

## Mechanical Stretch Stimulates Integrin $\alpha$ V $\beta$ 3-mediated Collagen Expression in Human Anterior Cruciate Ligament Cells

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The abbreviations used are: ACL, anterior cruciate ligament; ECM, extracellular matrix; **FAK**, focal adhesion kinase; G3PDH, glyceraldehydes-3-phosphate dehydrogenase; **TGF**, transforming growth factor

Key words: Anterior cruciate ligament; Collagen; Integrin  $\alpha$ V $\beta$ 3; Interface; Mechanical stretch

Word count: 2644 words

## Abstract

Biomechanical stimuli have fundamental roles in the maintenance and remodeling of ligaments including collagen gene expressions. Mechanical stretching signals are mainly transduced by cell adhesion molecules such as integrins. However, the relationships between stress-induced collagen expressions and integrin-mediated cellular behaviors are still unclear in anterior cruciate ligament cells. Here, we focused on the stretch-related responses of different cells derived from the ligament-to-bone interface and midsubstance regions of human anterior cruciate ligaments. Chondroblastic interface cells easily lost their potential to produce collagen genes in non-stretched conditions, rather than fibroblastic midsubstance cells. Uni-axial mechanical stretches increased the type I collagen gene expression of interface and midsubstance cells up to 14- and 6-fold levels of each non-stretched control, respectively. Mechanical stretches also activated the stress fiber formation by shifting the distribution of integrin  $\alpha$ V $\beta$ 3 to the peripheral edges in both interface and midsubstance cells. **In addition, integrin  $\alpha$ V $\beta$ 3 colocalized with phosphorylated focal adhesion kinase in stretched cells.** Functional blocking analyses using anti-integrin antibodies revealed that the stretch-activated collagen gene expressions on fibronectin were dependent on integrin  $\alpha$ V $\beta$ 3-mediated cellular adhesions in **the interface and midsubstance** cells. These findings suggest that the integrin  $\alpha$ V $\beta$ 3-mediated stretch signal transduction might have a key role to stimulate collagen gene expression in human anterior cruciate ligament, especially in the ligament-to-bone interface.

## 1. Introduction

Anterior cruciate ligament (ACL) injuries usually occur in the femoral attachments of the ligament-to-bone interface (Zantop, 2007). The structure of ACL has the three histological zones consisting of round, fusiform, and ovoid cells (Duthon, 2006; Murray, 1999). Round and ovoid cells, which are observed in the ligament-to-bone interfaces, seem to have the character of chondroblasts (Wang, 2006; Wang, 2007; Duthon, 2006). However, the cellular functions of these chondroblastic interface cells are unknown. Here, we hypothesized that the poor healing potential of ruptured ACL might be caused by low cellular responses of ligament-to-bone interface cells. To test our hypothesis, we isolated fibroblastic and chondroblastic cells from the midsubstance and ligament-to-bone interface zones in human ACL. In addition, we compared cellular responses between the midsubstance and interface cells including integrin-mediated attachments in stretched conditions.

Mechanical stimuli are important for ligament homeostasis (Henshaw, 2006). Ligaments have stress-oriented structures of collagen bundles (Duthon, 2006). Type I collagen is the main component of extracellular matrix (ECM) in ligaments (Almarza, 2008). Type II and III collagens have important roles for the maturation of ligament-to-bone interface zones (Nawata, 2002; Wang, 2006). Biomechanical studies have shown that intraarticular ACLs are exposed to 5% strains during normal activities of the knee joints (Hsieh, 2000; Hsieh, 2002; Lee, 1996). Uni-axial cyclic stretch increases the gene expressions of type I and III collagens by mediating the autocrine secretion of transforming growth factor (TGF)- $\beta$ 1 in human ACL cells (Kim, 2002). Uni-axial cyclic stretching also stimulates the cellular proliferation and type I collagen expression of human patellar tendon fibroblasts *in vitro* (Yang, 2004). However, the detailed mechanisms of stress-induced collagen expressions are still unclear in ligament fibroblasts.

Stretching treatments are transduced as intracellular signals by cell adhesion relating molecules. Integrins are not only the cell surface receptors recognizing ECM but also the signal transducers. Mechanical stress activates **the phosphorylation of focal adhesion kinase (FAK) and** the Rho-dependent assembly of focal adhesion complexes through the integrin-ECM contacts (Geiger, 2001). Cytoskeletal tractional force triggers intracellular signals such as MAP kinase and NF- $\kappa$ B pathways (Shyy, 1997). In human ligament fibroblasts, cAMP and Ca<sup>2+</sup>/phospholipid signaling pathways seem to have important roles in the integrin-mediated adhesions on fibronectin (Sung, 1996).

In this study, we investigated the effects of uni-axial mechanical stretch on the interface

and midsubstance ligament cells derived from human ACL. The present study also demonstrates that the mechanical stretch increased collagen gene expressions by modulating the integrin  $\alpha$ V $\beta$ 3-dependent focal adhesion in human ACL cells.

