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Bringing tendon biology to heel: Leveraging mechanisms of tendon development, healing, and regeneration to advance therapeutic strategies

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Abstract

Tendons are specialized matrix-rich connective tissues that transmit forces from muscle to bone and are essential for movement. As tissues that frequently transfer large mechanical loads, tendons are commonly injured in patients of all ages. Following injury, mammalian tendons heal poorly through a slow process that forms disorganized fibrotic scar tissue with inferior biomechanical function. Current treatments are limited and patients can be left with a weaker tendon that is likely to re-rupture and an increased chance of developing degenerative conditions. More effective, alternative treatments are needed. However, our current understanding of tendon biology remains limited. Here, we emphasize why expanding our knowledge of tendon development, healing, and regeneration is imperative for advancing tendon regenerative medicine. We provide a comprehensive review of the current mechanisms governing tendon development and healing and further highlight recent work in regenerative tendon models including the neonatal mouse and zebrafish. Importantly, we discuss how present and future discoveries can be applied to both augment current treatments and design novel strategies to treat tendon injuries.

Keywords

connective tissues; stem cells; injury; repair

1. Introduction

The musculoskeletal system is an interconnected network of cartilage, muscle, and bone that coordinates movement. Central to the proper function of this network are tendons, specialized uniaxial connective tissues that bridge muscle and bone. Tendons are viscoelastic, highly ordered, extracellular matrix (ECM)-rich tissues that can transmit mechanical forces as large as 12.5 times the human body weight.[1,2] Their dynamic role in daily movement and athletic activities also makes them prone to injury. Although tendons are proliferative during embryonic development and postnatal life, adult tendons exhibit little to no cellular turnover and extremely poor healing capacities.[3] Tendon injuries can

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therefore be especially devastating for an athlete's career as well as a strain on the quality of life for patients of all ages in the general population.

Injuries to tendons and other joint tissues including ligaments, which connect bones, comprise an estimated 45% of the approximate 32 million musculoskeletal injuries reported annually in the United States.[4] Although ligaments are considered molecularly indistinguishable from tendons during development, they are functionally distinct. While injuries to ligaments such as the anterior cruciate ligament (ACL) are also common, in this review we will focus on tendons. Tendon injuries are often caused by overuse, athletic activity, and/or aging in predominantly heavy load-bearing limb tendons and can range from chronic pain-inducing disorders called tendinopathies to severe acute pain induced by partial or full ruptures.[5–7] Despite their prevalence, treatment options for tendon injuries remain limited and largely rely on surgical re-attachment or replacement followed by a slow recovery period lasting several months. Trofa et al. (2017)[8] reported that 30% of professional athletes across a variety of sports were unable to return following Achilles tendon injury, and those that did return following treatment performed significantly worse than prior to injury within the first year. Hence, even with treatment, injured tendons form scar tissue with inferior biomechanical properties that can only sustain a maximum of 60% of the force of their healthy counterparts, leading to a high chance of re-rupture and a gradual progression of other musculoskeletal diseases including osteoarthritis.[9–11] Likewise, chronic pain-inducing tendon disorders called tendinopathies can also lead to ruptures,[12] altogether contributing substantially to a growing financial healthcare burden and underscoring the pressing need for the development of better alternative therapeutics for tendon injuries and disorders.

Although adult mammalian tendons exhibit limited reparative capacity, recent studies have demonstrated that tendons in neonatal mice and zebrafish can fully regenerate. Understanding the mechanisms driving successful regeneration in these models will yield valuable insights into the restoration of ordered matrix-rich tissues that can be applied towards designing new therapeutic approaches to stimulate regenerative outcomes in patients. In this review, we provide an overview of our current understanding of tendon development and repair and how tendon regeneration models can be exploited as powerful systems that can open up new frontiers in tendon regenerative medicine.

2. The molecular and cellular architecture of tendons

As a force transmitting and mechanosensing tissue, the tendon harbors a unique structural morphology critical to its function.[13,14] Tendons are hypocellular, ECM-rich tissues. The ECM is tightly packed with collagen I fibrils that run parallel to the tendon axis from muscle to bone (Figure 1A). Collagen I fibrils are comprised of a triple helical arrangement of collagen I polypeptides, which are ultimately assembled into successively higher order structures, i.e. fibers and then fascicles. Each fascicle is separated by a connective tissue layer called the endotenon. Although collagen I is the predominant ECM component and comprises approximately 80% of the tendon dry mass, other ECM proteins are present and required for the structural integrity of higher order collagen I arrangement.[15] For instance, other collagens (COL5A1, COL12A1, COL14A1, COL22A1) and ECM proteins including

Decorin (DCN), Fibromodulin (FMOD), Laminin 2 (LAMA2), and Tenascin C (TNC), crosslink and act as structural scaffolds between collagen I fibrils.[16] Altogether, these ECM components construct a hierarchically ordered structure with properly aligned collagen I fibrils, which is key to providing strength, durability, and stability to the tissue during force transmission.

Interspersed amongst collagen I fibrils are tendon cells, called tenocytes, which are arrayed in parallel rows along the tendon axis (Figure 1B–C). Tenocytes have stellate morphologies with thin, elongated cellular protrusions extending in all directions. These protrusions form cell-cell contacts with neighboring tenocytes from adjacent rows and within the same row, building an intricate tenocyte network. Gap junction proteins including Connexin-43 (CX43) are expressed at these cell-cell contacts and are thought to regulate the transfer of molecules between tenocytes.[17,18] However, the significance of these gap junction proteins in regulating intercellular communication as well as what molecules pass between the cells is not well understood. Tenocytes and collagen fibrils are encased in an outer cellular sheath called the peritenon that is composed of two layers, the epitenon and the paratenon.[5] In addition, while the interior of the tendon is largely devoid of vasculature or innervation, blood vessels and nerve endings penetrate and run through the epitenon and paratenon.[19,20]

Tendons are not a homogenous tissue and contain regions that are specialized for their function at their muscle and bone attachments. Tendons can broadly be broken into three general sections along their axes: the muscle-tendon attachment, termed the myotendinous junction (MTJ), the tendon midbody, and the tendon-bone attachment, called osteo-tendinous junction or the enthesis. While the structure of the tendon midbody is as described above, the MTJ and enthesis have distinct structural, cellular, and molecular characteristics required for muscle and bone attachment.

The MTJ displays a “zipper”-like morphology, where skeletal muscle fibers and collagen I fibrils in the tendon interdigitate in an alternating manner. At the tendon-muscle interface, the cytoskeletal network and basement membrane of the adjacent bundle of muscle fibers, or sarcolemma, directly attaches to collagen I fibrils in the tendon through binding interactions between different ECM proteins that are highly enriched at the MTJ including Thrombospondin 4 (*Tsp4*) and various laminins and integrins.[16] Notably, the interdigitating pattern at the MTJ maximizes contact surface area, which strengthens the connection point between the two tissues and ensures stable transfer of mechanical forces.[21,22]

Tendon-bone attachment sites are composed of either fibrous or fibrocartilaginous connections.[23] In fibrous entheses, dense tissue directly attaches to the bone or the periosteum via a large surface area, which helps to distribute forces and minimize stress levels. In contrast, the more common fibrocartilaginous enthesis has a comparatively narrow insertion site with a characteristic gradient comprised of four transition zones that contain increased mineralization and proteoglycan levels from the tendon towards the bone. The first zone includes an area that resembles the tendon midbody with linearly aligned type I collagen fibers surrounding fibroblast cells and it connects to the second zone of unmineralized fibrocartilage, which is mostly composed of types I, II, and III collagen and aggrecan. The third zone consists of mineralized fibrocartilage containing type I and II

collagen, type X collagen (*ColX*) and aggrecan and joins to the fourth zone of bone and mineralized type I collagen. This gradual change in structure reduces stress levels exerted on two tissues with different biomechanical properties (tensile tendon and brittle bone), while effectively transferring the muscle force to the bone.[24]

