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# Intracellular stress transmission through actin stress fiber network in adherent vascular cells

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**Abstract:** Intracellular stress transmission through subcellular structural components has been proposed to affect activation of localized mechano-sensing sites such as focal adhesions in adherent cells. Previous studies reported that physiological extracellular forces produced heterogeneous spatial distributions of cytoplasmic strain. However, mechanical signaling pathway involved in intracellular force transmission through basal actin stress fibers (SFs), a mechano-responsive cytoskeletal structure, remains elusive. In the present study, we investigated force balance within the basal SFs of cultured smooth muscle cells and endothelial cells by (i) removing the cell membrane and cytoplasmic constituents except for materials physically attaching to the substrate (i.e., SF–focal adhesion complexities) or (ii) dislodging either mechanically or chemically the cell processes of the cells expressing fluorescent proteins-labeled actin and focal adhesions in order to examine stress-release-induced deformation of the basal SFs. The result showed that a removal of mechanical restrictions for SFs resulted in a decrease in the length of the remaining SFs, which means SFs bear tension. In addition, a release of the preexisting tension in a single SF was transmitted to another SF physically linked to the former, but not transmitted to the other ones physically independent of the former, suggesting that the prestress is balanced in tensed SF networks. These results support a hypothesis regarding cell structural architecture that physiological extracellular forces can produce in the basal SF network a directional intracellular stress or strain distribution. Therefore, consideration of the coexistence of the directional stretching strain along the axial direction of SFs and the heterogeneous strain in the other cytoplasmic region will be essential for understanding intracellular stress transmission in the adherent cells.

**keyword:** Stress fiber, Intracellular stress, Endothelial cell, Smooth muscle cell, Mechanotransduction.

## 1 Introduction

Mechanical forces inherent in blood vessel such as blood flow or vascular wall stress are experienced by adherent vascular cells, i.e., endothelial cells (ECs) and smooth muscle cells (SMCs) to convert into biochemical and genetic responses (Davies, 1995). Although the exact mechanisms of the adherent cell mechanochemical signal transduction remain elusive, force transmission through cytoskeletal network within the cytoplasm has been implicated in affecting activation of localized mechano-sensing sites such as focal adhesions as a mechanical signaling pathway along with biochemical pathways (Davies, 1998; Helmke et al., 2001, 2003; Davies et al., 2003; Ingber, 2004; Hu et al., 2003, 2004).

To elucidate the intracellular stress-transmission-pathway, cytoplasmic strain due to extracellular forces has been measured using fluorescent proteins-tagged fiducial markers (Helmke et al., 2003; Hu et al., 2003). Helmke et al. (2001, 2003) studied displacements of vimentin intermediate filaments in ECs exposed to unidirectional fluid shear stress and then calculated cytoplasmic strain field. Also, He et al. (2003) examined movement of mitochondria, which is linked to microtubules, in SMCs subjected to a controlled external mechanical stimulus. In those experiments, heterogeneous spatial distributions of the displacement and the strain were detected. It is, however, uncertain to what degree such intermediate filament- or mitochondria-displacement and strain are actually associated with force transmission over the cytoplasm because (i) the strain fields were not calculated on the basis of the stretching or compressing strain of the cytoskeletal filaments themselves, and also (ii) the tracked organelles

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will be easily deformed or bent (Kurachi et al., 1995; Haga et al., 2000; Coughlin and Stamenovic, 2003), either of the reasons indicating in terms of structural mechanics that the deformation or the strain field based on those analyses can be produced without substantial force transmission, as would a crumpled soft paper.

We speculated that another strain field with a directional preference may coexist with the heterogeneous and non-directional strain field in the cytoplasm, and that the directional strain field may be actually related to substantial intracellular force transmission because such directional mechanical factors would be critical to explain how cells can sense the direction of externally applied forces. More specifically, conversion of changes in mechanical strain or tension in subcellular components with a directional preference into biochemical responses will be available for detecting the force direction (Naruse et al., 1998; Li et al., 2002).

With respect to this point, stress fiber (SF), which is a contractile bundle of actin filaments, will be a good candidate for force direction sensor because of their following well-known configuration and behaviors: (i) SFs often run between distant portions, i.e. focal adhesions that is an anchorage to the substrate or apical focal adhesion-like complexities (Kano et al., 2000), (ii) traction force measurements (Balaban et al., 2001; Tan et al., 2003) implicated there is isometric tension of $\sim$10 nN in SFs located along the bottom of adherent cells, the magnitude of which is much greater than the microtubules' ability to bear force (i.e., $\sim$1 pN) (Kurachi et al., 1995), (iii) SFs exhibit force direction-dependent dynamics (Davies, 1995; Naruse et al., 1998; Li et al., 2002; Kano et al., 2000; Byers and Fujiwara, 1982), and (iv) adherent cells exhibit anisotropic mechanical properties depending on the direction of SFs (Hu et al., 2004). These experimental results suggest a hypothesis that there is a stretching strain along the axial direction of each SF, which is different from the heterogeneous strain field in the other cytoplasmic region (Helmke et al., 2003; Hu et al., 2003), and physiological forces produce a direction-dependent change in the strain or tension level, leading subsequent directional responses.

So far, force transmission via the SFs or actin bundles running across the apical and the basal cell membrane or the nucleus membrane has been investigated (Hu et al., 2003, 2004; Maniotis et al., 1997). However, most of such vertically transverse SFs might be developed owing to the application of antibodies-coated beads onto the cell surface; therefore, it is unclear whether the cell behaviors embody a physiologically essential force transmission. In fact, SFs are rich along the basal portions of the cytoplasm in intact cells (Kano et al., 2000); yet, the force transmission among the basal SFs remains elusive despite potential physiological significance.

