SCIENTIFIC ARTICLE

Tendon Tissue Engineering: Mechanism and Effects of Human Tenocyte Coculture With Adipose-Derived Stem Cells

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Purpose Adipose-derived stem cells (ASCs) are a potential candidate for cell-based therapy targeting tendon injury; however, their therapeutic benefit relies on their ability to interact with native tenocytes. This study examines the mechanism and effects of coculturing human tenocytes and ASCs.

Methods Tenocytes (T) were directly cocultured with either ASCs (A) or fibroblasts (F) (negative control) in the following ratios: 50% T/50% A or F; 25% T/75% A or F; and 75% T/25% A or F. Cells were indirectly cocultured using a transwell insert that allowed for exchange of soluble factors only. Proliferation and collagen I production were measured and compared with monoculture controls. Synergy was quantified using the interaction index (II), which normalizes measured values by the expected values assuming no interaction (no synergy when II = 1). The ability of ASCs to elicit tenocyte migration was examined *in vitro* using a transwell migration assay and *ex vivo* using decellularized human flexor tendon explants.

Results Compared with monoculture controls, II of proliferation was greater than 1 for all tenocyte and ASC direct coculture ratios, but not for tenocyte and fibroblast direct coculture ratios or for tenocyte and ASC indirect coculture. The ASCs elicited greater tenocyte migration *in vitro* and *ex vivo*. The II of collagen I production was greater than 1 for direct coculture groups with 25% T/75% A and 75% T/25% A.

Conclusions Direct coculture of ASCs and tenocytes demonstrated synergistic proliferation and collagen I production, and ASCs elicited tenocyte migration *in vitro* and *ex vivo*. These interactions play a key role in tendon healing and were absent when ASCs were replaced with fibroblasts, supporting the use of ASCs for cell-based therapy targeting tendon injuries.

Clinical relevance When ASCs are delivered for cell-based therapy, they directly interact with native tenocytes to increase cell proliferation, collagen I production, and tenocyte migration, which may enhance tendon healing. *(J Hand Surg Am. 2017;■(■):1.e1-e9. Copyright © 2017 by the American Society for Surgery of the Hand. All rights reserved.)*

Key words Coculture, flexor tendons, tendon repair, tendon tissue engineering, tissue engineering.

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Tendon injuries, which afflict approximately 17 million people in the United States, present a significant source of morbidity.[1] Currently, the surgeon's options for acute injuries include primary surgical repair or secondary tendon reconstruction with tendon autograft. Despite significant advances in the past few decades, the intrinsic healing of tendons remains poor; thus, leaving patients with biomechanically suboptimal repairs that are inevitably weaker than the native, uninjured tendon.[2,3] This, coupled with the scarcity of donor tendons and concerns regarding donor site morbidity, presents a need for alternative options in tendon repair.

Cell-based therapy, a tissue engineering strategy, is a promising solution[4] that can potentially address both the scarcity and the suboptimal functional outcomes of using autologous donor tendons.[2] Delivering tenocytes could facilitate therapeutic action because tenocytes, through depositing collagen and remodeling the extracellular matrix (ECM),[5] are responsible for intrinsic tendon healing.[1,6] However, tenocyte use has been limited by the scarcity of donor tendons from which tenocytes can be cultured, the difficulty of isolating tenocytes, and the loss of phenotype of isolated tenocytes during *in vitro* expansion.[7,8]

In contrast, adipose-derived stem cells (ASCs) can be harvested with low donor site morbidity, are easily isolated, are found in abundance, and have the ability to differentiate along several lineage pathways.[9,10] This makes them an attractive option for regenerative cell-delivery therapies, with various potential applications in the field of reconstructive surgery.[11] The ASCs are particularly promising for tendon repair because they have been shown to enhance primary tendon healing,[12] exhibit tenocyte-like phenotype *in vitro* and *in vivo*,[13] modulate inflammatory responses during tendon healing,[14] and upregulate tenocytic markers when cocultured with tenocytes.[15]