3. Applying lessons from tendon development to augment existing treatments

Current tendon injury and disorder treatments are limited in the clinic with varying success rates. In this section, we will discuss known mechanisms underlying tendon development as well as how this knowledge can be applied to enhance current mainstream treatments or advance novel stem cell-based treatment strategies. As injuries most often occur in the tendon midbody and tendon-bone attachment, we will focus on these two areas of the tendon. For a comprehensive review of MTJ development, we refer the reader to the following reviews: Subramanian and Schilling, 2015[16] and Charvet et al. 2012.[25]

3.1. Tendon and tendon-bone attachment development

Tendons are found in craniofacial, axial, and limb regions of the body, each with distinct developmental origins. Craniofacial tendons are derived from the neural crest,[26–28] whereas axial and limb tendons are derived from the paraxial/somitic and lateral plate mesoderm, respectively.[29–31] Tendon development begins during embryogenesis and continues through post-natal periods. During early embryonic stages, tendon progenitors are specified and aggregate, forming primordia adjoining developing muscle and skeletal elements.[32] These progenitors proliferate[33] and differentiate throughout later stages of embryogenesis, secreting collagen I and other ECM proteins including Tenomodulin (*Tnmd*)[34,35] that are required for proper collagen I matrix assembly and structural integrity. The process of tendon maturation continues through postnatal stages, in which tenocytes continue to proliferate and collagen I fibrils grow vastly in diameter and length.[36,37] The expansion of the ECM during matrix maturation significantly decreases cell density and is accompanied by a decrease in proliferation as well as the adoption of the elongated, stellate morphology of mature tenocytes.[3,37] Proliferation levels continue to decrease through adulthood, in which very limited cellular turnover is observed.

Our understanding of transcriptional regulation of tendon development is limited to the identification of a small subset of transcription factors.[38] The basic-loop-helix transcription factor Scleraxis, *Scx*,[32] is highly enriched in tendon progenitors regardless of function and anatomical location. *Scx* is also expressed in the adult tendon, as well as some other tissues such as the heart valves.[39] Developmental studies in mice and zebrafish have determined that *Scx* is dispensible for the initiation of tendon formation, but required for the maintenance and maturation of tendon cell fate and matrix during later stages of development.[40,41] Canonical Transforming Growth Factor β (TGF-β) signaling through intracellular SMAD2/3 can promote expression of *Scx* and other tendon markers and is required for maintenance of tendon cell fate. However, $Tgf\text{-}\beta2^{-/-};Tgf\text{-}\beta3^{-/-}$ double null and *Prx1*-Cre;$Tgf\beta r^{flox}$ mutant embryos exhibited defects in tendon maturation, not specification,[42,43] suggesting other TGF-β ligands and/or additional signaling pathways

regulate tendon fate induction. Consistent with these findings, TGF-β superfamily members including Myostatin (*Gdf-8*) are also involved in tendon maturation.[44]

The transcription factors Mohawk (*Mkx)* and Early Growth Response 1 (*Egr1*) and *2* (*Egr2*) also play a role in tendon development. Although *Mkx* and *Egr1/Egr2* are highly expressed in many other tissues, loss of function animal models for these genes suggest they regulate specific aspects of tendon matrix development. $Mkx^{-/-}$ null mice display defects in the ECM maturation during late stages of embryogenesis as well as post-natal development and have lower expression of tendon genes (*Tnmd*, *Dcn*, and *Fmod*), resulting in hypoplastic tendons.[45,46] $Egr1^{-/-}$ and $Egr2^{-/-}$ null mice both display lower expression of many tendon-enriched collagens, leading to weaker tendons.[47,48] Interestingly, tendons from $Egr1^{-/-}$ null mice exhibit lower *Scx* expression, whereas those from $Mkx^{-/-}$ null mice have unchanged *Scx* expression, indicating *Mkx* regulates tendon maturation either downstream or through a parallel mechanism to *Scx*. While all three transcription factors regulate *Col1a1* transcription, none are master regulators of tendon cell fate.

Tendons can be divided into force-transmitting (energy storing or positional)[49] or anchoring connective tissues. In general, limb and craniofacial tendons are largely force transferring and directly involved in musculoskeletal movement, whereas axial tendons such as those that connect the intercostal muscles to the ribs anchor and dissipate forces to prevent displacement of the muscle from the bone. Evidence suggests that there may be discrete differences in their developmental programs. For example, $Scx^{-/-}$ null mouse mutants display developmental defects specifically in force transmitting tendons, but not anchoring tendons, due to impaired tendon elongation,[40,50] indicating *Scx* function may vary depending on the functional role and developmental program of the tendon. Interestingly, despite differences in developmental origin, both craniofacial and limb tendons do not require the presence of muscle to initiate tendon specification during embryogenesis, whereas axial tendons do.[27,51–53] Furthermore, Chen et al. (2020)[54] discovered that skeletal progenitors can be re-specified to a tendon cell fate via the inhibition of the mevalonate pathway during zebrafish cranial and fin, but not axial, tendon development. Together, these data interestingly suggest that similarities in the mode of regulation between craniofacial and limb tendons may outweigh the importance of their developmental cellular origins.

Neighboring muscle and skeletal tissues play significant roles in tendon development. Both the presence and movement of muscle is required for the maintenance of tendon cell fate and maturation.[28,30,32,55,56] In particular, the dependence of tendon development on muscle movements has been tied to mechanically-induced changes in TGF-β and Fibroblast Growth Factor (FGF) signaling.[55,56] Tendon induction in the developing autopod (hand region) has also been shown to be dependent on developing cartilage in mice.[57] Furthermore, evidence suggests that similar extracellular signals can induce different cellular fate outcomes in developing muscle, tendon, and bone. For example, high levels of Bone Morphogenetic Signaling (BMP) signaling promote cartilage formation,[58] but inhibit tenogenesis,[32] illustrating the existence of discrete genetic regulatory circuits in neighboring tissues originating from a gradient of extracellular signals.

Skeletal and tendon development are intricately linked during formation of the tendon-bone attachment. While muscle, tendon and cartilage first appear during embryonic development, the murine enthesis forms distinctly from these structures and matures during early postnatal stages. Unique multipotent progenitors at the tendon-bone interface specify the position of the bone eminence for the attachment site.[59] These progenitors express both *Scx* and the transcription factor SRY-Box Transcription Factor 9 (*Sox9*), which is required for skeletal development. Lineage tracing studies have determined that $Scx^{+}/Sox9^{+}$ cells give rise to the tendon-bone attachment unit and the associated bone eminence.[60] Tenocytes and chondrocytes further from the enthesis are derived from Scx^{+} and $Sox9^{+}$ single-positive progenitors, respectively. Early formation of the bone eminence, specifically the tendon-humoral tuberosity, requires BMP4 expression from tendon cells as deleting BMP4 in *Scx*-expressing cells affected the formation of the enthesis and associated bone ridge.

Although muscle loading is not involved in early phases of enthesis development, it is crucial for its maturation. Tatara et al. (2014)[61] showed that reducing muscle loading in the maturing enthesis during postnatal stages leads to impaired mineral deposition, reduced fibrocartilage formation, changes in the bone architecture and consequently inferior mechanical properties. Recent studies have begun to reveal molecular signals that translate the mechanical stimuli exerted from the muscle to the tendon-bone insertion site. Schwartz et al. (2015)[62] examined hedgehog (Hh) signaling during enthesis formation and found a Hh-responsive Zinc finger protein Gli1-expressing cell population at the developing enthesis, which is distinct from tenocytes and epiphyseal chondrocytes before mineralization. Interestingly, muscle paralysis during postnatal stages increased the number of $Gli1^{+}$ cells at the enthesis while ablation of these cells disrupts fibrocartilage formation in the mature enthesis. Dyment et al. (2015)[63] further showed that Hh signaling drives mineralization via these $Gli1^{+}$ cells in the unmineralized fibrocartilage. In particular, *Indian Hedgehog* (*IHh*) is a likely candidate Hh ligand involved in mineralization. Mineralization proceeds from the bone towards the tendon midbody and is characterized by expression of alkaline phosphatase and *ColX* in the mineralized fibrocartilage. The mature enthesis retains very few of these $Gli1^{+}$ cells within the area of unmineralized fibrocartilage which has been associated with its poor healing capabilities (Schwartz et al. 2017)[64].

