Front. Biol. 2018, 13(4): 263–276
https://doi.org/10.1007/s11515-018-1504-4

REVIEW

Origin of tendon stem cells *in situ*

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BACKGROUND: Adult stem cells are surveillance repositories capable of supplying a renewable source of progenitors for tissue repair and regeneration to maintain tissue homeostasis throughout life. Many tissue-resident stem cells have been identified *in situ*, which lays the foundation for studying them in their native microenvironment, i.e. the niche. Within the musculoskeletal system, muscle stem cells have been unequivocally identified in the mouse, which have led to considerable advances in understanding their role in muscle homeostasis and regeneration. On the other hand, for bone and tendon progenitor cells, mesenchymal stem cells have been used as the main *in vitro* cell model as they can differentiate into osteogenic, chondrogenic and tenogenic fates. Despite considerable efforts and employment of modern tools, the *in vivo* origins of bone and tendon stem cells remain debated. Tendon regeneration via stem cells is understudied and deserves attention as tendon damage is noted for a bleak, time-consuming recovery and the repaired tendon seldom regains the structural integrity and strength of the native, uninjured state.
OBJECTIVE: Here we review the past efforts and recent studies toward defining adult tendon stem cells and understanding tendon regeneration instead of tendon development. The focus is on adult tendon resident cells *in situ* and the uncertainty of their roles in regeneration.
METHODS: A systematic literature search using the Pubmed search engine was conducted encompassing the seminal papers in the tendon field.
CONCLUSIONS: Investigation of tendon stem cells *in situ* is in its infancy mainly due to lack of necessary tools and standardized injury model. We propose a concerted effort toward establishing a comprehensive cell atlas of the tendon, making genetic tools and choosing a reliable injury model for coordinated studies among different laboratories. Increasing our basic understanding should aid future therapeutic innovations to shorten and enhance the tendon repair/regeneration process.

Keywords Tendon, stem cells, midsubstance, sheath, injury

Introduction

Tendons are specialized, dense connective tissue that connects skeletal muscles and bones. They are mechanical, force-transmitting conduits for muscles to affect the position of their attached bones for posture and locomotion. As such, they are continually under tension and capable of withstanding tremendous forces. For example, the Achilles tendon can bear up to 7 times the weight of the human body (Shah et al., 2015). A related tissue type that connects the ends of bones together is ligament. In the clinical setting, overstretching and tearing of tendons and ligaments present from recreational overuse, trauma, or age-associated degenerative diseases. Reconstruction surgery of the anterior cruciate ligament is a commonly known procedure to restore knee function (Ateschrang et al., 2017; Petersen et al., 2017; Zampeli et al., 2017). Continuous improvement of orthopedic surgical procedures for tendons and ligaments seemingly outpaces our basic understanding for the natural process of their repair/regeneration. Given a long and productive history of studying tendon development and contrasting that to the tremendous advancement of the stem cell field at large, we are at the time to implement powerful techniques used in other developmental and stem cell systems to define and investigate tendon stem cells *in vivo*. With the constant mechanical stresses tendons bear, we imagine that they and their stem cells use unique strategies to maintain a homeostatic state as

Received December 8, 2017; accepted May 24, 2018
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well as their repair/regeneration mechanisms. Studying them should therefore gain new biological principals and concepts of tissue repairs.

Tendon development

'Regeneration recapitulates development' is a generally held concept (Hoffman and Cleveland, 1988; Imokawa and Yoshizato, 1997). Herein, we recount a few developmental events that may provide a frame of references to the process of adult tendon regeneration. For comprehensive developmental accounts, please see other reviews (Edom-Vovard and Duprez, 2004; Huang et al., 2015; Subramanian and Schilling, 2015; Gaut and Duprez, 2016). An important milestone in tendon development is the cloning and characterization of the *Scleraxis* gene (*Scx;* Cserjesi et al., 1995; Schweitzer et al., 2001; Brent et al., 2003). *Scx* encodes a bHLH transcription factor. Its expression defines the syndetome, a unique compartment located in the paraxial mesoderm that gives rise to the entire trunk musculoskeletal system. In the somite, myotome FGF signaling prefigures the *Scx* expression domain in the sclerotome (Brent et al., 2003), which is regulated by the restricted expression of *Pea3* and *Erm* (Brent and Tabin, 2004). In the limb, *Scx* is expressed the subectodermal mesenchyme in early limb bud stages in the mouse embryo. *Scx* expression prefigures future tendon and ligament cells and continues into differentiated cells in these perspective tissues. It has since been used as a definitive marker for tendon progenitors and tenocytes. While not pivotal for induction *in vivo,* TGFβ signaling, particularly through type II TGFβ receptor, is essential for maintaining *Scx* expression in tendon progenitors (Pryce et al., 2009).

To follow *Scx*-expressing cells, a transgenic mouse line that harbors the regulatory elements of *Scx* to drive GFP expression, i.e. the ScxGFP mouse, was made (Pryce et al., 2007). GFP-expression pattern of this transgene generally corresponds to endogenous *Scx* expression and this transgenic mouse has been widely used in the field. The authors did note that ScxGFP expression appears in a slightly larger domain than that of the endogenous Scx gene in early tenogenic regions. Subsequently, ScxGFP expression has been found in tissues outside of the tendon (Levay et al., 2008; Agarwal et al., 2017). These data reflect either ectopic expression of the transgene or differential expression levels (i.e. detection sensitivity) between endogenous gene versus transgene. As transcriptional regulation is tightly controlled at the endogenous locus and miRNA-mediated post-transcriptional regulation via 3′ untranslated region (3′UTR) is prevalent, a mouse knock-in (KI) allele with direct fusion between Scx and GFP (or with an intervening 2A-peptide) and intact 3′ UTR would be a better tool and deserves to be made in the future.

To investigate the role of *Scx* in tendon development, germ line mutant and conditional knockout of *Scx* have both been reported. The first report used the *Prx1Cre* driver, which directs Cre activity in the limb mesenchyme, to inactivate the *Scxflox* allele (Murchison et al., 2007). This limb-specific conditional mutant reveals the essential role of *Scx* in the maturation/differentiation of force-transmitting and intramuscular tendons, but not muscle-anchoring tendons. When ScxGFP was used to monitor mutant cells, it appears that they arrive at the normal location but arrested at a progenitor stage.

