improved cell alignment, increased ultimate stress and a higher Young's modulus [79].

In an attempt to further improve the positive effects of TSPCs on tendon healing different scaffolds or pre-treatments of cells were used. For example, transduction with Scx prior to implantation enhanced the expression of tendon-related markers in comparison to Mock-TSPCs. Scx-TSPCs in a fibrin construct, Mock-TSPCs in a fibrin construct or the fibrin construct only, were transplanted in a patellar tendon defect. In comparison to the Mock-TSPC and fibrin construct only group, healing was improved in the tendons implanted with Scx-TSPCs. They exhibited improvement in fiber arrangement and decreased vascularity and showed no signs of ossification. Regarding biomechanical properties, only at week four did the Scx-TSPC treated tendons show increased ultimate stress. At week eight, no differences between the three groups were observed and Young's modulus did not differ between groups at four or eight weeks after surgery. The authors try to explain these results with the usage of older cells for transplantation, which might have lower differentiation potential. Furthermore, this study does not address the question of whether if the transplantation of Scx-TSPCs increases the production of tendon specific ECM in vivo [80].

Along these lines, recent studies compared native TSPCs derived from tendon or periodontal ligament as well as tenogenically enforced BMMSCs via viral Scx over-expression versus BMMSCs for their potential to repair 3 mm complete defect in a rat Achilles injury model and showed that implantation of tendon cells is beneficial for late tendon repair in terms of matrix composition and reduced ossification [81,82].

In vitro treatment with connective tissue growth factor (CTGF) and ascorbic acid led to increased tenogenic proliferation, therefore the effect of such a pre-treatment on TSPCs tendon healing potential was analyzed in a patellar tendon injury model. The pre-treatment of TSPCs led to accelerated tendon healing, eight weeks post-implantation. At 16 weeks, no differences between tendons implanted with pre-treated TSPCs or untreated TSPCs could be observed. At 8 weeks, larger and better aligned fibrils were formed and biomechanical properties were increased, indicating that the pro-proliferative effect of CTGF and ascorbic acid also occurs in vivo and could be useful in accelerating tendon healing [83]. In another study with TSPCs, CTGF and ascorbic acid focussed on forming a TSPC cell sheet by rolling up, which was then named an engineered scaffold-free tendon tissue (ESFTT) [84]. The ESFTT was implanted subcutaneously in nude mice and in a patellar tendon window defect in rats. The subcutaneous implantation of ESFTT led to formation of neotendon 12 weeks after surgery.

In the patellar tendon window defect, the implantation of ESFTT improved tendon healing significantly. Implanted tendons had augmented ECM production, better collagen fiber alignment, as well as increased ultimate stress and Young's modulus at two, four and eight weeks after surgery [84].

Shen et al., combined allogeneic TSPCs with a knitted silk-collagen sponge scaffold and implanted it in rabbit rotator cuff tendons after creating a surgical defect. The implants did not cause an immunological reaction but led to increased fibro elastic cell ingrowth and reduced infiltration of lymphocytes, 4 and 8 weeks after surgery. At 12 weeks after implantation, the allogeneic TSPC-treated group exhibited increased collagen deposition and had better structural and biomechanical properties compared to the control group in which silk-collagen scaffolds were implanted without TSPCs. How the implanted scaffolds affected the production and organization of ECM has yet to be clarified [85].

As mentioned TSPCs, being a tendon native cell population, hold great promise for understanding tendon cell biology and for being a cell target for therapeutic implantation in tendinopathy and possibly in tendon rupture. A remarkable development in this direction was achieved by research on human autologous tenocyte implantation, where tendon cells were isolated from healthy tendon needle biopsy. After in vitro expansion, the cells were injected into the central tendinopathy of extensor tendons under ultrasound guidance on a single occasion. A 5 year follow-up with 16 patients showed significantly improved clinical and MRI tendinopathy scores concluding a long-term positive effect of the implanted cells [86]. A similar pilot study focusing on treatment of chronic recalcitrant gluteal tendinopathy concluded that autologous tenocyte implantation is safe, with improved and sustained clinical outcome up to 24 months after surgical intervention [87]. At present, this technology is already approved in Australia (Orthocell).

Despite the promising results indicating the capability of tendon-derived cells to improve and accelerate tendon healing in experimental models and to positively influence tendinopathy in humans, there is still a great need to analyze how exactly the cells mediate their beneficial effects to surrounding tissue and how these effects could be further improved.

2.2. Mesenchymal stem cells

Tendon-derived stem cells are not the only cell source that can be used for tissue engineering approaches to improve tendon healing, but also stem cells from other origins have proven to be promising. Amongst the different populations of stem cells, the mesenchymal stem cells (MSCs) have received most interest in musculoskeletal tissue engineering. MSCs are stem cells capable of differentiating into cells of one germ line, mesenchyme, i.e. osteoblasts (bone), chondrocytes (cartilage), tenocytes (tendon), myocytes (skeletal muscle) or adipocytes (fat).

In the late 1980s, the existence of MSCs in adult tissues was proposed on the basis that subcutaneously or intramuscularly implanted demineralized bone matrix caused the accumulation of multipotent progenitors that formed cartilage and/or bone in adult animals [88].

Since then, tissue engineering studies have focused on fabricating tissue ex vivo, using MSCs of different origins, (e.g. bone marrow, adipose tissue or periodontal ligament) often in combination with suitable scaffolds. According to the International Society for Cellular.

Therapy, cells must fulfill three criteria to be acknowledged as MSCs. First, MSCs must be adherent to plastic when maintained in culture. Second, MSC populations must be positive for several antigens such as CD105, CD73 and CD90 but must lack the expression of hematopoietic antigens like CD45, CD34 and markers for monocytes, macrophages and B cells. Third, the cells must be able to differentiate at least to osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions [89].

2.2.1. Bone marrow-derived MSC

Recent studies have highlighted the ability of BMMSCs to differentiate into various connective tissue types and their usability for tissue

repair (reviewed in [90–92]). Approximately 0.001–0.01% of the total cell population of the bone marrow are MSCs [93]. They can be easily obtained via - bone marrow aspirates, e.g. the iliac crest or long bones. To trigger differentiation of BMMSCs into the tenogenic lineage, treatments with different growth factors, mostly growth differentiation factors (GDF)/BMPs have been used, since it was shown that implantation of GDF-5, 6 and 7 leads to ectopic formation of neotendon tissue in vivo [94]. Treatment with GDF-5 (BMP-14) or GDF-7 (BMP-12) was shown to induce the expression of tendon-related markers, including Tnmd and Scx in human and horse BMMSCs, indicating that this treatment commits BMMSCs into tenogenic lineage in vitro [95,96]. Gene transduction of BMP-2 and active Smad8, GDF-5 or GDF-7 had similar effects [97,98], but also the transduction with Scx cDNA induced tenogenic differentiation in human BMMSCs [99].

In a rat Achilles tendon defect model, the injection of BMMSCs increased overall tendon healing significantly. Treated tendons showed increased production of collagens and a higher ultimate failure load at 1, 2 and 4 weeks after injury. Nevertheless, this study also stresses a higher capability of TSPCs to improve tendon healing [100]. Various other studies also used a rat Achilles tendon defect model to test the capacity of human or rat BMMSCs to improve tendon healing (Table 2). While three studies reported improved tendon healing with increased biomechanical capacities and collagen production [101 — 103], other studies could not demonstrate an effect of BMMSCs on tendon healing, stressing that there was only differences in biomechanical capacities at 2 but not 4 weeks after surgery and no differences in collagen production or ECM organization. Authors argue that the kind of Achilles tendon defect that was created (punch versus incision or collagenase induced lesion) affects the aptitude of BMMSCs for tendon healing [104,105]. Probably, BMMSCs have a temporarily beneficial effect especially in the early phases of tendon healing. In accordance to this hypothesis, Chong et al., reported improved tendon healing after BMMSC injection in a rabbit Achilles tendon injury model, 3 weeks but not 6 or 12 weeks after injury [106].