3.2. Common tendon injuries/disorders and mainstream treatments

Tendon ailments encompass acute injuries (partial or full ruptures) or tendinopathies, chronic disorders that cause prolonged pain in patients. Tendon injuries/disorders most frequently occur in the midbody or enthesis of major force-bearing limb tendons including the Achilles, patellar, supraspinatus, and flexor/extensor tendons (Figure 2). Like acute injuries, tendinopathies also affect the major limb tendons. However, while less studied, they also occur in craniofacial tendons including the temporalis tendon which is involved in jaw movement. Tendinopathies affecting the enthesis are called enthesopathies. One example is lateral epicondylitis, commonly called “tennis elbow”, which specifically causes pain around the extensor tendon insertions into the lateral epicondyle.[65]

The typical treatments for tendon injuries and tendinopathies are conservative, non-invasive strategies, which predominantly seek to alleviate pain rather than treat the tendon condition

itself. These include rest, immobilization of the injured area, anti-inflammatory drugs, as well as physical, cryo-, ultrasound, or laser therapy. Injections of growth factors such as platelet-derived growth factor (PDGF) or via platelet-rich plasma (PRP) are also available courses of treatment.[66,67] However, the efficacy of all of these treatments varies substantially depending on the mode and severity of injury or tendinopathy. Oftentimes conservative treatments fail and surgical intervention is required, especially in cases where the tendon has fully ruptured or in advanced tendinopathic patients. For partial and full tears, surgical repair of the ruptured tendon entails suturing the ruptured ends of the tendon together or re-attaching the tendon to the bone for enthesis tears. Overall, surgical intervention in general leads to lower rates of re-rupture and improved biomechanical performance compared to conservative treatments.[68] In severe cases of full tears or advanced tendinopathies, the damaged tendon is replaced with a healthy tissue graft. Tissue grafts can be autografts (usually quadriceps or hamstring tendon from the patient depending on which tendon is injured), allografts (from donor cadavers), or xenografts (from large animals). Each of these methods has associated complications. Autografts induce donor site morbidity, while allografts and xenografts run the risk of immune rejection. Surgical intervention is followed by functional rehabilitation with several months of physical therapy. However, even following treatment, repaired or reconstructed tendons in patients may function at as low as only a third of the tensile strength of a healthy tendon.[5]

3.3. Emerging cell-based treatments and engineering strategies

In the last two decades, tendon regenerative medicine has increasingly moved towards the development of stem cell approaches to treat tendon injuries and tendinopathies.[69] In particular, the use of mesenchymal stem cells (MSCs) for tendon treatments has become popularized.[70,71] The exact definition and nature of MSCs has been controversial and evolved over time (reviewed in Bianco, 2014[72]). The term MSC was originally coined by Caplan (1991)[73] to describe cells isolated from the bone marrow in seminal studies from the 1960–80s that exhibited multipotency *in vivo* and *in vitro*, with the ability to differentiate into various skeletal cell types including fat, bone, and cartilage (reviewed in Friedenstein, 1990[74]). At the time, these cells were also separately referred to as osteogenic[74] or stromal stem cells[75]. Caplan (1991) along with subsequent work further implied these cells could also generate and replace non-skeletal tissues including skeletal muscle and tendons/ligaments[76]; however, this notion became rooted in evidence generated *in vitro*, rather than *in vivo*. While the aforementioned bone marrow-derived stem cells have now been re-defined as skeletal stem cells based on their demonstrated function in bone maintenance, the hematopoietic niche, fracture healing, and ability to form fat, cartilage, and bone *in vivo* (reviewed in Ambrosi et al., 2019[77]), the identification of an MSC as per the original definition that differentiates into tissues derived from different germ layers during normal development and homeostasis has yet to be shown *in vivo*. For the purposes of this review, we will discuss MSCs in the context of tendon research, which includes cells isolated from various adult tissues including bone marrow, adipose tissue, and umbilical cord blood that exhibit the potential to differentiate into different cell types *in vitro*.

MSCs have been considered an ideal cellular source clinically because of their relative ease of extraction from adult tissues with minimal manipulation. Treatments using MSCs were

not regulated by the Food and Drug Administration (FDA) until recently. MSC injections have been shown to improve tendon repair in animal models *in vivo*[78–82] and some studies have also reported beneficial effects of MSC injections on human tendon healing including pain reduction and improved healing.[83] At present, there are several ongoing clinical trials investigating whether autologous and allogeneic MSCs derived from bone marrow or adipose tissue can effectively treat tendon injuries and tendinopathies predominantly in the rotator cuff.[71] MSCs have been shown to exhibit tenogenic differentiation potential *in vitro* through the modulation of various pathways involved in tendon development and healing including FGF, TGF-β, BMP, and Wnt[84,85] and the overexpression of *Scx, Mkx*, and *Egr1*.[48,86,87] While these cells can augment tendon healing in different animal models, less work has been done to demonstrate how well these findings translate to human MSCs in the clinical setting. Several studies have also identified heterogeneity in the molecular signature, differentiation capacity, and proliferative potential within individual MSC populations or between MSCs isolated from different sources.[88] How these differences may impact tenogenic differentiation potential and tendon healing outcomes is unknown. Furthermore, ectopic ossification of tendons is commonly observed in patients with tendon injuries or tendinopathies.[89] As transplanted MSCs have been shown to form ectopic bone during tendon healing,[90] the multipotent differentiation potential of MSCs may call into question of whether transplantation of committed tendon progenitor cells with restricted differentiation capacities would be a better treatment strategy. While MSCs show therapeutic potential, many questions remain unanswered surrounding the mechanisms underlying their beneficial effects. For instance, whether MSCs directly differentiate into tenocytes and contribute to human tendon healing *in vivo*, indirectly exert beneficial effects through the secretion of anti-inflammatory cytokines and growth factors, or both remains unclear. Long-term engraftment of MSCs *in vivo* has proven to be challenging and has yet to be demonstrated, indicating MSCs may indirectly affect tendon healing.[91,92] Indeed, a role for MSCs as a cellular source of signals that promote healing of the endogenous tissue has been underscored by a recent effort to rename the cells as medicinal signaling cells.[93]

As an alternative to adult stem cell-based cell therapies, studies in the last decade have shown the promise of directed stem cell differentiation approaches for cell replacement therapies to treat a variety of diseases and conditions.[94–96] Devising directed differentiation protocols to generate adult tendon tissue *in vitro* from human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) is a tenable goal to improve and replace modern tendon grafts as well as provide an alternative model system for studying mechanisms of human tendon biology. However, these cell sources come with their own associated concerns clinically. The potential tumorigenicity of hESCs and iPSCs, risks of immune rejection with the use of hESCs, and safety issues with virally induced pluripotency in iPSCs remain primary concerns. However, the use of these cell sources for other disease treatments will likely pave the way for their more widespread use[97].

