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In vitro mutual interaction between tenocytes and adipose-derived mesenchymal stromal cells

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Abstract

Background aims. Tendon is a complex tissue with a reduced regenerative ability. Nowadays, little or nothing is known about the regenerative effect of adipose-derived mesenchymal stromal cells (ADSCs) on tendons. *Methods.* The study aimed to evaluate the *in vitro* mutual interaction of ADSCs and tenocytes in standard culture conditions and a microwound healing model. Tenocyte viability, microwound recovery and the expression of genes encoding for the main extracellular matrix components and ADSC viability, differentiation and growth factor gene expression were evaluated. *Results.* The effects of ADSCs on tenocytes were observed more in the microwound healing model, in which the rate of microwound healing and the expression of decorin, tenascin and collagens were significantly increased. The influence of tenocytes on ADSCs was also found in standard culture conditions: ADSCs were directed toward a tenogenic lineage, and growth factor expression increased. *Conclusions.* This study clarifies some aspects of the mutual interaction of ADSCs and tenocytes and provides *in vitro* evidence for a possible future application of ADSCs as a therapeutic strategy for tendon repair.

Key Words: *adipose-derived mesenchymal stromal cells, gene expression, mutual interaction, tendon healing, tenocytes*

Introduction

Tendon is a complex tissue that connects and translates motion from muscle to bone. It is composed of a dense extracellular matrix (ECM) of collagen fibrils, produced by specialized tendon-specific fibroblasts (tenocytes) [1].

Tendon inflammation, degeneration and then rupture are due to several factors, including overuse and sudden increased strain. The avascular nature, relatively poor content of tenocytes (highly differentiated cells with limited proliferation rate) and reduced cellular metabolic rate are responsible for the poor tendon self-healing ability that results in poor tendon quality and reduced mechanical properties [2]. The repair process is slow and a scar tissue forms with reduced mechanical properties, thus making it prone to re-injury [3]. If not treated, knee tendon damage might cause instability and lead to the early onset of osteoarthritis, pain and disability [4]. Conventional therapies aim to restore the biological and biomechanical status of the native tissue. These therapies include drugs, physiotherapy and surgery, which mainly involves autografts, allografts, xenografts or the use of prosthetic devices (in severe tendon injuries) [5]. Combined donor site morbidity, high failure rates and the risk of re-injury impede long-term functional recovery. Thus, finding a successful therapeutic approach is still a challenge and a biological strategy is required to improve the quality of the tissue. Mesenchymal stromal cells (MSCs) have already been used in the regeneration of several musculoskeletal tissues [6], thanks to their ability to differentiate *in situ* toward the desired cell lineages. Moreover, MSCs are able to secrete trophic molecules, such as growth factors (GFs) and chemotactic molecules, which can recruit additional reparative cells into the lesion site [7]. Bone marrow–derived MSCs (BMSCs) are the most commonly used cells in tissue engineering, and the implantation of BMSCs into tendon injury has provided promising results in several *in vivo* studies, thus indicating that the microenvironment and cellular interactions play an

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important role in MSC differentiation [8]. Among other potential sources of MSCs [9], adipose-derived MSCs (ADSCs) appear to be the most promising stem cell population to have clinical relevance as an alternative for tendon repair. In addition, it has been reported that ADSCs have numerous advantages over BMSCs because they are readily available, easy to extract and isolate and do not produce postoperative complications [10]. The *in vitro* growth and efficacy of ADSCs has been reported in preclinical and clinical studies [11]. Some previous *in vitro* studies showed the ability of ADSCs to undergo tenogenic differentiation in the presence of appropriate biological stimuli, tensile strain, oxygen tensions [12–15], scaffolds [16,17] and when co-cultured with tenocytes as in a 3-dimensional high-density system [18,19].

Several studies have analyzed the molecular events that occur after tendon injury and during the healing process [20–23], but preclinical research on the ability of ADSCs to regenerate tendons is still in its infancy. Until now, the few *in vivo* studies that have shown the regeneration potential of ADSCs on tendon transections and tendonitis have not clarified the exact molecular mechanisms of action [24–28].

Starting from the hypothesis that there might be a mutual interaction between ADSCs and tenocytes, the main purpose of the present study was to verify whether ADSCs improve tenocyte viability and gene expression in an *in vitro* indirect co-culture system that allows the exchange of soluble factors between the two cell types without cell-cell contact. An *in vitro* tenocyte microwound healing model was also adopted as described in a previous study [29]. In addition, tenocyte influence on ADSCs was assessed in both standard culture conditions and in the microwound healing model.

It was observed that ADSCs not only improved the *in vitro* tenocyte viability and gene expression and the microwound repair, but also produced a mutual effect between tenocytes and ADSCs in both culture conditions.

Methods

The study protocol fell within the scope of other projects on bone, not affecting tendons, adipose tissue and ADSCs. These studies were approved by the Rizzoli Orthopaedic Institute Ethical Committee and by the Italian Ministry of Health and were performed strictly following the current laws on animal experiments.

Cell isolation

The Achilles tendons of three healthy 5-month-old female Sprague Dawley rats were dissected under sterile conditions. The explants were cut into 2 × 2-mm fragments and were cultured in growth tenocyte medium (TM), composed of a 1:1 mixture of HAM'S F12:Dulbecco's modified Eagle's medium (Sigma Aldrich, St Louis, MO, USA), 10% fetal bovine serum (Lonza, Verviers, Belgium), 1% penicillin-streptomycin solution (Gibco, Invitrogen, Carlsbad, CA, USA) and 25 µg/mL ascorbic acid (Sigma Aldrich). The cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. After 7 days, the tenocytes were observed in the cultures and then were grown until 90% confluence. The tenocytes were used for the experimental study after the first passage (P1).

