Talin is required to increase stiffness of focal molecular complex in its early formation process.

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Abstract

For cellular adaptation in mechanical environments, it is important to consider transmission of forces from the outside to the inside of cells via a focal molecular complex. The focal molecular complex, which consists of integrin, talin, vinculin and actin, is known to form in response to a force applied via the extra-cellular matrix (ECM). In the early formation process of the complex, the complex–actin connection is reinforced. These structural changes of the nascent complex result in an increase in its mechanical integrity and overall stiffness, possibly leading to the maturation of the nascent complex by enhancing force transmission. In this study, we hypothesized that the complex component talin is a crucial factor in increasing the stiffness of the nascent complex. To test the hypothesis, we used atomic force microscopy (AFM) to measure the stiffness of the nascent complex using a probe coated with fibronectin. Stiffness measurements were conducted for intact and talin knocked-down cells. Our results demonstrated that talin was required to increase the stiffness of the nascent complex, which could be caused by the reinforced connection between the complex and actin filaments mediated by talin.

1. Introduction

Cell adaptation to mechanical environments is triggered by force transmission from the outside to the inside of the cells [1]. Force is transmitted via a focal molecular complex that connects the extra-cellular matrix (ECM) and the cytoskeleton [2]. The focal molecular complex, consisting of integrin, talin, vinculin, and actin [3], is formed when the cell is subjected to a force via the ECM [4]. With regard to the formation of the nascent complex, early molecular events are particularly important because the complex is formed based on its initial structure. A previous study suggested that the nascent complex matures by reinforcing the complex–actin connection, and this complex disappear without such a reinforcement [5]. Therefore, the reinforced connection is thought to play a key role in the transmission of force generated outside of cells.

During force transmission, the stiffness of the complex, which is a measure of its mechanical integrity, is regarded as a pivotal factor. During its initial formation stage, a structural change in the complex can certainly lead to a change in its stiffness. Thus, an increase in the stiffness of the nascent complex enhances the force transmitted from the ECM to the cytoskeleton, possibly leading to maturation of the nascent complex through the opening of mechanosensitive ion channels [6] and/or the activation of signaling molecules via deformation of the cytoskeleton [7].

In a cell adhesion region, component proteins form a nascent complex by binding with each other within 50 s under an external force transmitted via the ECM [5,8,9]. For complex formation, the ECM-integrin binding first causes conformational changes in the integrin molecule [10], and the outside-in

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signaling initiates complex formation [11]. Then, talin and actin localize into the cell adhesion region

to form an integrin–talin–actin complex [12]. Eventually, to bind to vinculin, talin changes its

conformation by exposing its cryptic vinculin-binding site [13–16]. These sequential molecular events

lead to a reinforced connection between integrin and the actin filament [16,17]. Since talin works as an

adapter protein to connect integrin with actin filament, this connection is closely related to a change in

the stiffness of the complex. In this study, we hypothesized that talin contributes to increasing the

stiffness of the nascent complex. To test the hypothesis, we investigated the role of talin on the

stiffness of the nascent complex using atomic force microscopy (AFM).

2. Materials and Methods

2.1. Cell culture

In this study, we used mouse pre-osteoblast MC3T3-E1 cells (Riken Cell Bank), which retains the

cell surface receptor integrin β1 [18]. Cells were cultured in a minimum essential medium α (MEMα;

Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic (Nacalai

tesque) in a humidified incubator at 37℃ under a 5% CO_2 atmosphere.

2.2. Modification of AFM probe

OMCL-TR400PSA (spring constant = 0.02 N/m, tip radius = 15 nm, Olympus) was used as the

AFM probe. To coat the probe with fibronectin (i.e., form an FN probe), organic matters on the probe

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tip were cleaned using a vacuum plasma processing system (STREX). Then, for silane coupling, the

tip was put in 2% 3-aminopropyl-triethoxysilane (APTES; Sigma-Aldrich)/pure water for 15 min, and

then rinsed with pure water. The tip was immersed in 1 mM linker protein maleimide-

polyethyleneglycol-N-hydroxyl-succinimide (Maleimide-PEG-NHS; NANOCS)/pure water for 30

min, and then rinsed. Finally, the tip surface was modified in 100 nM fibronectin (EMD

Millipore)/PBS for 60 min, and then rinsed. Afterwards, to quench the remaining Maleimide residues,

the tip was placed in 2-mercaptoethanol (Wako) for 60 min. For a negative control group, an AFM

probe coated with BSA (BSA probe) was fabricated by modifying 4% BSA after Maleimide-PEG-

NHS.

