Control of microtubule assembly by extracellular matrix and externally applied strain

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Putnam, A. J., K. Schultz, and D. J. Mooney. Control of microtubule assembly by extracellular matrix and externally applied strain. Am J Physiol Cell Physiol 280: C556–C564, 2001.—A number of studies have suggested that externally applied mechanical forces and alterations in the intrinsic cell-extracellular matrix (ECM) force balance equivalently induce changes in cell phenotype. However, this possibility has never been directly tested. To test this hypothesis, we directly investigated the response of the microtubule (MT) cytoskeleton in smooth muscle cells to both mechanical signals and alterations in the ECM. A tensile force that resulted in a positive 10% step change in substrate strain increased MT mass by 34 ± 10% over static controls, independent of the cell adhesion ligand and tyrosine phosphorylation. Conversely, a compressive force that resulted in a negative 10% step change in substrate strain decreased MT mass by 40 ± 6% over static controls. In parallel, increasing the density of the ECM ligand fibronectin from 50 to 1,000 ng/cm² in the absence of any applied force increased the amount of polymeric tubulin in the cell from 59 ± 11% to 81 ± 13% of the total cellular tubulin. These data are consistent with a model in which MT assembly is, in part, controlled by forces imposed on these structures, and they suggest a novel control point for MT assembly by altering the intrinsic cell-ECM force balance and applying external mechanical forces.

cytoskeleton; mechanotransduction; smooth muscle cells; tubulin; tensegrity

MECHANICAL SIGNALS play an essential role in both normal and pathological development of a variety of tissue types, including bone (19), muscle (54), and vascular tissue (46, 60). These mechanical signals occur in vivo in the context of a complex extracellular matrix (ECM), which, by itself, is a critical determinant of the cellular microenvironment. Mechanical signals have been shown to alter cell proliferation (61, 62), production of ECM proteins (4, 8), and gene expression (53), often in an ECM-dependent manner (38, 41, 62). Likewise, signals from the ECM itself have been shown to regulate integrin-mediated signaling events during normal development and neoplastic transformation, generating signals that control growth, differentiation, motility, apoptosis, and matrix synthesis and remodeling (1, 2, 26, 31). Although the chemical composition of the ECM is clearly important, there is increasing evidence that mechanical cues from the matrix may be just as important (10, 32).

Previous studies have shown that altering the mechanics of the matrix without changing the chemical composition is enough to alter cellular behavior (9, 23, 28, 36). The mechanisms utilized by cells to transmit this mechanical information into a change in phenotype remain poorly understood and the subject of ongoing debate. Two dominant, although not necessarily mutually exclusive, paradigms have emerged to explain the responses of cells to changes in the mechanical microenvironment. In the first hypothesis, changes in the mechanics of the ECM trigger a cascade of soluble chemical second messengers, likely derived from the cluster of signaling proteins at the sites of cell adhesion (46, 52). In the second hypothesis, based on models of tensegrity architecture first put forth by Fuller (24, 33, 48), cells exist in an equilibrium balance of forces. According to this hypothesis, altered mechanics of the ECM could directly induce changes in this preexisting cellular force balance, resulting in alterations in the mechanics of the cytoskeleton. These changes may then directly trigger changes in gene expression or influence a variety of signaling pathways (6, 10, 32). An abundant amount of evidence in support of both models has led to increased debate on the subject of mechanotransduction. The cytoskeleton is clearly involved in the response to mechanical forces given that it is altered in response to a variety of externally applied mechanical signals in many different cell types (42, 43, 49, 51, 59). Furthermore, changing the flexibility of an adhesion substrate has been shown (47) to regulate cell migration and focal adhesion structure in a manner that is dependent on the force-generating capabilities of the actin cytoskeleton. In another study (12), an optical trap was used to simulate an increased ECM rigidity, causing strengthening of interaction between integrins and the cytoskeleton proportional to the restraining force of the trap. Likewise, applying forces to integrin ligand-coated beads increased the stiffness of the cytoskeleton in a linear relationship to the amount of stress applied (59).
Microtubules (MTs), one element of the cytoskeleton, may be involved in the mechanotransduction response by altering their assembly in response to compressive forces (5, 34, 49). Two theoretical models predict that altering compressive forces on MTs can alter their state of assembly (5, 30) and hence provide a testable hypothesis that forms the basis of this study. These models suggest that a pseudoequilibrium among tubulin monomers, MTs, and compressive forces inherent on the MTs could be altered by changing the amount of force on the MTs. In this study, the mechanical microenvironment was altered to test these theoretical models directly. We demonstrated control of MT assembly in smooth muscle cells (SMCs) both by applying single external mechanical forces and by changing the density of ECM ligand used for cell adhesion. MT assembly was induced by applying a positive step change in strain to the cell culture surface, imparting a tensile force to the adherent cells. MT disassembly was induced after the application of a negative strain to the substrate, effectively imparting a compressive force to the adherent cells. Changing the density of adsorbed fibronectin in static culture also shifted the pseudoequilibrium set point between tubulin monomeric subunits and MTs, with an increased density of ligand leading to an increased mass of MTs. Combined, these findings support previously published models predicting that MT assembly is regulated by imposed compressive forces.

