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Citation	Journal of Biomechanics, 40(10), 2184-2190 <a href="https://doi.org/10.1016/j.jbiomech.2006.10.023">https://doi.org/10.1016/j.jbiomech.2006.10.023</a>
Issue Date	2007
Doc URL	<a href="http://hdl.handle.net/2115/30163">http://hdl.handle.net/2115/30163</a>
Type	article (author version)
File Information	JBM40-10.pdf



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Antibody neutralization of TGF- $\beta$  enhances the deterioration of collagen fascicles in a tissue-cultured tendon matrix with *ex vivo* fibroblast infiltration

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Abstract. A tissue-cultured tendon matrix infiltrated with cultured fibroblasts can be regarded as an ideal tissue-engineered tendon model. To clarify the role of TGF- $\beta$  in a tissue-cultured tendon matrix during *ex vivo* cellular infiltration, the present *ex vivo* study was conducted to test the following hypothesis that antibody neutralization of TGF- $\beta$  enhances weakening of the collagen fascicles of the patellar tendon matrix in response to *ex vivo* fibroblast infiltration. In skeletally mature female rabbits, fibroblasts were isolated from the right patellar tendons using an explant culture technique, and the left patellar tendons underwent multiple freeze/thaw treatment with liquid nitrogen to obtain an acellular tendon matrix. Each acellular tendon was placed in a collagen gel containing cultured fibroblasts and then incubated with or without anti-TGF- $\beta$ 1 antibody for 6 weeks. We found that antibody neutralization of TGF- $\beta$  enhanced the decrease in the tensile strength and tensile modulus of the collagen fascicles of the patellar tendon matrix in response to *ex vivo* fibroblast infiltration. **The present study indicates a possibility that TGF- $\beta$  may have a role in suppressing the material deterioration of the fascicles in the tendon during *ex vivo* cellular infiltration.**

Recently, development of tissue-engineered tendon/ligament tissues is one of the important foci in the field of anterior cruciate ligament (ACL) reconstruction (Vunjak-Novakovic et al., 2004). Dunn et al. (1995) reported that fibroblastic cells could attach to collagen fibers in the scaffold, and that the cells could function *ex vivo*. Bellincampi et al. (1998) described how a fibroblasts-seeded collagen fiber scaffold showed promising biological results in their implantation study. However, much remains unclear concerning *ex vivo* remodeling of the collagen fiber scaffold in tissue engineering procedures. To clarify the mechanism of *ex vivo* remodeling of the collagen fiber scaffold, a tissue-engineered tendon model has been needed. Previously, King et al. (9) developed the freeze-thaw treatment to create an acellular tendon matrix. Namely, they repeatedly froze the patellar tendon by immersing it in liquid nitrogen and then thawed it by immersing it in saline solution. The acellular tendon matrix be regarded as a type of collagenous scaffold. Recently, the authors developed a method of inducing cultured fibroblasts to infiltrate the repetitively frozen-thawed tendon matrix in an *ex vivo* state (Ikema et al., 2005). The tissue-cultured tendon matrix infiltrated with cultured fibroblasts can be regarded as an ideal tissue-engineered tendon model. In this tissue-engineered tendon model, we have found that *ex vivo* infiltration of fibroblasts significantly deteriorates the mechanical properties of the collagen fascicles composing

the necrotized tendon matrix (Ikema et al., 2004). It is considered that this mechanical deterioration may occur in actual tissue-engineering procedures for the tendon and ligament tissue.

