

## CELL SPREADING CONTROLS BALANCE OF PRESTRESS BY MICROTUBULES AND EXTRACELLULAR MATRIX

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### 1. ABSTRACT

The controversy surrounds the cellular tensegrity model. Some suggest that microtubules (MTs) must bear a significant portion of cell contractile stress (prestress) if tensegrity is a useful model. Previously we have shown that for highly spread airway smooth muscle cells (areas > 2500  $\mu\text{m}^2$ ) MTs balance a significant but small portion (average 14%) of the prestress. To further explore if controlling the degree of cell spreading could modulate the portion of the prestress balanced by MTs, we utilized a recent method by which tractions are quantified in cells that are constrained within micropatterned adhesive islands of defined sizes on the surface of flexible polyacrylamide gels containing fluorescent microbeads. The prediction is that if MTs balance a portion of the contractile stress, then, upon their disruption, the portion of the stress balanced by MTs would shift to the substrate, causing an increase in traction and strain energy. We first activated the cells maximally with histamine and then disrupted the MTs with colchicine. Histamine resulted in an increase in intracellular calcium whereas ensuing colchicine addition in the presence of histamine did not change intracellular calcium concentration, suggesting there was no additional net increase in contractile stress inside the cell. We found that following disruption of MTs the increase in traction and strain energy varied with the degree of cell spreading: as the cell projected areas increased from 500  $\mu\text{m}^2$  to about 1800  $\mu\text{m}^2$ , the percent increase in tractions decreased from 80% to about a few percent and the percent increase in strain energy decreased from 200% to almost zero percent, indicating the portion of the prestress balanced by MTs decreased as the cells increased spreading. These findings demonstrate that complementary role of the extracellular matrix and the MTs in balancing the prestress is controlled by the degree of cell spreading.

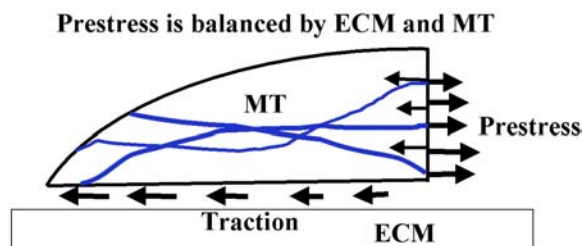
### 2. INTRODUCTION

Microtubules (MTs) are one of the major structural components of the cytoskeleton (CSK) that

determines cell shape and polarity, and other functions such as transport (1). Mechanical measurements in isolated MT polymers show that MTs have high flexural rigidity, suggesting that they may support substantial longitudinal mechanical compression (9). According to the cellular tensegrity hypothesis (15), the tensile stress in the cell should be balanced by compression-supporting elements, which could be internal components such as MTs or hydrostatic pressures and external components such as extracellular matrix (ECM). Recently we have demonstrated that the pre-tensile stress a cell generates is a major determinant of cell shape stability (28, 29), thus supporting the a priori prediction of the stress-supported structures including tensegrity structures. However, there is still controversy on how the prestress is balanced. Based on the arguments put forward by some investigators (12), the tensegrity model is a useful model only if intracellular MTs balance a significant portion of the contractile stress.

Published work shows that MTs of living cells do buckle during cell migration and after chemical stimulation, suggesting that they carry compressive stresses (9, 31). We have shown recently that MTs buckle under mechanical loading (28). Furthermore, we have shown that MTs balance about 14% of the contractile stress in highly spread airway smooth muscle cells (23). These experimental observations were supported by theoretical calculations using the strain energy method (23). However, 14% is only a small portion. Hence the question remains whether the portion of the prestress supported by the MTs can be changed by altering the physical environment to which the cells attach and whether the ECM and the MTs can play complementary roles in balancing the prestress.