Because ASCs remain viable for a relatively short period of time when delivered *in vivo*,[16,17] even in an immunocompromised model,[18] the therapeutic benefit of delivering ASCs to sites of tendon injury depends on ASCs' interactions with native tenocytes.[19] One method to examine these interactions is through coculture systems that allow for different forms of cell-to-cell interaction. In these coculture systems, there is building evidence that ASCs can not only differentiate along desirable lineages but also secrete paracrine signals that target surrounding cells, promoting proliferation, synthetic activities, and migration.[20–22] These interactions facilitated by stem cell coculture have already been explored in various tissues including cartilage,[23–25] bone,[26–29] vasculature,[10,30] lung,[22,31] kidney,[32] liver,[33–35] and nerve.[36–38]

Because these interactions have been well documented in other tissues, we hypothesize that ASCs will similarly promote proliferation, synthetic activities, and migration when cocultured with tenocytes. Although ASC-tenocyte coculture has been shown to promote ASCs' differentiation toward a tenogenic lineage,[15] there is limited understanding of the nature, mechanism, and potential therapeutic benefit of the interaction between these 2 cell types. Thus, in this study, we aimed to explore the nature of this interaction by examining proliferation, collagen production, and cell migration in an *in vitro* and *ex vivo* environment and the mechanism of this interaction by comparing 2 different coculture systems. In addition, human cells were used to expedite eventual translation. The goal is to optimize cell-based therapy as a therapeutic option for augmenting intrinsic tendon healing.

MATERIALS AND METHODS

Cell culture

Human ASCs (Lonza, Walkersville, MD) were cultured in adipose-derived stem cell growth medium (ASC-GM) (Lonza). Human tenocytes were primarily harvested from human flexor tendon specimens obtained through a surgical biopsy (institutional review board–approved protocol) of an unscarred area to avoid cell harvest from pathological specimens. Briefly, specimens were minced into 1-mm segments, trypsin-bathed (Sigma-Aldrich, St. Louis, MO) samples were incubated at 37°C on a rotator, the suspension was centrifuged, and the supernatant was removed. Tendon samples were then incubated in collagenase type I (Sigma-Aldrich), the suspension was passed through a cell strainer, the filtrate was centrifuged, and the remaining cell pellet was resuspended and cultured in F-12 media (Invitrogen, Waltham, MA). Adult skin derived Normal Human Dermal Fibroblasts (NHDF-Ad) (Lonza) were grown in fibroblast basal media (Lonza). All media were supplemented with 10% fetal bovine serum, and all cells were grown to 90% confluence at 37°C in a humidified tissue culture chamber with 5% carbon dioxide content.

Direct coculture

Tenocytes (T) and ASCs (A) were directly cocultured in triplicate with 10,000 cells per well in a 24-well plate at various ratios (50% T/50% A; 25% T/75% A; 75% T/25% A) and compared to monoculture controls (100% T, 100% A). The F-12 media and

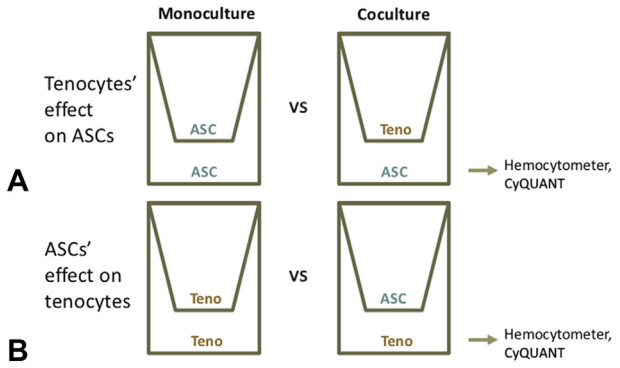


FIGURE 1: Indirect coculture system. **A** To determine tenocytes' effect on ASCs, ASCs were seeded in the bottom and tenocytes were seeded in the insert, and this was compared with a monoculture system in which ASCs were seeded in both chambers. **B** To determine ASCs' effect on tenocytes, tenocytes were seeded in the bottom and ASCs were seeded in the insert, and this was compared with a monoculture system in which tenocytes were seeded in both chambers. Cells in the bottom chamber were used for analysis, given the difficulty of extracting cells from the insert. Teno, tenocytes.