It is well known that TGF- $\beta$ 1 stimulates the synthesis of collagen and other proteins by fibroblasts (Marui et al., 1997; Deie et al., 1997, DesRosiers et al., 1996). In addition, we found that local administration of TGF- $\beta$ 1 inhibits mechanical deterioration of the ACL after the *in situ* freeze/thaw procedure during fibroblast infiltration following fibroblast necrosis *in vivo* (Nagumo et al., 2005). Therefore, there is a possibility that TGF- $\beta$  has a role in suppressing the material deterioration of the fascicles in the tendon during *ex vivo* cellular infiltration. Our overall goal is to clarify the role of TGF- $\beta$  in the remodeling of tendon matrix during *in vitro* cellular infiltration. The knowledge of the role of TGF- $\beta$  in the remodeling of tendon matrix *in vitro* is basically important not only for the development of tissue-engineered tendon grafts but also for a better understanding of the remodeling of tendon autografts used for the reconstruction of injured ligaments. To achieve this overall goal, we then hypothesize that antibody neutralization of TGF- $\beta$ 1 enhances the deterioration of collagen fascicles in the tissue-cultured tendon matrix with *ex vivo* fibroblast infiltration. The purpose of using a rabbit model in this study is to test this hypothesis.

## **Materials and Methods**

This study involved 24 skeletally mature female Japanese White rabbits weighing  $3.4 \pm 0.4$  kg (mean  $\pm$  SD). In each rabbit, fibroblasts were obtained from the right patellar tendon using an explant culture technique, and the left patellar tendon underwent multiple freeze/thaw treatment with liquid nitrogen to obtain an acellular tendon matrix (King et al., 1995; Ikema et al., 2005). Each acellular tendon matrix was paired with cultured fibroblasts derived from the patellar tendon in the opposite knee of the same animal. The following two experiments were conducted in the present study. All surgical procedures on the animals were carried out at the Institute of Animal Experimentation, Hokkaido University School of Medicine, under the Rules and Regulations of the Animal Care and Use Committee, Hokkaido University School of Medicine.

## **Experimental Design**

***Experiment 1: the effects of cellular infiltration.*** In the first experiment, 14 rabbits were used to clarify the effect of *ex vivo* cellular infiltration on the mechanical properties of collagen fascicles in the tendon matrix. In the cell-culture group, seven

acellular tendons that had been prepared using the freeze/thaw treatment described in the following section were placed in collagen gel containing cultured fibroblasts and then incubated in culture medium for 6 weeks. In the control group, the remaining seven acellular tendons were incubated in collagen gel without any cells under the same conditions as those in the cell-culture group. For each group, three specimens were used for confocal laser microscopic observation and four specimens were used for the mechanical evaluation of patellar tendon collagen fascicles. We also evaluated another four acellular tendons without incubation (time-0).

***Experiment 2: the effects of TGF- $\beta$  neutralization.*** The remaining ten pairs of fibroblasts and acellular tendons were used to determine the effect of neutralization of TGF- $\beta$ 1 with its antibody. In the A-TGF $\beta$  group (n=5), 2  $\mu$ g/mL of monoclonal anti-human TGF- $\beta$ 1 antibody (R&D, Minneapolis, MN, USA) was supplemented in the culture medium with 10% fetal bovine serum (FBS) during incubation of acellular tendons in gel containing cultured fibroblasts. In the non-A-TGF $\beta$  group (n=5), antibody was not supplemented during incubation of acellular tendons in the gel with cultured fibroblasts. At 6 weeks, collagen fascicles of the tendons were biomechanically evaluated in the same manner as in Experiment 1..

### **Isolation of Tendon Fibroblasts**

We isolated fibroblasts from the patellar tendons of the right knees. The patellar tendons were harvested under aseptic conditions. The outer synovial layer was removed by sharp dissection and these tendons were cut into 2- to 5-mm pieces that were then placed on the dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Ikema et al., 2005; Nagineni et al., 1992). The dishes were incubated at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere (95% air) in a cell-culture incubator. A confluent monolayer formed in 2 weeks. Cells from the second passage were used for all experiments.