To selectively manipulate tendon/ligament progenitors by genetic means, two transgenic Scx-Cre drivers (Sugimoto et al., 2013) as well as a KI ScxCre allele have been generated (Yoshimoto et al., 2017). The transgenic Scx-Cre lines were said to be more effective in marking early progenitors than the *ScxCre* KI allele as assessed by Cre reporter expression. Because the *ScxCre* allele is also a null allele, *ScxCre/Cre* mutant mice were analyzed for phenotypes outside of the limbs (constrained by *Prx1Cre* in the first report). Not only were the authors able to confirm previously described defects in the limb, they also found defects in tendons and ligaments elsewhere, including those in the diaphragm and intervertebral disks, supporting a general requirement of *Scx* in tendon and ligament development. Using the cell marking capacity of this *ScxCre* null allele, it was again shown that marked mutant cells were present at the tendon location and reduced in cell number but did not express a full complement of tendon differentiation matrix genes. Thus, *Scx* function is not important for specifying early progenitors, but rather for terminal differentiation of tenocytes. Should this KI *ScxCre* allele become publicly available, the field at large will be able to investigate gene function in tendons and ligaments systematically.

Subsequently, two other genes, *Mohawk* (*Mkx*, encoding a homeobox containing transcription factor; Anderson et al., 2006; Ito et al., 2010) and *Early growth response 1* (*Egr1*, encoding a zinc-finger transcription factor; Léjard et al., 2011; Guerquin et al., 2013) were identified as markers for differentiating tenocytes. *Mkx* and *Egr1* mutant mice develop tendons with reduced mass and reduced expression of tendon matrix proteins. Intriguingly, *Egr1* and *Scx* expression are regulated by mechano-loading (Maeda et al., 2011; Gaut et al., 2016). Together with *Scx*, their temporal expression and genetic function define two main stages of tendon development: *Scx* for the transition from progenitor to differentiated tenocytes, while *Mkx* and *Egr1* for tenocyte to be fully mature in producing matrix proteins. Whether there are additional tendon developmental stages, especially progenitor and stem cell states, is an open question.

The transcriptional properties and target genes of these three transcription factors have been studied primarily in mesenchymal stem cells and reviewed extensively, and not a focus herein. Suffice it to say that they participate in activating genes that encode tendon extracellular matrix proteins, notably *Col1a1* (collagen 1a1; Col1), *Col1a2*

(collagen 1a2) and *Tnmd* (tenomodulin) (Shukunami et al., 2006; Léjard et al., 2007; Bagchi and Czubryt, 2012). For example, Tnmd expression is almost completely absent in *ScxCre/Cre* mice, and diminished in *Mkx* and *Egr1* mutant mice. Curiously, Col1 is still detectable in *ScxCre*/Cre mice at the correct location. It is possible each of these transcription factors can partially compensate for each other in activating terminal differentiation matrix genes. In addition, the Smad3 transcription factor acting downstream of TGFβ signaling has been shown to be indispensable for tenogenic gene expression during tenocyte differentiation (Berthet et al., 2013). Despite the advances and wealth of information accumulated to date from studying these key transcriptional regulators, the primary molecular driver(s), beyond TGFβ signaling and *Scx,* for tendon progenitor specification and expansion during embryonic development remain elusive.

As outlined above many studies have been centered around the Scxexpressing (Scx^{+}) cells, but there are other cells in and around the tendon. *Tubulin Polymerization-Promoting Protein Family 3 (Tppp3)* has been observed by *in situ* hybridization to be expressed in a thin layer surrounding individual tendons (Staverosky et al., 2009) and its expression persists in adulthood (Wang et al., 2017). Additionally, RNA-seq on FACS-isolated $ScxGFP^{+}$ cells from various staged developing mouse limbs uncovered over 1500 genes with expression changes at distinct time windows (Liu et al., 2015). In the future, it will be interesting to see how they coordinate with Scx^{+} cells during development to generate the overall tendon structure as well as any role they may have during regeneration.

Mature tendon is compartmentalized

To date, most of our understanding of tendon organization is derived from electron microscopy data (Franchi et al., 2007; Richardson et al., 2007; Starborg et al., 2013; Buschmann and Bürgisser, 2017) (Fig. 1). Despite the generally agreed upon structural organization and anatomical positions of resident cells, quite a deal of uncertainty remains over the function of these cell types as well as whether more cell types exist. During development, tendon and ligament progenitors migrate to their respective positions and condense into discrete elements/nodules during mid-gestation stages in the mouse (Perez et al., 2003; Watson et al., 2009). At late embryogenesis, each of these elements take shape. They continue to grow in size after birth until adulthood, indicative of active progenitors at perinatal stages (Liu et al., 2012; Dyment et al., 2014; Dyment et al., 2015). A fully formed tendon element contains two main sub-compartments: the midsubstance in which the matrix producing tenocytes reside, and the tendon sheath which encases the midsubstance. The two ends of a tendon are further specialized: the end that connects to the bone is the enthesis, while the end that connects to the skeletal muscle, the myotendinous junction.