Only a few studies have begun to explore tenogenic induction from hESCs and human iPSCs. One strategy that has been demonstrated is the derivation of multipotent mesenchymal progenitors including MSCs connective tissue progenitors (CTPs), or neural crest cells from hESCs or iPSCs to improve tendon healing.[98–101] These approaches primarily rely on priming the multipotent progenitors to form tendon-like structures *in*

vitro via modulating mechanical stimulation, *Scx* overexpression, or cellular density and inducing terminal tenogenic differentiation and maturation post-transplantation *in vivo*. Alternatively, Dale et al. (2018)[102] demonstrated that modulation of BMP signaling induces the differentiation of tendon-like cells from hESCs; however, *Scx* expression was notably not induced in these cells. While not in human, Komura et al. (2020)[103] have also devised a stepwise differentiation protocol differentiating mouse iPSCs into tenocyte-like cells that improve tendon healing when implanted *in vivo*, highlighting the potential applicability of a similar strategy for human clinical applications.

Stem cell-based therapeutic approaches can be further enhanced when combined with tissue engineering strategies. Maximizing cell survival, long-term engraftment, and tenocyte differentiation potential of transplanted cells have been formidable hurdles to the practical application of stem-cell based treatments. To this end, bio-engineering approaches aimed at producing cellular scaffolds that re-create endogenous tendon or tendon healing microenvironments have shown promise.[104] For example, seeding multipotent progenitors primed for the tenogenic lineage in artificially engineered or decellularized ECM tendon scaffolds from animals including pig and horses has led to improved engraftment of transplanted tissue, tenocyte differentiation, and healing.[66,105] Transplantation of MSCs embedded in a collagen matrix together with growth factors such as BMP-12 has also been shown to increase M2 macrophage infiltration and accelerate flexor tendon healing.[106] It is therefore likely that the success of directed differentiation approaches will rely on merging tissue engineering principles with developmental and stem cell biology.

Other bioengineering-based strategies are being heavily explored as potential treatment options, which we briefly summarize here. For more in depth discussion on these approaches, we refer the readers to the following reviews: Docheva et *al.*, 2015[107] and Freedman and Mooney, 2019.[104] Some methods aim to generate artificial constructs that mimic native tendon to replace injured tendon including silk-based scaffolds or FDA-regulated biodegradable synthetic materials including poly(lactic-co-glycolic acid), or PLGA. These materials can mimic collagen fiber alignment and tendon mechanics as well as promote cellular ingrowth and healing post-implantation. However, as tendon matrix is heterogeneous along the tendon axis, accurately re-creating the natural viscoelastic properties with synthetic materials, especially at the muscle and bone attachment points, has proven challenging. Alternatively, surgically replacing only the damaged area of the tendon with synthetic grafts including commercially available devices like GraftJacket™ or Artelon® has shown promise in enhancing endogenous healing instead of replacing the entire tendon.[108,109] Another therapeutic avenue that has been long explored is the implantation of engineered scaffolds or surgical sutures embedded with biologics including *Connective Tissue Growth Factor* (CTGF)[110] or PRP[111] into the damaged area that may enhance tendon healing. Recent efforts have also focused on an integrative approach in which materials are designed to elicit proper cellular responses to form more complex tissues such as the enthesis.[112]

3.4. Augmenting stem cell-based strategies by building a complete picture of tendon development

While stem cell differentiation approaches have great potential, major challenges lie ahead in terms of generating functional tendon tissue *in vitro*. These hurdles are rooted in fundamental gaps in our knowledge of mechanisms underlying tendon development *in vivo*. First and foremost, tendon development is a complex multidimensional process dependent on coordinated signals from developing muscle and bone as mentioned above. While some important signaling pathways involved in tendon development have been identified including TGF-β and FGF, many others have yet to be deeply explored. Elucidating these signaling mechanisms will greatly aid the design of directed differentiation protocols to generate tendon tissue *in vitro* going forward. Moreover, our understanding of how each of these pathways is spatiotemporally integrated in the development of the entire musculoskeletal circuit remains incomplete.[105,113,114] Not to mention, mechanical force is also important for tendon development and maturation at both embryonic and postnatal stages. While much work has been done in assessing how various mechanical stimulation regimens, substrates, and growth factors can affect tenogenic differentiation potential *in vitro*,[115] understanding how these pathways and mechanical signals can be integrated in 3-D *in vivo* during healing will require a combination of developmental biology and bioengineering approaches. This interdisciplinary approach will facilitate the combination of innovative engineering solutions and stem-cell based tendon replacement strategies. Lastly, the lack of many tendon-specific molecular markers presents perhaps the biggest challenge in terms of assessing the exact molecular identity, maturation stage, and functionality of tendon tissue generated *in vitro*. While the structure of the tendon is critical to its function, many questions remain as to the cellular heterogeneity within the tendon as well as the functional relevance of tendon subpopulations. Recent studies have unearthed tendon subpopulations in both adult mouse[116,117] and human[118] tendon via single cell RNA-sequencing approaches; however the functional importance of these subpopulations particularly during homeostatic activities including running have yet to be explored. These discoveries may provide vital insight into how tendon tissue generated in a lab may accurately recapitulate the cellular composition and functionality of a natural tendon. In all, while applying the knowledge we currently harbor serves as a great starting point, a complete roadmap of tendon development *in vivo* will be paramount to aid in efforts to generate human tendons in a dish.

4. Major questions surrounding mechanisms underlying tendon healing and disorders

The development of effective, alternative treatments for tendon injuries and tendinopathies requires a mechanistic understanding of tendon healing *in vivo*. A bulk of tendon research has thus primarily been focused on understanding the cellular and molecular mechanisms underlying tendon healing both in natural and surgical repair contexts in animal models to improve existing treatments for patients. In this section, we review current overarching themes of ongoing tendon healing and repair research.

4.1. General overview of tendon healing

Tendons generally heal in three sequentially semi-overlapping phases following acute injury to either the midbody or enthesis: inflammation, cellular proliferation, and remodeling.[11,119] Within the first week post-injury, there is a large influx of leukocytes to the wound site. Similar to other wound healing contexts, researchers have shown in both canine and rat tendon injury and repair models that neutrophils are the first immune responders to arrive on site, peaking at approximately 1 day post-injury, and followed by monocyte-derived macrophages in the subsequent days.[120,121] Pro-inflammatory and chemotactic cytokines including Interleukin 1-β (*Il1-β*) and Tumor Necrosis Factor α *(Tnfα)* as well as collagen-degrading matrix metalloproteinases (*Mmp-1, 3, 13*) are highly expressed during this early period as the collagen matrix begins to degrade. VEGF expression also increases, stimulating angiogenesis into the wound area.[19] Towards the end of the inflammatory phase, cells begin to proliferate and migrate towards the injury site. Unlike neutrophils, macrophages remain on site during most of the tendon healing process to aid in cell proliferation and ECM deposition.[122] During this 2 to 3-week period, new collagen III fibrils are deposited at the wound site as cells accumulate. Finally, cells re-differentiate into mature tenocytes, elongate, and align longitudinally as collagen I fibrils gradually replace collagen III fibrils throughout the remodeling phase. Vascularity in the repaired tendon tissue also decreases during these later stages. Matrix maturation entails the assembly of collagen I fibrils into larger bundles and can take up to a year, ultimately resulting in fibrotic scar tissue with inferior biomechanical properties to a healthy tendon.

4.2. The cellular basis of tendon healing

To date, studies have shown that genes involved in tendon development and matrix maturation including *Scx* and *Tnmd* are also required for regulating various aspects of tendon healing (cell proliferation, vascular infiltration, matrix assembly, immune cell accumulation etc.).[5,7,123] Yet, while the general progression of mammalian tendon healing and repair has been well-documented, many key questions remain unanswered as to the cellular and molecular mechanisms controlling this process. In particular, the cellular origins of the healed scar tissue post-injury have been debated.