Here, we studied in two parts the role of basal SFs in cultured SMCs and ECs as an intracellular stress-bearing component. First, we identified the presence of preexisting tension in single SFs themselves by removing the cytoplasmic constituents except for the SF–focal adhesion complexities with chemical treatments. If the cytoplasm works as mechanical restrictions for SFs, and accordingly the SFs are initially being tensed even in statically cultured cell, then the length of the SFs would decrease after the removal of the surrounding materials, as would a tensed violin string. We found that the length of SFs was reduced after the removal of the cytoplasm in an ATP-independent manner. Second, a part of the preexisting strain was released by mechanically or chemically dislodging the cell processes that contain basal SFs to examine how the released tension was transmitted over the cytoplasm and specify the subcellular components bearing the tension. We observed several SFs formed an interconnected network, and a released tension was only transmitted to the SFs connected to the dislodged SF but not to non-associated SFs in single cells. These behaviors are consistent with the hypothesis regarding the cell structural architecture that extracellular forces can produce in the basal SF network a directional intracellular stress or strain distribution.

## 2 Materials and Methods

### *2.1 Cell preparation*

SMCs and ECs were obtained from bovine thoracic aortas according to the reported techniques (Shasby and Shasby, 1987; Yoshida et al., 1988). Cell culture was done in Dulbecco's modified Eagle medium (Invitrogen, NV Leek, The Netherlands) containing 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS) and 1% each of penicillin and streptomycin in an incubator at 37 $^\circ$C and 5% $CO_2$/95% air. Passages 5–10 SMCs and 5–12 ECs were seeded in 35-mm-diameter glass base culture dishes (Asahi Techno Glass, Tokyo, Japan) for experiments. Cells were co-transfected

with pEGFP-actin vector (GFP-actin, Clontech, Palo Alto, CA) and pdsFP593-focal adhesion targeting (FAT) vector (RFP-FAT, a gift of Dr. N. Mochizuki, National Cardiovascular Center, Japan) using a liposomal method according to the manufacturer's protocol (Invitrogen). FAT is an amid acid sequence at C-terminus of focal adhesion kinase (Hildebrand et al., 1993). Cells (∼50% confluence) were incubated with a mixture of the DNA plasmids, Lipofectamin (Invitrogen), and Plus Reagent (Invitrogen) in a serum-free medium (Opti-MEM, Invitrogen) for 5 h. After the mixture was replaced with DMEM, cells were incubated at 37$^o$C in a humidified 5% $CO_2$ atmosphere overnight. Cells transiently expressing GFP-actin and RFP-FAT were used for experiments 1 or 2 days after the transfection.

### 2.2 *Observation of stress fiber extraction*

The dish with the GFP-actin- and RFP-FAT-co-expressed cells was fixed on a stage of an inverted microscope (IX-70, Olympus, Tokyo, Japan). Chemical treatments described below were carried out to extract SFs. The cells were washed with a buffer consists of 10 mM imidazole (Wako, Osaka, Japan), 100 mM KCl, and 2 mM EGTA (Wako) (pH 7.2) (Katoh et al., 1998). The cells were then incubated in the same buffer with 25 $\mu$g/ml saponin (Wako) for 8 min at 37$^o$C to wash away intracellular ATP and cations (Pourati et al., 1998), which induce actomyosin-based contraction of SFs (Katoh et al., 1998). After washing the cells three times with a $Ca^{2+}$- and $Mg^{2+}$-free PBS including 1 $\mu$g/ml each of leupeptin (Wako) and pepstatin (Wako) and cooled to 4$^o$C before use, SFs were extracted according to the previously reported technique (Katoh et al., 1998). Briefly, the cells were treated with an ice-cold low-ionic-strength extraction solution consisting of 2.5 mM triethanolamine (Wako) and 1 $\mu$g/ml each of leupeptin and pepstatin in distilled water. The extraction solution was changed ∼10 times for 20–30 min. Extracted cells were then treated with 0.05% ice-cold Nonidet P-40 (NP-40, Wako) in the PBS (pH 7.2) for 5 min. The cells were squirted by a gentle stream of the PBS with 0.05% ice-cold Triton X-100 (Wako) (pH 7.2) using a pipette under phase-contrast microscopy to remove the apical and the basal membranous portions of cells. SFs, which were extracted up to this point and still attached to the dish, were then washed gently with the PBS ∼5 times.

Fluorescence images of both SFs and FATs (or focal adhesions) were obtained by using a digital CCD camera (C4742-95ER, Hamamatsu Photonics, Hamamatsu, Japan) whenever the extraction solutions were changed. The medium was replaced by fresh ones several times to maintain pH. The change in the lengths of SFs during the chemical treatment processes was determined by tracing manually peripheral thick SFs (given by green color) in the images from one end FAT to the other end FAT (given by red color points) with the NIH image software.

### 2.3 *Cell dislodging*

To observe the movements of SFs during cell retraction, focal adhesions that work as an anchor to the ground were dislodged from the substrate surface with either mechanical or chemical way. The dish with the cells expressing the fluorescent proteins was fixed on the stage of the inverted microscope for experiments.

