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Modulation of Nuclear Shape by Substrate Rigidity

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Abstract—The nucleus is mechanically coupled to the three cytoskeletal elements in the cell *via* linkages maintained by the LINC complex (for Linker of Nucleoskeleton to Cytoskeleton). It has been shown that mechanical forces from the extracellular matrix (ECM) can be transmitted through the cytoskeleton to the nuclear surface. Here we quantified nuclear shape in NIH 3T3 fibroblasts on polyacrylamide gels with a controlled degree of cross-linking. On soft substrates with a Young's modulus of 0.4 kPa, the nucleus appeared rounded in its vertical cross-section, while on stiff substrates (308 kPa), the nucleus appears more flattened. Over-expression of dominant negative Klarsicht ANC-1 Syne Homology (KASH) domains, which disrupts the LINC complex, eliminated the sensitivity of nuclear shape to substrate rigidity; myosin inhibition had similar effects. GFP-KASH4 over-expression altered the rigidity dependence of cell motility and cell spreading. Taken together, our results suggest that nuclear shape is modulated by substrate rigidity-induced changes in actomyosin tension, and that a mechanically integrated nucleus-cytoskeleton is required for rigidity sensing. These results are significant because they suggest that substrate rigidity can potentially control nuclear function and hence cell function.

Keywords—Nucleus, LINC complex, Substrate rigidity, Mechanosensing, Polyacrylamide gels.

INTRODUCTION

Cells can sense and respond to a diverse variety of mechanical cues from their environment including shear forces,[13] matrix strain[46] and matrix rigidity.[15] In particular, the rigidity of the matrix has emerged as a key parameter for controlling cell function for diverse applications in regenerative medicine.[25] Cell motility,[33,40,41,65] adhesion,[16,40,65] spreading[49,65] and differentiation[16,17] have been shown to vary significantly between soft and rigid substrates. Rigidity sensing is mediated by the intracellular cytoskeleton. On rigid substrates that can support larger mechanical stresses, intracellular tension is high and cells are able to assemble clear stress fibers and focal adhesions.[21,32,44,60,65] Cells on very soft substrates are unable to assemble stress fibers and therefore generate much lower levels of tension. Because intracellular tension is balanced in part by the nucleus and can induce nuclear shape changes,[11,34,37,48] this raises the possibility that nuclear shape could be sensitive to substrate rigidity. It may also be possible that nuclear-cytoskeletal linkages are required for rigidity sensing, given that disrupting cytoskeletal linkage with the nuclear surface disrupts cellular reorientation in response to applied substrate strain[11] and alters myoblast mechanotransduction and differentiation under strain.[7]

The cytoskeleton is mechanically linked to the nucleus by the LINC complex (Linker of Nucleoskeleton to Cytoskeleton) of nuclear envelope embedded proteins.[8,14,50,61] Since the cytoskeleton itself is linked to cell–matrix adhesions, there is a continuous mechanical link between the nucleus, the cytoskeleton and the extracellular matrix.[50,61] These linkages might cooperate with chemical signal transduction pathways in the reciprocal cross-talk between the nucleus and the extracellular matrix.[2–4] Lamins, the class V intermediate filament family proteins that form the nuclear lamina under the inner nuclear membrane, are key components of the LINC complex. The nuclear lamina is connected to chromatin (primarily transcriptionally silent heterochromatin[54]). The SUN1/SUN2 homotrimers[67] bind to lamins[24] and cross the inner nuclear membrane. The SUN proteins in turn are connected to the KASH-domain containing nesprin family of proteins that cross the outer nuclear membrane[52,56] and bind to the cytoskeleton. Nesprin proteins bind through dynein or kinesin motors to the microtubules,[43] actin filaments,[51] or through plectin to intermediate filaments,[28,58] thereby

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creating a molecular link between the cellular cytoskeleton and the structures of the nuclear periphery.[34,43,58,66]

In this work, we found that the nucleus in NIH 3T3 fibroblasts was of a spherical shape on soft substrates and a flattened ellipsoid on rigid substrates. Rigidity dependence of the nuclear shape was abolished upon LINC complex disruption, myosin inhibition, or in cells cultured on very thin gels. LINC complex disruption also eliminated the rigidity dependence of cell motility and spreading. Collectively, our results suggest that nuclear shape is sensitive to substrate rigidity, and an integrated nucleus-cytoskeleton is required for rigidity sensing in NIH 3T3 fibroblasts.

