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## Tendon and Ligament Regeneration and Repair: Clinical Relevance and Developmental Paradigm

Guang Yang<sup>#</sup>, Benjamin B. Rothrauff<sup>#</sup>, and Rocky S. Tuan<sup>1</sup>

Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219, USA

<sup>#</sup> These authors contributed equally to this work.

### Abstract

Tendon and ligament (T/L) are dense connective tissues connecting bone to muscle and bone to bone, respectively. Similar to other musculoskeletal tissues, T/L arise from the somitic mesoderm, but they are derived from a recently discovered somitic compartment, the syndetome. The adjacent sclerotome and myotome provide inductive signals to the interposing syndetome, thereby upregulating the expression of the transcription factor Scleraxis, which in turn leads to further tenogenic and ligamentogenic differentiation. These advances in the understanding of T/L development have been sought to provide a knowledge base for improving the healing of T/L injuries, a common clinical challenge due to the intrinsically poor natural healing response. Specifically, the three most common tendon injuries involve tearing of the rotator cuff of the shoulder, the flexor tendon of the hand, and the Achilles tendon. At present, injuries to these tissues are treated by surgical repair and/or conservative approaches, including biophysical modalities such as physical rehabilitation and cryotherapy. Unfortunately, the healing tissue forms fibrovascular scar and possesses inferior mechanical and biochemical properties as compared to native T/L. Therefore, tissue engineers have sought to improve upon the natural healing response by augmenting the injured tissue with cells, scaffolds, bioactive agents, and mechanical stimulation. These strategies show promise, both *in vitro* and *in vivo*, for improving T/L healing. However, several challenges remain in restoring full T/L function following injury, including uncertainties over the optimal combination of these biological agents as well how to best deliver tissue engineered elements to the injury site. A greater understanding of the molecular mechanisms involved in T/L development and natural healing, coupled with the capability of producing complex biomaterials to deliver multiple growth factors with high spatiotemporal resolution and specificity, will allow tissue engineers to more closely recapitulate T/L morphogenesis, thereby offering future patients the prospect of T/L regeneration, as opposed to simple tissue repair.

### Keywords

tendon development; tendon regeneration; clinical treatment; tendon tissue engineering

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<sup>1</sup>Correspondence: Dr. Rocky S. Tuan Center for Cellular and Molecular Engineering Department of Orthopaedic Surgery McGowan Institute for Regenerative Medicine University of Pittsburgh School of Medicine 450 Technology Drive, Room 221 Pittsburgh, PA 15219 T: 4126482603 F: 4126245544 rst13@pitt.edu.

## INTRODUCTION

Decades of research on tendon and ligament (T/L) injuries have yielded extensive knowledge of the mechanical and biological properties of these dense connective tissues, translating into advances in surgical and conservative therapies that can prevent major disability. However, T/L injuries remain a persistent clinical challenge. In the U.S. alone, tendon, ligament, and joint capsular injuries account for 45% of the 32 million musculoskeletal injuries each year (Butler et al., 2004), with rates rising due to increasing sports participation and an aging population. Unfortunately, current treatment strategies fail to restore the functional, structural, and biochemical properties of repaired T/L to those of native tissue. Consequently, the principal elements of tissue engineering – cells, scaffolds, and bioactive molecules – have been explored in an effort to improve T/L healing. Both *in vitro* and *in vivo* studies have expanded the understanding of T/L biology while demonstrating the utility of tissue engineering in enhancing the healing of musculoskeletal tissues. Nevertheless, no tissue engineered construct thus far has achieved complete regeneration of T/L. In response, tissue engineers are looking to the emerging understanding of T/L development in an effort to recapitulate the embryonic events that establish the native structure (Thomopoulos et al., 2010). While researchers are only beginning to explore methods of integrating developmental biology into the design process, such efforts may advance the field of T/L tissue engineering towards its ultimate goal, full restoration of normal mechanical and biological properties (Lenas et al., 2009a). In this review, we will begin with the present understanding of tendon development. Ligament development has not received as much attention as that of tendon, but lessons learned from the latter should be applicable to the former, as tendons and ligaments possess similar ultrastructure and physiology, as well as fulfilling similar functional roles (Tozer and Duprez, 2005). The natural healing cascade of T/L will be summarized, as current therapeutic approaches to the three most common tendon injuries – tears of the rotator cuff, Achilles tendon, and flexor tendon of the hand – will be reviewed. Next, an overview of the current tissue engineering strategies to improve tendon healing, including cells, growth factors, scaffolds, and mechanical stimulation, will be provided. In closing, we will briefly explore the current limitations to tendon and ligament regeneration before offering future directions to address these challenges.

## TENDON DEVELOPMENT

Despite the relative simplicity of tendon structure, understanding of tendon development was limited by the absence of a tendon-specific lineage marker. However, identification of several markers selectively associated with tendon and musculoskeletal tissues has made it possible to trace the formation and maturation of tendons (Figure 1A). Scleraxis (Scx), initially discovered as a sclerotome marker (Cserjesi et al., 1995), and subsequently in all muscle-to-bone attachment sites in chick embryos, is expressed in both tendon progenitor cells and mature tenocytes (Schweitzer et al., 2001). Scx binds a short *cis*-acting element known as tendon specific element 2 (TSE2) to form the Scx/E47 heterodimer, which in turn activates the collagen type I $\alpha$ 1 (COL1  $\alpha$ 1) proximal promoter (Carlberg et al., 2000; Lejard et al., 2007). The overexpression of Scx in tendon fibroblasts upregulates the expression of the tenomodulin (Tnmd) gene. This latter gene encodes a transmembrane protein that is

expressed selectively in tendons and ligaments, and is considered a late marker of tendon formation (Shukunami et al., 2006). In situ hybridization analysis of the somitic mesoderm that forms along the anterior-posterior axis of the developing embryo in segmented animals provided further insight into the origin of tendon progenitors (Brent et al., 2003; Schweitzer et al., 2001). Scx-expressing progenitor cells first appeared between the myotome and sclerotome (Figure 1B-D). The localization pattern indicated that cells expressing Pax1, an early sclerotome marker, occupy a similar somitic domain as that of Scx-expressing cells. However, Pax1 is expressed more ventromedially while Scx is restricted to those cells nearest the myotome. On the other hand, Scx expression does not overlap with MyoD expression in the myotome. Rather, Scx-expressing progenitor cells are seen immediately adjacent to MyoD-positive cells. They constitute a fourth somitic compartment, syndetome, associated with myotome and sclerotome (Figure 1 A-F).

Further resolution of the origin of Scx-expressing tendon progenitors was found by using a chick-quail chimera model. When quail sclerotomal cells were implanted into chick embryos, quail cells generated Scx-expressing progenitors. Conversely, in embryos transplanted with quail dermamyotomes, quail cells did not contribute to the Scx-expressing progenitors. Although cells of the developing muscle-tendon-bone complex occupy distinct spatial regions, the generation of Scx-expressing progenitors requires signals from both the sclerotome and myotome. Overexpression of Pax1 in sclerotome blocked Scx induction, suggesting that Pax1-positive sclerotome is unable to generate the tendon progenitors. Additionally, no induction of Scx was observed in somites with the dermamyotome removed prior to myotome formation, indicating that the myotome plays a critical role in Scx induction.

Fibroblast growth factor 8 (FGF8), secreted by the myotome, is partly responsible for inducing Scx expression through the Ets transcription factors *Pea3* and *Erm* (Brent and Tabin, 2004). It also functions to downregulate Pax1 in Scx-positive domains in the sclerotome, although such repression alone does not result in Scx expression (Brent et al., 2003). Other FGF family members, such as FGF4, were also found to positively regulate induction of tendon progenitors. FGF4 transcripts are located at the extremities of muscles, close to the attachment sites of tendons in the embryonic chick wing, and the overexpression of FGF4 induces ectopic expression of Scx and tenascin in wing buds (Edom-Vovard et al., 2002).

In addition to FGFs, members of the transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily are involved in regulating tendon development, as has been found in various species. For example, TGF $\beta$ -2/3 ligand and its receptors were detected throughout the tertiary bundles in the tendon midsubstance and endotenon during the intermediate stages of tendon development in the chick embryo (Kuo et al., 2008). During mouse patellar tendon development, all cells in the tendon were found to respond to TGF $\beta$  and BMP signaling at all stages examined, including embryonic and postnatal periods (Liu et al., 2012). In vitro micromass culture of chick mesodermal cells with TGF $\beta$  demonstrated significant upregulation of tendon markers Scx and *Tnmd*, with concurrent reduction in cartilage markers. This trend was lost with the addition of a Smad2/3-specific inhibitor, indicating that the tenogenic effect of TGF $\beta$  was mediated by the canonical Smad signaling pathway

(Lorda-Diez et al., 2009). Likewise, using a ScxGFP transgenic reporter, disruption of TGF $\beta$  signaling in a *Tgfb2*<sup>-/-</sup> *Tgfb3*<sup>-/-</sup> mouse model was found to result in the loss of most tendons and ligaments (Pryce et al., 2009) (Figure 1 G,H).

