Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions

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Abstract

We studied the effect of cyclic mechanical stretching on the proliferation and collagen mRNA expression and protein production of human patellar tendon fibroblasts under serum-free conditions. The role of transforming growth factor-β1 (TGF-β1) in collagen production by cyclically stretched tendon fibroblasts was also investigated. The tendon fibroblasts were grown in microgrooved silicone dishes, where the cells were highly elongated and aligned with the microgrooves. Cyclic uniaxial stretching with constant frequency and duration (0.5 Hz, 4 h) but varying magnitude of stretch (no stretch, 4%, and 8%) was applied to the silicone dishes. Following the period of stretching, the cells were rested for 20 h in stretching-conditioned medium to allow for cell proliferation. In separate experiments, the cells were stretched for 4 h and then rested for another 4 h. Samples of the medium, total cellular RNA and protein were used for analysis of collagen and TGF-β1 gene expression and production. It was found that there was a slight increase in fibroblast proliferation at 4% and 8% stretch, compared to that of non-stretched fibroblasts, where at 8% stretch the increase was significant. It was also found that the gene expression and protein production of collagen type I and TGF-β1 increased in a stretching-magnitude-dependent manner. And, levels of collagen type III were not changed, despite gene expression levels of the protein being slightly increased. Furthermore, the exogenous addition of anti-TGF-β1 antibody eliminated the increase in collagen type I production under cyclic uniaxial stretching conditions. The results suggest that mechanical stretching can modulate proliferation of human tendon fibroblasts in the absence of serum and increase the cellular production of collagen type I, which is at least in part mediated by TGF-β1.

Keywords: Mechanobiology; Stretching; Tendon fibroblasts; Proliferation; Collagen; TGF-β1; Alternatives

1. Introduction

Tendons consist of a complex matrix of parallel collagen fiber bundles, adhesion proteins, and proteoglycans with fibroblasts aligned with the collagen fibers. Collagen fibers are composed mostly of types I and III collagens and are the main structural component of tendons (Hayem, 2001; Kannus, 2000). The primary function of the tendon is to transmit muscle forces to bone, permitting body movement and joint stability. Thus, collagen structure provides the tensile strength needed to perform these functions. A large number of tendon fibroblasts must be present in tendon to repair and remodel the tendon matrix by producing extracellular proteins under mechanical loading conditions. Therefore, to better understand tendon mechanobiology and pathophysiology due to repetitive mechanical loading conditions, such as tendinopathy (Khan and Maffulli, 1998), it is important to determine the effect of mechanical loading on proliferation and collagen production of tendon fibroblasts.

Numerous studies have shown that mechanical loading induces various cellular responses in many types of cells. For example, biaxial mechanical stretching increases the expression level of transforming growth factor β1 (TGF-β1) in ligamentum flavum and smooth
The purpose of this study was two-fold: first, we wanted to examine proliferation, collagen gene expression and production of human patellar tendon fibroblasts (HPTFs) under cyclic uniaxial mechanical stretching conditions in vitro; second, we were interested in determining whether TGF-β1 gene and protein production increase in cyclically stretched tendon fibroblasts, and if so, whether TGF-β1 mediates collagen production by tendon fibroblasts under cyclic uniaxial stretching conditions. To isolate the effects of mechanical stretching on the tendon fibroblasts, serum-free medium was used in all cell stretching experiments. The results of this study show that cyclic uniaxial mechanical stretching only slightly increases the proliferation of tendon fibroblasts, but markedly increases the gene expression and protein levels of collagen type I and TGF-β1. Also, this study showed that TGF-β1 is a mediator of collagen type I production by HPTFs under cyclic uniaxial stretching as well as non-stretching conditions.

2. Materials and methods

2.1. Culture of tendon fibroblasts

HPTFs were isolated from fresh tendon samples donated by three healthy male subjects (15, 20, and 40 years old) who underwent ACL reconstruction using a patellar tendon autograft. The protocol for obtaining the tendon sample was approved by the University of Pittsburgh Institutional Review Board (IRB#0108109). The tendon sample was minced aseptically, transferred to a polystyrene Petri dish, and cultured with 10 ml of Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (50 U/ml). The culture was maintained at 37°C in an incubator with a humidified atmosphere of 5% CO2. To obtain enough tendon fibroblasts for stretching experiments described below, the cells were sub-cultured up to four times. These sub-cultured cells did not show apparent changes in morphology or doubling time.