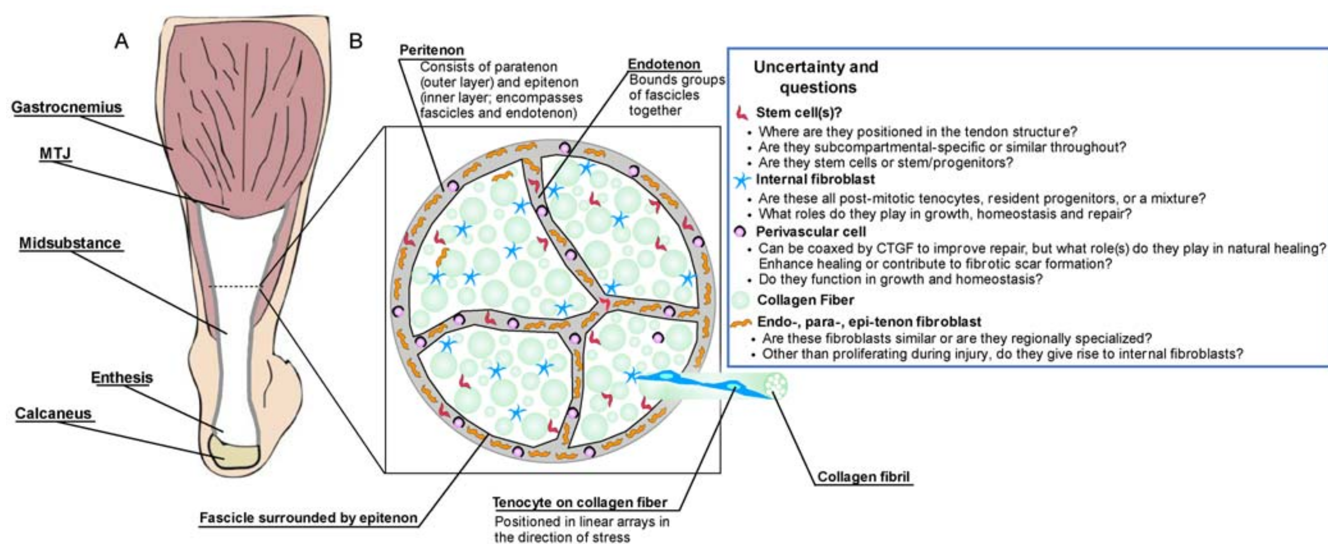

Figure 1 Brief overview of adult tendon: A) Cartoon of Achilles tendon and outstanding questions (b) in the tendon regeneration field. Achilles tendon structure shown intact with the Gastrocnemius muscle and inserted into the calcaneus bone. Transitional zones: myotendinous junction (MTJ); pink-white, and enthesis; gray-white, are depicted as gradients. B) Cross-section view of the tendon with anatomical position of resident cell types specified and questions surrounding them; individual tendon fascicles (white units) that contain collagen fibers of varying diameter sizes (green circles). Internal fibroblasts (blue stars) lie in between fibers; zoomed out view depicted. Putative locations of tendon stem cells (red) based off of LRC studies. Perivascular cells (black) surrounding blood vessels (pink circle) are found in the loose connective tissue sheaths (gray) along with fibroblasts (orange). Unsolved and outstanding questions are stated as such in the figure.

Although midsubstance is the main compartment of a given tendon, injuries can occur to any of these compartments, and each of them may have different strategies for regeneration/repair. Below is a brief overview of these compartments.

The midsubstance

Within the midsubstance lies 90-95% of the tendon cellular components—tenoblasts and tenocytes (Kirkendall and Garrett, 1997), responsible for the synthesis and deposition of matrix precursors—collagen, elastin and proteoglycans. Tenoblasts are immature tendon cells. As they mature, tenoblasts elongate and transform into tenocytes. Tenocytes have a lower metabolic demand and are noted for their decreased nuclear to cytoplasmic ratio. The collagens produced by tenocytes form dense fibers that align approximately parallel to the tendon's long axis, which is the direction of mechano-transduction and tenocyte alignment (Kirkendall and Garrett, 1997). The structural unit of a tendon is a collagen fibril, which is bundled hierarchically into a fascicle. Groups of fascicles compose a tendon (Elliott, 1965).

The peritenon/sheath, surrounding loose connective tissue layers

The tendon midsubstance is surrounded by a loose sheath of cells called the peritenon, or simply the sheath. This peritenon sheath is thought to allow tendons to glide against surrounding tissues (Elliott, 1965). Peritenon can be subdivided into an outer paratenon and an inner epitenon. While the paratenon cells are loosely organized, the epitenon is a thin layer of cells directly covering the midsubstance and continuing into its interior as the endotenon. The endotenon envelops individual fascicles, carrying small blood vessels, lymphatics and nerves (passing through the outer paratenon) (Kirkendall and Garrett, 1997). Intriguingly, perivascular cells associated with the blood vessels were recently shown to be a source of expandable progenitors via CTGF delivery during repair (Lee et al., 2015). Whether these cells naturally participate in the healing process requires lineage-based studies (see below). Depending on the tendon, the peritenon varies in proportion and organization of para- versus endo-tenons. These variations likely reflect the mechanical loads of a given tendon to support the movement of the particular bone and muscle they insert into (Kirkendall and Garrett, 1997). Peritenon/epitenon has been documented to alter Col1 synthesis in response to mechanical loading (Mendias et al., 2012), perhaps due to its close association with the force bearing midsubstance. In general, peritenon is thought to insulate and cushion the tendon from friction and acts as an interface between tendon proper and surrounding tissues as well as systemic factors.

The enthesis, where tendon meets the bone

Entheses are the interface between tendons (or ligaments) and bones. These interconnecting joints are essential for movement. The bone has a mechano-modulus on the order of 200 GPa whereas the tendon (or ligament) has a modulus on the order of 200 MPa (Lu and Thomopoulos, 2013). To alleviate/absorb such a large modulus mismatch, entheses have specialized transitional zones with a gradual change in structure, composition and mechanical behavior (Thomopoulos *et al*., 2003). They are classified in two categories based on how their collagen fibers attach to the bone (Benjamin and Ralphs, 1998). Direct insertions, also known as fibrocartilaginous entheses, are composed of four zones from tendon, uncalcified fibrocartilage, calcified fibrocartilage, and directly to the apophysis of the bone. Examples of these include patellar and Achilles tendons. On the other hand, indirect insertions, also known as fibrous entheses, have little structural/compositional transitions and attach to periosteal components on the shaft (i.e. metaphysis or diaphysis) of long bones, e.g. the deltoid tendon (Lui et al., 2010).

Myotendinous junctions, where tendon meets the muscle

Myotendinous junctions (MTJs) are the dedicated force transmission interface where skeletal muscle engages tendon. At the MTJ, tendon collagen fascicles match to muscle fiber fascicles for structural engagement. From the muscle side, finger-like projections of sarcolemma composed of bundled actin-rich microfilaments, emanate from the last Z-line toward the tendon. This is thought to increase the contacting surface area of the MTJs for effective force transmission. Two major muscle transmembrane systems have been described for engagement with the protein matrix between myofibers and tendons, the dystrophin-associated protein complex (Ibraghimov-Beskrovnaya et al., 1992) and $\alpha 7\beta 1$ integrin complex (Bao et al., 1993; Miosge et al., 1999). Both constitute a structural link between cytoplasmic actin and tendinous ECM proteins via Laminin 211. These ECM proteins tether the basement membrane to the collagen fibril matrix (Hall et al., 2007; Charvet, Ruggiero, Le Guellec, 2012). Secreted by muscle cells, Thrombospondin 4 (Thbs4; Subramanian et al., 2007; Subramanian and Schilling, 2014; Frolova et al., 2014) is a specialized ECM protein for the MTJ (Tidball and Lin, 1989). Whether there exists unique ECM protein(s), other than the generic Col1 and Tenascin-C, made by tenocytes is unknown.