Abdominal adipose tissue (7.73 ± 2.53 mg), harvested from the same donors, was digested with 0.075% collagenase II (Sigma Aldrich). The enzymatic reaction was stopped by the addition of complete medium (CM), that is, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Lonza), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, Invitrogen) and 5 µg/mL plasmocin (InvivoGEN, San Diego, CA, USA). The pellet was treated with 160 mmol/L NH_4Cl (Sigma Aldrich) for erythrocyte lysis. The nucleated cells were then seeded in CM. At subconfluence, the adherent cells (ADSCs) were used for the experiments. Before the setup of the indirect co-culture system, the ADSCs were characterized through the evaluations of surface epitopes, clonogenicity and osteogenic differentiation [21].

Indirect co-culture (standard culture condition)

The tenocytes were plated at the bottom of 24-well plates at a concentration of 1 × 10^4 cells/well in TM. ADSCs were seeded in 24-well 0.4-µm pore size transwells (Millipore, Carrigtwohill, Country Cork, Ireland) at a concentration of 3 × 10^3 cells/transwell in CM. At nearly 80% of confluence, ADSC-containing transwells were transferred into the wells with tenocytes and the co-cultures were maintained for up to 7 days in CM. Single cultures of tenocytes or ADSCs were used as controls. All the experiments were performed in triplicate and evaluations were performed after 24 hours and 3 days and 7 days of cultures.

In vitro tendon microwound healing model

In parallel, an *in vitro* microwound healing model was performed [29]. Briefly, once a confluent monolayer of tenocytes had been grown, a sterile Eppendorf tip was used to produce an artificial wound of 900-µm width by scraping the cell layer (time zero, T0). The transwells containing ADSCs were then transferred to the wells and evaluations were performed after 1, 4, 8, 24 and 48 hours (T1, T4, T8, T24 and T48). Empty transwells were transferred to the wells as controls, and all the experiments were performed in triplicate.

Table I. Detailed list of primers for quantitative polymerase chain reaction analysis.

Gene	Primer forward	Primer reverse	Amplicon length	Annealing Temperature
Gapdh	5′-CTCTACCCACGGCAAGTTCAAC-3′	5′-GACATACTCAGCACCAGCATCAC-3′	136 bp	60°C
Col1a1	5′-GCAAGAGGCGAGAGAGGTTT-3′	5′-GGACCAATGGGACCAGTCAG-3′	290 bp	57°C
Col3a1	Rn_Col3a1_1_SG		75 bp	55°C
Scx	Rn_Scx_1_SG		67 bp	55°C
Dcn	Rn_Dcn_1_SG		147 bp	55°C
Tnc	Rn_Tnc_1_SG		106 bp	55°C
Runx2	Hs_RUNX2_1_SG[a,b]		101 bp	55°C
Pparg	Rn_Pparg_1_SG[a]		146 bp	55°C
Tgfb1	Rn_Tgfb1_1_SG[a]		145 bp	55°C
Fgf2	Rn_Fgf2_1_SG[a]		138 bp	55°C
Nos2	Rn_Nos2_1_SG[a]		100 bp	55°C
Vegfa	Rn_RGD:619991_1_SG[a]		68 bp	55°C

[a]QuantiTect Primer Assay (Qiagen).
[b]Designed for human *Runx2,* also detects rat transcript.

Cell viability, microwound healing rate and average of cell migration speed

In both the standard culture condition and microwound healing model, four experimental groups were defined as follows: TEN-Co group: tenocytes co-cultured with ADSCs; TEN group: tenocytes in single culture (control); ADSC-Co group: ADSCs co-cultured with tenocytes; ADSC group: ADSCs in single culture (control).

Viability was evaluated in the standard culture condition by the WST1 colorimetric reagent test (WST1, Roche Diagnostics GmbH, Mannheim, Germany). A 1:10 dilution of WST1 reagent was added to the cells and incubated at 37°C for 4 h. Supernatants were quantified at 450 nm, with a reference wavelength of 625 nm. Results of the WST1 test are reported as optical density.

In TEN-Co and TEN groups, the microwound was observed by means of an inverted microscope (IX70, Olympus, Munster, Germany) at T1, T4, T8, T24 and T48. Three images per well were analyzed at ×10 magnification to measure the whole length of the wound. Image analysis was performed with the use of Cell^{B} imaging software (Olympus), and the width of the artificial wounds was measured by a blind investigator (10 measures per image). The percentage of healing was calculated according to the formula: (T0 − final time width) × 100/T0 width. In addition, the average migration speed was calculated by dividing the average width values between different time points by time-point hours.