### **Multiple Freeze/thaw Treatment for Preparation of an Acellular Tendon Matrix**

The patellar tendons were harvested from the left knees under aseptic conditions. We devitalized these patellar tendons using multiple freeze/thaw treatment (King et al., 1995; Ikema et al., 2005). Briefly, the patellar tendons were immersed in liquid nitrogen for 1 minute and then thawed in saline solution at 37°C. This procedure was repeated five times. Each tendon was then stored at -80°C. Before each tendon was cut, it was partially thawed overnight at 4°C and then completely thawed at room temperature. Each tendon was divided into three portions along the fiber direction and each portion was trimmed



into 3 x 1 x 15 mm blocks.

### ***Ex vivo* Infiltration of Fibroblast into the Acellular Tendon Matrix**

Confluent fibroblasts from the second passage were detached from the dishes by treatment for 2 minutes with trypsin-EDTA. Seven acellular tendon matrices were placed in 0.5 ml collagen gel (CELLGEN, Koken Co., Tokyo, Japan) including cultured fibroblasts at a final concentration of  $5 \times 10^6$  cells/ml (Ikema et al., 2005). These matrices in collagen gel were incubated in DMEM containing 10% FBS (GIBCO), 2 mM L-glutamine (GIBCO), 0.2 mM ascorbic acid (Sigma), and penicillin-streptomycin (each 100 U/mL; GIBCO) at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere for 6 weeks. We changed the medium every three days. At each medium change, we added TGF- $\beta$  antibody into the medium in the A-TGF $\beta$  group.

### **Evaluation of Cellular Distribution in Tendons**

After incubation, the nuclei of the fibroblasts in the incubated tendons were labeled with propidium iodide (Molecular Probes, Eugene, OR, USA). The tendons were washed with 37°C phosphate-buffered saline (PBS) three times and then fixed with OCT (optimum cutting temperature) compound to make frozen tissues. The tendons were

sliced sagittally into slices of 25- $\mu\text{m}$  thickness. These slices were stained with 1 ml PBS containing 0.5  $\mu\text{g}$  propidium iodide for 30 minutes. We evaluated the cellular distribution in the tendon with a confocal laser microscope (MRC-1024; BIO-MED Laboratories, Tokyo, Japan).

### **Mechanical Testing for Tendon Fascicles**

After incubation, the specimen was removed from the dishes after treatment of *ex vivo* cellular infiltration. Collagen fascicles with a diameter of approximately 300  $\mu\text{m}$  and with a length of 15 mm were very carefully dissected with a surgical knife, parallel to the axis of the patellar tendon. During the dissection, the substance was kept moist with PBS at room temperature. The cross-sectional area of the collagen fascicle was measured with the following non-contact method developed by Yamamoto et al. (1999). Briefly, the lateral image of a collagen fascicle immersed in physiological saline solution at 37°C was enlarged by a low-magnification microscope (SMZ-2Z, Nikon, Tokyo), taken with a CCD camera (WV-BD400, Panasonic, Tokyo, Japan), and then processed with a video dimension analyzer (Percept Scope C3160, Hamamatsu Photonics, Tokyo, Japan). The diameter was measured from 36 directions, while the fascicle was intermittently rotated with a stepping motor at an angular interval of 5°. The cross-sectional area was

calculated from averaging these diameters, assuming that the cross-section was circular. These measurements were taken 2 mm proximal position from the middle and 2 mm distal position to the middle. An average cross-sectional area was obtained from these two sets of measurements.

After the cross-sectional area measurement, we evaluated the mechanical properties of the collagen fascicles in the patellar tendons using a micro-tensile tester designed by Yamamoto et al. (1999). Acrylic blocks were attached to both ends with cyanoacrylate adhesive. One of the blocks was attached to a load cell (LVS-1KA, Kyowa, Tokyo, Japan) and the other one was attached to the crosshead of a linear stage (LU09200AKL1-P5Z0, Nihonseiko, Tokyo, Japan) that a microprocessor-controlled stepping motor (UPD566TG-A, Oriental Motor, Tokyo, Japan) moved at a crosshead speed between 0 and 20 mm/min. Tensile tests were carried out by moving the stage with the stepping motor. For the measurement of strain, two markers were drawn on a collagen fascicle with Nigrosine stain about 5 mm apart; the strain measured with this method is the strain on the specimen surface (Yamamoto et al., 1999). The distance between the markers was measured with the above-mentioned video dimension analyzer. Before the tensile tests, the samples were preconditioned with ten cycles of loading and unloading between 0 and 2% strain at a strain rate of 1.5% per second. The strain rate of