Likewise one long term study in horses reports a beneficial effect of BMMSCs in spontaneous lesions of superficial flexor digitorum longus tendons (SFDLT). Treated horses had improved ultrasound images, returned to full exposure more quickly and had a lower risk of re-rupture than untreated animals as long as 2 years after injury [107]. None of these studies reports if BMMSCs undergo tenogenic differentiation in vivo. The main reasons why tenogenic differentiation could not be proven are that the majority of the implanted cells do not survive and integrate athat fate tracking of the implanted cells was not carried out. A recent study has proposed a smart solution by non-invasive in vivo imaging of technetium-99 m labeled BMMSCs [108].

Since BMMSCs are thought to be hypo immunogenic, allogenic transplantation might not require immunosuppression. Another benefit of this cell types is that they can exert a positive influence on various blood cell types leading to an anti-inflammatory milieu during tissue repair by suppressing tissue necrosis factor (TNF)-α and interferon (INF)-γ, while stimulating the expression of suppressive cytokines like interleukin (IL)-10 [109].

Clinical trials investigating the effects of BMMSCs on tendon healing are scarce. Improved UCLA (University of California, Los Angeles) score was shown in 14 patients with complete rotator cuff tear after injection of non-fractioned iliac-derived BM mononuclear cells, 12 months after injury. Additionally, MRI showed improved tendon healing and integrity. Only one patient in this study showed aggravation of tendon strength and pain after 1 year, indicating that the procedure is safe and provides better functional outcomes than would usually be expected for such a lesion [110].

One problem with using BMMSCs for tendons, apart from the painful BM harvesting procedure, is the formation of calcification that could hamper tendon biomechanical properties. Furthermore, lengthy periods of cell expansion can lead to a phenotypic drift to the osteogenic lineage and donor age reduces quality of BMMSCs [111].

To further evaluate the feasibility of BMMSCs for tendon repair, multicenter clinical trials should be initiated, since BMMSCs are already approved for human use in graft versus host disease and in other human clinical trials.

2.2.2. Adipose tissue-derived mesenchymal stem cells (ADMSCs)

Due to painful harvesting procedures and the low content of MSCs in BM aspirates, researchers investigated other possible sources of MSCs, which should be easy to obtain, create minimal patient discomfort and yield higher numbers of MSCs to eliminate time consuming expansion steps in vitro. Adipose tissue presents itself as a good option.

In 2004, Zuk et al., first described the existence of MSCs in adult mesenchymal tissue. They isolated cells from lipoaspirates and gave evidence that these cells exhibit multilineage potential in vitro, differentiating towards the adipogenic, osteogenic, chondrogenic, and myogenic lineages when cultured in the presence of established lineage-specific differentiation factors [112]. Recently, it was found that a population of Tnmd-positive ADMSCs exists. These cells exhibit a phenotype very similar to that of TSPCs and can be biochemically induced towards the tenogenic lineage. They express higher levels of tenogenic genes (Scx, Tnmd, Tn C and Dcn), as well as Col I and Col III [113]. This finding makes adipose tissue an even more promising source of stem cells for the treatment of injured tendon.

In comparison to BMMSCs, MSCs from adipose tissue exhibit less potential for osteogenic and chondrogenic differentiation but are superior regarding adipogenic potential [114,115] To drive tenogenic differentiation of ADMSCs in vitro, insulin like growth factor (IGF)-1 or transforming growth factor (TGF)-β in co-culture with primary tenocytes and GDF-5 have been used successfully [116,117].

Injection of ADMSCs in a rabbit Achilles tendon injury model leads to ameliorated tendon healing. In comparison to untreated groups, tendons of treated rabbits exhibited more organized ECM deposition [118], while a second study also showed increased tensile strength, Col I, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) expression and reduced expression of TGF-β [119]. A similar effect was also reported in a rat Achilles tendon injury model. Col III/Col I ratio was reduced and tendons showed lower levels of degeneration and higher density of collagen fibers in ADMSC-treated group up to 12 weeks after collagenase induced lesion [120]. In contrast, Mora et al., reported that the transplantation of ADMSCs in a rotator cuff injury in rats did not beneficially effect biomechanical properties or collagen production, but treated tendons showed less inflammation which could result in more elastic repair and less scarred healing [121].

Race horses often suffer from superficial flexor digitorum longus tendon (SFDLT) lesions, due to high load during training and racing. There is evidence that the injection of ADMSCs after spontaneous or collagenase-induced SFDLT lesion significantly improves healing. Treated horses showed shorter periods of lameness and better organization of collagen fibers [122]. Since studies focusing on horses often have very long follow up examinations and most often do not sacrifice animals at the end of the study, they are especially interesting regarding long term outcomes of MSC treatments and re-rupture rates.

Like BM-MSCs, AD-MSs are already in clinical trials for other indications and should also be used in clinical trials analyzing tendon repair.

2.3. Perivascular cells

Perivascular cells (PCs) have drawn attention to tissue engineers in recent years. They can be isolated from multiple tissues and play important roles in tissue repair, vascular homeostasis and angiogenesis. PCs can be distinguished mainly in two subtypes: vascular smooth muscle cells (SMCs) and pericytes. SMCs mainly surround large vessels like arteries and veins and are separated from endothelial cells by the basement membrane and the inner elastic lamina, while pericytes surround smaller vessels and capillaries and are in direct contact with ECs. While pericytes are CD146, NG2, PDGFRb, aSMA, CD90,CD73,

CD105, CD44, ALP, nestin and vimentin positive in vitro, SMCs are positive for CD34, CD90, CD73, CD105, CD44 and vimentin. Clonal cultures can be grown from both subtypes [123,124]. Due to their property to secret high amounts of growth factors like heparin-binding epidermal growth factor (HB-EGF), bFGF and VEGF (reviewed in [125]), which are known to enhance tissue repair, PCs make a highly promising candidate for stem cell treatments for tendon, muscle or bone regeneration. Despite that, studies examining the possible application of PCs for tissue engineering are scarce. In 2008, Crisan et al., published proof of the existence of PCs with mesenchymal stem cell characteristics, showing that cultured PCs possessed chondrogenic, adipogenic and osteogenic capacities and expressed MSC markers. Furthermore, they stated that human pericytes, injected in hind limbs of a mouse model of muscular dystrophy, form new human myofibers and do so better than skeletal myoblasts [123]. Adipose tissue is another possible origin of PCs [126] but also PCs of the tendon express stem cell-related markers in vitro and in vivo as well as pericyte-related levels of SMA [55]. Mienaltowski et al., described two different TSPC populations, one located in the tendon proper and one in the peritenon. TSPCs of the peritenon seemed to be of a more vascular origin, again indicating the existence of perivascular TSPCs [65].