In mechanical dislodging, a fine glass needle (∼2 $\mu$m in tip diameter) was made by drawing a glass capillary tube with a glass-electrode puller and placed on a gripper of a hydraulic micromanipulator (MMO-203, Narishige, Tokyo, Japan) before experiments. Immediately prior to experiments, cells were incubated with a buffer consists of 10 mM imidazole, 100 mM KCl, 2 mM EGTA, and 25 $\mu$g/ml saponin (pH 7.2) for 8 min at 37$^o$C to wash away intracellular ATP and cations. Under illuminations from halogen and mercury light sources, the fine glass needle was placed underneath the basal surface of the cell. Focal adhesions, given by the red color fluorescence markers, under long cell processes extending from the cell body were rapidly dislodged by displacing slightly the glass needle, similar with the previously reported technique (Pourati et al., 1998; Albrecht-Buehler, 1987). The dislodged cell process was retracted toward the cell center, and then the fluorescence images were taken by the CCD camera.

In chemical dislodging, 0.05% trypsin-EDTA (Wako) was added to the cells in the PBS with a pipette at 37$^o$C to disconnect the focal adhesions from the substrate. Time-lapse images were acquired at every 1 s under the trypsinization by using a confocal laser scanning microscopy system that consists of an argon ion laser sources (Model 532-BS-AO4, Melles Griot, CA), a 60x/1.45NA Plan Apochromat objective lens (Olympus), the CCD camera, a confocal scanning unit (CSU-10, Yokogawa, Japan), and image analysis software (IPLab, Scanalytics, VA).

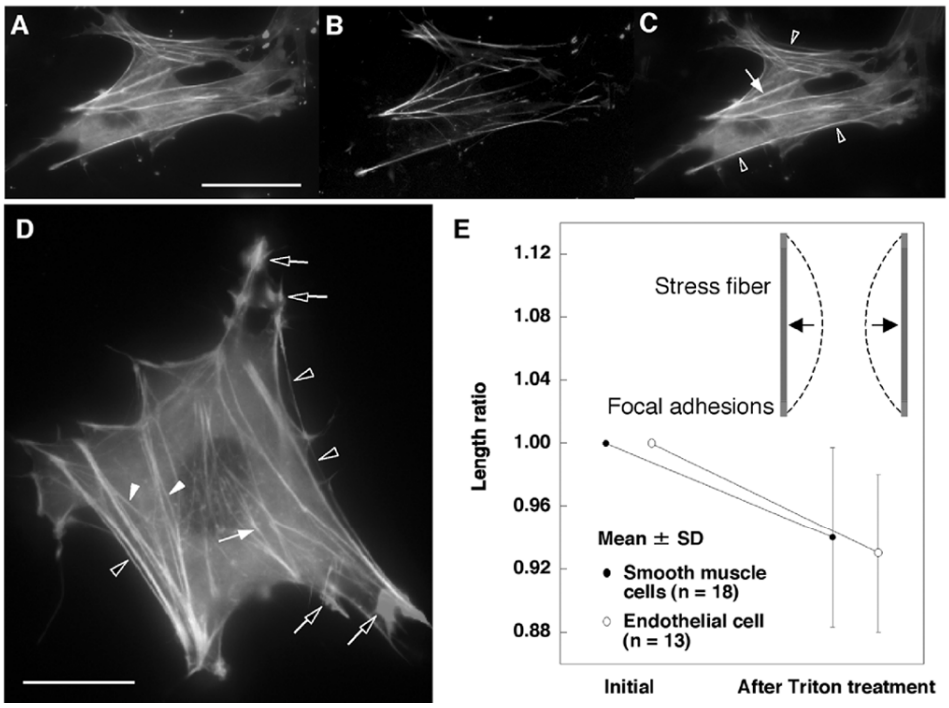


**Figure 1** : Preexisting strain of single SFs. See the color illustrations in the on-line version. (A and B) An SMC before (A) and after (B) the extraction treatments. GFP-labeled actin and RFP-labeled FAT were pseudocolored green and red, respectively. (C) A superimposed image of GFP-actin images in A (pseudocolored green) and B (pseudocolored red). The pixels show yellow when green and red overlap. Note that thick and originally inward-curved SFs (arrowheads) displaced outward from the cell center after the cytoplasmic constituents were removed. Originally straight SFs (arrow) did not deform. (D) A similar result with C showing superimposition of GFP-actin images of an SMC acquired before (pseudocolored green) and after (pseudocolored red) the extraction treatments. Note that some SFs (open arrowheads) were removed after the treatment, and thick SFs (closed arrowheads) pivoted outward. The positions of focal adhesions located at the terminal of SFs remained unchanged as shown by yellow color (see text for details). Scale bars = 20 $\mu$m. (E) Changes in the length ratio at the initial and just after the Triton treatment. The schematic diagram illustrates exaggeratedly a typical morphological change of SFs, i.e., the removal of cytoplasmic constituents resulted in a deformation from initially inward-curved morphology into a relatively straight line.

### *2.4 Electron microscopy*

Transmission electron microscopy was performed to observe the cells including SFs in the planes. The cells were fixed with fresh 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.5% tannic acid in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h at 4 °C, rinsed with 0.1 M sodium cacodylate buffer for 1 h at 4 °C, scraped off from the dish, post-fixed with 1% osmium tetroxide in the same buffer for 2 h at 4 °C, dehydrated using an ethanol series (60, 70, 80, 90 and 100%) with two changes of 30 min at each concentration, infiltrated with QY-1 (n-butyl glycidyl ether), embedded in Epon812, and cut with ultra-microtomes (Reichert Jung, Germany). Ultra-thin sections from selected blocks were mounted onto copper grids, stained with aqueous uranyl acetate and lead citrate, and observed by a transmission electron microscope (H-7100, Hitachi, Japan) at an accelerating voltage of 75 kV.