the tensile tests was also 1.5% per second. During tensile testing, specimens were immersed in physiological saline solution at 37°C. Tensile load and the distance between the above-mentioned two markers were recorded on a personal computer (PC386-GE, Epson, Nagano, Japan). From the stress-strain data, we obtained the tensile strength, the tangent modulus, and the strain at failure of the fascicles. The tangent modulus of the tendon fascicles was defined as the slope of the stress-strain curve between 5% and 10% strain using a least-square method.

### **Statistical Analysis**

For Experiment 1, we compared the mechanical properties of the collagen fascicles in the cell-culture group to those in the control group using unpaired  $t$ -tests. For Experiment 2, we used an unpaired  $t$ -test to compare mechanical properties of the collagen fascicles between the A-TGF $\beta$  and non-A-TGF $\beta$  groups. The significance limit was set at  $\alpha = 0.05$ .

### **Results**

*Experiment 1: the effects of cellular infiltration on-tendon fascicles.* In the cell-culture group, confocal laser microscopic observations revealed that a number of fibroblasts

were found entirely within the patellar tendon matrix at 6 weeks after the incubation of the acellular patellar tendon in the gel with cultured fibroblasts, while no fibroblasts were observed in the control group (Fig. 1). During tensile testing, all fascicle specimens failed between the two markers drawn on a collagen fascicle. Concerning the mechanical properties of fascicles of the tendon, the cell-culture group had a tensile strength of  $7.2 \pm 2.2$  MPa and a tangent modulus of  $63 \pm 6$  MPa, while the control group had a tensile strength of  $13.0 \pm 1.9$  MPa and a tangent modulus of  $78 \pm 5$  MPa. The collagen fascicle of the frozen-thawed tendon at the beginning of the culture, time-0, had a tensile strength of  $18.2 \pm 4.6$  MPa and a tangent modulus of  $106 \pm 19$  MPa. These parameters of the cell-culture group were significantly lower than those of the control group at 6 weeks (the tensile strength:  $p=0.0082$ ; the tangent modulus  $p=0.039$ ), while we could not find statistical differences in the control group between time-0 and 6 weeks (the tensile strength:  $p=0.415$ ; the tangent modulus  $p=0.109$ ). There were no significant differences in the strain at failure between the cell-culture group,  $18.9 \pm 5.6\%$ , and the control,  $20.7 \pm 3.7\%$  ( $p=0.506$ ). The strain at failure of the collagen fascicle of the frozen-thawed tendon at time-0 was  $18.2 \pm 4.6\%$ .

***Experiment 2: the effects of TGF- $\beta$  neutralization.*** During tensile testing, all fascicle

specimens failed between the two markers drawn on a collagen fascicle. The tensile strength and the tangent modulus of the A-TGF $\beta$  group, the group with neutralizing antibody, were significantly lower than those of the non-A-TGF $\beta$  group, the group without neutralizing antibody (the tensile strength:  $p=0.0028$ ; the tangent modulus:  $p<0.0001$ ) (Fig. 2). The average strain at failure of the A-TGF $\beta$  group was higher than that of the non-A-TGF $\beta$  group, while we did not find statistical difference in the strain at failure between the A-TGF $\beta$  and the non-A-TGF $\beta$  groups ( $p=0.1025$ ).