In this study, we attempted to determine whether the degree of cell spreading would regulate the portion of the prestress balanced by the MTs. We plated human airway smooth muscle (HASM) cells on micropatterned islands coated with saturated amounts of collagen-1 on



**Figure 1.** A schematic of force balance of an adherent cell. The contractile stress (the prestress) of the cell is balanced internally by stresses in compression-supporting structures microtubules (MT) and possibly other components and externally by tractions on the extracellular matrix (ECM).

polyacrylamide gels to control for the degree of cell spreading. In this manner the ECM molecules per unit area and cell plating time were fixed. We performed quantitative measurements of indices of MT compression in HASM cells by utilizing the traction microscopy technique (24). We found that in these HASM cells how much prestress is balanced by MTs is inversely related to the degree of cell spreading.

### 3. MATERIALS AND METHODS

Traction force microscopy has been used to measure cell traction at the cell-substrate interface (5, 24). From these measurements we can calculate the tractions and the elastic energy stored in a flexible substrate during cell contraction (3). In our previous study, we used this technique to measure traction of cultured HASM cells in response to histamine, a contractile agonist (29). In the present study, we combined this technique with the micropatterning method to assess compression in MTs in the cells whose spreading was tightly controlled (30).

Our working hypothesis is that cell contraction is balanced partly by traction at the cell-substrate interface and partly by compression of MTs (Figure 1). To test this hypothesis, we used traction microscopy to measure cell traction (root mean square) and strain energy in histamine-maximally-stimulated adherent HASM cells prior to and after MTs were disrupted by colchicine. If this hypothesis holds, then, for a given state of contractile stress, we predict that following disruption of MTs the traction would increase due to a transfer of the part of the contractile stress balanced by MTs to the substrate. The strain energy stored in the substrate would increase due to transfer of the compression energy of MTs to the substrate.

#### 3.1. Cell culture

HASM cells were cultured ( $10^5$  cells/cm<sup>2</sup>) with Hams/F12 media supplemented with 10% fetal bovine serum, 50 mg/ml gentamicin, and 2.5  $\mu$ M amphotericin B. Cells at passages 3-8 were used for all experiments. After reaching confluence, cells were serum deprived for 48 h and then plated in serum free defined medium. Cells were plated sparsely on a 70  $\mu$ m-thick polyacrylamide elastic gel block coated with collagen type-I (0.2 mg/ml). More details about cell culture have been published previously (29). Microfluorimetric quantitation of

intracellular calcium levels was performed using Fura-2 together with ratio imaging.

#### 3.2. Micropatterning the polyacrylamide gels

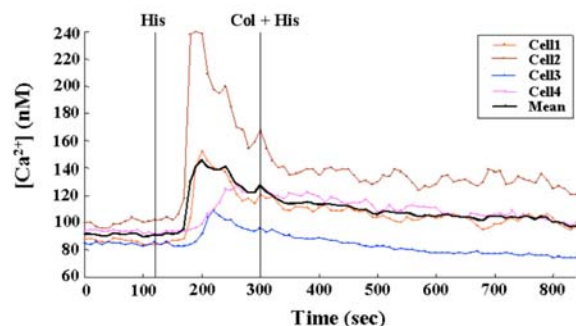
We used a recently developed approach to micropattern different shapes and different sizes on the polyacrylamide gel (30). In brief, polydimethylsiloxane (PDMS) membranes were made using a soft photolithography method (19). Then PDMS membranes were oxidized in a plasma cleaner to make the membranes compatible with the polyacrylamide gel so that tight seals could be formed between the membrane and the gel after the membrane was placed on top of the gel. The specific areas of the gel were activated with chemicals and then were exposed to saturated amounts of collagen-1 molecules (0.2 mg/ml) through the holes in the membrane (30). With this approach it was possible to constrain the cells to different sizes for the same amount of plating time. We plated cells on micropatterned islands of 25- $\mu$ m or 50- $\mu$ m diameter circles, corresponding to areas of 500 or 1960  $\mu$ m<sup>2</sup>. All cells were plated overnight (about 18 hrs) before experiments. Cell spreading areas were determined by tracing the contours of the cell in phase contrast.