ASC-GM were combined in a 1:1 ratio and changed every other day.

To assess whether the observed results are ASC-dependent, we repeated this experiment by replacing ASCs with fibroblasts (F), coculturing in various ratios (50% T/50% F; 25% T/75% F; 75% T/25% F), and comparing with monoculture controls (100% T, 100% F). The F-12 media and fibroblast basal media were combined in a 1:1 ratio and changed every other day.

All experiments were conducted separately 3 times.

Indirect coculture

We used 0.4-μm pore transwell inserts (Corning Incorporated, Corning, NY) to create an indirect, bilayer coculture system allowing for exchange of soluble factors but not cells. In a 24-well plate, the bottom chamber was seeded at a density of 10,000 cells per well while the insert was seeded at a density of 5,000 cells per insert. Cells in the bottom chamber were used for analysis, given the difficulty of extracting cells from the insert. To determine tenocytes' effect on ASCs, ASCs were seeded in the bottom and tenocytes were seeded in the insert, and this was compared with a monoculture system in which ASCs were seeded in both chambers (Fig. 1). To determine ASCs' effect on tenocytes, tenocytes were seeded in the bottom and ASCs were seeded in the insert, and this was compared with a monoculture system in which tenocytes were seeded in both chambers. For all groups, F-12 media and ADSC-GM were combined in a 1:1 ratio and changed every other day.

Measuring cellular proliferation

On day 5, when wells reached 70% to 90% confluency, proliferation was measured in direct and indirect coculture systems with a hemocytometer and confirmed with CyQUANT Cell Proliferation Assay (Invitrogen), which was used according to the manufacturer's protocol. Synergy was quantified using interaction index (II), which normalizes measured coculture values by the expected values assuming no interaction (no synergy when II = 1).[39] For example, the II of proliferation in ASC-tenocyte coculture was calculated as follows:

$$\mathrm{II} = \frac{\mathrm{Proliferation}_{\mathrm{Measured}}}{\mathrm{Proliferation}_{\mathrm{Expected}}},$$

where

$$\begin{aligned}&\mathrm{Proliferation}_{\mathrm{Expected}}\\&= (\%\ \mathrm{ASCs})(\mathrm{Proliferation}_{100\%\ \mathrm{ASC}})\\&+ (\%\ \mathrm{Tenocytes})(\mathrm{Proliferation}_{100\%\ \mathrm{Tenocyte}}).\end{aligned}$$

In vitro tenocyte migration

To assess ASCs' ability to elicit the migration of tenocytes, we used a 24-well, 8-μm pore, cell migration plate (Cell Biolabs, San Diego, CA). Migratory cells can pass through the insert's pores in the polycarbonate membrane to cling on the underside. Tenocytes were seeded in the upper chamber, and either tenocytes (negative control) or ASCs were seeded in the lower chamber. We added 100 μL and 500 μL of a 0.5 million cells/mL cell suspension to the upper and lower chambers, respectively.

After cells were allowed to migrate for 12 hours at 37°C, cell detachment buffer (Cell Biolabs) was added to dissociate migratory tenocytes from the membrane into a fresh 24-well plate, CyQUANT GR fluorescent dye (Cell Biolabs) was added, and fluorescence was read with a fluorescence plate reader at 480 nm/520 nm. This experiment was performed in duplicate for each condition.

Ex vivo tenocyte migration

We assessed ASCs' ability to elicit tenocyte migration in an *ex vivo* system by reseeding cadaveric human flexor tendons that were harvested from fresh, frozen cadaver forearms (Science Care, Phoenix, AZ) and decellularized using a previously published protocol.[40] Tendons were transected into 0.5-cm segments, and pairs of 2 segments were skewered onto a 25-gauge needle (Becton Dickinson & Co,