Gene expression analysis (RNA isolation and quantitative reverse transcription polymerase chain reaction)

Total RNA was isolated from tenocytes and ADSCs in both the standard culture condition and the microwound healing model. RNA was extracted with the use of TRIzol Reagent (Invitrogen) and reverse-transcribed with the use of the Superscript VILO complementary DNA Synthesis kit (Invitrogen), following the manufacturer's instructions. Complementary DNA was quantified with the use of the Quant-iT Pico-Green dsDNA assay kit (Invitrogen) and diluted to the final concentration of 5 ng/mL. Quantitative polymerase chain reaction analysis was performed in a LightCycler Instrument (Roche Diagnostics GmbH) with the use of the QuantiTect SYBR green polymerase chain reaction kit (Qiagen, Hilden, Germany). The protocol included a denaturation at 95°C for 15 minutes, 25–40 cycles of amplification (95°C, 15 seconds, appropriate annealing temperature for each target as detailed in Table I for 20 seconds and 72°C for 20 seconds) and a melting curve analysis to check for amplicon specificity. The threshold cycle was determined for each sample and used for calculation of relative expression by means of the $2^{-\Delta\Delta Ct}$ method. Table I summarizes the genes under analysis.

Statistical analysis

Statistical evaluation of the data was performed with the use of the software package SPSS/PC+Statistics TM 21 (SPSS Inc., Chicago, IL, USA). The study is the result of three independent experiments, and data are reported as mean ± standard deviation at a significance level of $P < 0.05$. After verifying the normal distribution of data and the homogeneity of variance, a one-way analysis of variance was applied for comparison between groups and between experimental times. Finally, a *post hoc* multiple comparison test (Dunnett) was performed to detect significant differences among groups and controls.

Results

Indirect co-culture (standard culture conditions)

ADSCs expressed high cluster of differentiation (CD) markers CD44, CD73, CD90 and CD105 and weak CD31, CD45 and CD34, nearly 5% of colony-forming units, and were able to differentiate toward an osteogenic lineage under osteogenic stimuli in the culture medium as shown in our previous study [21].

The TEN-Co group showed increased viability at both 3 ($P < 0.05$) and 7 days ($P < 0.0005$), when compared with the TEN group. Moreover, the viability of both groups increased over time ($P < 0.0005$) (Figure 1A). Co-cultured ADSCs showed increased viability at 3 hours in comparison to 24 hours ($P < 0.0005$) and 7 days ($P < 0.005$). When compared with ADSC-Co, the ADSC group showed increased viability at 24 hours ($P < 0.005$) and 7 days ($P < 0.0005$) (Figure 1B).

The TEN-Co group showed an increased expression of Decorin (*Dcn*) at 24 h ($P < 0.0005$), 3 days ($P < 0.005$) and 7 days ($P < 0.05$) and Tenascin C (*Tnc*) at 24 hours ($P < 0.005$) and 3 days

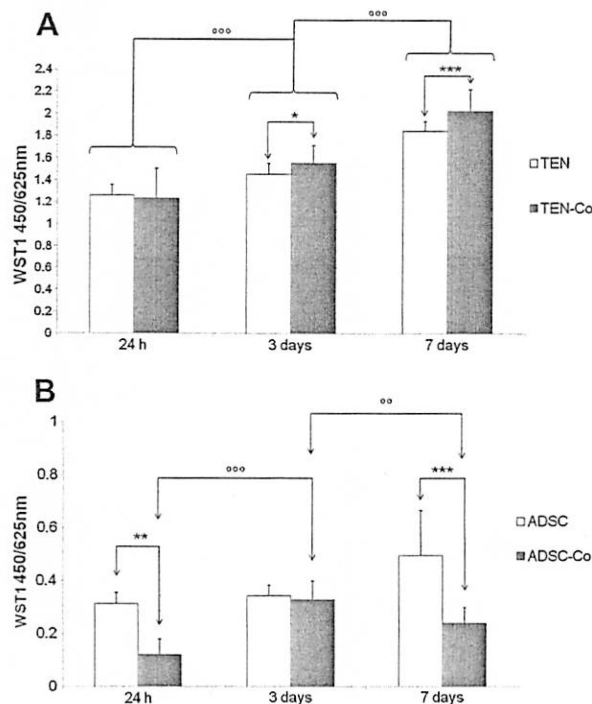

Figure 1. Viability of cells in single cultures and co-cultures, in standard culture conditions, at 24 hours and 3 and 7 days. Data are represented as mean ± standard deviation, $n = 3$ replicates. (A) Tenocytes: *$P < 0.05$: TEN-Co versus TEN at 3 days; ***$P < 0.0005$: TEN-Co versus TEN at 7 days. °°°$P < 0.0005$: 3 days versus 24 hours and 7 days versus 3 days (TEN and TEN-Co). (B) ADSCs: **$P < 0.005$: ADSC versus ADCS-Co at 24 hours; ***$P < 0.0005$: ADSC versus ADCS-Co at 7 days. °°°$P < 0.0005$: 3 days versus 24 hours (ADSC-Co); °°$P < 0.005$: 3 days versus 7 days (ADSC-Co).

($P < 0.05$) in comparison with the TEN group. Conversely, Scleraxis (*Scx*) expression was reduced in the TEN-Co group at 24 hours ($P < 0.05$) and 3 days ($P < 0.005$). For both the TEN-Co and TEN groups, *Dcn* expression was increased at 3 days in comparison with 24 hours ($P < 0.005$). The expression of Collagen I and III (Col1a1, Col3a1) and their ratio did not show significant differences at any experimental time (Figure 2A–C).

