

Tendon-Derived Progenitor Cells With Multilineage Potential Are Present Within Human Patellar Tendon

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Background: Progenitor cells serve as a promising source of regenerative potential in a variety of tissue types yet remain underutilized in tendinopathy. Tendon-derived progenitor cells (TDPCs) have previously been isolated from hamstring tendon but only as part of a concomitant medical procedure. Determining the presence of TDPCs in patellar tendon may facilitate clinical utilization of these cells because of the relative accessibility of this location for tissue harvest.

Purpose: To characterize TDPCs in human patellar tendon samples.

Study Design: Descriptive laboratory study.

Methods: Human patellar tendon samples were obtained during elective knee surgery. TDPCs were isolated and seeded at an optimal low cell density and subcultured to confluence for up to 2 passages. Flow cytometry was used to analyze for the expression of CD90+, CD105+, CD44+, and CD31-, CD34-, and CD45- markers. The multilineage differentiation potential of TDPCs was tested in vitro via adipogenic, osteogenic, and chondrogenic culture with subsequent cytochemical staining for Oil Red O, Alizarin Red, and Alcian Blue, respectively. Enzyme-linked immunosorbent assay was used to quantify the amount of adiponectin, alkaline phosphatase, and SRY-box transcription factor 9 secreted into cell culture supernatant for further confirmation of lineage differentiation. Results were analyzed statistically using the 2-tailed Student *t* test.

Results: TDPCs demonstrated near-uniform expression of CD90, CD105, and CD44 with minimal expression of CD34, CD31, and CD45. Adipogenic, osteogenic, and chondrogenic differentiation of TDPCs was confirmed using qualitative analysis. The expression of adiponectin, alkaline phosphatase, and SRY-box transcription factor 9 were significantly increased in differentiated cells versus undifferentiated TDPCs ($P < .05$).

Conclusion: TDPCs can be successfully isolated from human patellar tendon samples, and they exhibit characteristics of multipotent progenitor cells.

Clinical Relevance: These data demonstrate the promise of patellar tendon tissue as a source of progenitor cells for use in biologic therapies for the treatment of tendinopathy.

Keywords: stem cell; progenitor cell; tendinopathy; tendon; patellar tendon; regeneration

INTRODUCTION

Tendinopathy is a degenerative tendon disorder characterized by chronic pain, decreased strength, localized tenderness, and reduced function.⁵⁶ Approximately 30% of all general practitioner musculoskeletal visits are attributed to symptoms of tendinopathy, costing an estimated US\$30 billion in annual health care utilization in the United States alone.^{2,37} Traditional treatments for tendinopathy include activity modification, physical therapy consisting mostly of eccentric exercises, injections, and extracorporeal

shockwave therapy.^{8,21,26,54,61} However, these modalities often do not resolve symptoms, as many patients are unable to return to their prior levels of physical activity and work.³⁹

Although biologic treatments have shown promise in other musculoskeletal conditions, biologic treatments for tendinopathy have not advanced to the same degree.²⁰ Human progenitor cells hold promise for their ability to regenerate many types of tissue and ameliorate disease burden. Bone marrow-derived and adipose-derived mesenchymal progenitor/stromal cells (BMSCs and ADSCs) have been the most studied in musculoskeletal regenerative medicine because of their relative ease of isolation and multilineage potential.^{3,4} Cells derived from these sources have been utilized to treat chondral defects, fractures, and osteonecrosis, among others.^{14,15,18,19,24,27,50}

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In order to regenerate tendon, however, it would seem most advantageous to harvest progenitor cells from a tendon source. This would take advantage of the fact that progenitor cells from tendons are more phenotypically committed or “primed” for tenogenesis than are cells from other tissues.¹³ Bi et al¹ demonstrated this when they first isolated tendon-derived progenitor cells (TDPCs) and reported that progenitor cells derived from tendon were phenotypically distinct from bone marrow-derived progenitor cells. Additional investigations have shown TDPCs to have the ability to regenerate tendon after in vitro expansion and subsequent implantation.^{1,25,36,42,45}

The majority of literature to date describing the isolation of TDPCs from human samples has utilized hamstring tendon as the source tissue.^{1,43,46} Hamstring tendon samples, however, are typically only available as part of a concomitant medical procedure, such as anterior cruciate ligament (ACL) reconstruction. The patellar tendon, in contrast, is nearly subcutaneous, could be easily accessed in both the operative and nonoperative settings, and has been described as part of autologous tenocyte injection treatment for lateral epicondylitis.^{51,52}

There are few reports of isolation of TDPC from human patellar tendon.^{22,38} If sufficient TDPCs exist within patellar tendon, these cells could be easily harvested and provide a biological regenerative advantage in the treatment of tendinopathy. The purpose of this investigation was to determine the presence of TDPCs in human patellar tendon samples. The authors hypothesized that TDPCs could be consistently isolated from human patellar tendon samples.