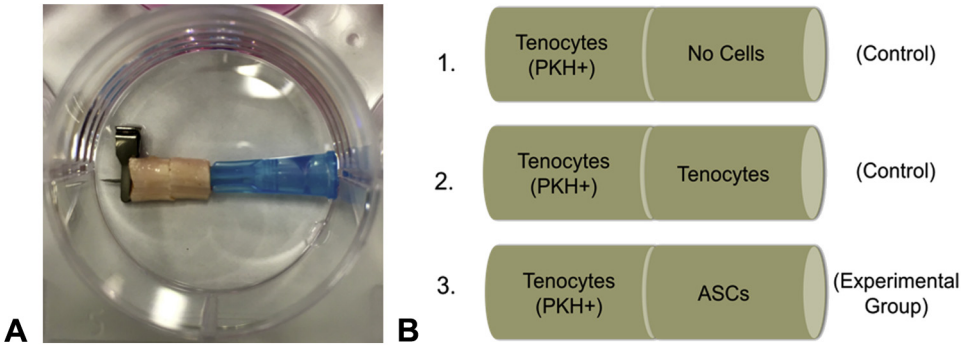


FIGURE 2: *Ex vivo* migration tendon construct and experimental groups. **A** The tendon construct included 2 tendon segments skewered onto a 25-gauge needle and a microvascular clamp that ensured that tendon ends remain in direct contact. **B** All groups included 1 PKH26-labelled tendon segment (left) and a second tendon segment (right) that was (1) not reseeded, (2) reseeded with unlabeled tenocytes, or (3) reseeded with unlabeled ASCs.

Franklin Lakes, NJ) to minimize manipulation of seeded tendons. A microvascular clamp (Accurate Surgical & Scientific, Westbury, NY) was placed on the needle end, immediately next to the tendon, to ensure that tendon ends remained in direct contact, and the entire construct was placed in a 6-well plate (Fig. 2A).

In all groups, 1 tendon segment was reseeded with tenocytes labeled by following the manufacturer's protocol for the PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich), which incorporates a fluorescent dye into cell membranes. The second tendon segment was (1) not reseeded, (2) reseeded with unlabeled tenocytes, or (3) reseeded with unlabeled ASCs (Fig. 2B). Conditions 1 and 2 served as negative controls, and 3 was the experimental group. To reseed tendons, each segment was placed in 1 mL of a 1.0 million cells/mL cell suspension, and underwent cell attachment for 8 hours in sealed tubes at 37°C on a rotating mixer (RPI Corp, Mount Prospect, IL).

On days 1, 5, and 9, the construct was imaged with a fluorescence microscope (Keyence BZ-X700, Itasca, IL). The TexasRed filter allowed for visualizing migration of PKH26-labelled tenocytes only. We then quantified migration of PKH26-labelled tenocytes by measuring the integrated density of fluorescence, a measurement of overall fluorescence intensity, with ImageJ Software (National Institutes of Health, Bethesda, MD).

Collagen I production

To quantify collagen I production in direct coculture systems, we used an enzyme-linked immunosorbent assay, On day 5, after fixing cells with 2% formalin, 0.3% hydrogen peroxidase in phosphate buffered saline was used to quench the endogenous peroxidase. The primary antibody, monoclonal anticollagen type I (Sigma-Aldrich), was added at a concentration of 1:1000 and incubated overnight at 4°C. The cells were washed and the peroxidase-conjugated secondary antibody (Sigma-Aldrich) was added at a concentration of 1:10,000 and incubated at room temperature for 1 hour. The concentration for both primary and secondary antibodies had been previously optimized by means of titration. The cells were washed again and 3,3',5,5'-tetramethylbenzidine liquid substrate system (Sigma-Aldrich) was added for 30 minutes. Then, 0.5 M sulfuric acid was added to stop the reaction, A 100-μL/sample was transferred to a 96-well plate, the plate was read on a microplate reader at 450 nm, and the optical density (OD) reading was recorded.