Growth and differentiation factors (GDF), members of the bone morphogenetic protein (BMP) family, are additional regulators of tendon development. Subcutaneous implantation of GDF-5, 6, and 7 leads to the formation of neotendon-like connective tissue in rats (Wolfman et al., 1997). Mice deficient in GDF-5 demonstrate inferior matrix composition and mechanical strength in their Achilles tendons (Mikic et al., 2001). Similarly, a null mutation of GDF-6 in mice is associated with substantially lower levels of tail tendon collagen content, which causes impaired mechanical integrity of the tissue (Mikic et al., 2009). However, GDF-7 deficiency does not affect the biochemical composition of mouse tail tendon fascicles, nor does it significantly affect the tensile material properties, suggesting differential effects of GDF isoforms in promoting tendon development (Mikic et al., 2008).

Upon aggregation, tendon progenitors of the developing embryo lay down small diameter collagenous fibrils (Banos et al., 2008; Connizzo et al., 2013), a process which continues after birth, with the fibrils growing in both the linear and lateral directions (Birk et al., 1995; Zhang et al., 2005). Fibrillar collagens, including types I, II, III, V, and XI, constitute the basic structural framework of tendons and ligaments. These collagens share a 300 nm-long triple helical domain comprised of three  $\alpha$  chains, each containing about 1,000 amino acid residues. Each chain is rich in glycine and proline. As the smallest amino acid, glycine allows the three helical  $\alpha$  chains to pack tightly together, while proline stabilizes the conformation due to its ring structure. Fibrillar collagens are synthesized in a precursor form, procollagen, that possesses a C- and N-terminal propeptide domain. Once secreted into the extracellular space, the propeptide domains are cleaved by specific telopeptidases. Mature fibrillar collagens generated from this modification spontaneously self-assemble into cross-striated fibrils in an entropy-driven manner, yielding a 67 nm repeat that characterizes the axial periodicity of collagen fibrils (Kadler et al., 1996). Collagen fibrils, 100-500 nm in diameter, are bundled into fibers between which tenocytes reside and maintain the extracellular matrix (ECM). The number and diameter of collagen fibers are highly variable among species, lower than 30  $\mu$ m in rat tail tendon while higher than 300  $\mu$ m in human tendons (Franchi et al., 2007). The collagen fibers are wrapped by a layer of connective tissue known as endotenon that contains blood vessels, lymphatics, and nerves, to form higher structural units called fascicles, which are surrounded by another connective tissue layer, epitenon, to form the tendon (Amiel et al., 1984; Kastelic et al., 1978) (Figure 2). Many of the tendons are further surrounded by loose areolar connective tissue called paratenon, which functions as an elastic sleeve and permits free movement of the tendon against the surrounding tissues.

Collagen type I is the predominant fibril-forming collagen in tendons and exists as a heterotrimer consisting of two  $\alpha$ 1 chains and one  $\alpha$ 2 chain. Collagen type I co-polymerizes with collagen type V, a known regulator of collagen fibrillar structure (Wenstrup et al., 2004). Non-fibrillar collagens, including the fibril-associated collagens with interrupted triple helices (FACIT) such as collagens type XII and XIV, also serve a regulatory role

during collagen fibrillogenesis (Ansorge et al., 2009). Collagen type XII has been postulated to integrate adjacent matrix components due to its ability to bind proteoglycans, fibromodulin, and decorin, while interacting with collagen type I fibrils (Zhang et al., 2005). Collagen type XIV integrates fibrils into fibers during development, while collagen type XII assumes the same structural and functional role in mature tendon (Connizzo et al., 2013).

Expression and deposition of small leucine-rich proteoglycans, including decorin, biglycan, fibromodulin, and lumican, also serves to organize fibril assembly and the resulting ultrastructure of the tendon. Decorin-deficient mice developed structurally impaired tendons with abnormal, irregular fibril contours. Moreover, biglycan expression increased dramatically in decorin-deficient mice, suggesting a functional compensation (Zhang et al., 2006). Fibromodulin deficiency alone leads to a significant reduction in tendon stiffness, with further loss in stiffness when combined with lumican deficiency. Fibromodulin might be required to stabilize small-diameter fibrils in early tendon development, with lumican assuming this role later (Chakravarti, 2002). In addition to their regulatory effect on fibrillogenesis, biglycan and fibromodulin were also found to maintain the niche of tendon stem cells. The expression of the tendon marker Scx and of collagen type I was decreased in tendon stem cells from biglycan- and fibromodulin-deficient mice, as compared to cells from wild-type mice (Bi et al., 2007). Glycoproteins are also important constituents of tendon. Tenomodulin (TNMD), the type II transmembrane glycoprotein identified as a marker of mature tenocytes, is positively regulated by Scleraxis. In fact, TNMD is among the most tendon-selective genes in both adult rat and human tendons, compared with other tissues examined (Jelinsky et al., 2010). Loss of Tenomodulin expression in gene-targeted mice abated tenocyte proliferation and led to a reduced tenocyte density. The diameters of collagen fibrils varied significantly and exhibited increased caliber (Docheva et al., 2005). Tenascin C (TNC), member of a family of four ECM glycoproteins in vertebrates, was employed as the primary tendon marker prior to the discovery of Scx (Shukunami et al., 2006). It is an ECM component directly regulated by mechanical stress; induction of its mRNA in stretched fibroblasts is rapid, both in vivo and in vitro (Chiquet et al., 2003). Congruent with these findings of gene expression, the degree of tenascin C deposition is greatest in areas of high mechanical loading (Chiquet-Ehrismann and Tucker, 2004).

As tendons mature, covalent cross-links are formed between collagen fibrils at the overlapping ends of adjacent collagen molecules (Fessel et al., 2012) Marturano et al. (2013) recently showed that collagen cross-links correlate significantly with the increasing elastic modulus of *in utero* tendon, whereas correlations between mechanical properties and collagen, glycosaminoglycan, and dsDNA content were all weak (Marturano et al., 2013). The formation of collagen type I cross-links in tendons is primarily driven by the enzyme lysyl oxidase, which acts on specific lysine and hydroxylysine residues and results in stable trivalent cross-links that enhance collagen interconnectivity and fibril stability (Bailey, 2001; Eyre et al., 2008). While mechanical properties, collagen cross-links, average fibril diameter, and the distribution of fibril diameter, have all been found to increase with age, the structure-function relationship between these biochemical characteristics and tendon mechanical properties remains inconclusive (Connizzo et al., 2013).

Although perhaps less so than bone and muscle, extensive research on the adaptation of tendons to mechanical stresses has been conducted (Killian et al., 2012b; Wang, 2006). Most investigations have focused on the tendon proper, finding that the nature of loading directs the homeostatic balance between anabolic and catabolic pathways in resident fibroblasts (Killian et al., 2012a). The role of mechanical stimulation in tendon healing is discussed below. In addition, researchers have recently explored the role of mechanical loading on the bone-tendon insertion site (Benjamin et al., 2006). Using a mouse model, Thomopoulos et al. showed that decreased muscle loading delayed maturation of the supraspinatus enthesis during postnatal development. While a fibrocartilage transition was identified in both experimental and control animals at 14 days post-birth, the mice with reduced muscle loading demonstrated less mineral deposition, impaired fibrochondrocyte and matrix organization, and inferior mechanical properties at later time points (Thomopoulos et al., 2010). This study, among others, reinforces the importance of proper mechanical loading in maintaining the health of the mature musculoskeletal system, but also in driving the appropriate maturation of these developing structures early in life. Should tendon injury occur, the implementation of physical rehabilitation protocols is equally important in regaining tendon function, though the innate healing process of tendons is quite poor, as highlighted below.

## TENDON INJURY AND NATURAL HEALING

Tendon injuries, broadly categorized as chronic degenerative tendinopathies or acute ruptures, are a common clinical problem. Degenerative tendinopathy often precedes acute ruptures, with the former considered a failed healing response that is characterized by hypervascularity, mucoid degeneration, ectopic bone and cartilage nodules, and disorganized extracellular matrix (Kannus and Jozsa, 1991; Riley, 2008). Given the temporal relationship between chronic and acute tendon injuries, it is arguable that research examining the interaction between both pathologies will provide insights into the common clinical scenario. However, most in vivo studies of the sequelae of acute tendon ruptures are performed in animal models with previously healthy tendons. Therefore the applicability of research findings to the human condition may be questioned. Nevertheless, the current understanding of the innate healing response following acute tendon injury follows.