The ADSC-Co group showed an increased expression of Transforming growth factor-β1 (*Tgfb1*) and Fibroblast growth factor 2 (*Fgf2*) at 24 hours ($P < 0.05$) and *Scx* at 3 days ($P < 0.005$) compared with the ADSC group. The contrary was observed for the expression of Nitric oxide synthase 2 (*Nos2*) at 3 days ($P < 0.005$), Peroxisome proliferator-activated receptor-γ (*Pparg*) at 3 days and 7 days and *Tgfb1*, *Fgf2* and Vascular endothelial growth factor A (*Vegfa*) at 7 days ($P < 0.05$) (Figure 3A–C).

In vitro tendon microwound healing model

The width of the microwound progressively decreased from 900 μm (at T0) to a mean of 100 ± 5 μm and 51 ± 2 μm (at T48) in the TEN and TEN-Co groups, respectively ($P < 0.0005$) (Figure 4A–C). The healing rate of the TEN-Co group was significantly higher at T4, T24, T48 ($P < 0.0005$) and at T8 ($P < 0.005$) than that of the TEN group, with a significant increase over time for both groups ($P < 0.0005$) (Figure 4D). TEN-Co cells migrated faster (average speed, 31.07 ± 5 μm/h) than did TEN cells (average speed, 23.62 ± 3 μm/h) at T4 and T8 ($P < 0.0005$), whereas the contrary was observed at T48 ($P < 0.005$). The TEN group had a higher migration speed at T8 than at T4 and T24 and higher at T24 than at T48 ($P < 0.0005$). For the TEN-Co group, the values were significantly higher at T8 than at T4 ($P < 0.005$) and T24 ($P < 0.0005$) and at T24 than at T48 ($P < 0.0005$) (Figure 4E).

Dcn at all time points and *Tnc* at T4 and T8 were expressed more by the TEN-Co group than by the TEN group ($P < 0.05$). Conversely, *Scx* was higher at T24 and T48 in the TEN group when compared with the TEN-Co group ($P < 0.05$). The expression of *Col1a1*, at T24, and *Col3a1*, at T48, was, respectively, higher and lower in the TEN-Co group than in the TEN group ($P < 0.05$) (Figure 5A–D).

In comparison to the ADSC group, ADSC-Co showed an increased expression of *Nos2* (T4 and T24), *Tgfb1* and *Fgf2* (T24) and *Vegfa* (T4 and T24) ($P < 0.05$). At T8, *Nos2* expression was greater in the ADSC group than that of the ADSC-Co group ($P < 0.05$).

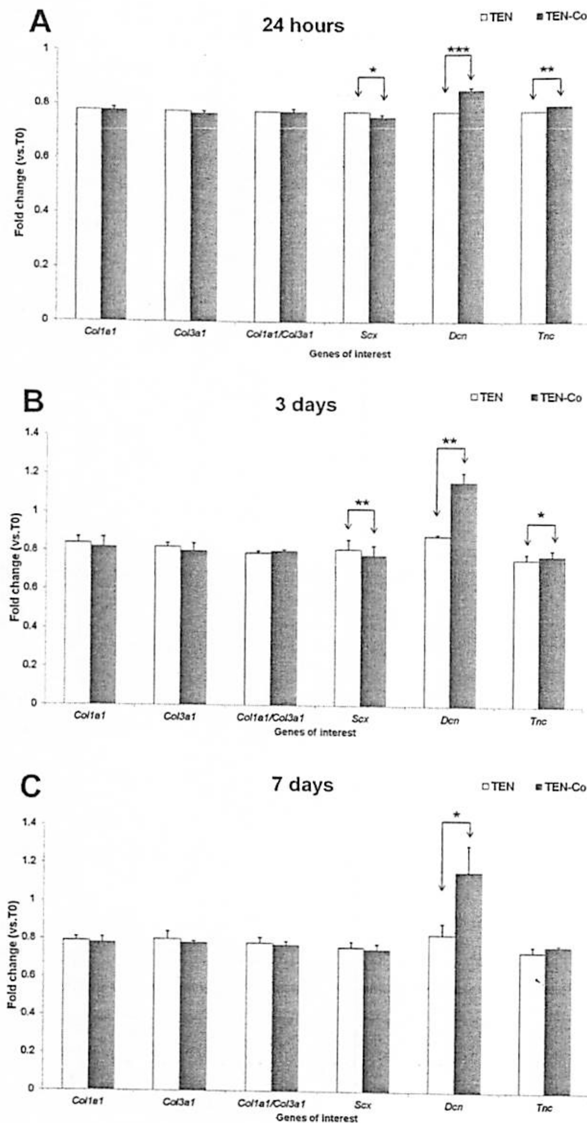

Figure 2. Gene expression analysis of tenocytes in single cultures (TEN) and co-cultures (TEN-Co) in standard culture conditions. Data are represented as mean ± standard deviation, $n = 3$ replicates. (A) At 24 hours: $*P < 0.05$, *Scx*: TEN versus TEN-Co; $***P < 0.0005$, *Dcn*: TEN-Co versus TEN; $**P < 0.005$, *Tnc*: TEN-Co versus TEN. (B) At 3 days: $**P < 0.005$, *Scx*: TEN versus TEN-Co; $**P < 0.005$, *Dcn*: TEN-Co versus TEN; $*P < 0.05$, *Tnc*: TEN-Co versus TEN. (C) At 7 days: $*P < 0.05$, *Dcn*: TEN-Co versus TEN.