Subcutaneous implantation of muscle-derived PCs in polyethylene glycol (PEG) fibrinogen constructs leads to endogenous formation of tissue that contains vessel-like structures and has muscle-like organization [127]. Regarding the osteogenic capacity of PCs, Tsang et al., reported that human PCs ectopically produce bone when transplanted subcutaneously in the back of mice [128]. In accordance, human PCs seeded on an apatite-coated poly(lactic-coglycolic acid) scaffolds significantly increased ossification in a murine calvarial defect and induced osteogenic growth factor release (BMP2, VEGF) [126]. In a mouse model, transection of the supraspinatus and the infraspinatus tendon leads to consistent muscle atrophy, fibrosis, and fatty infiltration, similar to those observed in human rotator cuff tears [129]. Injection of PCs in the supraspinatus muscles of mice with rotator cuff tear led to reduced muscle atrophy and fibrosis, indicating that PCs might be capable of diminishing fibroadipogenic degeneration [130].

There is evidence that PCs engage in tendon healing and might be able to ameliorate it. The epitenon contains a cell pool, which is positive for P75 (a marker for neural crest stem cells) and SMA. These cells increase in number after tendon injury and suturing of the epitenon promotes tendon healing [131]. One additional study reported that CD146 + TSPCs, which are mainly located perivascularly, pretreated with CTGF, are capable of improving tendon healing in a rat patellar tendon injury model, showing in a reconstructed collagen structure. Transplantation of CD146 + TSPCs did not result in improved collagen structure [132].

3. Influence of growth factors on stem cells in tendon healing

As mentioned above, various growth factors are involved in the activation and concertation of cellular processes during the different phases of tendon healing (Table 1 and graphical abstract). Tendon injury leads to the production of multiple growth factors, resulting in increased cellularity and tissue volume [24]. Numerous studies have been published, with the aim of understanding the influence of growth factors on tendon biology in vitro and on tendon healing in vivo, some of them, specifically addressing the question on how growth factors influence stem cells (reviewed in [31]). Specific growth factors that are especially important in tendon healing are, TGF-β, CTGF, BMP-12, −13, −14, bFGF, platelet derived growth factor (PDGF), IGF-1and VEGF [133–135]. A summary of their influence on stem cells during the tendon healing process either in vitro or in vivo is summarized in Table 3.

3.1. TGF-β

TGF-β is active at all stages of tendon healing, as it has multiple and variable effects, while it is also expressed by most cells involved in tendon healing [136,137]. It stimulates extrinsic cell migration, regulates proteinases, terminates cell proliferation and stimulates collagen production [138–140]. Furthermore, mechanical loading placed on tendon induces TGF-β expression and TGF-β-pathway that in turn, regulates ECM and protease expression in the tendon. Thus, TGF-β signaling pathway is crucial in tendon's adaptation to mechanical loading [141]. On the other hand, TGF-β expression is up-regulated in tendinopathy and overuse (excessive physical activity) is the major predisposing factor for the development of tendinopathy [141]. Thus, TGF-β has been directly implicated in tendon disorders, especially tendinopathy [141]. It is well established that TGF-β is the growth factor responsible for scar and fibrous adhesion formation in all tissues in response to injury [142].TGF-β is required for the transformation of normal fibroblasts to contraction-capable myofibroblasts, that produce the scar tissue [143]. Interestingly, large number of tendon cells in tendon sheets and tendon proper, transform to myofibroblasts in chronic tendinopathy [144,145]. The myofibroblasts are especially abundant in fibrotic paratenon that contracts around the tendon tissue in chronic tendinopathy [144,145]. It has been postulated that the shrinkage of the fibrotic paratenon around diseased tendon is driven by myofibroblasts and this process could hamper the vascular supply of the tendon in tendinopathy causing hypoxia that persists in tendinopathy [144,145]. In mammals, three different isoforms (TGF-β1, 2 and 3) of the 25 kDa homodimer are expressed, and in mice, the knockout of each isoform gives rise to a distinct phenotype [146]. TGF-β1 levels increase very shortly after tendon injury and stay elevated up to 8 weeks in the healing rat patellar ligament [137]. In flexor tendon repair in the mouse, a biphasic pattern of TGF-β expression was found, with an early peak of TGF-β1 and a late peak of TGF-β3, accompanied by an upregulated expression of TGF-β receptors [147]. In vitro, all isoforms of TGF-β were able to upregulate the production of Col I and Col III by tenocytes isolated from the tendon sheath, the endotenon and the epitenon [148]. The production and organization of new collagen fibers is essential for tendon to heal, thus TGF-β seems to play an essential role in tendon healing. Contrarily, there is also data, stating that TGF-β induces fibrotic scar formation, resulting in adhesion formation [149,150]. Specifically, TGF-β1 appears to be responsible for scar and adhesion formation, as treatment with TGF-β1 antibody increased range of motion within a rabbit model. A combined infiltration of TGF-β1 and 2 antibodies did not have beneficial effects [136], leading to the conclusion that TGF-β1 and 2 might have detrimental effects on scar and adhesion formation. These findings were further underscored by a study that reports a beneficial effect of antisense oligonucleotide treatment against TGF-β1, Smad3 and CTGF on flexor tendon healing without negative effects on tendon biomechanics within a mouse model [151]. Therefore, the right concentration and combination of different isoforms of TGF-β has to be found to improve tendon healing.

TGF-β signaling is highly active in tendon cells during development [152,153] and disruption of TGF-β signaling in TGF-β2 and 3 double knockout mice, or through deletion of TGF-β receptor type II (TGF-βRII) results in the loss of almost all tendons in the limbs, trunk, tail and head [154]. These findings hint at a possible role of TGF-β in tenogenic differentiation of TSPCs and other stem cells. It was shown in one study that TGF-β significantly enhances proliferation of TSPCs in vitro [155], but a second study reported the opposite effect for TGF-β1 [156]. Since the first study does not state which isoform of TGF-β was used, it is possible that different isoforms influence TSPC proliferation in a contrarious manner. Literature agrees that treatment of TSPCs with TGF-β leads to an increase in Scx expression [156,157], indicating that TGF-β might induce tenogenic differentiation of TSPCs. Similar results have been found for other types of MSCs. Various studies state that TGF-β treatment induces tenogenic differentiation in BMSCs [158–162]and two studies report the same effect on ESC [163,164]. In vivo transplantation of BMSCs transfected with TGF-β enhanced tendon healing in a rabbit Achilles tendon injury model, resulting in raised collagen production, faster ECM production and organization and creation

Table 3
Response of stem cells to growth factors.