#### 3.3. Calculation of traction and strain energy

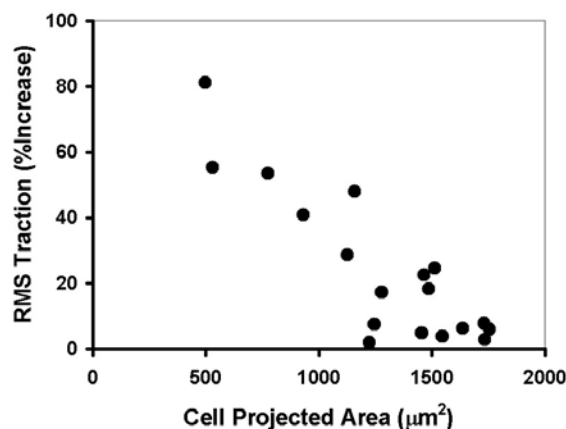
Detailed description of this technique is given in Wang *et al.* (29). Briefly, the polyacrylamide gel substrate was used as a strain gauge to measure the interfacial cell-substrate traction. Many fluorescent microbeads (0.2  $\mu$ m-diameter) embedded near the gel apical surface serve as markers whose displacements are recorded as the adhering cell contracts. The bottom surface of the gel was covalently bonded to a flat rigid plate and the lateral surfaces were free. From bead displacements and known elastic properties of the gel (Young's modulus of 1,300 Pa and Poisson's ratio of 0.48), the traction was calculated (29). The mean traction was obtained as the root-mean square of traction over the cell projected area. The strain energy stored in the substrate was calculated as the work of traction on displacement.

#### 3.4. Protocol

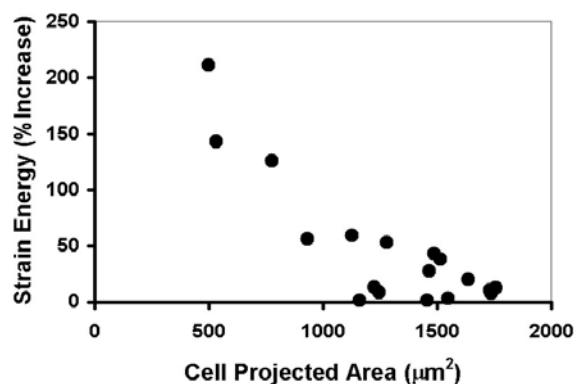
HASM cells plated on a micropatterned polyacrylamide gel block were first treated with 10  $\mu$ M histamine, then with 1  $\mu$ M colchicine plus 10  $\mu$ M histamine and finally with trypsin. This dose of histamine was shown to produce maximum increases in cell stiffness (14) and in myosin light chain phosphorylation (28). Histamine was added with colchicine in order to maintain the saturated bath concentration. Colchicine disrupted a large portion of the integrity of the microtubules 10 min after addition as determined by immunofluorescent staining (23). Trypsin was added until a cell was completely detached from the substrate. Images of fluorescent microbeads were taken at baseline, after addition of histamine, then after colchicine and finally after trypsin. The image after trypsin was used as the reference (force-free) image. The displacement field was calculated as follows: at baseline, after histamine, and after colchicine. In some cells intracellular calcium concentration was measured using Fura-2 under baseline condition, after histamine, and after colchicine treatments. In some cases, the calcium concentration was measured



**Figure 2.** Intracellular calcium concentration as a function of time under different experimental conditions. Zero to 120 sec: baseline calcium concentration; His: addition of histamine (10  $\mu$ M), a contractile agonist; Col + His: addition of colchicine (1  $\mu$ M), a microtubule disrupting agent, in the presence of histamine. N=4 cells.



**Figure 3.** Percent increase in RMS (root mean square) traction from controls as a function of cell projected areas after colchicine treatment. Controls: RMS traction after histamine stimulation and just before colchicine treatment. Each symbol represents one cell. N=18 cells.



