Lena Afeyan BP205 Proposal

Strategies for exploring of the contribution of the extracellular matrix to the tendon stem/progenitor cell niche

Abstract

Limited recovery of tendons following injury, lack of availability of tissue for engraftment, and donor site morbidity, are just a few reasons for the persisting need for therapeutic strategies to regrow tendons. Stem cell therapy has emerged as a potentially promising alternative, but will require a better understanding of the processes by which tenocytes are made. A significant recent advance to this end has been the discovery of a tendon stem/ progenitor cell (TSPC) population. In order to understand how these cells proliferate and differentiate *in vivo*, it will be critical to characterize their stem cell niche. In this study, an *in vitro* RNAi screen for BMP signaling in TSPCs, and *in vivo* knock down of extracellular matrix factors will be used to explore potentially important components of the stem cell niche. These experiments will contribute to the *in vitro* recapitulation of this niche and will be critical for its study and manipulation both *in vivo* and *in vitro*.

Introduction

Tendons are specialized tissue connecting bone to muscle. Tendons are made up of collagen fibrils, which cross-link to make fibers (Sharma and Maffulli, 2006). Tenocytes, or tendon cells, are located between parallel fibril chains. These cells synthesize a unique tendon extracellular matrix (ECM), made up of collagens, large proteoglycans, and small leucine-rich proteoglycans. The force transmitted by these structures allows for body movement.

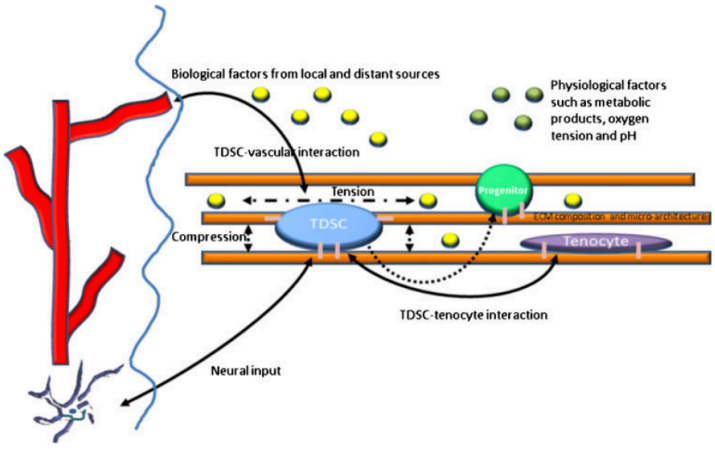
Tendon injury, often caused by excessive force, overuse, or age-related degeneration, is a widespread and persistent clinical problem. Our current solutions largely aim to relieve pain and use physical therapy to ease back into motion (Sharma and Maffulli, 2006). Injured tendon heals very slowly and in most cases, the end result of recovery is still diminished structural integrity and mechanical strength (Bi et al., 2007). Standard of care in treating common tendon-related injuries generally includes adding structural support during the healing process, in some cases through surgery. In the case of severe loss of tissue tendon or ligament tissue from other parts of the body are engrafted (Sharma and Maffulli, 2006). In these cases, however, there is low availability of engraftable donor tissue and allogenic transplant often causes immunogenic responses. Donor site morbidity, such as infection or debilitating injury in the site from which the tissue was taken, is another key limitation of these transplants (Mastrokalos et al., 2005).

For these reasons, in recent years, the possibility of regenerating tissue with stem cell technology has been particularly appealing for tendon injuries. Progress to this end has been slow because of a lack of understanding of how tenocytes are made and how the ECM is implicated in development and healing. Until recently, researchers had not isolated the progenitor cells responsible for the tendon lineage. The precursor for the tissues surrounding and closely related to tendons, namely chondrocytes, osteocytes, and adipocytes, was known to be mesenchymal stem cells. For this reason, research efforts focused largely on evaluating the potential of these cells, alongside embryonic stem cells, for tendon healing. While transplanting these cells was shown to promote healing in tendon tissue, their multipotential capacity often led to the formation of tumors over time *in vivo* (Lui and Chan, 2011).