Growth factor	Cell type	Study type	Result	Source
TGF-β	MSCs and TSPCs	In vitro	Increased tenogenic potential, upregulation of Scx	[157]
	TSPCs	In vitro	Enhanced proliferation	[155]
	ESCs	In vitro	Expression of tendon associated genes, tenocyte lineage differentiation	[163,164]
	ADMSCs	In vitro	No induction of tenogenic potential	[116]
	BMSCs	In vitro	Increased Scx expression and collagen production	[160]
	BMSCs	In vitro	Increased proliferation, increased production of Col I and Col III	[159]
	BMSCs	In vivo	Ameliorated tendon healing, increased collagen production and biomechanical features	[161]
	BMSCs	In vivo	Ameliorated tendon healing, increased Col I production, more rapid matrix remodeling, larger fiber bundles	[162]
			Loss of stemness, increased expression of Scx, Dcn, TN-C, Col I, Col II and osteonectin, decreased proliferation	
	TSPCs	In vitro		[156]
TGF-β + VEGF	BMSCs	In vivo	Accelerated alignment of reconstructed ligament	[202]
	BMSCs	In vivo	Ameliorated tendon healing, increased collagen production and biomechanical features	[161]
CTGF	TSPCs	In vivo	Better fibril organization, larger fibril size	[83]
	TSPCs	In vitro	Increased expression of Scx, Tnmd, Col I and Tn-C	[165]
	TSPCs	In vivo	Increased number of CD146 + TSPCs at injury site and enhanced tendon healing	[132]
			Production of abundant ECM, formation of a cell sheet	
	TSPCs	In vitro		[84]
			Increased Col I and TN-C synthesis, failure to show osteogenic or chondrogenic differentiation	
	BMSCs	In vitro		[166]
BMP 13	BMSCs	In vitro and in vivo	Increased expression of Scx and Tnmd in vitro, neotendon formation and promoted tendon healing in vivo Increased expression of Scx and Tnmd in vitro, formation of neo tendinous tissue	[173]
	BMSCs	In vitro and in vivo		[174]
BMP 12	BMSCs	In vitro and in vivo	Increased expression of Scx and Tnmd in vitro, enhanced tendon healing with increased matrix production and elevated expression of tendon markers in vitro	[171]
			Upregulated expression of Scx, Tnmd, Col I and TN-C in vitro, neo tendon formation in vivo	
			Upregulated expression of Scx, Tnmd, Col I and TN-C in vitro, neo tendon formation in vivo	
	BMSCs	In vitro and in vivo	Induction of tenogenic differentiation, expression of Tnmd and Dcn	[172]
			Induction of tenogenic differentiation, expression of Scx and Col I	
	ADMSCs	In vitro and in vivo		[172]
	BMSCs	In vitro		[95]
	BMSCs	In vitro		[167]
BMP 14	MDMSCs	In vitro	Increased failure strength and stiffness of repaired tendon	[175]
			Increased proliferation, induction of expression of tenogenic markers and ECM components	
	ADMSCs	In vitro		[117]
			Loss of stemness, elevated expression of Dcn, Scx and osteonectin, reduced expression of TN-C, Col I and Col II, no effect on proliferation	
	TSPCs	In vitro		[156]
BMP 14 + VEGF	BMSCs	In vitro	Enhanced tenogenic differentiation	[158]
BMP14 + TGF-β	BMSCs	In vitro	Enhanced tenogenic differentiation	[158]
bFGF	TSPCs	In vivo	Increase in MSCs at injury site, increased expression of Scx and Tnmd, higher histologicals scores and significant improvement in mechanical strength	[180]
			No beneficial effect on tendon healing, no differences in biomechanical capacities or histology	
			Induction of tenogenic differentiation, increased expression of Scx and TN-C	
	BMSCs	In vivo		[104,105]
			Induction of tenogenic differentiation, increased expression of Scx and TN-C	
			Enhanced proliferation	
	AFSCs	In vitro		[113]
	ADMSCs	In vitro	Promotes maintenance of differentiated cells, reduction of number of TSPCs	[113]

(continued on next page)

Table 3 (*continued*)

Growth factor	Cell type	Study type	Result	Source
	TSPCs	In vitro		[155]
	TSPCs and tenocytes	In vitro		[179]
PDGF	ADMSCs	In vitro	Induction of tenogenic differentiation, increased expression of Scx and TN-C	[181]
	ADMSCs	In vitro	Enhanced proliferation, tenogenic differentiation, shown by increased expression of Scx and Tnmd Increased cellularity and hypervascularity 3 weeks after injury, increased production of Col I and Col III	[278]
	BMSCs	In vivo		[187]
IGF-1	PBMSCs	In vitro	No influence on proliferation, no effect on expression of TN-C and Dcn	[279]
	BMSCs	In vivo	No improvement in tendon healing and biomechanical properties, no differences in gene expression Preservation of stemness, slight increase in Scx expression, slight decrease in Col I expression, downregulation of Col II expression, no effect on proliferation	[195]
	TSPCs	In vitro		[156]
IGF-1 +	TSPCs	In vitro	Adipogenic differentiation, increased expression of PPAR-γ	[196]

of larger fiber bundles, compared to implantation of un-transfected BMSCs. Mechanical strength of tendons treated with TGF-β BMSCs was also higher than in BMSC treated controls [162]. A combination of MSC transplantation and TGF-β administration seems to be a promising approach to treat ruptured tendon, but further studies, especially focusing on possible negative effects of TGF-β (e.g. adhesion and scar formation) are needed to ensure that TGF-β really and safely augments tendon healing. One also needs to keep in mind that the clinical trials with TGF-β for indications outside of the tendon had to halt due to excessive scar formation [142].

3.2. CTGF/CCN2

Connective tissue growth factor (CTGF/CCN2), a downstream mediator of TGF-β, and member of the CCN family, showed persistent upregulation over 21 days during healing in chicken flexor tendons [134], while it was moderately expressed at all time points in a rat supraspinatus injury model [133]. Despite these findings, very little is known about the role of CTGF in tendon healing and its influence on tenocytes. When treated with CTGF and ascorbic acid in vitro, TSPCs produce an abundant ECM and thereby form a cell sheet, which can be used as an engineered tendon tissue transplant [84]. Furthermore, CTGF is capable of inducing tenogenic differentiation of TSPCs in vitro, by upregulating the expression of Scx, Tnmd, Col I and TN-C [165]. In vivo implantation of TSPCs pretreated with CTGF ameliorated tendon healing in a rat patellar tendon window defect. CTGF TSPC transplanted tendon exhibited better collagen fiber organization and larger fibril size [83,132]. BMSCs react to CTGF treatment in vitro with an upregulation of Col I and TN-C expression, and more interestingly fail to undergo chondrogenic or osteogenic differentiation [166].

3.3. BMPs

BMP-12, −13 and −14 (also known as GDF-7, −6 and −5) also belonging to the TGF-β family, are known to stimulate mitogenesis and tenogenic differentiation of MSCs in vitro [167] and in vivo [94]. During tendon healing BMPs are elevated at early stages and decrease gradually over time [133]. While BMP-12, −13 and 14 mainly induce tenogenic differentiation, BMP-2 drives osteogenic differentiation, assigning it an important role in enthesis, meaning tendon to bone healing. BMP-2 is even able to induce new bone formation within tendon, which is not desired in intratendinous healing [168–170].

Treatment with BMP-12 induces tenogenic differentiation of BMSCs and ADMSCs in vitro, showing an upregulated expression of typical tendon markers, such as Scx, Tnmd, Col I, TN-C and DCN, while this effect was also observed in vivo [95,168,171,172]. Pre-treatment of human BMSCs with BMP-12 followed by transplantation in a rat calcaneal tendon defect led to better tendon repair than implantation of untreated BMSCs. BMP-12 treated cells were largely spindle shaped and produced well organized ECM, while in the untreated group only minimal tendon-like morphology was exhibited [171]. Furthermore, BMSCs and ADMSCs produce neotendinous tissue in a nude mouse model after adenoviral transfection with BMP-12 [173]. BMP-13 promotes tenogenic differentiation of BMSCs in vitro and also induces neotendon formation by this cell type [174,175]. Combination of an engineered tendon matrix with BMP-13 and BMSCs was shown to significantly improve tendon healing, resulting in improved fiber alignment, increased ultimate stress and Young's modulus [174].

BMP-14 is the only BMP whose effect on TSPCs has been investigated so far. It appears to have no effect on proliferation but leads to the loss of stemness of TSPCs. On the other hand, it elevates expression of Dcn, Scx and osteonectin, but reduces expression of TN-C, Col I and Col II [156]. In ADMSCs, it increases proliferation and induces tenogenic differentiation with an upregulation in tenogenic markers and tendon specific ECM components [117]. In a tendon in vitro healing model using muscle-derived MSCs on a gel patch treated with BMP-14, failure strength and stiffness of the repaired tendon was significantly higher than in non-cell groups. Interestingly, BMSCs treated with BMP-14 had no positive effect on biomechanical capacities of the repaired tendon [175].