Considering four domains of a given tendon, the questions naturally arisen are 1) do they have the same developmental cell origin? If so, what drives them to diversify and adopt

specialized roles? 2) If they have different cell origins, where are they and how to find them? For the purpose of this review, we pose related questions: 1) How do tendon stem cells become a reserved population of cells during embryonic development to adult transition? 2) For tendon regeneration after injury, are all four domains capable of regeneration? If so, do they use the same source of stem cells? 3) If each domain has a distinct stem cell population, how do these stem cells differ in nature? 4) Perhaps the most fundamental questions are, where are they located *in situ*, how do they behave during regeneration, and what molecular underpinnings control their regenerative potential? Efforts toward answering these questions have been probed and discussed below.

Tendon stem/progenitor cells defined by clonogenic assays *in vitro*

Clonal expansion followed by lineage-specific differentiation of cells isolated from a specific tissue is considered the basic level of success in uncovering putative stem/progenitor cells (Liu and Martin, 2003; Bajpai et al., 2012). Prospective cell isolation based on surface markers by FACS, followed by transplantation into the same or a different host has long been used to define a stem cell population, prior to using engineered genetic tools. Culturing cells from dissociated tendons has a long history. In these cultures, fibroblastic cells eventually become a dominant population. In specialized culture medium, mouse and human tendon cells dissociated from the midsubstance were able to form colonies in the midst of the fibroblastic cells (Bi et al., 2007). These colonies contained multi-potential stem/progenitor cells that can be induced to differentiate into osteogenic, adipogenic, and tenogenic cells *in vitro*. They were coined as tendon progenitor/stem cells, TPSCs. Profiling of these cells using a large panel of surface markers by FACS revealed slightly different marker atlas between mouse and human TPSCs. In particular, ~ 96% of mouse TPSCs were positive for Sca1 (Sca1^{+}), and ~ 75% of them, CD44^{+}. Importantly, these cells can form ectopic tendon-like structures after transplantation together with bone matrix into the mouse. They express Biglycan and Fibromodulin, two proteoglycans of the midsubstance. Mice mutant for these genes have smaller tendons (Bi et al., 2007). Curiously, these mutants develop ossification near the tendon, presumably due to increased BMP2 signaling that induces them toward osteogenic fate. How they respond to tendon injury was not reported. Zhang and Wang (2010) used rabbit Achilles and patellar tendon cells to extend such *in vitro* system using additional markers. They found that the cultured TPSCs were reactive to Nucleostemin, Oct-4, and SSEA-4 through continuous passage, while tenocytes were unreactive to all three markers.

Given that Biglycan and Fibromodulin are globally distributed in the tendon midsubstance (Bi et al., 2007), it is unclear whether all midsubstance cells have regenerative potential and the culturing condition selects a few to expand. Alternatively, only a sub-population has the intrinsic regenerative potential. Immunostaining was performed for Sca1, CD44, Nucleostemin, Oct4, and other markers and found in midsubstance and cells surrounding the midsubstance, i.e. the sheath. Surprisingly, a separate study using sheath-derived cells (Wang et al., 2017), were also found to display similar multi-differentiation potential *in vitro*, suggesting both midsubstance and sheath are of heterogeneous cell composition and contain stem/progenitors (see below). In some tissues (e.g. liver and intestine), excellent congruence of ‘stem/progenitor cell-identification’ between clonogenic assays and lineage tracing has been documented (Dorrell et al., 2011; Sato et al., 2009), though in the case of prostate stem cells discordance between these assays has been observed (Liu et al., 2010). Establishing an *in vitro* culture condition to coax and keep stem cells competent for desired tissue regeneration upon transplantation is a technical achievement toward the goal of cell-based therapy. Whether it teaches the normal physiologic process awaits proof from a more definitive methodology such as *in vivo* lineage tracing.

Three-dimensional (3D) studies for tendon *in vitro*

In addition to culturing tendon progenitors on 2D plastic dishes, efforts have been made to culture tendon/ligament struts tethered by silk sutures or hydroxyapatite (Calve et al., 2004; Paxton et al., 2009; Paxton et al., 2010) in 3D configuration. This system is useful to model tendon organization and mechano-load. More recently, Chien et al. (2017) demonstrated the feasibility of coaxing isolated, embryonic fibroblast-derived ScxGFP^{+} cells to form 3D constructs *in vitro* upon treatment with TGFβ2. The authors also provided proof-of-principal studies for gene manipulations using viral vehicles in this setting. Because these are ScxGFP^{+} cells of embryonic origin, the relevance to adult tendon stem cells is unclear. We acknowledge several other *in vitro* systems, beyond the scope of this review, aimed at therapeutics have been reported. For more details, please see the references provided: engineering biomaterial scaffolds (Wu et al., 2017a; Wu et al., 2017b) and, 3D hydrogels (Rubio-Azpeitia et al., 2015; Yin et al., 2018), and directing MSCs toward tendon stem/progenitors (Leong and Sun, 2016; Veronesi et al., 2017). As a method to identify and isolate definitive adult tendon stem cells becomes available, we envision ‘tendon organoid’ will be a useful *in vitro* system. The ability to organoids from stem cells *in vitro* has advanced considerably. For example, Gjorevski et al. (2016) was able to decipher critical regulators of gut stem cell lineage progression/restriction using polyethylene glycol gels as a supportive material. In the future, similar strategy can be applied to tendon stem cells to screen for regulators of their

activities, including proliferation, cell death, self-renewal, and differentiation.