## 2. Materials and methods

### *Cells and cell culture.*

Human ACL fibroblasts were isolated from intact ACL-bone samples (n=5) obtained at total knee arthroplasties in patients suffering osteoarthritis (mean, 70 years old). This study received the approval of our institution and the informed consents for this research were obtained. Surrounding synovial tissues and attached bones were carefully removed from ACL samples. The interface cells were isolated from 5-mm-segments of ACL-to-bone junctions (femoral and tibial interfaces). The remaining ligament was used as midsubstance cells. The ligament samples were minced, and incubated with collagenase (Sigma, St. Luis, MO) for 30 min at 37°C. The mixtures were filtered through 70  $\mu$ m cell strainers (BD Biosciences, Bedford, MA). The flow through were cultured in Dulbecco's Modified Eagle Medium (DMEM, Wako, Osaka, Japan) containing 10% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin/streptomycin (Sigma). Attached cells were incubated at 37°C in 5% CO<sub>2</sub> in a humidified atmosphere and subcultured at a density of 2,500 cells/cm<sup>2</sup> on non-coated polystyrene tissue culture dishes (BD Biosciences). The medium was changed every 3 days. Cells between passage 3 and 6 were used for experiments.

### *RT-PCR and quantitative real-time PCR analyses.*

Total RNA was isolated using ISOGEN reagent (Nippon Gene, Toyama, Japan). RNA samples (500 ng) were reverse-transcribed with ReverTra Ace (Toyobo, Osaka, Japan). The cDNAs underwent PCR amplification in the presence of 10 pmol of each specific primer using ExTaq DNA polymerase (Qiagen, Germantown, MD). The following specific primer sets were used (Furumatsu, 2003): 5'-GTGGT TCGTG ACCGT GACC-3' and 5'-GAGTG GCACA TCTTG AGGTC-3' for  $\alpha$ 1 chain of type I collagen (*COL1A1*), 5'-AATTC CTGGA GCCAA AGGAT-3' and 5'-AGGAC CAGTT GCACC TTGAG-3' for  $\alpha$ 1 chain of type II collagen (*COL2A1*), 5'-CTGGA AAGAG TGGTG ACAG-3' and 5'-TCTTC CTGGT CTGGC TGGTA-3' for  $\alpha$ 1 chain of type III collagen (*COL3A1*), 5'-CGACG AGCAC TGTTT CTC-3' and 5'-CTAAA TTCAG ATTCA TCCCG-3' for integrin  $\alpha$ V, 5'-GAAAA GATTG GCTGG AGGAA-3' and 5'-GGCAT ACCCC AACT CAAA-3' for integrin  $\beta$ 3, 5'-GACCA AGGCA GAA G GCAGC-3' and 5'-CTCAG CTCAA GCCTC CAGC-3' for integrin  $\alpha$ 5, 5'-GTGGT TGCTG GAATT GTTCT-3' and 5'-CCCTC AACT TCGGA TTGAC -3' for integrin  $\beta$ 1,

5'-CATCA AGAAG GTGGT GAAGC AG-3' and 5'-CGTCA AAGGT GGAGG AGTGG-3' for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*). The cycle number was selected in the linear part of the amplification curve. For all the RT-PCR fragments, the reactions were allowed to proceed for 27 cycles in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR analyses were performed using LightCycler ST-300 instrument (Roche Diagnostics, Mannheim, Germany) and FastStart DNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's protocol. Amplification of *G3PDH* was used for normalization. The final expression value was calculated dividing each expression level of non-stretched cells. These experiments were run in triplicate and similar results were obtained.

#### *Stretching experiments and functional blocking assays.*

The midsubstance and interface cells were seeded onto stretch chambers, each having a culture surface of  $2 \times 2 \text{ cm}^2$ , at the concentration of 50,000 cells/chamber. **And then, the cells were incubated on fibronectin-coated chambers for 24 h before stretching experiments.** The chamber was coated using human fibronectin (Chemicon, Temecula, CA) as described previously (Naruse, 1998). Uni-axial cyclic mechanical stretch (0.5 Hz, 7%) was applied for 2 h using a ST150 (STREX, Osaka, Japan). Non-stretched ACL cells culturing on the stretch chambers were used as controls. To inhibit the function of integrin  $\alpha 5$  subunit or  $\alpha V\beta 3$  in stretching experiments, anti-human integrin  $\alpha 5$  and  $\alpha V\beta 3$  functional blocking antibodies (4  $\mu\text{g/ml}$ , Chemicon) were used as described previously (Furumatsu, 2002). These concentrations of blocking antibodies did not directly affect the cellular attachment in the following procedure. Cells were incubated with each antibody for 10 min before seeding on fibronectin-coated chambers. Cells were allowed to attach for 2 h, then mechanical stretch (0.5 Hz, 7%) was applied for 2 h. RNAs were immediately collected after stretching experiments.

#### *Histology and immunohistochemistry.*

To investigate the cell morphology, we observed the overview of human ACL. Specimen were fixed with 4% paraformaldehyde solutions for 24 h and embedded in paraffin. These sections were cut into 5  $\mu\text{m}$ -thickness, stained with hematoxylin and eosin for standard light microscopy. Next, to investigate the cellular attachments responding to mechanical stretch, we observed the distribution of integrins, **phosphorylated FAK**, and F-actin fibers in both midsubstance and interface cells. Cells were stretched for 2 h on fibronectin-coated chambers, then fixed with 1% paraformaldehyde solutions. Chambers were incubated with anti-integrin  $\alpha V\beta 3$  antibody (20  $\mu\text{g/ml}$ , Chemicon) **and phosphorylated FAK (Tyr 925, 4  $\mu\text{g/ml}$ , Santa Cruz, CA)** for 1 h. Bovine serum albumin-containing solutions without primary antibodies were used

as negative controls. Alexa Flour 488-conjugated antibody (10 µg/ml, anti-mouse), Alexa Flour 647-conjugated antibody (10 µg/ml, anti-goat), Alexa Flour 568-conjugated phalloidin (2 µg/ml) (Invitrogen, Carlsbad, CA), and Hoechst 33342 (10 µg/ml) (ICN Biomedicals, Aurora, OH) were used for detections. Samples were examined under a fluorescence microscope (Leica, Wetzlar, Germany). Then we used x20 objective lens for detection.