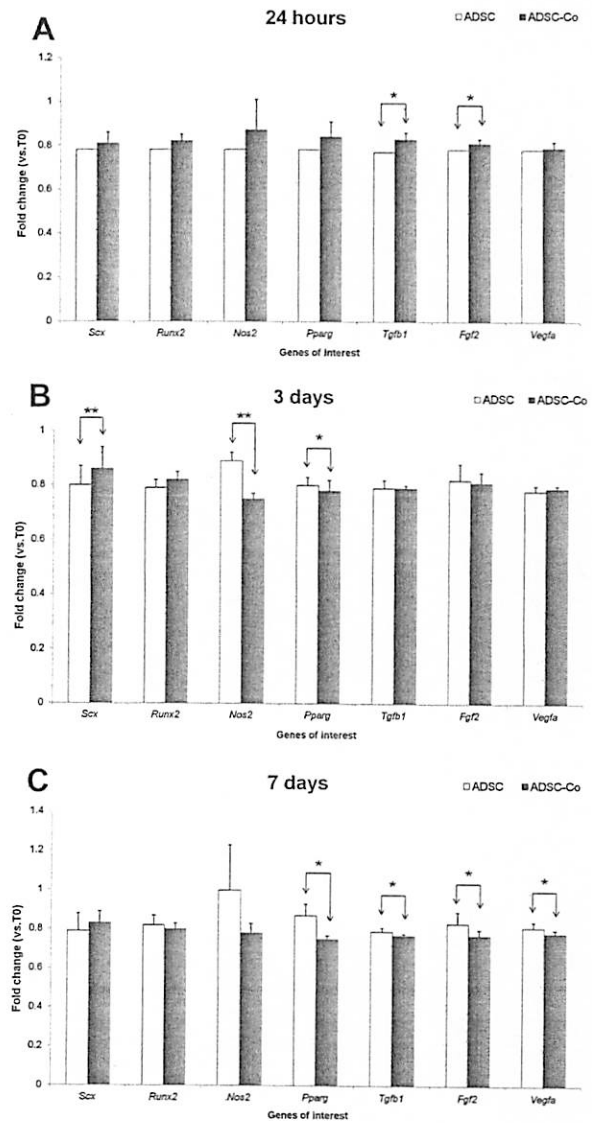

Figure 3. Gene expression analysis of ADSCs in single cultures (ADSC) and co-cultures (ADSC-Co) in standard culture conditions. Data are represented as mean ± standard deviation, $n = 3$ replicates. (A) At 24 hours: $*P < 0.05$, *Tgfb1* and *Fgf2*: ADSC-Co versus ADSC. (B) At 3 days: $**P < 0.005$, *Scx*: ADSC-Co versus ADSC; $**P < 0.005$, *Nos2*: ADSC versus ADSC-Co; $*P < 0.05$, *Pparg*: ADSC versus ADSC-Co. (C) At 7 days: $*P < 0.05$, *Pparg*, *Tgfb1*, *Fgf2* and *Vegfa*: ADSC versus ADSC-Co.

At T48, no significant differences were observed among groups or experimental times for all genes (Figure 6A–D).

Discussion

Tendon injuries are a major socio-economic problem, and, because of the poor healing properties of tendons and the limited efficacy of current therapeutic treatments, research is more often focused on the use of MSCs for tendon regeneration [17,19].

The leading hypothesis of the present *in vitro* study was that ADSCs might be suitable for tissue engineering–based strategies for the treatment of tendon injuries. In recent years, ADSCs have been widely studied in pre-clinical research for the regeneration of musculoskeletal tissue [10] as an alternative to BMSCs, but, until now, only few studies have been performed on tendon tissue. Most studies are *in vivo* evaluations and highlight the role of injected autologous or allogenic ADSCs in improving tendon mechanical strength, healing, functional recovery, collagen fiber organization and