METHODS

SMC culture. Vascular SMCs were isolated from thoracic rat aortas with an adaptation of a previously published technique (49, 50). Routine SMC culture was performed with medium 199 (M199) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Gibco, Gaithersburg, MD). Immunofluorescence localization of a smooth muscle-specific α-actin marker confirmed these cells were SMCs (data not shown). SMCs between passage 4 and 15 were used in all studies. For serum-free experiments in this study, cells were cultured in standard serum-containing medium until they were ready to be used for an experiment. Cells were then harvested with trypsin followed by either serum-containing M199 or a soybean trypsin inhibitor solution (0.75 mg/ml) in PBS with 5 mM EDTA. After centrifugation, cells were washed with PBS for removal of any residual serum, centrifuged again, and subsequently resuspended in a completely defined serum-free medium (CSC SF Media Kit supplemented with Rocket-Fuel additive (1:50); Cell Systems, Kirkland, WA).

Application of external step changes in strain to the cultured SMCs. Cells were plated out at densities between 20,000 and 30,000 cells/cm² on six-well culture dishes made of silicon rubber (BioFlex plates; Flexcell, Hillsborough, NC). These plates, initially untreated, were coated with a theoretical density of 1 μg/cm² (accounting for the additional surface area created by the volume of the liquid) of either type I collagen (Vitrogen 100; Cohesion, Palo Alto, CA) or fibronectin (human plasma fibronectin; Gibco). ECM proteins were adsorbed with the use of a carbonate/bicarbonate buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.4). Cells cultured on these substrates were allowed to attach and spread for ~24 h. Dishes were exposed to step changes in strain with the use of a custom-built device that we have previously described (49). We have previously characterized this device and shown that the strain is linear with respect to vertical displacement and that the strain is a true biaxial strain.

Extraction of MT and total tubulin fractions from cultured cells. MTs and total tubulin were differentially extracted from cultured SMCs as previously described (7, 44, 49). Briefly, to isolate polymeric tubulin (MTs), cells were washed in an MT stabilization buffer (MTSB) and incubated once for 15 min with MTSB plus 0.1% Triton X-100. This treatment permeabilizes the cell membrane, flushing out soluble cytoplasmic proteins, including monomeric tubulin. The remaining cytoskeletal ghosts were subsequently solubilized in a lysis buffer. Total tubulin was extracted by adding lysis buffer directly to intact cells. All extraction steps were performed at 37°C with prewarmed reagents to prevent any cold depolymerization of MTs. We have previously validated these methods by quantifying the distribution of tubulin in SMCs exposed to either nocodazole or paclitaxel, accurately quantifying nocodazole-induced MT depolymerization and paclitaxel-induced MT assembly (49).