#### *Statistical analysis.*

All experiments were repeated at least three times and similar results were obtained. Data were expressed as means S.D. Differences among groups were compared by using the Mann-Whitney *U*-test. Statistical significance was established at  $p < 0.05$ .

### **3. Results**

#### *The ACL-to-bone interfaces contain round cells packaged with hematoxylin-stained ECM.*

Firstly, we analyzed the structure of human ACL including ligament-to-bone junctions (Fig. 1A). Midsubstance area consisted of fusiform cells and parallel fibers (Fig. 1B). However, the cells in interface regions (femoral and tibial interfaces) formed ovoid or round shapes. In addition, the ECM structure of interfaces was different from that of midsubstance (Fig. 1B). To assess the morphological differences between the midsubstance and interface zone of human ACL, we isolated two types of cells from each region. The shape of midsubstance cells showed spindle and fibroblastic cellular morphologies *in vitro* (Fig. 1C, middle panel). On the other hand, the interface cells isolated from femoral and tibial insertions displayed chondroblastic appearances such as small and triangular morphologies (Fig. 1C, upper and lower panels).

#### *Cultured interface cells loose their phenotypes in collagen gene expressions.*

Next, we investigated the expression patterns of collagen genes in tissues and isolated cells to assess the different cellular behaviors between three-dimensional (Tissues, *in vivo*) and two-dimensional (Cultured cells, *in vitro*) conditions. The expressions of *COL1A1*, *COL2A1*, and *COL3A1* genes were detected in the tissue RNAs of interface zones (Fig. 2, Tissues, I). However, these expressions were decreased in cultured interface cells (Fig. 2, Cultured cells, I). In midsubstance cells, the expression of *COL1A1* gene was equally detected in both tissues and cultured cells. The expression of *COL2A1* gene was not detected in both tissues and cultured midsubstance cells. Interestingly, the *COL3A1* gene expression was observed in cultured midsubstance cells (Fig.2, Cultured cells, M). On the other hand, the expressions of integrin  $\alpha V$  and  $\beta 3$  subunits, which recognize collagens and fibronectin, were decreased by two-dimensional conditions in the interface cells (Fig. 2, Cultured cells, I). These results indicated that the

phenotypes of both ACL cells were changed by **ECM environments and cellular adhesions**, especially in the interface cells.

*Mechanical stretch reproduces the expression of COL1A1 and COL3A1 genes in cultured ACL cells.*

To maintain the phenotype of midsubstance and interface cells, we applied uni-axial cyclic mechanical stretches on cultured cells. The *COL1A1* expression of cultured ACL cells was dramatically increased by mechanical stretch (Fig. 3). Mechanical stretch stimulated the *COL1A1* expression of midsubstance and interface cells up to 6- and 14-fold levels of each non-stretched control, respectively (Fig. 3, B and C). The *COL3A1* expressions were also enhanced up to 1.8-fold levels of controls by stretching treatment in both cells (Fig. 3, B and C). **In addition, the mechanical stretch increased the integrin  $\alpha V$  and  $\alpha 5$  expression in cultured midsubstance and interface cells, respectively (Fig. 4).** These findings suggest that mechanical stretch **and integrins might have key roles** to maintain the collagen gene expressions and the phenotypes of ACL cells.

*Mechanical stretch activates the integrin  $\alpha V\beta 3$ -mediated stress fiber formation on fibronectin.*

Here, we analyzed the relationships among mechanical stretch, cellular adhesion, and stress fiber formation in ACL cells. Integrin  $\alpha V\beta 3$  was shifted to the peripheral edge of cells by stretching treatments (Fig. 5A, Integrin  $\alpha V\beta 3$ , green signals). In addition, mechanical stretch changed the integrin  $\alpha V\beta 3$ -dependent stress fiber formation in both ACL cells on fibronectin (Fig. 5A, red signals). However, the stress fiber orientations were not parallel, **and not perpendicular**, to the stretching direction. **Integrin  $\alpha V\beta 3$  also colocalized with phosphorylated FAK in stretched ACL cells (Fig. 5B, Merged).**

*Stretch-activated collagen gene expressions depend on the integrin  $\alpha V\beta 3$ -mediated cellular adhesions.*

To assess the role of integrin for stretch-induced collagen gene expression, we performed functional blocking assays using anti-integrin antibodies. Stretch-activated *COL1A1* and *COL3A1* expressions were inhibited by the functional blocking antibody for integrin  $\alpha V\beta 3$ , without influencing the attached cell number, in the midsubstance and interface cells (Fig. 6). However, the functional blocking for integrin  $\alpha 5$  did not suppress the stretch-induced *COL1A1* and *COL3A1* expressions in both ACL cells (Fig. 6). These results suggest that stretch-activated intracellular signals for collagen syntheses might be transduced by integrin  $\alpha V\beta 3$ , not by integrin  $\alpha 5$ , in human ACL cells.

#### 4. Discussion

ACL injuries are difficult to cure conservatively because of their low healing potentials. Ligament healings are influenced by several factors including cellular proliferation, migration, adhesion, neovascularization, and growth factors (Marui, 1997; Frank, 1999). Mechanical strain also plays a fundamental role in the maintenance and remodeling of ligament structure (Woo, 1987). The present study demonstrates that the integrin  $\alpha V\beta 3$ -mediated stress signal transduction has a key role to stimulate *COL1A1* gene expression in human ACL cells. This is the first report to investigate the **stretch-mediated** cellular response in the ACL-to-bone interface cells.