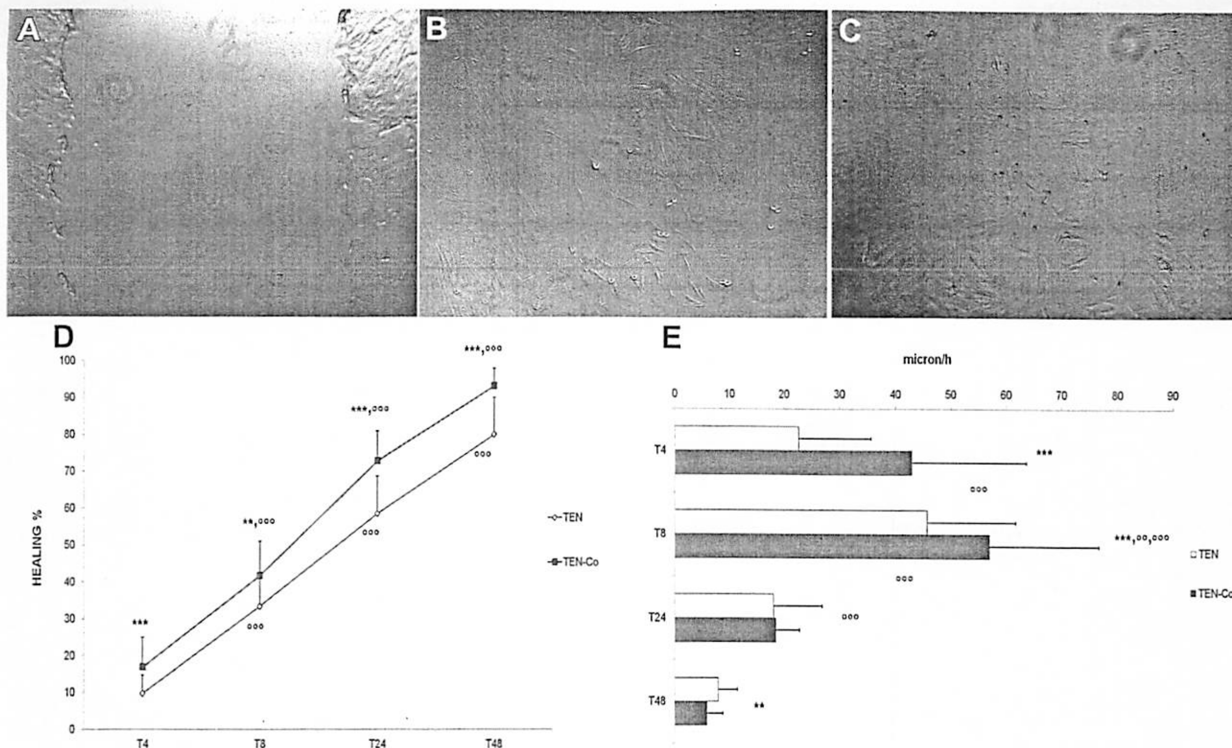

Figure 4. Healing percentage (%), microscopic images and average of migration speed (μm/h) of tenocytes in single cultures (TEN) and co-cultures (TEN-Co) at different culture time points (T4, T8, T24 and T48). Data are represented as mean ± standard deviation, $n = 30$ measures for three replicates. (A) Microscopic image of microwound at T0. Magnification ×10. (B) Microscopic image of microwound at T48 for TEN group. Magnification ×10. (C) Microscopic image of microwound at T48 for TEN-Co group. Magnification ×10. (D) Percentage of microwound healing: $^{***}P < 0.0005$, at T4, T24 and T48: TEN-Co versus TEN; $^{**}P < 0.005$, at T8: TEN-Co versus TEN; $^{\circ\circ\circ}P < 0.0005$: T8 versus T4, T24 versus T8 and T48 versus T24. (E) Average of cell migration speed: $^{***}P < 0.0005$, at T4 and T8: TEN-Co versus TEN; $^{**}P < 0.005$, at T48: TEN versus TEN-Co; $^{\circ\circ\circ}P < 0.0005$, TEN: T8 versus T4 and T24, T24 versus T48; $^{\circ\circ}P < 0.005$, TEN-Co: T8 versus T4; $^{\circ\circ\circ}P < 0.0005$, TEN-Co: T8 versus T24, T24 versus T48.

preventing the progression of the lesion and inflammatory infiltrate [24–28]. However, the molecular mechanisms that govern the healing process of tendons are still poorly understood in comparison with those of other tissues. These analyses may be important to identify the most appropriate treatment strategies.

Therefore, the main purpose of the present *in vitro* study was to investigate the viability and the molecular response of tenocytes under the influence of ADSCs in both standard culture conditions and a microwound healing model. For this purpose, the viability of tenocytes and their expression of the most important tenogenic markers and ECM proteins were analyzed in an indirect co-culture system. This co-culture model, in the absence of cell contact, aims to evaluate the effect of soluble factor exchange between the two cell types and, as affirmed by Kraus *et al.* [18], is applicable in translational research. Moreover, the influence of tenocytes on ADSC differentiation and GF expression was also assessed because the majority of *in vitro* studies induced ADSC differentiation with GFs, scaffolds and tensile strain [12–17], and little is known about ADSC tenogenic differentiation under the effect of tenocytes [18,19].

In the present study, in standard culture conditions, the addition of ADSCs increased tenocyte viability and the expression of *Dcn* and *Tnc* in comparison with the single cultures. Decorin and tenascin C are, respectively, a proteoglycan and a glycoprotein involved in the assembly and in the remodeling of tendon ECM. These proteins confer elasticity to tendons during the development and in the repair processes; their absence decreases the mechanical properties of the tissue and disrupts the collagen fibril architecture [30]. Albeit small, the significant decrease in *Scx* expression, an essential transcription factor for tenocyte differentiation, was observed in tenocytes co-cultured with ADSCs, which is consistent with the *Scx* characteristic of an early marker of tendon development, which is more predominant in tendon progenitor cells than in differentiated ones [20]. As observed also by another literature study, *Scx* is also expressed in mature differentiated tenocytes [31]. However, in this study, the tenocyte phenotype was maintained, given the high levels of *Dcn*, *Tnc* and the constant expression of collagens.