## Discussion

The present study was conducted to test the hypothesis that antibody neutralization of TGF- $\beta$ 1 enhances the weakening of the collagen fascicles of the patellar tendon matrix in response to *ex vivo* fibroblast infiltration. First, the present study confirmed that *ex vivo* tendon-derived fibroblasts can infiltrate a frozen/thawed patellar tendon matrix. The findings of the present experiment suggested that antibody neutralization of TGF- $\beta$  enhances the decrease in the tensile strength and tensile modulus of the collagen fascicles of the patellar tendon matrix in response to *ex vivo* fibroblast infiltration. Therefore, these findings suggest that TGF- $\beta$  has a role in suppressing the weakening of the fascicles in the tendon during *ex vivo* cellular infiltration. The average

strain at failure of the collagen fascicles treated with antibody to TGF- $\beta$  was higher than that without antibody to TGF- $\beta$ , although we failed to obtain statistical difference because of small sample size and large variation in the strain at failure. This indicates a possibility that the treatment with antibody to TGF- $\beta$  may make tendon more extensible.

In the present study, we used an acellular tendon matrix created by a repetitive freeze/thaw procedure. Therefore, the dead cells in the tendon matrix after the repetitive freeze/thaw procedure might affect the infiltration of fibroblasts into the tendon matrix. However, we matched the source animal of the cells to that of the tendon matrix in the present study. Therefore, the effect of the dead cells inside the matrix on infiltrating tendon fibroblasts was considered to be relatively minimal. In addition, the present tendon matrix has the advantage that the matrix is physiological to tendon cells.

In the present study, we confirmed that cell infiltration leads to decreases in tensile strength and tangent modulus of the tendon matrix. We previously reported that cells that had infiltrated the devitalized patellar tendon synthesize type III collagen at least for 12 weeks with weakening of the mechanical properties of the patellar tendon *in vivo* (Tohyama et al., 2006). Therefore, *ex vivo* cell infiltration might also weaken the tendon matrix with the increase in type III collagen.

Recently, several *in vivo* studies were reported on the application of an antibody

to TGF- $\beta$ 1 for the prevention of scar formation during wound healing (Shah et al., 1992; Nath et al., 1998). Their findings indicate that administration of an antibody to TGF- $\beta$ 1 reduces collagen synthesis of fibroblasts. In addition, Yamauchi (2002) reported that application of an antibody to TGF- $\beta$  suppressed fibroblast proliferation in an *ex vivo* state. We have not confirmed the cross-reactivity of the anti-TGF- $\beta$ 1 antibody used in the present study with rabbit TGF- $\beta$ . At the present, it is impossible to obtain recombinant rabbit TGF- $\beta$  1 because the complete code of rabbit TGF- $\beta$  1 is not determined. Therefore, it is very difficult to examine the cross-reactivity of this anti-TGF- $\beta$ 1 antibody with rabbit TGF- $\beta$ . However, the manufacturer provided data showing that the anti-TGF- $\beta$ 1 antibody used in the present study would neutralize TGF- $\beta$ 1 and - $\beta$ 2 from human, mouse, rat, and other species. The data also showed that cross-reactivity of this antibody with TGF- $\beta$ 3 is less than 2%. Therefore, this antibody was considered to neutralize rabbit TGF- $\beta$ 1 and  $\beta$ 2. These findings suggest the possibility that antibody neutralization of TGF- $\beta$  inhibited the collagen synthesis of fibroblasts that infiltrated the tendon matrix in an *ex vivo* state and reduced the mechanical strength of the tendon matrix. However, it remains unknown whether the anti-TGF- $\beta$ 1 antibody inactivates endogenous TGF- $\beta$  of infiltrated fibroblasts, exogenous TGF- $\beta$  in culture medium with 10% serum, or both.



