IN VITRO CHARACTERIZATION AND IN VIVO ASSESSMENT OF EQUINE TENDON-DERIVED PROGENITOR CELLS

BY

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ABSTRACT

Tendinitis is a common cause of breakdown injury in equine athletes and accounts for 30% to 50% of all racing injuries. The last decade has seen significant development in mesenchymal stem cell (MSC)-based therapies in tendon repair. The focus on tendon-derived progenitor cells (TDPCs) for tendon healing is based on the rationale that stem cells obtained from tendons are more phenotypically-committed or 'primed' for tenogenesis than cells from other tissues. The overall objective of this body of research is to characterize and evaluate equine tendon-derived progenitor cells for tendon healing in horses.

TDPCs were isolated via a differential adhesion preplating screen that has been successfully used to isolate skeletal muscle-derived stem cells. Cell suspensions obtained via collagenase digestion of equine lateral digital extensor tendon (n=4) were serially transferred into adherent plates every 12 hrs for 4 days. TDPCs obtained from the initial, third and seventh preplates were used for subsequent analyses. Growth/proliferation and basal tenogenic gene expression of the three TDPC fractions were largely similar. Preplating and subsequent monolayer expansion did not alter the immunophenotype ($CD29^+$, $CD44^+$, $CD90^+$, and $CD45^-$) and trilineage differentiation capacity of TDPC fractions. Overall, TDPCs were robustly osteogenic but exhibited comparatively weak adipogenic and chondrogenic capacities. These outcomes indicate that preplating does not enrich for tendon-derived progenitors during in vitro culture, and 'whole tendon digest'-derived cells are as appropriate for cell-based therapies.

In vitro growth characteristics of matched equine TDPCs and bone marrow MSCs (BM-MSCs) during monolayer expansion were assessed (n=6). Subsequently, third passage TDPCs and BM-

MSCs were cultured on acellular tendon matrices for 7 days with or without insulin-like growth factor supplementation. Matrix production and matrix gene expression were analyzed at the end of in vitro culture. During monolayer expansion, at each passage, the yield of TDPCs was 3-fold higher than the matched BM-MSCs. The viability of TDPCs on acellular tendon matrices was 1.6-2.8 fold higher than BM-MSCs. New collagen and glycosaminoglycan syntheses were significantly greater in TDPC groups and in IGF-I–supplemented groups. The mRNA concentrations of collagen type I and III, and cartilage oligomeric matrix protein (COMP) were not significantly different between TDPC and BM-MSC groups. These in vitro results demonstrated that TDPCs may offer a useful resource for cell-based therapies for tendon healing.

Lastly, the efficacy of TDPCs in an in-vivo equine flexor tendinitis model was evaluated. Collagenase-induced tendinitis was created in both front superficial digital flexor (SDF) tendons (n=8). Four weeks later, the forelimb tendon lesions were treated with 1×10^7 autogenous TDPCs or saline. Twelve weeks after forelimb TDPC injections, tendons were harvested for assessment of matrix gene expression, biochemical, biomechanical and histological characteristics. Collagen I and III, COMP and tenomodulin mRNA levels were similar in both TDPC and saline groups and higher than normal tendon. Yield and maximal stresses of the TDPC group were significantly greater than the saline group's and similar to normal tendon. However, the elastic modulus of the TDPC and saline groups were not significantly different. Histological assessment of the repair tissues with Fourier transform-Second Harmonic generation imaging demonstrated that collagen alignment was significantly better in TDPC group than in the saline controls. In summary, TDPC administration improved the histological and biomechanical properties of collagenase-induced tendinitis lesions.

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CHAPTER 1: INTRODUCTION

Tendon injuries range from acute tendon rupture to chronic tendinopathy, and are among the most common orthopedic problems. Achilles tendon injuries, rotator cuff degenerative tendinopathy, flexor tendon injuries are common causes of disability and pain among people and sport/performance horses, respectively. Further, long-term disability is a frequent consequence, due to prolonged healing time and high rate of recurrence following the initial injury. Tendons are highly specialized connective tissues that transmit tensile forces between muscles and bones. Tendons are relatively hypocellular and hypovascular tissues, with little or no intrinsic regenerative capacity. Current therapies involve conservative management and/or surgical debridement and repair, depending on the location and severity of the pathology. Irrespective of the approach used, the resultant repair tissue is biomechanically inferior to healthy tendon and re-injury is common despite prolonged rehabilitation.

Cell-based approaches to tendon healing have been widely investigated in experimental models of tendinitis with the goal of improving the quality of repair tissue. Mesenchymal stem cells (MSCs) derived from different sources, including tendon tissue, have been evaluated in both in vitro and in vivo models of tendon repair with promising results. The focus on tendon-derived progenitor cells (TDPCs) for tendon regeneration is based on the rationale that cells derived from the tendons will be phenotypically and biosynthetically more capable of stimulating functional repair than MSCs derived from other tissue sources.

The objective of this body of research is to characterize equine TDPCs in vitro and subsequently assess their healing properties in an in vivo equine collagenase model of tendinitis. This thesis is

composed of a literature review and three original research chapters. The literature review (chapter 2) focuses on the biology of tendon-derived stem/progenitor cells (TDPC), their in vitro and in vivo characteristics, and their efficacy in improving healing in experimental models. This review was accepted for publication by the Journal of Stem Cell Research and Medicine [1].

The first experimental study (chapter 3) addressed the MSC characteristics of equine TDPCs isolated from the lateral digital extensor tendon via differential adherence preplating screen. In this chapter, in-vitro proliferation during monolayer expansion and immunophenotype of equine TDPCs were determined prior to trilineage differentiation. We then assessed the in vitro osteogenic, adipogenic and chondrogenic potential of equine TDPCs. The manuscript from this study has been accepted for publication by the journal Tissue Engineering: Part C Methods [2].

The second study (chapter 4) compared the in vitro growth characteristics of matched equine TDPCs and bone marrow MSCs (BM-MSCs) during monolayer expansion. Subsequently, equine TDPCs and BM-MSCs were cultured on acellular tendon matrices and matrix production and matrix gene expression were analyzed. This study was published in the American Journal of Veterinary Research [3].

The third study (chapter 5) evaluated the efficacy of autogenous TDPC injections in an in vivo collagenase model of equine superficial digital flexor tendinitis. The healing potential of TDPCs was assessed using transcriptional, biochemical, histological and biomechanical outcomes. The manuscript that describes this study was recently accepted for publication by the Journal of Orthopaedic Research [4].

References:

1. Durgam, S.S., Stewart, M.C. (2016). Tendon-derived progenitor cells: In vitro characterization and clinical applications for tendon repair. Journal of Stem Cell Research and Medicine, 1, pp. 8-17.

2. Durgam, S., Schuster, B., Cymerman, A., Stewart, A., Stewart, M. 2016. Differential adhesion selection for enrichment of tendon-derived progenitor cells during in vitro culture. Tissue Engineering Part C Methods, August 1, [Epub ahead of print].

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4. Durgam, S.S., Stewart, A.A., Sivaguru, M., Johnson, A.J.W., Stewart, M.C. 2016. Tendon-derived progenitor cells improve healing of collagenase-induced flexor tendinitis. Journal of Orthopaedic Research, doi: 10.1002/jor.23251, [Epub ahead of print].

CHAPTER 2: LITERATURE REVIEW

This chapter was recently accepted for publication as a review article by the Journal of Stem Cell Research and Medicine.[a]

Tendon injuries range from acute tendon rupture to chronic tendinopathy, and are among the most common orthopedic problems. Achilles tendon injuries account for up to 50% of all sports-related injuries [1,2], while rotator cuff degenerative tendinopathy is a common cause of shoulder pain/disability [3]. Flexor tendon injuries are linked to several occupations in people [4] and in sport/performance horses [5]. Further, long-term disability is a frequent consequence, due to prolonged healing time and high rate of recurrence following the initial injury [6,7]. Consequently, the financial impact of tendon injuries is considerable.

Tendons are highly specialized connective tissues that transmit tensile forces between muscles and bones. Tendons are relatively hypocellular and hypovascular tissues, with little or no intrinsic regenerative capacity. Current therapies involve conservative management and/or surgical debridement and repair, depending on the location and severity of the pathology [8,9]. Irrespective of the approach used, the resultant repair tissue is biomechanically inferior to healthy tendon and re-injury is common despite prolonged rehabilitation. Consequently, cell-based approaches to tendon healing have been widely investigated in experimental models of tendinitis with the goal of improving the quality of repair tissue. Mesenchymal stem cells

[a] Durgam, S.S., Stewart, M.C. 2016. Tendon-derived progenitor cells: In vitro characterization and clinical applications for tendon repair. Journal of Stem Cell Research and Medicine, 1, pp. 8-17.

(MSCs) derived from different sources have been evaluated in both in vitro and in vivo models of tendon repair and the outcomes have been recently reviewed [10-15].

This review will specifically focus on tendon-derived stem/progenitor cells, their in vitro and in vivo characteristics, and their efficacy in improving tendon healing in experimental models. Several different terms have been used in the literature to describe the stem/progenitor cell populations within tendons. In this review, the term 'tendon-derived progenitor cells' (TDPCs) is used to refer to these cells.

Tendon Structure and Function:

Tendons are dense collagenous tissues that connect muscles to bones and are composed of a hierarchical arrangement of predominantly collagenous subunits **(Figure 2.1)**. Morphologically, tendons contain a variable number of fascicles, which are comprised of multiple collagen fiber bundles. The fiber bundles contain many collagen fibrils [16,17]. These collagen units are oriented in the direction of the predominant tensile load. Tendon fascicles are bound together by a loose areolar connective tissue, the endotenon, which becomes confluent with the outer epitenon. The epitenon is surrounded by the peritenon; a fine connective tissue sheath which functions as an elastic sheath to permit free movement of the tendon against the surrounding structures. The collagen molecules are stabilized by intermolecular chemical crosslinks resulting in high tensile strength [18].

Histologically, tendons have a highly organized and anisotropic structure **(Figure 2.2)**. Collagen fibers are aligned along the longitudinal axis in each fascicle. Tenocytes are located both within

and between the fascicles, arranged in rows along the direction of the collagen fibers [19]. A characteristic crimp pattern of collagen fibers is a typical ultra-structural feature of tendons **(Figure 2.3)**. The crimps function as a buffer to provide immediate longitudinal elongation in response to physiological tensile loads [17].

Tendons can be classified as positional or energy-storing tendons. All tendons transmit forces from muscle to bone; however, energy-storing tendons have the additional function of extension and recoil to increase the efficiency of locomotion [19]. Tendons respond to tensile loads at multiple structural levels [20,21]. Crimp elongation provides a relatively modest (approximately 3%) strain response to load. Elastic 'sliding' between adjacent fibers, fibrils and fascicles, rather, than direct 'unit' extension, provides the majority of tensile strain. In addition, recent evidence suggests the presence of helical substructures within the tendon fascicles, which provides a mechanism for efficient extension under load and recoil when unloaded [22]. Therefore, the collective mechanical properties of both the interfascicular and intrafascicular matrices are responsible for the functional capacity of tendons.

Endogenous Tendon Healing:

Healing tendons undergo the traditional phases of an initial inflammatory response, a proliferative phase and a remodeling phase [5]. The reactive inflammatory phase lasts for about a week, in the absence of ongoing injury, and is characterized by a marked increase in cross-sectional area at and around the site of injury, consequent to local hemorrhage and edematous swelling. This is followed by inflammatory cell infiltration, primarily neutrophils and macrophages. The proliferative phase overlaps with the latter half of the inflammatory phase and

peaks in about 2-3 weeks. Neovascularization, local synthesis of chemokines, trophic factors and proliferation of fibroblasts are dominant features of this phase. These processes culminate in tenocyte proliferation and collagen synthesis leading to formation of immature fibrovascular tissue. The remodeling phase is characterized by formation of fibrous tissue. During this stage, the healing tissue undergoes changes in size and shape. This phase is divided into consolidation and maturation processes [23]. The repair tissue transitions from predominantly cellular to fibrous in nature, as new matrix is synthesized at the injury site. However, increased vascularity, neuronal infiltration and cellularity persist for up to 3 months post-injury [6,24,25] and the collagen architecture remains disorganized for several months **(Figure 2.3)**. This results in reduced extensibility and elasticity of the repair tissue and predisposes the site to re-injury.

Tendon-Derived Progenitor Cells:

The focus on TDPCs for tendon regeneration is based on the rationale that cells derived from the target tissue (in this case, tendon) will be phenotypically and biosynthetically more capable of stimulating functional repair than MSCs derived from other tissue sources. The existence of MSCs in tendon tissue was first reported in 2003 [26]. Tendon-derived cell lines expressing tendon-phenotype related genes such as scleraxis, cartilage oligomeric matrix protein (COMP), and type I collagen (in addition to osteopontin and Runx2), were developed from transgenic mice. Bi et al. (2007) identified and characterized a unique cell population, termed tendon stem/progenitor cells from mouse and human tendon samples [27]. These cells demonstrated universal stem cell characteristics of clonogenicity, multipotency and self-renewal capacity. This study also showed that the bioactivity of TDPCs, like other MSCs, is heavily dependent on their local environment/ matrix interactions (‘niche’). Comprehensively characterizing

stem/progenitor cells in tendons and their role in tendon responses to injury is paramount for developing effective regenerative therapies.

In vitro Characteristics of TDPCs:

TDPC isolation: TDPCs have been isolated from fetal [28] and adult human, murine [27], rat [29], lapine [30] and equine [31,32] tendons. TDPCs are typically isolated via collagenase type I digestion followed by low-density plating. Most studies have used an initial seeding density of $5x10^2$ cells/cm^2, however the optimal seeding density for TDPC isolation has not been established. Cell surface epitope-based selection ($CD90^+$, $CD73^+$, $CD105^+$ and $CD45^-$) for TDPC isolation from tendon digests has also been attempted. However, this technique does not separate tenocytes and fibroblasts from TDPCs, as markers specific for tenogenic lineage are lacking [33,34]. Other techniques of TDPC isolation include cell migration from tendon explants [27], differential adhesion of isolated cells [32,35], colony isolation [30] and selective substrate adhesion to fibronectin [31]. No clear benefits of the latter techniques over standard low-density plating have yet been identified. Currently, enrichment of TDPCs relies on multiple passage sub-culture, to enrich for rapidly and persistently proliferative stem cells from initial heterologous tendon digest populations [36].

Donor age may influence the number, proliferative and multi-lineage capacities of TDPCs. The total number of TDPCs in aged rats decreased by 70% compared to young rats [37]. In human isolates, proliferation and clonogenicity of TDPCs from aged tendons was decreased although their multi-lineage potential was retained [38]. Age did not affect in vitro characteristics in

equine TDPCs [31], suggesting that species-specific differences in age-dependent in vitro characteristics of TDPCs exist.

TDPCs have been isolated from rat and murine Achilles and patellar tendons, lapine Achilles tendon, human patellar, Achilles, rotator cuff and biceps tendons, and equine extensor and flexor tendons. Clear differences in the characteristics of TDPCs isolated from different tendons have not been demonstrated, but the number and biosynthetic activity of TDPCs are increased in response to physiologic loading and exercise, [39], and the stem/progenitor cell populations derived from the peritenon and tendon proper are distinct [40-42]. Tendon proper-derived TDPCs were more permissive for in vitro tenogenic differentiation than peritenon-derived progenitor cells. Further investigation into this aspect of TDPC biology is warranted, since these sub-populations may have distinct functions and efficacies in intrinsic and extrinsic tendon healing.

In vitro expansion and proliferation: Using TDPCs to treat tendon injuries is dependent on efficiently expanding these cells to clinically relevant numbers while maintaining their 'stemness' and therapeutic value during multiple passages. TDPCs, like other MSCs, proliferate more rapidly than terminally differentiated tenocytes during in vitro expansion [30]. Culturing human TDPCs in reduced oxygen conditions increases proliferation [43-45], metabolic rates and biosynthetic activities [40] but in one of these studies [43], 2% oxygen levels reduced their multi-lineage potency. Similarly, equine TDPCs isolated via low-density plating and cultured under hypoxic conditions had higher proliferation rates than TDPCs cultured in normoxic conditions [31]. The effect of passage on the in vitro characteristics of TDPCs has received little

attention, and the existing data is not consistent. Zhang et al. (2010) reported that the proliferation of porcine TDPCs decreased as the passage number increased [44]. In contrast, Tan et al. (2012) found that the proliferation rate and colony-forming ability of rat TDPCs increased with subsequent passages [46]. More research will be necessary to clarify this issue and determine species-specific consequences of multiple passages on subsequent activities.

Immunophenotypic profile: TDPCs share common stem cell markers identified in MSCs from other tissue sources. Specific markers that distinguish TDPCs from terminally differentiated tenocytes are poorly defined. TDPCs express CD44, CD90, CD146, CD73, Sca-1, Stro-1, nucleostemin, Oct-4, SSEA-4 and are negative for CD31, CD34, CD45, CD144, CD106 [27-31,40,44]. These markers cannot distinguish TDPCs from other MSCs and some these MSC markers are, in fact, fibroblast markers (CD44 and CD90). On the other hand, certain markers that distinguish TDPCs from bone marrow MSCs have been identified. Murine and human TDPCs lacked CD18 expression, which is expressed by bone marrow MSCs [27]. CD106 is expressed by human and rat TDPCs but is absent in bone marrow MSCs.

The specific source of TDPCs may also affect their immunophenotype. Approximately 90% of TDPCs from tendon proper expressed Sca-1 whereas only 70% of peritenon-derived TDPCs expressed this marker [41]. As expected, TDPCs isolated from peritenon had higher expression of CD133 (a pericyte marker) than TDPCs from tendon proper. A similar study found that TDPCs from peritenon were $CD146^{+}$, $CD34^{-}$; TDPCs from interstitial tissue were $CD146^{-}$, $CD34^{+}$; and TDPCs from tendon proper were $CD146^{-}$, $CD34^{-}$. All three populations were $CD44^{+}$, $CD31^{-}$and $CD45^{-}$ [40]. These findings must be interpreted with caution as these results

were largely derived from rat patellar tendon-derived TDPCs and the study sample sizes were low. Further research addressing the regional immunophenotypic characteristics of TDPCs is required to understand the link between immunophenotype and clinical efficacy.

Due to a relatively small proportion of progenitor cells in tendon, most studies analyze the immunophenotype of TDPCs after a short period of sub-culture. The immunophenotype of TDPCs, as with other MSCs, changes during in vitro passaging. One study reported the Expression of CD146, CD73 and CD90 in freshly isolated rat patellar TDPCs is lost after in vitro culture [46,47]. Kowalski et al. (2015) reported that although in vitro passaging altered the expression of CD34 and CD44 in the three sub-populations of TDPCs, their overall pattern of expression was unchanged [40]. These findings suggest that any protocol for TDPC selection based on immunophenotype will need to accommodate the alterations that occur with in vitro culture.

Multipotency of Tendon-derived Progenitor Cells:

In vitro differentiation: TDPCs, like other MSCs, are able to differentiate into adipogenic, osteogenic and chondrogenic lineages when exposed to appropriate stimuli (**Figure 2.4**). Allowing for inter-species variability, the reported in vitro tri-lineage differentiation potential of TDPCs isolated from healthy tendons has been inconsistent across studies. Earlier seminal studies characterizing TDPCs reported equivalent differentiation along adipogenic, osteogenic and chondrogenic pathways [26,27,48]. In contrast, more recent studies have reported restricted adipogenic capacity of TDPCs isolated from normal tendons [31,49]. In vitro culture conditions can also affect the differentiation potential of TDPCs. Hypoxia during in vitro culture enhanced

the differentiation capacity of human TDPCs [45] although equine TDPCs were unaffected by hypoxia [31]. Further, in vitro passage decreased the adipogenic and chondrogenic differentiation of TDPCs while their osteogenic capacity was increased [50].

The comparative differentiation potentials of TDPCs and bone marrow MSCs have also been investigated. de Mos et al. (2007 and Randelli et al. (2013) demonstrated that the tri-lineage potential of TDPCs and bone marrow MSCs were similar [33,48], whereas Tan et al. (2012) showed that TDPCs had a higher adipogenic, osteogenic and chondrogenic potential than bone marrow MSCs [50]. TDPCs have higher BMP receptor expression and are more responsive to BMP-2-induced osteogenic differentiation than bone marrow MSCs [36].

Data on the influence of donor age on TDPC multipotency is not consistent. TDPCs isolated from aged rat tendon underwent adipogenesis more readily and expressed higher levels of adipogenic markers (PPARγ, leptin) than their younger counterparts, whereas the osteogenic and chondrogenic capacity of TDPCs was unchanged [37]. However, more recent study that compared activities of human hamstring and Achilles tendon-derived TDPCs did not identify an effect of donor age on tri-lineage differentiation characteristics of TDPCs [38,51]. It is likely that species- and donor site-specific differences in the respective TDPC populations contributed to the disparities in outcome. Regardless, the influence of age on TDPC activity requires further investigation, given that degenerative tendinopathy is more prevalent in older individuals.

The in vitro differentiation potential of TDPCs isolated from healthy tendon tissue and pathological tissue are markedly different. TDPCs isolated from injured tendon had a higher in

vitro chondrogenic potential than TDPCs from normal tendon [52]. These cells were implicated in chondro-degeneration noted during tendon healing and were characterized as $CD105^-$ cells. A follow-up study by the same group demonstrated that in vitro culture of TDPCs isolated from injured tendon treated with IL-1b decreased their trilineage differentiation potential. Further research on this aspect of TDPC pathobiology is required to determine whether cells isolated from pathological tissue during reparative surgeries can be used for consequent cell-based therapies [53]. Further, given the detrimental effects of inflammatory cytokines on TDPC activities, it will be critical to define the optimal time (following injury) for cell delivery to avoid aberrant responses of stem cells implanted in an active inflammatory milieu.

