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Tendon Stem Progenitor Cells: Understanding the biology to inform therapeutic strategies for tendon repair

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Abstract

Tendon and ligament injuries are a leading cause of healthcare visits with significant impact in terms of economic cost and reduced quality of life. To date, reparative strategies remain largely restricted to conservative treatment or surgical repair. However, these therapies fail to restore native tendon structure and function; thus, the tissue may re-rupture or degenerate with time. To improve tendon healing, one promising strategy may be harnessing the innate potential of resident tendon stem/progenitor cells (TSPCs) to guide tenogenic regeneration. In this review, we outline recent advances in the identification and characterization of putative TSPC populations, and discuss biochemical, biomechanical, and biomaterial methods employed for their culture and differentiation. Finally, we identify limitations in our current understanding of TSPC biology, key challenges for their use, and potential therapeutic strategies to inform cell-based tendon repair.

Keywords

Tendon; Stem Cell; Progenitor; TSPC; Tendon Repair

Introduction

Clinical interest in tendons and ligaments is primarily focused on injury and degeneration, since there are very few associated congenital disorders. In the United States, musculoskeletal diseases impact ~50% of people over 18 years and ~75% of people aged 65 years. A recent survey found that of 126.6 million musculoskeletal conditions reported, >3 out of 5 incidents were due to injuries. Importantly, the second highest cause of musculoskeletal injuries was related to tendons and ligaments.¹ To date, surgical interventions that physically reconnect damaged tissues remain the leading technique to repair a tendon or ligament rupture. However, despite some restoration of tissue integrity, these connective tissues lack the intrinsic ability to regenerate to their pre-injury state. Instead, healing occurs via fibrosis and disorganized scar formation, which compromises mechanical and structural functionality; the risk of tendon or ligament re-rupture despite surgery therefore remains high (particularly for intra-articular tissues such as the

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supraspinatus tendon of the rotator cuff or the anterior cruciate ligament of the knee). Repeated injuries to tendons/ligaments can have a significant impact on the patient's mobility and quality of life while adding to an ever-increasing healthcare burden.

Given the limitations of surgical repair, one therapeutic modality with exciting potential is the use of stem cells (including tissue specific stem cells), to repair damage. In several self-renewing and regenerative tissues, such as muscle, intestine, and hair follicles, definitive markers have been established to enable identification and characterization of resident stem cell populations and their *in vivo* activity.^{2–10} Similarly, major advances have been made for bone marrow derived mesenchymal stem cells (BMSCs), adipose stem cells, and periosteal cells.^{11–19} In contrast, while stem/progenitor cells have been identified for tendons (frequently termed TSPCs), there remains a paucity of information regarding distinguishing markers, making it challenging to study these cells *in vivo*. Given the molecular and structural similarities between tendons and ligaments,^{20–22} we will focus this review on tendon and TSPCs, since the majority of the literature is concentrated on tendon biology. We expect some of these findings will likely be applicable to ligaments as well.

The ability to identify, isolate, culture, expand, and functionalize TSPCs efficiently is key to harnessing their potential for therapeutic strategies to improve tendon repair. In this review we describe recent efforts to identify and characterize TSPC populations and discuss advances made toward the culture/expansion of TSPCs and induction of tenogenic cell fate (using molecular and mechanical factors). We also evaluate current state of the art techniques to generate functional tendon constructs using TSPCs. Finally, we outline current barriers to our understanding of TSPCs and discuss strategies for future research efforts and clinical translation.