Quantification of tubulin mass. A competitive ELISA technique (58) was performed as previously described (49). A mouse monoclonal anti-β-tubulin antibody (diluted 1:500; Boehringer Mannheim, Indianapolis, IN), followed by an alkaline phosphatase-conjugated goat anti-mouse IgG (diluted 1:2,000; Bio-Rad, Hercules, CA), was used to assay the tubulin content in both Triton X-100-insoluble and total cellular lysates. A purified bovine brain tubulin standard (Molecular Probes, Eugene, OR) was assayed in parallel to generate a standard curve. After the substrate for the enzyme (Bio-Rad) was added to generate a colored reaction product, plates were read at 405 nm with a microplate reader (Vmax kinetic microplate reader; Molecular Devices, Sunnyvale, CA). Absorbance values over a range of dilutions were used to determine the tubulin concentration in the experimental samples to ensure that the absorbance values were in the linear region and that the signal was not saturated. All values for tubulin mass determined in this study are presented as values relative to control conditions.

Inhibition of tyrosine phosphorylation with genistein. Certain experiments were performed with cells cultured in the presence of a tyrosine phosphorylation inhibitor, genistein (Sigma). SMCs cultured for 1–2 days on static six-well culture dishes in normal serum-containing M199 were subsequently serum starved for 18–36 h to reduce any basal levels of tyrosine phosphorylation caused by the presence of serum. After serum starvation, cells were subjected to genistein (between 10 μM and 1 mM final concentration) for 15 min before serum was added back to their culture environment. The original lophophiled genistein stock was solubilized in DMSO, so an amount of DMSO equivalent to that in the 500 μM genistein-treated sample was added to the untreated cultures to confirm that DMSO alone had no effect on tyrosine phosphorylation. After 15 min of exposure to the serum-containing environment, cells were lysed. Equal protein amounts (1.5 μg of total cellular protein) of these samples were separated by gel electrophoresis. Western blots for phosphotyrosine (using a mouse monoclonal anti-phosphotyrosine antibody, clone PY-20; BD Transduction Laboratories, Lexington, KY) were used to determine the optimal dosage of genistein that reduced total cellular tyrosine phosphorylation without affecting cell adhesion and morphology.

Controlling cell-matrix interactions and quantification of spread cell area. For experiments investigating the role of ECM density, SMCs were cultured on different densities of fibronectin on bacteriologic six-well culture dishes in a com-
Microtubule assembly is modulated by externally applied strain. In the first part of this study, we directly tested the hypothesis that tensile forces applied to the adhesion substrate lead to an increase in net MT assembly. SMCs were cultured in 10% serum-containing medium on silicon rubber substrates precoated with type I collagen. After 24 h, cells were exposed to a single externally applied 10% step change in strain and held in the strained position for finite time periods ranging from 1 min to 1 h. Triton X-100-insoluble (n = 6) and total cellular lysates (n = 6) were obtained from cells held in the stretched position. Quantification of MT mass with the use of a competitive ELISA revealed that a positive step change in external strain drives increased MT assembly (Fig. 1). This response occurred rapidly (after 1 min of tensile load) and peaked at ~15 min after application of the strain, with a statistically significant 34 ± 10% increase (P < 0.05) in MT mass over unstretched controls. The response may occur even faster, but the limitations of the protein extraction methods make resolution of <1 min impossible at this time. The MT mass remained elevated before returning to near unstretched levels after 1 h. In all experiments conducted in this study, quantification of total cellular tubulin levels revealed no change (data not shown). This finding suggests that the time scale of these experiments was short enough to prevent de novo protein synthesis from influencing the level of total cellular tubulin within the cell. A constant level of total cellular tubulin suggests that MT assembly occurred from the preexisting cytoplasmic pool of α- and β-tubulin. *P < 0.05 vs. conditions at time 0 (t = 0).