METHODS

Cell Culture

NIH 3T3 fibroblasts were cultured in DMEM with 4.5 g/L glucose (Mediatech, Manassas, VA) supplemented with 10% donor bovine serum (Gibco, Grand Island, NY), 1% Penn-Strep (Mediatech) and maintained at 37 °C in a 5% CO_2 environment. The GFP-KASH4 plasmid construct was used as described previously,[43] and was transfected into living cells for 6 h using Lipofectamine™ 2000 Reagent and OPTI-MEM media (Invitrogen, Carlsbad, CA). Cells were allowed to grow on substrates for 24 h before fixation and imaging. For experiments with cells exposed to 100 μM blebbistatin (EMD Biosciences, La Jolla, CA), the treatment was given for 1 h prior to fixation at 24 h of total growth.

Hydrogel Preparation, Functionalization, and Characterization

Polyacrylamide (PAA) hydrogels of known stiffness were prepared on glass-bottom dishes according to the methods in Pelham and Wang.[40] Briefly, glass-bottom dishes were treated with APTMS (Aldrich, St. Louis, MO) and glutaraldehyde (Fisher, Waltham, MA), prepolymerized polyacrylamide (PAA) was dropped onto the dish, and a coverslip was laid over to flatten the gel during polymerization. Upon removal of the coverslip, each gel was rinsed with 200 mM HEPES buffer (Mediatech), functionalized *via* Sulfo-SANPAH (Fisher) treatment, and human fibronectin at 5 μg/mL (Fisher) was coated overnight before seeding cells. Four different ratios of acrylamide and bis-acrylamide (Fisher): 50:1, 40:1, 20:1, and 12.5:1 were chosen to make gels with Young's modulus of 0.4 kPa, 24.5 kPa, 38.7 kPa and 308 kPa. The Young's modulus of the polyacrylamide gels was measured with an AR-G2 rheometer (TA Instruments). 1 mL of pre-polymerized PAA was pipetted onto the bottom plate, and a 60 mm diameter 1° steel cone with gap size 30 μm was lowered onto the solution until it became fully polymerized. 10% strain was applied to the gels and storage modulus (G') values were recorded over a range of angular frequencies of 0.1–10 rad/s. Plateau values of G' were taken at 1 rad/s and used to calculate $E = 2 * G' (1 + \upsilon)$, where the Poisson's ratio $\upsilon = 0.48$.[5] Rheometer values were very close to those reported by Putnam and coworkers[41] for the 50:1, 40:1 and 20:1 crosslinked gels; the rheometer measurements were unreliable for the 12.5:1 gel. For this gel, we used a Young's modulus of 308 kPa based on experimental measurements in Peyton and Putnam[41] for the same crosslinking concentration.

Gels of controlled thickness were cast by forming a drop of known volume of acrylamide solution onto a glass-bottom dish and flattened by a coverslip of known diameter during the polymerization process. This allowed us to make gels of 5, 10, 15, and 20 μm nominal thickness. Gels were dried overnight under gently flowing air, and their actual thickness was measured using spectral reflectance on a Filmetrics F40 photospectrometer. Heights are reported in μm ± SEM from 5 thickness measurements per gel.

Immunostaining

Cells were fixed in 4% formaldehyde (Red Bird Service, Batesville, IN) for 20 min at room temperature, rinsed with PBS (Mediatech), and then permeabilized with 0.1% Triton X-100 in 1% bovine serum albumin solution. For imaging of actin, cells were incubated with Alexa Fluor 594 phalloidin (Invitrogen) for 1 h. Nuclei were stained with Hoechst 33342 (Invitrogen) diluted 1:100 for 1 h. To check fibronectin coating density, polyacrylamide gels were coated with 5 μg/mL fibronectin overnight at 4 °C. Gels were then incubated with mouse monoclonal primary antibody against fibronectin (Abcam, Cambridge, UK) at 1:100 dilution. Samples were washed then treated with secondary antibody goat anti-mouse 488 nm (Invitrogen) at 1:500 dilution. The fluorescence intensity of labeled fibronectin on the different gels was quantified from images taken under constant imaging conditions. No significant difference in intensity levels was seen suggesting that fibronectin adsorption does not change appreciably on the different gels (Fig. S1).