Tendon contains label retaining cells expressing generic stem cell markers *in vivo*

Prior to engineered tools to define adult stem cells, retention of pulse-labeled DNA during replication in a cell after a long chase period was considered a generic way to identify stem cells *in situ* (Potten and Hendry, 1975). The assumption for label retaining cells (LRCs) being stem cells is that stem cells divide rarely and therefore retain incorporated DNA labels for a long period, or that stem cells asymmetrically segregate and retain old DNA strands permanently, i.e. the immortal strand hypothesis (Cairns, 1975). Direct testing of these hypothetical proposals by comparing LRCs with definitive stem cell markers in various tissues have, however, discredited LRCs as an inherent property of stem cells (Barker et al., 2007; Snippert et al., 2010; Li and Clevers, 2010). Nevertheless, several publications have described the presence of LRCs in the tendon (Bi et al., 2007; Kurth et al., 2011; Runesson et al., 2013). Due to a lack of credibility for LRCs being definitive stem cells, we describe these studies below to highlight the proliferation dynamics during tendon growth and regeneration.

During post-natal growth period, three consecutive pulses of BrdU (a nucleotide analog to label DNA) administration to 3-day old mouse pups labeled ~40% of patellar tendon cells, supporting rapid proliferation during post-natal tendon growth. After chased to 14 weeks (adult), ~6% of cells retained the BrdU-label. Regardless whether these 6% of LRCs may or may not be tendon stem cells, the reported profile of BrdU labeling and retention indicate a robust proliferation of tendon cells after the pulse-labeling period that dilutes the LRCs at 14 weeks. The patterns of LRCs at both time points were not noted to have a particular spatial signature. A subsequent study used the rat patellar tendon for IdU (another nucleotide analog) labeling at 1-day of birth to determine LRCs (Tan et al., 2013). It was found that LRCs become a stable population between 6 and 8 weeks. Here, more LRCs were noted in peritenon and enthesis than in the midsubstance (ranging from ~10-4%), and some associated with the blood vessel (CD146$^+$). In the midsubstance, some but not all LRCs are CD44$^+$ or Sca1$^+$, but it was unclear on the extent of their overlap. A large population of non-LRCs is also CD44 + or Sca1$^+$. Whether Sca1$^+$CD44$^+$ LRCs correspond to the *in vitro* cultured TPSCs cannot be ascertained.

After injury to the rat patellar tendon at 6 weeks, pre-labeled IdU$^+$ LRCs were found to increase by ~5 fold during the first 7 day regeneration (Tan et al., 2013). Sca1$^+$ and CD44$^+$ populations were not quantified and did not appear concordant by *in situ* pattern. At 14 days post-injury, LRCs disappeared, likely reflecting dilution of pre-labeled IdU during transient expansion for repair/regeneration. As the IdU detection limit is unknown, the replicative cycles of these LRCs were not known. Many LRCs in uninjured control samples were said to express Scx or Tnmd. Given that *Tnmd* is a target gene of Scx, these LRCs were presumably both Scx$^+$ and Tnmd$^+$. Expanding LRCs at 7 days post injury were also reported to be Scx + Tnmd +. It was unclear whether the Scx$^+$Tnmd + LRCs found at 6 weeks (prior to injury) were differentiated tenocytes incorporating IdU at their last cell cycles, and whether the expanding Scx$^+$Tnmd$^+$ LRCs found at 7 days post-injury were derived from Scx-Tnmd- LRCs, or whether some Scx$^+$Tnmd$^+$ tenocytes were proliferative. Without a second replication label and lineage relationship established between these cells, these results should be weighed carefully. Four indicators for generic stem cell markers, Oct4, Nanog, Sox2, and Nucleostemin, was further employed to correlate the stem-ness of LRCs in uninjured and injured-expanding LRCs. CD146, a marker for pericyte, was also examined. None of them were expressed by LRCs in uninjured controls, but all were in expanding LRCs.

When tendon midsubstance was dissociated for culture, expanding cells diluted out IdU, lost CD146, but maintained the other four markers – Sca1 and CD44 were not assayed. It therefore appears that these four generic stem cell markers stained transit amplifying cells instead of quiescent stem cells – that is, they indicate proliferation competence rather than stem-ness per se. Whether a given transit amplifying cell expresses only 1 or all 4 of these markers is unknown. Critically, the 'expanding' LRCs at day 7 after injury might not be of midsubstance origin, as there were other pre-labeled LRCs outside the midsubstance that could potentially migrate into the injured region.

In another study to survey the injury/regeneration process in the Achilles tendon, Runesson et al. (2015) employed immunostaining for CD45 (for leukocytes), Dynamin 2 (a proxy for migrating cells), as well as for Nucleostemin and Oct4. Infiltration of CD45$^+$ cells 7 days after injury was found and expected. In contrast to Tan et al. (2013), they noted Nucleostemin$^+$ and Oct4$^+$ cells in uninjured tendon midsubstance. Nucleostemin$^+$ cells increased from ~10% in uninjured tendon to ~60% in injured area in the first 2 weeks post injury, and returned to ~10% by 8 weeks, depicting a dynamic pattern of this population of cells. When regional differences of Nucleostemin$^+$ cell fractions were quantified, the surrounding connective tissue (CT) region had the smallest, while the 'alcian blue rich region' had the largest percentages. Few Oct-4$^+$ and Dynamin2$^+$ (presumed to label migratory) cells were found in the peritenon and MTJ but not in the midsubstance in uninjured controls, but they appeared in injured/regenerated midsubstance. It was inferred that the Oct4$^+$ and/or Dynamin2$^+$ cells in the regenerating midsubstance migrated from peritenon or MTJ. Without support from lineage tracing data, it is hard to exclude the possibility that the regenerative cells within the midsubstance turn on

these markers as would be suggested by data from Tan et al. (2013). Although the above *in vivo* studies reviewed here have limitations to pinpoint the origin(s) of tendon stem cells *in situ*, they do provide invaluable spatial and temporal information of markers expression in uninjured and injured tendon compartments.