## 3 Results

### *3.1 Observation of extraction process of SFs*

The process of the extraction of basal SFs was observed in GFP-actin- and RFP-FAT-co-expressed cells. The NP-40 and Triton treatment removed the cell membrane and some cell organelles (Fig. 1A and B; see the color illus-

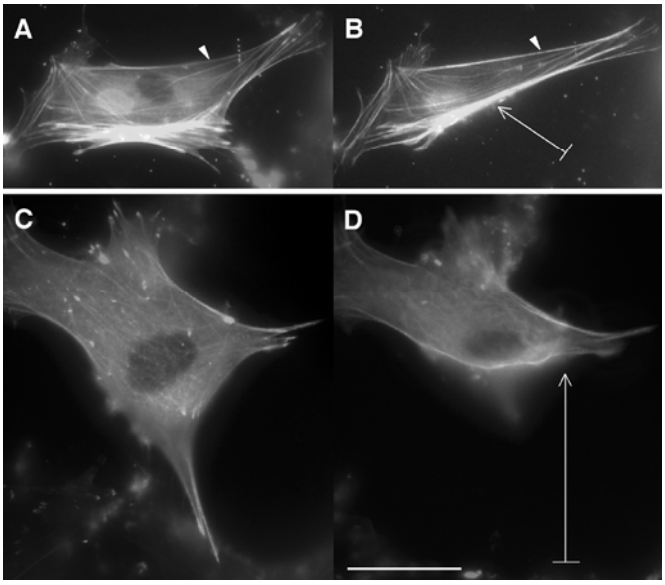


**Figure 2** : Retraction of GFP-actin (pseudocolored green)/RFP-FAT (pseudocolored red)-co-expressed SMCs after local detachment at terminals of SFs contained in the cell processes. See the color illustrations in the on-line version. (A–D) Detached focal adhesions were moved toward the cell center until they reached the straight line between non-detached focal adhesions (B) or the nucleus (D). Arrows mean extent of focal adhesion displacements. The local detachment was often followed by the similar deformation into linear shape of another SF that located at the other edge of the cell (arrowheads). Scale bar = 20 $\mu$m.

trations of Fig. 1 in the on-line version). We confirmed in separate fluorescence microscopy that EGFP-tubulin (Clontech)-detected microtubules, SYTO 13 (Molecular Probes, Eugene, OR)-detected nuclei, and Calcein-AM (Molecular Probes)-detected other cytoplasmic constituents such as mitochondria were removed almost entirely from the cells in the SF extraction process. In contrast, basal SFs were still attached to the substrate surface and clearly seen even after the chemical treatments. The superimposition of GFP-actin images before and after the treatments showed that shapes of a few SFs (Fig. 1C, arrowheads) changed from an inward-curved arch (green color) to a relatively straight line (red color). A similar result (Fig. 1D) showed that when the cell membrane was disrupted and the cytoplasmic constituents were flown away, some SFs (Fig. 1D, open arrowheads, green color), which cross-link thick SFs, were also removed together with the cell membrane or disappeared probably because they were mechanically severed. The thick SFs, meanwhile, still stayed on the substrate because they firmly attached there and were not severed when the cytoplasmic constituents were flown away. Some of such fine and fragile actin bundles or actin lattice were detached at one end or entangled around focal adhesions (Fig. 1D, closed arrows, red color). As a consequence, a number of the thick and originally inward-curved SFs (Fig. 1C and D, closed arrowheads) pivoted outward from the cell center around the focal adhesion sites located at the both ends while the positions of the both ends remained unchanged, and the configurations approached straight lines (Fig. 1E, schematic diagram showing a typical morphological change of SFs). In contrast, originally straight SFs did not change the curvature unless their end focal adhesions were detached (Fig. 1C and D, open arrows). We confirmed the similar behaviors were observed in an enzyme-freed cell dissociation buffer (Invitrogen) containing a $Ca^{2+}$chelater at 37 $^{o}$C instead of the PBS. We conducted the experiments for fifteen SMCs and eight ECs and observed the similar results for every cell. Significant differences in the SF shape changes were not found between the cultured SMCs and ECs.

We examined how much the length of the SFs was changed before and after the shape change due to the removal of the cytoplasmic constituents. We measured the length ratio defined as the length after the chemical treatments divided by its original length. After the Triton treatment, the length ratio became 0.94 $\pm$ 0.057 for SMCs (mean $\pm$ SD, n = 18 SFs) and 0.93 $\pm$ 0.05 for ECs (mean $\pm$ SD, n = 13 SFs) (Fig. 1E), indicating that the SFs were being stretched in the intact cytoplasm with, on averagFe, $\sim$6.4% (= (1 – 0.94) / 0.94 x 100) stretching strain magnitude for SMCs and $\sim$7.5% strain for ECs.

### *3.2 Cell retraction by mechanical manipulation*

The leading edge of long cell processes extending from adherent cells was dislodged from the substrate by manipulating the glass needle. Detached focal adhesions moved toward the cell center in $\sim$10–60 s until they reached on the straight line between non-detached focal adhesions (Fig. 2A and B; see the color illustrations of Fig. 2 in the on-line version) or around the nucleus (Fig. 2C and D). The local detachment was often rapidly followed by the similar deformation into linear shape of another SF that located at the other edge of the cell (Fig.