Tendon structure and cell markers

Tendons are contractile tissues that connect muscle to bone. Their primary function is to transmit tensile forces, thereby enabling movement of the skeleton. Although the exact composition of specific tendons differs according to anatomic location (likely due to distinct mechanical loading environments), in general, all tendons share a similar structure composed primarily of aligned type I collagen fibers (65–80%). In addition to collagen, minor extracellular matrix (ECM) components include elastin and small leucine rich proteoglycans such as decorin, biglycan, and fibromodulin.²² Collagen ECM in tendons is hierarchically arranged; collagen fibrils are assembled into fibers, which are then organized into fascicles. Each of these fascicles is encased by a thin connective tissue layer, called the endotenon, which enables movement of individual fascicles and provides a source of nutrient supply via blood vessels.^{23–25} The entire tendon is then enveloped by the epitenon, a fine sheath of connective tissue (similar in composition to endotenon). In some tendons, the epitenon is surrounded by a paratenon, a loose connective tissue which facilitates the movement of tendons and sustains the vascular network that penetrates the endotenon and epitenon (Figure 1).²⁵ While the complex architecture described is a distinct feature of tendons in humans and larger animals, in mice, tendons are composed of a single fascicle surrounded by epitenon only, with no observable endotenon.

The cells that give rise to tendons were once considered fairly homogeneous and distinguished largely by fibroblastic morphology and location. In recent years, sophisticated lineage tracing efforts and single cell RNA technology now suggest the presence of distinct populations within the resident cells.^{26; 27} These exciting efforts remain in early stages and functional differences and differentiation potential have not been established or tested. For differentiated tendon cells, one well-established marker is the transcription factor *Scleraxis* (*Scx*), which is first expressed in embryonic progenitors and remains expressed during postnatal stages.²¹ Use of the reporter *ScxGFP* has greatly facilitated the detection and analysis of tendon cells.^{21; 28} Other markers for tendons include the transcription factors *Mohawk* (*Mkx*),^{29; 30} *Early growth response protein 1 and 2* (*Egr1*, *Egr2*),³¹ and the glycoprotein *Tenomodulin* (*Tnmd*).³² In addition, key ECM components such as type I collagen (*Col1a1*) and decorin are frequently used as markers for differentiated tendon cells. For a more detailed review on the functional requirements for these transcription factors and key signaling pathways during tendon development, please see Huang et al.³³

Identification and Characterization of TSPC populations

TSPCs derived from tendon fascicle.

The first study to identify TSPCs suggested that their niche reside within the tendon fascicle and is maintained by biglycan and fibromodulin.³⁴ In addition to cell surface markers indicative of 'stemness', TSPCs expressed tendon-specific markers such as *Scx* (as well as higher expression of tendon-related markers *Col1a1* and *Tnc*), suggesting that TSPCs represent a subpopulation of resident tendon cells.³⁴ In this study, TSPCs were derived from both human hamstring and mouse patellar tendons under normoxic conditions and displayed hallmark traits of stem/progenitor cells *in vitro* such as self-renewal, clonogenicity, and multi-lineage potential. While TSPCs expressed many of the same cell surface markers as BMSCs, mouse TSPCs also expressed CD90.2, which was not expressed by BMSCs. Human TSPCs on the other hand, expressed BMSC markers including Stro-1, CD146, CD90 and CD44, but were negative for the BMSC cell surface receptor, CD18.³⁴

TSPCs derived from epitenon.

Although this groundbreaking study indicated that TSPCs may reside within the tendon, other studies that challenge tendons via injury or overloading suggest that the epitenon may be another source of TSPCs.^{26; 35; 36} During mouse development, epitenon cells expressing the marker *Tppp3* are not observed surrounding tendons until after distinct tendons are formed.³⁷ Postnatally, the epitenon can be identified based on detection of laminin, α -smooth-muscle-actin (α SMA), and platelet derived growth factor receptor α (PDGFR α), markers that are not normally expressed by differentiated tendon cells under homeostasis.^{36; 38} Analysis of *ScxGFP* further confirmed that tendon and epitenon cells represent separate populations.³⁵ After tendon injury, lineage tracing showed that α SMA-lineage and *Prg4*-lineage progenitor cells are recruited to mediate tendon healing, although the exact origin of these cells remains unclear since several tissues are labeled by these markers.^{26; 39} Interestingly, *Scx*-lineage (*Scx^{lin}*) tendon cells are not recruited to repair adult tendon after injury, but undergo abnormal differentiation toward cartilage.³⁵ In contrast, neonatal *Scx^{lin}* tendon cells undergo recruitment into the injury site and tendon-specific differentiation.