Aberrant TDPC Differentiation in Tendinopathy: Fatty degeneration, chondrogenic dysplasia and ectopic calcification within the repair tissue of chronically injured tendons are well-documented [54-63]. Ectopic chondro-ossification in the mid-substance of Achilles and patellar tendons occurred as a consequence of endochondral ossification [63,64], reflecting a major phenotypic shift within the tendon cell population. The underlying pathogenesis for these metaplastic changes in chronic tendonopathy is poorly understood. Abnormal matrix deposition likely occurs from extrinsic cells that migrate to the site of injury [65] or from native tenocytes and/or TDPCs that undergo trans-differentiation to non-tenogenic phenotypes.

Experimental evidence indicates that alterations in matrix components within tendon repair tissue can profoundly impact the phenotype of TDPCs. TDPCs isolated from the biglycan-fibromodulin double knockout mice had increased collagen type II and aggrecan expression compared to wild-type TDPCs [27]. In vivo, TDPCs isolated from these mice formed bone in

addition to tendon-like tissue, while the wild type TDPCs formed tendon-like tissue only. Asai et al. (2014) showed that TDPCs in injured tendons trans-differentiate into chondrogenic cells and induce chondro-degenerative lesions [52]. The molecular pathogenesis of aberrant TDPC differentiation in tendinopathy has not yet been fully elucidated, but several recent studies have implicated inflammatory cytokines, TGF-β/BMP signaling, extracellular matrix changes and altered biomechanical stimuli in these phenotypic shifts.

Inflammatory cytokines and biological factors: The pro-inflammatory cytokines IL-1β, IL-6 and TNFα are up-regulated in both acute and chronic strain type injuries [66-68], inducing expression of inflammatory mediators Cox-2, PgE2, and collagenases MMP-1 and -13. All these factors are known to be involved in tendon matrix degradation [69]. TDPCs isolated from injured tendon and cultured in the presence of IL-1β irreversibly lose their tenogenic identity and increase their chondrogenic and osteogenic capacities [53].

TGFβ signaling is critical to fibrosis and scar formation in connective tissues and has also been implicated in pathogenesis of tendon injury [57,70,71]. TGFβ signaling activity, from TGFβ-1 in particular, is increased in injured tendon, particularly in regions of chondrogenic metaplasia and heterotopic ossification [63,64]. Excessive TGFβ signaling also stimulates pro-inflammatory effects and tenocyte apoptosis [70]. TGFβ alters the metabolic activities of tenocytes during healing, increasing collagen secretion and consequent scar tissue formation, providing a therapeutic rationale for TGFβ signaling blockade. In support of this concept, attenuation of TGFβ signaling by targeting TGFβ-1, CTGF and Smad 3 with anti-sense oligonucleotides reduced scarring and adhesion formation in a murine flexor tendon repair model [72]. Further,

the chondro-degenerative lesions induced by injured TDPCs in healing tendons are dependent on TGFβ signaling [52].

Chondro-osteogenic BMPs, such as BMP-2, BMP-4, BMP-6 and BMP-7, promote cartilage, bone and bone-tendon junction repair [73-77]. Several lines of evidence implicate dysregulation of BMP activity in tendinopathy as a cause of ectopic calcification. Ectopic overexpression of BMPs is observed in naturally occurring calcifying tendinopathy and experimental models of tendinitis [78,79], and intra-tendinous administration of rhBMP-2 results in ectopic calcification [80]. Murine TDPCs exposed to BMP-2 during in vitro culture prior to subcutaneous implantation into immuno-compromised mice generated enthesis-like elements comprised of both tendon-like and osseous tissues [27].

Altered tendon matrix composition: Disorganized collagen matrix, increased non-collagenous ground substance and an increased number and rounded morphology of the tenocytes are hallmarks of injured tendon [81,82]. During the healing process of experimental and naturally occurring tendinitis, the levels of large proteoglycans and sulfated glycosaminoglycans (characteristic of cartilage matrix) increase within tendon matrix [62,83-85]. These changes reduce the elasticity and tensile strength of the repair tissue. Proteoglycans such as biglycan, decorin, fibromodulin and lumican, although constituting a very small portion of tendon ECM, are active participants in collagen fibrillogenesis [86-89] and can also bind and sequester growth factors such as TGFβ and IGF-I [52,70,90-92] to modulate TDPC activities.

Altered tendon biomechanics: TDPCs, like terminally differentiated tenocytes, are sensitive to repetitive tensile loading in vitro [30,93]. Short-term treadmill exercise increased the yield of TDPCs from murine Achilles and patellar tendons. Further, the TDPCs isolated from exercised mice had higher biosynthetic activities than control mice. In vitro exposure to 4% tensile strain promoted tenogenic differentiation, whereas 8% tensile strain induced osteogenic differentiation [30]. TDPCs exposed to in vitro mechanical loading increased BMP-2 expression and had a higher osteogenic potential compared to unloaded TDPCs [93]. A recent study demonstrated that applying in vitro biaxial mechanical stress induces the expression of the proteoglycans, fibromodulin, lumican and versican in TDPCs [94]. These findings provide a mechanistic explanation for ectopic calcification that occurs as a result of mechanical overloading. The combination of excessive loading, BMP up-regulation and alterations in non-collagenous protein expression could generate conditions favoring TDPC chondro-osteogenesis, at the expense of tenogenic differentiation.

Applications of Tendon-derived Progenitor Cells in Tendon Regeneration:

Stem cell implantation has improved tendon healing in most studies (reviewed in 10,11,14,15]. Accepting the experimental benefits of stem cell-based therapies, several factors must be considered while choosing a particular cell type to treat tendon injuries. The tissue source must be easily accessible with acceptable donor site morbidity. The requirements for vitro manipulation (expansion and phenotypic modulation) should be minimized, to mitigate the risks of contamination and 'chain of custody' lapses. Finally, the phenotypic and reparative activities of the therapeutic cell type should closely match the target tissue. In this respect, tenogenically-

committed stem/progenitor cells derived from tendons seem more appropriate for tendon regeneration than MSCs obtained from other tissue sources.

In vitro evidence supporting TDPCs for tendon regeneration:

Several recent studies have evaluated the tenogenic potential of TDPCs under in vitro conditions. Acellular tendon has been used in tissue-engineering studies as a scaffold in cell-based approaches for flexor tendon injuries in murine models [95,96]. Decellularization of tendons was carried out by freeze-thaw cycles followed by trypsin digestion. Co-culturing TDPCs with acellular tendon matrix in vitro significantly increased their tenogenic marker expression and subsequent tenogenic differentiation. This acellular tendon-matrix model has been used by our group for comparative analyses of equine TDPCs and bone marrow for tendon healing [97,98]. TDPCs were more viable and showed superior integration into acellular tendon matrices than bone marrow MSCs (**Figure 2.5**). In addition, TDPCs had significantly higher collagen and proteoglycan synthesis levels than bone marrow MSCs. A follow-up study showed that supplementing FGF-2 during monolayer expansion of TDPCs potentiated the biosynthetic activities of TDPCs compared to bone marrow MSCs during in vitro culture with pulverized acellular tendon matrix [35]. Human fetal TDPCs cultured in aligned nanofibrous scaffold supported tenogenesis and suppressed osteogenic differentiation [28]. Collectively, these results indicate that TDPCs respond to tendon matrix components by adopting a biosynthetically active tenogenic phenotype, supporting the strategy of implanting these cells into healing tendon lesions.

In Vivo Evidence Supporting TDPCs for Tendon Regeneration:

The benefits of cell-based treatments for experimental tendon defects were first reported in 2002. Autologous tenocyte constructs were used to bridge partial flexor tendon defects in adult chickens [99]. Fourteen weeks following implantation of tenocytes, the histologic structure and biomechanical properties of the tenocyte-treated tendons were significantly improved compared to the untreated controls. Since the discovery and characterization of TDPCs, several studies have evaluated the reparative activity of TDPCs in in vivo models of tendon injury. These studies are summarized in **Table 2.1**.

Ni et al. (2012) were the first to investigate the in vivo healing characteristics of TDPCs in a rat patellar tendon window defect model [100]. GFP-labeled TDPCs in a fibrin glue matrix were injected at the defect. TDPC numbers at the site of injection decreased over time and were completely absent by four weeks after implantation. TDPC implantation improved the histologic, biomechanical and ultrasonographic characteristics of patellar tendon healing. No ectopic bone formation was detected at 4 and 16 weeks post-injury, which were the end-points of this two-part study. A subsequent study by the same group evaluated the effect of rat patellar tendon-derived TDPCs transduced with scleraxis (SCX) in the same model [101]. The histological and biomechanical characteristics of TDPC-Scx treated tendons were significantly better than tendons treated with TDPCs transduced with empty viral vector, suggesting that genetically priming TDPCs for tenogenic differentiation is clinically beneficial, although an alternative to viral delivery will likely need to be developed prior to approval for clinical applications.

A recent study by our group evaluated the effect of autologous TDPCs in a collagenase-induced equine flexor tendinitis model [102]. The TDPCs were implanted four weeks after

tendinitis induction, and improved the biomechanical and histological characteristics of the tendons, 12 weeks following administration. The biochemical and transcriptional outcomes were not significantly influenced by TDPC injection. Similar to the findings of Ni et al. (2012) [100], TDPCs were not detected at the injection sites beyond 4 weeks after treatment. Significant improvements in collagen micro-architecture have also been reported in this equine model, following bone marrow MSC [103], adipose-derived MSC [104] and fetal-derived embryonic stem cell [105] administration. The biomechanical consequences of these other progenitor types have yet to be determined and the relative merits of these stem cell populations for tendon repair have yet to be defined in direct comparative studies.

Accepting the multipotency of TDPCs, priming TDPCs for tenogenic differentiation during in vitro culture and expansion has been investigated to avoid abnormal matrix deposition within the healing tissue [106]. Connective tissue growth factor (CTGF) is highly expressed during early stages of tendon repair [107]. In addition, CTGF stimulates tenogenic differentiation of TDPCs in vitro, when supplemented with ascorbic acid [108]. Rat patellar tendon-derived TDPCs were cultured with CTGF and ascorbic acid for two weeks to produce a thin cellular sheet, before being transplanted into patellar tendon window defects [109]. The biomechanical and histologic characteristics of the TDPC-treated tendons at 2, 4, and 8 weeks were improved compared to un-treated controls. A recent follow-up study by the same group included an additional experimental group in which untreated TDPCs in a fibrin glue matrix (i.e cultured without CTGF and ascorbic acid) were implanted into the patellar tendon window defect [110]. The biomechanical, histological and ultrasonographic characteristics of tendons defects treated with TDPCs that were

exposed to CTGF and ascorbic acid were significantly better than defects treated with control TDPCs, suggesting that pre-implantation 'priming' of TDPCs will be clinically beneficial.

Autologous products like platelet-rich plasma (PRP) can also be administered with TDPCs to enhance tendon repair. Intralesional PRP administration alone has improved the overall healing characteristics of repair tissue in experimental models of tendinitis [111,112]. In vitro, platelet-rich clot releasate stimulates tenogenic differentiation of TDPCs while inhibiting osteogenic differentiation [113]. TDPCs and PRP synergize to stimulate collagen gene expression of healing rat Achilles tendons [114]. In a follow up study by the same group, passage 2 TDPCs were cultured with platelet-rich clot releasate for 3 days prior to in vivo administration [115]. TDPCs supplemented with PRP prior to intralesional administration significantly improved the overall healing characteristics of the Achilles tendon compared to control TDPCs. These findings support the hypothesis that PRP likely augments the local trophic factor synthesis and cytokine modulatory effects of TDPCs and improves reparative effects, while also acting as a bio-compatible delivery vehicle.

TDPCs in Tissue Engineering:

TDPC activities are heavily influenced by extracellular matrix. Given that there is gross disruption of tendon matrix in acute injuries and major changes in extracellular matrix composition in chronic tendinopathy, incorporating TDPCs in an appropriate 'teno-inductive' scaffold may improve tissue repair, in comparison to direct injections of TDPCs. Biological scaffolds seeded with differentiated tenocytes and bone marrow MSCs have improved repair of

tendon defects in several in vivo models [reviewed in 13]. Similar approaches using TDPCs have been evaluated in a few studies.

Zhang et al. (2009) prepared decellularized matrix by pulverizing and nuclease-digesting tendon. This matrix promoted proliferation and tenogenic differentiation of human and rat patellar tendon-derived TDPCs in vitro [95]. Subsequently, TDPCs cultured with decellularized tendon, or TDPCs alone were implanted subcutaneously, along the dorsal midline, and into patellar tendon window defects of nude mice. Interestingly, TDPCs cultured with tendon matrix synthesized neo-tendon tissue whereas naïve TDPCs did not form recognizably tendon-like tissue at either site. A decellularized matrix prepared from dermal fibroblasts was used to support rat and human patellar tendon-derived TDPCs for one week prior to in vivo implantation in a similar in vivo model [116]. Co-culturing TDPCs with dermal fibroblast-derived matrix promoted tenogenic differentiation in vitro and neo-tendon formation in vivo, whereas these effects were not seen with control TDPCs. Given that matrices from both tendinous and non-tendinous sources support proliferation and tenogenic differentiation of TDPCs, an appropriate bio-matrix could optimize the therapeutic value of these cells after in vivo delivery, although implantation of semi-solid cell-matrix composites is clearly more invasive and complicated than percutaneous injection.

Table and Figures:

TENDON	SPEC-IES	MODEL	SOURCE OF TDPCs	IN VITRO CULTURE AND EXPANSION	VEHICLE USED	DURA-TION OF THE STUDY	Ref.
Patellar	Rat	Patellar tendon window defect	Rat patellar tendon-derived	Low density plating	Fibrin glue	4 weeks	[104]
Patellar	Rat	Patellar tendon window defect	Rat patellar tendon-derived	Low density plating	Fibrin glue	16 weeks	[98]
Patellar	Rat	Patellar tendon window defect	Rat patellar tendon-derived	Low density plating + CTGF (25 ng/mL) and Ascorbate (25 uM)	Scaffold-free cell construct	8 weeks	[112]
Patellar	Rat	Patellar tendon window defect	Rat patellar tendon-derived	Low density plating +/- CTGF (25 ng/mL) and Ascorbate (25 uM)	Fibrin glue	16 weeks	[113]
Patellar	Rat	Patellar tendon window defect	Rat patellar tendon-derived	Low density plating and lentiviral-induced Scleraxis overexpression	Fibrin glue	8 weeks	[119]
Patellar	Rat	Patellar tendon window defect	Rat and Human patellar-tendon derived	Low density plating + in-vitro culture with decellularized tendon matrix	Tendon Matrix gel	8 weeks	[99]
Patellar	Rat	Patellar tendon window defect	Rat and Human patellar-tendon derived	Low density plating + in-vitro culture with dermal fibroblast matrix	Dermal fibroblast matrix	8 weeks	[120]
Achilles	Rat	Achilles transection	Rat Achilles-derived	Low density plating	Collagen sponge	2 weeks	[116]
Achilles	Rat	Collagenase	Rat Achilles-derived	Low density plating + 10% PRP releasate to P2 TDPCs for 3 days	PRP	8 weeks	[117]
SDFT	Horse	Collagenase	Lateral digital extensor tendon-derived	Differential adhesion selection	Saline	12 weeks	[105]

Table 2.1: Summary of TDPC-based tendon repair in in vivo models.

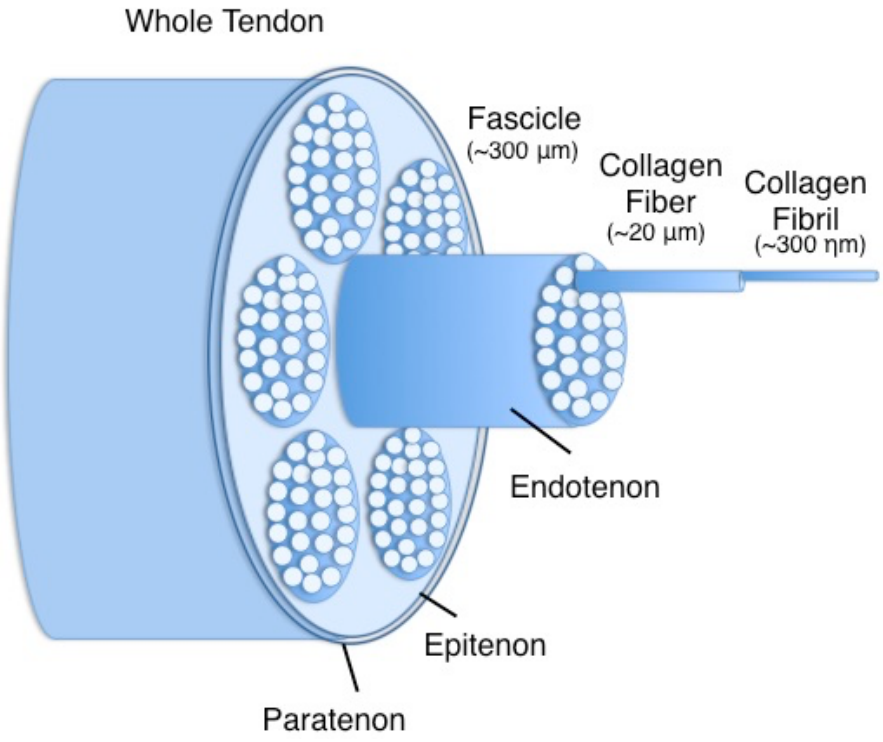

Figure 2.1: Schematic representation of hierarchical structure in tendons.

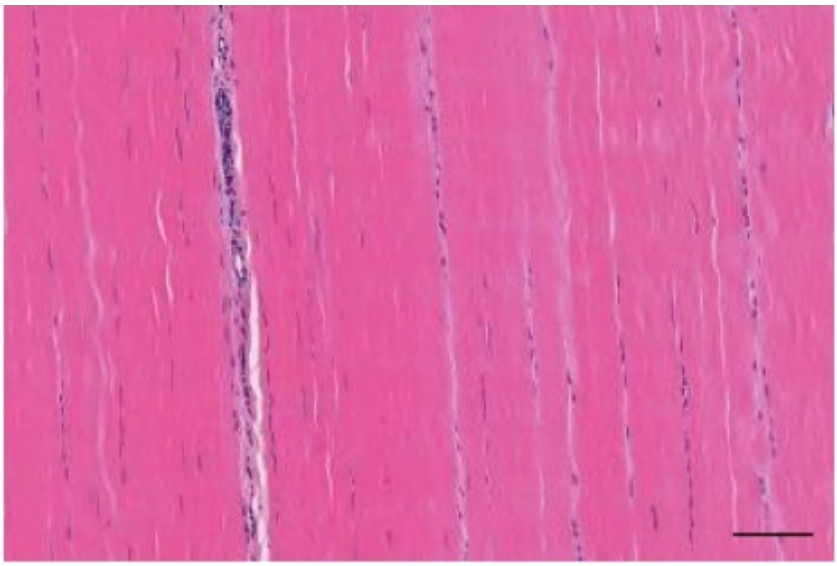

Figure 2.2: Hematoxylin and eosin stained, bright-field microscopy image of a longitudinal section of normal equine tendon. Scale bar = 100 microns.

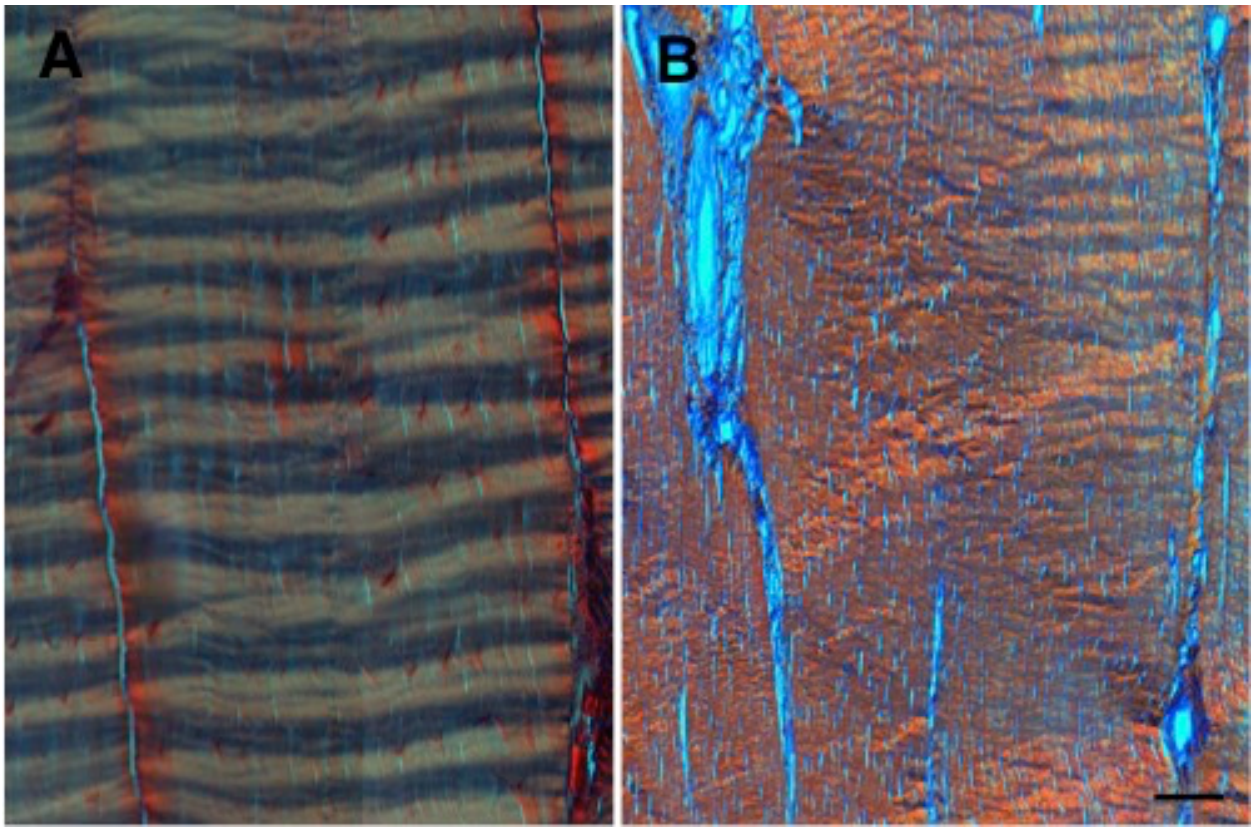

Figure 2.3: Picro-Sirius Red stained, polarized light microscopy image of a longitudinal section of (A) normal and (B) chronically injured (16-weeks post collagenase-injection) equine superficial digital flexor tendon. Scale bar = 100 microns.