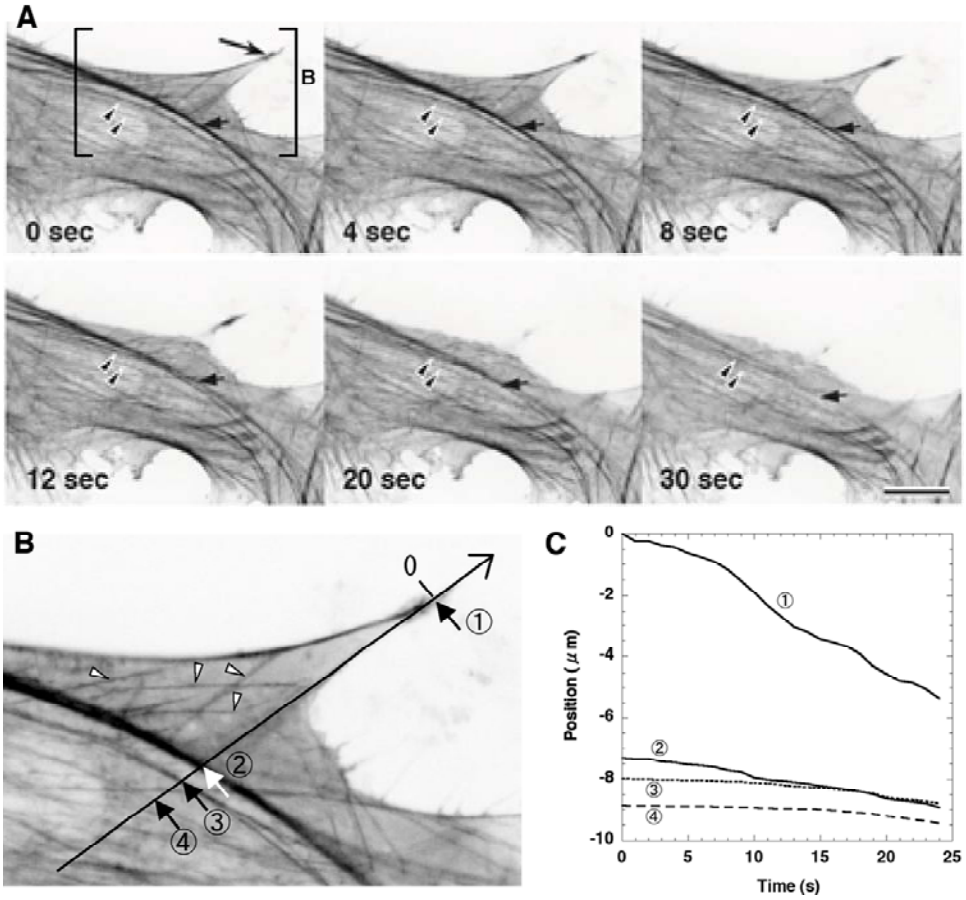


**Figure 3** : Retraction of a GFP-actin-expressed SMC with a treatment of trypsin. (A) Time-lapse confocal images of the detachment of focal adhesions (long arrow) under the trypsinization. The color was inverted. The tension in a thick SF (short arrow) was partly relaxed owing to the relaxation of tensions in the cross-linking thin SFs. The thick SFs displaced toward the cell center to a greater degree than the other adjacent SFs (arrowheads). In 30 s, the thick SFs passed by the adjacent SFs. Scale bar = 10 $\mu$m. (B) A magnified view of a part of A at 0 s. A number of apparently thin actin bundles (arrowheads) cross-link the thick SFs (arrow 1 and 2). (c) Time course of SFs displacement. SFs physically associated with each other (arrow 1 and 2 in C) displaced analogously, different from the other non-connected SFs (arrow 3 and 4 in C), suggesting tension transmission among the interconnected SFs.

2A and B, arrowhead). We conducted the experiments for eight SMCs and ten ECs, and the similar behaviors were observed for every cell. Significant differences in the cell behavior were not found between the cultured SMCs and ECs.

### *3.3 Cell retraction by trypsinization*

Time-laps fluorescence images of cells under trypsinization showed that the cells changed their shape in ∼20–60 s while SFs seemed disappeared or buckled (Fig. 3A). The disappearance of SFs was probably caused by the actin depolymerization or deviation from the focal plane (Sato et al., 2005). When a focal adhesion (Fig. 3A, a long arrow) that located at the end of a thick SF cross-linked to another thick SF (Fig. 3A, a short arrow) through thin SFs (Fig. 3B, arrowheads) was detached, the two thick SFs approached each other. Then, one of the thick SFs (Fig. 3A, the short arrow), whose tension was relaxed in part because of the relaxation of the tension in the cross-linking thin SFs, displaced toward the cell center to a greater extent compared to the other adjacent SFs (Fig. 3A, arrowheads; Fig. 3B). To show the behavior clearly, changes in the positions of four selected SFs (Fig. 3B, arrows) in the acquired images were computed by using image analysis software (IMAQ Vision with LabVIEW 7 Development System, National Instru-