The ACL-to-bone interface has been reported to be consisted by four different zones: bone, mineralized interface, non-mineralized interface, and ligament (Wang, 2006; Nawata, 2002). The interface region shifts the structure from proliferative to hypertrophic zone of growth plate during development. Age-dependent changes in collagen deposition (from type II collagen to type X collagen) are observed at the interface (Wang, 2006). Our study indicated that **the cells in the interface zone** of aged ACLs still expressed *COL2A1* gene **in three-dimensional conditions** (*in vivo*, Fig. 2, Tissues). However, non-stretched interface cells easily lost their potential to produce collagen genes **in two-dimensional conditions** (*in vitro*, Fig. 2, Cultured cells). These results suggest that the cartilaginous interface cells and **surrounding ECMs** have important roles for connecting ligament-to-bone by neutralizing tensile strain. **We demonstrated that mechanical stretch increased the expression of *COL1A1* and *COL3A1* gene in the midsubstance and interface cells (Fig. 3 and Supplemental Fig. 2). However, the expression of *COL2A1* was not fully recovered by mechanical stretch in the interface cells (Supplemental Fig. 2). In porcine articular chondrocytes, *Col2a1* expression is temporarily increased by cyclic tensile strain (0.5 Hz, 10%, 3 h) and gradually drops to 30% of initial level (Huang, 2007). These findings suggest that the cyclic stretch has an important role to activate the fibroblastic phenotype of ACL cells. The chondroblastic property of interface cells might be enhanced by a different mechanical stress, such as pellet- and micromass-cultured conditions.**

Mechanical stress-induced collagen gene expression has a crucial role to activate cellular behaviors. Several intracellular signals that transduce ECM forces have been reported. ERK 1/2, protein kinase C, and tyrosine kinase activations are necessary for stretch-induced *COL1A1* expression in cardiac fibroblasts (Papakrivopoulou, 2004; Husse, 2007). Tyrosine kinase-mediated TGF- $\beta 1$  production is also important for stress-induced *COL1A1* expression in vascular smooth muscle cells (Joki, 2000). In stretched mesangial cells, *COL1A1* expression is



increased by phosphatidylinositol-3-kinase- and epidermal growth factor receptor-mediated Akt activations (Krepinsky, 2005). However, the stretch-mediated regulation of collagen expression is not fully elucidated in ligament cells. Mechanical strain increases the expression of integrin subunits in smooth muscle cells (Wilson, 1995), human umbilical vein endothelial cells (Aikawa, 2001), cardiac fibroblasts (Butt, 1995), osteosarcoma cells (Carvalho, 1995), and hepatocytes (Nebe, 1995). In ACL fibroblasts, several authors have described the expression pattern of integrins in response to mechanical stretch (Henshaw, 2006; Hannafin, 2006). Integrin  $\alpha 5\beta 1$ , which serves as the fibronectin-specific receptor, has been reported to be increased in stress-deprived rabbit ligament fibroblasts using immunohistochemical analyses (AbiEzzi, 1997). Semi-quantitative RT-PCR analyses show that the expression of  $\beta 1$  integrin subunit is higher in ruptured (non-strained) ACL than in intact human ACL (Brune, 2007). On the other hand, 5% cyclic strain increases the cell surface expressions of  $\alpha 5$  and  $\beta 1$  integrin subunits on fibronectin-coated plates using flow cytometrical analyses. In addition, mechanical stretch-induced expression of integrin  $\beta 3$  is only influenced on laminin, not on fibronectin, in canine ACL fibroblasts (Hannafin, 2006). In cardiac myocytes, stretch-induced upregulation of the cardiac gap junction protein is mediated by  $\beta 1$  integrin on native type I collagen, not by  $\beta 3$  integrin (Shanker, 2005). These different observations might be caused by the followings: i) cell species, ii) stretching conditions and coating ECs, iii) detection methods, and iv) the expression time-lag between mRNAs and proteins. **The present study demonstrated that mechanical stretch increased the expression of *integrin  $\alpha V$*  in ACL cells (Fig. 4). Functional blocking analyses revealed that the stretch-induced *COL1A1* expression depended on integrin  $\alpha V\beta 3$ -mediated cellular adhesions in ACL cells (Fig. 6). In addition, integrin  $\alpha V\beta 3$  colocalized with phosphorylated (activated) FAK by stretching treatments (Fig. 5B). These findings suggest that the stretch-induced *COL1A1* expression may be upregulated by the increase of *integrin  $\alpha V$*  expression and the activation of integrin  $\alpha V\beta 3$  in ACL cells.** Further studies will be required to investigate the relationships among mechanical stress, integrins, and collagen expression in ligament fibroblasts.

## Acknowledgements

We are grateful to our colleagues at the Department of Orthopaedic Surgery for their great support during this study. This work was supported by Japan Society for the Promotion of Science (Nos. 18890115 and 20791040, Furumatsu) and by the Japanese Foundation for Research and Promotion of Endoscopy (Furumatsu). This work was also supported by Child Health and Development from the Ministry of Health, Labor and Welfare (20-3, Nishida) and System Cell Engineering by Multi-Scale Manipulation (No. 17076006, Naruse) from the

Ministry of Education Science Sports and Culture, Japan.

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## Figure legends

### Fig. 1.

#### The difference of cell morphologies between the midsubstance and interface cells in human ACL.

(A) The overview of hematoxylin and eosin-stained human ACL. The indicated upper, middle, and lower boxes represent the region of femoral interface, midsubstance, and tibial interface, respectively. *Bar*, 10 mm.