Overall, BMP-12, −13 and −14 are potent inducers of tenogenic differentiation in different types of MSCs, but due to a lack of in vivo studies it has yet to be clarified how they affect TSPCs in tendon healing and to what extent they beneficially influence healing capacities of other MSCs.

3.4. bFGF

bFGF is a member of the heparin-binding growth factor family and is known to be a potent stimulator of angiogenesis and cellular migration in vitro and in vivo [176]. bFGF also stimulates proliferation of tendon

fibroblasts, shown by an in vitro wound closure model of Chan et al., [177]. In a later study, it was found that bFGF mRNA is upregulated in mature tenocytes, fibroblasts and inflammatory cells surrounding the healing site in the tendon sheath [178]. Regarding the time course of tendon healing, bFGF levels are elevated at the early stages [133], leading to the conclusion that bFGF might promote early events in tendon healing.

In vitro, bFGF enhances proliferation of TSPCs [155] but also promotes the maintenance of differentiated cells and reduces the number of TSPCs in a TSPC-tenocyte co-culture [179]. Hence, it is likely that bFGF drives differentiation of TSPCs to tenocytes. In a rat rotator cuff injury model, implantation of a bFGF hydrogel leads to increased ultimate strength and higher histological scores. In addition, the number of MSCs was significantly increased in the bFGF treated group and that these cells expressed increased amounts of Scx, indicating that more tenogenic progenitor cells were generated at the healing sites [180].

While bFGF stimulates tenogenic differentiation of amniotic fluid stem cells and ADMSCs [181], it seems to have no beneficial effect on the ability of BMSCs to enhance tendon healing. In two studies using a rat Achilles tendon defect model, BMSCs lentivirally transfected with bFGF failed to ameliorate healing of the defect. No differences in biomechanical properties or histological appearance could be observed between treated and untreated groups [104,105].

Since there are very little studies on the effects of bFGF on MSCs with a focus on tenogenic differentiation and tendon healing, it would be of interest to analyze if different concentrations of bFGF might generate different effects. Furthermore, since bFGF is known to be angiogenic, analyzing the effects of bFGF on formation of neovessels in healing tendon would be advisable.

3.5. PDGF

PDGF is a potent mitogen for cells of mesenchymal origin, including fibroblasts, smooth muscle cells and glial cells [182,183]. In the healing canine digital flexor tendon, PDGF was upregulated suggesting a possible role in tendon healing [184]. Expression of PDGF receptor β is also elevated in healing tendon and this elevation persists for over 6 months. It was also shown that PDGF administration has a beneficial effect in a rat patellar tendon defect. Treatment with PDGF increased tendon healing when it was administered seven days after injury, but not 3 days after injury [185]. Thus, PDGF might not be essential for the first stage of tendon healing. In vitro, PDGF plays an important part in tissue remodeling. It was observed to stimulate collagen, non-collagen protein production and DNA synthesis in a dose dependent manner in different types of rabbit tendons [186] Therefore, PDGF might play an important role in the later stages of tendon healing, where an increase in ECM production was observed.

Furthermore, PDGF increases tenogenic differentiation of ADMSCs in vitro, causing an upregulation in expression of Scx, Tnmd and TN-C. PDGF transfected BMSCs seeded on an irradiated Achilles tendon allograft were investigated for their effect on anterior cruciate ligament reconstruction. It was shown that the implantation leads to accelerated cellular infiltration, enhanced collagen production and the initial promotion of angiogenesis [187]. These results also indicate that PDGF could have beneficial effects on tendon healing after injury.

3.6. IGF-1

IGF-1 is involved in multiple processes in normal body growth and healing. It mediates all stages of wound healing, especially the inflammatory and the proliferative phase [188]. It is also upregulated locally during and after inflammation following soft tissue injury, accompanied by upregulation of its receptors [188–191]. During ligament healing, IGF-1 mRNA was found to be most upregulated 3 weeks after injury in the rabbit [188] but in horse flexor tendon, IGF-1 levels had decreased by approximately 40% after 2 weeks following injury. Increased levels were found after 4 and 8 weeks within this model [192]. Since the time course of tendon healing in rabbit and horse is different, these results are not controversial. IGF-1 mainly seems to stimulate proliferation and migration of fibroblasts and other cells at the injury site and to increase production of collagens and other ECM components in these cells [193,194].

Due to its effects on collagen and ECM production, IGF-1 seems to be most important in the formation and remodeling stages of healing.

Treatment of TSPCs with IGF-1 led to preservation of stemness, a slight increase in Scx expression, a slight decrease in Col I expression and downregulation of Col II expression in vitro. It had no effect on their proliferation [156]. It is not known how IGF-1 influences TSPCs in vivo.

Adenoviral gene transfer of IGF-1 in BMSCs did not increase their ability to improve tendon healing. Transplantation of non-transduced BMSCs was as effective in increasing biomechanical properties and ECM production as transplantation of IGF-1-BMSCs [195].

Treatment or transfection of TSPCs with IGF-1 might actually have negative effects on tenogenic differentiation. Liu et al., reported, that combined treatment of rat TSPCs with IGF-1 and BMP-2 significantly increases adipogenic differentiation and that they mediate prostaglandin E (PGE) 2 induced adipogenic differentiation of TSPCs. PGE 2 is suggested to be involved in the pathological changes associated with tendon overuse, including osteogenic and adipogenic changes [196].

3.7. VEGF

The angiogenic factor, VEGF, is almost completely downregulated in healthy tendon but its expression reoccurs during tendinopathy and tendon healing [197,198]. Neo-angiogenesis could be observed in human Achilles tendon disorders [199] but no link to VEGF expression has been made. Despite VEGF having an effect on stromal cells, it is a highly specific growth factor for endothelial cells [200], has only a little direct role in early tendon healing per se, potentially stimulating cell migration and proliferation. It is most active during the proliferative and remodeling phase, where it stimulates angiogenesis [197,201]. The role of neoangiogenesis in tendinopathy and tendon healing will be discussed more intensely in the latter part of this review.

The influence of VEGF on MSCs in the context of tendon healing has mostly been investigated in combination with other growth factors. In combination with BMP-14, VEGF enhances tenogenic differentiation of BMSCs in vitro [158]. Two in vivo studies have examined the effect of BMSCs treated with VEGF and TGF-β on ligament or tendon healing. In the first study, ACL grafts were seeded with TGF-β, VEGF, or TGF-β/VEGF transfected BMSCs. Three weeks after surgery cellularity and vascularity were increased in the VEGF and VEGF/TGF-β BMSC grafts. Cellularity and vascularity decreased in all groups over time. At 12 and 24 weeks after surgery, biomechanical properties of VEGF and VEGF/TGF-β were better than in untreated groups. Co-expression of both growth factors led to the best outcome for all parameters investigated [202]. In the second study, human BMSCs were transduced either with an adenovirus carrying TGF-β, VEGF or both cDNAs. In the co-expression group, maximum failure load, tendon stiffness and elastic modulus of the healing tendons were significantly increased and there were signs of accelerated lesion remodeling. VEGF transduced BMSCs had a negative effect on tendon healing in this study, showing an increased vascularity and decreased collagen content. Biomechanical properties were not affected by the implantation of VEGF-BMSCs [161].

Since an increase in vascularity is necessary for tendon healing, the ambivalent actions of VEGF have to be investigated carefully, if it is to be used as a potential enhancer of tendon healing.