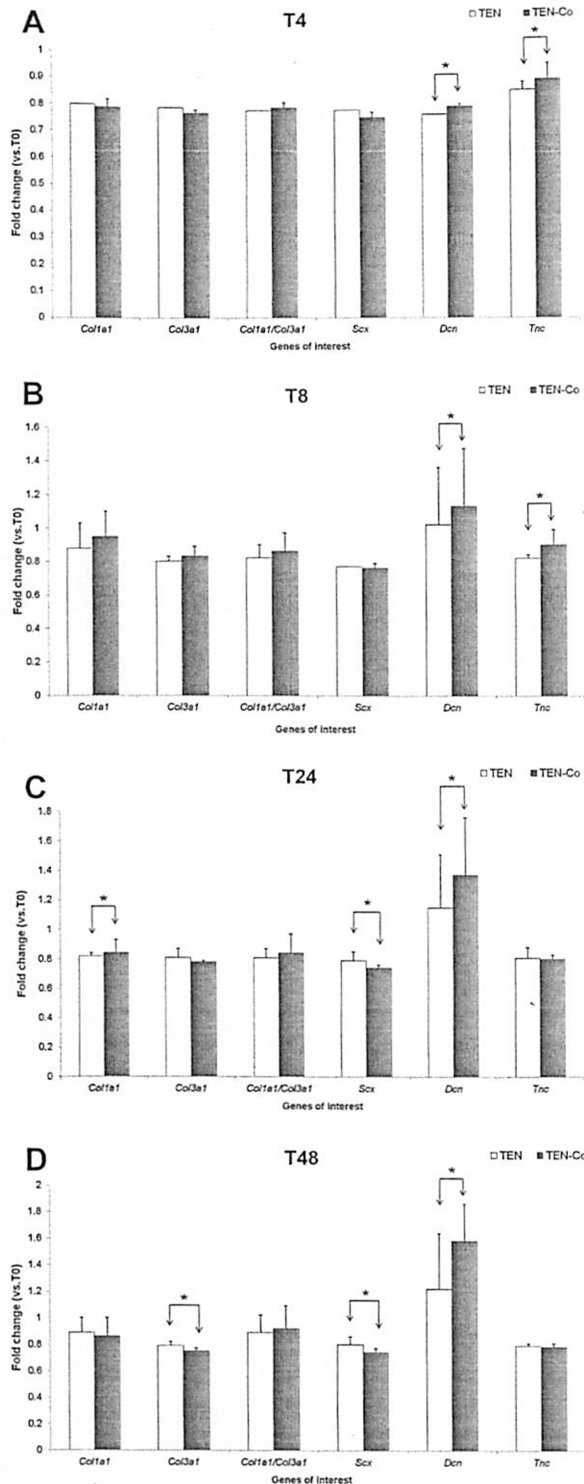

Figure 5. Gene expression analysis of tenocytes in single cultures (TEN) and co-cultures (TEN-Co) in the microwound healing model. Data are represented as mean ± standard deviation, $n = 3$ replicates. (A) T4: $*P < 0.05$, *Dcn* and *Tnc*: TEN-Co versus TEN. (B) T8: $*P < 0.05$, *Dec* and *Tnc*: TEN-Co versus TEN. (C) T24: $*P < 0.05$, *Scx*: TEN versus TEN-Co; $*P < 0.05$, *Dcn*: TEN-Co versus TEN; $*P < 0.05$, *Col1a1*: TEN-Co versus TEN. (D) T48: $*P < 0.05$, *Scx*: TEN versus TEN-Co; $*P < 0.05$, *Dcn*: TEN-Co versus TEN; $*P < 0.05$, *Col3a1*: TEN versus TEN-Co.