The recent isolation and characterization of a population of cells in both human and mouse tendon, now termed tendon stem/ progenitor cells (TSPC) by Bi et al. has therefore represented a significant advance toward the development of a tendon therapy (Bi et al., 2007). These authors showed that this population was distinct from related stem cells including bone marrow stem cells and mesenchymal stem cells. They also showed that these cells shared characteristic markers with both other stem cells and tenocytes and had multidifferentiation potential. Since their discovery in 2007, efforts have focused on finding more markers for these cells and developing strategies to more efficiently engraft these cells *in vivo* and find them in other animals. These cells have been shown to create functional tendon *in vivo*, and have been isolated, cultured, and transplanted consistently by many groups (Lui, 2015b; Lui and Chan, 2011).

Still, a key limitation to the manipulation of these cells is a lack of understanding of the signals that create them during development and steer them toward tenogenesis. Despite our ability to isolate and study these cells *in vitro*, we still have a limited sense of how to recapitulate their local environment in tissue (Lui, 2015b; Lui and Chan, 2011). Such an understanding will be critical for tissue engineering and therapy development. Thus, it will be important to study their stem cell niche. A stem cell niche is the microenvironment surrounding a stem cell population including signals like growth factors and cell configurations such as the structure of the intestinal crypt (Moore and Lemischka, 2006). This environment comprises the extrinsic signals the cells follow in balancing self-renewal and differentiation. These niches are specific to each adult stem cell population and define how these populations coordinate to give rise to complex tissues in a temporally controlled manner during development (Jones and Wagers, 2008). An important step in the discovery of any new stem cell population is the characterization of the components that make up its niche and the cells that control the signals it receives (Moore and Lemischka, 2006).

From initial TSPC studies, the tendon ECM, alongside structural components such as fibrils, is believed to make up the signals of the tendon stem cell niche. The figure here outlines components that surround TSPCs (also called TDSCs), all of whose contribution should be evaluated (Lui and Chan, 2011). These cells have been traced to the ECM space and an initial knockout of 2 proteoglycans that make up most of the ECM seemed to alter their ability to engraft and form functional tendon (Bi et al., 2007). The levels of many ECM components have also been shown to change in response to tendon stress or damage, as in the case of tendinopathy. While structural contributions such as tensile stress and oxygen tension toward TSPC differentiation have been evaluated, signaling proteins or molecules in their environment have not been identified (Lui and Chan, 2011). Could these environmental changes be involved in the TSPC response to tendon damage? To what extent, if at all, may tenocyte products direct the function of TSPC cells?



To address these questions, I aim to explore the effect of perturbing the ECM on TSPC function *in vitro* and *in vivo*. The goal of this study will be dissect the ECM to find important components of the tendon stem cell niche in order to recapitulate this niche *in vitro*. In this study mouse models will be used as they have been shown to be closely related to human phenotypes in tendon (Bi et al., 2007). This stem cell niche will be explored through a series of 3 aims. In the first two, relevant tenocyte products for the stem cell niche will be identified *in vitro* and characterized *in vivo*. Specifically, in *Aim 1*, I describe an *in vitro* screen for tenocyte products that impact TSPC differentiation related signaling. In *Aim 2*, important ECM components, potentially informed by the result of the previous screen, will be knocked out *in vivo* in mice to observe their impact on tendon formation and TSPC expression profiles. Lastly, in *Aim 3,* ECM factors found to be important in this and previous studies will be added to *in vitro* cultured TSPCs and their impact on proliferation, differentiation, and expression profile will be measured.

Specific Aims

AIM 1- To identify tenocyte products that affect a key TSPC signaling pathway.

Summary: The presence of tenocytes is known to signal to various stem cell lineages, through previously uncharacterized pathways, to differentiate (Kraus et al., 2013). This aim will dissect these signals by evaluating the effect of knocking down individual tenocyte products on TPSC BMP signaling. Specifically, tenocyte cultures with individual ECM components knocked down via shRNA transfection will be made. These tenocytes will be co-cultured with TSPCs containing a BMP-luciferase reporter because BMP signaling is important in TSPCs and has been implicated in differentiation fate (Bi et al., 2007; Lopez-Rovira et al., 2002; Lui and Chan, 2011). Thus impact of these knockouts on BMP signaling with BMP2 treatment as compared to the wildtype control can serve to screen for tenocyte signals potentially involved in TSPC tenogenesis. It is important to note here that this BMP signaling readout simply indicates that these factors could affect important stem cell pathways in TSPCs, but will require further exploration in the next aims to tie factors to the stem cell niche.