**Figure 4.** Percent increase in strain energy in the substrate from controls as a function of cell projected areas after colchicine treatment. Controls: strain energy in the substrate after histamine treatment and just before colchicine treatment. Each symbol represents one individual cell. N = 18 cells.

under baseline condition and after colchicine treatment alone.

#### 4. RESULTS AND DISCUSSION

First we measured intracellular calcium concentration in response to saturated amounts of histamine (10  $\mu$ M). Consistent with previously published work (20), histamine addition elevated intracellular calcium in these airway smooth muscle cells (Figure 2). However, a follow-up treatment with colchicine (1  $\mu$ M) that disrupted MTs in these cells (23) failed to induce intracellular calcium elevation (Figure 2), although colchicine treatment alone did increase intracellular calcium concentration (not shown). Previously we showed that colchicine treatment after maximal activation with histamine did not further increase myosin light chain phosphorylation (28). All these results demonstrate that under these conditions disruption of MTs with colchicine after maximal activation with histamine did not further increase the contractile stress in the cell. Thus, any observed increase in traction and strain energy after colchicine should be the result of the loss of the compression-supporting capacity MTs, which balanced a part of cell contractile stress prior to their disruption and the result of a shift of load and strain energy from MTs to the substrate. It is important that these results were not consistent with MTs bearing tension because it would lead to a decrease in traction if MTs were tension-bearing elements.

We quantified tractions after colchicine treatment and compared them with tractions just before addition of colchicine. We found that colchicine treatment resulted in an increase in traction, and the percent increase in tractions varied with cell spreading areas: the smaller the spreading area, the higher the percent increase in tractions (Figure 3). In other words, it appears that portion of the contractile stress balanced by MTs is inversely proportional to cell spreading area. Since the rest of the contractile stress must be balanced by ECM, these results indicate that the portion of the contractile stress balanced by ECM increases with cell spreading areas. These data suggest that MTs and ECM play a complementary role in balancing the contractile stresses generated inside the CSK.

We further computed strain energy in response to colchicine treatment. We found that the elastic energy stored in the elastic gel increased with disruption of MTs: the higher the spreading area, the smaller the percent increase in strain energy (Figure 4). The percent increase in strain energy varied from 200% to almost 0% as the spreading areas increased from 500  $\mu$ m<sup>2</sup> to 1800  $\mu$ m<sup>2</sup>. Although we have attributed the traction elevation after MT disruption to shift of load from MTs to ECM, there could be other alternative explanations. One is the potential role of caldesmon. It is known caldesmon modulates actomyosin interactions independent of myosin light chain kinase. It is not known if MT disruption by colchicine alters caldesmon phosphorylation which in turn might affect contractile stress generation. Another is the potential role of Rho-kinase mediated calcium independent actomyosin contraction (6). Yet another possibility is the role of

CSK remodeling after MT disruption, i.e., the rearrangements of the remaining CSK filaments and linking proteins. These might potentially alter the force distribution and balance intracellularly and manifest in alterations in tractions. Further experiments are needed to determine if these play a role or not, and if they do, to what degree.

A major assumption in this study is that the mechanical equilibrium was maintained among the contractile stress bearing elements, MTs and the ECM substrate. However, it is possible that other cellular structures may also contribute to the balance of forces and strain energy. One possibility is the stress fibers. However, stress fibers isolated from fibroblasts and endothelial cells on average shorten to about 23% of their initial lengths, suggesting that under normal physiological conditions stress fibers are indeed in tension (17). Similarly, intermediate filaments appear to play the roles of tension elements and of preventing MT buckling (2, 15, 26). Another is the hydrostatic pressure in the cytoplasm. However, since the cytoplasm is virtually incompressible, the hydrostatic pressure's contribution to strain energy should be almost zero. Thus our finding of a large increase in strain energy after colchicine cannot be explained by the hydrostatic pressure difference across the cell membrane. This is consistent with our previous finding that there is little change in cell stiffness after membrane permeabilization that abolishes this pressure difference (21, 27). Other contributions such as elastic energy stored in the myosin head appear to be very small (23).