Histological and *in vivo* transplantation studies from the 1970s and 80s in large animal models of flexor tendon repair including canines and rabbits suggested that both tenocytes and cells from the surrounding sheath possess the capacity to respond to injury.[11] With the discovery of resident stem cells that drive tissue homeostasis and repair in a variety of adult tissues including the gut, skin, and muscle,[124–127] the idea that such a population may also exist in the tendon became an attractive hypothesis. Although homeostatic proliferation levels and cell turnover rates are extremely low in adult tendons, researchers have indeed identified subpopulations of cells isolated from either interior tendon tissue or peritenon that exhibit stem-cell like characteristics *in vitro*. Commonly referred to as tendon stem progenitor cells (TSPCs), these cells can expand clonally and differentiate into multiple lineages *in vitro*, as well as generate tendon tissue upon subsequent transplantation *in vivo*.[117,128–132] Yet to date, only a few studies have taken advantage of Cre-based lineage tracing methods in murine models to examine whether such a stem or progenitor cell exists *in vivo* and participates in natural tendon homeostasis and healing. Collectively, these

findings have pointed to cells both in the surrounding peritenon and within the tendon proper as participating in tendon healing, but the functional significance of each distinct population remains unclear.

Pre-existing tenocytes in the main tendon body have been shown to contribute to tendon healing in certain contexts. Sakabe et al. (2018)[133] showed that *Scx* is necessary for tendon healing in a partial Achilles tendon transection injury. Best and Loiselle (2019)[134] performed conditional lineage tracing of *Scx*$^+$ tenocytes during flexor digitorum longus (FDL) tendon repair and demonstrated their contribution to healed scar tissue. A separate study from the same group also demonstrated that the fibrosis-promoting factor S100a4 is expressed in resident tenocytes and that these cells also contribute to the formation of scar tissue following FDL repair.[135] Lineage tracing of both populations following FDL repair intriguingly revealed largely separate contributions to the scar tissue, with *Scx*$^+$ tenocytes mainly contributing to the aligned bridging tendon tissue and S100 Calcium Binding Protein A4-positive (*S100a4*$^+$) tenocytes comprising the majority neighboring fibrotic tissue (Figure 3A). Ackermann et al. (2019)[135] further found that *S100a4*-haploinsufficiency and *S100a4*-expressing cell ablation during FDL repair improved tendon healing outcomes. However, other cell types including macrophages that infiltrated the repair site following injury also expressed *S100a4*, making it unclear whether impairing *S100a4* production from resident tenocytes was the prime cause for this improvement. Finally, Moser et al. (2018)[136] showed that a subpopulation of tenocytes expressing *Axin2* contributes to healed scar tissue in a full tear enthesis injury and repair model of the supraspinatus tendon. These studies clearly show contribution of *Scx*-lineage cells to tendon repair, however whether their contribution to healing is mediated via dedifferentiation to a progenitor-like state or proliferation of differentiated tenocytes remains unknown. Notably, these studies performed surgical re-attachment post-injury and the mechanisms governing healing in natural healing contexts may differ. For example, Howell et al. (2017)[137] demonstrated that *Scx*$^+$ tenocytes do not contribute to healed scar tissue following full transection of the mouse Achilles tendon (Figure 3B), illuminating distinct healing mechanisms potentially due to differences in tendon type or load-bearing following surgical repair versus natural healing.

Recent studies in natural tendon healing models have demonstrated that cells derived from the peritenon harbor injury-responsive progenitor populations that comprise a majority of healed tendon tissue (Figure 3C). Dyment et al. (2014)[138] identified an α-smooth muscle actin (αSMA)-expressing progenitor population that contributed to both patellar tendon growth and healing in a full length, central third patellar tendon injury model. The authors found that αSMA-expressing progenitors in the paratenon expand upon injury, migrate to bridge the defect, and differentiate into *Scx*$^+$ tenocytes (Figure 3B). Additionally, Wang et al. (2017)[131] identified a progenitor population derived from Osteocalcin$^+$ *(Bglap*$^+$) tendon sheath cells that proliferates and contributes to both tibialis anterior tendon and Achilles tendon healing following partial injury in mice. They further demonstrated that active Hedgehog (Hh) signaling was both sufficient to induce proliferation in the tendon sheath and necessary for proper sheath-mediated repair. Most recently, an injury-responsive progenitor population co-expressing Tubulin polymerization promoting protein 3 *(Tppp3)* and Platelet-derived growth factor receptor A (*Pdgfra*) was identified in the patellar tendon sheath of mice. Harvey et al. (2019)[117] demonstrated that *Tppp3*$^+$*Pdgfra*$^+$ progenitor cells

contribute to healed tendon tissue following a partial biopsy punch-induced injury in the patellar tendon. They further showed that these cells self-renew in the sheath, a stem cell property that was not examined in prior studies. Interestingly, these cells did not contribute to tenocytes in the interior portion of the tendon tissue during homeostasis, suggesting they are only activated upon injury. The authors further demonstrated that PDGF signaling was necessary for stimulating proper pro-reparative responses from *Tppp3*$^{+}$*Pdgfra*$^{+}$ cells. However, PDGF signaling also appeared to augment fibrotic responses from a separate population of *Pdgfra*$^{+}$ only cells, indicating it may be involved in coordinating multiple aspects of tendon healing. Altogether, the identification of these populations collectively represents significant advances in the field, and provides a starting point to assess whether similar populations exist and respond to injury in other tendons.

It is important to note that each of these studies traced tendon sheath-derived subpopulations using different markers (*Sma, Tppp3*, and *Bglap*), making it unclear whether the same subpopulation responded in each model or whether the cellular origin of the healed tissue differed depending on the type of injury and/or tendon. In fact, the type of injury appears to be an important factor in determining which cellular populations respond. For example, *Axin2*$^{+}$ tenocytes contribute to healed tissue only following a full, but not partial, enthesis injury in the supraspinatus tendon following repair.[136] Moreover, midbody versus enthesis injuries involve different cell types and may differ in repair mechanisms. As previously mentioned, calcified fibrocartilage formation depends on Hh signaling mediated by *Gli1* expression in the mouse enthesis. Though this *Gli1*$^{+}$ cell population persists in mature entheses of adult mice, the number of cells expressing the transcription factor is strongly diminished.[62] Schwartz et al. (2017)[64] showed that this *Gli1*$^{+}$ cell population contributes to enthesis regeneration after injury and maintains high levels of expression throughout the healing process in postnatal stages. In contrast, mature entheses show an even lower number of *Gli1*$^{+}$ cells right after injury and no contribution to the healing from this cell type. The authors speculate that this may be one of the reasons why mature entheses are incapable of regenerating and do not restore their original morphology after injury.

To complicate the issue further, transcriptional heterogeneity across different tendons and within tenocytes has been reported.[134,139,140] For instance, *Bglap* was reported to be a reliable marker of adult tendon sheaths in mice,[131] but was not detected within the adult patellar tendon sheath.[117] Whether and to what extent these differences are important for tendon development, function, and repair remains relatively uncharacterized. Another limitation of these studies is that at present there are no markers that fully differentiate between the midsubstance and the peritenon. For instance, while *Scx* or *αSMA* expression predominantly labels tenocytes in the midsubstance or cells in the peritenon, respectively, their expression can also label a small subset of cells in the peritenon or midsubstance. Both the heterogeneous nature of gene expression within different tendons and the lack of robust markers for tendon subpopulations has made generalizing conclusions challenging within the field. While tendons have been relatively understudied compared to other musculoskeletal tissues, the burgeoning set of sequencing technologies and molecular tools to study tendon tissues will aid in our understanding of tendon healing and repair in the future.[141,142]