An estimated 300,000 tendon and ligament repair surgeries are performed annually in the U.S (Pennisi, 2002). In spite of surgical intervention, the natural healing process of tendons is still slow, due to their hypocellular and hypovascular nature (Liu et al., 2011). Even after one year, the structure and function of the resulting tissue remain inferior to uninjured tendons. The healing response is predictable, and is traditionally divided into three overlapping stages – (1) inflammation, (2) proliferation/repair, and (3) remodeling (Hope and Saxby, 2007). In the inflammatory stage, the blood clot that forms immediately following rupture of tendon vessels activates the release of chemoattractants and serves as a preliminary scaffold for invading cells. Inflammatory cells including neutrophils, monocytes, and lymphocytes migrate from surrounding tissues into the wound site, where necrotic debris is digested by phagocytosis (Voleti et al., 2012). Additionally, the recruitment and activation of tenocytes begins, but peaks in the subsequent stage. The second stage, known as the proliferative or reparative phase, begins roughly two days after

the injury occurs. Fibroblasts of the paratenon or surrounding synovial sheath are recruited to the wounded area and proliferate, particularly in the epitenon (Garner et al., 1989). Likewise, intrinsic tenocytes located in the endotenon and epitenon migrate to the wound site and begin proliferating. Both sources of tenocytes are important in synthesizing an extracellular matrix and in establishing an internal neovascular network (James et al., 2008). Concurrently, neutrophil levels decline while macrophages continue to release growth factors that direct cell recruitment and activity (Voleti et al., 2012). In this early stage of healing, the matrix synthesized by tenocytes is composed of increased amount of collagen type III (Juneja et al., 2013). Water content and glycosaminoglycan concentrations remain elevated during this stage (Sharma and Maffulli, 2006). Lastly, the remodeling phase commences 1–2 months after injury. Tenocytes and collagen fibers become aligned in the direction of stress. A higher proportion of collagen type I is synthesized (Abrahamsson, 1991), with a corresponding decrease in cellularity and collagen type III and glycosaminoglycan contents (Sharma and Maffulli, 2006). After 10 weeks, fibrous tissue gradually changes to scar-like tendon tissue, a process that continues for years. The repaired tissue never completely regains the biomechanical properties it had prior to injury and the biochemical and ultrastructural characteristics remain abnormal even at 12 months (Miyashita et al., 1997).

Numerous bioactive molecules are involved in orchestrating the cellular response during tendon repair (Andia et al., 2010). A variety of growth factors are markedly upregulated following tendon injury and are active at multiple stages of the healing process, including insulin-like growth factor-I (IGF-I), TGF- $\beta$ , bFGF, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), BMP, and connective tissue growth factor (CTGF) (Chen et al., 2008; Kobayashi et al., 2006; Molloy et al., 2003; Wurgler-Hauri et al., 2007). IGF-1 is an important mediator during the inflammatory and proliferative phase of tendon healing. IGF-1 mRNA levels were more than 5-fold higher compared with control groups 3 weeks after injury to the rabbit medial collateral ligament (MCL) (Sciore et al., 1998). In a collagenase-induced lesion created in equine flexor tendons, IGF-1 expression and protein levels increased following injury and peaked in weeks 4–8 (Dahlgren et al., 2005). Similarly, TGF- $\beta$  shows high levels of gene expression and activity throughout the tendon-healing period (Chang et al., 2000; Chen et al., 2008; Natsu-ume et al., 1997). Rabbit flexor tendons subjected to transection and repair exhibited increased signal for TGF- $\beta$ 1 mRNA in both resident tenocytes and infiltrating cells from the tendon sheath (Chang et al., 1997). In a rat tendon injury model, a biphasic pattern was observed in which TGF- $\beta$ 1 expression was highest in the early phases of healing and gradually decreased thereafter, whereas TGF- $\beta$ 3 was upregulated later (Juneja et al., 2013). TGF- $\beta$  receptors are also upregulated during tendon healing. In a rabbit zone II flexor tendon wound, immunohistochemical staining of transected and repaired tendons demonstrated increased TGF- $\beta$  receptor type I (TGF- $\beta$  RI), TGF- $\beta$  RII, and TGF- $\beta$  RIII protein levels in the epitenon and along the repair site (Ngo et al., 2001). bFGF, a potent mitogenic and angiogenic growth factor, is also involved in tendon healing. Tendons subjected to transection and repair exhibited an increased signal for bFGF mRNA in both resident tenocytes and in the fibroblasts and inflammatory cells located in the tendon sheath (Chang et al., 1998). Moreover, bFGF significantly accelerated wound closure of a rat patellar tendon defect in

vitro (Chan et al., 1997), due in part to its effect on cell proliferation. PDGF was detected in canine digital flexor tendons 3, 10, and 17 days after tendon repairs (Duffy et al., 1995). It is a potent chemoattractant for macrophages and fibroblasts and may stimulate these cells to express other growth factors, including TGF- $\beta$ , which directly stimulates new collagen synthesis (Pierce et al., 1989). VEGF has been shown to be a potent stimulator of angiogenesis during tendon healing (Petersen et al., 2003) and the importance of angiogenesis in tendon healing is widely recognized (Fenwick et al., 2002). VEGF activity peaks after inflammation, most notably during the proliferative and remodeling phases. Significant expression of VEGF occurred at the canine flexor tendon repair site at 7 days post-operatively, with expression localized to cells within the repair site itself, as opposed to cells of the epitenon (Bidder et al., 2000; Boyer et al., 2001). CTGF showed a high level of gene expression over a 21-day period of early tendon healing in a chicken flexor digitorum profundus tendon injury (Chen et al., 2008). Likewise, in a rat model of supraspinatus tendon detachment and repair, CTGF was moderately expressed across all time points in both the insertion and midsubstance (Wurgler-Hauri et al., 2007). In the same study, BMP-12, -13, and -14, were dramatically elevated at 1 week and gradually decreased thereafter. These BMPs are established tenogenic factors, capable of driving differentiation of mesenchymal stem cells in vitro (Park et al., 2010) and forming neo-tendon tissue in vivo (Wolfman et al., 1997).

While many studies have investigated the spatial and temporal distribution of growth factors in tendon healing, several other bioactive molecules have been recently recognized as integral mediators of repair. For instance, regulators of extracellular matrix accretion and degradation, including matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), are responsive to tendon injury and repair (Garofalo et al., 2011). In an oft-cited study examining gene expression in a rat flexor tendon laceration model, MMP-9 and MMP-13 expression peaked between days 7 and 14, while MMP-2, MMP-3, and MMP-14 expression remained elevated for 28 days (Oshiro et al., 2003), suggesting that the former pair participates in collagen degradation while the latter three mediate both collagen degradation and remodeling (Sharma and Maffulli, 2006). Markers of reinnervation show an equally complex pattern of expression during tendon healing. In a rat model of acute Achilles tendon rupture, neuronal markers for regenerating (growth associated protein 43, GAP-43) and mature (protein gene product 9.5, PGP9.5) fibers were found in the paratenon of both the ruptured and intact contralateral tendons. Conversely, only the ruptured tendon expressed these markers in the tendon proper. Immunoreactivity was evident as early as 1 week post-injury and peaked at week 6, before declining (Ackermann et al., 2002). As a last example, and in accordance with VEGF expression described above, nitric oxide appears to play a role in tendon healing. Nitric oxide synthase (NOS) levels peaked at 7 days following Achilles tendon tenotomy in a rat model, before returning to baseline at day 14. Furthermore, inhibition of NOS decreased the cross-sectional area and the failure load of healing tendons (Murrell et al., 1997).

A growing understanding of the molecular mediators of tendon healing has led to the supplementation or inhibition of these bioactive molecules as a means of enhancing the quality of the repaired tissue, as discussed below. Others have sought to modulate the inflammatory process itself, thereby affecting the innate healing cascade that produces



fibrotic scar in place of regenerated tendon. Unfortunately, the utility of blocking inflammation post-injury remains a topic of debate. For instance, the systemic administration of non-steroidal anti-inflammatory drugs (NSAIDs) for 7 days following Achilles tendon transection in a rat yielded a healing tendon with inferior mechanical properties and a reduced cross-sectional area (Dimmen et al., 2009). Likewise, Virchenko et al. found a similar effect when parecoxib was administered immediately followed surgery (Virchenko et al., 2004). On the other hand, tendon healing was improved when the drug was administered starting 6 days post-surgery. Therefore, the early inflammatory cascade may be needed for the restoration of native tendon characteristics, while late inflammation is detrimental.

An equally debated issue concerns which elements of the adult tissue impair tendon regeneration. Bayer et al. demonstrated embryonic-like fibrillogenesis by adult human tendon fibroblasts when cultured in a fibrin gel contracted around a suture, suggesting that the hormonal/mechanical milieu inhibits the regenerative potential of adult tendons (Bayer et al., 2010). Conversely, Favata et al. transplanted fetal tenocytes contracted around a suture into an adult environment and performed a partial tenotomy on the engineered tendon construct. As compared to adult fibroblasts, the fetal tenocytes filled the defect without scar, suggesting that the adult environment is *not* an impediment to scarless tendon regeneration, but that such dysfunctional healing may be intrinsic to aged cells (Favata et al., 2006). Likewise, a recent study found that the frequency of tendon-derived stem/progenitor cells (TSPCs) decreases with age. Additionally, the aged TSPCs possess a decreased proliferation rate but an increased propensity for adipogenic differentiation (Zhou et al., 2010). Taken together, it remains unclear why adult tendons fail to regenerate, though such a fact creates a clinical challenge for physicians and patients alike, as outlined below.

## CURRENT CLINICAL TREATMENTS FOR COMMON TENDON INJURIES

While tendons anchor every muscle of the body to bone, the most common injuries involve the rotator cuff tendons, Achilles tendon, and flexor tendons of the hand. Furthermore, these three tendons present different challenges to repair, as they possess unique anatomy, function, biomechanical properties, healing capacities, mechanisms of injury, and approaches to rehabilitation (Gott et al., 2011). Current injury rates and clinical treatment strategies for these three tendons are discussed.