(B) The difference of cell morphology among each zone was observed with hematoxylin and eosin staining. The ovoid and round cells were detected in the interface zones (upper and lower panels). The fusiform cells were abundantly observed in the midsubstance area (middle panel). The ECM of interface region was highly stained by hematoxylin, rather than that of midsubstance. *Bar*, 100  $\mu\text{m}$ .

(C) The midsubstance and interface cells showed fibroblastic and triangular morphologies, respectively. Cellular morphologies of femoral interface, midsubstance, and tibial interface are shown in upper, middle, and lower panels, respectively. *Bar*, 100  $\mu\text{m}$ . The expressions of collagen genes in ACL-to-bone interfaces are shown in Supplemental Fig. 1. No differences of collagen gene expressions were observed between femoral and tibial interfaces (Supplemental Fig. 1).

### Fig. 2.

#### The different expression of collagen genes between tissues and cultured cells (three- and two-dimensional environments) of human ACLs.

RT-PCR was performed using specific primers as described. The expression of *COL1A1* gene was detected in both midsubstance and interface tissues. The *COL2A1* and *COL3A1* genes expressed in the tissues of interface zones. However, the expressions of these collagen genes were decreased in cultured interface cells. In cultured midsubstance cells, the *COL3A1* expression was additionally detected. The expressions of *integrin  $\alpha\text{V}$*  and  *$\beta\text{3}$*  subunits were decreased in cultured interface cells. These experiments were run in triplicate from five different ACL samples and similar results were obtained. M, midsubstance. I, interface.

### Fig. 3.

#### Mechanical stretch stimulates the *COL1A1* expression in cultured midsubstance and interface cells.

(A) *COL1A1* and *COL3A1* expression of midsubstance cells were slightly increased by mechanical stretch in RT-PCR analyses. In the interface cells, *COL1A1* gene expression was

strongly induced by mechanical stretch. M, midsubstance. I, interface.

(B and C) In real-time PCR analyses, mechanical stretch also increased the *COL1A1* expression of midsubstance and interface cells. Relative mRNA levels of each collagen gene were normalized using *G3PDH* controls and calculated by the expression levels of non-stretched cells. Data were shown as the mean S.D. of triple determination. Relative *COL1A1* and *COL3A1* expression of midsubstance cells was increased up to 6.1- and 1.8-fold levels of non-stretched cells by mechanical stretch, respectively. In the interface cells, mechanical stretch stimulated relative *COL1A1* and *COL3A1* expression to 14.4- and 1.8-fold levels of non-stretched-cells, respectively. \* Statistical significances ( $p < 0.05$ ) were observed between indicated bars using Mann-Whitney *U* test. *Error bars*, S.D.

**Fig. 4.**

**Mechanical stretch modulates the integrin gene expressions in cultured ACL cells.**

Stretch-mediated changes of cell adhesion molecules (integrins) were investigated by RT-PCR analyses. Mechanical stretch increased the expression of *integrin  $\alpha V$*  and  *$\alpha 5$*  in the midsubstance cells. However, the expression of *integrin  $\beta 1$*  and  *$\beta 3$*  subunits was not influenced by stretching treatment (Midsubstance). In the interface cells, mechanical stretch stimulated the expression of *integrin  $\alpha V$* . No significant increase was observed in the expression of *integrin  $\alpha 5$* ,  *$\beta 1$* , and  *$\beta 3$*  subunits (Interface).

**Fig. 5.**

**Mechanical stretch modulates the F-actin fiber formation by shifting the distribution of integrin  $\alpha V\beta 3$  on fibronectin.**

(A) Rearrangements of F-actin fibers, detected by phalloidin reagents, were induced by mechanical stretch for 2 h (red signals). The expression of  $\alpha V\beta 3$  integrin was localized at the edge of perisomatic filopodia in both midsubstance and interface cells on fibronectin-coated stretch chambers (Integrin  $\alpha V\beta 3$ , green signals). Cells incubated in the absence of primary antibodies were denoted as control. Merged images are shown. Arrows denote the stretching directions. *Stress fibers randomly aligned*. *Bar*, 100  $\mu\text{m}$ .

(B) Integrin  $\alpha V\beta 3$  (green signals) colocalized with phosphorylated FAK (pFAK, red signals) in stretched midsubstance and interface cells. Merged images of magnified areas denote the colocalization (yellow signals) on fibronectin. *Bar*, 10  $\mu\text{m}$ .

**Fig. 6.**

**Mechanical stretch increases the *COL1A1* and *COL3A1* expressions in an integrin  $\alpha V\beta 3$ -dependent manner.**

The functional blocking antibody against integrin  $\alpha 5$  did not inhibit the increase of collagen gene expression in both midsubstance (A) and interface cells (B). On the other hand, the increase of stretch-activated collagen expression was not observed in the presence of anti-integrin  $\alpha V\beta 3$  antibodies (A and B).

(C) In real-time PCR analyses, uni-axial cyclic strains increased the *COL1A1* expression up to 3- and 10-fold levels of controls in the midsubstance and interface cells, respectively, even in the presence of the functional blocking antibody against integrin  $\alpha 5$ . However, the anti-integrin  $\alpha V\beta 3$  antibody prevented the mechanical stretch-dependent increase of *COL1A1* and *COL3A1* expressions in both ACL cells (C and D). Functional blocking antibody against integrin  $\alpha V\beta 3$  inhibited the filopodia formation of ACL cells on fibronectin (Supplemental Fig. 3). Relative mRNA level of each collagen gene was normalized using *G3PDH* control and calculated by each expression level of non-stretched cells. Data were shown as the mean standard deviation of triple determination. \* Statistical significances ( $p < 0.05$ ) were observed between indicated bars using Mann-Whitney *U* test. Error bars, S.D.