To quantify synergy, the II of collagen I production in ASC-tenocyte coculture was calculated as follows:

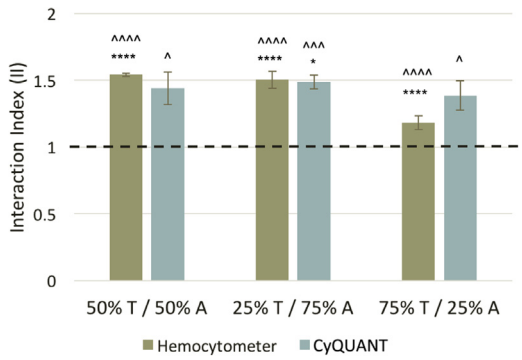
$$\mathrm{II} = \frac{\mathrm{OD}_{\mathrm{Measured}}}{\mathrm{OD}_{\mathrm{Expected}}},$$

where

$$\mathrm{OD}_{\mathrm{Expected}} = (\%\ \mathrm{ASCs})(\mathrm{OD}_{100\%\ \mathrm{ASC}}) + (\%\ \mathrm{Tenocytes})\left(\mathrm{OD}_{100\%\ \mathrm{Tenocyte}}\right).$$

Statistical methods

Results are reported as mean ± SD, and differences were evaluated with an unpaired *t* test with significance set at *P* less than .05. A post hoc power analysis given an alpha set at .05, 2-tailed test, and the effect size observed in the present study was conducted.[41] Post hoc power analysis for ASC-tenocyte coculture hemocytometer studies revealed that we would need a minimum of 6 samples, demonstrating that our study was sufficiently powered to detect the



(*) indicates statistical significance against tenocyte control (100% T) and (^) indicates statistical significance against ASC control (100% A), with *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; and ****$P \leq .0001$.

FIGURE 3: Direct coculture of tenocytes and ASCs. The interaction index of hemocytometer counts and CyQUANT assays were greater than 1 for all coculture ratios, demonstrating statistically significant synergistic proliferation of all coculture ratios compared with 100% T and 100% A controls (II = 1). A, ASCs; T, tenocytes; error bars represent SD.

observed differences in proliferation between coculture and monoculture controls for ASCs and tenocytes. However, we were underpowered to detect a small difference, should one exist, in our negative control group (fibroblast-tenocyte coculture).

RESULTS

Direct coculture: proliferation

In tenocyte and ASC direct coculture, the II of hemocytometer counts were greater than 1 for all coculture ratios (50% T/50% A = 1.54 ± 0.01; 25% T/75% A = 1.50 ± 0.06; 75% T/25% A = 1.18 ± 0.05), as were those of CyQUANT assays (50% T/50% A = 1.44 ± 0.12; 25% T/75% A = 1.48 ± 0.05; 75% T/25% A = 1.39 ± 0.11), demonstrating statistically significant synergistic proliferation of all coculture ratios compared with 100% T and 100% A controls alone (II = 1) ($P < .05$) (Fig. 3).

Comparisons across the 3 coculture groups for hemocytometer counts demonstrated that 75% T/25% A resulted in less synergistic proliferation than 50% T/50% A ($P = .001$) and 25% T/75% A ($P < .05$); however, there was no difference between the 50% T/50% A and the 25% T/75% A groups ($P > .05$). Comparisons across coculture groups for CyQUANT assays demonstrated no differences in synergistic proliferation ($P > .05$).

In tenocyte and fibroblast direct coculture, II of hemocytometer counts for coculture ratios were as follows: 50% F/50% T = 0.73 ± 0.11; 25% F/75% T = 0.91 ± 0.16; and 75% F/25% T = 0.77 ± 0.06. The II of CyQUANT assays were as follows: 50% F/50% T = 0.98 ± 0.12; 25% F/75% T = 1.01 ± 0.00; and 75% F/25% T = 0.93 ± 0.05. No coculture ratios demonstrated statistically significantly greater II compared with 100% T and 100% F controls alone (II = 1) ($P > .05$). Comparisons across coculture groups for hemocytometer counts and CyQUANT assays demonstrated no differences in II ($P > .05$).

Indirect coculture: proliferation

In tenocyte and ASC indirect coculture, there was no significant difference ($P > .05$) between all coculture and monoculture conditions for both hemocytometer and CyQUANT assay results (Fig. 4). This was true for experiments measuring tenocytes' effect on ASCs as well as ASCs' effect on tenocytes, suggesting that secreted soluble factors alone are not sufficient to elicit the synergistic effect.