These data indicate that progenitor cells from the tendon proper may be biased toward non-tenogenic differentiation in the adult wound environment.³⁵ Consistent with these studies, a tendon overloading model also showed minimal activation of tenocytes, but dramatic proliferation and matrix deposition by epitenon cells.⁴⁰ Treadmill exercise also activated *ScxGFP* expression in epitenon cells, suggesting an ability to differentiate toward a tendon-specific fate.⁴¹

Comparative *in vitro* studies of tendon cells derived from tendon fascicle and epitenon/paratenon show that while both populations contain TSPCs, those from the epitenon have less colony forming ability relative to tendon fascicle-derived TSPCs. More tendon-derived cells expressed the stem cell marker, Sca-1 as well as fibroblast markers CD90.2 and CD44 compared to epitenon cells (Figure 1). Tendon-derived TSPCs also exhibited greater levels of tenogenic marker expression for *Scx* and *Tnmd* compared to epitenon progenitors. Interestingly, TSPCs from both regions of the tendon formed *in vitro* tendon-like structures and were multipotent, suggesting that there may be two sources of distinct progenitor/stem cells for tendon.^{42; 43} This is further supported by work identifying Cd105⁺ and Cd105⁻ progenitors within tendon, with differing chondrogenic potentials after injury.⁴⁴ However, the source of these Cd105^{+/+} subpopulations was not investigated.

TSPCs derived from vasculature.

Recent studies suggest that pericytes or perivascular cells derived from the surrounding vasculature may be the source of epitenon TSPC progenitors. Perivascular cells have long been established in many tissues as multipotent, with the capacity to differentiate into cartilage, fat and muscle;^{45; 46} some pericytes also express α SMA.⁴⁷ Although tendon fascicles are avascular, blood vessels that supply nutrients can be found within the non-tenogenic compartments (epitenon, endotenon, or paratenon). TSPCs derived from paratenon show higher expression of vascular and pericyte markers such as *Endomucin* (*Emcn*), *Musashi1* (*Msi1*), and *Cd133* relative to tendon-derived TSPCs.⁴² Recently, a novel marker *Nestin* (*Nes*), was identified in TSPCs from the epitenon/paratenon, particularly in perivascular niches. *Nes*⁺ TSPCs co-expressed *Scx* and demonstrated greater clonogenic ability and tenogenic potential compared to *Nes*⁻ TSPCs.⁴⁸ Immunostaining of human tendon and perivascular TSPCs isolated from human tendon microvessels confirmed the presence of *Msi1*, *Scx*, *Nes*, α SMA, and *Cd133* in perivascular TSPCs.⁴⁹ Co-expression of perivascular stem cell markers with *Scx* is an interesting finding since *Scx*⁺ cells are typically not observed in the epitenon or paratenon regions. Whether this suggests low levels of *Scx* in these cells or that the cells may be 'primed' to express *Scx* during culture is still unclear.

Collectively, these results suggest there may be two or more distinctive populations of TSPCs, however, most studies rely on *in vitro* characterization of these cells. Definitive lineage tracing experiments have not been carried out due to the absence of specific Cre lines and well-defined, specific markers for TSPCs. Despite these limitations, the use of existing inducible Cre lines such as *ScxCreERT2*, which targets intrinsic tenocytes, may be useful to separate TSPC populations associated with the tendon proper vs the epitenon. We show that both *Scx*^{lin} and non-*Scx*^{lin} TSPCs can be isolated and expanded from mouse

Achilles tendons labeled by *ScxCreERT2/RosaT* (Figure 2). However, whether these populations have distinctive differentiation potentials remains an open question. The recent identification of *Nes* as a potential TSPC marker may also overcome some of these challenges and allow fate-mapping of this intriguing progenitor population. Since the epitenon, endotenon, and tendon fascicle are very closely associated spatially (in mice the epitenon is only 1–2 cell layer thick and impossible to manually dissect), the risk of cross contamination from these distinct tissue compartments is also relatively high, which may lead to heterogeneous cultures and complicated interpretation of bulk RNA analysis. Although *ScxCreERT2* has the potential to separate these populations, tamoxifen recombination is never 100% efficient and some tendon proper cells may not be labeled. The use of single cell RNA techniques will therefore enable classification of all cell populations and perhaps allow identification of definitive markers for not only TSPCs, but also for differentiated tendon and epitenon/endotenon cells.