On the basis of results from the first part of this study, we hypothesized that mechanical control of MT assembly was dependent on the direction of the external force applied. To test this hypothesis, we subjected cultures to a compressive force that results in a negative strain on the substrate, the opposite of the stimulus provided in Fig. 1. Silicon rubber substrates with attached SMCs were stretched 10% and held in a stretched position for 24 h. After that initial 24-h period, the strain in the substrate was released, effectively imparting a negative strain to the substrate and to the cells attached to it. Again, Triton X-100-insoluble (n = 5) and total cellular lysates (n = 5) were obtained from the cells. Quantification of microtubule mass with the use of a competitive ELISA revealed that a negative strain drives net MT disassembly (Fig. 2A) in a time frame similar to that of the response to the positive strain. The MTs disassembled continually throughout the time course studied in these experiments (30 min), ultimately resulting in a 40 ± 6% decreased MT mass after 30 min. Statistical analysis...
(one-way ANOVA) revealed a strong statistical significance among all data points ($P < 0.0001$). Parallel quantification of the total cellular tubulin revealed no statistical difference in the level of total tubulin within the cells during these experiments (Fig. 2B).

**Strain-driven microtubule assembly occurs to the same extent on both type I collagen and fibronectin.** We next examined whether strain-driven MT assembly is a widely applicable mechanotransduction response, observed in a variety of culture and matrix conditions. To address this hypothesis, we investigated the dependence of strain-driven MT assembly on the ECM ligand. Cells cultured on 1 μg/cm² fibronectin or type I collagen in a defined, serum-free medium were subjected to a single positive strain, as in the first set of experiments. Triton X-100-insoluble ($n = 6$) and total cellular lysates ($n = 6$) were extracted 15 min after a 10% strain was initiated in the substrate. Quantification of tubulin in these two types of lysates by competitive ELISA revealed that the MT mass increased in response to the increased strain for cells cultured on both ECM molecules studied (Fig. 3A). The magnitude of the MT assembly response was similar to that seen for the conditions used in the first set of experiments (i.e., collagen-coated substrate in a serum-containing medium). The assembly of MTs in response to the tensile strain occurred from the cytoplasmic pool of monomeric tubulin, as evidenced by the $\sim 80\%$ decrease in the amount of available monomer between the static and stretched conditions (Fig. 3B). Nearly identical results were obtained in all serum-free studies whether serum-containing M199 or a soybean trypsin inhibitor solution was used to inhibit the action of trypsin after cells were harvested to be plated out for these experiments (data not shown). These findings demonstrate that a positive step change in strain drives net MT assembly for SMCs cultured on either type I collagen or fibronectin, suggesting that the chemical identity of the matrix may not be critical in triggering strain-driven MT assembly.

**Strain-driven microtubule assembly occurs independently of tyrosine phosphorylation.** A number of studies in the literature have determined that some cellular responses to applied mechanical forces are dependent on a number of soluble signaling cascades. Many signals from growth factors and the ECM converge via phosphorylation of tyrosine residues on various proteins (31, 52), and many tyrosine phosphorylation events occur in response to mechanical signals (46, 51, 60). To eliminate the influence of these events, we used the inhibitor genistein. Control experiments were first conducted to establish the stability of the MT pool. Triton X-100-insoluble and total cellular lysates ($n = 5$) were extracted 15 min after a positive 10% step change in strain for cells cultured on both ECM molecules in a serum-free medium. The MT content was quantified by competitive ELISA. A positive step change in strain drove a significant increase in the MT mass for cells cultured on both ECM molecules studied (Fig. 3A). The magnitude of the MT assembly response was similar to that seen for the conditions used in the first set of experiments (i.e., collagen-coated substrate in a serum-containing medium). The assembly of MTs in response to the tensile strain occurred from the cytoplasmic pool of monomeric tubulin, as evidenced by the $\sim 80\%$ decrease in the amount of available monomer between the static and stretched conditions (Fig. 3B). Nearly identical results were obtained in all serum-free studies whether serum-containing M199 or a soybean trypsin inhibitor solution was used to inhibit the action of trypsin after cells were harvested to be plated out for these experiments (data not shown). These findings demonstrate that a positive step change in strain drives MT assembly for SMCs cultured on either type I collagen or fibronectin, suggesting that the chemical identity of the matrix may not be critical in triggering strain-driven MT assembly.