### Rotator Cuff Tendon

The rotator cuff is comprised of the interdigitating tendons of four muscles – subscapularis, supraspinatus, infraspinatus, and teres minor – that attach on the lateral aspect of the humeral head, serving important roles in both stabilizing and mobilizing the shoulder. Rotator cuff tears are a common cause of debilitating pain, reduced shoulder function, and weakness, affecting more than 40% of patients older than 60 years of age and resulting in 30,000 to 75,000 repairs performed annually in the United States (Ricchetti et al., 2012). Novel surgical techniques improve repair strength at time 0, but fail to provide superior clinical scores when compared against older approaches (Dines et al., 2010; Lorbach and Tompkins, 2012). Likewise, rehabilitation protocols implementing early mobilization have been developed with the hope of improving the rate of healing while minimizing long-term

stiffness. Unfortunately, such approaches show little benefit over more conservative protocols (Kim et al., 2012; Parsons et al., 2010).

Despite advances in surgical techniques and in the understanding of shoulder pathology, chronic tears fail to heal in 20-95% of cases (Derwin et al., 2010; Galatz et al., 2004). In particular, the bone-tendon interface that forms following surgical repair fails to recapitulate the native enthesis, with a fibrovascular scar forming in place of the complex fibrocartilage transition seen between native tendon and bone (Newsham-West et al., 2007). The intra-articular environment may partly explain this poor outcome (Bedi et al., 2009), as the synovial fluid, which contains the anti-adhesive protein lubricin, has been shown to inhibit bone-tendon healing (Funakoshi et al., 2010; Sun et al., 2012).

Tendinopathy and partial tears of the rotator cuff tendons are often treated conservatively with physical therapy and corticosteroid injections. A randomized, controlled study in which patients with full-thickness rotator cuff tears received subacromial injections of triamcinolone reported improved pain relief for at least 3 months, as compared to controls (Gialanella and Prometti, 2011). However, a recent systematic review concluded that there was little reproducible evidence to support the efficacy of subacromial corticosteroid injections in managing rotator cuff disease (Koester et al., 2007). Of further concern, Zhang et al. reported non-tenogenic differentiation (i.e. adipogenic, chondrogenic, osteogenic) of tendon-derived stem cells when treated with dexamethasone (Zhang et al., 2013).

Given the high re-tear rates experienced with current surgical approaches, scaffolds designed to provide mechanical augmentation or enhance biological healing have been investigated in both animal and human studies. Only two prospective randomized, controlled trials have been performed using commercially available devices consisting of decellularized extracellular matrices (Barber et al., 2012; Derwin et al., 2006; Iannotti et al., 2006). While one of these studies found improved subjective clinical scores in patients with scaffold-augmented repairs (Barber et al., 2012), animal models with the same augmented repair did not recapitulate the native bone-tendon interface (Dejardin et al., 2001). Concurrently, many surgeons have augmented rotator cuff repairs with platelet-rich plasma (PRP), an autologous source of concentrated growth factors of known importance in wound repair (Foster et al., 2009). As borne out in multiple prospective clinical studies (Castricini et al., 2011; Ruiz-Moneo et al., 2013), PRP does not have an effect on the overall re-tear rates or shoulder-specific outcome scores after arthroscopic rotator cuff repair (Chahal et al., 2012). As with the anterior cruciate ligament of the knee, PRP may be an insufficient carrier of growth factors as the plasmin found within synovial fluid may quickly degrade the fibrin matrix, thereby allowing diffusion of growth factors away from the repair site (Murray et al., 2009).

### **Achilles Tendon**

The Achilles tendon is the largest and strongest tendon in the body, but one of the most likely to be injured (Calleja and Connell, 2010). Whereas rotator cuff tears are increasingly prevalent with age, Achilles ruptures are most commonly seen in men aged 30-50 (Longo et al., 2009). Achilles tendinopathy is increasingly common due to increasing participation in recreational sports, with Achilles pathology accounting for 30-50% of all sports-related

injuries (Sadoghi et al., 2013). Nevertheless, Achilles tendon ruptures are also seen in elite athletes and, despite the research interest this garners, this injury is notorious for its poor quality and slow rate of healing (Maffulli et al., 2011).

Just as full-thickness rotator cuff tears are almost always preceded by partial tears and tendon degeneration (Oh et al., 2011), noninflammatory tendinosis and chronic tendinopathy predispose the Achilles tendon to complete rupture (Hess, 2010). In examining biopsy samples of patients undergoing open repair for torn Achilles tendons, Tallon et al. found that ruptured tendons were significantly more degenerated than tendinopathic tendons (Tallon et al., 2001). Therefore, concurrent research efforts have been made towards promoting healing of both tendinopathic and ruptured tendons.

While a detailed discussion of the etiology and histopathological characteristics of Achilles tendinopathy exceeds the scope of this review, it is worth noting that conservative approaches – including reduced activity, cryotherapy, eccentric loading, deep friction massage, orthotics, and therapeutic ultrasound – produce good to excellent outcomes in up to 75% of cases. In recalcitrant Achilles tendinopathy, surgical excision of adhesion, removal of degenerative nodules, and tenotomies intended to promote angiogenesis, can be performed (Maffulli et al., 2004). Likewise, complete Achilles ruptures can be treated both conservatively and surgically with satisfactory results. However, a recent Cochrane Review found that open surgical repair significantly reduced the re-rupture rate (4.4%) compared with nonoperative treatment (10.6%) (Jones et al., 2012). A percutaneous surgical approach did not reduce the re-rupture rate compared with open repair, but did result in significantly fewer postoperative infections.

As the typical patient suffering from Achilles injuries is often young and active, there is significant research interest in accelerating the healing rate. Recent trends towards earlier weight-bearing and active strengthening have produced comparable or superior results to cast immobilization (Garrick, 2012; Kearney and Costa, 2012). As with rotator cuff tears, surgeons have applied PRP to the surgical site to bolster repair. While Sanchez et al. (Sanchez et al., 2007) reported an accelerated return to sport and a smaller cross-sectional area of PRP-treated surgical repairs in a small cohort of athletes, the only randomized trial to date found no difference in mechanical properties or subjective function scores when comparing PRP-treated to untreated patients (Schepull et al., 2011). Given the long duration for recovery (9-12 months) and the residual impairment following injury and repair, research aimed at improving outcomes is still needed.

## Flexor Tendon

Tendons of the flexor pollicis longus, flexor digitorum profundus, and flexor digitorum superficialis muscles attach to the distal and middle phalanges of the hand, allowing flexion at the distal and proximal interphalangeal joints, respectively. Portions of the flexor tendons are enveloped in synovial sheaths, which condense at certain locations to create an arrangement of pulleys that act as fulcrums for the tendon (Griffin et al., 2012). Located immediately beneath the palmar skin and fascia, the flexor tendons are prone to laceration and crush injuries. Rupture of intrasynovial flexor tendons presents unique repair challenges for three reasons: (1) ruptures do not heal without surgical intervention, (2) careful

postoperative management of healing tendons is necessary to prevent adhesions and improve gliding function between the tendon and sheath, but mobilization increases the risk of re-rupture, and (3) hypertrophy of healing tendon must be avoided so as to minimize gliding resistance (Griffin et al., 2012).

Since the first report of flexor tendon repair in 1917, suture techniques to optimize surgical results have been extensively explored in animal and cadaveric models (Griffin et al., 2012; Kleinert et al., 1995; Nelson et al., 2012). In light of this body of research, Kim et al. suggested the following surgical techniques to improve upon historical methods: (1) 8-core suture strands with a high-caliber suture material, (2) a purchase length of approximately 1.2 cm, (3) a locking-loop configuration with the knot placed outside the repair site, and (4) a peripheral suture placed deep into the tendon and far away from the cut end (Kim et al., 2010). An equally extensive series of studies examining rehabilitation protocols for flexor tendon repairs also exists (Chesney et al., 2011; Small et al., 1989; Thien et al., 2010). Both early active motion protocols and regimens combining passive flexion with active extension result in low rates of tendon re-rupture and good range of motion following repair. Nevertheless, there exists no universally accepted gold standard for suture material or technique, nor rehabilitation protocol. A recent meta-analysis found rates of re-operation of 6%, re-rupture of 4%, and adhesion formation of 4% (Dy et al., 2012). Consequently, many researchers have explored tissue engineering approaches, as discussed below, aimed at enhancing tendon repair (Chang, 2012; Manning et al., 2013). While several strategies show promise in reducing adhesion formation and increasing tensile strength in animal models of flexor tendon injury, none of these regenerative medicine approaches has been implemented in human clinical trials to date.

## TENDON TISSUE ENGINEERING

In cases of severe tendon injury, surgical treatments may be used to repair or replace the damaged tendon with autografts, allografts, xenografts, or prosthetic devices (Lui et al., 2011). However, the clinical outcomes remain unsatisfactory due to limitations including donor site morbidity, high failure rates, risk of injury recurrence and limited long-term function recovery (Klepps et al., 2004; Krueger-Franke et al., 1995; Voleti et al., 2012). These limitations have spurred the development of tissue engineering strategies, which apply a combination of cells, scaffolds, bioactive molecules, and ex vivo mechanical stimulation to create functional replacements or to bolster the innate healing of tendon defects (Kuo et al., 2010). Ultimately, tissue engineering aims at improving the quality of healing in order to promote full restoration of tendon function.