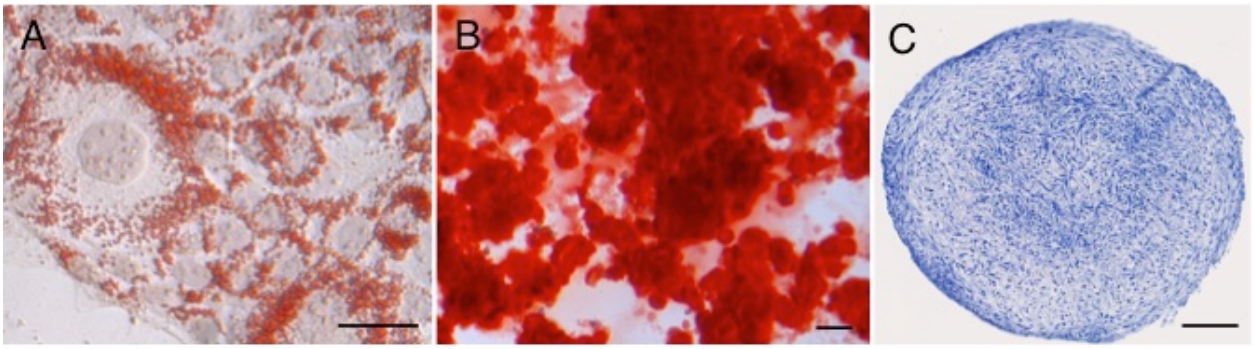

Figure 2.4: Trilineage differentiation of equine TDPCs. (A) Oil-Red-O staining of TDPCs after 14 days in adipogenic medium. (B) Alizarin Red staining of TDPCs after 14 days in osteogenic medium. (C) Toluidine blue-stained TDPC pellet at day 20 in chondrogenic medium. In all panels, bar = 100 microns.

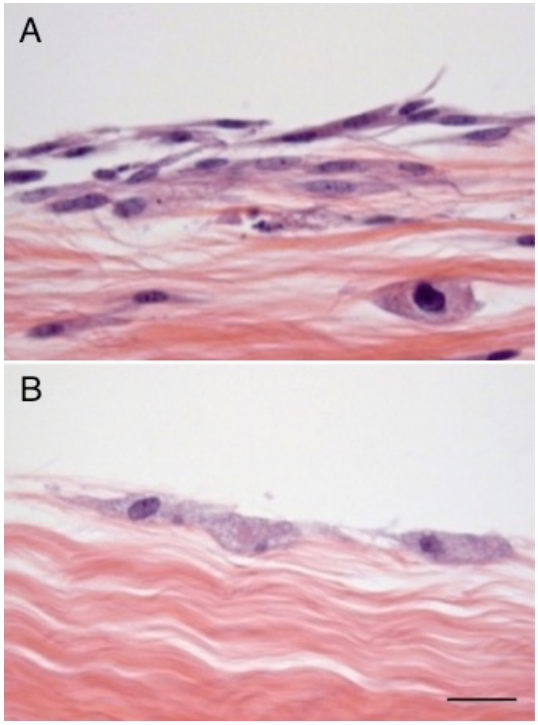

Figure 2.5: (A) TDPCs and (B) bone marrow MSCs seeded on acellular tendon matrices demonstrate differential colonization of tendon explants and some penetration by TDPCs into the underlying matrix. Scale bar = 100 microns.

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CHAPTER 3: DIFFERENTIAL ADHESION SELECTION FOR ENRICHMENT OF TENDON-DERIVED PROGENITOR CELLS DURING IN VITRO CULTURE

This chapter was accepted for publication as an original research article in the journal Tissue Engineering Part C Methods. 2016 August 1, [EPub ahead of print].[b]

Introduction:

Tendinitis is a common career-limiting injury in athletes. In general, tendons have a poor healing capacity, and the resultant repair tissue is biomechanically inferior to healthy tendon [1,2]. Re-injury is common despite prolonged rehabilitation [3]. Cell-based therapies show promise to improve the quality of tendon repair and consequent functional restoration. The efficacy of intra-lesional mesenchymal stem cell (MSC) administration for tendon healing has been evaluated in experimental models of tendinitis. These studies have consistently have demonstrated improved histologic and biomechanical repair indices [4-7].

Although the benefits of MSC-based therapies for tendinitis and other musculoskeletal injuries have been well established, their clinical use depends on identifying a suitable cell source, isolating a homogenous progenitor population, and efficiently expanding the cells to clinically applicable numbers while maintaining therapeutic efficacy. Currently, several techniques to enrich MSCs from primary cell isolates have been developed. These include cell separation based on size and shape [8,9], expansion from initial low density plating [10], differential attachment to plastic or other substrates [11,12] and/or specific cell surface markers [13-15].

[b] Durgam, S., Schuster, B., Cymerman, A., Stewart, A., Stewart, M. 2016. Differential adhesion selection for enrichment of tendon-derived progenitor cells during in vitro culture. Tissue Engineering Part C Methods, August 1, [EPub ahead of print].

Tendon-derived progenitor cells (TDPCs) constitute a promising resource for tendon regeneration [16,17]. Several recent studies have characterized TDPCs on the basis of previously established MSC cell surface markers [18-20]. In all these studies low-density plating and subsequent colony formation was used to isolate TDPCs. Immunophenotyping TDPCs and selective enrichment using MSC markers has proved to be problematical as markers exclusive to the tenogenic phenotype are limited [21,22]. Currently, isolation of homogeneous TDPCs relies on serial passage sub-culture, to enrich for rapidly and persistently proliferative stem cells from initial heterologous tendon digest populations. Time-dependent adherence, or 'preplating', is a marker-independent isolation method, which segregates rapidly adherent fibroblasts from the less adherent progenitor cells. This approach has been successfully used to isolate stem cells from skeletal and cardiac muscle tissues [23-25]. Muscle and tendon tissues are both mesenchymally derived tissues with relatively low cell densities and a hierarchically arranged, predominantly fibrillar extracellular matrix [26,27]. Further, the cellular compartments of both tissues contain heterogeneous populations with very few progenitor cells [18,23]. The objective of this study was to determine if preplating enriches TDPCs from tendon digests prior to monolayer expansion. To test this hypothesis, preplate-selected TDPC sub-populations were characterized via immunophenotyping and standard trilineage differentiation assays.

Methods:

TDPC Isolation and Preplating: All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Hindlimb lateral digital extensor (LDE) tendon was harvested from four young adult horses (2- 4 years of age) euthanized for reasons unrelated to musculoskeletal disease. A 1-2 cm length of LDE tendon specimen was diced into 0.25-cm^3

pieces and digested in 0.2% collagenase (Worthington) in DMEM supplemented with 2% fetal bovine serum (Gemini Biomedicals) at 37^{o}C for 16 hours [28,29]. The cells were isolated by filtration and centrifugation and the cells were seeded at 500 cells/cm^2 in monolayer cultures in high-glucose DMEM supplemented with 20% fetal bovine serum, 37.5 μg/ mL of ascorbic acid, 300 μg of L-glutamine/mL 100 U of sodium penicillin/mL, and 100 μg of streptomycin sulfate/mL. TDPCs were isolated using a differential attachment protocol established for isolating stem cells from skeletal muscle digests [23-25]. Primary cells from tendon digests were seeded onto adherent flasks, and the culture medium containing unattached cells was serially transferred to fresh culture flasks every 12 hrs, on seven occasions. The cells that adhered during initial plating were designated as TPP0; and the cells that adhered after 36 (third transfer) and 84 hrs (seventh transfer) were designated as TPP3 and TPP7, respectively. Subsequently, TDPCs obtained from TPP0, TPP3 and TPP7 were expanded in monolayers for two passages and used for subsequent analyses.

Monolayer expansion and cell proliferation: TDPC fractions isolated by preplating were trypsinized at approximately 80% confluency. Viability was determined via trypan blue exclusion and the resultant cells were seeded at 1 x 10^4 cells/cm^2 and expanded in monolayer cultures in high-glucose DMEM supplemented with 10% fetal bovine serum, 37.5 μg/ mL of ascorbic acid, 300 μg of L-glutamine/mL, 100 U of sodium penicillin/mL, and 100 μg of streptomycin sulfate/mL (complete DMEM) incubated at 37^{o}C in 4% CO_2, and 95% humidity conditions [28]. Passage 0 TDPCs were counted and replated at 10,000 cells/cm^2 and expanded in monolayers for two passages. Cell proliferation during first and second passages (P1 and P2) of each TDPC fraction was calculated as population doublings using the formula: Log_2

(harvested cell number/seeded cell number). Population doubling times during P1 and P2 of each TDPC fraction were calculated by dividing the time of each passage by the corresponding population doubling value. Cells obtained from confluent passage 2 monolayers of TPP0, TPP3, TPP7 (passage 3 TDPCs) were used for immunophenotyping and differentiation assays.

Flow Cytometry and Immunophenotyping: Third passage TDPCs from TPP0, TPP3, TPP7 were used for single-color flow cytometry. Aliquots of 1 x 10^6 TDPCs were fixed in 4% paraformaldehyde and then blocked with 1% BSA in high-glucose DMEM for 20 minutes. Cells were washed in PBS and resuspended in either fluorescent conjugated or unconjugated primary antibodies and incubated at 4^{o}C for 30 minutes. Flow cytometry was used to evaluate the MSC immunophenotype (CD29, CD44 and CD90) during monolayer expansion, following previously published protocols [30]. CD45 was included as a negative control for hematopoietic progenitors. The following antibodies were used according to the manufacturers' recommendations: anti-human conjugated anti-CD29-Alexa 488 (BioLegend); anti-horse conjugated anti-CD44-RPE (AbD Serotec, BioRad); anti-horse non-conjugated anti-CD90-Alexa 647 (Accurate Chemical and Scientific Corporation) and anti-human conjugated anti-CD45-Alexa 488 (AbD Serotec, BioRad) [30]. The following filters were used in a flow cytometry analyzer (Accuri C6, BD Biosciences) to isolate the emission wavelength of the conjugated fluorochromes: FL-1 (510 nm and 545 nm wavelengths of light) for CD29 (519 nm emission) and CD45 (519 nm emission), FL-2 (560-580 nm wavelength) for CD44 (578 nm emission) and FL-4 (665-695 nm wavelength) for CD90 (668 nm emission). After the emission analysis on "FCS express (Flow Research Edition)", data were expressed as 'percentage of deviation from the control antibody groups'. Bone marrow-derived MSCs were used as reference controls. Cells

in the absence of antibody and in the presence of secondary antibody only were used as controls. A threshold gating out at least 95.5% of control cells was used.

Tenogenic Gene Expression: Three million third passage TDPCs from TPP0, TPP3 and TPP7 were stored for RNA isolation prior to trilineage differentiation experiments. Basal expression of tenogenic genes, scleraxis (Scx) and tenomodulin (Tnmd) was assessed by quantitative PCR, as detailed below.

Trilineage differentiation and phenotypic staining:

Osteogenic culture: Passage 3 TDPCs from TPP0, TPP3 and TPP7 were plated at 5 x 10^3 cells/cm^2 in 6-well plates and cultured in complete DMEM until they reached 80 % confluence. Complete DMEM was then substituted with osteogenic media (complete DMEM supplemented with 10 mM β glyceraldehyde-3-phosphate, 50 μg/mL ascorbic acid, 100 ηM dexamethasone). The media were replaced every 3 days [11,20]. The cultures were maintained for 14 days.

Alizarin Red staining was used to assess mineralized matrix deposition. The cell-matrix layer was washed with PBS and fixed with 70% ethanol, and stained with 2% Alizarin R stain for 10 mins. Low-magnification (10x) images were obtained prior to osteogenic differentiation and at days 7 and 14 of osteogenic culture. Up-regulation of osteogenic genes was also assessed by quantitative PCR, as detailed below.

Adipogenic culture: Passage 3 TDPCs from TPP0, TPP3 and TPP7 were cultured in 6-well plates with complete DMEM until they reached 80% confluence. Complete DMEM was then substituted with adipogenic media (high-glucose DMEM containing 10% rabbit serum, 100 U of

sodium penicillin/mL, 100 μg of streptomycin sulfate/mL and supplemented with 1 mM dexamethasone, 100 mM indomethacin, 10 mg/mL insulin, and 500 mM isobutylmethylxanthine) [11,20]. Media were replaced every 2 days. These cultures were maintained for 10 days.

Oil-Red-O staining of monolayers was used to detect intracellular lipid accumulation. Cell monolayers were washed with PBS, fixed with 70% ethanol, and stained with 0.3% Oil Red O stain for 1 hour. Low- (10x) and high-magnification (50x) images were obtained prior to adipogenic differentiation and at days 3 and 10 of adipogenic culture. Up-regulation of adipogenic genes was also assessed by quantitative PCR, as detailed below.

Chondrogenic culture: Pellet cultures were established in microcentrifuge tubes from passage 3 TDPCs by resuspending 5 x 10^5 cells/mL in chondrogenic media (high-glucose DMEM supplemented with 100 ηM dexamethasone, 25 μg/ml ascorbic acid, 10 ηg/ml TGF-β1, 1% ITS media supplement), and pelleting 500 μl aliquots of the cell suspensions at 400 rcf [11,20,30]. Chondrogenic cultures were maintained for 20 days. Media were replaced every 3 days.

Representative pellet sections were stained with toluidine blue to assess sulfated glycosaminoglycan (sGAG) deposition [30]. After 20 days, cell pellets were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Six micron-thick sections were stained with toluidine blue. High-magnification (50x) histological images were acquired with LEICA Q500MC microscope (Leica Cambridge Ltd). Up-regulation of chondrogenic genes was also assessed by quantitative PCR, as detailed below.

RNA Isolation and Quantitative RT-PCR: Total RNA was isolated using a previously described protocol [28,30]. The samples were homogenized in a guanidinium thiocyanate-phenol-chloroform solution reagent (TRIzol, Invitrogen) according to manufacturer's suggested protocol. RNA isolation from the chondrogenic pellets included the high-salt precipitation variation recommended by the manufacturer, to minimize co-precipitation of proteoglycans [30]. The resultant pellet was purified using RNeasy silica columns that included on-column DNase digestion. One μg of RNA from each sample was reverse-transcribed (Superscript II, Invitrogen) using oligo(dT) primers. Equine gene-specific primers were designed from published sequences in Genbank, and using ClustalW multiple sequence alignment (available at www.ebi.ac.uk) (**Table 3.1**). Primer specificity was confirmed by cloning and sequencing the amplicons during optimization experiments, as previously described [28,30]. PCR amplifications were catalyzed by Taq DNA polymerase (BioRad iCycler, Bio-Rad Laboratories) in the presence of Sybr green. Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, normalized to expression of the reference gene, elongation factor-1α (EF1α) [31].

Statistical Analysis: The normality of distribution of quantitative data (relative mRNA expression) was confirmed using the Kolmogorov-Smirnov test using SigmaStat 4 software (Systat Software). Data are expressed as 'mean ± standard error'. One-way ANOVA was used to assess the effect of preplating on cell proliferation and differentiation, in the three fractions of TDPCs (TPP0, TPP3, TPP7). A p value of ≤ 0.05 was considered significant.

Results:

Cell Culture and Proliferation: The time from initial plating of each preplated TDPC subpopulation to 80% confluence was significantly less in TPP0 (4 $\pm$ 0.9 days; $p = 0.034$) than in TPP3 (9.8 $\pm$ 0.9 days) and TPP7 (12.3 $\pm$ 1.2 days). In addition, the more slowly adherent TDPC sub-populations were quiescent for the first 5-6 days after transfer, prior to cell division and colony formation. Population doubling time and population doublings during P1 and P2 were not significantly different between the TDPC fractions (**Figure 3.1**).

Immunophenotype: More than 90% of cells from all TDPC fractions were immunopositive for CD29, CD 44 and CD90; surface markers that characterize equine MSCs. All fractions of TDPCs were negative for the hematopoietic marker, CD45 (**Figure 3.2**). There were no significant differences ($p = 0.7$) in the immunophenotypes of TPP0, TPP3 and TPP7.

Tenogenic Gene Expression: The basal mRNA expression of tenogenic markers, Scx and Tnmd was similar in all TDPC fractions ($p > 0.1$) (**Figure 3.3**).

Tri-lineage Differentiation:

Osteogenesis: Alizarin Red staining of the mineralized matrix in day 7 osteogenic TDPC cultures showed minimal stain uptake in all fractions. By day 14, intense staining of mineralized nodules, indicative of robust osteogenic differentiation, was evident in all TDPC fractions (**Figure 3.4**). There were no noticeable differences in the intensity of Alizarin Red stain uptake between the TDPC fractions. Basal mRNA expression of osteogenic transcription factors osterix and Runx2 was similar in all TDPC fractions prior to osteogenic differentiation. In osteogenic medium, expression of osterix and Runx2 transcripts was significantly increased (10-12 fold;

$p<0.05$) by day 14 in all TDPC fractions and the expression was not significantly different between them. At day 14 of osteogenic induction, changes in mRNA levels of genes linked to osteogenic phenotype (osterix, osteonectin, alkaline phosphatase) corroborated the Alizarin Red staining outcomes. Expression of osterix, Runx2, osteonectin and alkaline phosphatase at day 14 of osteogenic culture was not significantly ($p=0.6$) different between the TDPC fractions (**Figure 3.4**).

Adipogenesis: Oil-Red-O staining of adipogenic cultures showed intracellular lipid droplet deposition in all TDPC fractions at day 3 that was higher at day 10 (**Figure 3.5**). Basal mRNA expression of the adipogenic transcription factor PPARγ was similar in all TDPC fractions. Adipogenic culture medium stimulated a marginal increase (2-3 fold) in PPARγ mRNA expression by day 10 in all three TDPC fractions; this increase was not significant ($p=0.4$). Changes in expression of adipogenic genes (PPARγ, FABP-4 and adiponectin) were not consistent (Figure 5B). Expression of adiponectin mRNA at day 10 of adipogenic culture was significantly ($p=0.042$) higher in TPP7 compared to TPP0 and TPP3; however, there was no significant difference in expression of FABP-4 in the three TDPC factions at day 10 of adipogenic culture (**Figure 3.5**).

Chondrogenesis: Toluidine blue staining intensity of day 20 chondrogenic pellets was minimal in all three TDPC fractions indicating low sulfated glycosaminoglycan (sGAG) content within the pellets (**Figure 3.6**). Basal mRNA expression of Sox-9 was similar in all TDPC fractions. As expected, chondrogenic stimulation significantly up regulated Sox-9 mRNA expression at day 20 compared to baseline, but Sox-9 expression was not significantly ($p=0.31$) different between the

three TDPC fractions. Chondrogenic matrix gene transcripts (collagen type II and aggrecan) were significantly ($p=0.002$) up regulated at day 20 of chondrogenic stimulation compared to baseline in all TDPC fractions (**Figure 3.6**).

Discussion:

This study investigated the value of preplating for TDPC enrichment from tendon digests. We hypothesized that a differential attachment preplating screen would enrich for TDPCs in the more slowly adherent fraction(s). Accepting the marked differences in initial seeding densities of the primary cultures, preplating and subsequent monolayer expansion did not alter the growth/proliferation or immunophenotypic characteristics of the TDPC sub-populations. Basal expression of tenogenic markers was similar across the preplate fractions and the osteogenic, adipogenic and chondrogenic differentiation capacities of the three TDPC fractions were also similar. These outcomes do not support the hypothesis.

Time to confluence was significantly shorter for TPP0 cultures, compared to TPP3 and TPP7. This finding was expected, given that a large proportion of the primary isolates attached during the first preplate. There were substantially fewer unattached cells after the first serial plating for subsequent transfers. The population doubling and population doubling times in subsequent passages were similar across the TDPC fractions, indicating that the 'per cell' proliferative capacities of the sub-populations were similar; consistent with results obtained for muscle-derived stem cells isolated via similar protocols [24,25]. Initial quiescence and heterogeneity in colony-forming units noted in this study during in-vitro isolation have also been reported in both mouse and human TDPCs [18] and is a common feature of MSCs from other tissue sources.

Allowing for species variations in stem/progenitor cell immunophenotypes, the cell surface marker profiles of equine TDPCs in our study were similar to other studies [11,20] and were characteristic of equine MSCs ($CD29^+$, $CD44^+$, $CD90^+$, and $CD45^-$) [30,32,33]. Preplating and subsequent monolayer expansion did not influence immunophenotype. As with this study, preplated skeletal muscle-derived stem cell populations did not differ in their MSC surface marker profile; however, preplating did enrich cells expressing markers specific to myogenic stem cells [23,24,34]. Scleraxis expression did not differ in the TDPC preplate groups in this study; further, unpublished data from our group indicate that Scx expression in TDPCs and bone marrow-derived MSCs (from the same donors) is equivalent under basal conditions. The transcriptional regulation of tenogenesis is less well characterized than for myogenesis or several other mesenchymal lineages, and Scx expression, by itself, might be inadequate as a selection marker [35]. In support of this possibility, equine TDPCs exhibit several tenogenic characteristics more strongly than their bone marrow-derived counterparts in in vitro models, despite highly similar basal profiles [28,36,37].