There are several limitations in this study. The first limitation is that we did not apply any tension to the tendon matrix. The reason why we did not apply mechanical loading to the tendon matrix was that it was difficult to determine the physiological load value for the tendon matrix *ex vivo*. In the present study, we found that after 6-week incubation under stress-deprivation conditions, the average tangent modulus of the collagen fascicles without seeding cells was 74% of the modulus at time-0. This finding suggests that the effect of the stress-deprivation on the mechanical deterioration of the tendon matrix is not negligible *ex vivo*. We previously found that stress-deprivation up-regulates TGF- $\beta$  expression of fibroblasts in the patellar tendon (Uchida et al., 2005). We also reported that an application of 0.05-mg anti-TGF- $\beta$ 1 antibody significantly reduced the tangent modulus and the tensile strength of the stress-shielded patellar tendon *in vivo* (Katsura et al., 2006). Therefore, in the present *in vitro* study, there is a possibility that stress-deprivation induced the TGF- $\beta$  production of fibroblasts. We should conduct further studies to clarify the role of TGF- $\beta$  on the loaded tendon matrix *ex vivo*.

The second limitation is that we did not measure the collagen and matrix metalloproteinase (MMP) synthesis of the infiltrated cells in the tendon matrix. The information about the collagen and MMP synthesis of the infiltrated cells should be helpful to understand the effects of the TGF- $\beta$ 1 antibody neutralization on the mechanical

properties of the collagen fascicles. In the present study, we confirmed that cell infiltration leads to decreases in the tensile strength and tangent modulus of the tendon matrix. We previously reported that cells that had infiltrated the devitalized patellar tendon synthesize type III collagen for at least 12 weeks with weakening of the mechanical properties of the patellar tendon *in vivo* (Tohyama et al., 2006). Therefore, *ex vivo* cell infiltration might also weaken the tendon matrix with an increase in type III collagen. It is also important to clarify the mechanism by which the addition of TGF- $\beta$ 1 antibody resulted in decreased mechanical properties of the tendon matrix in view of MMP regulation of the infiltrating cells. As we stated above, in the present study, there is a possibility that stress-deprivation induced the TGF- $\beta$  production of fibroblasts. Previously, we reported that TGF- $\beta$  down-regulates mRNA of MMP-13 of rat fibroblasts that had infiltrated the patellar tendon matrix *in vivo* (Tohyama et al., 2002). Therefore, addition of TGF- $\beta$ 1 antibody might prohibit TGF- $\beta$ -induced down-regulation of MMP production by fibroblasts that had infiltrated the tendon during their infiltration of the tendon matrix, resulting in the deterioration of the mechanical properties of the tendon matrix. On the other hand, Philips et al. (2003) reported that anti-TGF- $\beta$  antibody induces an increase in TGF- $\beta$  production and a decrease in MMP-1 production of human dermal fibroblasts. Therefore, there is a possibility that the adverse effect of anti-TGF- $\beta$ 1

antibody on the mechanical properties of the tendon matrix during cellular infiltration is independent of MMP production of the infiltrating cells. Therefore, further studies should be conducted to clarify the effect of TGF- $\beta$ 1 antibody on MMP production of fibroblasts during their infiltration of the tendon matrix.

Third, we did not attempt to measure the concentration of TGF- $\beta$ 1 in the medium containing 10% FBS. Because anti-bovine TGF- $\beta$ 1 antibody or recombinant bovine TGF- $\beta$ 1 is not available for calibration, it is difficult to measure the concentration of TGF- $\beta$ 1 in FBS by commercially available TGF- $\beta$ 1 immunoassays. Based on the study by Kropf et al. (1997), the average concentration of TGF- $\beta$ 1 in human serum is approximately 40 ng/ml. Therefore, we assume that approximately 4 ng/ml TGF- $\beta$ 1 existed in the medium containing 10% FBS.

Fourth, we obtained fibroblasts from the normal patellar tendon. However, previous studies demonstrated that the responsiveness of fibroblasts to TGF- $\beta$ 1 depends on the source of cells (Yoshida and Fujii, 1999; Schmidt et al., 1995; Spindler et al., 1996). Therefore, the results of the present study may not be applicable to fibroblasts derived from other kinds of tendons or ligament tissues. Fifth, we did not attempt to evaluate the effect of antibody neutralization of TGF- $\beta$  on the cellular distribution in the tendon. Our pilot study could not find any obvious effects of antibody neutralization of TGF- $\beta$ 1 on

cellular infiltration into the tendon.