METHODS

Institutional review board approval was obtained before study initiation. Samples of patellar tendons were obtained during elective knee surgery from 2 healthy human donors undergoing ACL reconstruction (1 man: age, 32 years; 1 woman: age, 26 years). To assess for the presence of TDPCs, we utilized the definition of multipotent mesenchymal stromal cells as put forth by the International Society of Cellular Therapy (ISCT): adherent to plastic when maintained in standard culture conditions, trilineage (osteoblasts, adipocytes, and chondroblasts) differentiation in vitro, and having the specified cell surface marker expression profiles.¹²

Cell Isolation and Expansion

Tissue was minced and washed twice in 50 mL of phosphate-buffered saline (PBS; Gibco) and 1% penicillin-streptomycin

(Gibco) under sterile conditions and centrifuged at 400g at 22°C for 5 minutes. The supernatant was collected, and the tissue fragments were digested in 3 mg/mL of collagenase type I from *Clostridium histolyticum* and 4 mg/mL of dispase II from *Bacillus polymyxa* (Sigma) in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) for 2 hours at 37°C, with gentle shaking every 30 minutes. The resulting cell suspension was diluted in PBS, filtered via a 75- μ M strainer to remove debris, and centrifuged at 400g at 22°C for 10 minutes to obtain a cell pellet. The supernatant was aspirated without disturbing the pellet. The cell count and viability were performed using a hemocytometer and trypan blue. The isolated viable nucleated cells were seeded at an optimal low cell density (500 cells/cm²) and resuspended in DMEM/Nutrient Mixture F-12 (DMEM/F-12; Gibco) supplemented with 20% fetal bovine serum (Gibco) and 1% penicillin-streptomycin. They were then cultured at 37°C and 5% CO₂ to form colonies for the isolation of TDPCs. Subcultured cells were expanded at 500 cells/cm² when they reached 90% confluence up to 2 passages and then subjected to the in vitro characterization following the previously described guidelines for the definition of mesenchymal stromal cells.¹²

Expression of Progenitor Cell Surface Markers

Flow cytometry was performed on cells obtained from the digestion after the second passage. An amount of 1×10^6 of cells were harvested using trypsin-EDTA (Gibco), filtered via a 70- μ m strainer, and washed in PBS. The cells were suspended in 100 μ L of PBS, stained with Zombie Aqua Fixable Viability (BioLegend), and incubated for 20 to 30 minutes in the dark. After the incubation, the cells were then resuspended in flow wash/stain buffer (Biosciences) and centrifuged. Pellets were collected in PBS (Invitrogen) and stained first with Human Fc Block (Biosciences) for 10 minutes and then using selected antibodies for 30 minutes in the dark. To define mesenchymal stem cells (MSC), we sorted for CD90+, CD105+, CD44+, and CD31-, CD34-, and CD45-markers. Cells were compared with unstained cells as a negative control. Samples were acquired via flow cytometric analysis (BD LSRFortessa), and data were further analyzed using FlowJo software (Version 10.6.2; Tree Star Inc).

In Vitro Multilineage Differentiation

The multilineage potential of the TDPCs was tested in vitro via osteogenic, adipogenic, and chondrogenic culture. To evaluate for trilineage differentiation, the treated cells, after a specific time period, were stained. The supernatants and

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the micropellets were collected before cytochemical staining in order to perform a separate enzyme-linked immunosorbent assay (ELISA) analysis. Samples were also stained with hematoxylin and eosin (H&E) to evaluate the morphology of the cells. Each sample was compared with unstimulated cells as a negative control. After the second passage, cells were harvested using trypsin-EDTA and seeded at 3000 cells/cm² in a different 6-well plate, and then they were cultured in adipogenic and osteogenic media for up to 3 weeks. For chondrogenesis, cells were collected after the second passage, and micropellets of 1×10^6 cells were created in 15-mL conicals with centrifugation for 5 minutes at 400g. The supernatant was aspirated, and the pellet was partially suspended in 100 μ L of culture media. From this suspension, we removed droplets of 6 μ L each and cultured them in 40 μ L of chondrogenic induction media up to 3 weeks to preserve the micropellet for the cytochemical assay. In parallel, micropellets with a cell density of approximately 1×10^6 viable cells per pellet were placed in 15-mL conicals using the same technique. The supernatant was aspirated, but the pellet was cultured in 4 mL of induction media in the 15-mL conicals for 21 days. After 21 days, the micropellets from the 15-mL conicals were collected, and the cells were lysed for the quantitative analysis.