Basal expression and induction profiles of osteogenic-, adipogenic- and chondrogenic-lineage genes in response to respective in vitro stimuli were similar in all TDPC fractions. However, the overall osteogenic, adipogenic and chondrogenic capacities of TDPCs were markedly different. All TDPC fractions underwent robust osteogenic differentiation, evident in both matrix staining and gene expression profiling. The induction of transcriptional adipogenic markers was inconsistent. PPARγ was only modestly up-regulated (approximately two-fold), while adiponectin induction was the only parameter assayed in this study that was responsive to preplating, exhibiting increased expression in later preplate sub-populations. In marked

contrast, there was a significant (over three logs) increase in FABP-4 expression above baseline expression. These somewhat contradictory results indicate that the indices of adipogenesis addressed in this study are not transcriptionally linked, and emphasize the value in evaluating several phenotypic indicators in differentiation studies. Similarly, chondrogenic differentiation of TDPCs was less impressive than seen in other equine progenitor populations [30,38]. It is not clear from the outcomes of the current study whether the impressive osteogenic capacity of TDPCs is an intrinsic property of these cells or is an aberrant consequence of extensive in vitro proliferation. TDPC-mediated ectopic ossification has been reported during tendon healing [39,40], providing support for the former possibility. The restricted adipogenic and chondrogenic profiles of TDPCs observed in our study are consistent with other studies that characterized TDPCs isolated from healthy tendon via low-density plating [41] and specific substrate adhesion [11]. Fatty and mucoid degeneration [42,43], chondro-dysplasia [44,45] and ectopic ossification [39,40] have been reported in both naturally occurring and experimental models of tendinitis. These changes reflect aberrant differentiation of progenitor cells and/or transdifferentiation of tenocytes within the tissue. Although the specific mechanism(s) that generates these aberrant phenotypes is undefined, it is likely that the differentiation of TDPCs under inflammatory/ healing and homeostatic conditions differ widely. In this respect, TDPCs isolated from healthy tendons with a restricted differentiation potential may be particularly suitable for cell-based therapies, provided a suitable source with minimal donor-site morbidity can be identified.

The developmental origin of TDPCs is largely unknown and research in this area is ongoing. Although markers specific for tenogenic lineage (tenomodulin, scleraxis, mohawk) have been identified, their expression is not limited to tenogenic cells and they are not particularly useful

for cell isolation/selection protocols [21]. Other techniques of MSC isolation such as selective substrate adhesion, hypoxic conditions during in-vitro culture also did not enrich for TDPCs [11]. Although the results of this study indicate that preplating confers no clear benefit for TDPC enrichment, these outcomes and the results of related studies indicate that 'whole tendon digest' cell stocks are of significant therapeutic value for tendon repair [16,29,46].

Conclusion:

In conclusion, differential adherence preplating did not enrich equine TDPC isolation during in vitro culture and monolayer expansion. Preplating did not alter the in vitro growth/proliferation characteristics of TDPCs. The immunophenotype and trilineage differentiation potential of the three TDPC fractions assessed in this study was similar. Overall, TDPCs had a robust osteogenic capacity and a minimal adipogenic and chondrogenic capacity. The results of this study indicate that whole digest tendon-derived cell stocks are adequate for enrichment of progenitor cells via monolayer expansion.

Table and Figures:

Gene		Sequence	Amplicon (bp)
Scleraxis (Scx)	S A	5’ GAC CGC ACC AAC AGT GTG AA 5’ TGG TTG CTG AGG CAG AAG GT	231
Tenomodulin (Tnmd)	S A	5’ CCC GTG ACC AGA ACT GAA AT 5’ GTT GCA AGG CAT GAT GAC AC	232
Osterix (Osx)	S A	5’ GGC TAT GCC AAT GAC TAC CC 5’ GGT GAG ATG CCT GCA TGG AA	207
Runx2	S A	5’ CAG ACC AGC AGC ACT CCA TA 5’ CAG CGT CAA CAC CAT CAT TC	177
Osteonectin (Osn)	S A	5’ AAC CTT CTG ACC GAG AAG CA 5’ TGG GAC AGG TAC CCA TCA AT	190
ALP	S A	5’ TGG GGT GAA GGC TAA TGA GG 5’ GGC ATC TCG TTG TCC GAG TA	221
PPAR	S A	5’ TGC TGT GGG GAT GTC TCA TA 5’ GGT CAG TGG GAA GGA CTT GA	212
Adiponectin (Adpn)	S A	5’ AGG ACA AGG CTG TGC TCT TC 5’ GAA GGA AGC CTG TGA AGG TG	202
FABP4	S A	5’ AGG ACA AGG CTG TGC TCT TC 5’ GAA GGA AGC CTG TGA AGG TG	202
Sox9	S A	5’ GAA CGC ACA TCA AGA CGG AG 5’ CTG GTG GTC TGT GTA GTC GT	304
Col II	S A	5’ AGC AGG AAT TTG GTG TGG AC 5’ TCT GCC CAG TTC AGG TCT CT	223
Aggrecan (Agg’n)	S A	5’ GAC GCC GAG AGC AGG TGT 5’ AAG AAG TTG TCG GGC TGG TT	202
EF1-alpha	S A	5’ CCC GGA CAC AGA GAC TTC AT 5’ AGC ATG TTG TCA CCA TTC CA	328

Table 3.1: Primers used for real-time PCR amplification.

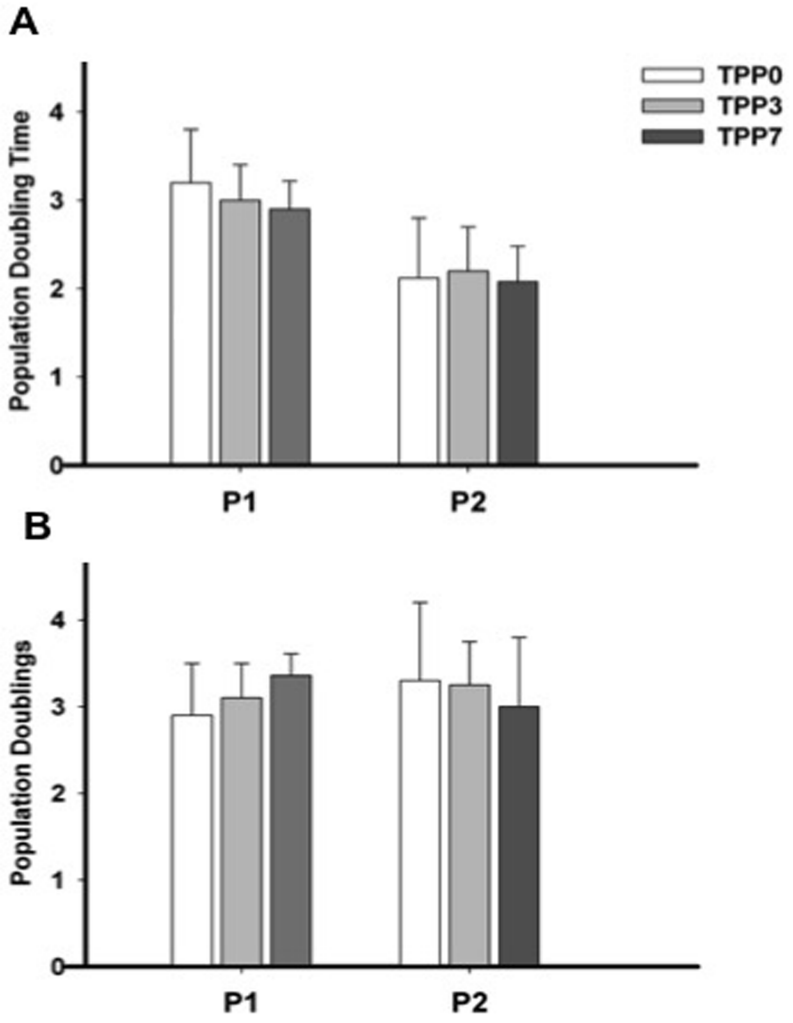

Figure 3.1: (A) Population doubling time and (B) population doublings of TDPC fractions, during the first (P1) and second (P2) passages of monolayer expansion (mean $\pm$ SE; n = 4).

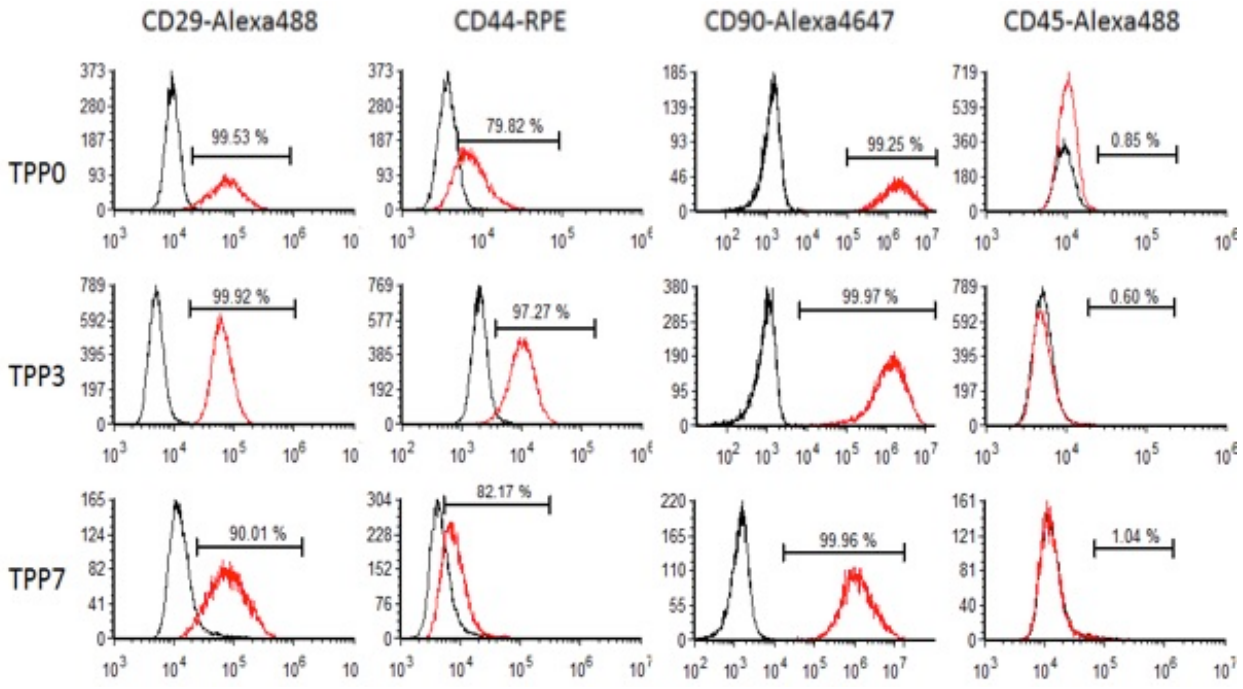

Figure 3.2: Immunophenotypic profile of third passage TDPC fractions.

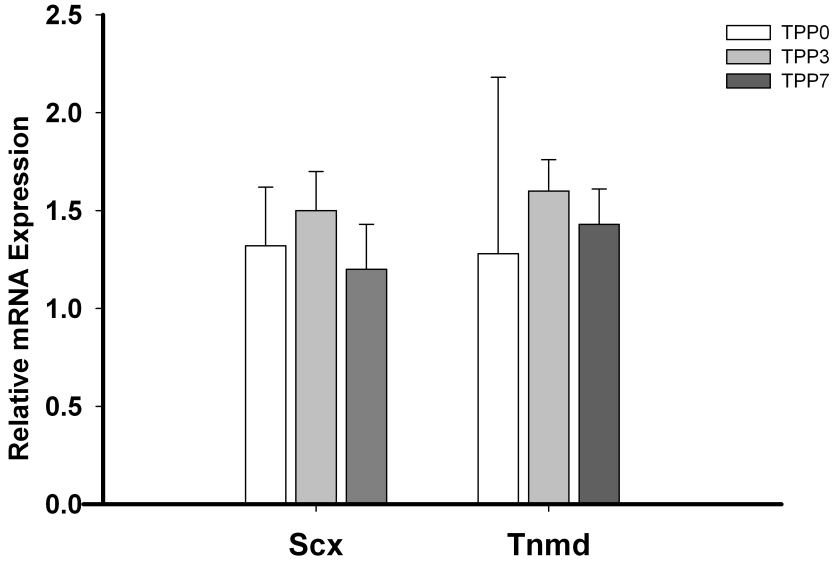

Figure 3.3: Relative mRNA expression of tenogenic genes, scleraxis (Scx) and tenomodulin (Tnmd) in TDPC fractions (mean $\pm$ SE; n = 4).

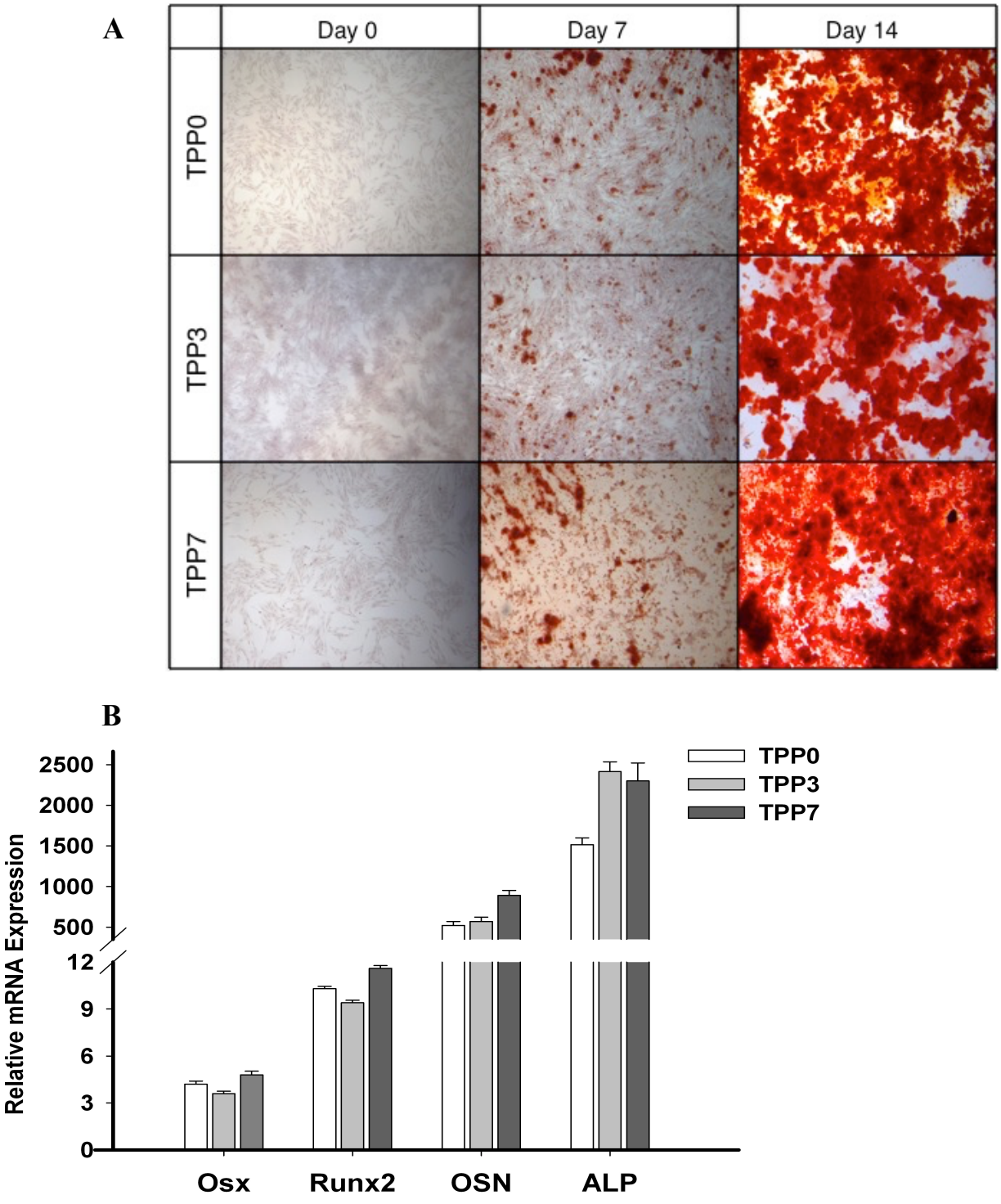

Figure 3.4: (A) Alizarin Red staining (X 10 magnification) of day 0, 7 and 14 osteogenic cultures. TPP0 (upper panels), TPP3 (middle panels), TPP7 (lower panels). Scale = 100 microns. (B) Relative mRNA expression of osteogenic genes; osterix (Osx), Runx2, osteonectin (Osn) and alkaline phosphatase (ALP) in day 14 TDPC cultures (mean $\pm$ SE; n = 4).

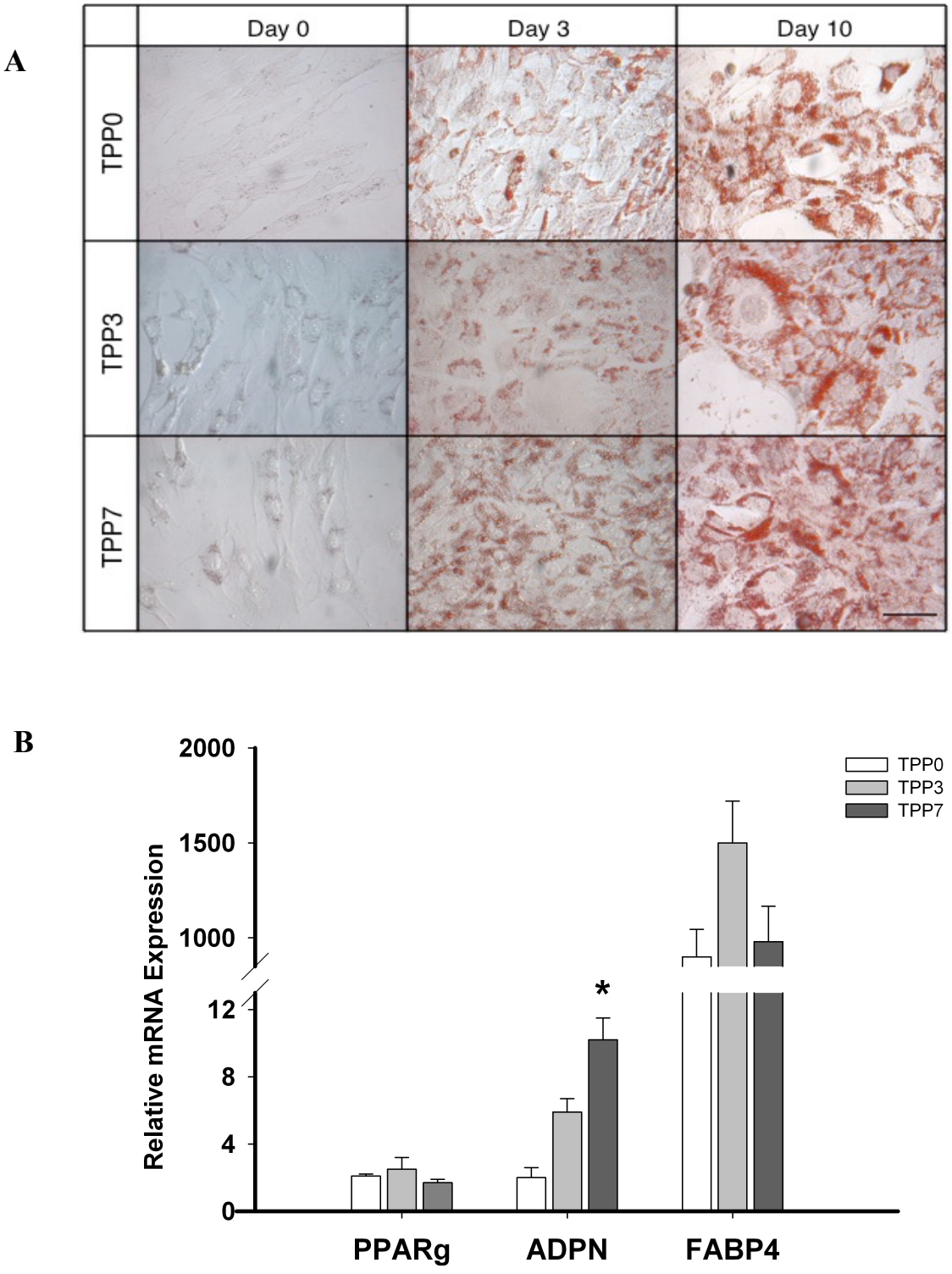

Figure 3.5: (A) Oil-Red-O staining (X 50 magnification) of day 0, 3 and 10 adipogenic cultures. TPP0 (upper panels) , TPP3 (middle panels), TPP7 (lower panels). Scale = 100 microns.
(B) Relative mRNA expression of adipogenic genes; PPARγ, adiponectin (ADPN) and fatty acid binding protein-4 (FABP4) in day 10 TDPC cultures (mean ± SE; n = 4).