2.2. Stretching experiments

The tendon fibroblasts were transferred to custom-made silicone dishes, which had been coated with 10 μg/ml of ProNectin-F (Sigma, St Louis, MO) to promote cell attachment on the silicone surface. The dishes, each having a culture surface of 3 × 6 cm², were transparent and non-toxic to cultured cells (Wang and Grood, 2000). A special feature of these dishes was that the culture surface was fabricated with microgrooves, with a ridge and groove width of 10 μm and a depth of 3 μm. The microgrooves in each dish were oriented along the stretching direction. With or without stretching, the tendon fibroblasts in the microgrooved surface of the silicone dish were highly elongated and aligned parallel to the microgrooves, which was also the direction of applied uniaxial stretching (Fig. 1).

A total of 2 × 10⁴ tendon fibroblasts were plated to each dish and grown in regular growth medium (DMEM plus 10% FBS and 1% P/S) for 36–48 h. Then, the medium was replaced by serum-free medium, which is the DMEM supplemented with 1% serum-replacement (Sigma, St Louis) and 25 μg/ml ascorbic acid to promote cellular collagen synthesis. After incubation for 12 h, the tendon fibroblasts were cyclically stretched using a custom-made stretching apparatus as described previously (Neidlinger-Wilke et al., 2001). The stretching magnitude varied from 0% (i.e., no stretch for control), to 4%, and to 8%, but stretching frequency and duration were kept constant at 0.5 Hz and 4 h, respectively. Note that the stretching magnitude was defined to be the percentage change in the length of the dish between two clamps, and therefore they were not the strains seen by the cells on the dish surface, which are generally much smaller than the applied substrate strains (Wang et al., 2001).

Following the end of stretching, the stretched tendon fibroblasts were incubated in stretching-conditioned medium for 4 h. One exception, however, was the cell proliferation experiments, where the incubation time was extended to 20 h after stretching, so that cells had
Table 1
Primer sequences for RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences (forward/reverse)</th>
<th>PCR product size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen-I</td>
<td>5'-TCCCCCAGCCACAAAGAGCTCACA-3'/ 5'-GTGATTGGGTCTCCCCGTCGTC-3'</td>
<td>155</td>
<td>58</td>
<td>Nakatani et al. (2002)</td>
</tr>
<tr>
<td>Collagen-III</td>
<td>5'-CTGCCATCTGAACCTGAGGTAAGG-3'/ 5'-CCATCCTCCAGAAGGGATGAGG-3'</td>
<td>447</td>
<td>58</td>
<td>Nakatani et al. (2002)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5'-CAACAAATCTGGCCTACCTCA-3'/ 5'-GGTAGTGACCCTGATGGTCCA-3'</td>
<td>199</td>
<td>58</td>
<td>Nakatani et al. (2002)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TCACCATCTCCAGGAGGCG-3'/ 5'-TGCTTACACCACCTCTTGA-3'</td>
<td>570</td>
<td>56</td>
<td>Adam et al. (1999)</td>
</tr>
</tbody>
</table>

...enough time for proliferation. Also, for those experiments that were to determine the role of TGF-β1 in collagen production by tendon fibroblasts, the anti-TGF-β1 antibody (R&D Systems, Minneapolis, MN) was added to the serum-free medium with a final concentration of 0.4 μg/ml. The cells were subjected to an 8% stretching magnitude with a stretching frequency of 0.5 Hz for 4 h. Our preliminary experiments had determined that this dosage of antibody resulted in non-detectable TGF-β1 levels in medium. For a negative control, the same concentration of chicken IgG antibody (Abcam Ltd., Cambridge, UK) was added to cell cultures in separate stretching experiments.