4. Delivery of stem cells to injured tendon

One of the fundamental issues restricting the use of cell-based therapies is the delivery of the cells to the target, as well as their

engraftment [203]. The stem cells have either been injected systemically into circulation or locally at the site of the injury. The systemic administration of stem cells has the problem that the cells do not accumulate at the site of the injury at therapeutically relevant numbers, whereas local injection might cause additional damage to the injured tissue and the stem cell survival is low after local injection due to engraftment problems [203]. The tendon rupture itself provides an appealing opportunity to target therapeutics, including stem cells, to the desired location by simple administration of cells to the circulation. Granulation tissue, (i.e. the early loose connective tissue that forms between the ruptured tendon ends) is made out of newly formed, tiny capillaries [33]. These newly formed angiogenic blood vessels have unique molecular structures on their surface and provide an opportunity for targeting ligands that bind to them in an organ-specific manner [204]. Due to this, a random peptide library (1.0×10^9 cyclic peptide ligands) was screened by in vivo phage display on ruptured Achilles and patellar tendons during the proliferation phase to find systemically administered peptides capable of homing to the injured sites [204]. The best vascular homing peptides identified, home to injured tendons up to 200-fold and have been used successfully for delivery of therapeutic agents, enhancing tissue regeneration [204–207] Following MSC "painting" of the surface with multiple copies of these vascular targeting peptides, MSCs delivery and engraftment were increased by 400% to the infarcted myocardium [206]. Thus, the vast microvascular network formed by a robust angiogenic response at the site of tendon rupture offers plenty of molecular targets for systemically administered ligands to home and deliver cargo to the ruptured tendon [208]. As these vascular homing peptides are capable of homing to ruptured tendons and the neovessels are also a hallmark of tendinopathy, the platform for utilizing the vascular homing peptide-technology to deliver stem cells to both tendinopathy and acute, traumatic tendon ruptures is essentially already in place [203, 206] (Fig. 3).

5. Effects of drugs and novel boosters on stem cells in tendon healing

To date, drugs to treat tendinopathy are mostly restricted to analgesics and anti-inflammatory agents (Table 4). Corticosteroid injections are commonly used to treat tendinopathy in the initial phase due to their ability to reduce inflammation and provide pain relief, even though there are several reports of spontaneous tendon ruptures following the use of local corticosteroid injections [209–211]. Furthermore, data on the utility of local steroid treatment is controversial [212,213]. Studies using animal models tried to explain the negative effects of corticosteroids on tendon healing, proposing inhibition of tendon cell proliferation, decreased collagen synthesis or increased collagen breakdown as possible explanations [214–216]. Poulsen et al., treated primary human tenocytes with dexamethasome (Dex) and reported that this led to the induction of senescence via the p53/p21 pathway and further showed that injections led to an increase in p53 and p21 positive cells in human supraspinatus tendons [217]. To elucidate to what extent corticosteroids influence TSPCs and how this could contribute to the poor outcome of tendon healing after local injection, further in vitro studies were performed. It became clear that Dex, one of the most commonly used corticosteroids, reduces TSPC proliferation in a dose-dependent manner [216,218]. Furthermore, it inhibits tenogenic differentiation of TPCS and pushes them into an adipogenic or chondrogenic lineage [218,219].

The implantation of Dex treated TSPCs in nude rats led to extensive forming of fatty, cartilage-like and bone-like tissue after three weeks [218]. The increase of adipogenic differentiation is most likely due to an increase in dickkopf1 expression which leads to inhibition of the classical WNT/β-catenin pathway [220]. Trimacinolon, another member of the corticosteroid drug family, is often used to treat pain after tendon injury. Like Dex, it also induces differentiation of human supraspinatus tendons into adipocytes and significantly reduces their proliferation. Unfortunately this study did not clarify if the extracted cells were tenocytes or TSPCs and is likely to be a containing both cell types [221]. In accordance, treatment of murine mesenchymal stem cells with Trimacinolon reduced cell proliferation and tenogenic differentiation, while adipogenic differentiation was enhanced [222]. These results lead to the assumption that Trimacinolon will also negatively influence TSPC proliferation and differentiation.

Bupivacaine, Ropivacaine and Morphine are other analgesics often used for pain relief (e.g. anterior cruciate ligament reconstruction) without knowing whether they affect tendon cells. Since it is of great importance not to compromise the viability or metabolism of TSPCs within the tendon graft, Haasters et al., investigated the effects of these three drugs on the viability and metabolism of TSPCs in vitro.

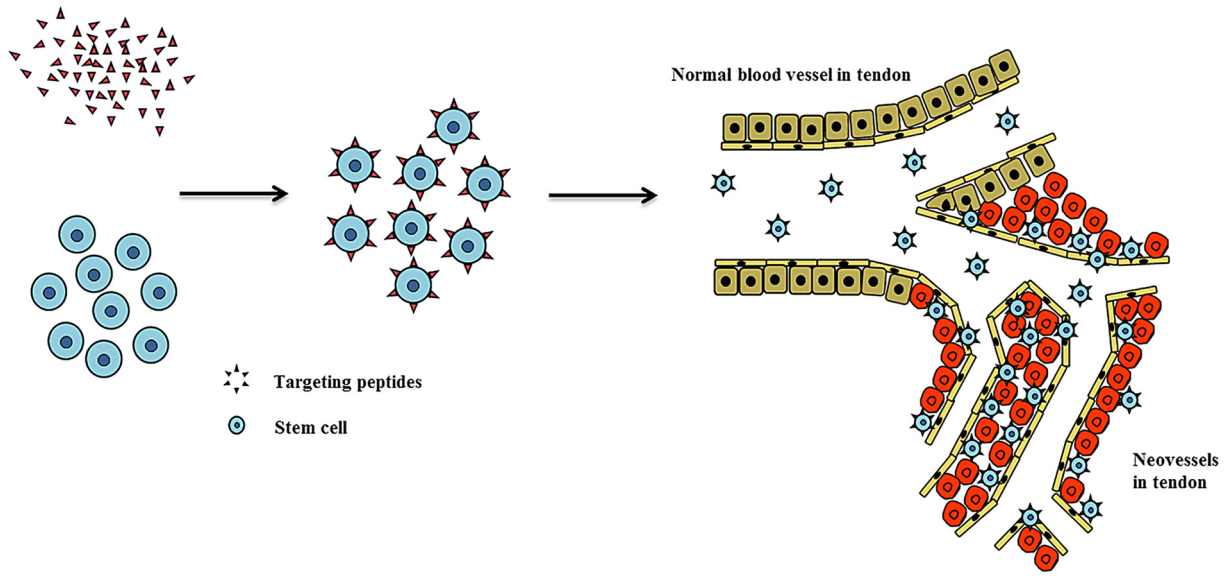

Fig. 3. A schematic drawing on targeted delivery of stem cells to neovessels in tendon injuries. Specific molecular structures on the surface of newly formed angiogenic blood vessels in the regenerating tendon after an acute rupture (or in tendinopathy) provide a platform for target organ-specific delivery of the systemically administered stem cells. Stem cells are conjugated with multiple copies of a vascular homing peptide that recognize the angiogenic blood vessels and work as an "address tag". The outcome of the targeted therapy is identical to topical application: increased number of the stem cells in the target tissue and reduced accumulation in the healthy organs.

Table 4
Effects of drugs on tendon stem/progenitor cells.