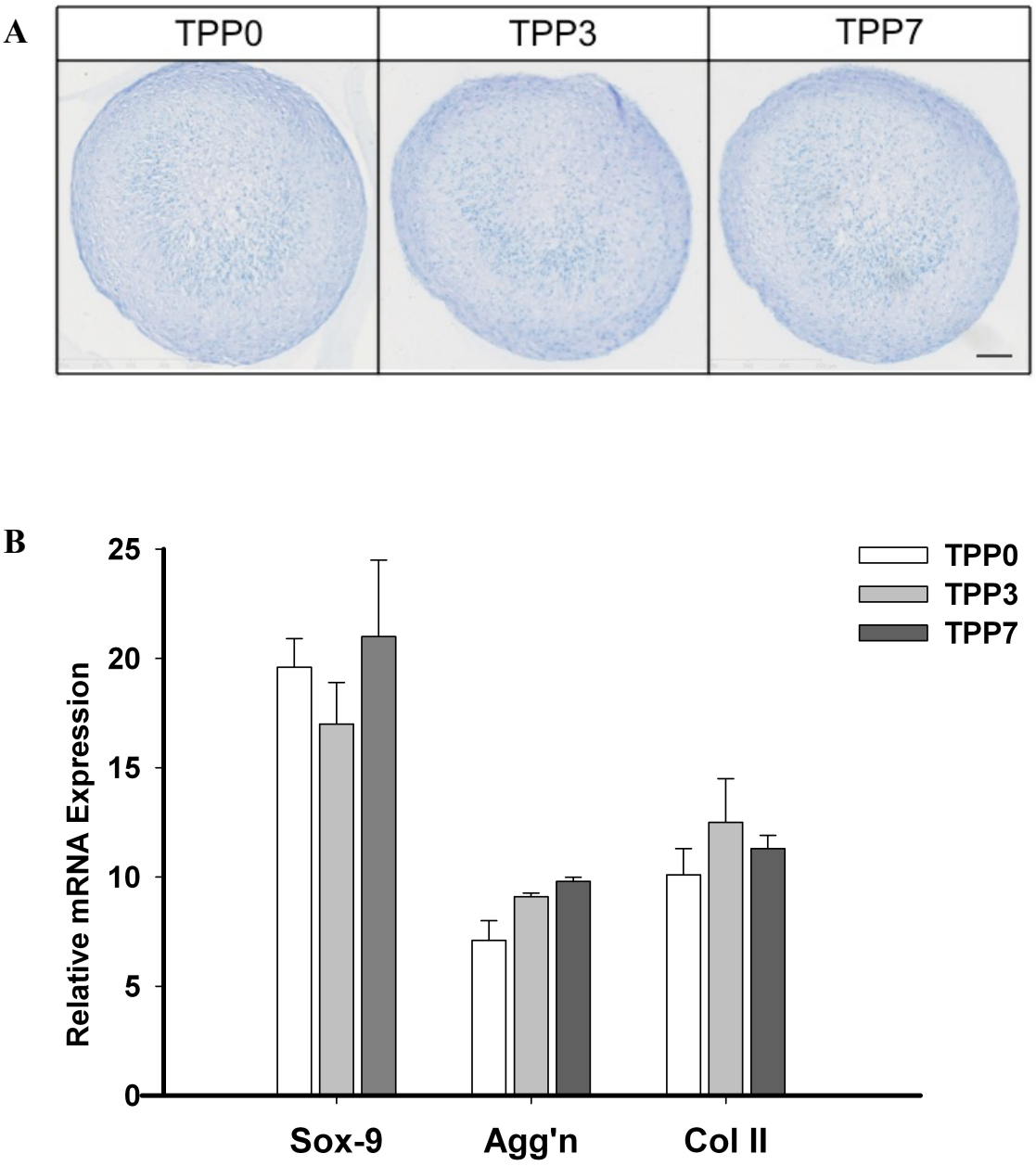

Figure 3.6: (A) Toluidine blue staining (X 10 magnification) of day 20 chondrogenic pellets. Scale = 100 microns. (B) Relative mRNA expression of chondrogenic genes; Sox9, aggrecan (Agg'n) and collagen type II (Col II) in day 20 chondrogenic pellets (mean $\pm$ SE; n = 4).

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CHAPTER 4: COMPARISON OF EQUINE TENDON- AND BONE MARROW–DERIVED CELLS CULTURED ON TENDON MATRIX WITH OR WITHOUT INSULIN-LIKE GROWTH FACTOR-I SUPPLEMENTATION

This chapter was published as an original research article in the American Journal of Veterinary Research in 2012.[c]

Introduction:

Tendinitis is a common cause of breakdown injury in equine athletes and accounts for 30% to 50% of all racing injuries [1-3]. Tendon injuries are often degenerative injuries, and the prevalence of tendinitis increases with age [3]. Despite improvements in early detection and monitoring, the tissue that is deposited during the process of tendon repair does not restore the original matrix organization and biomechanical properties [4-8]. As a consequence, reinjury is common (up to 43% of tendon strain injuries) [1].

Mesenchymal stem cells are characterized by their ability to maintain considerable proliferative activity and differentiate along several tissue lineages (ie, multipotency). These characteristics have generated research interest for use in addressing the potential of MSCs in tissue regeneration [9-13]. Most of these studies [11,12,14] have focused on MSCs derived from bone marrow aspirates. Both bone marrow– and adipose-derived cells have been used empirically for the treatment of tendonitis in horses. Only few controlled studies [15-19] have been reported.

[c] Durgam, S.S., Stewart, A.A., Pondenis, H.C., et al. 2012. Comparison of equine tendon- and bone marrow-derived cells cultured on tendon matrix with or without insulin-like growth factor-I supplementation. American Journal of Veterinary Research, 73, pp. 153-161.

It has been indicated in several studies [19-22] that alternative sources of progenitor cells might also be beneficial for specific therapeutic applications, which include treatment of tendon injuries. In another study [22] conducted by our research group, it was found that tendon- and muscle-derived cells proliferated more rapidly in vitro and had better viability and greater tenogenic matrix production, compared with results for bone marrow–derived cells.

Tendons are composed of longitudinally oriented fibrillar collagen, predominantly collagen type I, that accounts for 75% of the tissue [8,23]. Tendons also contain collagen type III, which comprises approximately 14% of total collagen in physiologically normal tendons [24]. The COMP is a glycoprotein secreted by tenocytes that helps regulate the diameter of collagen fibrils [25]. Synthesis of both collagen and proteoglycan are increased in healing tendons [29,30]. Specifically, collagen type III and COMP concentrations are increased in the acute stage of healing, and both participate in collagen type I fibrillogenesis [25-29]. Independent of the matrix synthesis responses, restoration of the normal architecture of the ECM is critical to effective tendon repair because a high degree of alignment of collagen fibrils is critical for the ability of flexor tendons to withstand high recurrent tensile loads.

In vitro and in vivo studies [31-34], investigators have reported benefits for the administration of growth factor to tenocytes. Exogenous injections and transfection of bone marrow–derived MSCs with IGF-I stimulated tendon healing in horses with tendinitis experimentally induced by the injection of collagenase [15,34]. In several studies [31-34], IGF-I stimulated matrix synthesis and ECM production in tendons and ligaments. In early stages of tendon repair, IGF-I protein concentrations decrease by 40%, compared with protein concentrations in physiologically

normal tendon; however, by 4 weeks after injury, tissue concentrations of IGF-I peak, although they remain elevated through 8 weeks after injury [30]. Therefore, supplementation with exogenous IGF-I during the early phases of tendon repair may provide a therapeutic advantage [15,30,33,34]. The plasma concentration of IGF-I ranges from 25 to 82 ng/mL in clinically normal horses [35]. In the study reported here, we used an IGF-I concentration of 100 ng/mL, which was selected on the basis of the results of an in vitro flexor tendon explant study [33] conducted by other investigators and a stem cell study conducted by our research group. This IGF-I concentration significantly increases in vitro cell number and matrix synthesis [31,33].

The objective of the study reported here was to compare in vitro growth characteristics of tendon- and bone marrow–derived cells during monolayer expansion. We also assessed matrix production and matrix gene expression of monolayer-expanded cells cultured on acellular tendon matrix. The effects of IGF-I supplementation on gene expression and matrix production were assessed in both cell populations. The hypothesis tested was that tendon-derived cells supplemented with IGF-I would grow and persist on matrix better and produce more tendon ECM than would cells derived from bone marrow.

Materials and Methods:

Samples: Samples of bone marrow and tendon were collected aseptically from 7 young horses (2 to 4 years of age) euthanized for reasons unrelated to musculoskeletal disease. Horses were sedated with xylazine hydrochloride (0.5 to 1.0 mg /kg, IV), and anesthesia was induced by IV administration of ketamine hydrochloride (2.2 mg/kg) and diazepam (0.1 mg/kg). Anesthesia was maintained by IV infusion of a solution of 5% guaifenesin, 1 mg of ketamine/mL, and 1 mg

of xylazine/mL. Bone marrow aspirates were collected as described elsewhere [36]. Then, all horses were euthanized by IV administration of sodium pentobarbital (104 mg/kg, IV). Immediately after the horses were euthanized, both superficial digital flexor tendons were harvested aseptically from each horse. All samples were obtained in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois.

Collection and processing of bone marrow–derived cells: The right tuber coxae was clipped of hair and aseptically prepared. A bone marrow biopsy needle was used to aspirate 15 to 20 mL of bone marrow into syringes that contained 1,000 U of heparin. The bone marrow aspirate was transferred to a centrifuge tube, diluted with 15 mL of PBS solution, and centrifuged at 300 X *g* for 10 minutes. Supernatant was removed, the pellet was resuspended in PBS solution, and centrifugation was repeated. Pelleted cells were resuspended in 12 mL of low-glucose DMEM supplemented with 10% FBS, 300 μg of L-glutamine/mL, 100 U of sodium penicillin/mL, 100 μg of streptomycin sulfate/mL, and 1mM sodium pyruvate/mL. Resuspended cells were placed in a 75-cm^2 flask and incubated at 37°C in a 5% carbon dioxide atmosphere with 90% humidity. To obtain adequate cell numbers for subsequent experiments, bone marrow–derived cells were passaged twice. Time to confluence and cell counts at time of trypsinization were recorded for all cell types at each passage.

Collection and processing of tendon-derived cells: A 6-cm X 1-cm^2 sample of each superficial digital flexor tendon was reserved for cell isolation. A 2-cm^3 sample was snap-frozen in liquid nitrogen for RNA isolation, and the remainder was cryopreserved for cell-free tendon matrix

production. The specimen for cell isolation was diced into small pieces and digested for 16 hours at 37°C in 0.2% collagenase-high-glucose DMEM supplemented with 1% FBS, 100 U of sodium penicillin/mL, and 100 μg of streptomycin sulfate/mL. After digestion, the isolated cells were passed through a 40-μm filter. The isolated cells were collected by centrifugation at 300 X *g* for 5 minutes. Supernatant was removed, and the cell pellet was resuspended in culture medium containing high-glucose DMEM supplemented with 20% FBS, 300 mg of L-glutamine/mL, 100 U of sodium penicillin/mL, and 100 μg of streptomycin sulfate/mL. Cell yields were determined by use of a hemacytometer, and viability was determined via trypan blue dye exclusion [37].

Culture of tendon-derived cells: Progenitor cells were collected from tendon samples by use of previously described preplating procedures [23,32]. Tendon-derived cells were seeded at 13,300 cells/cm^2 in culture flasks in high-glucose DMEM supplemented with 20% FBS, 300 mg of L-glutamine/mL, 100 U of sodium penicillin/mL, and 100 μg of streptomycin sulfate/mL. Rapidly adherent fibroblast-like cells were excluded by differential attachment whereby the culture medium and unattached cells were serially transferred to fresh culture flasks every 24 hours during the first 72 hours of culture [22,38]. Tendon-derived cells that adhered after the transfer at 72 hours were expanded in monolayer for subsequent experiments.

Tendon matrix culture: Samples of superficial digital flexor tendons were cut longitudinally with a dermatome to produce uniformly flat 0.5-mm-thick tendon sheets. These sheets were cut into 1 X 1-cm square explants. The explants were subjected to 4 rounds of freeze-thaw cycles at –80° and 4°C to kill endogenous cells. These cell-free tendon explants (matrix only) were

maintained in culture without the addition of cells to serve as negative control samples or seeded with aliquots (125,000 cells) of the monolayer-expanded bone marrow– or tendon-derived cells.

Experiments comprised 5 treatment groups (matrix only, matrix and bone marrow–derived cells, matrix and bone marrow–derived cells with IGF-I, matrix and tendon-derived cells, and matrix and tendon-derived cells with IGF-I). Each treatment group had 14 replicates. The 14 replicates were distributed into 3 samples for cell numbers, 3 samples for collagen synthesis, 5 samples for GAG synthesis, and 2 samples for histologic evaluation. The mean of all replicates was calculated to yield a single data point for each horse. Total RNA was isolated from 3 of the 7 horses. Treatment groups established for RNA isolation had an additional 12 replicates. There were 70 to 130 samples for each of the 7 horses.

Cell-seeded tendon matrices were supplemented with 1 mL of tenogenic media that contained 0 or 100 ng of IGF-I. Tenogenic media consisted of high-glucose DMEM supplemented with 10% FBS, 37.5 μg of ascorbic acid/mL, 300 μg of L-glutamine/mL, 100 U of sodium penicillin/mL, and 100 μg of streptomycin sulfate/mL. Media were changed every 2 days. All culture samples were collected on day 7 (day 0 was the first day of cell culture).

Cell numbers on matrices: Three cell matrices from each treatment group were used to measure cell number on day 7 via a mitochondrial metabolic assay, which was used in accordance with the manufacturer's instructions. In brief, 50 μL of the assay reagent containing tetrazolium was added to fresh media in each well, and wells were incubated at 37°C for 2.5 hours. One hundred microliters of media from each well was transferred to a 96-well plate, and absorbance was

measured at 492 nm in a microplate reader to detect concentrations of the metabolic product, formazan. All samples were assayed in duplicate, and a mean value was calculated to provide a single data point. These optical density values were converted to cell numbers by reference to standard curves determined from plated tendon- and bone marrow–derived cells from each horse.

Collagen synthesis: Collagen synthesis was determined by measuring [^{3}H] proline^o incorporation by use of a published protocol [39]. On day 6, 3 cell matrices of each treatment group were radiolabeled with 50 μCi of [^{3}H] proline/mL of tenogenic medium and incubated for 24 hours. The samples were washed 3 times with 0.5 mL of PBS solution containing 1mM proline and stored at –80^{o}C. Radiolabeled samples were freeze-thawed 3 times, digested, and homogenized to disrupt cells and matrix prior to RNase treatment. Total protein was precipitated with tricholoroacetic acid and washed 3 times with L-proline to remove traces of unincorporated [^{3}H] proline. The resulting pellets were digested with purified collagenase, and centrifuged at 3,220 X *g* for 10 minutes. Supernatant and the pelleted material were separated and added to scintillation liquid, and radioactivity was counted by use of a scintillation counter. Newly synthesized collagen was detected on the basis of radioactivity in the sample supernatants following collagenase digestion [39]. Collagen synthesis was normalized for background amounts by subtracting the number of disintegrations per minute of the explant-only control samples. Values were expressed as the number of disintegrations per minute per explant seeded with 125,000 cells.

GAG synthesis: Synthesis of GAG was determined by measuring $^{35}SO_4$ incorporation into each sample. Five cell matrices of each treatment group were radiolabeled with 10 μCi of ^{35}S-labeled

sodium sulfate/mL during the last 24 hours of the experiment. Samples were washed 3 times with PBS solution and then digested in 1 mL of buffer that contained 0.5 mg of papain at 65°C for 16 hours [40]. Aliquots (25 mL) of ^{35}S-labeled papain-digested tendon matrices were placed in multiwell punch plates, precipitated with alcian blue dye, and counted by use of scintillation. All CPM values were adjusted for radioisotope decay from the time of radiolabeling to assay. Proteoglycan synthesis was normalized for background amounts by subtracting the CPM of the explant-only control samples. Values were expressed as the number of CPM per explant seeded with 125,000 cells.

RNA isolation: Sufficient numbers of cells were generated from 3 horses to support transcriptional analyses. Twelve cell matrices from each treatment group were pooled, snap-frozen in liquid nitrogen, and stored at –80°C for RNA isolation. The RNA was isolated by use of a protocol adapted from a technique for cartilage RNA isolation [41]. Briefly, tissues were pulverized under liquid nitrogen, homogenized in guanidinium isothiocyanate lysis buffer, extracted with phenol-chloroform, precipitated with isopropanol, and purified by use of a commercially available column-based procedure. This procedure included an on-column DNase treatment to exclude contamination of the genomic template.

Real-time PCR gene expression: One microgram of RNA from each sample was converted to cDNA by use of a commercial reverse transcription kit and oligo(dT) primers. Target cDNAs were amplified via real-time PCR assay by use of *Taq* DNA polymerase and gene-specific primers designed by use of a multiple sequence alignment program from published sequences available in GenBank. Primer specificity was confirmed by cloning and sequencing the PCR

products. Real-time quantitative PCR was performed in triplicate for the equine specific primers collagen type I, collagen type III, and COMP mRNAs and the reference gene, elongation factor-1α. The generated amplicons were quantified by incorporation of a fluorescent dye by use of a commercial fluorescent PCR detection system. To relate in vitro expression to in vivo expression, RNA from freshly collected, snap-frozen tendon samples was used as a reference for gene expression analyses. Relative gene expression was quantified by use of the $2^{-\Delta\Delta CT}$ method [42].

Gene expression on northern blots: Northern blot analyses of collagen type I, collagen type III, and COMP mRNAs were performed on samples obtained from 1 horse. Gel electrophoresis and northern blot analyses of RNA samples were performed in accordance with standard protocols [43]. Radiolabeled probes were synthesized from gel-purified partial cDNA templates by use of ^{32}P-dCTP and random 9-mer primers and were purified through spin columns. Consistency of RNA sample loading was assessed via electrophoresis followed by ethidium bromide staining of the gels. The RNAs were transferred to nylon hybridization membranes via capillary transfer by use of a high-salt buffer, as described elsewhere [43]. Elongation factor-1α was used for normalization of gene expression. Prehybridization, hybridization, and wash conditions were as described in protocols recommended by the manufacturer of the nylon membranes. Northern blot data were quantified by use of a computer workstation and commercially available software.

Histologic examination: Two tendon samples from each treatment group were fixed in 4% paraformaldehyde and embedded in paraffin in accordance with routine protocols. Sections

(thickness of 6 μm) were stained with H&E for evaluation of the cell layers colonizing the matrix surfaces.

Statistical analysis: Mean ± SE value for each variable was calculated for each cell type and IGF-I supplementation status for tendon samples obtained from each of the 7 horses in the study. Background values detected in the matrix-only group were subtracted from values for the other groups. Data for cell number, GAG synthesis, and collagen synthesis were logarithmically transformed to accommodate between-horse variability. In addition, collagen type I, collagen type III, and COMP mRNA expression were normalized to expression of the reference gene, elongation factor-1α. The effects of cell type and IGF-I supplementation were evaluated by use of generalized estimating equations, which is a method robust to violation of assumptions required for a repeated-measures ANOVA [44]. When group differences were detected, pairwise multiple comparisons were conducted by use of nonparametric tests. A statistical program was used to perform statistical analyses. Values of $P \leq 0.05$ were considered significant. Mean ± SE values were determined for days to confluence and cell number following monolayer expansion of bone marrow– and tendon-derived cells.

Results:

Cell isolation and expansion: After the completion of preplating procedures and cell attachment, the cells from bone marrow and tendon proliferated in focal clones of tightly packed cells with fusiform morphologic characteristics. During the first passage, bone marrow–derived cells reached focal confluence significantly ($p = 0.006$) more rapidly than did tendon-derived cells (**Table 4.1**). However, during the second passage, tendon-derived cells reached confluence

significantly ($p = 0.018$) more rapidly than did bone marrow–derived cells. After 2 passages, there was no significant ($p = 0.12$) difference for the number of days to confluence between bone marrow– and tendon-derived cells. Both cell types required 17 to 19 days to complete 2 passages. Cell numbers at the first and second passage were significantly ($p = 0.01$ and 0.004, respectively) higher for tendon-derived cells than for bone marrow–derived cells. Tendon-derived cell expansion yielded significantly ($p = 0.004$) more cells (mean of approximately 6.7 X 10^6 more cells), compared with the number of cells in bone marrow–derived cell cultures.

Cell numbers on matrices: After culture for 7 days, the mean $\log_{10}$ numbers of cells adherent to the acellular tendon matrices were significantly ($p < 0.001$) increased in the tendon-derived cell groups, compared with the numbers in the bone marrow–derived cell groups (**Figure 4.1**). In addition, cell numbers in the IGF-I–treated groups were significantly ($p = 0.002$) increased, compared with the cell numbers in cell groups cultured without IGF-I. Evaluation of cell numbers for the cell-free matrix-only group revealed that there were no viable cells in these control cultures. Results of statistical analysis for transformed and untransformed data were summarized (**Table 4.2**).

Collagen synthesis: Mean $\log_{10}$ collagen synthesis was significantly ($p < 0.001$) increased in the tendon-derived cell groups, compared with synthesis in the bone marrow–derived cell groups (**Figure 4.2**). Treatment with IGF-I significantly ($p = 0.01$) increased the mean $\log_{10}$ collagen synthesis of tendon-derived cells. There was no significant effect of IGF-I supplementation on collagen synthesis by bone marrow–derived cells. Results of statistical analysis for transformed and untransformed data were summarized (**Table 4.2**).

GAG synthesis: Mean $\log_{10}$ GAG synthesis was significantly ($p = 0.001$) increased in the tendon-derived cell groups, compared with GAG synthesis in the bone marrow–derived cell groups (**Figure 4.3**). Treatment with IGF-I significantly ($p < 0.001$) increased the mean $\log_{10}$ GAG synthesis in both the tendon- and bone marrow–derived cell groups. Results of statistical analysis for transformed and untransformed data were summarized (**Table 4.2**).

mRNA expression in the ECM: No mRNA was isolated from the acellular control tendon matrices that were subjected to 4 freeze-thaw cycles. Sufficient quantities of RNA for further analyses were obtained from only 3 of 7 horses in the study because of inherent difficulty in isolating RNA from a relatively small number of viable cells within a large volume of dense, acellular matrix. Quantitative PCR analyses of collagen type I mRNA expression in the cell-matrix groups revealed no significant differences between bone marrow– and tendon-derived cell groups ($p = 0.08$) or in response to IGF-I administration ($p = 0.08$; **Table 4.3**). Similarly, there was no significant difference in collagen type III expression between tendon-derived cell groups ($p = 0.95$) or in response to IGF-I ($p = 0.32$). Although tendon-derived cells expressed 3- to 5-fold more COMP mRNA than did bone marrow–derived cells, the expressions did not differ significant ($p = 0.16$). Supplementation with IGF-I did not significantly ($p = 0.80$) affect COMP mRNA expression.

Northern blot analyses of collagen types I and II and COMP mRNAs from total RNA isolated from cells collected from 1 horse had expression profiles that were consistent with the real-time PCR results (**Table 4.4**).

Histologic examination: In all cell-treated groups, the cells were predominantly adherent to the surface of the autogenous matrices (**Figure 4.4**). Tendon-derived cells were present in higher numbers than were bone marrow–derived cells, and IGF-I treatment increased matrix-associated cell numbers in both cell types, which was consistent with the cell count data. In addition, tendon-derived cells appeared to have a more elongate tenocyte-like appearance, compared with the appearance of the bone marrow–derived cells.