2.3. MTT assay for measuring fibroblast proliferation

To determine the number of viable cells in silicone dishes, the MTT assay was used (Voytik-Harbin et al., 1998). Briefly, 200 μl of MTT solution (5 mg/ml) was added to the medium of each silicone dish. The dishes were then incubated at 37 °C with 5% CO₂ for 3 h. After incubation, the dishes were centrifuged for 5 min at 1900 RPM. The supernatant was removed, and 2 ml of extraction buffer (15 ml DMF, 14.1 ml H₂O, and 6 g SDS) was added to each dish and incubated. After overnight incubation at 37 °C, the solution was mixed thoroughly. Duplicate samples of 200 μl were aliquotted into a 96-well plate, and the absorbance, or optical density (OD) value, was measured using a microplate reader (Spectra MAX 190, Molecular Devices, CA) at 550 nm. The obtained OD value represents the number of viable cells in each sample.

2.4. RT-PCR for measuring collagen type I, collagen type III, and TGF-β1 mRNA levels

Oligonucleotide primers were designed according to the published sequences of collagen type I, type III, and TGF-β1 genes (Nakatani et al., 2002). A primer was also created for GAPDH (Adam et al., 1999), a housekeeping gene, whose mRNA level was determined to assure that equal amounts of RNA from each sample were loaded. These primers were synthesized by the University of Pittsburgh DNA Synthesis Facility (Table 1).

For the RT-PCR assay, total RNA was extracted from tendon fibroblast lysates using the RNAeasy Mini Kit (QUIAGEN, Valencia, CA). In a final volume of 20 μl containing 1 μg total RNA, 5 mM MgCl₂, 1 × reverse transcription buffer, 1 mM dNTP, and 1 U/μl recombinant RNaseOUT™ (Invitrogen Life Technologies, Carlsbad, CA), synthesis of cDNA was carried out at 42 °C for 55 min, then at 70 °C for 15 min. The synthesized cDNA was amplified using the primers listed in Table 1. For collagen type I, collagen type III, and TGF-β1, the amplification was done at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. For GAPDH, however, the amplification was carried out at 94 °C for 50 s, 56 °C for 40 s, and 72 °C for 40 s. A total of 25 cycles were used, which is within the linear region of the assay (Nakatani et al., 2002). After electrophoresis was performed in a 2% agarose gel containing ethidium bromide, the cDNA bands were visualized, scanned and quantified by image analysis. Then, the density of each band was normalized to that of GAPDH.

2.5. ELISA for measuring collagen type I and TGF-β1 levels in medium

An enzyme immunoassay kit (Takara Bio Inc., Japan) was used to measure the levels of carboxy-terminal procollagen type I propeptide (PIP) in medium. Briefly, 100 μl of horseradish peroxidase-labeled anti-PIP conjugate solution was pipetted into each well of an antibody-coated plate. Then, 20 μl of each sample (1:15 dilution ratio), or the standard solution for the standard curve, were added to the wells and incubated for 3 h at 37 °C. Then, after removing the solution, the well was washed four times with 1 × PBS. Next, 100 μl of substrate solution was added to each well and incubated for 15 min at room temperature, followed by the addition of 100 μl stop solution (1 N H₂SO₄). The
concentration of collagen type I was obtained by measuring the absorbance at 450 nm on the microplate reader (Spectra MAX 190, Molecular Devices).

The levels of TGF-β1 in medium were measured using a commercially available kit (R&D systems, Minneapolis, MN) according to a protocol supplied by the manufacturer. The kit consisted of a microplate pre-coated with TGF-β soluble receptor type II, which binds TGF-β1. For assaying, 200 μl taken from the culture medium of each sample was pipetted into the wells of the plate, activated by the addition of 1 N HCl and 1.2 N NaOH (sample:HCl:NaOH = 5:1:1), and incubated for 3 h at 37°C. A corresponding amount of standard solution was used to create the standard curve. After washing, horseradish peroxidase-linked polyclonal antibody specific for TGF-β1 was added to the wells and incubated for 1.5 h at room temperature. After washing again, a substrate solution was added to each well and incubated at room temperature for 20 min. Then, a stop solution was added to each well to terminate reaction. The plate was put into the microplate reader, and the TGF-β1 concentration of each sample was measured at 450 nm.