2.3. Immunofluorescence imaging of integrin on AFM probe

To visualize the integrin attached on the tip of the FN probe after it was in contact with the cells,

the tip surface was fixed with paraformaldehyde and blocked with 3% BSA/PBS. Subsequently, the tip

surface was coated with antibodies for integrin staining and then rinsed with PBS. The rat anti-mouse

integrin β1 antibody (Abcam) and Alexa Fluor 488-conjugated donkey anti-rat IgG antibody (Abcam)

were used as the primary and secondary antibodies, respectively. Total internal reflection fluorescence

microscopy (TIRFM; Olympus) was employed to detect fluorescence of the antibody, and the results

were finally analyzed using the image processing software ImageJ (NIH).

2.4. Talin 1 knock down

To inhibit expression of talin in cells, we added plasmids (pSilencer 3.0-H1) to knock down talin 1

and plasmids (pCAGGS-EGFP) to express the green fluorescent protein (GFP) by using a transfection

reagent FuGENE HD (Promega) dissolved in Opti-MEM (Gibco). The GFP positive cells were used as

talin 1 knocked-down cells in the experiments. The culture medium was then replaced by a

transfecting mixture solution. After 2 h of incubation in the transfecting mixture solution at 37°C in a

5% CO_2 atmosphere, the cells were cultured in a fresh normal culture medium for 46 h at 37°C with

5% CO_2. The culture time was set such that the highest transfection efficiency could be obtained as

described in a previous study [19].

2.5. AFM stiffness measurement

Stiffness measurement for the focal molecular complex using AFM (NanoWizard 3, JPK) was

performed on cells cultured for more than 2 h. The AFM probe approached the cell surface at 1 μm/s

by extending a piezo actuator with a constant indentation force ($F = -100$ pN) using a feedback control

system (Fig. 1 A, black). Then, the probe and the cell surface were kept in contact for different contact

time T [s] (0 s, 5 s, 10 s, 15 s, 20 s, 25 s, and 30 s) (Fig. 1 B). After a contact time T had passed, the

probe was pulled away from the cell surface at 1 μm/s by retracting the piezo actuator (Fig. 1 A,

purple). Accordingly, a force against piezo height curve (i.e. force curve) was obtained. For each

contact time T, force curves were obtained from 8–10 cells. A 25-point grid was drawn on the cell

within an area of 10 μm × 10 μm, and measurements were taken at each point on the grid.

To determine the stiffness of the complex, the force curves were analyzed using JPKSPM Data Processing (JPK). The probe tip height was determined from the piezo height, force and its spring constant. The tilt of the curves caused by thermal drift was corrected by subtracting the slope of the curves after rupture. Then, the force after rupture was set to $F = 0$ as the origin. The origin of extension ΔL [nm] was defined as the tip height when the force reached 0 pN. After these processes, the force curves (as shown in Fig. 1 C) were analyzed. A positive curve, in which the focal molecular complex was successfully formed, was defined using a threshold of a maximum force F_{max} [pN]. The threshold was determined as $\mu + 2\sigma$ (μ: mean, σ: S.D.) (67.5 pN) at 0 s contact. The maximum force F_{max} was obtained from the curve within 500 nm of ΔL, which is several times as long as the length of the complex (~100 nm) [3]. The stiffness of the focal molecular complex was defined as stiffness K [pN/nm] that is the slope of curves (0 nm ≤ ΔL ≤ 100 nm) (Fig. 1 D). For these calculations, the numerical computational software Scilab (INRIA) was used.

3. Results

3.1. Formation of fibronectin–integrin binding

We examined whether fibronectin–integrin binding occurred when the FN probe came into contact with the cells. To confirm the presence of integrin residue on the tip of the post–contact FN probe, TIRFM imaging was conducted. Fluorescence images in Figs. 2 A and B represented fluorescence

spots on the tip of non– and post–contact FN probes, respectively. Fluorescence intensity on the tip of

the post-contact FN probe was higher than that on the non-contact FN probe (Fig. 2 A, B lower),

indicating that integrin was detected on the tip of the post-contact probe. This implies that fibronectin–

integrin binding occurred when the FN probe came into contact with the cells.