Discussion:

In the study reported here, initial yields of tendon-derived cells were significantly higher than were yields from bone marrow aspirates, and tendon-derived cells were easier to culture during first passage. Tendon- and bone marrow–derived cell cultures both required approximately 17 to 19 days to reach second passage confluence. However, after 2 passages, sufficient numbers of tendon-derived cells were generated for most cell-based treatments that have been used in the treatment of tendinitis [15,19]. These results are similar to those of another study [22] in which more cells were obtained at confluence from tendon-derived cell cultures than from bone marrow–derived cell cultures. It is possible that bone marrow–derived cells could have provided higher cell yields with different isolation techniques, such as density gradient centrifugation and initial RBC lysis [45,46]. Although greater numbers of tendon-derived cells can be obtained in a shorter period, donor site morbidity remains a concern. These concerns were addressed in a recent study in which our research group evaluated tendon-derived cells obtained from the lateral digital extensor tendon as a clinically relevant sample. To our knowledge, there have been no adverse effects of collecting tissue from the lateral digital extensor tendon for at least 4 months

after tenectomy. Longer follow-up evaluation of the lateral digital extensor tenectomy site in clinically normal horses is warranted prior to use in clinically affected horses.

In the present study, tendon-derived cells persisted on the acellular matrix and continued to proliferate during the 7-day culture period. In contrast, bone marrow–derived cell numbers did not change from the original seeding density. Supplementation with IGF-I increased (2-fold increase) tendon- and bone marrow–derived cell numbers, compared with cell numbers for unsupplemented cultures. The IGF-I–mediated increase in cell numbers could have resulted from an increase in in cell survival rate or cell proliferation. Investigators in several other in vitro studies [31-33] have detected similar increases in tenocyte cell numbers with IGF-I supplementation. In a recent study [47] it was found that IGF-I supplementation increases cell numbers by reversing cell cycle arrest, which suggests that cell proliferation was a factor in the increase in cell numbers in the study reported here.

Considerable increases in collagen and GAG synthesis were detected in tendon-derived cells, compared with results for bone marrow–derived cells, which was consistent with results of another study [22]. Supplementation with IGF-I further increased matrix synthesis by tendon-derived cells, compared with synthesis in unsupplemented control samples. These results are similar to those of several in vivo and in vitro studies [15,31-33] in which investigators detected IGF-I enhancement of tendon matrix synthesis. Some of these in vitro anabolic effects of IGF-I are mediated through increases in mitogenesis, with consequent increases in population-wide matrix synthesis. However, it should be mentioned that the matrix synthesis results in the present study were expressed in terms of cell numbers at the start of the experiments and did not account

for possible changes in cell numbers as a consequence of differential persistence or proliferation during the course of the experiments. Further evaluation is required before specific conclusions can be drawn regarding the biosynthetic capacities of these cell types and their responses to IGF-I supplementation.

In the present study, there were no significant differences in expression of collagen type I, collagen type III, and COMP mRNAs by cell type or in response to IGF-I supplementation. However, values of $P > 0.05$ but < 0.10 were obtained for collagen type I (with and without IGF-I supplementation) and collagen type III (without IGF-I supplementation). The mRNA data in the present study were derived from samples of only 3 horses, and this may have been an insufficient sample size to adequately assess differences in mRNA concentrations because of low statistical power. In addition, results from another study [22] conducted by our research group revealed significantly greater collagen type III expression by tendon-derived cells than by bone marrow–derived cells (data derived from samples of 4 horses). In that study [22], there was no difference in collagen type I or COMP mRNA expression in these cell types. Furthermore, the results of the present study are similar to those of other studies [33,48] in which IGF-I supplementation had minimal effects on ECM gene expression despite overall increases in tendon matrix synthesis. Regardless, increases in matrix synthesis per explant are a more important outcome variable than are changes in mRNA expression.

Results from the present study support the hypothesis that tendon-derived cells supplemented with IGF-I grow and adhere to acellular matrix better and produce more ECM than do cells derived from bone marrow. These results suggest that tendon-derived cells may be a better

source for cells used in repair of tendon injuries. Supplementation with IGF-I enhances cell persistence and proliferation and matrix synthesis. Results of this study support further in vivo evaluation of tendon-derived cells and IGF-I for use in tendon repair.

Tables and Figures:

Variable	Bone marrow	Tendon
No. of days to passage 1	12.8 ± 0.5	17.3 ± 0.3*
No. of days to passage 2	4.5 ± 1.0	2.3 ± 0.3*
Total No. of days to passage 2	17.3 ± 0.9	19.5 ± 0.5
No. of cells at passage 1 (X 10^6)	0.28 ± 0.75	1.18 ± 0.22*
No. of cells at passage 2 (X 10^6)	0.94 ± 0.06	6.30 ± 0.99*
Total No. of cells for passage 1 and 2 (X 10^6)	1.21 ± 0.11	7.95 ± 0.86*

Table 4.1—Mean ± SE values for number of days to passage 1 and 2 and number of cells at the time of passage during monolayer culture of bone marrow– and tendon-derived cells.

Values represent results for samples obtained from 7 horses.
*Value differs significantly ($P \leq 0.05$) from the value for bone marrow–derived cells.

Variable	Bone marrow Without IGF-I	Bone marrow With IGF-1	Tendon Without IGF-1	Tendon With IGF-I
No. of cells/explant	85,649 ± 43,619	190,924 ± 87,173	215,430 ± 100,923	472,970 ± 105,612
Log_{10} No. of cells/explant	4.81 ± 2.60	8.21 ± 2.36*	10.03 ± 1.85†	12.93 ± 0.21*†
Collagen synthesis (DPM/ explant)	52,361 ± 21,870	42,545 ± 11,574	61,418 ± 11,956	99,987 ± 20,172
Log_{10} collagen synthesis (Log_{10} DPM/explant)	10.37 ± 0.52	10.40 ± 0.37	10.87 ± 0.29†	11.29 ± 0.37*†
GAG synthesis (CPM/explant)	6,232 ± 1,574	12,776 ± 5,191	25,605 ± 8,764	49,133 ± 16,520
Log_{10} GAG synthesis (Log_{10} CPM/explant)	8.41 ± 0.39	8.98 ± 0.42*	9.86 ± 0.30†	10.53 ± 0.30*†

Table 4.2—Mean ± SE values for number of cells, collagen synthesis, and GAG synthesis for equine bone marrow– and tendon-derived cells cultured with and without IGF-I on a tendon matrix for 7 days.

Values represent results for samples obtained from 7 horses.
*Within a row, value differs significantly ($P \leq 0.05$) from the value without IGF-I. †Within a row, value differs significantly ($P \leq 0.05$) from the corresponding value for bone marrow–derived cells.
DPM = Disintegrations per minute.

Variable	Tendon Without IGF-I	With IGF-I	Bone marrow Without IGF-I	With IGF-I
Collagen type I	1.00 ± 0	1.76 ± 0.22	2.12 ± 0.36	2.09 ± 0.56
Collagen type III	1.00 ± 0	1.27 ± 0.62	0.96 ± 0.54	1.07 ± 0.48
COMP	1.00 ± 0	1.15 ± 0.50	0.373 ± 0.28	0.213 ± 0.10

Table 4.3—Results for real-time PCR evaluation of mRNA expression of bone marrow– and tendon-derived cells after culture with and without IGF-I on a tendon matrix for 7 days.

Values reported are mean ± SE; values for mRNA expression of collagen type I, collagen type III, and COMP were normalized on the basis of expression for elongation factor-1α. Values represent results for samples obtained from 3 horses. A physiologically normal tendon was used as a positive control sample.

Variable	Control tendon*	Tendon Without IGF-I	With IGF-I	Bone marrow Without IGF-I	With IGF-I
Collagen type I	1.06	1.64	1.57	1.49	2.11
Collagen type III	0.39	3.66	4.32	4.32	3.54
COMP	0.81	0.83	0.70	0.82	0.89

Table 4.4—Results for northern blot evaluation of mRNA expression for bone marrow– and tendon-derived cells after culture with and without IGF-I on a tendon matrix for 7 days.

Values reported are the mean mRNA expression of collagen type I, collagen type III, and COMP after normalization on the basis of expression for elongation factor-1α. Values represent results for samples obtained from 3 horses.
*Physiologically normal tendon (positive control sample).

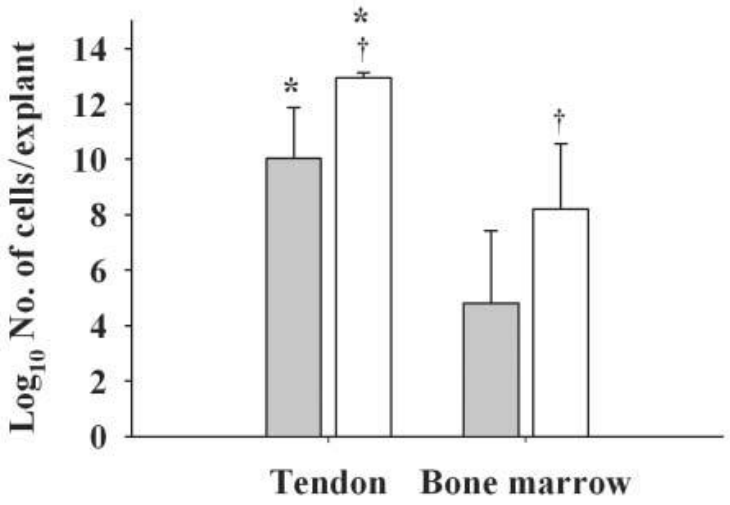

Figure 4.1—Log_{10} mean ± SE cell numbers for tendon- and bone marrow–derived cells cultured on tendon matrix for 7 days with IGF-I (100 ng/mL [white bars]) or without IGF-I (gray bars). Values represent results for samples obtained from 7 horses.

*Value differs significantly ($P \leq 0.05$) from the corresponding value for the bone-marrow–derived cells. †Within a cell type, value differs significantly ($P \leq 0.05$) from the value for cells cultured without IGF-I.

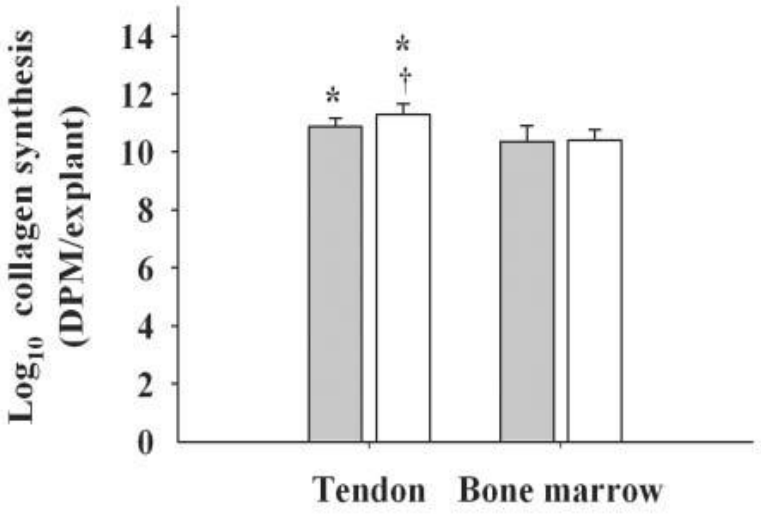

Figure 4.2—Log_{10} mean ± SE disintegrations per minute (DPM) as a measure of incorporation of [^{3}H] proline into collagen of the tendon matrix after seeding with tendon- and bone marrow–derived cells and culture for 7 days.

Values represent results for samples obtained from 7 horses.

See Figure 4.1 for key.

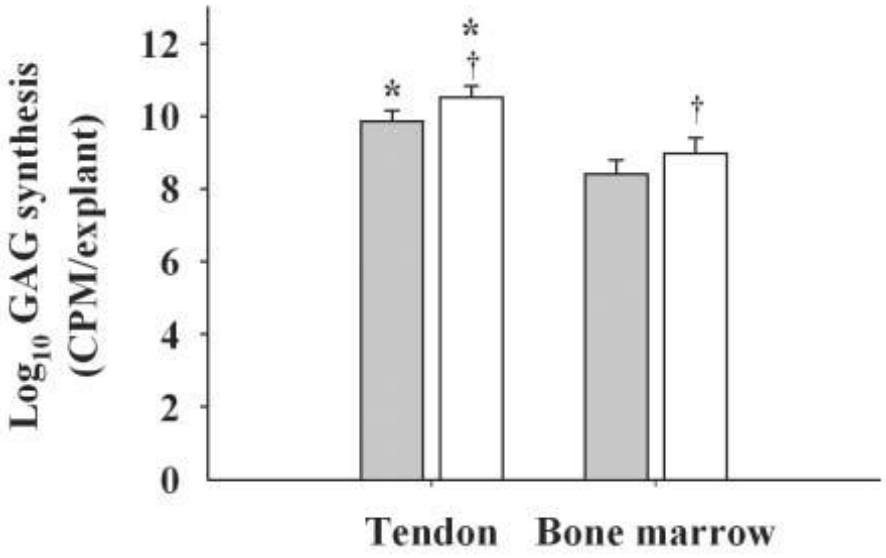

Figure 4.3—Log_{10} mean ± SE CPM as a measure of incorporation of ^{35}S-labeled sodium sulfate into GAG of the tendon matrix after seeding with tendon- and bone marrow–derived cells and culture for 7 days.

Values represent results for samples obtained from 7 horses.
See Figure 1 for key.

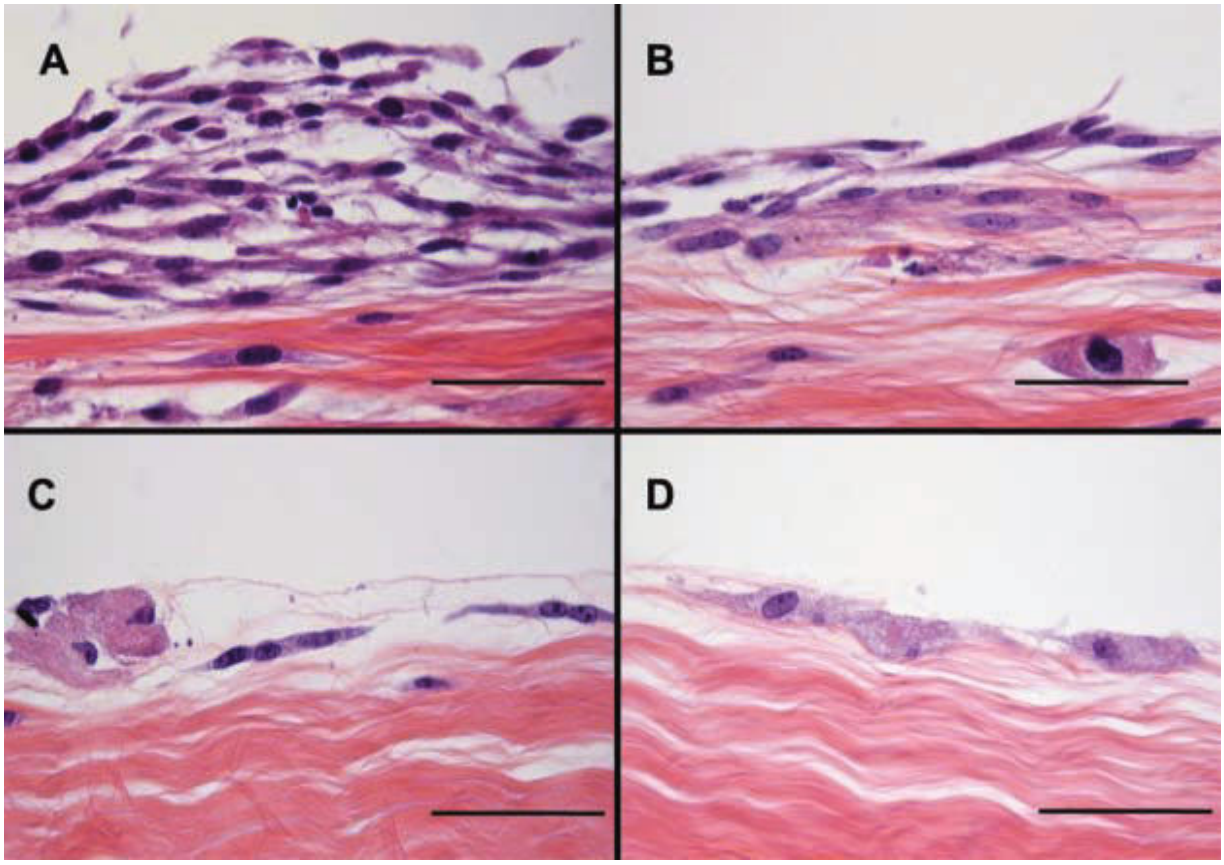

Figure 4.4—Photomicrographs of sections of tendon matrix seeded with equine bone marrow–derived cells and cultured with (A) and without (B) IGF-I for 7 days or with tendon-derived cells and cultured with (C) and without (D) IGF-1 for 7 days. All cells are adhered to the surface of the acellular matrix. Subjectively, tendon-derived cells had a higher number of cells arranged in a linear fashion, compared with results for bone marrow–derived cells. Tendon-derived cells cultured with IGF-I had the highest number of cells, compared with results for the other treatment groups. H&E stain. Bar = 50 μm.

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CHAPTER 5: TENDON-DERIVED PROGENITOR CELLS IMPROVE HEALING OF COLLAGENASE-INDUCED FLEXOR TENDINITIS

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Introduction

Tendinitis/tendinopathy is a common and debilitating injury in athletes, manifesting acutely or as chronic strain type injury. Achilles tendons in humans and superficial digital flexor (SDF) tendons in horses are functionally analogous and have a high-energy storing capacity in tension. At high speeds, these tendons function close to their maximal capacity and are, therefore, prone to strain injury [1]. SDF tendon injuries in equine athletes are a common cause of lameness with incidence rates of 11-30% [2]. Similarly, up to 75% of Achilles injuries in humans are related to athletic activities [3,4]. Tendons in general have a poor healing capacity, and require prolonged rehabilitation after treatment of the acute injury.[5] Regardless, the resultant repair tissue is biomechanically inferior to the original tendon, and therefore recurrence rates can be as high as 30% [1,4,5]. This has stimulated a number of studies focused on cell-based and/or biologic therapies to improve tendon healing.

Although progenitor cells share common characteristics of clonogenecity, multipotentiality and self-renewal, they also retain source-specific characteristics which likely influence their responses in target tissues [6,7]. Intuitively, progenitor cells derived from the 'low-cell, collagen-

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rich' environment of tendons may be more efficacious for treating tendinitis than other cell populations [8]. Tendon-derived progenitor cells (TDPCs) have been characterized in humans[8] and rodents [8,9]. Allogeneic TDPC administration in rodent models of tendon injury improved histologic and biomechanical indices of repair [10,11]. In vitro, equine TDPCs exhibited greater colonization, proliferation and matrix synthesis than bone marrow-derived mesenchymal stem cells (BM-MSCs) when cultured on acellular tendon matrices [12]. The objective of this study was to evaluate the efficacy of autogenous TDPC injections in a large animal model of flexor tendinitis using transcriptional, biochemical, histological and biomechanical outcomes.

Methods:

Study Design: All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Eight young adult horses (2-4 years of age) free of musculoskeletal disease were used for this 16-week study. On the first day of the study, both forelimb SDF tendons were injected with collagenase, as detailed below. Four weeks later, one randomly assigned forelimb SDF tendon was injected with TDPCs, and the contralateral SDF tendon was injected with saline. In each horse, a randomly selected hindlimb SDF tendon was used to monitor the survival and distribution of TDPCs, 1, 2, 4 and 6 weeks after administration (2 horses at each time point; **Figure 5.1**). The remaining untreated hindlimb SDF tendon in each horse was used as a within-animal 'normal' control. The study was terminated after 16 weeks; corresponding to 12 weeks after the forelimb TDPC/saline injections.

Collagenase Model: Both forelimb SDF tendons were injected with sterile collagenase (Worthington Biochemical Corporation, Lakewood, NJ). One thousand units of collagenase were

injected at 2 sites, 3 cm proximal and 3 cm distal to the center of the metacarpal region, with ultrasonographic guidance, as previously described [13-15]. Within 24 hours of collagenase injections, there was marked swelling of the SDF tendon, and the tendons were painful on palpation. Horses were lame at a walk for the first post-injection week; however, the lameness and local signs of pain responded favorably to systemic anti-inflammatories and was resolved by the second week. All horses' distal limbs were bandaged and the horses were stall-rested for the subsequent two months.

Collagenase lesions were also created in a randomly selected hindlimb SDF tendon of each horse at 5, 6, 8 and 10 weeks before the termination of the study (two horses at each time point). These time points were chosen to assess survival and distribution of TDPCs at the site 1, 2, 4 and 6 weeks after injection.

TDPC Isolation and Culture: At the time of forelimb collagenase injections, lateral digital extensor (LDE) tendon was harvested from one of the hindlimbs, under regional analgesia.[16] A 4-cm linear skin incision was made on the lateral surface of the proximal metatarsus starting at the distal insertion of the LDE tendon and extended proximally. A 0.5 x 1.0 cm^2 section of LDE tendon was collected and diced into 0.25-cm^3 and digested in 0.2% collagenase in DMEM supplemented with 2% fetal bovine serum at 37^oC for 16 hours [16]. The cells were isolated by filtration and centrifugation and the cells were seeded at 10,000 cells/cm^2 in monolayer cultures in high-glucose DMEM supplemented with 20% fetal bovine serum, 37.5 μg/ mL of ascorbic acid, 300 μg of L-glutamine/mL 100 U of sodium penicillin/mL, and 100 μg of streptomycin sulfate/mL. The rapidly adherent, fibroblast-like cells were excluded by transferring the

unattached cells to fresh culture flasks after 24 hrs. The transferred cells were grown to confluence, and then passaged twice. TDPCs from the confluent, second passage cultures were used for intra-lesional injections. The multipotentiality of these expanded cells was tested using standard adipogenic, osteogenic and chondrogenic assays, as previously described [8,9].

Cell Labeling: For the TDPC distribution and survival assessment, 5 µL of 1,1'dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine percholate (DiI); (Molecular Probes, Eugene, OR) was added to each 1 x 10^6 cell aliquot in 1 mL of HBSS and incubated for 30 minutes at 37 ^{0}C.[17] The cells were then washed four times with HBSS to remove excess dye prior to injection. Post-labeling cell viability was confirmed via trypan blue (Sigma-Aldrich, St. Louis, MO) exclusion test.