Adipogenesis. To determine adipogenesis, TDPCs were collected and cultured using the StemPro Adipogenesis Differentiation Kit (Gibco) for up to 2 weeks and stained with Oil Red O to visualize the accumulation of lipid vacuoli after embedding with 4% paraformaldehyde. To quantify adiponectin secreted in the culture medium of adipo-induced cells, we used a standard adiponectin ELISA (Abcam). This assay uses an antibody specific for human adiponectin coated on a 96-well plate. Standards and samples are pipetted into the wells, and adiponectin present in a sample is bound to the wells via the immobilized antibody. The color was developed in proportion to the amount of adiponectin bound and measured at 450 nm using a spectrophotometer (SpectraMax M5; Molecular Devices).

Osteogenesis. To diagnose osteogenesis, cells were cultured using the StemPro Osteogenesis Differentiation Kit (Gibco), and on day 21, osteogenic differentiation of TDPCs was visualized using the intensity of Alizarin Red staining on micro-copy. In addition, the alkaline phosphatase (ALP) activity was quantified using ELISA (Alkaline Phosphatase Assay Kit; Colorimetric Inc) in the cell culture supernatants. This kit uses *p*-nitrophenyl phosphate as a phosphatase substrate that turns color when dephosphorylated via ALP. The optical density was measured at 405 nm using a spectrophotometer (SpectraMax M5).

Chondrogenesis. To diagnose chondrogenesis, micropellets of TDPCs in 6-well plates were created and then cultured using a chondrogenesis differentiation kit (StemPro). For analysis, the pellets in the 6-well plates were embedded in 4% paraformaldehyde and then stained with Alcian Blue to detect the presence of acidic polysaccharides such as glycosaminoglycans in cartilage cells. For the quantitative assay, the micro cell pellets were lysed, and the presence of SRY-box transcription factor 9 (SOX9) was detected using ELISA. The ELISA uses an affinity

tag-labeled capture antibody and a reporter-conjugated detector antibody that immunocaptures the sample analyte in solution. Signal was generated proportionally to the amount of bound analyte, and the intensity was measured at 450 nm using a spectrophotometer (SpectraMax M5).

Data Analysis

Sample data output generated from flow cytometry was analyzed using FlowJo software. Gating was applied to exclude debris, multicellular aggregates, and dead cells by selecting the central live population of single cells with highly similar morphologic and cytometric characteristics. Isotype control readings were used against fluorescence readings to determine fluorescence shift. Compensation was conducted using FlowJo software using single-stain analysis readings of monoclonal antibodies. Characterization of TDPCs is based on the expression of MSC-associated surface markers (CD90, CD105, CD44) and non-MS surface markers (CD31, CD34, CD45). Positive and negative percentages from different samples were combined to obtain a mean \pm standard deviation for positive and negative markers. All experiments were performed in duplicate, and TDPC differentiation was performed using cells from each donor.

Statistical Analysis

To evaluate the differences in the presence of adiponectin, ALP, and SOX9 in comparison with the negative control, a 2-tailed Student *t* test was performed. Data were analyzed using R Foundation for Statistical Computing (version 4.0.0; R Development Core Team). *P* values $< .05$ were considered to be statistically significant. All experiments were performed in duplicate. These differentiations were performed using cells from different donors.