Drug	Effect on TSPCs	Source
Dexamethasone	Increased synthesis of dickkopf1, inhibition of the classical WNT/β-catenin pathway, differentiation of TSPCs to adipocytes	[220]
	Inhibition of differentiation to tenocytes, downregulation of Scx expression	[219]
	Low concentrations increase, high concentrations reduce cell proliferation, upregulation of non-tenogenic differentiation, formation of fatty tissues, cartilage-like tissues, and bony tissues	[218]
	Dose-dependent inhibition of proliferation, collagen production, colony formation and expansion	[216]
Trimacinolone	Reduced proliferation, increased adipogenic differentiation	[221]
Bupivacaine	Reduced cell viability and metabolism, induction of apoptosis	[62]
Ropivacaine	Reduced cell viability and metabolism, induction of apoptosis at higher concentrations	[62]
Morphine	No cytotoxic effect, no effect on cell metabolism, survival or apoptosis	[62]
Sclerosin agents	Sclerosin injections result in sclerosis, shrinkage of neovessels in various tendinopathies and have positive effect on the tendon tissue	[280–285]
Bevacizumab	Alone or in combination with platelet rich plasma accelerates and improves tendon healing in rodent models	[286,287]
Digoxin	Impedes calcification and promotes tenogenesis of TSPCs in vitro through inhibition of HIF-2alpha	[288]

Table 5
Novel boosters of tenogenesis.

Component	Effect on stem cell	Source
Ephrin A4, Ephrin B2	Eph A4 increases proliferation of aged TSPCs, Eph A4 and Eph B2 increase cell motility, rescue migration deficit of aged TSPCs and improve their actin turnover.	[64]
Mohawk	Overexpression of Mkx impairs adipogenic and osteogenic differentiation of MSCs, increases Scx expression and leads to the formation of cell sheets with larger Col fibril diameters.	[225]
	Implantation of Mkx-TSPCs in rat Achilles tendon injury model leads to increased amounts of mature Col and better biomechanical capacities.	
Retinoic acid receptor agonists	RAR agonists induce Scx expression in TSPCs and block differentiation to adipocytes, osteocytes or tenocytes, while maintaining expression of stem cell markers.	[224]
Rho/Rock agonists	Inhibition of Rho/Rock signaling leads to loss of Scx and Tnmd expression and the tenocyte-like phenotype of MSC under tenogenic conditions.	[226]
	Inhibition of Rho/Rock signaling impedes stretch induced morphological changes of MSCs and upregulation of tenogenic gene expression	[227]

They found that Bupivacaine and Ropivacaine are both cytotoxic for TSPCs and reduce their metabolism, while Morphine had no such effect, suggesting that morphine is the drug of choice in clinical practice [62].

Overall, there are few drugs used for the treatment of tendinopathy or tendon injury, despite use in pain relief. However, little is known about how these drugs affect viability, proliferation and expression profiles of TSPCs. Hence, there is a great need of in vitro studies analyzing if drugs have undesirable effects on TSPC survival, proliferation and tenogenic differentiation to enhance tendon repair.

More recently, studies focusing on new cellular boosters that could enhance tenogenic differentiation of stem cells and therefore increase the success of stem cell transplantation after tendon rupture have been published (Table 5). Popov et al., analyzed the effects of age on Ephrin (Eph) A4 and Eph B2 expression and their effect on self-renewal, migration and actin turnover of young and aged TSPCs. The motivation for this study was that aged TSPCs change their expression of different Eph members, enter senescence earlier and have a self-renewal deficit as well as dysregulated actin dynamics, cell motility and cell matrix interactions [63]. In aged TSPCs, Eph A4 and Eph B2 were downregulated significantly. They further showed that treatment with Eph A4 and Eph B2 can overcome the migration deficit of aged TSPCs and that Eph A4 increases proliferation of aged TSPCs, almost to the level of young TSPCs [64]. In accordance, another study found that a disturbed Eph A signaling is related to age-associated senescence in human cardiac progenitor cells [223]. These findings indicate that Eph signaling is important for the maintenance of stem cell characteristics from different origins. Regarding tendon rupture, implantation of TSPCs overexpressing Eph is potentially a promising approach.

One problem with working with TSPCs is that they undergo spontaneous differentiation in vitro. Hence the culture steps necessary between isolation and re-implantation, might lead to the loss of TSPC stemness. One possible way to circumvent this problem might be the administration of retinoic acid receptor (RAR) agonists. A study using high throughput screening identified RAR agonists as strong inducer of Scx expression in TSPCs. Further analysis by the authors found that treatment with a RAR agonist does not influence cell viability or morphology, but that it leads to an upregulation of Scx expression and blocks differentiation into osteocytes, adipocytes or tenocytes. In addition, the treatment also preserved the expression of stem cell markers in vitro [224]. Implantation of TSPCs that have not undergone differentiation during in vitro cultivation, might help to unleash their complete regeneration potential.

In terms of stem cells of other origins from tendon regeneration, Mohawk (Mkx) might be a promising candidate for inducing tenogenic differentiation. Overexpression of Mkx in MSCs impaired adipogenic and osteogenic differentiation of MSCs and TSPCs. Furthermore, MSCs overexpressing Mkx formed cell sheets with a larger collagen fibril diameter in vitro and implantation of these cells in a rat Achilles tendon injury model, increased the amount of mature collagen and enhanced the biomechanical capacities of the repaired tendon in comparison to tendons treated with mock-MSCs [225].

Activation of the Rho/Rock signaling pathway could be another way of efficiently inducing tenogenic differentiation in MSCs, since inhibition of this pathway leads to a loss of the elongated phenotype of tenocytes, a reduction in the expression of Scx and Col I and stretch induced morphological changes seen in tenogenic differentiation are impeded [226,227].

Despite the fact that the Rho/Rock signaling pathway has been suggested to be involved in the differentiation of MSCs [228,229] there are no studies investigating the effect of activators of this signaling pathway on the tenogenic differentiation potential of MSCs.

Overall, we have to deepen our knowledge of molecules and signaling pathways involved in tenogenic differentiation (Fig. 2) to be able to identify adequate boosters of this process, which can then enhance the healing potential of MSCs applied in tendon injuries.

6. Effects of drugs on vasculature and endothelium in tendon healing

In the healthy tendon, blood vessels enter the tendon from the myotendinous junction, the bone insertion site and from the paratenon. In sheathed tendon, blood vessels only enter the tendon at a few distinct points, while in tendons containing a paratenon, vessels pass through the tissue more frequently. Since tendons are extended by mechanical load during movement, the vasculature must be compliant to being stretched. Hence, the vessels form curves within the tendon tissue [230]. Due to their limited metabolism and their mechanical function, tendons contain only very little vasculature. In the adult tendon, the relative avascularity is simply caused by the fact that the metabolic rate of tendon is almost non-existent [18,231].

Persistent hypoxia is the major driver of tendinopathy [30]. The histological outcome of tendinopathy, (i.e. tendinosis), is the outcome of persistent hypoxia in the tendon tissue [30,35]. Hypoxic changes are even more severe in tendon rupture than tendinopathy and the hypoxic changes are the major predisposing factor for acute tendon ruptures [34,232]. To survive under hypoxia, the cells secrete angiogenic growth factors (e.g. VEGF) in the hope of inducing angiogenesis to the area requiring oxygen [200,233,234]. Thus, neoangiogenesis is a typical symptom of chronic tendinopathies and tendon rupture, accompanied by an increase in VEGF expression [235–237]. The role of neovessels for degenerative tendon disorders is poorly understood (reviewed in [231]). One theory associates the area of newly grown microvessels with pain in tendinopathy, since nerve structures are often in close relation [199, 238]. This view is supported by the fact that VEGF also stimulates nerve (axon) growth and could be the reason why nerves generally follow blood vessels in the human body [239]. Thus in chronic tendinopathies, hypervascularity is a sign of attempted repair and might be a contributory factor to pain while in an acute injury, the increased vascularity seems to be essential for tendon repair [26]. When considering the role of neovessels in tendinopathy, it is fundamental to understand that the neovessels are non-functional blood vessels, which do not have proper perfusion and do not deliver oxygen and nutrients to the cells needing them, resulting in hypoxia persisting in the tissue surrounding them [233,234] So, the chronic persistence of neovessels seen in tendinopathy should be always considered a sign of failed repair.