In vitro tenocyte migration

When tenocytes were seeded in both the upper and the lower chamber, tenocyte migration as measured by a fluorescent plate reader was 191.11 ± 38.17 relative fluorescence units. In contrast, when ASCs were seeded in the lower chamber and tenocytes in the upper chamber, tenocyte migration was 499.14 ± 153.65 relative fluorescence units ($P < .01$) (Fig. 5), signifying that ASCs stimulate more tenocyte migration than the control.

Ex vivo tenocyte migration

On day 1, no group demonstrated migration of PKH26-labelled tenocytes (Fig. 6), with integrated density of fluorescence (IDF) of 616,203, 634,551, and 634,363 for groups 1, 2, and 3, respectively. On day 5, group 3, with an ASC-seeded tendon in contact with labeled tenocyte-seeded tendon, demonstrated limited tenocyte migration (IDF 830,636), and groups 1 and 2, with acellular tendon (IDF 596,618) and tenocyte-seeded tendon (IDF 682,764) in contact with labeled tenocyte-seeded tendon, respectively, demonstrated no tenocyte migration. On day 9, group 1 continued to demonstrate no tenocyte migration (IDF 636,871), group 2 demonstrated limited tenocyte migration (IDF 879,408), and group 3 demonstrated considerable tenocyte migration compared with other groups (IDF 1,337,563).

Direct coculture: collagen I production

In tenocyte and ASC direct coculture, the II for collagen production was as follows: 50% T/50% A = 1.08 ± 0.11; 25% T/75% A = 1.13 ± 0.05; and 75% T/25% A = 1.13 ± 0.03. Compared with the 100% T and 100% A monoculture controls (II = 1), the 2

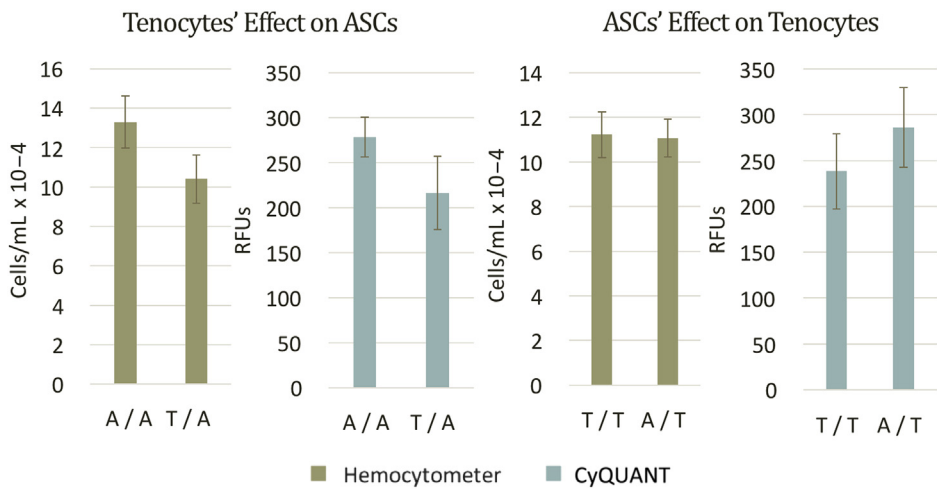


FIGURE 4: Indirect coculture of tenocytes and ASCs. Compared with indirect monoculture controls, indirect coculture of tenocytes and ASCs did not demonstrate greater proliferation for either hemocytometer or CyQUANT assay results. RFU, relative fluorescence units; T, tenocytes; error bars represent SD.