RESULTS

Cells Isolation and Expansion

Subcultured TDPCs were cultured onto tissue-treated culture flasks for several passages and adhered to the plastic flask when maintained in standard culture conditions. These cells demonstrated a fibroblastic-like appearance and exhibited distinct colony formation. The TDPCs showed constant proliferation during the expansion and continued to be adherent with a spindle-like cell morphology appearance typical of MSCs (Figure 1), satisfying the first criterion described from the ISCT.¹²

Expression of MSC Surface Markers

The other minimum criterion for the identification of MSCs is the presence of MSC-associated surface markers, such as CD90, CD105, and CD44, and simultaneous low expression or the absence of non-progenitor cell-associated surface markers, such as CD34, CD31, and CD45. TDPCs



Figure 1. Light microscopy (4 \times) image demonstrating proliferation and colony formation of spindle-like tendon-derived stem cells.

demonstrated near-uniform expression of CD90, CD105, and CD44, with minimal expression of CD34, CD31, and CD45 (Figure 2). These results demonstrated that the TDPCs satisfied the surface antigen expression criteria of the ISCT.¹²

In Vitro Multilineage Differentiation Assay

To determine whether our isolated TDPCs were capable of adipogenic, osteogenic, and chondrogenic differentiation, cytochemical staining and ELISA were performed.

Adipogenesis. Cells demonstrated many intracellular lipid vacuoles typical of adipocytes after staining with Oil Red O (Figure 3C). Furthermore, H&E staining demonstrated that TDPCs changed in morphology from spindle-shaped to larger, more rounded cells (Figure 3, A and B). Analysis using ELISA revealed significantly increased expression of adiponectin as compared with the untreated TDPCs ($P < .001$) (Figure 4A).

Osteogenesis. Alizarin Red staining demonstrated the abundant formation of calcium on the differentiated TDPCs, which was not observed in the undifferentiated cells (Figure 3, D and F). The H&E staining also demonstrated a rounded cell morphology, different from that of undifferentiated TDPCs (Figure 3E). The quantitative analysis showed significantly higher ALP expression in the induced cells compared with the noninduced control group ($P = .035$) (Figure 4B).

Chondrogenesis. Culture in chondrogenic medium resulted in the production of glycoproteins of the cartilage matrix as seen via Alcian Blue staining (Figure 3, G-I). The expression of SOX9 was significantly increased in the cells that underwent chondrogenic culture differentiation versus undifferentiated TDPCs ($P < .001$) (Figure 4C).

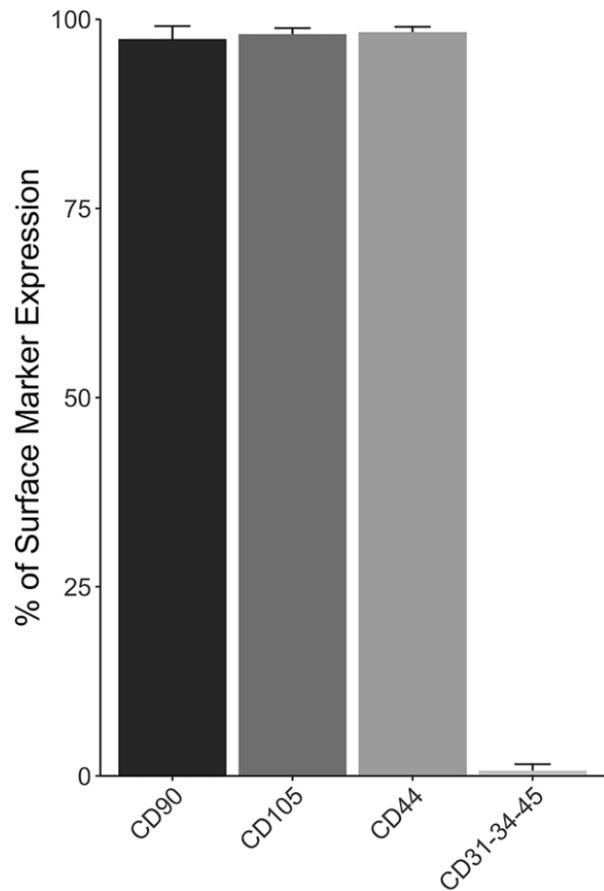


Figure 2. Percentage of tendon-derived progenitor cell surface immunophenotype at second passage. Data are presented as mean \pm SD.