3.2. Formation of integrin–talin–actin filament complex

We verified the formation of integrin–talin–actin filament complex inside the cell after the FN

probe came into contact with the cells. To evaluate the complex formation, positive curves, in which

the complex was successfully formed, were obtained based on maximum force F_{max} following the two

procedures discussed: First, a threshold of maximum force F_{max} was defined as $\mu + 2\sigma$ (μ: mean, σ:

S.D.) at 0 s contact (Fig. 3 A). As contact solely for 0 s is extremely short, the fibronectin–integrin

binding did not occur, and hence, the complex could not be formed. The positive curve was then

defined as a curve in which the maximum force F_{max} exceeded the threshold. The complex formation

was evaluated as a time-dependent variation in proportion of positive curves to total force curves.

For intact cells, the proportion of positive curves was linearly increased as fitted by the black line

($p = 0.0033$, Fig. 3 B), which we confirmed that the complex was successively induced by interaction

with fibronectin. We then compared the proportion of intact and talin knocked-down cells to verify the

formation of the talin–containing molecular complex on the intact cells. The proportion for talin

knocked-down cells (Fig. 3 B, gray bar) was evidently lesser than that for intact cells. This result

indicates that talin was present in the molecular complex formed inside the intact cells. Moreover, we tested whether the complex formation was triggered by fibronectin-integrin binding. For the negative control group, the proportion for BSA probe (Fig. 3 B, white bar) was evidently lesser than that for FN probe, indicating that fibronectin–integrin binding was required for the complex formation. Taken together, these results imply that the integrin–talin–actin filament complex was formed after the probe-cell contact.

3.3. Increase in stiffness K requiring talin

To investigate the effect of talin on the stiffness of nascent focal molecular complex, stiffness K (Fig.1 D) was measured for each contact time T on the intact cells and talin knocked-down cells (Fig. 4). Regarding the intact cells, stiffness K for 10-to-30-s contact significantly increased compared with that for 5 s contact (Two-way ANOVA test followed by Tukey-Kramer test, $p < 0.001$). Stiffness K for talin knocked-down cells, in contrast, was less than that for the intact cells (Two-way ANOVA test, $p < 0.001$). These data demonstrated that talin was required to increase the stiffness of nascent focal molecular complex.

4. Discussion

Our novel method using AFM stiffness measurements first revealed that talin was required to increase the stiffness of the complex in its early process. The maturation of focal molecular complex

has a major influence on cellular adaptation to the mechanical environment [20–22]. A previous

research suggested that the maturation of the nascent complex depends on the reinforced complex-

actin filament connection [4]. This reinforcement is thought to enhance a force transmission function

of the complex to open stretch-activated ion channels [5] and to send biochemical signals via the

deformation of cytoskeleton [6], leading to the maturation of the complex. Although the stiffness of

the complex plays a central role on the force transmission, previous studies have not focused on the

stiffness of the nascent complex. This study first reported the involvement of talin in the increase in

the stiffness of the complex during its early formation process.

A previous study suggested that talin led to the reinforced connection between nascent focal

molecular complex and actin filament via its conformational changes. The molecular dynamics

simulation demonstrated that a talin rod undergoes conformational changes to expose hidden vinculin

binding sites under external tensile force [14]. In addition, an *in vitro* molecular experiment suggested

that the talin-actin complex binds to vinculin by applying tensile force on the complex [15]. Moreover,

a cellular experiment indicated that vinculin was colocalized with talin in a stretched cell [16]. These

studies suggested that the connection between focal molecular complex and actin filament could be

reinforced, by connecting more actin filaments to talin via vinculin [23,24]. In our experiment, talin,

vinculin and actin began to localize in the adhesion area within 10 s which is consistent with the

observations made in previous studies [4,9]. Taken together, the focal nascent complex-actin filament

connection in our experiment was probably reinforced after conformational changes of talin molecule.