4.3. Emerging views on the pathology of tendinopathies

Acute tendon injuries can oftentimes be the end result of tendinopathies, chronic pathological tendon disorders that lead to increased morbidity. Tendinopathies comprise approximately 30% of general practice musculoskeletal consultations and are clinically diagnosed following patient presentation of chronic pain, reduced activity-based movement, and swelling in some cases coupled with either ultrasonography or magnetic resonance imaging (MRI).[12,143] In contrast to healthy tendons which are white and exhibit a firm matrix, tendinopathic tendons are discolored, have a loose, disorganized collagen matrix, are hypercellular, and develop increased vasculature and innervation, as well as chondroplasia in some cases.[7,144] Notably, many of these characteristics resemble early stages of tendon healing following acute injury including collagen I matrix degradation, deposition of collagen III, as well as neoangiogenesis. These morphological changes are thought to occur in three progressive stages according to the continuum model originally proposed by Cook and Purdam (2008)[145]: reactive tendinopathy, tendon dysrepair, and degenerative tendinopathy. According to the model, acute tensile or compressive overloading triggers tendon thickening via excess cell proliferation and matrix growth in early reactive tendinopathy. If overloading becomes repetitive, more severe matrix degeneration, cell infiltration and proliferative responses occur in what mirrors a tendon healing response. Finally, in degenerative or advanced tendinopathy, the tendon exhibits acellular areas, neurovascular infiltration, disorganized matrix, dysfunction, and higher levels of cell death. Tendinopathies are therefore believed to be a result of the improper resolution of healing and gradual ECM degradation triggered by the initial formation of microtears in the tendon tissue caused by excessive mechanical loading.

Although the principal causes of tendinopathy are generally accepted as overuse, aging, and genetics,[144,146] the molecular mechanisms driving disease initiation and progression remain poorly understood. Most patient biopsy samples taken for research are typically obtained from advanced stages of the disease when surgical intervention is necessary, making study of early stages of human tendinopathy difficult. In fact, many patients are asymptomatic during early stages of tendinopathy.[6,147] These limiting circumstances have altogether made it challenging to devise experimental injury models that replicate the general progression of human tendinopathy. Nevertheless, various methods have been widely implemented in animal models to mimic certain aspects of tendinopathy such as ECM degradation in different tendons including treadmill running to simulate overuse/fatigue or chemical induction of tendinopathy via injection of collagenase, TGF-β, or Substance P.[148–151] Although artificially induced, experimental tendinopathic animal models have been valuable for uncovering general mechanisms of tendon disease. In some cases, comparison between animal overuse models and patient tissue has even spurred new human models of tendinopathy. For instance, Millar et al. (2010)[152] demonstrated that matched intact subcapularis tendon in patients with torn supraspinatus tendons demonstrated signs of early tendinopathy including matrix degeneration, increased apoptosis, and increased expression of inflammatory cytokines. As these characteristics reflected similar disease progression in a rat supraspinatus overuse tendinopathic model, the authors proposed that matched intact subcapularis tendon in patients with full thickness rotator cuff tears were a suitable model for early stages of human tendinopathy.

The role of inflammation in chronic tendon disorders has been particularly controversial and represents perhaps the most striking shift in the tendinopathy field over the last decade. Historically, tendinopathy was primarily considered a chronic degenerative condition that was non-inflammatory. This notion stemmed from several older histological studies reporting a lack of infiltration of immune cells in tendinopathic tissue[153–156] as well as lack of clinical diagnostic evidence, as patients typically did not present with signs of inflammation including redness of the skin.[12,157] However, more recent studies incorporating modern molecular techniques and cell-type specific markers have shown that immune cells do indeed infiltrate tendinopathic tissue in patients, particularly during earlier stages of disease. Schubert et al. (2005)[158] provided one of the first pieces of experimental evidence demonstrating the infiltration of myeloid cells and lymphocytes in Achilles tendinopathy patients. Many studies have since corroborated and extended these findings in human patellar, Achilles, and rotator cuff tendinopathies[6,152,159–168] and animal models.[121,151,169–172] Comparison of Achilles tendinopathic tissue with spontaneously ruptured Achilles tendons revealed higher levels of granulocytes in ruptured tendons, while macrophages, mast cells, and lymphocytes were more prevalent in diseased tendons, suggesting immune-related events differ between tendinopathy versus spontaneous acute injury.[158]

Likewise, inflammatory signatures appear to evolve as tendinopathy progresses. In a comparative study between healthy and tendinopathic human supraspinatus tendon, Dakin et al. (2015)[162] showed that early and intermediate-stage tendinopathic tendons displayed a distinct inflammatory signature compared to that of advanced stages, indicating separate treatments may be needed to effectively treat different stages of disease. The authors further revealed that two well-known modulators of resolution of inflammation, formyl peptide receptor 2 (*FPR2*) and chemokine-like receptor 1 (*ChemR23*), were highly expressed in early and intermediate, but not advanced tendinopathic samples, collectively suggesting that diseased tendons can initiate, but not sustain a response to resolve inflammation. Moreover, a clear link between pain and inflammation in tendinopathy was demonstrated as biopsy samples from patients who still exhibited pain post-surgical treatment displayed continual activation of inflammatory interferon signaling, while tendon biopsies from patients in which pain resolved post-treatment expressed higher levels of two mediators of inflammatory resolution, arachidonate 15-Lipoxygenase (*ALOX15*) and cluster of differentiation 206 (*CD206*). Given *CD206* is a common surface marker for M2 macrophages, which are generally polarized to an anti-inflammatory, pro-reparative phenotype (Roszer et al., 2015, Watanabe et al., 2019), these data suggest a link between failure to resolve inflammation and chronic pain. Notably, tendon fibroblasts derived from diseased versus healthy tendons *in vitro* responded to inflammatory insults in a distinct manner compared to their healthy counterparts. Adding to this framework, Stolk et al. (2017) performed macrophage co-cultures with tenocytes derived from ruptured human supraspinatus tendons and demonstrated that tenocytes change their secretory inflammatory profile in response to immune stimulation, which can in turn alter the phenotypic polarization state of macrophages. Taken together, these findings suggest that tenocytes can directly influence inflammatory response outcomes including augmenting or resolving inflammation *in vivo*. Importantly, similar phenomena were observed in human Achilles

tendinopathic tissue as well[167], collectively suggesting that chronic inflammation key for the early progression of human tendinopathy.

How inflammation fits into the larger picture of tendinopathy progression remains less clear. As overuse is the most common immediate cause of chronic tendinopathy in patients, genetic studies in mice have examined mechanisms that may link mechanical overloading in the tendon to the onset of inflammation. Mechanical forces in the tendon are critical for homeostasis and physical disruptions in normal tensile forces lead to changes in morphology due to stress/necrosis, tenocyte apoptosis, matrix degeneration, and increased expression of inflammatory markers.[173,174] Recent studies have demonstrated that alarmins are important triggers for the onset of tendinopathy. Alarmins are ubiquitous, endogenous molecules that are released from cells upon stress or necrosis and act as 'danger' signals to the innate immune system.[175,176] Several classes of canonical alarmins including cytokines, heat shock proteins (HSPs), S100 proteins, and high mobility group box 1 (*HMGB1*) are highly up-regulated in early human tendinopathic patients.[164,177–179] Genetic mouse studies have identified important roles for two alarmins in tendinopathy progression, Interleukin-33 (*Il-33*) and *Hmgb1*. *IL-33* is highly expressed in endothelial cells and tenocytes during early stages of human tendinopathy and acts as both a molecular switch from collagen I to collagen III production in resident tendon fibroblasts and an inflammatory signal to the immune system[164]. *Hmgb1* also plays a key role in the initiation and progression of tendinopathy in mice[180]. Upon mechanical overloading, *Hmgb1* is highly induced and released into the matrix. Ectopic application of *Hmgb1* in normal tendons triggered degenerative tendinopathic changes including hypercellularity, the infiltration of immune cells, and neovascularization. Most notably, chemical inhibition of HMGB1 activity with glycyrhizzin (GL) was sufficient to inhibit the onset of tendinopathy in overloaded mice by lowering levels of pro-inflammatory molecules and matrix degrading enzymes in addition to reducing chondroplasia. The release of alarmins from stressed and necrotic cells is therefore a vital event in the onset of tendinopathy. As alarmins serve multi-functional roles in different cell types, understanding the molecular interactions between damaged tenocytes, immune cells, as well as other cells including endothelial cells will be essential for elucidating targetable aspects of tendinopathy for treatment. More broadly, these studies provide a molecular basis for how mechanical disruption and cellular stress in the overloaded tendon can initiate multiple facets of tendinopathy progression including inflammation.