DISCUSSION

The present investigation demonstrated successful isolation of TDPCs from human patellar tendon. These data demonstrate the promise of patellar tendon tissue as a source of TDPCs for use in biologic therapies for the treatment of tendinopathy. Prior investigations have reported the presence of TDPC in human tendon samples, but TDPCs have been predominantly isolated from hamstring tendons. Bi et al,¹ who were the first to report on the isolation of TDPCs, utilized pediatric hamstring samples from children undergoing hamstring tenotomy for knee flexion contracture. Ruzzini et al⁴³ and Stanco et al⁴⁶ were also able to isolate tendon-derived progenitor cells, but they utilized hamstring tendon samples from patients undergoing ACL reconstruction. The use of patellar tendon, which is more superficial in nature, provides a more accessible source of TDPC for the typical patient with tendinopathy who does not require ACL reconstruction. In fact, needle biopsy of the patellar tendon for other indications has been described in multiple publications.^{35,51,52} Even for those patients with signs and

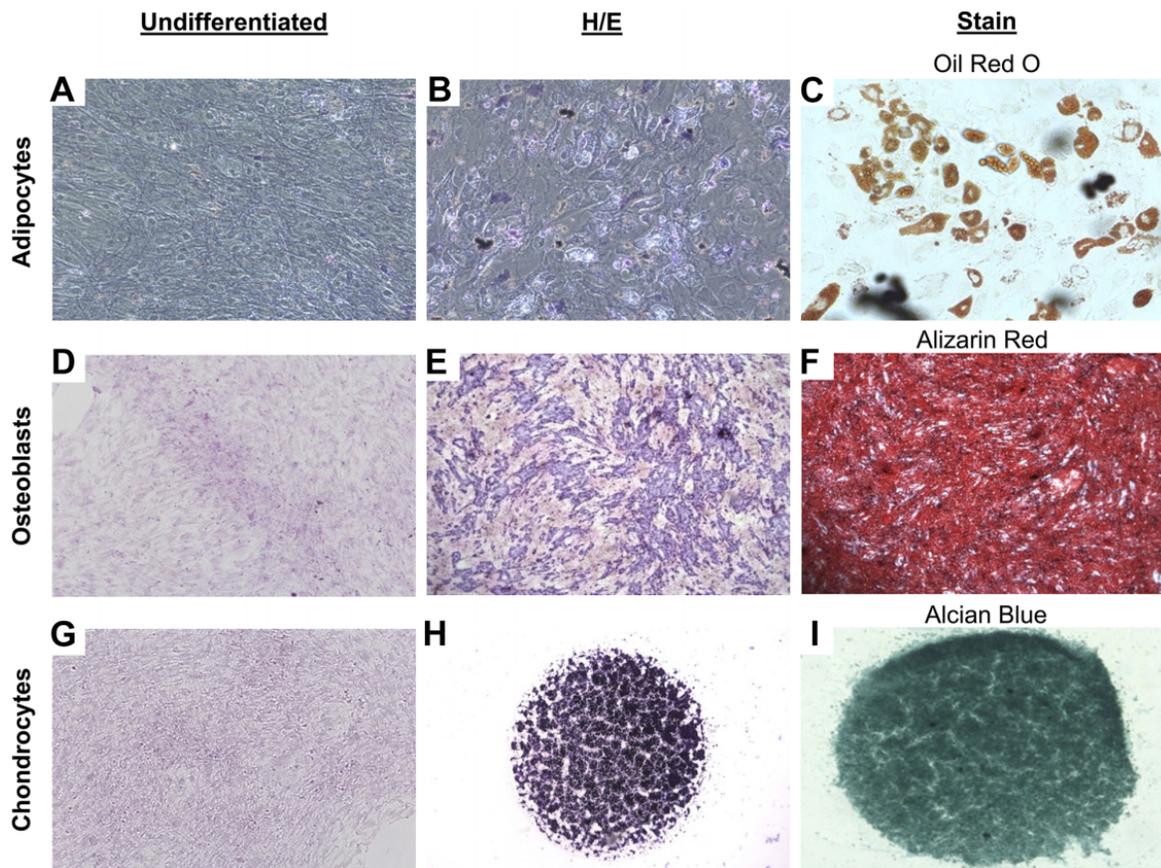


Figure 3. Multilineage differentiation potential of tendon-derived progenitor cells at second passage. (A) Negative control for adipogenesis (10 \times); cell differentiation into adipocytes stained with (B) hematoxylin and eosin (H&E) and (C) Oil Red O (20 \times). (D) Negative control for osteogenesis (10 \times); cell differentiation into osteoblasts stained with (E) H&E and (F) Alizarin Red (2 \times). (G) Negative control for chondrogenesis (2 \times); cell differentiation into chondrocytes stained with (H) H&E and (I) Alcian Blue (2 \times).

symptoms of patellar tendinopathy, tendon samples could be acquired from the contralateral limb or distally in the patellar tendon away from the typical location of proximal patellar tendinopathy.