Details:

1.1 Choosing candidate ECM component genes: A variety of ECM factors produced by tenocytes have been implicated in tendon stress response. Specifically, several groups have observed changes in levels of certain proteins or peptides in the ECM in tendon injury, tendinopathy and increased mechanical burden. Examples of such ECM factors include matrix metalloproteinases, tissue inhibitor metalloproteases, biglycan, fibromodulin, prostaglandin E2, and bone morphogenic proteins 2, 4, 6, and 7 (Zhang et al., 2016). To date many studies have focused on phenotypic impacts of injury and stress through exercise in mice on TSPCs, but how changes in TSPC function arise from changes in the ECM, or in other words, from signals from tenocytes, remains unknown. However, multiple recent reviews have suggested that the aforementioned ECM factors may contribute to the stem cell niche of these TSPCs (Bi et al., 2007; Lui and Chan, 2011; Zhang et al., 2016). Following these criteria, around 20 candidate genes will be chosen based on strength of literature precedent tying their differential expression to tendon stress (Lui and Chan, 2011). These genes will be individually knocked down in tenocyte culture. Here, it is important to note that limitations in the scalability of tenocyte *in vitro* culture systems will be a deciding factor in the number of genes that can be screened for this study. Future work can expand upon this method to include a broader range of tenocyte products.

1.2 Preparing mouse tenocytes: Mouse primary tenocytes will be harvested from mouse Achilles tendon tissues and expanded in culture following the methods described, validated and optimized

by Shimada et al. (Shimada et al., 2014). These harvested tenocytes will be embedded in collagen gel for 10 days of continuous culture, followed by gel digestion, passage, separation into multiple dishes, and expansion. The numbers of mice used will depend upon the reproducibility of this method and its optimization for the creation of independent tenocyte cultures for shRNA transfection.

1.3 RNAi design and tenocyte knockdown: The genes above will be knocked down via transfection of shRNAs designed for each gene. The method for design, synthesis, and transfection of this library follows that of Lu et al. in studying siRNA engineered tenocytes for tissue engineering (Lu et al., 2011). Each gene will have 2 shRNAs designed based on their sequence using a tool such as the online Ambion siRNA designer. SiRNAs will be transfected via lipofectamine according to the Thermofisher Scientific L2000 protocol. Individual dishes of expanded tenocytes will be transfected with each shRNA separately such that one gene is knocked down in each culture dish.

1.4 TSPC preparation: TSPCs will be isolated from mouse tendon as described by Bi et al. and validated by multiple other groups (Bi et al., 2007; Lui and Chan, 2011). Overall, tendon tissue is minced and digested with collegenase type I, followed by the isolation of nucleated cells that remain quiescent and attached to the plate for several days with trypsin. The identity of these cells is confirmed via established surface markers (Lui, 2015a). A BMP responsive luciferase reporter construct pID1-lux, which contains a fragment of human Id1 gene minimal promoter with BMP downstream signal smad binding sites (Lopez-Rovira et al., 2002) will be added to the cells via transient transfection with the Amaxa Nucleofactor system. Transfection conditions will have to be optimized to these TSPCs as described in Bi et al.

1.5 Co-culture and identification of candidate ECM factors: TSPCs with the BMP signaling reporter will be added to the prepared tenocyte knockdown cultures and, following overnight treatment with BMP2, luciferase activity will be measured using the Promega dual reporter assay system (Bi et al., 2007) with activity normalized to relative renilla activity within each dish. Co-cultures in which the BMP signaling is significantly lower or higher than the non-targeting siRNA control (see below) tenocyte-TSPC co-culture will be identified as potential ECM candidates for the tendon stem cell niche.