TDPC Injections: Four weeks following collagenase injections, one randomly selected forelimb of each horse was injected with 5 x 10^6 TDPCs in 0.15 mL of sterile phosphate buffered saline (TDPC group) at the two sites (1 x 10^7 cells in total) corresponding to collagenase injection, under ultrasonographic guidance [13,15]. The contralateral forelimb SDF tendon was injected with an equal volume of saline (saline group) in a similar manner. For the following month, the horses were hand-walked for 5 minutes once a day. During the fourth month, all horses were hand-walked for 10 minutes once a day.

The lesions in the hind SDF tendons were injected with 5 x 10^6 DiI-labeled TDPCs (as described above) at 1, 2, 4 or 6 weeks (n=2 at each time point) prior to termination of the study.

Tendon Harvesting and Sample Allocation: All horses were euthanized with pentobarbital overdose 12 weeks after the forelimb TDPC and saline injections. The SDF tendons were collected sterilely immediately after euthanasia. Both forelimb and the normal hindlimb SDF tendons were axially divided. One half of each tendon was frozen at -80^0 C for biomechanical testing. The repair tissue in the remaining hemi-tendon was sectioned transversely into 4 specimens, all from within the grossly pathological tissue, and allocated to endpoint analyses as depicted in **Figure 5.2**. The specimens were snap-frozen in liquid nitrogen for RNA isolation, biochemical assays, fixed in OCT compound or 4% paraformaldehyde for histologic processing. Macroscopically injured regions of the hind SDF tendons (injected with the DiI-labeled TDPCs) were sectioned transversely and fixed in OCT compound for histologic processing.

RNA Isolation and Quantitative RT-PCR: RNA was isolated by a previously described protocol [12]. Briefly, tendons were pulverized under liquid nitrogen, homogenized in guanidinium isothiacyanate lysis buffer, extracted in phenol-chloroform and precipitated in isopropanol. The resultant pellet was purified using RNeasy column-based protocol that included an on-column DNase digest. One μg of RNA from each sample was reverse-transcribed (Superscript II, Invitrogen, Carlsbad, CA) using oligo(dT) primers. Gene-specific primers for collagen types I and III, cartilage oligomeric matrix protein (COMP) and tenomodulin were designed from published sequences in Genbank, and using ClustalW multiple sequence alignment (available at www.ebi.ac.uk). Primer specificity was confirmed by cloning and sequencing the amplicons during optimization experiments, as previously described (**Table 5.1**) [16]. PCR amplifications were catalyzed by Taq DNA polymerase (BioRad iCycler, Bio-Rad Laboratories, Hercules, CA) in the presence of Sybr green. Relative gene expression was

quantified using the $2^{-\Delta\Delta CT}$ method, normalized to expression of the reference gene, elongation factor-1α (EF1α) [18].

Biochemical Assays: Ten mg of tendon tissue was digested overnight in 0.1% papain (Sigma, St. Louis, MO) at 65° C. Sulfated glycosaminoglycan (sGAG) content was measured by the dimethylmethylene blue (DMMB) dye-binding assay, as previously described, using ovine chondroitin sulfate to generate a standard curve [19]. DNA content was determined by fluorometric measurement of Hoechst 33258 (Sigma, St. Louis, MO) dye incorporation [19].

Total soluble collagen content was determined using a commercially available kit (Biocolor, Carrickfergus, County Antrim, UK) following overnight digestion of 10 mg of tendon samples in 1.3mg/mL pepsin [14].

Biomechanical Testing: The tendon specimens were thawed to room temperature prior to testing. Each tendon was sectioned axially in the longitudinal plane to generate two samples (each comprising one quarter of the tendon), to ensure secure anchorage and assess biomechanical variability. Mean cross-sectional area of each sample was calculated by averaging three separate measurements taken at the injured region with vernier calipers. The quarter-tendon specimens were tested individually and the data were averaged to provide single values for each sample. The tendon ends were secured in a hydraulic testing device (Instron 8511, Norwood, MA) using custom-designed, serrated clamps, with the lesion centered between the clamps.[21] The SDF tendon specimens used for biomechanical testing ranged from 35-40 cm. Between 6 and 8 cm at each end was engaged in the serrated clamps, leaving 10-15 cm gauge length. The

gross tendon lesions were 8-10 cm in length, comprising 60-80% of the tissue between the clamps. All tendons were pre-conditioned at 1% strain for 10 cycles prior to tensile testing. The tendons were loaded to failure in tension at a strain rate of 2.5%/second. Load-displacement values were acquired at 10 Hz. Displacement was measured as change in grip-to-grip distance. These values were normalized to the mean cross-sectional area of the samples to obtain stress-strain data. Yield stress and maximum stress (N/cm^2 = MPa) were calculated by dividing the yield load (N) and maximum load (N) by the mean cross-sectional area (cm^2). The slopes of the linear portion of the stress-strain curves were used to calculate elastic modulus.

Histology: The paraformaldehyde-fixed samples were paraffin-embedded using standard protocols. Sections were cut to 6-μm and stained with Picro-Sirius Red (Sigma, St. Louis, MO) or Toluidine Blue (Sigma, St. Louis, MO). The OCT-preserved samples were sectioned at -20^0C to 10-μm using a cryostat (Leica CM3050S, Ramsey, MN) for SHG, DiI and DAPI imaging.

Fluorescence microscopy: The cryo-sections from the hind SDF tendons injected with DiI-labeled TDPCs were mounted with diamidino phenyl indole (DAPI; Vector Laboratories, Burlingame, CA) for nuclear fluorescence. The sections were imaged with a fluorescence microscope (Zeiss Axiovert 200M, Peabody, MA) with the apotome-structured illumination optical sectioning system. Excitation wavelengths of 358 nm and 459 nm were used for DAPI and DiI respectively.

Second Harmonic Generation (SHG) microscopy and Fourier Transformation (FT): SHG images were obtained from longitudinal cryosections via confocal microscopy as previously

described [22]. Five cryosections were made from the mid-sections of each tendon specimen (normal, saline and TDPC) from each horse. SHG images were obtained from 5 random fields in each section. Collectively, 200 data points were used to compare SHG-derived indices of collagen alignment in the three groups. A modified confocal system (Zeiss LSM 710, Peabody, MA) equipped with a tunable Ti:Sapphire laser source that produced 70 fs pulses (80MHz repetition rate) was used to image the samples. An excitation wavelength of 780 nm was used to generate SHG emission from collagen fibers at all orientations. The beam was reflected by a short-pass 760 nm dichroic mirror and focused onto the sample using a 40X water-immersion objective. The backward SHG emissions were collected by the same objective. The images were Fourier-Transformed using imageJ software (http://rsweb.nih.gov/ij/). A gray-scale image representative of spatial distribution of collagen fiber orientation was obtained, as previously described [22]. A single value (in degrees) for the mean orientation of the collagen fibers, was calculated from each group, with '90^{0}' representing consistent longitudinal alignment.

Statistical Analyses: All data were normally distributed. Descriptive statistics were reported as mean and standard error of the mean (SE). The treated tendons were compared to each other as well as the control tendon using repeated measures one-way analysis of variance (ANOVA) and, where indicated, Holm-Sidak post hoc comparisons. For all analyses, SigmaStat 4 software (Systat Software, San Jose, CA) was used. The p values reported in our results represent differences obtained from post-hoc comparisons. A p value ≤ 0.05 was considered statistically significant.

Results:

Cell Survival and Distribution (Figure 5.3): DiI fluorescence of the labeled TDPCs was visualized along with nuclear DAPI fluorescence in the hindlimb SDF tendon cryosections. The TDPCs were present in large clusters at the injection sites, 1 week after administration. At 2 weeks, there were substantially fewer TDPCs remaining at the injection sites, and they appeared to be linearly distributed along with the native tenocytes. Very few labeled TDPCs were present at the 4-week time point, and no labeled cells were detectable by 6 weeks.

Gene Expression: Collagen type I, COMP and tenomodulin mRNA levels were significantly increased in both TDPC and saline groups compared to the normal tendon (**Table 5.2**). There were no significant differences between the experimental groups. Collagen type III mRNA levels were also substantially higher in the TDPC (approximately 12-fold) and saline (approximately 20-fold) groups but, because of considerable inter-animal variability, these differences did not reach statistical significance (**Table 5.2**).

Biochemical Analyses: DNA content, reflecting cellularity, was significantly higher (approximately 20%) in both the TDPC and saline groups, compared to normal controls. However, the total collagen and sGAG contents of the experimental groups were not different from those of normal tendon (**Table 5.3**).

Biomechanical Properties: The results of the quarter-tendon tests from each specimen were highly similar. All tendons failed within the gauge length at the healthy-damaged tissue interface. Grossly, failure occurred through longitudinal extension of the interfaces between

collagen bundles until complete separation occurred (Supplementary video), as opposed to an abrupt transversely oriented failure. The mean cross sectional area of the normal tendons (0.8 $\pm$ 0.01 cm^2) was significantly less than the areas of the TDPC (2.29 $\pm$ 0.2 cm^2; $p = 0.02$) and saline (2.57 $\pm$ 0.28 cm^2; $p = 0.024$) groups. Yield stresses of the TDPC-injected and normal tendon were statistically similar ($p = 0.3$) and significantly higher than the saline group ($p = 0.005$; **Figure 5.4**). Similarly, the maximum stresses of the TDPC-injected and normal tendon were statistically similar ($p = 0.07$) and significantly higher than the saline group ($p < 0.001$; **Figure 5.4**). The elastic modulus of TDPC and saline groups were statistically similar ($p = 0.2$) and both were significantly lower than normal tendons ($p = 0.002$, and $p < 0.001$, respectively; **Figure 5.4**). Similarly, the stiffness of TDPC (10,200 $\pm$ 546 N/cm) and saline (9,600 $\pm$ 432 N/cm) groups were significantly lower than normal tendons (14,032 $\pm$ 945 N/cm; $p < 0.001$ in both comparisons) and not significantly different ($p = 0.2$) from each other.

Histology: As shown in Picro Sirius Red-stained sections, the collagen microarchitecture of the TDPC tendons was more longitudinally aligned and the crimp pattern more organized than the saline group (**Figure 5.5**). Sulfated GAG distribution in the Toluidine Blue-stained sections of the TDPC group tendons was comparable to normal tendons, whereas metachromasia was more apparent in the saline-injected tendons (**Figure 5.6**). Consistent with these observations, Fourier Transform analyses from the SHG images (**Figure 5.7**) demonstrated that collagen fibers were significantly ($p = 0.03$) more aligned in TDPC sections (mean = 105.67 $\pm$ 8.3 degrees) than in saline sections (mean = 114.7 $\pm$ 16.2 degrees) and similar ($p = 0.1$) to values from normal tendons (mean = 98.4 $\pm$ 6.2 degrees).

Discussion:

This study was carried out to determine the effects of equine TDPCs on tendon healing in a collagenase model of tendinitis. We hypothesized that autogenous TDPCs will improve tendon healing, as assessed by transcriptional, biochemical, biomechanical, and histologic outcomes. Intralesional TDPC injections did improve the tensile strength of the repair tissue, reflected by increased yield and maximum stresses, and also significantly improved collagen fiber alignment in the repair tissue, as measured by Fourier transform SHG (FT-SHG). In contrast, the biochemical and transcriptional outcomes were not significantly influenced by TDPC injections. These outcomes are somewhat limited because relatively small samples of the affected tissues were used (**Figure 5.2**) and these may not represent the overall status of the healing tissues, given the spatial variability in the pathology generated by collagenase injections and subsequent reparative responses; an intrinsic limitation of this large animal model.

Normal hind limb SDF tendons were used as 'within animal' controls in this study, in addition to the matched contralateral, saline-injected tendons. Accepting that loading and extension are higher in fore limb SDF tendons than in hind limbs [23], we consider that hind limb tendon provided satisfactory matched reference values to compare healing in fore limb SDF tendons. The biomechanical data were normalized to tendon cross-sectional area, to exclude variability derived from morphometric differences.

The effects of autogenous TDPCs in this study are similar to the outcomes of studies investigating other cell-based therapies for experimental tendinitis, such as BM-MSCs [14,24,25], adipose-derived nucleated cells [14] and fetal-derived embryonic stem cells [15].

Although collagen gene expression was increased in TDPC ($p = 0.02$) and saline ($p = 0.03$) groups compared to normal tendon, there was no significant difference in the collagen contents. This 'disconnect' between transcriptional and translational outcomes has been reported in other cell-based tendinitis studies [14,15] and is likely due to gene up-regulation without subsequent translation and/or rapid turnover of newly synthesized matrix proteins. Significant improvements in collagen micro-architecture were reported in all these studies, using semi-quantitative histologic analyses. Despite the improved matrix organization, Chong et al. [26] and Schnabel et al. [14] found only marginal improvements in biomechanical strength.

In the current study, the tensile and material properties of the entire tendon (both healthy and 'repair' regions) were measured, similarly to other studies [14,26]. The grossly pathological regions in the saline and TDPC groups comprised 60-80% of the gauge length, while normal tendon was engaged in the serrated clamps at each end. Accepting that mechanical testing of the 'repair' region in isolation would provide more specific biomechanical characterization of the healing tissue, the entire tendon was tested in this study to determine the overall/global biomechanical properties of the weight-bearing structure. The yield stress (point of initial collagen fiber disruption) and maximum stress (point of complete tendon disruption) of the TDPC group were not significantly different from those of normal tendons, whereas these parameters were significantly lower in the saline group. In contrast, the elastic modulus of the TDPC group was significantly less than that of normal tendons (about 50% of normal values), although higher than the saline controls. Elastic modulus is a sensitive indicator of energy-storing capacity and elastic recovery after loading. Incomplete restoration of elastic modulus in damaged tendons reflects the high recurrence rates of these injuries. Given the prolonged time

frame of recovery in clinical tendonitis cases (8-12 months), it is possible that elastic modulus continues to improve over time frames longer than the current study (12 weeks) [27]. Further long-term studies will be necessary to determine the long-term effect of TDPCs on the biomechanical properties of healing tendon and functional recovery in clinical cases.

Custom-made plastic, serrated jaw clamps adapted from a previous study [21] were used for rigid gripping of tendons in this study. Tensile failure tests with equine tendons can be confounded by the clamp design, the interface properties between the fixed points and the tendon tissue, and amount of normal tendon present between the grips [14]. The set-up used in this study was uncomplicated, did not require freezing or PMMA anchoring of the tendon ends, and supported tensile testing to failure at high loads. All tendons failed at the healthy-injured tendon interface, rather than the clamp-tendon interface. Equine tendons absorb tensile load through fascicle extension and sliding between collagen bundles rather than fiber extension and rupture alone, prior to failure [28,29]. This 'sliding' mechanism of failure was noted during testing of the tendon samples in the current study. This indicates that restoration of tertiary hierarchical structure is critical to functional tendon repair, in addition to recovery of a characteristic collagen fiber crimp pattern.

The clinical use of autologous TDPCs for treating tendon injuries is dependent on identifying a suitable donor site. In this study, TDPCs were isolated from a small portion of the LDE tendon and was collected with little difficulty at the time of the collagenase injections. Tenectomy of the LDE is used to treat refractory stringhalt in horses, with minimal post-surgical morbidity. Nevertheless, a tenectomy procedure is more invasive than bone marrow aspiration or adipose

tissue collection. Additional outcome analyses will be required to determine whether any clinical benefits derived from TDPCs justify the more complex collection procedure. Tenocytes from extensor tendon are less proliferative than digital flexor tenocytes and have a lower matrix-synthesis rate [30]. It is not clear whether these differences in primary tenocyte populations are retained through the extensive in vitro selection and expansion protocol used to generate TDPCs, but progenitors derived from digital flexor tendons may prove to be more clinically effective than LDE-derived cells, provided an innocuous flexor tendon donor site can be identified. TDPCs have a relatively high in vitro proliferation capacity, so only a small amount of autogenous tendon is required to generate clinically useful cell numbers [16,31]. In human patients, healthy tendon autografts are routinely obtained from patellar tendon and palmaris longus tendon, for cruciate [32,33] and ulnar collateral ligament [34] reconstruction respectively. These could also be used as sources for autogenous TDPC isolation. Pathologic tendon tissues excised during surgical repair of Achilles tendons [35] and rotator cuff injuries [36] could also be used to isolate autogenous TDPCs; however, the characteristics of TDPCs from diseased tissue would need to be evaluated before this option can be considered feasible. Alternatively, allogeneic TDPCs avoid potential donor-site complications and extended culture intervals required for autogenous cells. Allogeneic primary TDPCs [37], adipose-derived nucleated cells [38], BM-MSCs [39] and fetal-derived embryonic stem cells [15] implanted within experimental tendon lesions had similar survival and distribution patterns as reported in this study. Histological improvements in collagen architecture were seen without adverse immune reactions. These findings support further studies in this regard.

FT-SHG imaging of cryosectioned tendon samples provided an objective and quantitative method to assess collagen fiber alignment in healing tendons and corroborate visual assessments of collagen alignment in the Picro-Sirius Red-stained sections (**Figure 5.5**). Over the last decade, SHG microscopy has been adapted to evaluate spatial distributions of fibrillar proteins, particularly collagen, utilizing non-centrosymmetric property of collagen molecules, whereby light at half the wavelength of incident light is emitted following interaction with collagen [40,41]. High-resolution images are produced without the need for special staining and associated chemical and/or thermal processing that might disrupt the integrity of the tissue samples. In this respect, FT-SHG constitutes a valuable tool for ongoing and future tendon repair studies, since the modality provides high-resolution, spatially distinct and quantitative indices of collagen fiber orientation throughout the repair site.

Improved collagen microarchitecture is a consistent finding across studies evaluating cell-based therapies. Accepting this, the mechanisms by which cell-based therapies improve the matrix organization of healing tendons are poorly understood. In vivo cell tracking studies have reported that very few MSCs remain around the injection site beyond a few weeks [42,43], indicating that their therapeutic impact likely occurs during their transient presence at the repair site and does not involve major direct contributions to new matrix synthesis [14-16, 36,39]. Local cytokine modulation and trophic factor synthesis have been proposed as likely mechanisms by which MSCs influence tissue regeneration [44-46]. Identifying soluble factors that mediate ‘stem cells’ therapeutic effects could simplify biologic therapy considerably, if cell-based therapies do influence repair through secreted proteins. Although the quantities and orientation of large fibrillary collagen molecules (types I and III) are routinely analyzed in tendon repair studies, it is

also possible that MSCs influence the intrinsic healing response by modulating non-fibrillar collagens and other small matrix molecules that are critical to formation of fibrillar collagen higher order structures [47,48]. In light of the findings of this study, further investigations on the clinical use mechanisms of action of TDPCs for the treatment of tendon injuries are warranted.

Tables and Figures:

Gene		Sequence	Amplicon (bp)
Col I	S A	5' GAA AAC ATC CCA GCC AAG AA 5' GAT TGC CAG TCT CCT CAT CC	231
Col III	S A	5' AGG GGA CCT GGT TAC TGC TT 5' TCT CTG GGT TGG GAC AGT CT	215
COMP	S A	5' TCA TGT GGA AGC AGA TGG AG 5' TAG GAA CCA GCG GTA GGA TG	223
Tenomodulin	S A	5' CCC GTG ACC AGA ACT GAA AT 5' GTT GCA AGG CAT GAT GAC AC	232
EF1-alpha	S A	5' CCC GGA CAC AGA GAC TTC AT 5' AGC ATG TTG TCA CCA TTC CA	328

Table 5.1: Primers used for real-time PCR amplification of genes of equine bone marrow– and tendon-derived cells.

Transcript	Saline	TDPC
Collagen type I	9.76 ± 2.90* $p = 0.03$	7.48 ± 1.50* $p = 0.02$
Collagen type III	20.79 ± 8.76 $p = 0.06$	12.81 ± 3.45 $p = 0.07$
COMP	3.17 ± 0.74* $p = 0.03$	3.13 ± 0.60* $p = 0.02$
Tenomodulin	18.23 ± 5.74* $p < 0.001$	23.32 ± 7.05* $p = 0.02$

Table 5.2. Quantitative PCR analysis of mRNA levels. Transcript abundance in the TDPC and saline groups was normalized to expression in the normal tendon within each horse, which was accorded a value of '1.00'. Fold changes are represented as mean $\pm$ SE (n = 8).

p values refer to the outcomes of Holm-Sidak post hoc comparisons to normal tendon values. * Indicates a significant difference.

Molecule	Normal	Saline	TDPC
DNA content μg/100 mg	21.80 ± 3.84	26.94 ± 3.13* p = 0.03	25.64 ± 3.24* p = 0.02
Total collagen content μg/100 mg	22.57 ± 5.54	23.98 ± 2.92 p = 0.70	23.71 ± 4.13 p = 0.56
Total sGAG content μg/100 mg	44.74 ± 4.23	43.44 ± 3.02 p = 0.43	44.96 ± 3.05 p = 0.21

Table 5.3. Biochemical analyses of DNA, sulfated glycosaminoglycans and collagen content of treated and normal tendons. Data are represented as mean ± SE (n = 8).

p values refer to the outcomes of Holm-Sidak post hoc comparisons to normal tendon values. * indicates a significant difference.