2.6. Western blot for measuring collagen types I and III levels in medium

To measure levels of collagen in the medium, 1 ml of medium was diluted with 1.0 M acetic acid (1:1). After letting it set for 4 h at room temperature, pepsin (100 μg/ml) was added, and the solution was kept at 4°C. After overnight digestion, salt powder was added to the pepsin digest to create a final concentration of 3.0 M NaCl and then stirred at 4°C for 18 h. The medium solution was dialyzed against 0.05 M NH₄CO₃. The dialysates were dried and analyzed by running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting (see below).

For Western blot experiments, the protein concentration of each sample was measured using a BCA protein assay kit (Pierce, Rockford, IL). A total of 20 μg protein from each sample was loaded into a 7% SDS-PAGE gel with loading buffer for electrophoresis at 125 V for 90 min. The separated protein was then transferred to a nitrocellulose membrane, using a Mini-Protean 3 cell and transfer model (Bio-Red System, Hercules, CA). The membrane was incubated overnight at 4°C in a 5% non-fat milk/PBS-Tween-20 solution. Then, the 5% non-fat milk PBS/Tween-20 was discarded. Next, the primary antibody, either rabbit polyclonal anti-collagen-I or mouse monoclonal anti-collagen-III (both from Abcam Ltd., Cambridge, UK), in 1% non-fat milk/PBS with 0.1% Tween-20 (1 g non-fat milk added to 100 ml PBS/Tween-20 solution), was added and incubated at room temperature for 2 h. The secondary antibody, either goat anti-rabbit IgG (H+L) for collagen type I or rabbit anti-mouse IgG for collagen type III, in 1% non-fat milk/Tween-20 (1:4000) was added and incubated for another 2 h. The membrane was washed three times with 0.1% PBS/Tween-20 for 15 min after the application of each antibody. The bands for types I and III collagens were detected by an ECL Detection Kit (RPN 2132, Amersham Biosciences, USA).

2.7. Statistical analysis

One-way ANOVA was used for statistical data analysis of the stretching effects on tendon fibroblasts, followed by Duncan’s test for multiple comparisons at a significance level of 0.05.

3. Results

After 4% and 8% stretching for 4 h followed by 20 h incubation, the tendon fibroblast proliferation was slightly increased by 1.8% and 7.9%, respectively (Fig. 2). However, only 8% stretching induced a significant increase in fibroblast proliferation compared with that of non-stretched cells (p = 0.01).

Cyclic stretching of the tendon fibroblasts increased the mRNA expression level of collagen type I in a stretching-magnitude-dependent manner (Fig. 3B). Specifically, at 4% and 8% stretching, the level of collagen type I mRNA expression in stretched tendon fibroblasts was increased by 10.4% (p < 0.001) and 22.3% (p < 0.001), respectively, compared to that of non-stretched cells. Also, the collagen type I mRNA level at 8% stretching was significantly higher than that at 4% stretching (p < 0.01). Compared with that of non-stretched cells, cyclic stretching at both 4% and 8% stretching slightly increased collagen type III mRNA expression levels, but the level of the increase was lower than that of collagen type I (Fig. 3B).

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**Fig. 2.** Cyclic mechanical stretching slightly increased the proliferation of human tendon fibroblasts. The cells were stretched at 0.5 Hz for 4 h at a magnitude of either 4% or 8%, followed by 20 h of incubation in stretching-conditioned medium. Note that the numbers of stretched tendon fibroblasts were normalized to that of non-stretched cells, and that two sets of separate experiments (n = 10) were carried out.
The expression of TGF-β1 mRNA showed a pattern similar to that of collagen type I. Specifically, the TGF-β1 mRNA expression level increased with stretching magnitude (Fig. 3). Compared to non-stretched tendon fibroblasts, the cells stretched at 4% and 8% increased TGF-β1 mRNA expression levels by 11.5% \( (p<0.001) \) and 24.6% \( (p<0.001) \), respectively.

Also, compared to that of non-stretched cells, 4% and 8% stretching of tendon fibroblasts increased the collagen type I level in medium by 11% \( (p=0.03) \) and 25% \( (p<0.001) \), respectively (Fig. 4A). The results from the ELISA assay were confirmed by Western blot analysis of collagen levels in medium (Fig. 4B). No significant changes in the collagen type III level in the medium, however, were found at either 4% or 8% stretching, as compared to non-stretched cells.