Multiple experimental paradigms for tendon injuries

To study tendon regeneration *in vivo*, many experimental paradigms have been devised to injure tendons (outlined in Table 1). In a systematic evaluation across many tendons, the patellar tendon has shown to be the least variable by numerous metrics (Beason et al., 2012). From a basic research standpoint, a reproducible experimental paradigm is of utmost importance. With the variety of injury paradigms and lack of agreement on model tendons to study, collective progress is difficult as reliable comparisons across laboratories cannot be made. We propose the field adopt the biopsy punch injury for the Patellar tendon as the initial standardized paradigm for it faithfully yields consistent, reproducible repair (Lin et al., 2006). More importantly, this paradigm provides a focal and precise context to study the repair/regeneration process in an otherwise intact tendon. By contrast, some injury models elicit fibrotic scar formation instead of tendon regeneration. For example, longitudinal mid-1/3 patellar tendon excision appeared to generate a ‘collagenous bridge’ covering the surface over the wounded

Table 1 Tendon injury paradigms

Injury paradigm	Clinical presentation modeling	Advantages	Disadvantages
Local collagenase injection (Dahlgren et al., 2002; Urdzikova et al., 2014)	Tendinopathy; Matrix/Collagen remodeling	Facilitates understanding matrix remodeling; can be locally injected making it minimally invasive	Difficult to control diffusion into tissues surrounding tendon, which may complicate the autonomy of the repairing tendon; hypervariable outcomes; not physiologically relevant
Achilles overloading by muscle ablation (Gumucio et al., 2014) or treadmill running (Runesson et al., 2013)	To study tension modulation and hypertrophic growth; mimics trauma victims (i.e. muscle injuries)	Allows for deciphering consequence of increased/decreased tension	Pleiotrophic effects from multi-tissue damage/repair process complicate understanding local response of tendon
Burn injury (Peterson et al., 2015)	Mimics tendinopathy; trauma victims	Non-invasive approach, not requiring surgery	Highly variable; damages many tissues complicating the autonomy of the repairing tendon
Mid-2/3 tendon incision (horizontal) or excision (Beason et al., 2012)	Acute tendon tear; fibrotic scarring	Mimics acute tears presented clinically; allows studying regeneration when tension is maintained; enables understanding of fibrotic scar formation	Regeneration success varies depending on which tendon performed; can affect mobility; requires surgery and anesthesia to perform.
Central 1/3 patellar tendon excision (longitudinal) (Dyment et al., 2014)	Acute tendon tear; Collagenous bridge formation	Maintains tension by lateral struts; often used for ACL reconstruction	Form a ‘collagenous bridge’ covering the surface over the wounded region without generating tendon fibrils—‘failure to regenerate’
Complete Achilles tendon tear or transection (Runesson et al., 2015) (Howell et al., 2017)	Mimics common clinical presentation	Facilitates understanding fibrotic tissue accumulation and scar formation	This injury affects mobility, sutures are often used to mechanically stabilize tendon stumps, functioning as a substrate/bridge for regeneration. Suturing technique can affect outcome (Mazzocca et al., 2005; Kim et al., 2006); results in fibrotic scar formation not regeneration.
Biopsy punch (Lin et al., 2006)	Mimics minor tear in otherwise intact tendon	Reproducible injury model; offers a window to study the repair process at a cellular l evel and have little impact on mobility	Requires surgery and anesthesia to perform
Needle punch (Schwartz et al., 2017)	Mimics tendon-bone injuries	For enthesis regeneration; different gauge needles can be used	Post-op requires microCT to confirm the location of the injury (outside or inside of the enthesis); requires large cohort given the nature of bony defects

region without generating tendon fibrils (Dyment et al., 2014; and see below). Complete horizontal transection of the Achilles tendon caused fibrotic tissue formation between the cut stumps without regeneration (Howell *et al*., 2017). Fibrotic tissue accumulation and scar formation after tendon injury are not unexpected. In fact, fibrosis likely presents a challenge toward uncovering stem cells *in situ*. Using punch injury, where fibrosis is limited, may pose an opportunity to identify them. This paradigm should serve as a platform to rigorously tease out the dynamics of repair/regeneration and tendon stem cell biology. Once determined, this knowledge should be applied to the other 'failure-to-regenerate' injury paradigms and may help explain why stem cells are unable to regenerate the tendon back to its original state.

Both robust and failed tendon regeneration models should greatly aid the findings for stimulatory and prohibitory factors, respectively, and help translate the findings toward alleviating unfavorable conditions for tendon regeneration clinically. In addition, exploiting the genetic basis for scar-free tendon regeneration in MRL/MpJ (Lalley et al., 2015) and LG/J (Arble et al., 2016) mice may help uncovering molecular players that circumvent regenerative hurdles encountered by tendons.

Lineage tracing studies for tendon regeneration

As mentioned above, lineage tracing is a way to track stem/progenitor cell behavior. It has been a gold standard in embryological studies to map the origins of different cell types (reviewed in Kretzchmar and Watt, 2012). Multiple lineage tracing methods have been invented and the best to date is the Cre-loxP system for indelible reporter expression (Soriano, 1999). For tendon development, *ScxCre* driver has been used to label the tendon and ligament lineage described above. In addition to tendon and ligament, a few cells in cartilage elements are also found. Using *Gdf5Cre* together with the Confetti reporters to mark descendant cells with multiple fluorescent proteins, the developmental pattern of fibrocartilage cells in the enthesis has also been documented (Dyment et al., 2015). In the adult, *Gdf5*-lineage occupies cartilage, fibrocartilage at the tendon enthesis, and many cells in the tendon midsubstance, and all cells in the ligament (Dyment et al., 2014). Because constitutive Cre-drivers such as *Gdf5Cre* records all cells at any given time expressing *Gdf5* prior to the assayed time, the adult lineage data can either be that there is a common Gdf5^{+} cell population for all described cell types, or that cells of different origins express *Gdf5* at different times. By extension, even though *ScxCre* and *Gdf5Cre* mark tendon, ligament, and cartilage, one can interpret that cells in the three tissues express these genes at one point of their life, instead of their common cell origin. Intriguingly, the *Bgn*/*Fmod* mutant mice develop ectopic bones near the tendon, and *ScxCre* labeled cells in mice harboring a specific form of ACVR1 directly contribute to ectopic ossification, indicating that tendon cells are capable of transfating to cartilage/bone cells under specialized conditions *in vivo* (Agarwal et al., 2017). Conditions that allow transfate may be captured in vitro where TPSCs can be differentiated to tendon, fat, cartilage, and bone cells (Bi et al., 2007).