It is known that smooth muscle cells are viscoelastic (8). Thus it is possible that after disruption of MTs by colchicine, a significant portion of the energy stored in MTs could be dissipated. Together with the possibility of incomplete disruption of MTs with colchicine, the percent increase in strain energy after colchicine could be an underestimate of the total energy stored in the MT in these intact cells.

Finally, the substrate stiffness might affect the balance of prestress by the substrate and MTs in three potential ways. First the total prestress might increase in response to elevated substrate stiffness. It has been shown by Yu-Li Wang's group that when substrate stiffness is increased by 2.5 times, the tractions a cell exerts on the substrate increase by about 50% (25); thus the total prestress might increase. Second it has been shown that elevating substrate stiffness increases cell spreading areas (7), which in turn could increase total prestress (30). Third it is possible that for a given degree of cell spreading, the higher the substrate stiffness, the higher the portion of the total prestress being balanced by the substrate. One interpretation is that as an adherent cell probes its environment via force-transmitting molecule integrins, a stiffer substrate would result in an increased deformation of the CSK until a new mechanical equilibrium is reached, which in turn will activate the cell to generate more force. However, CSK filaments can only sustain a finite strain before structure failure; the increased force is balanced by being partitioned to the substrate and by strengthening the CSK via remodeling to maintain the stress on the CSK

relatively constant. In our current study, we plated cells on a medium stiff substrate of 1300 Pa. Our preliminary study with cells on a very stiff substrate of 30,000 Pa shows that the prestress increases by about 2-3 fold when compared with that on the 1300 Pa substrate. Importantly, the prestress and stiffness of the same cell increases or decreases proportionally in response to contractile agonists or relaxation agents. Thus our recent finding that the prestress dictates cell stiffness (29) is confirmed for cells on very stiff substrates. The above suggests that as the substrate stiffness increases, the portion of the prestress balanced by MTs might decrease and the portion of the prestress balanced by the substrate might increase for the same degree of cell spreading. However, a living adherent cell has a very dynamic CSK that undergoes rapid processes of polymerization, depolymerization, bundling, severing, and remodeling. How the cell manages to maintain the balance between this dynamic process and the cell shape stability remains to be determined.

## 5. CONCLUSIONS

Our data demonstrate that the portion of the prestress balanced by compression-supporting network MTs is inversely proportional to the degree of cell spreading. The higher the cell spreading, the less prestress being balanced by MTs. Since the ECM substrate plays a complementary role, it suggests that the higher the cell spreading, the more prestress being balanced by ECM. Our current work clarifies an issue in the controversy of cellular tensegrity. The cellular tensegrity idea, put forward by Ingber (15, 16), extends the classical mechanical tensegrity definition to include both intracellular and extracellular elements in balancing the CSK prestress. We believe, however, that the most important feature of the cellular tensegrity hypothesis is that the cell shear stiffness must increase with the CSK prestress, no matter how the prestress is balanced: either by the substrate or by intracellular compression-supporting elements. This feature has been demonstrated by our recent studies in which the prestress is altered via chemical agents (28, 29), external stretching (21), or overexpression of myosin light chain kinase (4) in smooth muscle cells, endothelial cells, and fibroblasts. Our finding of prestress dictating cell shape stability extends the work of Harris *et al.* who show the existence of contractile prestress in cell locomotion on flexible substrates (11). Recently we have also shown that the prestress is important in force transmission within the CSK (13). Externally applied strains and the prestress have been shown to result in changes in MT assembly (22, 18). Since MTs also play important roles in other cell functions, it remains to be seen how complementary roles of ECM and MTs in balancing the prestress might affect these functions such as intracellular transport.

## 6. ACKNOWLEDGMENTS

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