How inflammation and matrix degeneration during mammalian tendinopathy ultimately lead to a chronic 'failed healing' response instead of tissue restoration is poorly understood. Studying animal models capable of tendon regeneration such as zebrafish and neonatal mice can therefore provide invaluable insight into answering these questions. Given their extensive appendage and organ regenerative abilities, it stands to reason that damaged cells in regenerative organisms/models may intrinsically respond in a different manner to tissue damage than their adult mammalian counterparts, driving restorative outcomes versus disease and scarring. In fact, some studies have determined that regenerative properties are likely linked to intrinsic cellular responses in the tendon, rather than extrinsic cellular and environmental cues. For example, fetal sheep tendons retain their regenerative properties even when transplanted into an adult microenvironment[181] and fetal murine

tendon fibroblasts are better at engrafting compared to those from adults when seeded in scaffolds for tendon tissue engineering.[182] Recent research has further attributed the loss of neonatal regenerative properties and cellular plasticity in other mammalian organ systems including the heart and brain to the deposition of ECM.[183,184] Given adult tendon is an ECM-rich tissue, similar mechanisms may underlie the transition from fetal and neonatal tendon regeneration versus adult disease and scarring in mammals.

5. Learning from tendon regenerative models to enhance endogenous tendon healing strategies in humans

It is clear that missing or misregulated cues underlie the inability of adult mammalian tendons to regenerate following injury. In contrast, tendons have been shown to perfectly regenerate in larval zebrafish and neonatal mice.[137,185] Howell et al. (2017) discovered that *Scx*$^{+}$ tenocytes proliferate and regenerate the Achilles tendon following full transection in neonatal, but not adult mice. A population of αSMA-expressing cells infiltrate the injury site during early stages post-injury in both neonatal and adult mice. However, while this population persists and eventually forms scar tissue in adults, these cells largely leave regenerating neonatal tendons as *Scx*$^{+}$ cells are recruited into the injury site to restore the tendon. Surprisingly, *Scx* expression was re-activated in tenocytes in the injured tendon stubs in the adult, though these cells underwent aberrant differentiation into cartilaginous nodules, reminiscent of ectopic calcification seen in patients. In light of the differences between the cellular basis of neonatal tendon regeneration versus adult scar formation uncovered in this study, it is tempting to speculate that the extrinsic αSMA-expressing cells originated from the exterior sheath as in other non-regenerative natural tendon healing models.[138] Although this was not addressed, such a finding would hint that the key to initiating proper regeneration may be to limit the contribution of cells from the sheath during later stages of healing or properly induce *Scx* expression in these cells. A recent follow up study further determined that TGF-β signaling is required for tenocyte recruitment during neonatal regeneration,[186] demonstrating functional mechanisms of regeneration may recapitulate tendon development.

Most recently, the zebrafish has emerged as a new model for tendon regeneration. Following genetic ablation of *scxa*-expressing tendon cells, larval zebrafish completely regenerate their tendons and tendon attachments to muscle and bone. Focusing on the craniofacial tendon which attaches the sternohyoideus muscle to the ceratohyal cartilage and functions in jaw movement during feeding, the authors demonstrated that tendon regeneration occurs via BMP signaling-mediated activation and recruitment of neighboring *SRY-box transcription factor 10*-positive (*sox10*$^{+}$) and *nk2 homeobox 5*-positive (*nkx2.5*$^{+}$) progenitors, which surround the cartilage and muscle, respectively. These findings highlight the plasticity of connective tissues in the context of injury and regeneration, similar to other regenerative models including the axolotl.[187–190] Interestingly, complete ablation of tendon cells led to defects in both cartilage and muscle morphology and function, which are restored upon regeneration. As the musculoskeletal system functions as an interconnected unit, these results further imply that aberrations in muscle and skeletal tissues in patients may pathologically stem from defects in tendon. Given the remarkable capacity of adult zebrafish

to regenerate other appendages and organs,[191] it will be important to examine whether tendon regenerative properties in larvae extend through to adulthood using various modes of injury. While the likelihood of developing an injury or tendinopathy increases with age in mammals, regenerative models including the zebrafish can retain their regenerative abilities throughout their lifespan.[192] Furthermore, immune cells including macrophages and T-cells are critical for successful regeneration of other organs in the zebrafish[193–195] and differences in immune responses have been linked to the success or failure of regenerative outcomes.[196–198] Whether zebrafish are resilient to developing chronic tendon disorders remains an unexplored space, therefore providing the tendon community with a unique model to investigate interesting questions pertaining to the roles of aging and inflammation in the onset and progression of tendinopathy.

Identifying the appropriate cues that are required for successful regeneration may lead to both the design of novel therapeutic strategies or the augmentation of existing biologics treatments to correct or supply missing signaling mechanisms at the appropriate times during tendon healing using pharmacological drugs or extracellular signaling molecules. As tendon regeneration is a dynamic and complex process, it likely relies on the execution of not one, but rather a sequence of required molecular mechanisms. It is thus possible that a temporally managed approach with shifting treatments may be most successful at improving healing outcomes in patients. This body of research may further identify the required signaling mechanisms in specific cell types, adding another layer of complexity to treatment design. Altogether, uncovering the molecular and cellular basis of tendon regeneration *in vivo* will be an invaluable step to advancing the combined efforts of bioengineers, biologists, and medical professionals in the orthopedic community towards designing effective strategies for treating tendon ailments.

6. Concluding remarks and future perspectives

We are at the precipice of an exciting era for tendon regenerative medicine. Many open questions remain as to the mechanisms underlying tendon development, healing and regeneration. Here, we have detailed our current understanding in each of these areas and further discuss how increasing our knowledge of these processes is a necessary prerequisite to the enhancement of current treatment strategies and design of novel therapeutic avenues. Notably, the study of emerging tendon regenerative models may also provide insight into mechanisms that may be leveraged for stimulating better outcomes in patients. The collective effort to build a complete molecular and cellular blueprint of tendon development, healing, and regeneration will ultimately accelerate advances in tendon regenerative medicine.