Concerning clinical relevance, the process of the grafted tendon remodeling is considered to be very slow after ligament reconstruction (Abe et al., 1993). To accelerate the remodeling process of the tendon matrix after ligament reconstruction, grafting tissue-engineered tendon matrix is one possible strategy (Cartmell and Dunn, 2004). However, this study suggested that, when we intend to develop tissue-engineered tendon/ligament tissues in the near future, we should take the *ex vivo* mechanical deterioration of tendon fascicles during infiltration of seeded cells into account, and that TGF- $\beta$  has a role in suppressing the material deterioration of the fascicles in the tendon during *ex vivo* cellular infiltration. To solve this problem, we should conduct further studies for inhibition of the material deterioration of the matrix in response to cellular infiltration *ex vivo*.

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## Figure Legends

**Fig. 1** Confocal laser microscopic findings of the patellar tendon matrix at 6 weeks after the incubation of acellular patellar tendons in the gel with cultured fibroblasts (a) and without fibroblasts (b). In the cell-culture group, a number of fibroblasts were found entirely within the patellar tendon matrix at 6 weeks after the incubation of the acellular patellar tendon in the gel with cultured fibroblasts. On the other hand, no fibroblasts were observed in the control group, while there were some non-specific backgrounds in the patellar tendon matrix.

**Fig. 2** Effects of TGF- $\beta$ 1 neutralization on mechanical properties of tendon fascicles at 6 weeks (a. stress-strain curves, b. the tensile strength, c. the tangent modulus, d. the strain at failure). The error bars in the figures represent one standard deviation. In the A-TGF $\beta$  group, 2  $\mu$ g/mL monoclonal anti-human TGF- $\beta$ 1 antibody was supplemented during incubation of acellular tendons in gel containing cultured fibroblasts, while antibody was not supplemented in the non-A-TGF $\beta$  group.