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To directly reveal the structural changes of the complex involving talin, vinculin and actin, their behavior should be visualized when the AFM probe comes into contact with the cells. By recruiting fluorescence microscopy and fluorescence probe in the AFM system, the localization of these proteins, conformational changes of the talin molecule and talin-vinculin binding can be visualized during the contact [25]. This helps verify the relation between the behavior of the proteins and stiffness of the complex. Thus, when exploring further studies, the introduction of a fluorescence system to our current AFM system will reveal the dynamics of the components underlying our results. Furthermore, the coupling of AFM to a fluorescence system will investigate modulation of force transmission by increase in the stiffness of the nascent complex and effects of the subsequent biochemical signaling on the complex maturation.

The AFM stiffness measurement for the nascent molecular complex is expected to help determine how its components affect various cell activities. For instance, the nascent focal molecular complex during cell migration generates more traction force than a matured complex [26]. During the development of the growth cone of a neuron, the cadherin-based nascent complex also generates the traction force [27]. As another cell activity, osteocyte senses the external force via the integrin-based molecular complex on narrow cell processes [28]. In cell behaviors represented above, the stiffness of the focal complex is thought to contribute to the force transmission for the cell traction or mechanotransduction. Moreover, the force transmission varies depending on the component proteins of the complexes (e.g. integrin β1 or β3) because each protein exhibits a different binding strength

[29,30]. Employing AFM stiffness measurement helps better understand the cell activities regarding

the force transmission function generated by different complexes components.

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Figure/Table Legends

Fig. 1 AFM stiffness measurement. **(A)** Schematic of AFM stiffness measurement. The AFM probe approached the cell surface by extending a piezo actuator. After the probe-cell contact, the probe was pulled away by retracting the actuator. **(B)** The contact state was retained using constant force (F = -100 pN) for contact time T [s]. **(C)** Typical force curve. The intersection point represents the origin of the curve. The origin of force F was determined as force after rupture. The origin of extension ΔL was defined as the tip height when force reached 0 pN. **(D)** Maximum force F_{max} [pN] was measured within 500 nm of ΔL. Stiffness K [pN/nm] was calculated by linear approximation of the curve (0 nm $\leq \Delta L \leq$ 100 nm).

Fig. 2 Integrin β1-fibronectin binding. Fluorescence images of **(A)** non- and **(B)** post-contact (contact time T = 30 s) FN probe. The fluorescence intensity in the both images was profiled along with the red lines. The yellow lines indicate the outline of cantilevers. The blue and red dashed lines indicate the position of the probe.

Fig. 3 Formation of integrin-talin-actin filament complex for contact time T. **(A)** Time-dependent variations in maximum force F_{max} [pN] for intact cells. Black points indicate the mean. Error bars indicate S.D.. A positive curve was defined as the force curve, in which maximum force F_{max} excesses μ + 2σ (μ = mean, σ = S.D.) at 0 s contact. **(B)** Proportion of positive curve to total curves was shown

with respect to each contact time T for intact cells, talin 1 knocked-down cells and BSA probe. Linear regression analysis indicated that the proportion for intact cells significantly increased with contact time T ($p < 0.01$).