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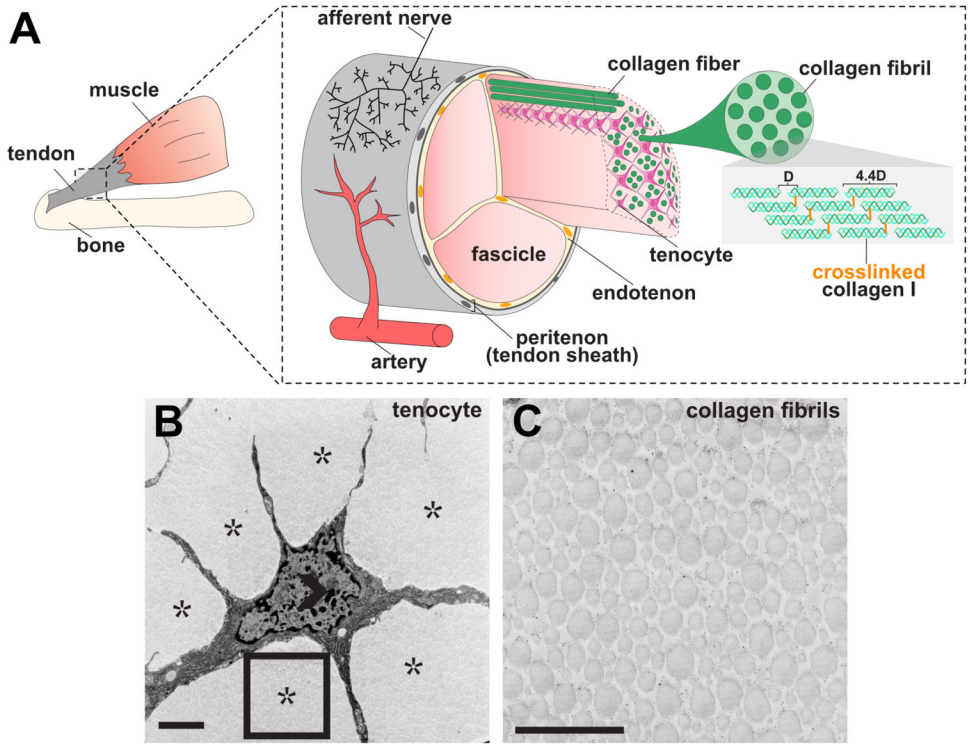

Figure 1. Tendon structure.

(A) Graphical representation of tendon morphology. The tendon midsubstance is comprised of collagen molecules which are spaced apart at a distance of 67 nm (letter D in diagram) and cross-linked to form stable fibrils[199]. Tenocytes are interspersed between collagen fibrils, which together generally form higher order bundles called fascicles. Fascicles are held together by connective tissue called the endotenon. The tendon midsubstance is encased in the peritenon, or tendon sheath, which is comprised of a basement membrane and epithelial cell layer.[200] (B–C) Transmission electron micrograph (TEM) of 6-week old mouse tenocyte (B) and collagen fibrils (C). Open arrowhead marks cell nuclei and asterisks mark collagen fibrils. Rectangle in B is shown at higher magnification in C. Scale bar, 1 um. Images in B–C were adapted from Kalson et al. (2015).[37]

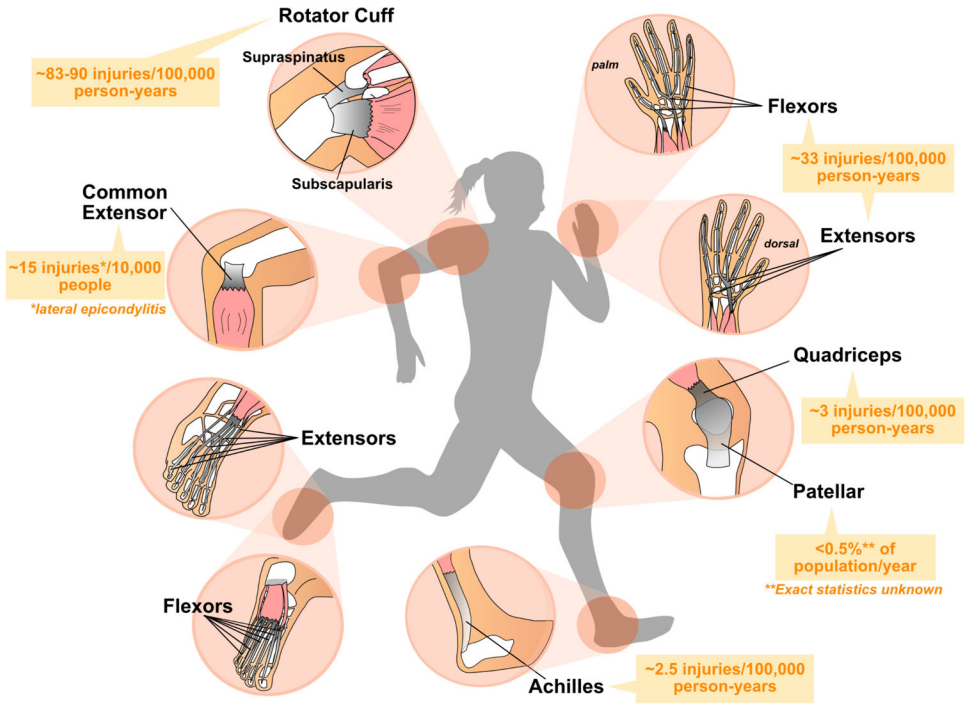

Figure 2. Major force-bearing limb tendons in the human body.

Diagram depicting the structure of major limb tendons that are frequently injured including the digital flexor and extensor tendons, common extensor, Achilles, rotator cuff, and patellar tendons. Muscles are depicted in pink, tendons in grey, and bone in white. Average incidence of injuries amongst the general population (predominantly US statistics) are shown in orange.[201–206] Note: Rates of tendon rupture or disease vary with age and population demographics of geographical location or study.

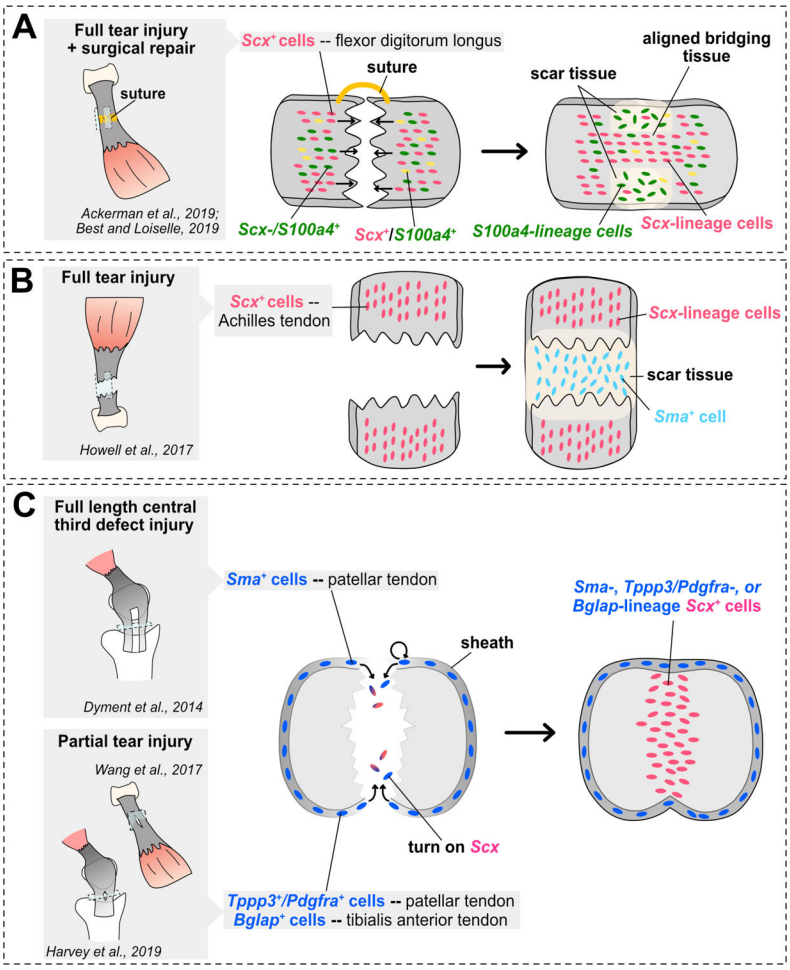

Figure 3. Summary of lineage tracing studies in mouse tendon healing models.

(A–C) Schematics illustrating main conclusions of mouse tendon healing genetic lineage tracing studies in various tendon midsubstance injury models and limb tendons. Studies assessing contribution of cells from the midsubstance are shown in A–B, while studies assessing contribution of cells from the tendon sheath are shown in C. Sagittal views are shown in the cartoons in A–B, while a transverse view is shown in C.