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performed to determine the optimal dosage of genistein for experiments in which SMCs were subjected to strain. A range of concentrations of genistein were screened for their ability to reduce serum-induced tyrosine phosphorylation in SMCs (Fig. 4A), and both 500 μM and 1 mM concentrations of genistein reduced the levels of serum-induced tyrosine phosphorylation (note the disappearance of bands above 132 kDa and near 55 kDa and the lower intensity of bands near 80 and 125 kDa). On the basis of these experiments, a concentration of 500 μM genistein was used for cells subjected to strain. It should be noted that serum starvation dramatically reduced the levels of tyrosine phosphorylation within these cells as well (first 2 lanes in Fig. 4A). SMCs were then plated on collagen-coated BioFlex plates in the serum-containing M199. After 24 h, cells were then serum-starved for an additional 24 h before being subjected to a positive strain in the presence of 500 μM genistein. Cells were pretreated with genistein for 15 min before being exposed to strain. Triton X-100-insoluble (n = 6) and total cellular lysates (n = 6) were extracted 15 min after the strain was initiated because this was the point at which a maximal response was seen in the preliminary experiments (see Fig. 1). MTs assembled (Fig. 4B, left) from the pool of monomeric tubulin (Fig. 4B, right) in the presence of genistein in the same fashion as control conditions.

Control of MT assembly via the ECM density. Previous studies have indicated that changes in ECM density alter cell shape via alteration of the cell-ECM force balance (36, 45). According to these studies, an increased ECM ligand density would support more binding between cells and their substrate, alter cell shape,

Fig. 4. Strain-driven MT assembly is independent of tyrosine phosphorylation. A: control experiments were performed to determine the optimal dose of genistein required to inhibit or significantly reduce serum-induced tyrosine phosphorylation. Samples exposed to the various conditions were lysed, and 1.5 μg of total cellular protein was separated by electrophoresis and transferred to a polyvinylidene difluoride membrane. Probing for phosphotyrosine levels demonstrated a reduction in a significant number of bands in the samples exposed to genistein. MW, molecular weight. B: polymeric tubulin content (left) increased in SMCs exposed for 15 min to a positive 10% step change in strain in the presence of the tyrosine phosphorylation inhibitor genistein (500 μM). Monomeric tubulin content (right) decreased in SMCs in these same experiments. Data represent means ± SD of a single experiment with n = 5 Triton X-100-insoluble fractions for both the strained and static conditions. *P < 0.05 vs. static controls.
and transfer inherent cytoskeletal prestress away from the cytoskeleton to the ECM. To address this question, we investigated the effect of ECM density on MT assembly. Cells were cultured on bacteriologic culture dishes in a completely defined serum-free environment. Fibronectin was preadsorbed onto the culture dishes at theoretical densities of 50, 100, 500, and 1,000 ng/cm² with a carbonate/bicarbonate buffer. After 24 h, Triton X-100-insoluble (n = 6) and total cellular lysates (n = 6) were extracted and quantified for tubulin by using the competitive ELISA. SMCs cultured on 50 ng/cm² fibronectin had 59 ± 11% of their total cellular tubulin in the form of MTs, while those cultured on 1,000 ng/cm² fibronectin had 81 ± 13% of their total cellular tubulin in the form of MTs (P < 0.05) (Fig. 5A). Quantification of spread cell area in these different conditions revealed that the increased MT mass correlated with a slight but significant increase (P < 0.05) in cell projected area across these different ECM conditions (Fig. 5B). To confirm that presenting increased density of ECM ligand leads to an increase in cell-ECM bonds, we performed immunofluorescence localization of the α5-integrin receptor. A qualitative increase in the number and organization of focal adhesions was found as ECM density was increased (Fig. 5C). SMCs cultured on a range of fibronectin densities were subsequently subjected to a 10% step increase in strain. In all matrix conditions studied (from 100 to 5,000 ng/cm²), the mass of MTs increased over static controls, although the extent of the net assembly of MTs was dependent on the ECM ligand density (Fig. 6). For cells cultured on 100 ng/cm² fibronectin, MT mass increased by 5 ± 10% over static