Controls:

Controls at the tenocyte transfection step will include a scrambled/ non-targeting siRNA and a no siRNA (L2000 only) transfection as negative controls and a siRNA targeting an essential housekeeping gene as a positive control (Lu et al., 2011). A non reporter transfected TSPC negative control will also be used.

AIM 2- To qualitatively and quantitatively observe the impact of the absence of ECM components on TSPC function in vivo.

Summary: In this aim, a small number of candidates, ideally informed by the hits from the screen above, will be evaluated for their *in vivo* impact on TSPCs. Specifically, these factors will be genetically inactivated in mice and their effect on tendon formation and TSPC expression profile through RNA-seq will be measured. In this way, this aim will characterize potential stem cell niche components in their *in vivo* phenotypic context and will begin to quantify the response of TSPC expression to perturbation of this niche.

Details:

2.1 Creating genetically inactivated mice: 5-10 genes will be chosen for *in vivo* analysis based either on hits from the *Aim 1* screen, or from the most promising candidates from the literature

that led to the 20 candidates tested in the first aim. This aim, therefore, will not rely on the success of the BMP signaling screen. The chosen genes will be knocked out (Shimada et al.) with a targeting replacement vector with homologous recombination in ES cells following a protocol such as that of companies like Taconic (Hall et al., 2009). Depending on the extent to which each chosen gene is essential to overall development of the mice, Cre inducible conditional knockouts or knockdown may need to be used.

2.2 Measuring KO impact on TSPCs. In these mice with specific genetic inactivations, tendon formation will be observed by ressecting the skin around major tendons in 4-month-old mice and imaging them alongside wildtype mice as described by Bi et al in one gene inactivated mouse (Bi et al., 2007). TSPCs will also be isolated from these mice as previously described and RNA sequencing will be used to compare their expression profiles to those of wild type mouse TSPCs. Total RNAs will be extracted (using eg the Qiagen RNeasy Micro Kit) and sequencing libraries generated with an Illumina kit for Hiseq 2000 will be mapped and aligned to the reference mouse genome mm9 as described and validated in detail by Liu et al (Liu et al., 2015). Here it is important to note that these cells cannot be perfectly isolated into a homogeneous population, and therefore average expression differences will be measured.

AIM 3- To begin to recapitulate the stem cell niche signals to TSPCs in vitro

Here, ECM factors will be added individually and in combination to cultured TSPCs and their proliferation and differentiation will be measured. Unlike the previous aims in which components of the niche were knocked out, this aim will connect the screen results and the *in vivo* data and the RNA sequencing profiles observed to begin to recapitulate the stem cell niche. Specifically, ECM components such as BMPs or proteoglycans will be applied directly to these cells in an *in vitro* culture. Population doubling assays and staining for tenogenesis and osteogenesis markers, both established and described in TSPCs by Bi et al., will be used (Bi et al., 2007). These assays will reflect the effect of these ECM factors on the two paths these cells can take *in vivo*, namely proliferation and differentiation. As described above, these cells will also be analyzed by RNA-sequencing and compared to untreated wild type TSPC expression profiles. The effect of the addition of these factors alone and in combination will be compared to the impact of knockouts *in vivo* to begin to construct a model for the stem cell niche and the signals controlling TSPC fate.

Conclusions

Ultimately, while each of these aims can build upon each other to create a better understanding of the TSPC niche, each can also stand alone in probing its components. Because the signaling that determines stem cell fate can involve many complex pathways, evaluating the impact of perturbation on multiple downstream TSPC phenotypes, including signaling, proliferation, differentiation, gene expression, and tendon formation. It is also important to note, however, that these individual phenotypes cannot stand alone in representing stem cell fate and the identification of implicated ECM components is just a simple step in narrowing the search for nodes of an undoubtedly intricate web of signals controlling TSPCs. However, for this reason methodical isolation of important components through loss of function *in vitro* and *in vivo*, combined with recapitulation of the niche and signal transduction *in vitro*, will provide insight from which to continue mechanistic studies of this stem cell niche. Important next steps will include probing protein interactions within the niche and extending these studies to the human TSPC niche.

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