### Cells

Cells widely used in tendon tissue engineering include tendon fibroblasts (tenocytes), dermal fibroblasts, and mesenchymal stem cells (MSC). Tenocytes, as reviewed in 'Tendon Development' above, synthesize the constituents of extracellular matrix such as collagen, proteoglycans and glycoproteins, and have great influence on collagen fiber formation (Canty and Kadler, 2005; Franchi et al., 2007). Isolated human tenocytes are able to synthesize collagen and upregulate expression of *Scx*, *Tnmd*, and *Dcn* in response to growth factors (Qiu et al., 2013). Tendon defects bridged by an autologous tenocyte-engineered

tendon in a hen model demonstrated improved mechanical strength and matrix deposition compared with the cell-free scaffold (Cao et al., 2002). Similarly, decellularized rabbit flexor tendons reseeded with autologous tenocytes had the same elastic modulus as normal tendons, but still possessed a decreased ultimate stress (Chong et al., 2009). Importantly, the seeded tenocytes reduced the degradation of a collagen scaffold incubated in culture medium (Tilley et al., 2012), a mechanism which may partly explain the superior mechanical properties of cell-seeded scaffolds.

Despite the advances in tenocyte-based tendon tissue engineering, harvesting autologous tenocytes may cause secondary tendon defects at the donor site. Therefore, dermal fibroblasts have been considered as an alternative cell source to address this limitation, for they are easily accessible and do not cause major donor site morbidity (Van Eijk et al., 2004). An *in vivo* study in a porcine model showed that dermal fibroblast- and tenocyte-engineered tendons were similar to each other in their gross morphology, histology, and tensile strength (Liu et al., 2006), indicating the potential of dermal fibroblasts for tendon engineering. Likewise, human tendon-like neotissue was generated by using dermal fibroblasts placed under static strain, producing longitudinally aligned collagen fibers and spindle-shaped cells. Additionally, the average collagen fibril diameter and tensile strength increased with time (Deng et al., 2009). Lastly, an injection of dermal fibroblasts suspended in autologous plasma improved the healing of refractory patellar tendinopathy, as shown in a recent clinical trial (Clarke et al., 2011).

Adult MSCs are another promising cell source for tendon tissue engineering. Due to their self-renewal and multi-lineage differentiation potential, MSCs from a variety of tissues, including bone marrow (Tucker et al., 2010), adipose, and tendon, have been applied to tendon tissue engineering. Bone marrow MSCs (BMSC) seeded in polylactide/glycolide (PLGA) suture material demonstrated higher collagen production and DNA content compared with anterior cruciate ligament fibroblasts (ACLF) and skin fibroblasts (Van Eijk et al., 2004). Similarly, rabbit BMSCs seeded in a silk scaffold showed significantly increased ligament-related ECM expression, including tenascin-C and collagen types I and III, as compared to ACLF-seeded scaffolds (Liu et al., 2008a). In another rabbit model, polyglycolic acid (PGA) sheets seeded with BMSCs had enhanced mechanical strength and expression of collagen type I when compared against the cell-free PGA scaffold (Yokoya et al., 2012).

While the tenogenic potential of BMSCs has been extensively studied, less attention has been paid to adipose-derived mesenchymal stem cells (ASCs). Compared to BMSCs, ASCs are (1) harvested by less invasive procedures, (2) available in greater quantities, and (3) demonstrate similar potential to differentiate along multiple mesenchymal lineages (Gimble et al., 2007). Recently, a report showed that GDF-5-treated rat ASCs expressed tendon-specific markers *Scx* and *Tnmd* (Park et al., 2010). Human ASCs seeded onto a mesh derived from hyaluronan (Hyalonect) and placed under mechanical stress formed a vascularized tendon-like structure (Vindigni et al., 2013). Rabbit Achilles tendon defects repaired by a platelet-rich plasma (PRP) gel mixed with ASCs showed improved tensile strength and collagen type I synthesis, as compared with PRP gels alone (Uysal et al., 2012). In another rabbit tendon defect model, cell proliferation was higher in ASCs than tenocytes,

though similar rates of collagen synthesis were found between cell types (Kryger et al., 2007).

Resident, tissue-specific, adult stem cells seem to have a higher regenerative potential for the tissue where they reside (Lui and Chan, 2011). In this regard, tendon-derived stem cells (TDSC) have been identified and applied to tendon tissue engineering. TDSCs are a unique cell population in tendons that have universal stem cell characteristics such as clonogenicity, multipotency and self-renewal capacity (Bi et al., 2007). They also possess a high regenerative potential, which is comparable with that of BMSCs (Randelli et al., 2013). TDSCs in fibrin glue, as compared to a fibrin glue alone, promoted superior tendon repair as measured both histologically and biomechanically (Lui and Chan, 2011). Despite the potential advantages of TDSCs, the isolation of autologous cells would present the same donor site morbidities as tenocytes.

## Scaffolds

Scaffolds are another critical factor for tendon tissue engineering. They provide biomechanical support to the healing tissue until endogenous cells have deposited native matrix, thereby preventing re-rupture. In addition, scaffolds with desired functionality can improve tendon healing by facilitating cell proliferation, promoting matrix production, and organizing the matrix into functional tendon tissues. Scaffolds can be further modified to improve tendon healing, including approaches such as cellular hybridization, surface modification, growth factor attachment, and mechanical stimulation-mediated cellular remodeling (Liu et al., 2008b). Three major categories of scaffolds are used: (1) native tendon matrices, (2) synthetic polymers, and (3) derivatives of naturally occurring proteins.

Scaffolds derived from tendon matrices could in theory retain both the normal biomechanical and biochemical properties, thereby serving as the ideal biomaterial to support tendon healing. However, before these tissues can be utilized, the native cells must be removed to prevent disease transmission and an immune response (Badylak et al., 2009; Deeken et al., 2011). Thereafter modified allografts or xenografts could be developed into mechanically functional delivery vehicles for cells, gene therapy vectors, or other biological agents. Optimized processing protocols have allowed acellular tendon scaffolds to maintain similar biomechanical properties compared against native tendon tissues (Pridgen et al., 2011). Moreover, a number of ECM proteins and growth factors are highly preserved in acellular tendon (Ning et al., 2012; Pridgen et al., 2011), suggesting potential biocompatibility and bio-functionality of these scaffolds. In support, extrinsic fibroblasts were successfully cultured on a tri(n-butyl)phosphate (TnBP)-treated patellar tendon in vitro, creating viable tissue-engineered grafts (Cartmell and Dunn, 2004). Similarly, detergent-treated (sodium dodecyl sulfate) rabbit semitendinosus tendons demonstrated a crimp pattern characteristic of native tendon, yet permitted integration of autologous dermal fibroblasts after cell seeding (Tischer et al., 2007). A great number of biodegradable and biocompatible polymers, in particular the  $\alpha$ -hydroxy-polyesters, have been used for tendon tissue engineering, including polyglycolic acid (PGA), poly-L-lactic acid (PLLA) and their copolymer polylactic-co-glycolic acid (PLGA), for their biodegradability and material characteristics. In a rabbit Achilles tendon defect model, knitted PLGA seeded with BMSCs

exhibited greater tensile strength and deposition of collagen types I and III, as compared to a cell-free scaffold (Ouyang et al., 2002; Ouyang et al., 2003). As reviewed earlier, PGA was used as scaffold and cell carrier to bridge a tendon defect in a hen model and a porcine model, respectively (Cao et al., 2002; Liu et al., 2006). PGA seeded with mouse skeletal muscle derived cells (MDCs) formed a tendon structure with mature collagen fibrils (Chen et al., 2012a). In a similar study, aligned PLLA scaffolds supported cellular proliferation and tenogenic differentiation (Yin et al., 2010).

Despite the advantages of polyesters, they also suffer from several limitations, including an absence of biochemical motifs for cellular attachment and an accompanying inability to fully regulate cell activity (Wan et al., 2003). Scaffolds made from natural proteins and their derivatives may address these issues. Since tendon ECMs are mainly composed of collagen type I, scaffolds based on collagen derivatives are highly biocompatible. Collagen derivatives also exhibit better bio-functionality by supporting cell adhesion and cell proliferation better than polyester materials. Collagen gels seeded with rabbit BMSCs contracted around sutures to improve neotissue formation in a rabbit patellar tendon defect (Awad et al., 2003). Similarly, tissue-engineered constructs created by seeding MSCs in a collagen gel/sponge blend improved the histological and mechanical properties in the same rabbit model (Juncosa-Melvin et al., 2006). Collagen-derived scaffolds are also further modifiable by cross-linking or co-fabricating with other materials to enhance the mechanical strength and water resistance (An et al., 2010; Awad et al., 2003; Fessel et al., 2012; Panzavolta et al., 2011).