Fig. 5. Increased fibronectin matrix density increases the level of polymeric tubulin in SMCs, correlating with an increased cell area and an increased number of adhesion sites. A: polymeric tubulin content increased after 24 h in SMCs spread on different densities of FN. Data represent means ± SD for a single experiment with n = 5 Triton X-100-insoluble fractions and 5 total fractions for each condition. *P < 0.05 vs. 50 ng/cm² controls. B: SMCs spread to a greater extent on the same 4 densities of FN matrix. Data represent means ± SD for n = 70 cells. *P < 0.05 vs. 50 ng/cm² controls. C: SMCs have an increased number of distinct focal adhesion sites as the density of FN matrix is increased. Immunofluorescence microscopy revealed α5-integrin receptor in cells cultured on (from left to right) 50, 100, 500, or 1,000 ng/cm² FN.

Fig. 6. Strain-driven microtubule assembly occurs to a greater extent for SMCs cultured on a higher density of FN. Strain-driven MT assembly in SMCs exposed to a 10% step increase in strain for 15 min occurred to a greater extent in SMCs cultured on 500 ng/cm² FN than in cells cultured on 100 ng/cm² FN. Data represent means ± SD of a single experiment with n = 5 Triton X-100-insoluble fractions for both the strained and static conditions. *P < 0.05 vs. static controls. Strain-induced MT assembly occurred to nearly the same extent in SMCs cultured on 2 higher Fn densities (1,000 and 5,000 ng/cm²) as for those cultured at 500 ng/cm² (data not shown).
controls. For cells cultured on 500 ng/cm² fibronectin, the net increase in MT mass was 27 ± 10%, significantly higher than the assembly on the 100 ng/cm² condition (P < 0.05). Strain-induced MT assembly occurred to the same extent in cells cultured on higher densities of fibronectin (1,000 and 5,000 ng/cm²) as in cells cultured on 500 ng/cm² (data not shown).

**DISCUSSION**

We have shown that MT assembly can be controlled in SMCs in a predictable fashion both by applying a step change in external strain and by altering the density of ECM ligands. Our model for this additional point of control for MT assembly is summarized in a schematic representation in Fig. 7. In this model, tensile forces leading to positive substrate strain promote net MT assembly in SMCs cultured on flexible substrates, while compressive forces imparting negative substrate strain drive net MT disassembly. In parallel, the equilibrium set point between monomeric and polymeric tubulin can be shifted simply by changing the amount of available ligand used for cell adhesion.

In this study, MTs responded to mechanical strain in the same manner for SMCs cultured on both fibronectin and type I collagen, independent of tyrosine phosphorylation. These findings support the idea that this response is due to the direct mechanical coupling between the ECM and the cytoskeleton. Although we have not exhaustively tested matrix molecules or signaling inhibitors to determine whether the MT response to strain is universal, the finding that the response is the same on fibronectin and collagen is distinct from other mechanical signaling pathways that show a dependence on the identity of the ECM (12, 22, 38, 41, 43). Stretch-induced extracellular signal-regulated kinase activation has been shown to occur only on a fibronectin matrix, suggesting that the particular integrin used to transmit the mechanical signal is critical in determining the fate of the cell (41). Similarly, strengthening of cytoskeleton-integrin linkages in response to an applied force has been shown to be regulated by the tyrosine kinase Src in an ECM-dependent fashion (22). We do not believe that our results are in opposition to these earlier studies. Instead, in response to a mechanical signal, a cell presumably receives many parallel mechanical and chemical signals, all of which must be integrated and understood to generate the appropriate response. Hence, altering the mechanics of the cytoskeleton may not be sufficient alone to explain the ECM-dependent mechanotransduction responses reported in numerous other investigations. However, directly altering the mechanics of the MT network may be permissive for other downstream signaling events, acting in concert with, and not instead of, numerous soluble signaling pathways.