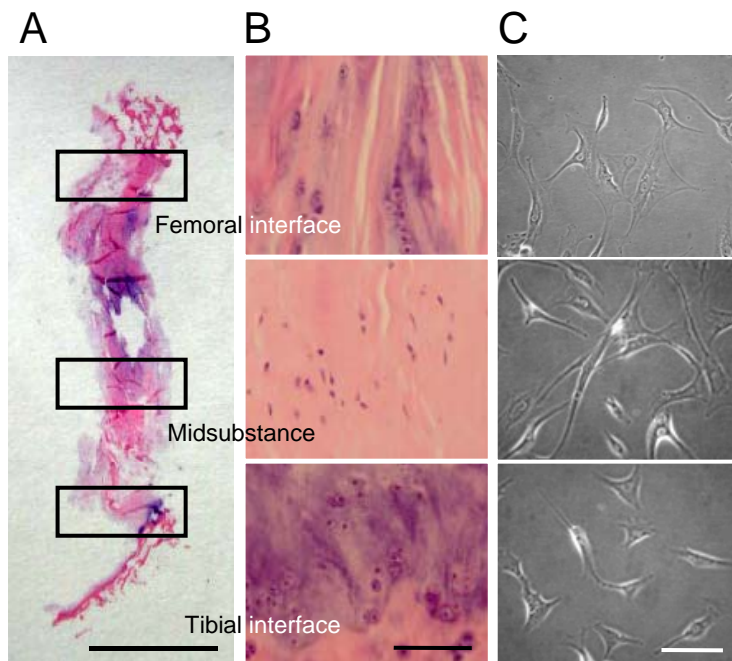


Figure 1.  
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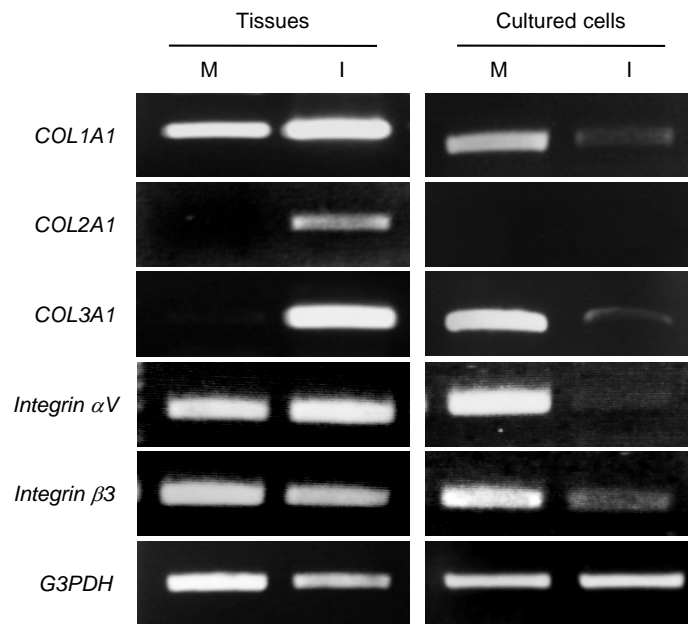


Figure 2.  
Tetsunaga et al.

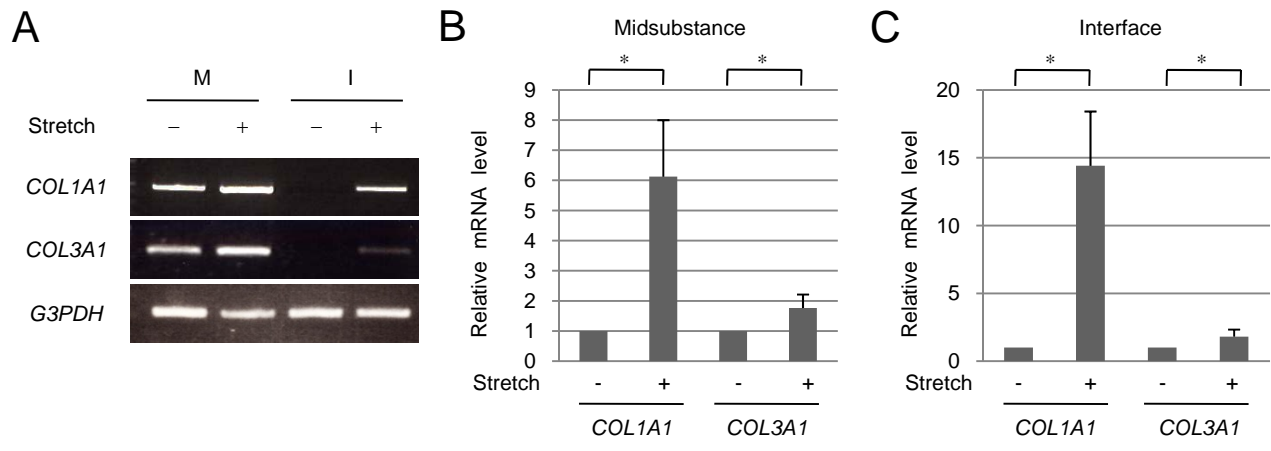


Figure 3.  
Tetsunaga et al.