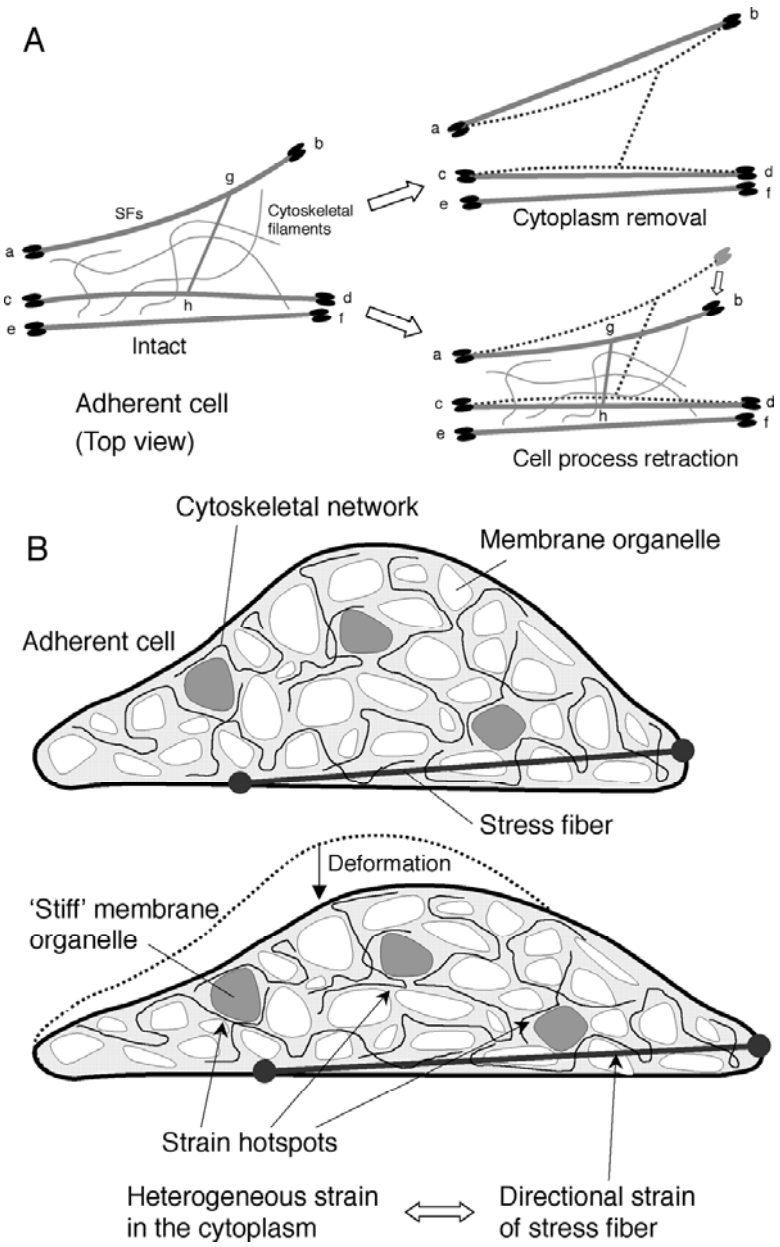


**Figure 4** : A model of intracellular force balance. (A) Force balance within SF network. Interconnection of SFs produce an inward-curvature of the SF morphology (a–b and c–d) while an independent SF (e–f) shows a straight shape between separate focal adhesions. Removal of cytoplasmic constituents causes mechanical cutting of relatively thinner SF (g–h), followed by a deformation of the remaining SFs into straight shapes owing to preexisting tension in the SFs. Local detachment of a cell process (b) causes the similar release of tensions in the interconnected SFs (a–b and c–d), while the non-connected SF (e–f) remains staying as they had been. (B) A probabilistic model of adherent cell at static (upper) and deformed (lower) states. Cell deformation due to shear stress or hydrostatic pressure will produce a heterogeneous cytoplasmic strain field determined by the organelles' stiffness since the existence of relatively stiff organelles causes local increase in the strain level around them or the surrounding cytoskeletal filaments such as intermediate filaments. Meanwhile, the existence of SFs, which are terminated at focal adhesions and generate isometric tension, will produce a directional strain in the cytoplasm.

ments, Austin, TX). The time course change (Fig. 3C) showed that mutually associated SFs (Fig. 3B, arrow 1 and 2) by virtue of the cross-linking actin bundles (Fig. 3B, arrowheads) were displaced analogously toward the cell center just after application of trypsin. Meanwhile, the other SFs (Fig. 3B, arrow 3 and 4), which were likely to be mechanically independent of the former SFs (Fig. 3B, arrow 1 and 2), were displaced after a lapse of $> 10$ s just for the whole cell rounding, indicating that the tensions in the former SFs were not transmitted to the latter (Fig. 3B, arrow 3 and 4). The similar behaviors were observed in four SMCs.

## 4 Discussion

The most significant finding of the present study is that removal of cytoplasmic constituents except for the materials physically connecting to the extracellular anchor (i.e., SF–focal adhesion complexities) resulted in a decrease in the length of the SFs remaining on the substrate, demonstrating the presence of preexisting stretching strain of single SFs (Fig. 4A, cytoplasm removal). In addition, the release of the prestress in a single SF was transmitted to another SFs physically linked to the former, but not transmitted to the other SFs physically independent of the former, suggesting that the preexisting tensions are balanced in tensed SF networks (Fig. 4A, cell process retraction). These results support the hypothesis regarding the cell structural architecture that extracellular forces can produce in the SF network a directional intracellular stress or strain distribution, which is different from the heterogeneous and non-directional distribution observed in the other cytoplasmic region (Helmke et al., 2003; Davies et al., 2003).