Endothelial function is regulated by a finely balanced equilibrium between vasorelaxing and vasoconstricting mediators. In these processes nitric oxide (NO) seems to have an important role. NO is produced by two enzymes, NO synthase 1 (NOS1) and NOS2. NOS1 is a constitutively expressed enzyme that produces tiny amounts of NO to keep blood vessels open ("dilated"). In turn, NOS2 is an inducible enzyme expressed in inflammation and produces large quantities of NO. NO derived from NOS2 reacts with toxic superoxides and stimulates the production of peroxynitrites and free radicals, eventually predisposing to endothelial dysfunction [240,241]. In normal, un-injured tendon there is little to no NOS present but after injury, expression of NOS 1, 2 and 3 was upregulated in a rat rotator cuff and Achilles tendon injury model with a peak, 7 days post injury [242,243] and feeding rats with a NOS inhibitor, significantly reduced tendon healing [244]. VEGF induces the expression of NOS2 and the increased NO concentrations encountered in the ruptured tendon after the injury are related to active angiogenesis and the VEGF-driven vasodilation as well as the inflammation cascade. In human rotator cuff samples, excised during surgical repair, NOS activity was found in 7 out of 10 samples [245]. Overuse of tendon also results in an over-expression of NOS isoforms and this might contribute to degenerative changes mediated by increased levels of metalloproteinases or cytotoxicity [246,247].

To what extent and how NOs influence endothelium in tendons and how this contributes to tendon injury are questions not yet completely understood. There have been attempts to treat tendinopathies with NO (reviewed in [244]). In three randomized clinical trials, NO was administered to the area of tenderness via a glyceril trinitrate (GNT) patch in three different conditions: tennis elbow, Achilles tendinosis and supraspinatus tendinosis. In all three conditions, the NO GNT patch led to enhanced clinical recovery that is demonstrated in reduced pain, increased range of motion and increased strength compared to a placebo GNT patch.

In summary, one needs to understand that there is neither tissue repair nor regeneration without oxygen. In turn, oxygen needs to be delivered to tissue undergoing repair by blood vessels. Thus, the blood vessels are crucial for any tissue repair after injury. The increased vascularity seen in the persistent neovessels in tendinopathy is a sign of failed repair. It is well established in cancer research that the neovessels are non-functional; they do not have proper perfusion and do not deliver oxygen and nutrients to the tissue The hypoxia persists in the tissue surrounding them [233,234]. Thus, the focus on tendinopathies should be on stabilizing the non-functional neovessels to provide adequate oxygen and nutrients supply to the tendon. Neovascularization is essential for the early stages of tendon healing. Hence it might be interesting to study the effect of drugs that influence vascularization at different time points after tendon injury and pursue strategies that stabilize the vasculature to a functional one [248,249].

Angiogenic inhibitors have been most extensively studied in the context of cancer, since cutting tumor blood supply is a promising approach. Many of them have passed or are close to passing FDA approval and are already used for therapy of cancer or age-related muscular degeneration [250]. However, anti-angiogenic drugs have been a major disappointment in the treatment of cancer as the survival benefit derived from them, has been rather minimal [233,234,248]. The mechanism of resistance to the currently available antiangiogenic therapies in tumors as well as in retinopathy are actually related to the eradication of the neovessels, which worsens the underlying ischemia and drives the formation of new neovessels by alternative molecular mechanisms [233,234,248]. Thus, the proposed molecular mechanism for future antiangiogenic therapies is one in which the angiogenic blood vessels are "normalized" to stable ones to alleviate the hypoxia [233,234,248, 249] The "normalized" blood vessels are functional, they have the proper perfusion inside them and can carry enough oxygen and nutrients for tissue regeneration to take place [233,234,248]. As described above, first animal studies using anti-VEGF treatment to accelerate tendon healing have reported promising results, hence the creation of a finely balanced VEGF levels seems to be desirable. However, it is well-established in other injury models, such as wound and fracture healing that tissue regeneration cannot be obtained if the injury is treated with VEGF-inhibitors [251,252].

Pro-anigiogenic substances are of great interest in the early stages of tendon healing and especially when it comes to transplantation of scaffolds, since in this instance, the formation of a robust new vascular network is essential for their incorporation to the healing tissue. Ideally, scaffolds could be directly loaded with short half-life angiogenic agents, such as growth factors and they should have desired release kinetics from the scaffolds.

7. Conclusion

Our understanding of the exact mechanisms of tendon healing and the precise roles that different cell types play in this process is still limited and requires forthcoming research focusing at solving concrete issues that have been formulated thanks to the research efforts of the past decades. At present, the result of treatments for ruptured tendon is often poor, but stem/progenitor cells hold a great promise for outcome improvement, although we need to further investigate how they can be forced or stabilized into the tenogenic lineage and to what extent they can be beneficial for early and late tendon healing stages. Stem/progenitor cells of the tendon tissues have been identified and studied in vitro and in vivo; however, we are still lacking tools to segregate with high level of purity the immature cells from terminally differentiated cells. Special attention should be given in the tendon research to identify surface markers for cell sorting and to standardize protocols for enriching and sustaining different tendon-derived cell populations. In the possible mode of using stem/progenitor cells to augment tendon healing decorating them with growth factors or supporting them by cellular boosters might be a good way to increase their healing potential. A very promising approach with regards to their homing to the site of injury is to equip them with vascular homing peptides that can guide them to neoangiogenic activity centers. Regarding the potential of stem/progenitor cells as a therapeutic agent, it is also very important to clarify their survival and integration rates as well as to follow their fate and function over longer periods of time in vivo. When stem/progenitor cells are implemented into various experimental animal models for tendinopathy or tendon injury, we have to carefully and critically

consider their reflection to the human conditions and tendon size dimensions. Future efforts to optimize or develop clinically relevant animal models have to be undoubtedly pursued in order to achieve preclinical models with valid translation to human and veterinary medicine. Another important future perspective for understand what influences the and influencing tendon healing process and tendinopathy is to decipher the current ambivalent roles of inflammation and inflammatory cells, and in the following steps to design and apply strategies to steer them in order to experimentally examine whether certain inflammatory pathways can result in beneficial or detrimental outcomes of tendon healing or can lead to amplification or resolution of tendinopathy. It is possible that in the next decade that specific inflammatory cell types with positive influence on tendon repair will be identified and hence, the field can move towards regenerative immunological strategies to treat certain forms of tendon diseases. There is already some clinical evidence where autologous implantation of tendon-derived cells for human tendinopathy shows positive effects in term of safety and clinical scores. It would be very interesting to investigate the exact engagement of the transplanted cells that are processed to repair and their cross-talk to endogenous cells, as well as if such strategy is foreseeable for treatment of tendon ruptures.

Taken together, Achilles would have been happy to know of the progress achieved so far but like any true hero, would seek further perfection by trying to find answers to the many open questions and exploratory possibilities in the field in order to shape up efficient rescue strategies for ruptured or diseased tendons. This review hopes to encourage scientists to engage in studies aiding in better comprehension of the potential of stem/progenitor cells to improve and speed up the healing of injured tendons.

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