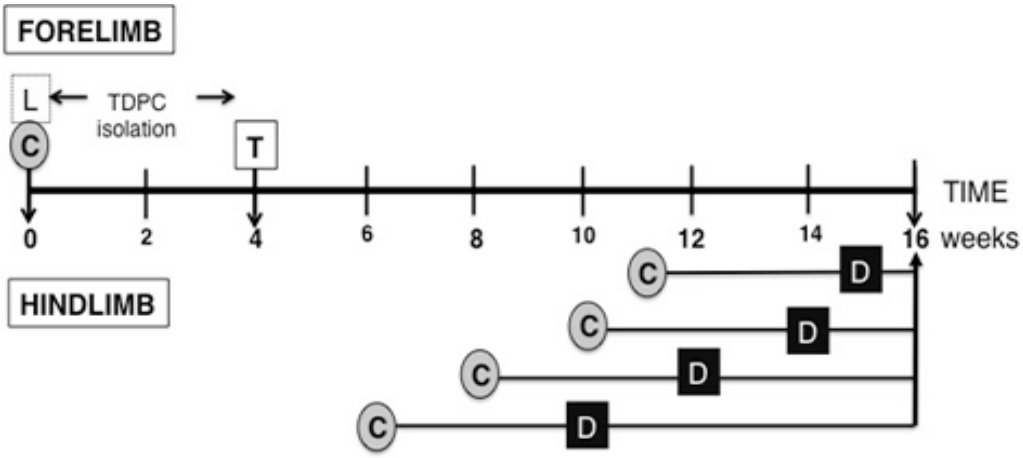


Figure 5.1: Schematic representation of the study design (over 16 weeks).
C - collagenase injection; L - LDE tenectomy; T - TDPC injection; D - DiI-labeled TDPC injection.

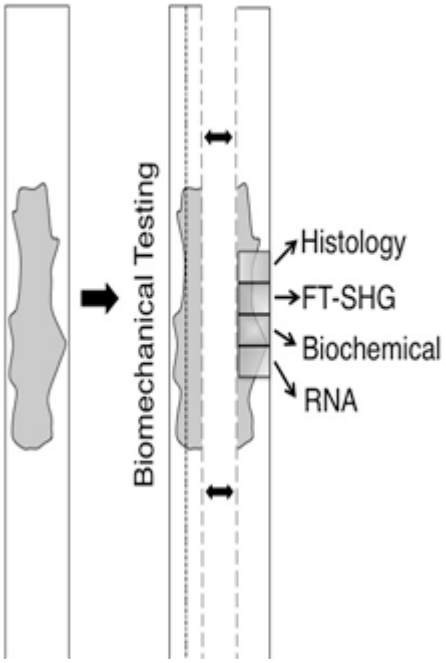


Figure 5.2: Schematic representation of sample allocation from normal, TDPC- and saline-treated SDF tendons. The irregular shaded region represents collagenase-injured tissue.

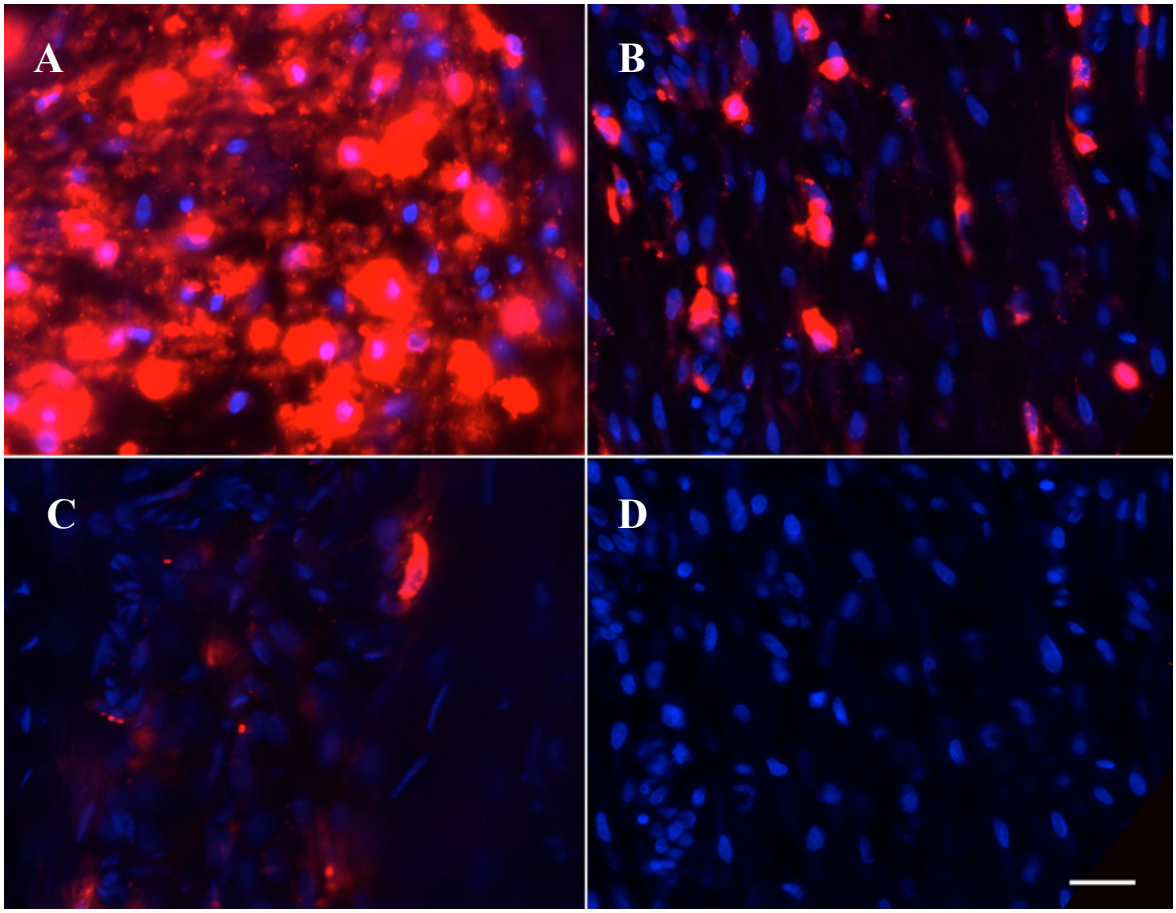
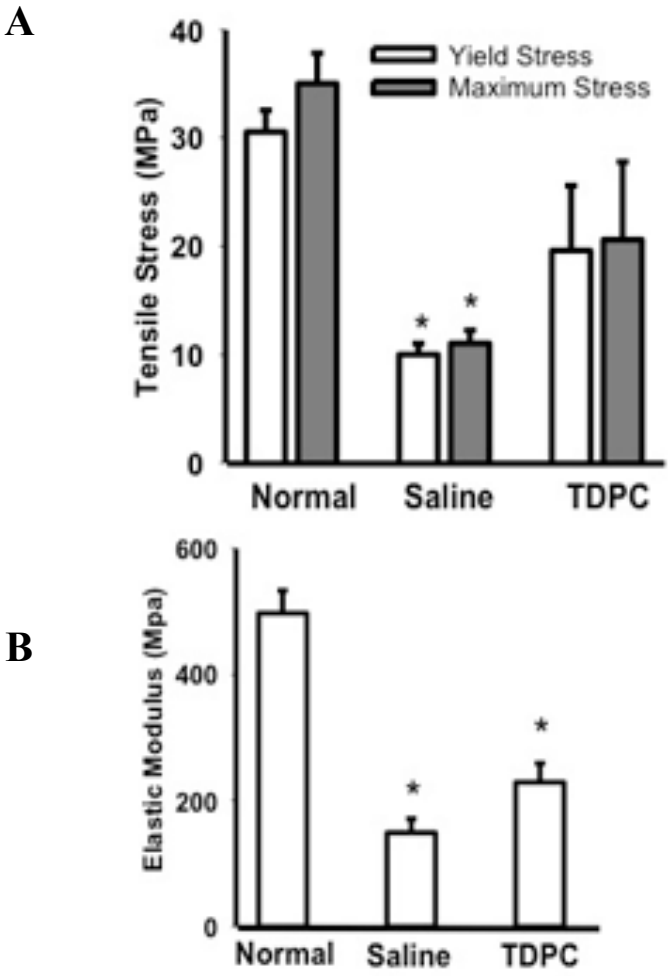


Figure 5.3: Fluorescence microscopic images of longitudinal cryosections of SDF tendons (A) 1week, (B) 2 weeks, (C) 4 weeks, and (D) 6 weeks after intra-lesional injections of DiI-labeled TDPCs. Bar = 100 microns.



F**igure 5.4: (A)** Mean tensile yield and maximum stresses (Mpa) of normal, saline and TDPC tendons. Bars represent mean $\pm$ SE (n = 8). * Indicates a significant difference from normal tendons. **(B)** Mean elastic modulus (Mpa) of normal, saline and TDPC tendons. Bars represent mean $\pm$ SE (n = 8). * Indicates a significant difference from normal tendons.

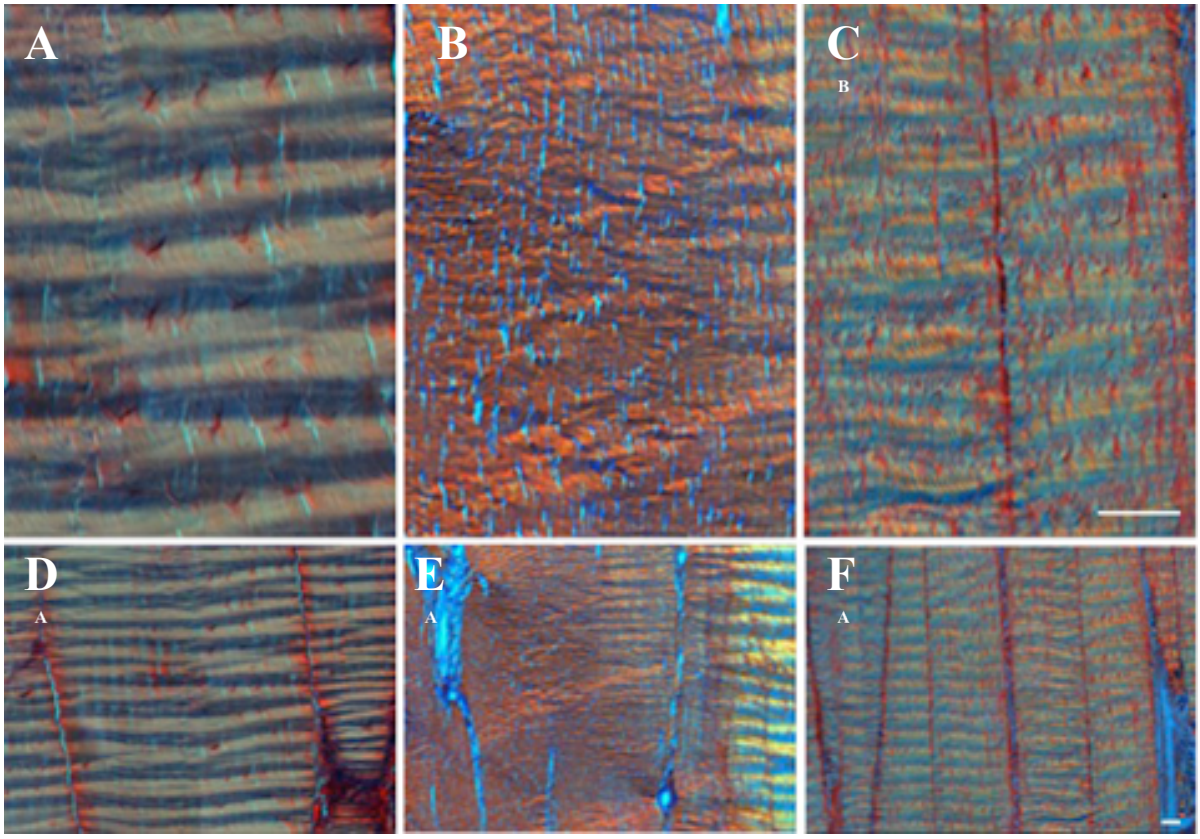


Figure 5.5: Picro-Sirius Red-stained longitudinal sections of (A, D) normal, (B,E) saline, and (C, F) TDPC tendons under polarized light. Panels A, B and C are high magnification images, while panels D, E and F are low magnification mages. In all panels, the scale bar = 100 microns.

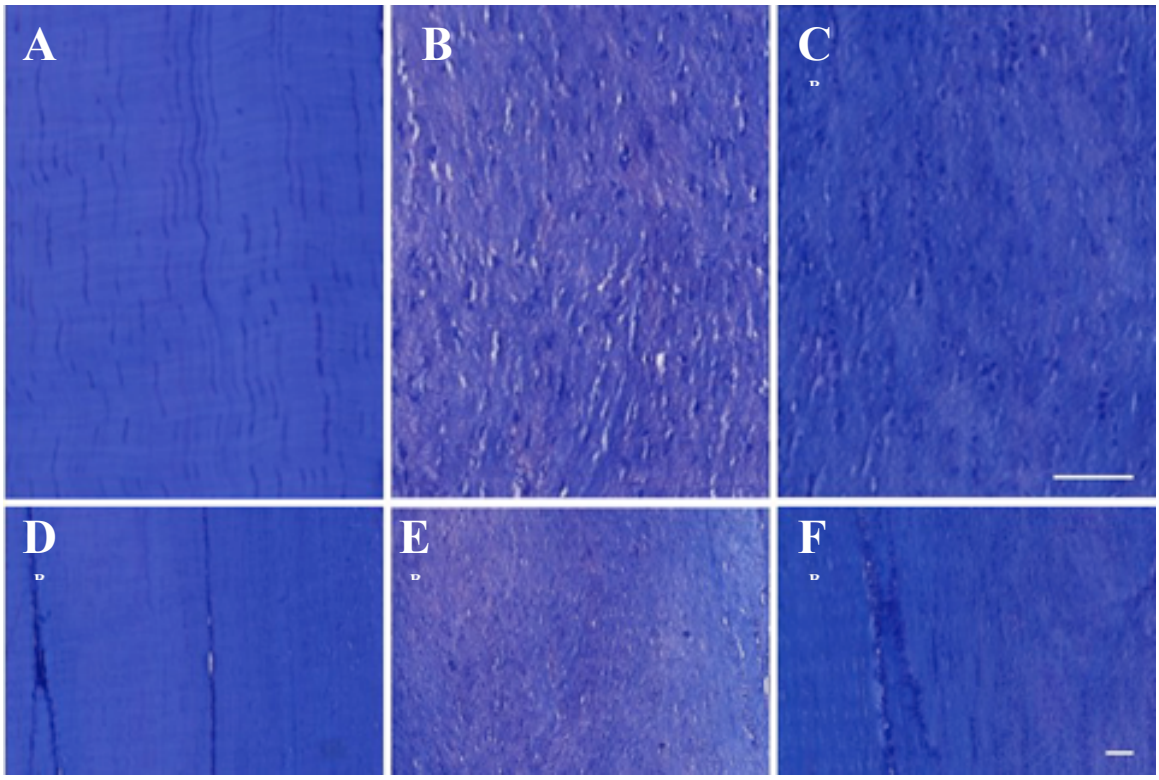


Figure 5.6: Toluidine Blue-stained longitudinal sections of (A, D) normal, (B, E) saline, and (C, F) TDPC tendons under polarized light under bright-field microscopy. Panels A, B and C are high magnification images, while panels D, E and F are low magnification mages. In all panels, the scale bar = 100 microns.

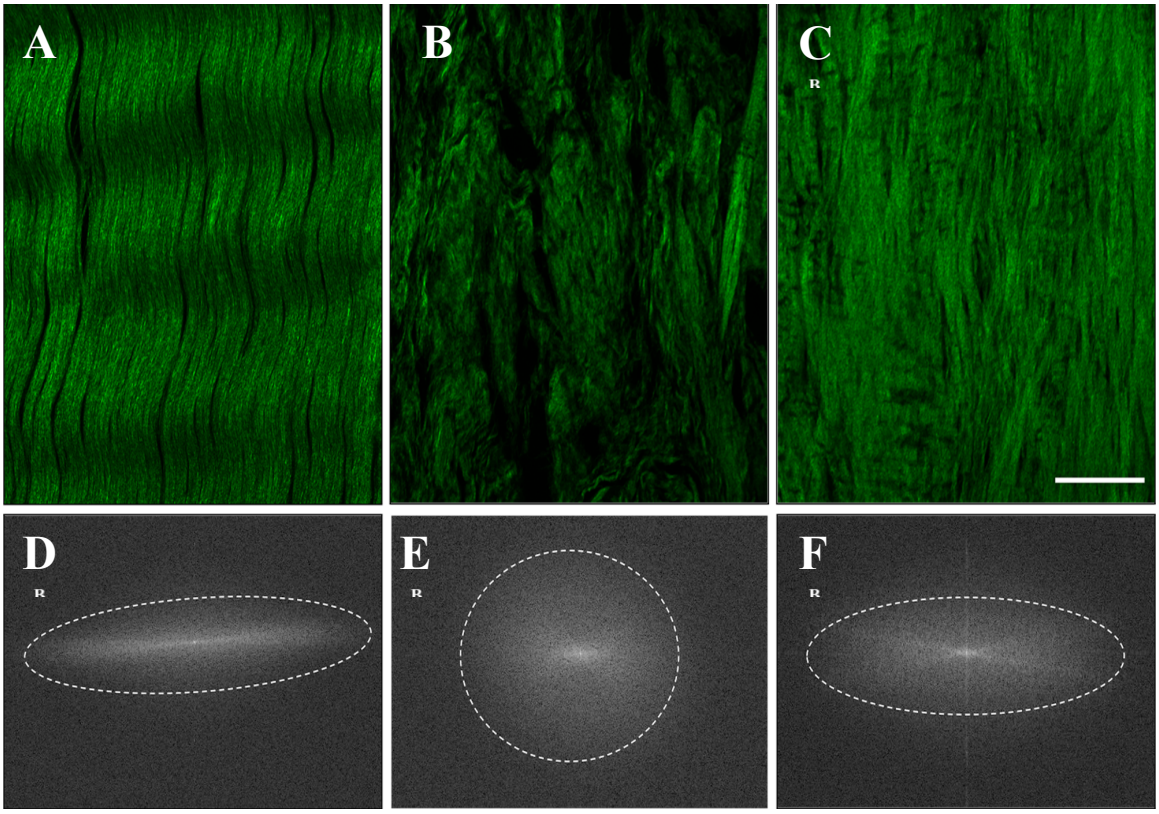


Figure 5.7: SHG images of longitudinal cryosections of (A) normal, (B) saline, and (C) TDPC tendons obtained by confocal microscopy. The corresponding Fourier distributions of collagen orientation are shown in panels (D), (E) and (F). In all panels, the scale bar = 100 microns.

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CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The first set of results presented in this dissertation (chapter 3) addressed the isolation and in vitro characterization of equine tendon-derived progenitor cells. Differential adherence preplating did not enrich equine TDPC isolation during in vitro culture and monolayer expansion. Preplating did not alter the in vitro growth/proliferation characteristics of TDPCs. The immunophenotype and trilineage differentiation potential of the three TDPC fractions assessed in this study was similar. Overall, TDPCs had robust osteogenic capacity but minimal adipogenic and chondrogenic capacity. These results indicate that whole digest tendon-derived cell stocks are adequate for enrichment of progenitor cells via monolayer expansion.

Understanding the basics of TDPC biology is critical for their successful application in tendon repair/regeneration. To date, markers specific to TDPCs and tenocytes in general are poorly defined, and a reliable in vitro tenogenic differentiation assay is still lacking, although research efforts in this area are ongoing. TDPCs are heterogeneous cells and therefore developing a single marker that can definitively identify TDPCs is likely not feasible. Studies focusing on identifying a panel of co-expressed markers to define TDPCs are more realistic. Self-evidently, determining whether any given immunophenotypic tendon-derived sub-population holds any therapeutic advantage will also need to be addressed in rigorous in vivo models.

The experiments addressing the in vitro tendon matrix synthesis revealed significantly higher capacity in TDPCs compared to donor-matched BM-MSCs (chapter 4). Based on these promising results, the in vivo healing capacity of equine TDPCs was evaluated in a collagenase model of superficial digital flexor tendinitis. Intralesional injections of autogenous TDPCs

improved the biomechanical and histological properties of healing tendon tissue (chapter 5). Tendon healing in experimental models has been substantially improved with cell-based and other biologic approaches, although these therapies do not completely restore the tissue microarchitecture. With the data from recent cell-tracking studies, it is well established that exogenous stem cells are cleared from the injection site within a few weeks and do not directly contribute to the pool of tenocytes and/or progenitor cell participating in tendon repair/regeneration. Future studies focusing on cytokines and/or trophic factors secreted by TDPCs that mediate their therapeutic effects could simplify therapy considerably. Optimizing stem cell delivery by combining cells with teno-inductive scaffolds may retain cells at the implantation site for longer periods of time, with correspondingly longer therapeutic actions.

Restoring the biomechanical function of repair tissue should be the ultimate goal of any regenerative therapy for treating tendon injuries. Tendons respond to tensile loads via elongation and sliding mechanism between each element of the hierarchical structure. As the gross and microscopic structure of tendons is disrupted in tendon injury, the ideal regenerative therapy must restore the hierarchical structure of tendons and the sliding mechanisms of the tendon components, in addition to improving collagen alignment to regain full biomechanical function. Currently, collagen fiber pattern along the long axis of the tendon is the major outcome parameter used to assess tissue morphology but this does not address restoration of tertiary structure. Developing non-invasive and histological techniques that comprehensively assess tendon histology at multiple levels of matrix organization will be vital to comprehensively evaluate new therapies.

Finally, clinical use of TDPCs for treating tendon injuries is dependent on identifying a suitable tissue source, with minimal donor site morbidity. In general, a tenectomy procedure for isolating autologous TDPCs is more invasive than bone marrow aspiration. In our in vivo study, autologous equine TDPCs were derived from the lateral digital extensor tendon with minimal post-operative morbidity. In human patients, healthy tendon autografts are routinely obtained from patellar and palmaris longus tendons, for cruciate and ulnar collateral ligament reconstruction surgeries, respectively. These sites could also be used for autogenous TDPC isolation. Further work is needed in this regard. Tendon tissues excised during reparative surgeries of Achilles tendon and rotator cuff injuries could also be used to isolate autogenous TDPCs; however, given the altered characteristics of TDPCs from pathological tissues, further research on these TDPCs is required before this possibility can be considered feasible. Allogeneic TDPCs can also be considered, as they avoid donor-site complications and culture delays that are required to generate clinically relevant autologous cell numbers. Although several in vivo experimental studies demonstrate the therapeutic benefits of allogeneic TDPCs, major regulatory and immunogenic concerns exists to prevent translating this option to human tendon injuries.