TSPC Culture and Differentiation: Growth factors, Small Molecules, and Epigenetic Regulators

Hypoxia improves long-term TSPC culture.

Therapeutic use of TSPCs for tendon healing requires standardized protocols for isolation, expansion, long term culture, and defined differentiation. One major drawback of extended tissue culture is phenotypic drift after longer passages *in vitro*. At later passages, TSPCs show reduced proliferation rates, decreased ECM production, and decreased expression of tendon and stemness markers.^{50; 51} To improve TSPC culture conditions and reduce phenotypic drift, a few recent studies achieved modest success using hypoxic conditions (Supplementary Table 1). In embryonic stem cells and BMSCs, hypoxia enhanced self-renewal and maintained proliferative capacity while reducing spontaneous differentiation and apoptosis.^{52–54} Consistent with these studies, TSPCs cultured at low oxygen tensions enhanced cell proliferation, increased expression of stemness and tendon marker genes, and reduced expression of osteogenic, adipogenic, and chondrogenic genes.^{55–57} Interestingly, TSPC differentiation toward osteogenesis increased under normoxia compared to hypoxia.⁵⁸ Collectively these studies suggest that oxygen tension may be an important regulator of TSPC maintenance and expansion, as well as differentiation potential.

Growth factors that enhance TSPC proliferation.

Growth factors that have been tested for TSPC proliferation include Connective tissue growth factor (CTGF), Platelet-derived growth factor BB (PDGFBB), basic fibroblast growth factor (bFGF), and Insulin-like growth factor-1 (IGF1) (Supplementary Table 1). In several cases, treatment with these growth factors also induced tenogenic gene expression *in vitro*, although *in vivo* implications for tendon biology are not always clear. Supplementation with CTGF enriched a CD146⁺ TSPC subpopulation (likely derived from a perivascular source) and stimulated proliferation and tenogenic differentiation.⁵⁹ This was consistent with several studies showing efficient tenogenic differentiation in TSPCs in parallel with downregulation of osteo-, chondro- and adipogenic gene expression.^{60–62} *In vivo* delivery of CTGF also resulted in recruitment of CD146⁺ cells and improved tendon

healing.⁵⁹ PDGFBB or bFGF delivered individually to TSPCs in tissue culture significantly increased cell proliferation as well as matrix and collagen synthesis.^{63; 64} Administration of PDGFBB in combination with bFGF resulted in an even higher rate of cell proliferation in a dose-dependent manner.⁶⁴ While combinatorial culture with multiple growth factors often results in additive or synergistic responses in proliferation or differentiation, application of more than one growth factor can also have unexpected effects. For example, PDGFBB or IGF1 significantly enhanced cell proliferation, tenocyte chemotaxis, and metabolic activity, however, PDGFBB decreased expression of tenogenic markers.⁶⁵ This is in contrast to IGF1 treatment alone, which reduced the loss of stemness markers and upregulated tenogenic gene expression in TSPCs.⁶⁶ On the other hand, treatment with bFGF or GDF5 (a member of the TGF β /BMP family discussed in greater detail below) upregulated *Scx* and *Tnc* expression, but did not significantly enhance tenocyte proliferation, chemotaxis or collagen synthesis. Interestingly, of all different pairwise combinations tested, IGF1+GDF5 showed the most promising increase in tenogenic marker expression and collagen synthesis.⁶⁵ Thus, activation of different signaling pathways can result in unexpected interactions that may not be necessarily predicted by the use of single growth factors in isolation.

Growth factors and small molecules that enhance TSPC tendon differentiation.