Beyond collagen, scaffolds made from silk have been used in tendon tissue engineering. Tenogenesis of MSCs is enhanced when seeded on aligned silk fibroin (SF) electrospun fibers, as evidenced by the upregulation of expression of tendon/ligament-related proteins (Teh et al., 2013). When subjected to mechanical stimulation *in vitro*, human embryonic stem cell-derived mesenchymal stem cells (hESC-MSCs) within a knitted silk-collagen sponge scaffold exhibited tenocyte-like morphology and positively expressed tendon-related gene markers (Chen et al., 2010). TDSCs within the same scaffold were found to improve rabbit rotator cuff healing, exhibiting increased collagen deposition and better structural and biomechanical properties, as compared to the control group (Shen et al., 2012).

In addition to mechanical properties, the topographical cues provided by scaffolds must be carefully considered when engineering tendon constructs. The micro-/nano-structure of material surfaces has been widely reported to modulate cellular behavior. Since tendon tissue is primarily composed of parallel collagen fibers, alignment is an important topographical characteristic to mimic in tendon tissue engineering. The expression of tenomodulin in tenocytes was rescued after being switched from a smooth to microgrooved silicone membrane (Zhu et al., 2010). Moreover, tendon specific markers such as scleraxis and tenomodulin were significantly increased on electrochemically aligned collagen (ELAC) threads when compared to randomly oriented collagen fibers (Kishore et al., 2012). ELAC scaffold-treated rabbit patellar tendon exhibited improved stiffness and fascicle formation (Kishore et al., 2011). In addition to collagen, aligned polyester materials have also been created for tendon tissue engineering. Cell alignment, distribution, and matrix deposition were found to match the nanofiber organization of a PLGA scaffold designed for rotator cuff

repair (Moffat et al., 2009). Likewise, TDSCs seeded on aligned PLLA nanofibrous scaffolds demonstrated increased tenogenesis and collagen deposition, together with suppressed osteogenesis (Yin et al., 2010).

### Bioactive molecules

Although numerous growth factors have been shown to be active in both tendon development and healing, the application of growth factors to clinical tendon repair remains challenging. Little is known about the synergistic and antagonistic interactions, nor the optimal spatial and temporal distribution, of growth factors that would produce the best effects. However, some success has been achieved in accelerating the healing process while subsequently enhancing the quality of repaired tissue. While the many bioactive mediators of tendon healing have been reviewed elsewhere (Bedi et al., 2012; Molloy et al., 2003), we highlight here the effects of exogenous application of several growth factor identified to be involved in innate tendon healing (see 'Tendon Injury and Natural Healing').

The application of IGF-1 to the site of carrageenan-induced inflammation in a rat Achilles tendon showed that IGF-1 was able to mitigate inflammation-induced functional deficits (Kurtz et al., 1999). Additionally, IGF-1 also stimulates the proliferation and migration of fibroblasts at the site of injury, and subsequently increases the production of collagens and other extracellular matrix structures during the remodeling stages. For example, recombinant human IGF-1 stimulated proteoglycan, collagen, noncollagen protein, and DNA synthesis in a dose-dependent manner in rabbit flexor tendons (Abrahamsson, 1997). In human tendon, the collagen fractional synthesis rate (FSR) and procollagen type I N-terminal propeptide (PINP) content were significantly higher in the IGF-I treated leg compared with the control leg ( $P < 0.05$ ) (Hansen et al., 2012). Like IGF-1, TGF- $\beta$  is involved in matrix synthesis in tendon healing. There is a significant increase in collagen types I and III production in rabbit tendon cells with the addition of these TGF- $\beta$  isoforms (Klein et al., 2002). Additionally, TGF- $\beta$  signaling is able to drive tenocyte differentiation through the induction of Scx, indicating its potential in stem cell-based tendon tissue engineering. Exposure of equine embryo-derived stem cells (ESCs) to TGF- $\beta$  in vitro produced an upregulation of Scx at the gene and protein level (Barsby and Guest, 2013). Other TGF- $\beta$  family members have also been used in tendon tissue engineering with some success. For example, GDF-5 (BMP-14)-treated rat ASCs expressed tendon-specific genes including Scx and Tnmd in both 2D and 3D electrospun matrix systems (James and et al., 2011; Park et al., 2010). Likewise, addition of these growth factors to a transected Achilles tendon improved the mechanical properties (Forslund and Aspenberg, 2001).

Another widely used growth factor in tendon tissue engineering is bFGF. A dose-dependent increase in proliferation and the expression of collagen type III was found 7 days post-injury in a rat patellar tendon treated with bFGF (Chan et al., 2000). Consistent with this finding, addition of bFGF significantly increased the number of equine tendon-derived cells and enhanced collagen type III levels (Durgam et al., 2012). A bFGF-releasing nanofibrous scaffold was developed to facilitate BMSC proliferation and tenogenic differentiation. The loaded bFGF was released over a week and successfully promoted tyrosine phosphorylation of seeded BMSCs, resulting in enhanced expression of tenogenic markers with concurrent



increases in the deposition of collagen and tenascin-C on the scaffold (Sahoo et al., 2010). As was seen with IGF-1, PDGF-BB stimulated collagen and noncollagen protein production and DNA synthesis in rabbit tendon. These effects occurred in a dose-dependent manner (Yoshikawa and Abrahamsson, 2001). When delivered by fibrin glue, PDGF-BB improved healing of the rabbit medial collateral ligament (MCL) (Hildebrand et al., 1998).

### Mechanical Stimulation

As tendons permit the transmission of force from muscles to bone, their mechanical properties have been extensively studied (Wang, 2006; Woo et al., 2006). In the context of tendon injury and repair, it is recognized that controlled mobilization of healing tendons is needed to improve outcomes, although the optimal timing and magnitude of loading is largely debated (Killian et al., 2012b). In animal models, the complete removal of load in healing tendons results in inferior mechanical properties (Galatz et al., 2009; Murrell et al., 1994), while increased loading in the form of exercise is also detrimental to tendon properties if implemented too quickly (Gimbel et al., 2007; Thomopoulos et al., 2003). Although strong clinical evidence for an optimal rehabilitation protocol for tendon injuries does not exist, it is well accepted that healing tissues should be loaded in a controlled manner to promote favorable remodeling and functional outcomes (Killian et al., 2012b).

More recently, the cellular and molecular basis for loading of healing tendons and ligaments has been explored (Wang et al., 2007). Increased cellular proliferation, collagen production, and tenogenic gene expression is found in both fibroblasts and mesenchymal stem cells exposed to static or cyclic uniaxial tension, with the latter promoting greater effects (Altman et al., 2001; Garvin et al., 2003; Kuo and Tuan, 2008; Yang et al., 2004). Expression of Scx was upregulated in a strain- and cycle-dependent manner in a mesenchymal cell line (C3H10T1/2) seeded in a collagen hydrogel (Scott et al., 2011). Mechanical stretch appears to induce tenogenesis through at least two mechanisms with partially independent downstream targets – integrin-dependent signaling and biochemical (i.e., TGF- $\beta$ ) pathways. Xu et al. showed that RhoA/ROCK and focal adhesion kinase regulate mechanical stretch-induced realignment of MSCs by regulating cytoskeletal organization, thereby driving tenogenesis (Xu et al., 2012). Likewise Maeda et al. found that active TGF- $\beta$  levels in tendons correlated directly when the extent of physical force and Scx expression. (Maeda et al., 2011). However, the disruption of cytoskeletal organization did not affect TGF- $\beta$ -mediated Scx expression in tenocytes, suggesting that TGF- $\beta$  regulates Scx expression independently of cytoskeletal tension. This was supported by Chen et al., who found that force and lentiviral-mediated Scleraxis overexpression synergistically promoted tenogenesis of ESC-MSCs (Chen et al., 2012b). Conversely, tendon overuse injuries are thought to result from upregulated expression of catabolic and inflammatory mediators (Riley, 2008; Wang, 2006). Yang et al. (2005) found 4% uniaxial stretching of fibroblasts decreased COX-2 and MMP-1 expression and PGE<sub>2</sub> production, while 8% stretching increased these inflammatory products. Likewise, cyclic stretching at 4% strain promoted tenogenic differentiation of TDCs, while 8% strain induced greater degrees of adipogenic, chondrogenic, and osteogenic differentiation (Zhang and Wang, 2010). Taken together, these in vitro studies support the clinical strategy of controlled mobilization, where progressively greater frequencies and magnitudes of loads are applied to healing tissues to promote tenogenesis of

endogenous progenitor cells, increase extracellular matrix synthesis, and minimize inflammatory and catabolic responses.

Beyond improving the biochemical and material properties of healing tendons, mechanical stimulation can be used to precondition tissue engineered constructs prior to implantation, in turn resulting in improved in vivo outcomes (Thorfinn et al., 2012). Cell-seeded decellularized extracellular matrices demonstrated higher ultimate stresses and elastic moduli when exposed to cyclic stretch in bioreactors (Androjna et al., 2007; Angelidis et al., 2010). The augmented mechanical properties are explained in part by cell-mediated remodeling of the extracellular matrix, with cyclic loading promoting increased deposition of collagen fibrils in the direction of strain (Gilbert et al., 2007; Nguyen et al., 2009). Through a series of studies, Butler et al. (2008) showed that matching the material properties of MSC-seeded collagen sponges with those of native tendon could improve the mechanical and histological characteristics of healed tendon in vivo. Interestingly, MSC-seeded collagen constructs produced better results than tendon autografts or constructs made stiffer through dehydrothermal crosslinking, emphasizing the importance of cellular and topographical composition of constructs in restoring native tendon qualities (Kinneberg et al., 2011; Nirmalanandhan et al., 2009).