Fig. 4 Increase in stiffness K requiring talin. Stiffness K for intact and talin 1 knocked-down cells was plotted with respect to each contact time T. On the right side of the plots, mean and S.D. are shown. ANOVA test indicated that talin 1 knock-down had a significant effect on stiffness K ($p < 0.001$). Tukey-Kramer test indicated that stiffness K for 10-to-30 s contact was significantly larger than that for 5 s contact in AFM stiffness measurements on intact cells (* $p < 0.001$).
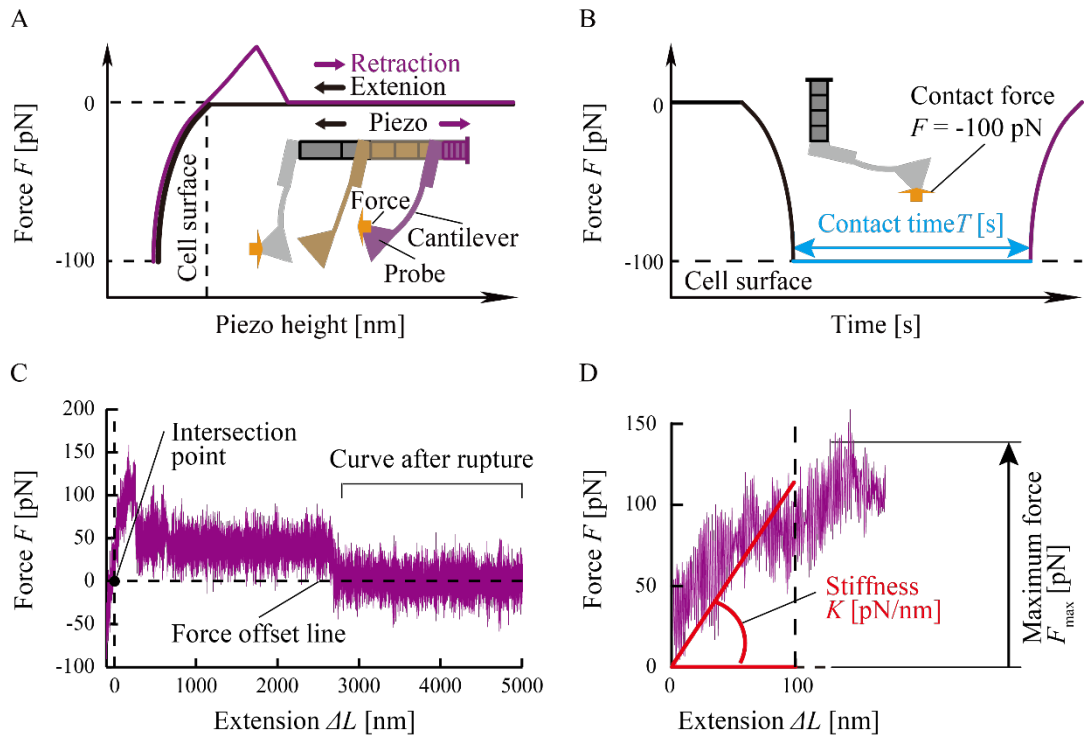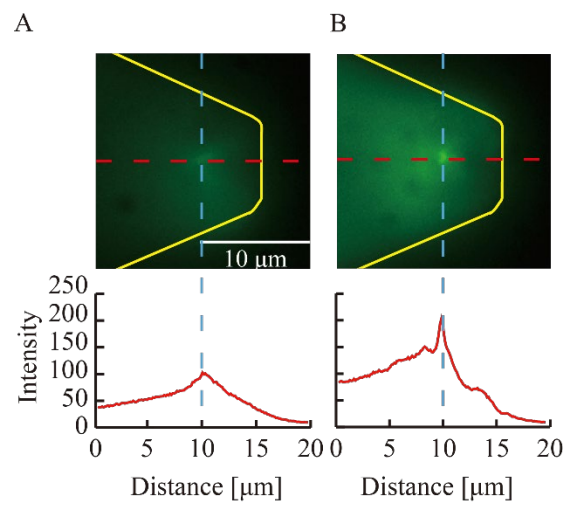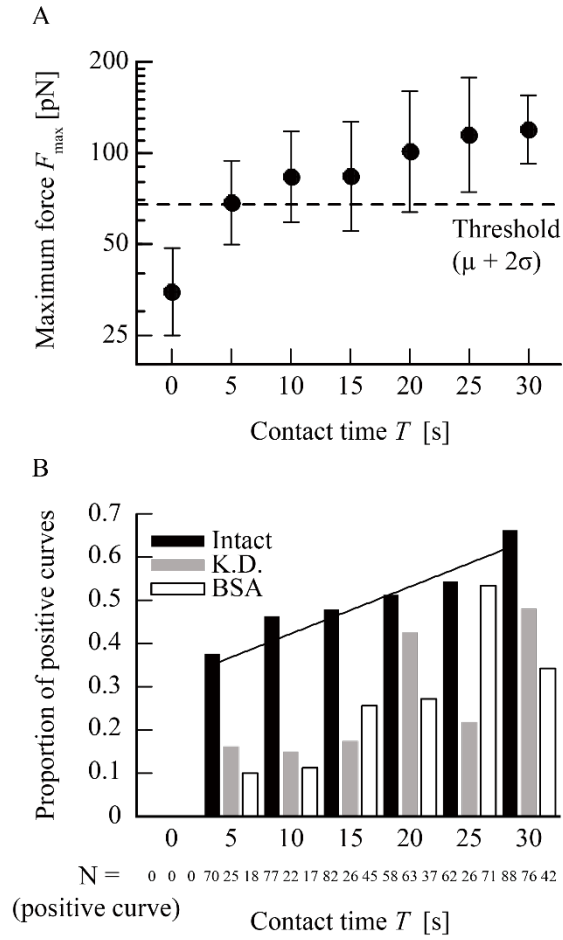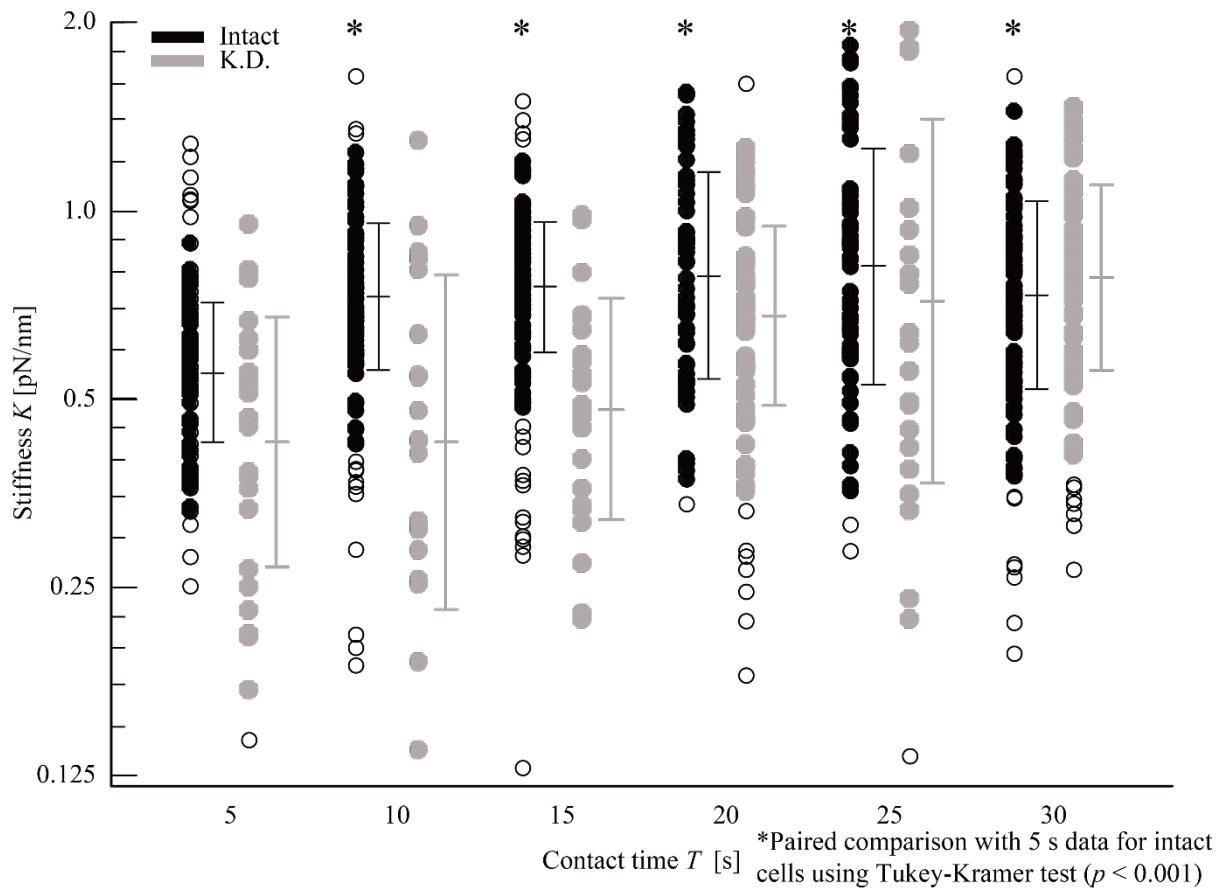
Fig. 1

Fig. 2

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