### *4.1 Preexisting strain of SFs*

SFs are anchored at the cell base to the underlying extracellular matrix through focal adhesions. Thick SFs or dense peripheral band (Wong and Gotlieb, 1986), which often locates around the cell periphery, will attach to the substrate firmly, and therefore most of them remained staying on the substrate even when the other cytoplasmic constituents including the cell cortex were disrupted or flown away during the chemical treatments (Fig. 1). On the other hand, just after the removal of the cell membrane and cytoplasmic constituents by the NP-40 treatment, some of the apparently thinner SFs were severed at somewhere along the fibers or detached from the substrate probably because of their weaker strength or adhesion to the ground compared with that of the thicker ones. The thick SFs had originally inward-curved shapes. The removals of the cell membrane etc. caused a change in the force balance among the remaining SFs, resulting in that the SFs pivoted outwards from the cell center around each end, and the length of the SFs shortened to reduce their mechanical strain energy (Fig. 1; Fig. 4, cytoplasm removal). This shortening in the extraction process occurred in the absence of ATP, i.e., the shrink was not based on the actomyosin contraction although the original preexisting tension would be generated by the actomyosin contraction. It is therefore likely that the SF shape change occurred because the removed cell components had worked as mechanical restriction for SFs. From a mechanical viewpoint, this result supports the hypothesis that SFs were being stretched to have preexisting tension in the adherent cells.

Pourati et al. (1998) reported that the adherent ECs, which were dissected across the cytoplasm with a microneedle, retracted to $\sim$30% of the initial length. The retraction was prevented by treatment with cytochalasin D, an inhibitor of the actin polymerization, suggesting that preexisting strain in actin lattice may be present in adherent cells and be essential for the retraction. In the present study, by contrast, the SFs shrank to $\sim$94% of the initial length when the restrictions were partly released, which would not be enough to induce such a great retraction. The difference between the Purati's and the present shortening magnitudes is due to a variety of reasons, e.g. (i) focal adhesions might bear considerable amounts of preexisting tension in actin-related structure (Ingber 2005; Balaban et al., 2001; Tan et al., 2003; Wang et al., 2001), (ii) all of the SF-interconnecting actin bundles were not severed or removed in the present experiment, and (iii) the surface tension in the cell membrane, which would work to minimize the surface area, might contribute to a part of the cell rounding in the Pourati's experiment.

In order to confirm the role of focal adhesions in bearing intracellular stresses, ATP-free and GFP-actin-/RFP-FAT-co-expressed cells were mechanically dislodged from the substrate (Fig. 2). The detached and freely movable focal adhesions were displaced toward the cell center together with the associated SFs, which ceased to move when their shapes became straight between the distant focal adhesions that still remained to attach to the

ground at the ends (Fig. 2A and B). The movement of the dislodged SF was prevented when it hit an obstruction that is the nucleus before reaching straight shape (Fig. 2C and D). These behaviors will be accounted for by that focal adhesions bore a considerable amount of preexisting tensions in SFs, and the detachment of the focal adhesions resulted in reduction of mechanical strain energy of the SFs until they encountered another mechanical restriction such as the nucleus that will be also able to bear intracellular stresses (Deguchi et al., 2005).

### *4.2 Tension transmission via SF network*

The local detachment of the SFs from the anchors was often rapidly followed by the similar deformation into linear shape of another SF that located at the other edge of the cell (Fig. 2A and B, arrowhead). This behavior may be due to that the release of tension in the SF was transferred to the other SF presumably interconnected with each other. To investigate the point more in detail, the time-lapse confcal microscopy was conducted for observing the cells under trypsinization (Fig. 3). The results showed that the magnitude of SF displacement was not spatially uniform when single cell processes were retracted. More specifically, the tension in an SF (Fig. 3A, long arrow) was clearly transmitted via a number of actin bundles (Fig. 3B, arrowheads) to the associated SF (Fig. 3A, short arrow) but not to non-linked SFs (Fig. 3A, arrowheads). The relative displacements of the SFs were certainly caused by differences in each tension level. The cross-linking actin bundles (Fig. 3B, arrowheads) would play a role in mechanically transmitting tension between the distant SFs and regulating the tension levels. The tension transmission in living cells as well as in extracted SFs (Fig. 1, Fig. 4A) suggests that interconnections of SFs mechanically stabilize the network and work as intracellular stress-bearing components as a whole.

Investigation of such mechanical pathways, i.e. how forces are distributed in the cytoplasm, is particularly inevitable to explain the mechanism by which cells can sense the direction of externally applied force and convert it into polarized responses (Davies et al., 2003; Ingber 2005; Naruse et al., 1998; Li et al., 2002) such that ECs adapt themselves to unidirectional shear stress-imposed condition to elongate and orient in the direction of the flow (Davies, 1995). One approach for revealing the mechanical pathway is to impose a force on to a cell and examine resultant cytoplasmic strain fields by detecting displacements of a targeted subcellular component that bears intracellular force (Helmke et al., 2001; Hu et al., 2003). Helmke et al. (2001, 2003) studied displacements of GFP-labeled vimentin intermediate filaments in ECs subjected to unidirectional fluid shear stress and resultant strain fields assuming that the cells were homogeneous continuum material. The results showed that complex and heterogeneous spatial distributions of the displacement and the cytoplasmic strain fields were detected just after onset of the shear stress. It is, however, uncertain how much such intermediate filament displacement and calculated strain are actually associated with substantial intracellular force transmission because the strain field was not obtained based on the stretching strain of the filaments themselves but on the Lagrangian strain tensor of an area determined from projected intermediate filament images. Also, what intermediate filaments can transmit is probably only tension but almost not bending moment (Wang and Stamenovic, 2000). Accordingly, a part of the strain field in the Helmke's result might reflect a deformation of very soft (Haga et al., 2000) membrane-bound organelles surrounded by intermediate filament and packed in the cytoplasm (Fig. 5, region around black arrow), e.g. mitochondria, which will have a variety of resistances to deformation and boundary conditions (i.e., restriction against displacements) with other subcellular components, thus certainly showing such heterogeneous and non-directional strain distributions by even unidirectional imposition of fluid shear stress or static pressure without substantial intracellular stress transmission (Fig. 4B).