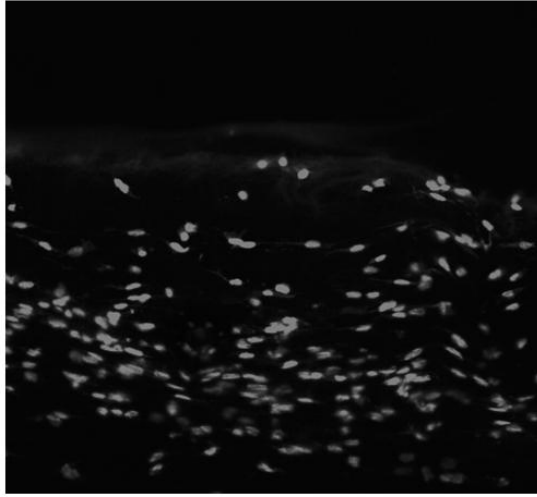


Fig. 1-a





Fig. 1-b

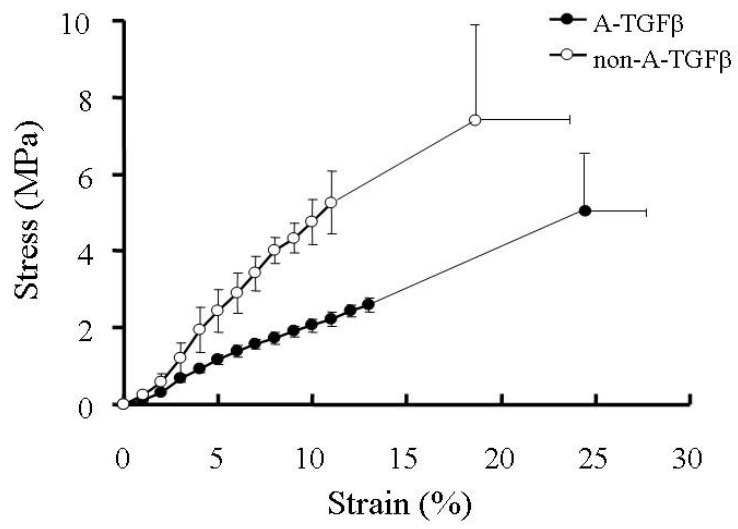


Fig. 2-a

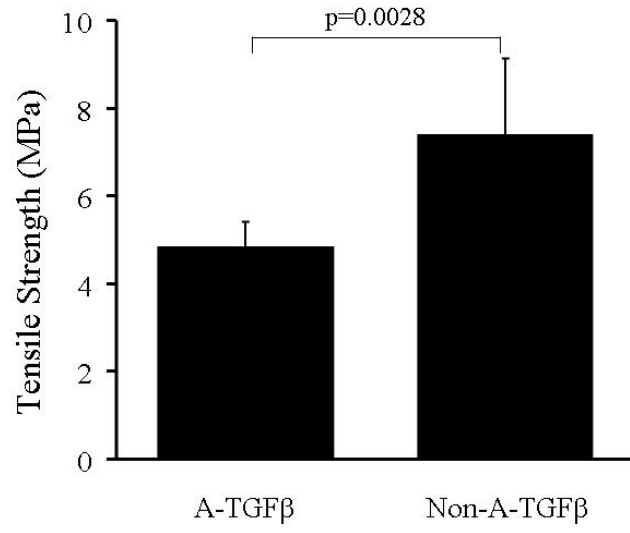


Fig. 2-b

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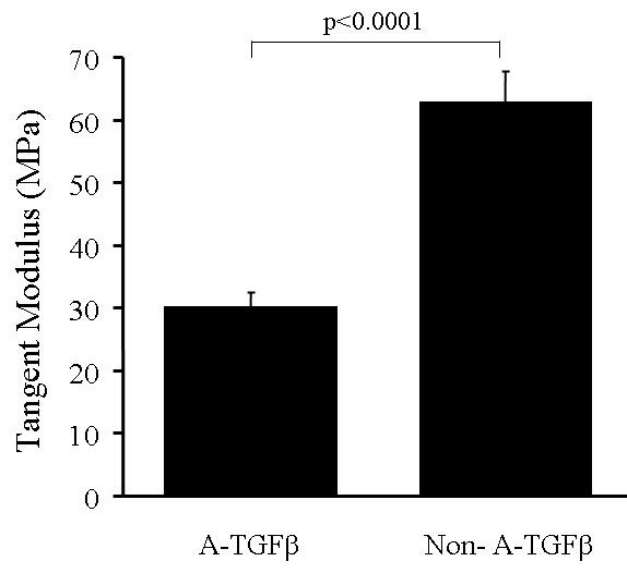


Fig. 2-c

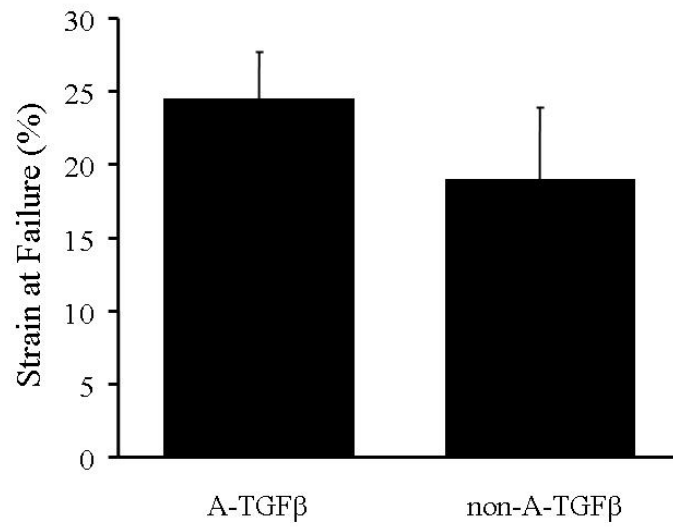


Fig. 2-d