As highlighted above, recent research has begun to elucidate the role of mechanical stimulation in improving natural tendon healing and in optimizing the properties of cell-seeded constructs fabricated ex vivo. However, the effects of mechanical loading on the integration and remodeling of tissue engineered constructs implanted in vivo are only beginning to emerge (Ambrosio et al., 2010b). Using a mouse model of muscle injury, Ambrosio et al. showed reduced fibrosis and improved integration of muscle-derived stem cells when mice performed treadmill running for 5 weeks following cell implantation (Ambrosio et al., 2010a). Likewise, the ability of growth factors to improve healing when injected into a tendon lesion is dependent upon the mechanical environment of the tissue following injection (Aspenberg, 2007; Forslund and Aspenberg, 2002; Virchenko and Aspenberg, 2006). Using decellularized porcine small intestinal submucosa for Achilles tendon repair in a rabbit model, Hodde et al. (1997) found that complete immobilization of the ankle joint following surgical repair impaired matrix remodeling. While many questions remain, these studies highlight the role of in vivo mechanical loading in promoting the integration and remodeling of tissue engineered constructs. A thorough understanding will be needed for the promise of regenerative medicine to be realized.

## CURRENT CHALLENGES AND FUTURE DIRECTIONS

Past research in tissue engineering for improved tendon healing, as highlighted above, took advantage of concurrent discoveries in molecular biology and material science to explore the benefit of applying cells, growth factors, or scaffolds, independently or in combination. Multiple animal studies confirm the possibility of these strategies. However, advances in biomaterials and nanotechnology now allow bioengineers to more closely replicate the complexities of native tissues, thereby enabling finer control of cellular behavior (Lutolf et al., 2009). Refinement of electrospun nanofibrous scaffolds can support tenogenic differentiation of seeded MSCs (Kishore et al., 2012) while approaching the mechanical

properties of native tendon (Moffat et al., 2009), thereby offering the prospect of scaffold-augmented tendon repair in the near future. However, the insertion of tendons into the stiffer bone presents a more complex engineering problem. This problem is being pursued through several approaches. Spalazzi et al. have developed triphasic scaffolds capable of supporting spatially-confined cellular phenotypes germane to tendon-bone insertions – fibroblasts, chondrocytes, and osteoblasts (Spalazzi et al., 2008). Others have adopted dual spinnerette pumps containing different polymer solutions to fabricate a gradual transition along an electrospun scaffold (Ramalingam et al., 2013; Samavedi et al., 2012), while Phillips et al. (Phillips et al., 2008) achieved similar results by controlling the spatial distribution of retrovirus encoding the osteogenic transcription Runx2/Cfba1 on an electrospun scaffold. Conversely, Ma et al. (Ma et al., 2012) have created bone-ligament-bone constructs with a functional enthesis by juxtaposing MSC-derived osteoblasts and fibroblasts that are self-contained in their deposited extracellular matrix. While these advances demonstrate the possibility of fabricating graded interfaces containing different cell phenotypes with surrounding matrices of divergent mechanical and biochemical properties, the integration of these ex vivo constructs with native tissue remains a largely unexplored challenge.

Despite the promise of regenerative medicine that has continued to grow since its inception over two decades ago, translation of basic research into clinical therapies has been frustratingly slow. Treatment of tendon injuries is no exception. Part of the challenge in implementing new regenerative approaches in clinical practice concerns economic and practical matters. In particular, cell-based therapies requiring ex-vivo expansion and manipulation of autologous cells require added expense and multiple procedures for tissue harvesting and re-implantation. Consequently, several researchers have argued that the promise of regenerative medicine will be most quickly and effectively realized by focusing on augmenting the natural healing response. This could be achieved by utilizing off-the-shelf scaffolds or growth factors that could recruit and direct endogenous reparative cells to restore native organ function (Evans et al., 2007; Jakob et al., 2012). Likewise, the concurrent application of biophysical modalities offers a non-invasive means of bolstering healing. For instance, low-intensity pulsed ultrasound (Lovric et al., 2013), pulsed electromagnetic fields (Strauch et al., 2006), and extracorporeal shockwave therapy (Chow et al., 2012), can improve the histological and mechanical properties of healing tendon-bone insertions. While biophysical modalities have long been part of rehabilitation therapy protocols for musculoskeletal injuries, recent research probing the effects of these physical agents on the molecular and cellular functions will allow their optimal use in combination with conventional surgical and tissue engineering approaches (Ambrosio et al., 2010b).

Efforts to facilitate endogenous repair by intraoperative or non-invasive means may overcome some of the hurdles currently impeding the translation from the bench to bedside, but basic research that integrates the growing knowledge of developmental biology into the design process will be needed to fully recapitulate native tissue structure and function (Thomopoulos et al., 2010). In particular, elucidation of the expression patterns of transcription factors involved in tendon development (Liu et al., 2011) would allow tissue engineers to apply growth factors in spatiotemporally defined patterns necessary to restore native structure. To that end, biomaterials capable of delivering multiple growth factors at specified times and locations are needed. While the fabrication of such complex biomaterials

is still in its infancy, recent advances in controlled delivery suggest the possibility of implementing these scaffolds in the near future (Richardson et al., 2001; Santo et al., 2013). Furthermore, the construction of mathematical models of biological control circuits, based in part on the burgeoning fields of systems biology and network science, could allow the evaluation of explicit hypotheses that would inform process design (Lenas et al., 2009a; b). This would allow regenerative medicine approaches, for tendons and other tissues, to move beyond a trial-and-error empirical endeavor and towards a technology-based discipline with modifiable design parameters.