One established strategy to induce tissue-specific differentiation *in vitro* is to activate or inhibit signaling pathways known to regulate tissue development *in vivo*. For tendon development, the TGF β signaling pathway is required for induction and maintenance of tendon progenitors in the embryo; loss of either the ligands or the receptor results in loss of all tendons.^{67; 68} Although TGF β ligands are frequently used to induce chondrogenesis *in vitro*,^{69–72} they are also potent inducers of tendon markers (such as *Scx*) in cell and organ cultures.^{67; 68} Treatment of TSPCs derived from tail, flexor, and patellar tendons consistently enhanced expression of tenogenic markers in response to TGF β ligands, including *Scx*, *Mkx*, *Col1a1*, *Col5a1*, and more (Supplementary Table 1).^{66; 73–76} These tenogenic effects are not observed when TGF β signaling is inhibited using the small molecule inhibitor SB431542.⁷⁶ In addition to TGF β s, treatment with Myostatin (another TGF β Smad2/3 subfamily member) resulted in similar pro-tenogenic findings.⁷⁷ While the remaining TGF β subfamily members (such as Activins) have not been investigated in detail, it is possible that the Smad2/3 subfamily of the TGF β superfamily may all have similar pro-tenogenic activities. Whether each of the TGF β subfamily ligands activate similar downstream signaling events (via Smads or non-Smad pathways) is not known.

The other subfamily within the TGF β superfamily is composed of bone morphogenetic proteins (BMPs), which are distinguished by their downstream signaling through Smad1/5/8. While several BMPs inhibit tendon induction during development and *Scx* expression in cell culture,^{21; 78; 79} a subset of BMPs (formerly known as Growth and differentiation factors GDFs 5, 6, and 7) may positively regulate tendon-specific differentiation.^{80–85} Like TGF β s, these BMPs generally improve tendon differentiation (Supplementary Table 1). Rat TSPCs treated with BMP14 (GDF5) increased expression of *Scx* while reducing adipogenic and chondrogenic potential.⁶⁶ BMP14 also increased cell proliferation in a time and dose dependent manner as well as matrix content.^{86; 87} Similarly, BMP12 (GDF7) resulted in upregulation of several tendon markers, including *Scx*, *Tnmd*, *Col1a1* and *Tnc*.⁸⁸ Although

combinatorial screens with these BMPs have not been extensively studied for TSPCs, stimulation with BMP12 in combination with overexpression of CTGF had an additive effect on the expression of tendon genes.⁸⁸ Similarly, TSPCs co-transfected with BMP12 and CTGF showed increased expression of *Scx*, *Col1a1*, *Col3a1* and *Tnc* with inhibition of osteo-, chondro- and adipogenic markers.⁸⁹

In addition to the TGF β superfamily members, a small molecule screen identified retinoic acid receptor agonists as *Scx* inducers in human Achilles tendon derived TSPCs.⁹⁰ A RAR β and RAR γ agonist called Tazarotene markedly enhanced nuclear SCX relative to total SCX, suggesting that the pro-tenogenic effect of RAR signaling is mediated via increased nuclear translocation of SCX. This may help maintain TSPCs in a tenogenic state by preventing spontaneous or erroneous differentiation to other lineages in culture. RAR signaling also modulates SCX nuclear localization via epigenetic modifications, specifically histone methylation.⁹⁰ Inhibition of histone deacetylases (HDAC) by trichostatin A (TSA) or valproic acid (VPA) in mouse TSPCs derived from *ScxGFP* tendons reactivated *ScxGFP* after extended time in culture.⁹¹ TSA or VPA treatment also upregulated tendon markers while suppressing osteogenic and chondrogenic differentiation to some degree. ChIP-Seq analysis of TSPCs treated with HDAC inhibitors revealed increased H3K27Ac peaks at the *Tgf β 1*, *Tgf β 2*, *Ctgf*, and *Fmod* regulatory regions but not at the *Scx* promoter.⁹¹ These results indicate that supplementation with epigenetic regulators can be an effective strategy for tenogenic differentiation of TSPCs (Supplementary Table 1).