Furthermore, cyclic stretching of the tendon fibroblasts also affected their TGF-β1 expression levels. The levels in medium at 4% and 8% stretching were significantly increased by 25% \( (p<0.002) \) and 64% \( (p<0.001) \), respectively, compared to that of non-stretched cells (Fig. 5). The TGF-β1 level at 8% stretching was also significantly higher than that at 4% stretching \( (p<0.001) \). Therefore, TGF-β1 production by stretched tendon fibroblasts increased in a stretching-magnitude-dependent manner.

Finally, the level of collagen type I in the medium at 8% stretching was found to be significantly increased by 29% \( (p<0.0001) \) compared to that non-stretched fibroblasts (Fig. 6). The addition of anti-TGF-β1
separate experiments (medium by stretched as well as non-stretched tendon fibroblasts. Two treatment significantly decreased the production of collagen type I in stretched but treated with the anti-TGF-

The third group was not stretched. Finally, the fourth group was not stretched in the presence of anti-TGF-

of cells were used. The first group was stretched at 8%, whereas the second group was stretched in the presence of anti-TGF-

expression of collagen type I in human tendon fibroblasts. Four groups antibody (0.4 μg/ml), however, significantly decreased collagen type I production by the stretched cells (p < 0.001) as well as non-stretched cells (p < 0.05). The antibody inhibition appeared specific since the same amount of chicken IgG antibody did not affect collagen type I gene expression (data not shown). Moreover, there was no statistical difference in the TGF-β1 levels between the antibody-treated and non-stretched cells (p = 0.39). Note that the treatment with anti-TGF-β1 antibody did not change cell proliferation.

4. Discussion

Human tendon fibroblasts are exposed to repetitive mechanical loading in vivo. Therefore, it is important to determine the effects of mechanical loading on tendon fibroblasts in order to better understand tendon mechanobiology as well as pathophysiology. Using an in vitro system that can control cell shape, alignment, and uniaxial stretching conditions of tendon fibroblasts in vivo (Fig. 1), this study shows that cyclic stretching of tendon fibroblasts in serum-free conditions slightly increased cell proliferation, but markedly increased mRNA and protein levels of collagen type I. However, the mRNA level of collagen type III in the stretched tendon fibroblasts was only slightly increased, and its protein level was not changed. Also, cyclic mechanical stretching induced a higher level of TGF-β1 mRNA expression and protein production. Finally, inhibition of TGF-β1 with antibody abrogated the stretch-induced increase in collagen type I level in medium. Taken together, these results indicate that cyclic uniaxial mechanical stretching in the absence of serum modulates proliferation, collagen type I and TGF-β1 gene and protein production of human tendon fibroblasts. The results also show that TGF-β1 is a molecular mediator of collagen type I production in stretched human tendon fibroblasts.

The results of this study are consistent with the notion that mechanical loading induces various cellular responses seen in many types of cells (Banes et al., 1995, 1999; Birukov et al., 1995; Li et al., 1998; Neidlinger-Wilke et al., 1995). Also, the small increase in number of tendon fibroblasts in response to cyclic mechanical stretching is not surprising. This is because fibroblasts exhibit low mitotic activities under normal physiological conditions (Ahmed et al., 1998) and our in vitro system mimics closely in vivo conditions of tendon fibroblasts (cell shape, alignment, and uniaxial stretching). Previous studies also show that cyclic stretching increased DNA synthesis of human flexor tendon fibroblasts (Almekinders et al., 1993) and proliferation of HPTFs (Zeichen et al., 2000). Moreover, cyclic mechanical stretching has also been reported to increase proliferation of other types of cells, including melanocytes (Kippenberger et al., 1999) and osteoblasts (Neidlinger-Wilke et al., 1995). However, the quantitative comparison of cell proliferation in this study with previous studies would be difficult, due to the different experimental conditions used, including (1) type of tissues where cells were derived from (e.g., human patellar tendons in this study vs. human flexor tendons in a previous study, Almekinders et al., 1993); (2) type of mechanical stretching (uniaxial stretching in this study vs. biaxial or mixed stretching in many other studies); and (3) serum-free medium in this study vs. serum-containing medium in most previous studies.