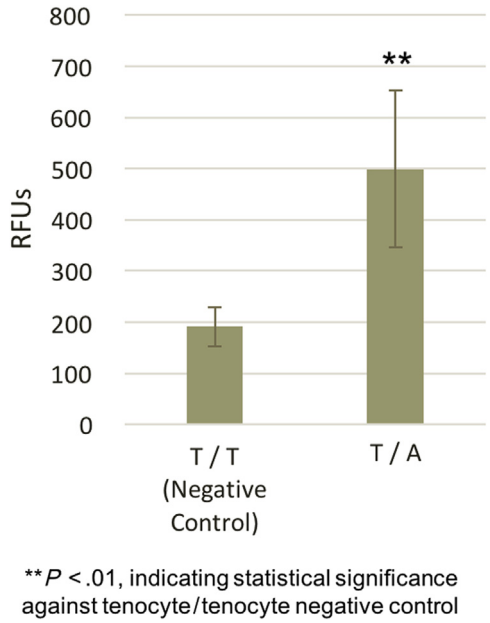


FIGURE 5: *In vitro* tenocyte migration. There was greater tenocyte migration through the upper chamber when ASCs were seeded in the lower chamber, as compared to tenocytes seeded in the lower chamber. RFUs, relative fluorescence units; T, tenocytes; error bars represent standard deviation)

latter coculture ratios resulted in synergistic collagen I production ($P < .05$). Comparisons across tenocyte-ASC coculture groups demonstrated no differences in II of collagen I production ($P > .05$).

In tenocyte and fibroblast direct coculture, the II for collagen production was as follows: 50% F/50% T = 1.00 ± 0.02; 25% F/75% T = 0.91 ± 0.07; and 75% F/25% T = 1.05 ± 0.14. Compared with the 100% T and 100% F monoculture controls (II = 1), no coculture groups resulted in synergistic collagen I production ($P > .05$). Comparisons across tenocyte-fibroblast coculture groups demonstrated no differences in II of collagen I production ($P > .05$).

Discussion

The ASCs are a promising cell type for cell-based therapies targeting tendon injuries[42,43] because they have been reported to enhance primary tendon healing[12] and promote tissue repair via paracrine signaling.[44–46] The success of cell-based therapy to target tendon injury relies on ASCs' ability to stimulate native tenocytes in ways that enhance these tenocytes' natural ability to repair tendons. The ASCs can accomplish this in 3 ways: (1) stimulating tenocyte proliferation, (2) stimulating synthesis of extracellular matrices (ECM), and (3) recruiting additional tenocytes to the site of injury. Whereas studies coculturing tenocytes and ASCs have shown that tenocytes are able to direct ASCs toward a tenogenic lineage,[15,47] the interaction between these 2 cell types, and thus ASCs' potential therapeutic effect, has remained unclear.

In this study, we demonstrated with 2 different proliferation outcomes analyses that direct coculture of ASCs and tenocytes leads to synergistic proliferation, which is supported by the literature.[15] However, further details of the interaction remained unknown. To elucidate the mechanism driving this synergistic proliferation, we cocultured ASCs and tenocytes in an indirect coculture system and demonstrated the absence of synergistic proliferation. This indicates that the stimulatory effect is mediated by direct cell-to-cell