One major limitation for effective and reproducible TSPC differentiation is the absence of defined media formulations tailored toward tenogenesis. While chemically defined media have been established for chondrogenesis,^{92; 93} majority of the literature still relies on media containing 10% fetal bovine serum during tendon differentiation. Since serum contains many growth factors (in unknown concentrations) and can vary considerably by batch or manufacturer, the use of serum is an impediment to culture of TSPCs for clinical translation. This can also result in conflicting results using biochemical additives, which may activate opposing signaling pathways.

Mechanical Stimulation and 3-dimensional TSPC Culture

Dynamic uniaxial and biaxial tensile loading.

Another strategy to induce tenogenic differentiation, while preventing phenotypic drift of TSPCs, is to expose cells to mechanical cues similar to those experienced in the native microenvironment.^{94; 95} Although specific tendons undergo specific loading depending on anatomical location, the general type of loading for most tendons is uniaxial tension. To determine the effects of mechanical conditioning on TSPCs, cells are typically subjected to uniaxial or biaxial cyclic strain. Biaxial stimulation of TSPCs plated on flexible silicone resulted in increased expression of ECM genes such as fibromodulin, lumican, and versican as well as expression of collagen-binding integrins α 1, α 2, and α 11.⁹⁴ Although biaxial loading had a clear effect, these genes are not tendon-specific. In contrast, direct comparison of uniaxial and biaxial stimuli showed that uniaxial loading of TSPCs was more strongly pro-tenogenic (and osteogenic) than biaxial loading, which induced a mixed osteogenic, adipogenic, and chondrogenic fate in TSPCs.⁹⁵ Uniaxial loading of 3-dimensional (3D)

TSPC constructs significantly enhanced tendon differentiation, mechanical properties, and neo-tendon formation.⁹⁵ Despite enhanced differentiation, it is unlikely that mechanical loading of naïve TSPCs in the absence of biochemical stimuli will be sufficient for inducing a mature, tendon-specific phenotype.

Scaffold-free TSPC constructs.

One popular method for creating a 3D microenvironment conducive to tendon differentiation is forming a dense sheet of confluent cells. In this method, TSPCs undergo tenogenesis, lay down ECM, and assemble into cell sheets upon treatment with growth factors or small molecules, including ascorbic acid (AA),⁹⁶ CTGF/AA,^{60; 62} and HDAC inhibitors TSA or VPA.⁹¹ In tendon cell sheets formed after CTGF/AA treatment, significant increases were observed for tendon-specific markers (*Scx* and *Tnmd*) and tendon-associated ECM genes (*Colla1*, *Dcn*, and *Bgn*). In the absence of fixed anchors, scaffold-free tendon sheets were characterized by immature loose and disorganized ECM structure (in contrast to the highly aligned organization of native tendon). Surprisingly, subcutaneous implantation of this disorganized tendon construct in nude mice resulted in formation of a neo-tendon tissue with mature and aligned collagen fibrils over a period of 12 weeks, suggesting that the *in vivo* environment may supply factors conducive to tendon maturation.⁶⁰ How this is achieved in the absence of mechanical tension, and the molecular signals required remains to be elucidated. Interestingly, application of HDAC inhibitors markedly improved the ability of TSPCs to turn on tendon-related genes and form cell sheets with stronger *ScxGFP* signal as well as more aligned collagen fibers.⁹¹ *In vivo* implantation of these growth factor or small molecule treated TSPC cell sheets into tendon defects increased tendon-specific marker expression, collagen deposition, and alignment with improved mechanical properties of the repaired tendon.^{60; 91; 96} These studies suggest that *in vitro* pre-differentiation within a 3D environment followed by *in vivo* delivery may be an effective therapeutic strategy for tendon repair.

3D scaffolds for TSPC culture.