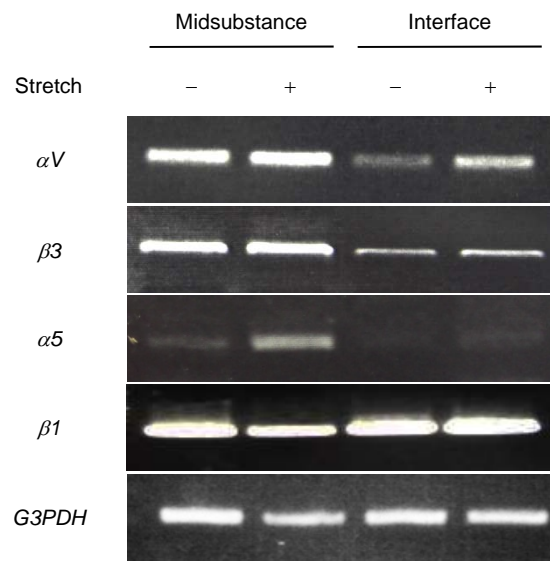


Figure 4.  
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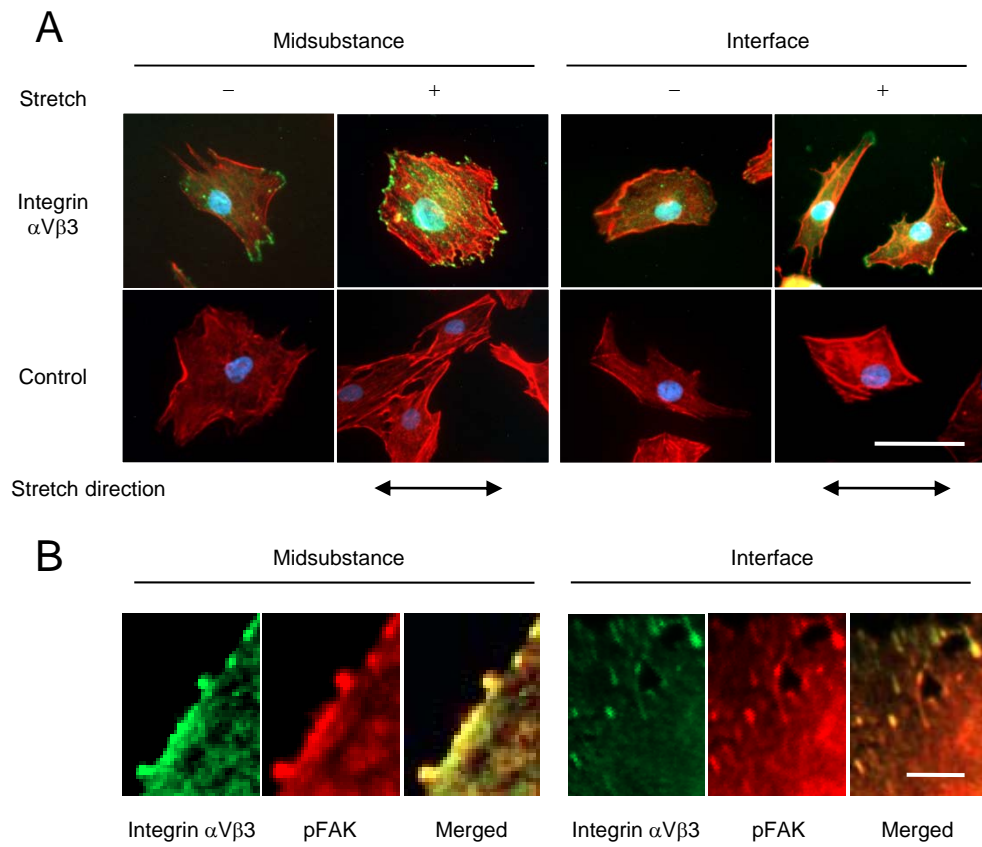


Figure 5.  
Tetsunaga et al.