If such non-directional cytoplasmic strain field was the unique strain filed existing in the cytoplasm, it is difficult to explain the mechanism of such directional remodeling of cells in terms of mechanics. In regard to this point, we hypothesized that there is another coexisting cytoplasmic strain field determined by basal actin SFs in addition to the heterogeneous strain field (Helmke et al., 2003; Davies et al., 2003) obtained from the observation of intermediate filaments. Since SFs run almost straight (Fig. 5, arrowheads) between distant focal adhesions with some branches (Fig. 5, arrows) and generate isometric tension (Katoh et al., 1998), the boundary conditions would quite differ from those of other cytoplasmic constituents such as intermediate filaments. Hence, strain of SFs at the cell scale will always develop along the direction of the SFs, and therefore the magnitude of

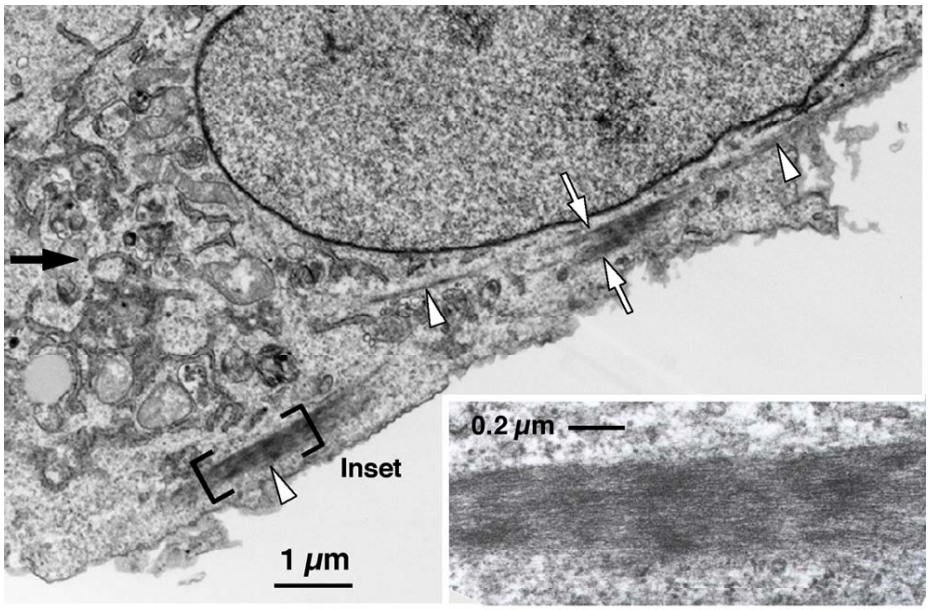


**Figure 5** : Transmission electron micrographs of an EC with SFs (arrowheads) including branching SFs (arrows). The coexistence of packed membrane organelles and tensed SFs supports the proposed cell structural model in Fig. 4. (Inset) A higher magnification view of an SF.

the extracellular force-induced stretching strain will be dependent on the direction of the force (Fig. 4B). This hypothesis is realistic in that (i) reorganization of SFs depends on the direction of fluid shear stress (Davies, 1995), and (ii) stiffness of adherent cells was dependent on the direction of SFs (Hu et al., 2003), either of them suggesting that SFs bear direction-dependent intracellular stresses. We presented the evidence supporting the hypothesis in the present study, which demonstrated intracellular stress transmission in tensed SF network just like a hammock or a spider's web as had been proposed in an intracellualr stress transmission analysis (Coughlin and Stamenovic, 2003). The coexistence of the directional stretching strain along the axial direction of SFs and the heterogeneous strain of the other cytoplasmic region would be the accurate view of adherent cell structure, which will be essential in interpretation of intracellular force transmission.

Quantitative evaluation of the force balance establishing the cell architecture will be the subject of future study. So far, micro fabrication techniques (Balaban et al., 2001; Tan et al., 2003) have allowed measurements of traction forces applied by adherent cells to the substrate at single focal adhesion sites, i.e., how much focal adhesion sites bear tensions. The results confirmed that traction forces of $\sim$10 nN, the direction of which was toward the long axis of SFs, were detected. Deguchi et al. (2005) showed with in vitro tests that single SFs isolated from cultured SMCs can bear tension of 10-nN order of magnitude. These studies implicate close association between tensions in SFs and extracellular matrix through focal adhesions. Bundling of a number of actin filaments in the basal SFs may produce such a high tension (Fig. 5, inset). By contrast, buckling force of single microtubules is $\sim$1 pN (Kurachi et al., 1995), suggesting the cytoskeletal components do not directly join the force balance at focal adhesion sites of 10-nN order of magnitude. Mechanical properties and tension-bearing abilities of both single intermediate filament bundles and single SFs in vivo remain elusive, which will be required to understand quantitatively the exact force transmission pathway.

## 5 Summary

Extracted basal SFs in adherent SMCs and ECs shortened in an ATP-independent manner after removal of the cell membrane and the other cytoplasmic constituents, demonstrating the presence of preexisting tension in the single SFs. A release of the tension was transmitted to another SF via cross-linking SF networks. These findings suggest that there is a tensed SF network, which will be useful to produce a directional cytoplasmic strain field

that is different from the heterogeneous strain field in the other cytoplasmic region.

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