In addition to scaffold-free cell constructs, the development of biomimetic scaffolds and biomaterials presents another attractive route for optimizing directed differentiation of TSPCs as well as their encapsulation and delivery. While much of this work has focused on BMSCs, a few biomaterials have now been tested on TSPCs, with encouraging results. Scaffolds can be used to direct cell shape by topographical cues that orient TSPCs towards an elongated cell type as seen in native tendon tissue.⁹⁷ TSPCs seeded on such microgrooved membranes showed stronger upregulation of tenogenic markers, compared to TSPCs seeded on smooth membranes. TSPC elongation also inhibited differentiation towards the osteo-, chondro- and adipogenic lineages.⁹⁷ Biomimetic scaffolds that mimic native tendon architecture (in terms of aligned fibers) and mechanical properties can also support TSPC culture, ECM deposition, and initiate a tendon-specific gene response.⁹⁸

Recent work developing biomaterials for TSPCs showed that seeding TSPCs in fibrin gels,^{43; 59; 61; 99} collagen hydrogels^{100; 101} or engineered tendon matrix (ETM) films or gels,¹⁰² can induce *in vitro* tenogenic differentiation. In addition, these scaffolds serve as successful *in vivo* delivery vehicles for TSPCs into a tendon defect. *In vitro* culture using soft

hydrogels frequently employs fixed static anchors as part of the culture system, to allow for cell-mediated contraction to form a linear, 3D tensioned construct.^{43; 103; 104} Even in the absence of dynamic cyclic loading, the linear 3D environment and uniaxial static tension is sufficient to align TSPCs along the axis of tension leading to aligned collagen deposition and upregulation of tendon markers. One limitation of many biomaterial models is that long-term culture is typically not possible in the absence of additional tenogenic growth factors such as TGF β .^{103–105} The collagen matrix produced by TSPCs *in vitro* also remains immature (small uniform fibrils) and mechanical properties can only approach that of embryonic tendons.^{43; 60; 105} However, these scaffolds support TSPC expansion, limited tenogenic differentiation *in vitro*, and *in vivo* implantation (alone or in combination with growth factor or small molecule treatments).^{59–61; 65; 91; 96–100; 102; 106} In general, several studies suggest that combining chemical supplementation (including growth factors such as CTGF or small molecules such as TSA) with 3D culture best enhances tenogenic differentiation and improves subsequent healing after implantation of constructs.^{60; 91; 96} Consistent with almost all tendon differentiation research however, the media formulations for 3D constructs are also serum-based and functional properties are rarely assessed.

Therapeutic Strategies for Tendon Repair

Devising an effective therapy for tendon repair/regeneration will likely require a multi-pronged approach that combines activation/delivery of an appropriate cell type, tailored differentiation (either *ex vivo* or *in vivo*), and a microenvironment conducive to regeneration. While this review focused on TSPCs since these tissue-specific cells may be ‘primed’ toward tenogenic differentiation, pioneering early research in tendon tissue engineering applied bone marrow-derived mesenchymal stem cells and tested strategies for optimizing their tenogenic potential via 3-dimensional and mechanical cues.^{107–113} Recent studies also highlight the exciting capacity of neighboring endogenous cells to be activated and recruited toward tendon repair *in vivo*, given the correct molecular signals.^{59; 114} The choice of cell type is an essential consideration which impacts the overall therapeutic strategy. For instance, the choice of bone marrow derived MSCs will require a compatible delivery vehicle and likely additional factors to induce tenogenesis. Patient specific MSCs will also require an additional procedure to harvest cells and expansion in culture. In contrast, TSPCs or other endogenous stem cells may be activated *in vivo* by simple delivery of a molecular factor. Other important considerations in selecting the appropriate cell source include age, availability, expansion capacity and ease of use as well as cost of culture.

For efficient tenogenic differentiation, TSPCs or other cell sources must be guided along the correct path via biochemical and mechanical stimuli. This may involve pre-differentiation of TSPCs *in vitro*, which enables precise control over the differentiation process. However, pre-differentiation may be limited by the high cost of culture (including growth factors) and time. Cells may also change their phenotype post-implantation and adopt alternative fates toward cartilage or bone. The use of undifferentiated TSPCs (either by *in vivo* activation or *ex vivo* expansion) is another option, however directed differentiation of these TSPCs *in vivo* will require additional research since targeted delivery of the necessary biochemical factors to a specific population of cells will likely be challenging.