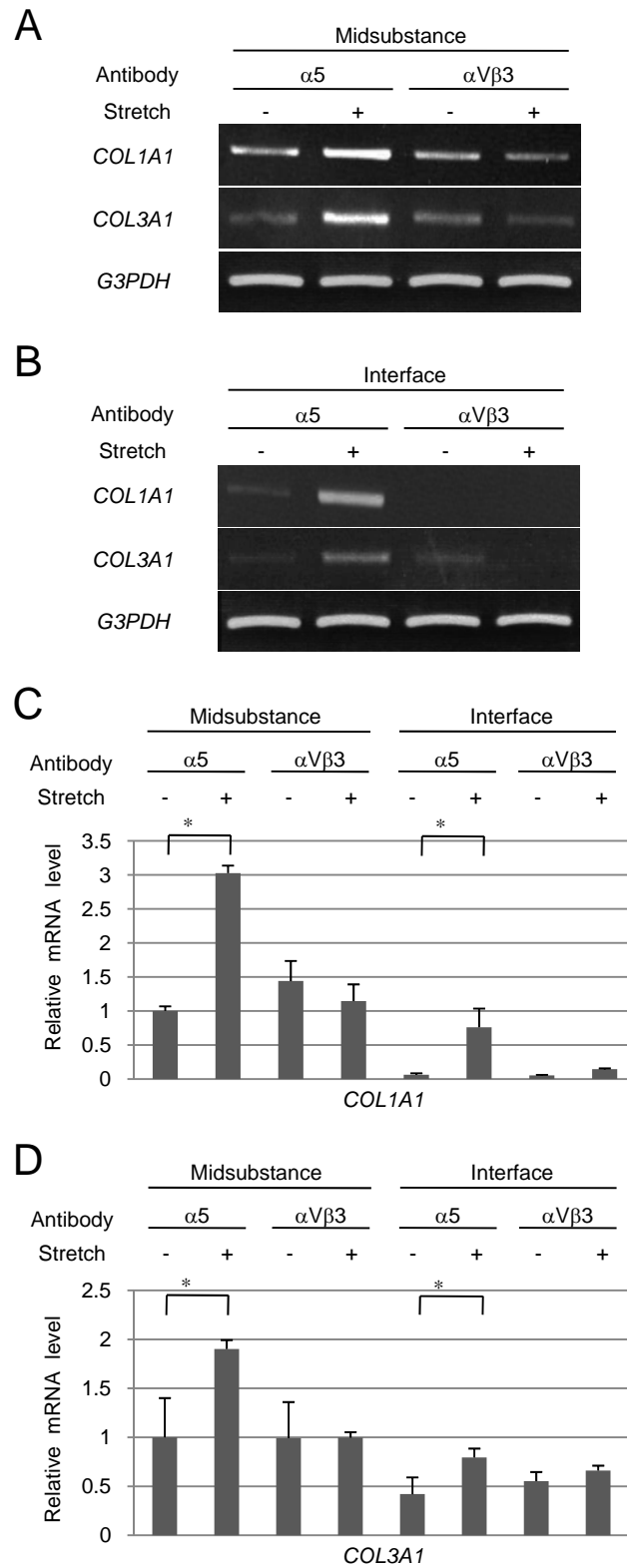
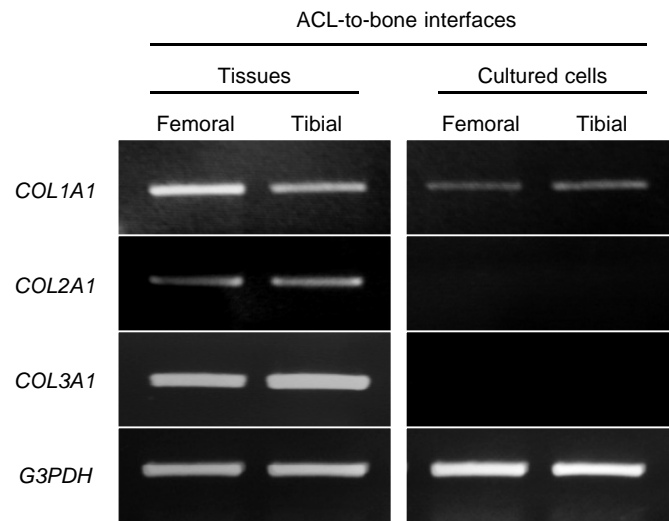
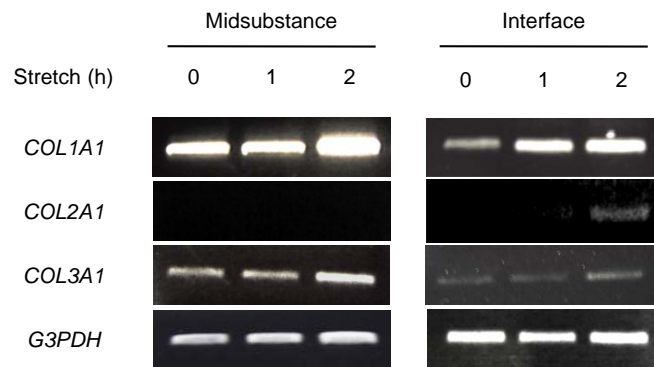


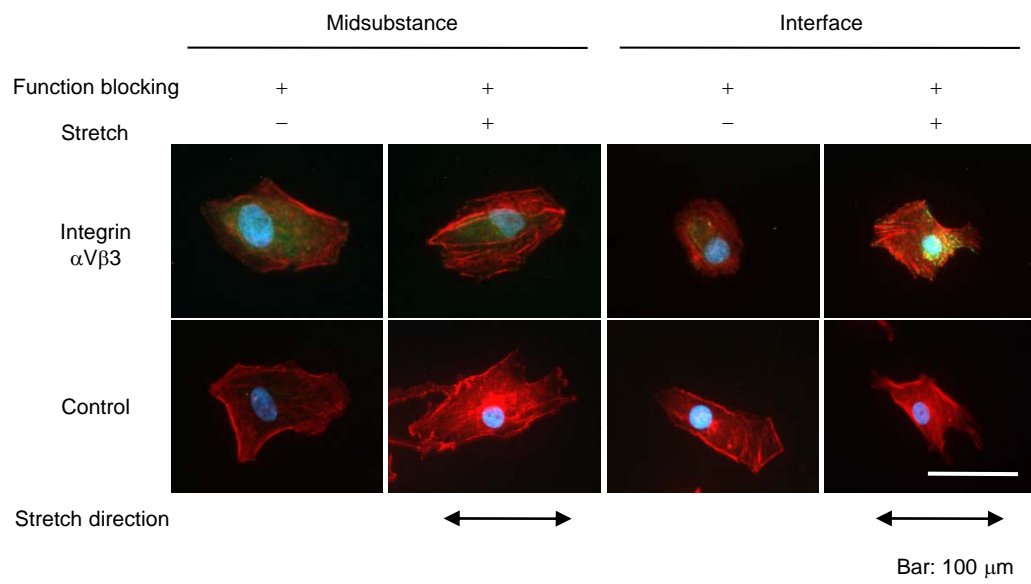
Figure 6.  
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Supplemental Figure 1.  
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Supplemental Figure 2.  
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Supplemental Figure 3.  
Tetsunaga et al.