This study also shows that the collagen type I mRNA level and corresponding protein level of human tendon fibroblasts increase in response to cyclic mechanical stretching. The changes in the mRNA expression of collagen type III, however, were small in comparison to the level of changes seen in collagen type I, and its protein level was not changed under our experimental conditions. These results contrast the fact that cyclic stretching of human ligamentum flavum cells at 10% for 48 h increases collagen type III but not type I gene expression (Nakatani et al., 2002). The difference between the two studies may be attributed to different types of cells used and other experimental conditions, such as serum-containing medium vs. serum-free medium in our study.

A previous study suggested that collagen synthesis depended on post-stretching rest time (Bosch et al., 2002). The rest time of tendon fibroblasts in this study was 4 h, at which we found that collagen type I gene and protein expression were markedly increased. It is likely, however, that with increased rest time after cyclic stretching, the stretched tendon fibroblasts would have a higher level of collagen mRNA expression and protein production. It is also possible that with increased rest time, the level of collagen type III protein levels might
also be increased. But the level of collagen type III would be expected to be lower than that of collagen type I, since fibroblasts in culture predominately produce type I collagen (Riederer-Henderson et al., 1983). Future studies are required to investigate the dependency of collagen gene expression and collagen production on rest time.

This study shows that cyclic stretching of human tendon fibroblasts increased TGF-β1 expression at both gene and protein levels. The result is consistent with that of previous studies with ligamentum flavum cells (Nakatani et al., 2002) and human tendon fibroblasts (Skutek et al., 2001). In the study by Nakatani and co-workers, endogenous TGF-β1 was found to increase collagen production. Also, the addition of anti-TGF-β1 antibody inhibits collagen gene expression in the ligamentum flavum cells and also decreases α(I) collagen expression in fetal smooth muscle cells (Gutierrez and Perr, 1999). We also showed that addition of anti-TGF-β1 antibody suppressed the increase of collagen type I production in human tendon fibroblasts under cyclic uniaxial stretching as well as non-stretching conditions. Therefore, the result is consistent with the notion that TGF-β1 is a mediator for collagen type I production in connective tissue cells.

In this study, several experimental conditions were tightly controlled. These include alignment, shape, and uniaxial stretching conditions of tendon fibroblasts, which mimicked the in vivo situation. Also, the potential complicating effects due to the presence of serum in medium were eliminated by using a serum-free medium. Nevertheless, there are a few limitations in this study. First, we had to sub-culture tendon fibroblasts to obtain enough cells for cell stretching experiments. Cells that were sub-cultured up to four times, however, did not change morphology and doubling time noticeably, which contrasts the fact that with serial passaging of the knee ligament fibroblasts, cell growth rates changed significantly (Hannafin et al., 1999). The reasons for this discrepancy may include differences in species (canine vs. young human subjects), tissue types (ligaments vs. patellar tendons), and culture conditions. It should be noted that the number of cell passages is an important factor when one compares the results of this study with those from other studies. Second, this study only examined the fibroblast responses to mechanical stretching at one stretching duration (4 h), one rest time (20 h for fibroblast proliferation, and 4 h for collagen and TGF-β1), and one stretching frequency (0.5 Hz). Therefore, future studies should investigate the effects of different stretching frequencies, durations, and rest times on the proliferation and collagen production of human tendon fibroblasts. Also, under serum-free condition, mechanosignaling pathways, such as stress-activated protein kinase (SAPK)/JNK (Skutek et al., 2003), production of inflammatory mediators (Almekinders et al., 1995; Wang et al., 2003) should also be investigated to provide a more complete picture of cyclic mechanical stretching effects on human tendon fibroblasts. Therefore, the molecular and cellular mechanisms of patellar tendinopathy (Khan et al., 2000) due to repetitive mechanical loading can be better understood.

In conclusion, this study shows that under serum-free conditions, cyclic uniaxial stretching of HPTFs increases proliferation, collagen type I and TGF-β1 gene expression and protein production. This study also shows that TGF-β1 at least partially mediates the production of collagen type I in the human tendon fibroblasts under cyclic uniaxial stretching conditions.

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