In comparison to isolation from hamstring tendons, TDPCs derived from patellar tendon samples have been characterized by fewer studies.^{22,38} In 1 investigation, Lee et al²² found that hypoxic conditions promoted proliferation of patellar tendon TDPCs but reduced the osteogenic, adipogenic, and chondrogenic differentiation. Further, Qin et al³⁸ utilized human patellar tendon TDPCs to demonstrate that TDPCs also have fibrochondrogenic differentiation potential. Both studies demonstrated that TDPCs had all characteristics of multipotent mesenchymal stromal cells. As such, combined with previous findings, the present investigation further demonstrates that TDPCs can be consistently isolated from the patellar tendon.

Currently, BMSCs and ADSCs provide most progenitor cells for clinical use for a variety of indications,^{29,47,48} but these cells may not be optimal for the treatment of tendinopathy because they are not “primed” for tendon

regeneration and have been shown to be phenotypically distinct from bone marrow–derived progenitor cells.¹ TDPCs may be superior because they are isolated from the native tendon source and express higher levels of tenogenic markers, such as tenomodulin and scleraxis (*Scx*), key regulators of tenocyte differentiation.^{1,11,23,48} Tan et al⁴⁸ demonstrated that TDPCs expressed higher levels of tenomodulin, *Scx*, and type I collagen compared with BMSCs. Additionally, the authors showed that TDPCs proliferate faster and have higher clonogenicity potential than do BMSCs. Further, Youngstrom and colleagues⁵⁷ evaluated the tenogenesis of TDPCs, BMSCs, and ADSCs in a dynamic bioreactor and found that of those cell types, TDPCs expressed the highest levels of *Scx* and type I collagen transcripts and resulted in the most mature tendon-like phenotype in vitro.

TDPCs have a multifactorial role in tendon repair. Studies have shown that TDPCs can spontaneously differentiate into tenocytes in vitro without induction medium.⁴⁹ Platelet-rich plasma supplementation in vitro can also promote TDPC differentiation into tenocytes and enhance in vivo tendon

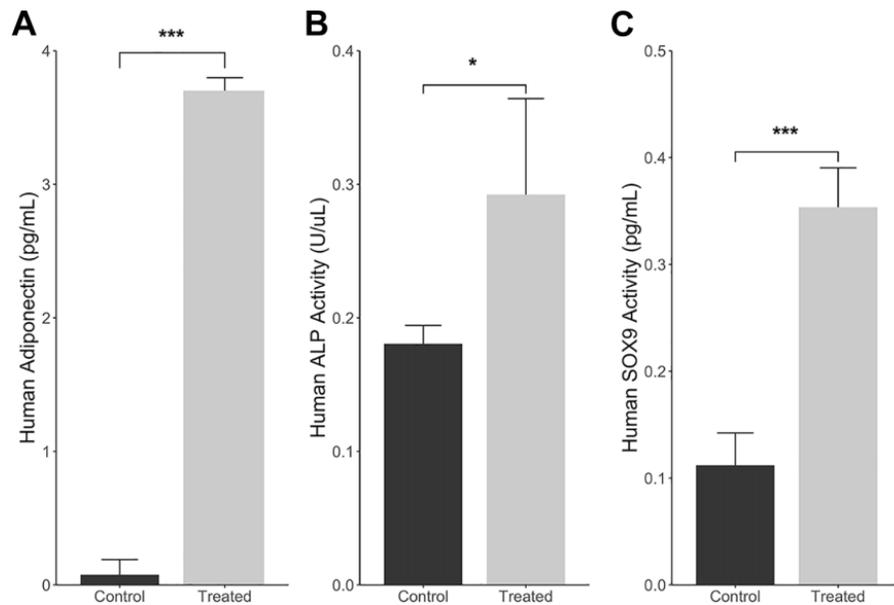


Figure 4. Enzyme-linked immunosorbent assay analysis of multilineage differentiation potential of tendon-derived progenitor cells. (A) Adiponectin concentration in cellular supernatant after induction of adipogenesis. (B) Alkaline phosphatase (ALP) concentration in cellular supernatant after induction of osteogenesis. (C) SRY-box transcription factor 9 (SOX9) concentration in cellular supernatant after induction of chondrogenesis. Data are presented as mean \pm SD. * $P < .05$; *** $P < .01$.