Finally, another key element of a tendon repair therapy is the choice of cell/molecular delivery vehicle or biomaterial scaffold used to create a sustained and appropriate microenvironment for tuning differentiation *in vivo*. The ideal vehicle or scaffold should have low toxicity, degrade over time as regeneration/repair proceeds, and maintain integration with the native tissue while achieving high enough mechanical properties to prevent early failure or re-rupture. While a biomaterial with native tendon properties is conceptually attractive, these properties may also hinder infiltration by endogenous cells or integrate poorly with host tissue. From a clinical perspective, an off the shelf product to supplement current surgical repair methods will likely be the most attractive option for tendon repair. Such a strategy would eliminate procedures that require additional harvests or *ex vivo* culture or preconditioning of cells. Thus, the ideal strategy may be a biomimetic scaffold with tunable mechanical properties that allows for sustained delivery of the appropriate molecular cues that activate/recruit/reprogram endogenous TSPCs.

Conclusion

To date, tendon biology remains a relatively nascent field, and definitive markers have not been completely identified for differentiated tendon or epitenon/endotenon cells. While cells with features characteristic of stem cells (TSPCs) have been isolated from tendons, the source and location of these cells, their markers, and *in vivo* function remain to be elucidated. Recent studies suggest there may be multiple populations of TSPCs within tendon, however their specific roles in regulating tendon growth, homeostasis, adaptation, and healing are still largely undefined. In the context of adult wound healing, it is unclear why TSPCs do not regenerate tendon as part of normal healing. Unlike regenerative tissues such as muscle or the intestine, which are regulated by tissue specific stem cells that fuel tissue replacement, tendons heal by scar and sometimes, abnormal differentiation toward cartilage or bone. Tendon is also fairly quiescent, with low cell and matrix turnover over the lifetime of the tissue. It is intriguing to speculate that some TSPCs may not serve as true tendon stem cells, but reflect an early progenitor population that fuels tendon growth during postnatal development and are subsequently lost after tendon maturation. The TSPC populations isolated during adult stages may therefore be more akin to ‘supporting cells’ that undergo scar formation to repair the tissue after injury. Clearly distinguishing the different subpopulations of TSPCs at different stages will be critical for understanding their distinct functions. The use of single cell sequencing technologies will be crucial for these efforts.

Despite the conundrum of their native *in vivo* activities, the potential of TSPCs for tenogenic differentiation under the right cues still enables their use for tendon therapies. The literature strongly supports the capacity of isolated cells to undergo tendon-specific differentiation given the appropriate biochemical, biomechanical, and microenvironmental signals. The most attractive strategy proposed for clinical translation is the delivery of signaling factors that target and recruit endogenous cells and guide them toward tenogenesis *in vivo*. Such a strategy eliminates concerns of donor site morbidity from cell harvest and extended (and expensive) *in vitro* culture prior to re-implantation. Optimization of culture conditions is still necessary to identify the most effective molecules and formulations for delivery. *In vitro* differentiation platforms can also be used to better understand basic tendon biology, directly test signaling pathways or identify novel small molecules for tenogenesis by high throughput

screening. Enhanced TSPC culture techniques may also benefit applications such as derivation of tendon cells from pluripotent sources such as induced pluripotent stem cells (iPSCs), which may be a more clinically relevant cell source for direct application. Given the paucity of TSPCs in the native tendon tissue as well as issues with expansion time and maintenance of TSPC stemness, iPSCs, which can be expanded long term with minimal phenotypic drift, represent a particularly attractive option for improving tendon healing strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic showing tendon architecture and potential sources for tendon stem progenitor cells (TSPCs) along with markers currently known to be expressed by each TSPC population. Purple cells represent paratenon/epitenon-derived TSPCs; blue cells represent tendon fascicle-derived TSPCs; green cells represent perivascular TSPCs.

Figure 2.
Expression of the *tdTomato* reporter in **A.** *Scx* lineage and **B.** non-*Scx* lineage cells isolated from mouse Achilles tendons labeled by *ScxCreERT2/RosaT*. Images are at 4× magnification.