An advanced Cre system, the CreERT or the second-generation CreERT2 system utilizes a tamoxifen inducible Cre for temporal control, enabling cell marking at a specified time (Feil et al., 1996; Feil et al., 1997). The advantage of using the inducible CreER over the constitutive Cre in lineage tracing has been demonstrated many times in the literature. In the developing joint, the original study using *Gdf5Cre* (Rountree et al., 2004) assigned all joint cells of a single Gdf5-expressing progenitor source, while the later study using *Gdf5CreER* (Schwartz et al., 2016) revealed that different joint cell lineages are derived from newly recruited *Gdf5*-expressing cells at different times. For the enthesis, a *Gli1CreERT2* driver was activated at a selected time point to mark and trace Hedgehog-responding fibrochondrocytes that mature from an unmineralized to mineralized state during tendon growth (Dyment et al., 2015), confirming prior work that Hedgehog signaling (*Gli1* is a direct target of Hedgehog signaling) is critical for enthesis development. When *Gli1*-expressing cells in the immature enthesis were marked at post-natal day 5 (P5), and the enthesis was injured at P7, proliferative pre-marked cells were found at P14 and presumed to regenerate enthesis (Schwartz et al., 2017). Cells pre-marked at P5 did not proliferate when injury was performed at P42, indicating that they progressed to a differentiated state and lost their regenerative potential by P42. However, when tamoxifen was used to mark Gli1-expressing cells after P42, small clusters of marked cells were found near the injury site, suggesting that they proliferated little (not directly assessed). These studies support that Hedgehog signals to early enthesis progenitors for their proliferation and differentiation, and that this signaling is diminished in adult enthesis, which may underlie their poor regenerative property.

This inducible lineage tracing strategy was also applied to document the tendon regeneration process using a transgenic αSMACreERT2 driver. This driver was originally developed to mark mesenchymal progenitors of adult bone (Grcevic et al., 2012), but was found to also mark a subset of cells in paratenon, midsubstance, and myotendinous junction (Dyment et al., 2014). Following 1/3 longitudinal excision of patellar tendon (described above), αSMA-lineage marked cells first accumulate over the injury site considerably, followed by expression of the ScxGFP transgene. Marked cells residing in the surface made collagen fibrils not characteristic of tendon cells, whereas those entering the injury site did not show longitudinally aligned collagen

second harmonic generation (SHG) signal typical of tendon midsubstance. The structure formed by these αSMACreERT2 marked cells were referred to as a 'collagenous bridge'. The presence of αSMA-descending ScxGFP$^+$ cells suggest that mesenchymal cells can be coaxed to express ScxGFP in this injury paradigm, but the lack of detectable SHG signal from these cells would suggest that they are not tendon cells, at least not fully differentiated. Furthermore, as this CreERT2 driver marks multiple cell types in and around the tendons, the origin of the 'collagenous bridge' cells is difficult to ascertain.

More recently, two other lineage tracing strategies were documented (Zhang et al., 2017; Wang et al., 2017). One used a constitutive Cre, Osteocalcin (*Bglap*)-Cre, which labeled sheath cells in the adult tendon (Wang et al., 2017). While they confirmed extrinsic cells in the tendon can proliferate in response to injury, suggested by data from treadmill-induced mechanical loading of ScxGFP tendons (Mendias et al., 2012), it is important to note that lineage marking by constitutive Cre can occur at any time up until assay. They demonstrated *Bglap* is upregulated in response to injury, thus, the stem cell, progenitor, differentiated cell, or a mixture of these could be labeled. While intriguing, future exploration of this gene using an inducible lineage tracing will offer greater precision and reach a definitive answer. *Keratocan* (Kera) has been reported to also be expressed in tenocytes (Rees et al., 2009; Zhang et al., 2017). Using the tetracycline (tet)-inducible system, Zhang et al. (2017) demonstrated a *Keratocan-IRES-rtTA (KerartTA*) in combination TH2B-EGFP reporter line enables spatiotemporal monitoring of tenocytes during development and adulthood. The role these cells play in regeneration, and whether they overlap with Scx$^+$ cells remain to be explored. Caution must be taken when using H2B-GFP as a lineage tracer given the serial dilution of GFP following each cell division.

A *ScxCreERT2* driver has also been generated (Agarwal et al., 2017). Exactly how this allele was made is unclear in the literature. Nevertheless, it was used to monitor *Scx*-expressing cell lineage after complete horizontal transection to the Achilles tendon (Howell et al., 2017). As mentioned above, adult Achilles tendon failed to regenerate in this injury paradigm. A few unexpected and illuminating findings were reported in this study. First, neonates that underwent the same complete transection procedure did regenerate. In the regenerated region, proliferated and Scx-lineage marked cells were found, likely reflecting the proliferative potential of peri-natal progenitors needed for tendon growth. There appeared many ScxGFP$^+$ cells that are not lineage marked by *ScxCreERT2* (by a red color lineage tracer). Whether they are derived from non-Scx-lineage or result from inefficient lineage marking is unclear. Second, increased ScxGFP signal was detected at the severed stump in adult Achilles. Intuitively, these areas should experience the least mechanical force after severing, suggesting that either *Scx* expression can be induced by low mechano-force, or by a biochemical factor (s) at the injured site. Third, in the fibrotic area, αSMA$^+$ cells were widespread and αSMA + blood vessels contain ScxGFP$^+$ cells.

Comparing this result to the 1/3 patella excision paradigm by Dyment et al. (2014) in which the αSMA-CreERT2 marked lineage turns on ScxGFP after covering the surface of the injury site, it seems that a αSMA lineage is capable of activating ScxGFP expression in the 'failed regeneration' condition, and that ScxGFP expression in itself does not signify tendon regeneration. Therefore, αSMA population(s) more likely represent cell source(s) for fibrotic scar formation. Lastly, in the tendon area next to the stump, ectopic cartilages formed with ScxGFP-expressing cells as well as *ScxCreERT2* lineage marked cells. Thus, in this failed regeneration condition, Scx-lineage can transdifferentiate into chondrocytes and maintain ScxGFP expression. Given that there are no regenerated tendons in this scenario, whether Scx-lineage can regenerate tendon cells in a permissive condition is not known. If Scx-lineage can regenerate tendon cells, whether all or selected few Scx$^+$ cells can do so, will be difficult to determine using this CreERT2 driver. As to why adult tendon in this paradigm does not regenerate, we suspect that low mechanical load and small size of the neonatal Achilles tendon allows the actively proliferating progenitor cells to migrate and join together, whereas without suturing the transected adult Achilles, the tendon stumps were pulled apart too far and without any linkage support, failed to regenerate.