healing in animal models.^{55,60} Further, mechanical loading also has an effect on TDPC outcome, as Zhang and Wang⁵⁹ determined that low mechanical stretching promoted tenogenic differentiation of TDPCs, whereas higher loading induced adipogenic, chondrogenic, and osteogenic changes. It has also been shown that prostaglandin E₂ decreases TDPC proliferation and induces osteogenic differentiation, leading to increased production of BMP-2. This mechanism may play a role in the pathogenesis of calcific tendinopathy.⁵⁸ Additionally, another study has reported that despite a higher number of total resident TDPCs in tendinopathic tissue, these TDPCs are unable to differentiate into tenocytes to regulate the extracellular matrix (ECM).⁵

The difficulty in treating tendinopathy lies in the multifactorial pathogenesis of the disease. The utilization of tendon derived stem cells (TDSC) as a biologic agent may address many of these aberrant pathways. One of the main pathological mechanisms in the development of tendinopathy involves repetitive tendon mechanical overload leading to microscopic collagen fibril failure.⁴¹ Given the poor intrinsic healing capacity of tendon, ECM damage may accumulate over time, leading to progressive structural injury and symptomatic disease.²⁸ Healthy tendons are predominantly composed of type I collagen, which gives them their mechanical strength. In tendinopathy, there is increased production of type III collagen, increased vascularity, and deposition of additional ECM proteins, leading to loss of collagen organization.^{9,32} In addition to mechanical overload, other mechanisms that may propagate the development of tendinopathy include matrix metalloproteinase and tissue inhibitors of metalloproteinase

imbalance,⁴⁰ hypoxia,³³ dysregulated apoptosis,¹⁷ and neuronal proliferation.¹⁰ Given their role as a central regulatory cell within the tendon environment,²⁵ the utilization of TDSCs may mitigate many of these pathological processes. For instance, the use of TDSCs as a biologic therapy may allow for the increased production of type I collagen over type III collagen, addressing a major difference in the composition of the ECM in tendinopathy versus healthy tendon.

Despite these varied mechanisms, it has become increasingly evident that a central component of tendinopathy is a chronic inflammatory response. Multiple studies have shown that inflammation is present in early tendinopathy,^{16,30-32} and current evidence supports the complex interplay between infiltrating immune cells, activation of resident stromal cells, and the innate immune system in response to tissue damage.³² Additionally, recent reviews have demonstrated that the immune response is a major player in both tendon healing and dysregulation.^{6,7} Tenocytes are the most abundant cell type in tendon, and in the setting of mechanical overload, these cells can produce altered ECMs and inflammatory cytokines, such as interleukin (IL)-1 β ,⁴⁴ IL-6,³⁴ and IL-17A,³⁰ that continue to promote the inflammatory conditions in tendinopathy. In addition to tenocyte differentiation, TDPCs can play a role in modulating inflammation in tendinopathy. TDPCs have been shown to secrete anti-inflammatory cytokines, such as IL-10, when delivered into animal models of tendon injury.^{45,49} Further, recent studies have demonstrated that exosomes derived from TDPCs may also play a role in controlling inflammation in tendinopathy and regulating the tendon ECM.^{53,62} Zhang

et al⁶² found that animal models of Achilles tendon injury treated using TDPC exosomes demonstrated decreased expression of IL-6 and CCR7, a marker for M1 pro-inflammatory macrophages, and increased expression of IL-10. Additionally, TDPCs combined with their exosomes upregulated tissue inhibitors of metalloproteinase-1 and type I collagen.

There were some limitations to the current study. As this was an in vitro study, the multilineage differentiation potential and clonogenicity exhibited by TDPCs in culture may not be representative of their capabilities in vivo. This study also included a relatively small sample size with tendon samples collected from 2 patients, which may limit the reliability and external validity of our results. Further, we could not determine if the amount and quality of TDPCs changed with increased age. Additionally, samples were only harvested from patients with healthy tendons, even though future TDPC treatment would target patients with tendinopathy. As such, TDPCs harvested from tendinopathic samples may have different characteristics. Finally, this investigation did not study interactions between other cell types and TDPCs. There could be synergistic interactions between TDPCs and tenocytes or MSCs derived from other sources.

CONCLUSION

Tendon-derived cells that exhibit characteristics of multipotent progenitor cells can be successfully isolated from human patellar tendon samples, offering an easily accessible source of cells for tendinopathy treatment and tendon regeneration.

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