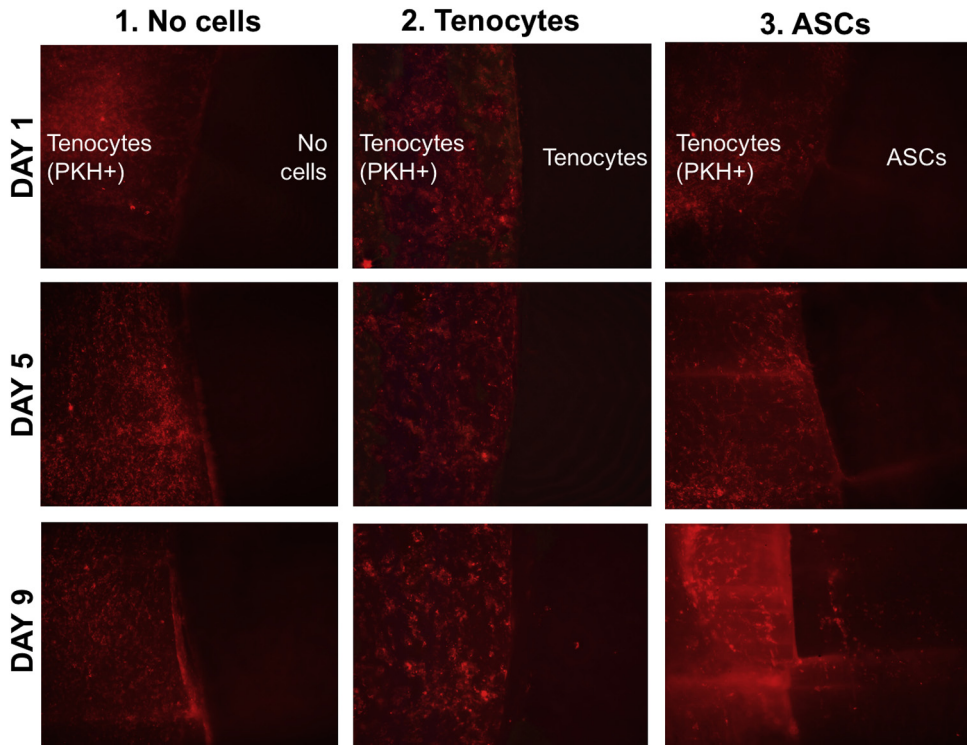


FIGURE 6: *Ex vivo* tenocyte migration. On day 1, all groups demonstrated no migration of PKH26-labelled tenocytes. On day 5, group 3 demonstrated limited tenocyte migration, and groups 1 and 2 demonstrated no tenocyte migration. On day 9, group 1 continued to demonstrate no tenocyte migration, group 2 demonstrated limited tenocyte migration, and group 3 demonstrated considerable tenocyte migration compared with other groups.

contact and that secreted soluble factors, even if contributory, are not sufficient alone. Further, although previous stem cell coculture studies have identified stem cells as the cell type responsible for stimulating proliferation,[44,48] this had yet to be confirmed with tenocytes. After directly coculturing tenocytes with fibroblasts, absence of a synergistic effect confirmed that ASCs play the critical role.

We further characterized the interaction between ASCs and tenocytes by demonstrating ASCs' ability to attract tenocytes in both an *in vitro* and an *ex vivo* environment. This provides evidence for ASC delivery as a promising therapeutic strategy because it would not only stimulate cell proliferation but also recruit tenocytes, the primary cell type facilitating native tendon healing,[49] to the site of injury.

Finally, directly cocultured ASCs and tenocytes exhibited synergistic collagen I production, and this effect was also lost after replacing ASCs with fibroblasts. This suggests that, once native tenocytes have migrated to the delivered population of ASCs, cell-to-cell interactions can potentially enhance synthetic capabilities. This is yet another advantage of ASC delivery because remodeling of ECM and deposition of collagen I, which makes up greater than 90% of the structural elements of normal tendon,[50] are critical to tendon repair.

There are several limitations to this study. First, it is unknown whether the cells being stimulated to undergo increased proliferation and collagen I production are tenocytes or ASCs. The dominant cell type undergoing the observed synergy has implications for whether collagen I is laid down into the organized collagen fibrils of tendons because tenocytes, but not ASCs, have the capacity to do this. Although future studies are needed to differentiate between these 2 cell types in direct coculture, previous studies demonstrating that ASCs differentiate into a tenogenic lineage in ASC-tenocyte coculture[15] suggest the possibility that, even if ASCs are the dominant cell type undergoing synergistic proliferation and collagen production, they can ultimately participate in tendon generation. Second, it is unknown whether the observed proliferation, collagen I production, and migration will also occur in an *in vivo* environment. Work is under way to answer this critical question.

Our results support the use of ASCs as the cell type for cell-based therapy targeting tendon injuries because of favorable interactions between delivered ASCs and native tenocytes. Specifically, our study

demonstrated that direct coculture of ASCs and tenocytes results in synergistic proliferation and collagen I production and that ASCs stimulate tenocyte migration *in vitro* and *ex vivo*. Given the key role that tenocyte proliferation and collagen deposition play in tendon healing, these cell-to-cell interactions may provide therapeutic benefit to patients with a variety of tendon injuries.

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