While often overlooked in the study of mechanotransduction, MTs have been studied extensively in the process of neurite extension, clearly contributing to their elongation (17, 18, 37, 55, 63). These findings provided much of the groundwork and initial support for a tensegrity-based model for the cytoskeleton. In a tensegrity model for the cell, the cytoskeleton exists in a dynamic balance of forces, with externally applied mechanical forces superimposed on this preexisting force balance (33–35). Reorganization of the cytoskeleton in response to mechanical forces has been widely reported, particularly for endothelial cells exposed to shear forces (11, 16, 25, 42, 57). Application of a restraining force via optical tweezers (12, 22) and twisting torques with a magnetic twisting device (59) both have been shown to result in a strengthening, or stiffening, of the cytoskeleton. Our findings in this present study support the possibility of MTs acting as compression-resistant struts. Presumably, applying a tensile strain to the substrate reduces the inherent compressive stress on the MTs, favoring a net increase in assembly. By contrast, applying a compressive strain to the substrate increases the compressive stress on the MTs, favoring a net disassembly of MTs.

Numerous investigations have studied MTs in the context of cell contractility (e.g., cell-generated forces) in place of the externally applied forces used in this study. These studies demonstrate that depolymerization of MTs with pharmacological agents (i.e., colchicine or nocodazole) induces formation of actin stress fibers and increased focal adhesions, the hallmarks of contractility (15, 20, 21, 40). The mechanism of increased contractility appears to involve greater myosin light chain phosphorylation, which results from drug-induced MT assembly (39). Other investigators have also demonstrated that changes in the contractility of...
the cytoskeleton are, in part, due to the small GTP-binding protein RhoA, a central signaling molecule that has been linked to many downstream responses (14, 56). Drug-induced depolymerization of MTs has been shown to trigger an increase in contractility and actin stress fiber formation (13, 20) via activation of Rho (21, 40). It is now believed that cross talk between Rho and Rac may influence the activity of myosin light chain by regulating the activity of myosin light chain phosphatase and kinase (3). All together, this study and past reports indicate MT assembly may be regulated by mechanical signals both directly and via chemical signaling pathways. Changes in MT assembly may influence a number of downstream signaling pathways that subsequently influence cell functions (27).

Our findings in this study disagree with those from a recent study by Heidemann et al. (29) in which local forces were applied to cells expressing green fluorescent protein-tagged MTs with the use of calibrated microneedles. We hypothesize that MTs in cells subjected to whole cell mechanical perturbations via integrin receptors (as in our study) will respond differently than will MTs in cells exposed to local mechanical manipulations. In our system, mechanical signals transduced via the ECM through integrin receptors act on an integrated cytoskeletal network, changing MT assembly. While we cannot rule out alternative explanations to describe how local perturbations in the mechanical microenvironment may affect the cytoskeleton, clearly MT assembly is influenced by mechanical forces in our system.

The pseudoequilibrium between tubulin monomers and MTs was altered in this study simply by changing the density of ECM ligand. Changes in the ECM density alone have been shown to trigger changes in cell shape for cultured hepatocytes, modulating the assembly of their MTs (44, 45). However, unlike results in those studies, our results demonstrate that changing fibronectin density causes only slight changes in cell shape for cultured hepatocytes, modulating the assembly. While we cannot rule out alternative explanations to describe how local perturbations in the mechanical microenvironment may affect the cytoskeleton, clearly MT assembly is influenced by mechanical forces in our system.

In summary, we have shown that MT assembly can be predictably controlled in SMCs by externally applied forces and changes to the ECM, suggesting that control of MT assembly is, in part, mechanical in nature. While the physiological relevance of mechanical control of MT assembly is unclear, changes in MT assembly may be critical to integrate the complex mechanical and chemical signaling events involved in a wide variety of cellular phenomena, including mechanotransduction and cell migration.

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