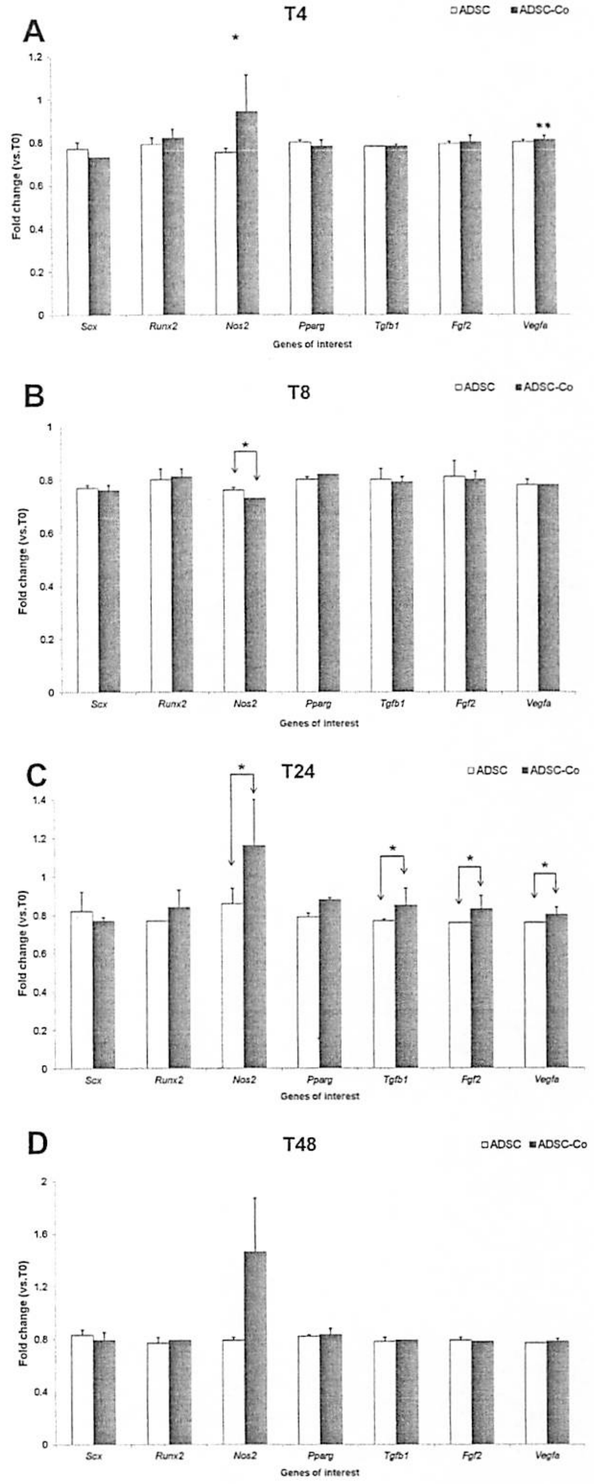

Figure 6. Gene expression analysis of ADSCs in single cultures (ADSC) and co-cultures (ADSC-Co) in the microwound healing model. Data are represented as mean ± standard deviation, $n = 3$ replicates. (A) T4: $**P < 0.005$, *Vegfa*: ADSC-Co versus ADSC; $*P < 0.05$, *Nos2*: ADSC-Co versus ADSC. (B) T8: $*P < 0.05$, *Nos2*: ADSC versus ADSC-Co. (C) T24: $*P < 0.05$, *Nos2*, *Tgfb1*, *Fgf2* and *Vegfa*: ADSC-Co versus ADSC. (D) T48: No significant differences.

ADSC viability increased over time but was significantly lower when ADSCs were co-cultured with tenocytes in comparison with the single ADSC culture, which was probably due to the induction of cell differentiation. Runt-related transcription factor 2 (*Runx2*) and *Pparg*, the master regulators of osteogenesis and adipogenesis [21], were respectively unchanged and lower in co-cultures than in single cultures. Conversely, the expression of *Scx* was higher when combined with tenocytes than the single culture at all experimental times and significantly at 3 days. These results highlighted that in the tenogenic microenvironment, induced by the presence of tenocytes, *Scx* expression in ADSCs is enhanced, whereas osteogenic and adipogenic differentiation lineages are downregulated. These *in vitro* results are in line with the increase in *Scx* expression observed by Luo *et al.* [22] in indirect co-cultures of BMSCs and tenocytes. For future *in vivo* use, it is important to restrict the possible commitment of ADSCs to an osteogenic or adipogenic lineage to avoid the pathological bone or adipose tissue formation during the healing of tendon tissue.

In addition, when ADSCs were co-cultured with tenocytes, *Tgfb1* and *Fgf2* showed the same trend with a higher expression after 24 hours and lower one at 7 days than that of the single culture. Transforming growth factor-β and basic fibroblast growth factor stimulate the production of ECM components, downregulate the expression of metalloproteinases and are also involved in tendon formation and healing. FGF signaling in particular is required for the early stages of differentiation in a number of lineages [24]. *Vegfa* expression, usually involved in tendon healing and the normal process of tissue development [24], is significantly reduced after 7 days in co-cultures compared with that of single standard culture conditions. Finally, the enzyme nitric oxide synthase (*Nos2*) is important during the healing phases of tendons because NO improves collagen synthesis and ECM gene expression [32]. This is probably the reason that in standard culture conditions *Nos2* expression did not change substantially.

With regard to the microwound healing model, ADSCs appeared to accelerate the healing process and, as expected, in co-cultures, the recovery speed was increased in the earlier stages (T4–T8) than at T24 and T48 because tenocytes reached confluence and the microwound was closed. Similar to what was observed in standard conditions, *Dcn* and *Tnc* expression was higher in co-cultures than in single ones, whereas for *Scx*, the opposite occurred. Converse to that observed in standard conditions, the addition of ADSCs in the wound model increased *Col1a1* at T24 and decreased *Col3a1* at T48. This finding is in agreement with the literature data that Collagen III is low in healthy tendons and is mostly synthesized after an injury [30].

During the microwound healing, no differences were observed in ADSC *Scx*, *Runx2* and *Pparg* expression, whereas the expression of the three GFs (*Tgfb1*, *Fgf2* and *Vegfa*) and *Nos2* increased under tenocyte influence.

In conclusion, this study highlighted the mutual interaction of ADSCs and tenocytes. The indirect co-culture system adopted showed the exchange of factors between the two cell types: ADSCs positively affect tenocyte behavior in standard culture conditions and much more during the microwound recovery, thus facilitating the recovery through the secretion of bioactive factors. Indeed, during microwound healing, not only the major tenocyte markers but also the *Col1a1* and *Col3a1* are, respectively, more and less expressed under ADSC influence. The influence of tenocytes on ADSCs is more evident in the standard culture condition than during microwound healing. In the latter case, this is probably because the lack of cell-cell and cell-matrix contacts disturbs the physiological behavior of tenocytes. Indeed, in standard culture conditions, tenocytes influence not only GF expression but also ADSC tenogenic differentiation, whereas during microwound healing, ADSC differentiation is not affected, even if GF gene expression increases under the influence of tenocytes. These results support the fact that the positive effects exerted by the injection of ADSCs in a tenogenic microenvironment are not—or not only—caused by their differentiation ability, but it is probably caused by their paracrine activity that induces the resident tenocytes to regenerate the compromised tissue. The data of the present *in vitro* study confirm that, as already observed for cartilage [33,34], the molecules present in the tendon microenvironment induce the differentiation of implanted MSCs.

In our opinion, this study is innovative because the mechanism of action of ADSCs in tendon regeneration and tenocyte metabolism is still unknown. After further *in vitro* and *in vivo* evaluations, our findings might lead to a future use of ADSCs in tendon lesions as a therapeutic strategy as the result of their paracrine activity. In future studies, it will also be useful to evaluate the mutual effect of ADSCs and tenocytes isolated from pathologic tendons caused by trauma, aging, endocrine diseases or drug administration.

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