Inducible lineage tracing coupled with molecular markers offers unparalleled power to define spatiotemporal relationships between stem cells and their descending cell types. In the case of using *Gli1CreERT2*, the constraint lies in Hedgehog-responsive cells. Non-Hedgehog responsive tendon enthesis stem cells, if exist, could not be examined. The *ScxCreERT2* driver is invaluable, but it has only been used in limited ways. The combination of *ScxCreERT2* and *Scxflox* alleles should allow future investigation of *Scx* function in maintaining tendon midsubstance and regenerating tendons after injury in the adult. However, one or two CreERT2 alleles are unlikely sufficient to understand tendon regeneration comprehensively, especially considering that the tendon has multiple compartments, not to mention numerous cells types already revealed by staining patterns of various antibodies.

Therapeutic interventions to improve tendon healing

Due to clinical relevance, many methods have been sought to improve tendon healing by supplying natural or synthetic biomaterials to serve as a structural scaffold (Sundar et al., 2009; Hexter et al., 2017). In addition, delivery of growth factors, such as IGF-1 (Kurtz et al., 1999; Dahlgren et al.,

2002), VEGF (Kaux et al., 2014), bFGF (Fukui et al., 1998; Chan et al., 2000), TGF-β (Chang et al., 2000), PDGF (Letson and Dahners, 1994; Hildebrand et al., 1998), GDF5/6/7 (Wolfman et al., 1997; Rickert et al., 2001), and platelet-rich plasma (PRP; Kajikawa et al., 2008; Lyras et al., 2009; Baksh et al., 2013), have been applied with variable degrees of success (Molloy et al., 2003). Whether and how the endogenous tendon stem cells respond to the above factors are not known due to the elusive identity of these cells. It is possible that these factors act in other supportive cells that are beneficial to tendon healing. Even so, they are still clinically relevant and inform us on new dimensions to investigate their roles in supporting tendon stem cell activity. How they may influence the proliferation and differentiation properties of the *in vitro* defined transplantation competent TSPCs (Bi et al., 2007) should be examined for a better understanding. Along this line, a different population of TSPCs identified by another surface marker CD146 has been found to be responsive to connective tissue growth factor (CTGF) toward tenogenic differentiation *in vitro* (Lee et al., 2015). A direct comparison between these new CD146$^+$ TSPCs (Lee *et al.*, 2015) and the Sca1$^+$/CD44$^+$ mouse TPSCs (Bi et al., 2007) is also lacking. Interestingly, ~ 91% of the *in vitro* defined human TSPCs are CD164$^+$ (Bi et al., 2007). Nevertheless, CTGF delivery *in vivo* enhanced healing, suggestive of a stem-like role of CD146$^+$ cells. CD146 + cells appeared rare in the tendon tissue *in situ* and were only found around CD31 + endothelial cells, which is consistent with CD146 being an ascribed pericyte marker (Shih IM, 1999; Covas et al., 2008). Could pericytes or a sub-population of them be the elusive tendon stem cells *in situ*, or they are coaxed to transfate to tendon progenitors by CTGF treatment? Unfortunately, CTGF knockout mice develop chondrodysplasia and are lethal, so the endogenous role of CTGF in adult tendon repair/regeneration is yet to be determined *in vivo* (Ivkovic et al., 2003). For therapeutic designs that target tendon stem cells to enhance their regenerative capacity, definitive identification of these cells, followed by molecular characterization, is very much needed.

Concluding remarks

Because molecular pursuit of tendon stem cells is at the beginning stage, the field should take this as an opportunity to plan future research strategically. As a starting point, one must tackle the current unmet needs in tendon stem cell biology: 1) attain a global assessment of gene expression patterns in adult tendon compartments and assign spatially distinct cell types; 2) generate tendon compartment-specific CreERT2 drivers to investigate the role of each compartment in tendon growth, injury and disease; 3) selective gene inactivation studies to determine crucial players in various aspects of tendon biology. To understand the biological complexity of cell types residing in the adult tendon, a gene expression atlas is of crucial importance. Gene expression of ScxGFP$^+$ cells has been profiled at different stages in the embryonic limb (Liu et al., 2015). We propose that a comprehensive profiling of all tendon cell populations, rather than one cell type, is needed. In addition to bulk RNA-seq, single cell RNA-seq has been shown to be powerful for uncovering novel markers and assembling single cell lineage trees (Zheng et al., 2017). Applying this technology to tendon and combined with marker analysis, a topographic expression map of the tendon can be constructed. Once a molecular atlas of tendon is drawn, CreERT2 alleles driven by genes in different compartments or specialized sub-compartment should then be made as essential genetic tools. For the making of new CreERT2 alleles, a knock-in design that preserves the 5′ and 3′ UTR of the gene to recapitulate post-transcriptional regulation is desirable. These alleles then can be used to determine cell location (marking) and the fate(s) of their descendant cells (tracing), i.e. whether any of them mark tendon stem cells for maintaining homeostasis throughout life and/or for regeneration after injury. Alternatively, screening pre-existing CreERT2 alleles deposited to JAX may be an immediate solution to obtain lineage marking tools for the tendon. Finally, the function of a given gene of interest should be tested by conditional inactivation in conjunction with lineage marking by choosing from the bank of tendon-specific CreERT2 alleles. Combination of cell marking and gene inactivation should allow for determining the critical action of the mutated gene.

We have proposed above to standardize injury paradigms, which should incentivize the re-examination of tendon injury-regeneration process with extensive time points by RNA-seq. A repository of these data sets will provide the molecular foundation to stage regenerative progression, instead of only assessing a few selected tendon-specific genes by qRT-PCR. Isolating selective lineage-marked cells after injury/regeneration for comprehensive expression profiling and comparing their profile to the tendon expression atlas in the future should substantially increase the accuracy to determine regenerated cell type(s). By developing new tools and applying modern genomic technologies, tremendous strides will be made to tackle the fascinating biology of the tendon. Of utmost importance, definitive proof of stem cell existence *in situ* by way of lineage tracing and transcriptome analysis will revolutionize the tendon field. Should the elusive tendon stem cell(s) be found, stem cell-based therapies may change the current clinical approaches for treating developmental, functional and disease-related issues.

Compliance with ethics guidelines

Authors declare no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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