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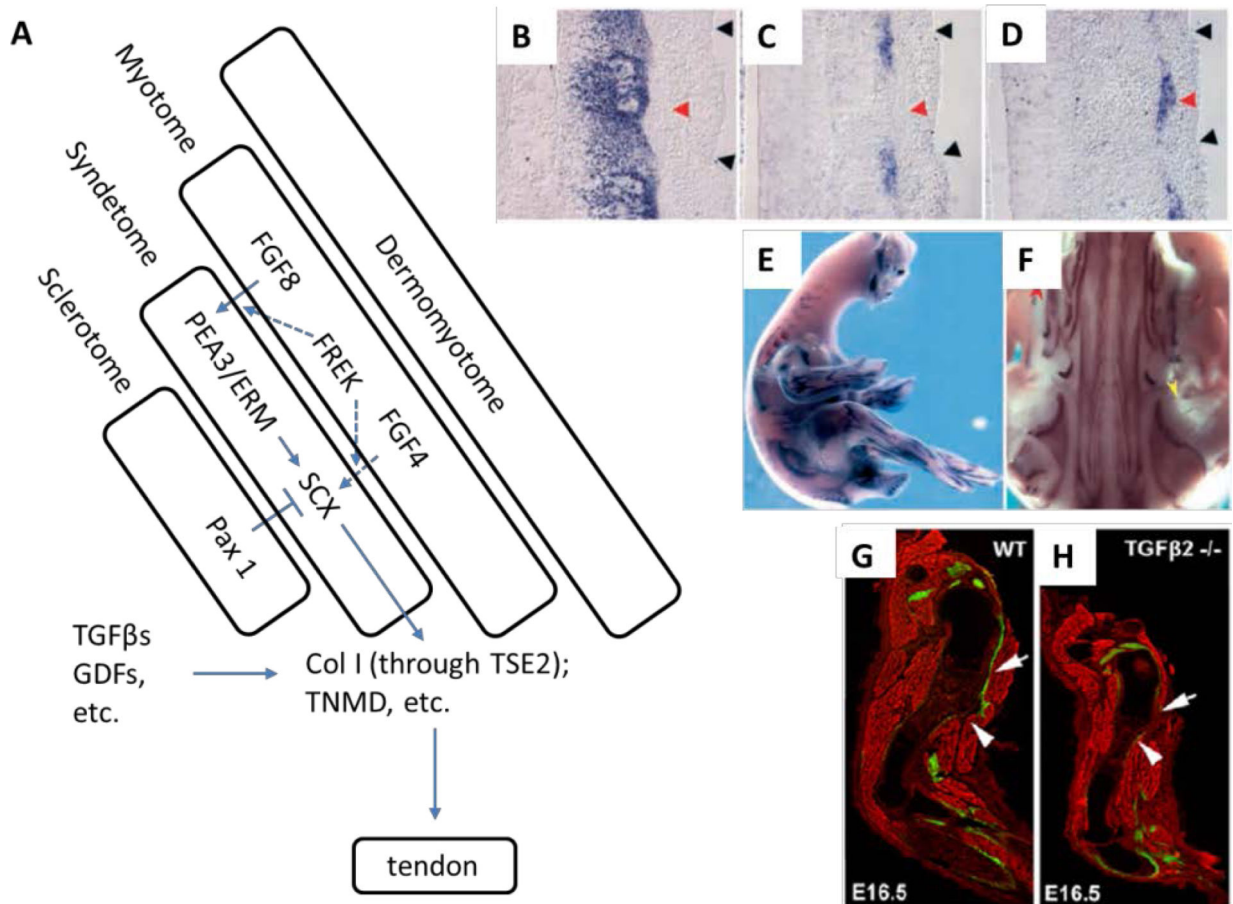
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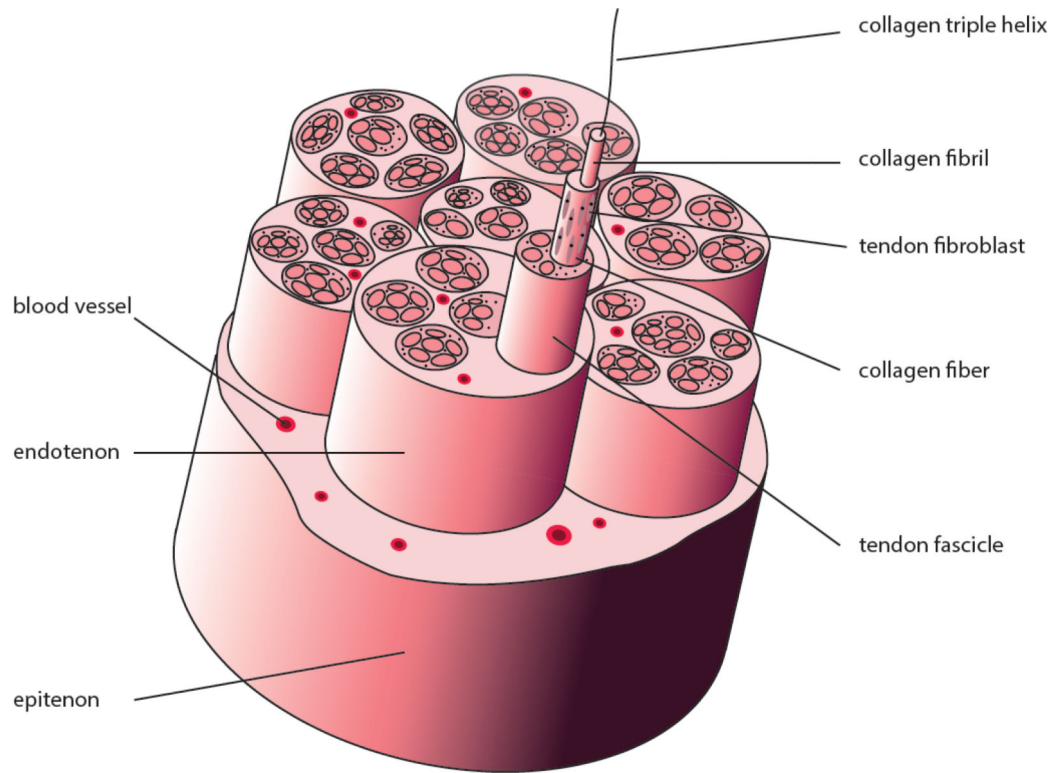
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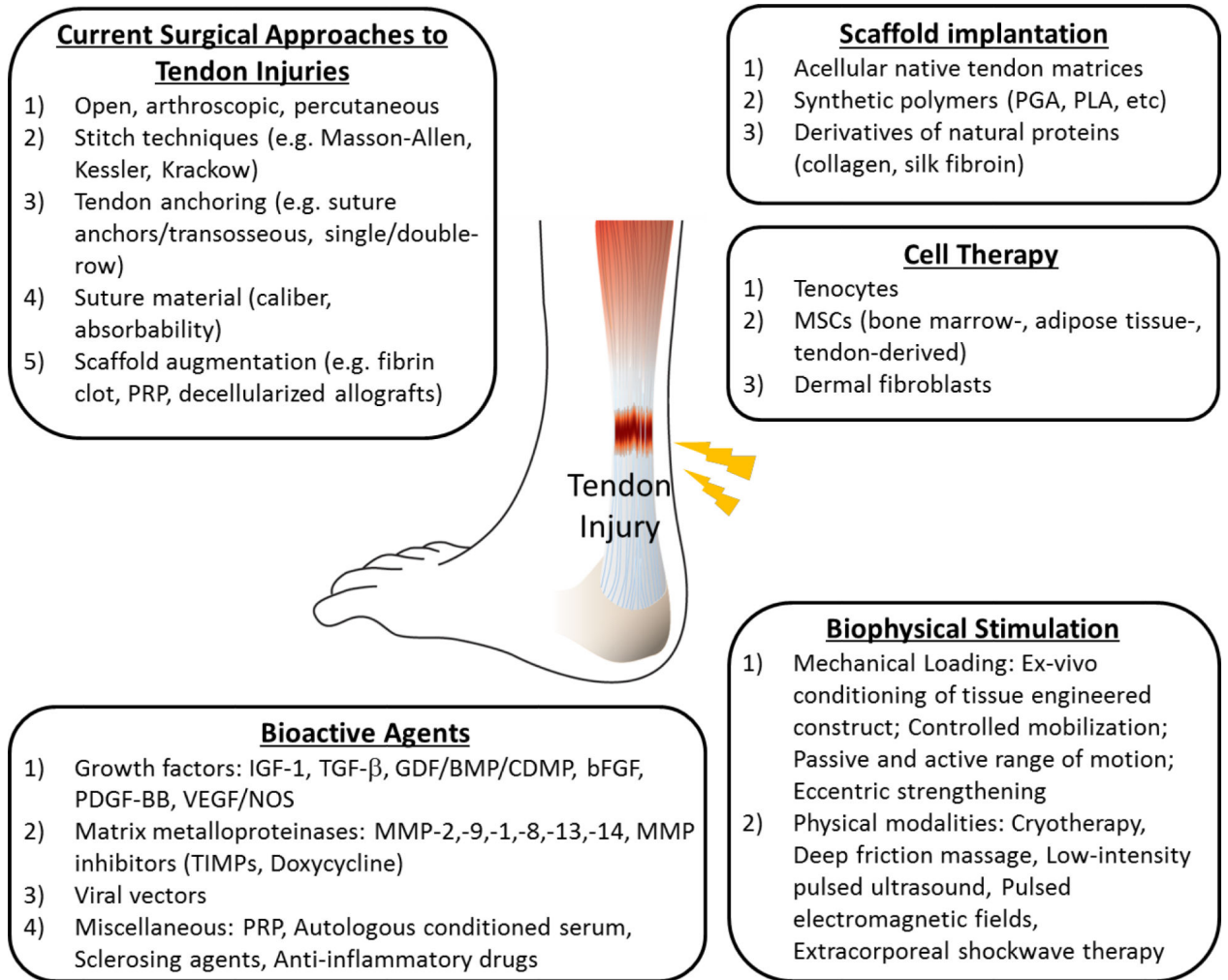
**Figure 1.**

An overview of tendon development. (A) *Scx*-expressing cells of trunk tendons appear between the myotome and sclerotome during early development, constituting a fourth compartment, syndetome, of the somite. Using a chimeric embryo model, syndetome is found emerging from sclerotome. FGF8 and 4 and their receptor, FREK, from myotome are involved in activating *Scx*-expression, while Pax1 from sclerotome suppresses *Scx* expression. *Scx* regulates downstream tendon-related genes including Col I and TNMD. (B-F) Visualized by in situ hybridization, *Scx* (C, blue) is found expressed in cells between myotomes (red arrowhead) of adjacent somites (black arrowheads), and its expression pattern does not overlap with that of Pax1 (B, blue) or MyoD (D, blue). *Scx* is expressed in all limb (E) and axial tendons (F) in chick embryos [Reproduced with permission from Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, Lassar A, Tabin CJ. 2001. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 128(19):3855-3866]. A variety of growth factors play critical roles in tendon development and maturation, including members of the TGF $\beta$  superfamily. (G, H) White arrows indicate the missing Deltoid tendon (green) in TGF $\beta$ 2 deficient (TGF $\beta$ 2<sup>-/-</sup>) mouse embryo compared with wild type (WT) [Reproduced with permission from Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dunker N, Schweitzer R. 2009. Recruitment and maintenance of tendon progenitors by TGFbeta signaling are essential for tendon formation. *Development* 136(8):1351-1361].



**Figure 2.**

The hierarchical architecture of tendon. Collagen triple-helices self-assemble into fibrils. Bundles of fibrils form fibers, which constitute tendon fascicle. Tendon fibroblasts (tenocytes) reside between collagen fibers. Fascicles are wrapped by endotenon, a layer of connective tissue containing blood vessels, nerves and lymphatics. Multiple fascicles are further surrounded by another connective tissue layer, epitenon, to form the tendon tissue.



**Figure 3.**

An overview of approaches for repair of tendon injuries. Briefly, surgical interventions and biophysical stimulation are currently employed in clinical care. Meanwhile, tissue engineering strategies are the cutting-edge of tendon healing and regeneration. Engineered replacement of injured tendon using a combination of cells, bioactive molecules, and scaffolds is under intensive investigation.