Measurement of Nuclear Dimensions

Cells on gels were imaged on a Leica SP5 confocal microscope equipped with a 63× oil immersion objective. To visualize the vertical cross-section of nuclei, z-stacks of 0.3 μm step size were taken, and 3D projections were made using Leica Application Suite

Advanced Fluorescence (LAS-AF) software. The major axis of a nuclear cross-section was taken as the longest diameter, and the minor axis was drawn perpendicular to the major axis. Nuclear dimensions (a, major axis; b, minor axis; h, height) were calculated from fluorescence profiles measured at points on a line drawn along the axes of symmetry of the nuclear cross-section. The full width at half maximum (FWHM) method was used to calculate the dimension[31]; briefly, each dimension was measured as the distance between two shoulder points which were at half of the maximum intensity. The area was calculated as $A = \pi ab$, and the volume as $V = (\pi/6)abh$. Nuclear height and volume are apparent measurements as the images were not calibrated with standards of known dimensions.

Cell Motility Assay

To measure cell motility, cells were cultured on fibronectin-coated gels for 6 h prior to imaging. Movies of 10 min intervals were taken for 12 h on a Nikon TE2000 microscope equipped with a 10× phase contrast lens. An enclosed chamber kept the cells in a humidified, 5% CO_2 and 37 °C environment during imaging. Images were processed using Nikon Elements software and ImageJ. Matlab was used to track centroid position of the cells in (x, y) coordinates, and to calculate the mean cell speed. Cell speed was calculated as the mean displacement between successive frames divided by the time interval of 10 min (frames were collected at 10 min intervals). A minimum of 8 cells were measured for each condition.

Statistical Analysis

All data are presented as mean ± SEM, all statistical comparisons were made with Student's t test.

RESULTS

Nuclear Shape is Sensitive to Substrate Rigidity

For controlling the rigidity of the substrate, we chose the polyacrylamide gel assay in which the Young's modulus is varied by changing the degree of cross-linking. The volume of the nucleus is expected to increase with the progression of the cell through the cell cycle.[64] To control for the fact that the rigidity of the polyacrylamide gels may affect cell cycle progression and the relative fractions of cells in G1, G2 and S, we first performed cell cycle analysis on the different substrates. Cells grown for 24 h on gels were suspended, fixed, stained with Hoechst 33342 and analyzed with a BD Biosciences LSR II flow cytometer to measure the populations in G1, G2 and S phase. As shown in Fig. S2, no significant differences in cell fractions of cell cycle phases were observed between the different substrates. The FACS analysis suggested that cell synchronization was unnecessary, and hence all experiments were performed with unsynchronized cells cultured in full growth medium.

A recent paper[55] argued that cells do not sense the rigidity in the polyacrylamide gel assay, but rather differences in adhesive ligand crosslinking owing to varying porosity in different gel substrates. To account for potential differences in adhesive ligand crosslinking, we used an assay in which the polyacrylamide crosslinking was kept constant and the thickness of the gel was varied. As the gels are cast on glass, cells are expected to start pulling on the underlying glass for a thin enough gel[9,10,47] such that a gel that is soft owing to a low degree of cross-linking will appear 'rigid'. If cells do not sense rigidity of the gel but rather the geometry of the porous matrix that controls adhesive ligand crosslinking, then decreasing the thickness is predicted to have no effect on cell and nuclear morphology.

Shown in Fig. 1a are x–z cross-sections of representative nuclei from cells grown on polyacrylamide gels of controlled rigidity and thickness. The x and y directions lie in the plane of the underlying substrate, and the z direction indicates the axis perpendicular to the substrate. Nuclear height was high on soft substrates and low on rigid substrates on the 22 μm thick gels. In addition, cells appeared rounded on soft substrates with few stress fibers, while on more rigid substrates, the mean spreading area of cells was high and the cells assembled visible stress fibers (Fig. S3). Shown in Fig. 1b is a quantification of nuclear heights from at least 30 cells per condition. There is a clear decrease in nuclear height from the soft to the stiff substrates.

On the 17 μm thick gel (Figs. 1a, 1c), there are still some differences in nuclear height between cells cultured on substrates of different moduli. However, on the 12 and 6 μm thick gels, nuclear height is insensitive to the Young's modulus (Figs. 1a, 1d and 1e). The average value of the nuclear height on thin gels (6 and 12 μm thick) is similar to the nuclear height on the most rigid thick gels used in this study (308 kPa). Cell spreading and stress fiber formation that is normally observed on rigid substrates was observed on 6 and 12 μm thin gels independent of the Young's modulus (Fig. S3). Together, these results strongly support the conclusion that cells are sensing rigidity in the polyacrylamide gel assay, and that nuclear shape is sensitive to substrate rigidity.

Effect of LINC Complex Disruption on Nuclear Geometry

In an effort to understand the mechanism underlying nuclear shape dependence on substrate

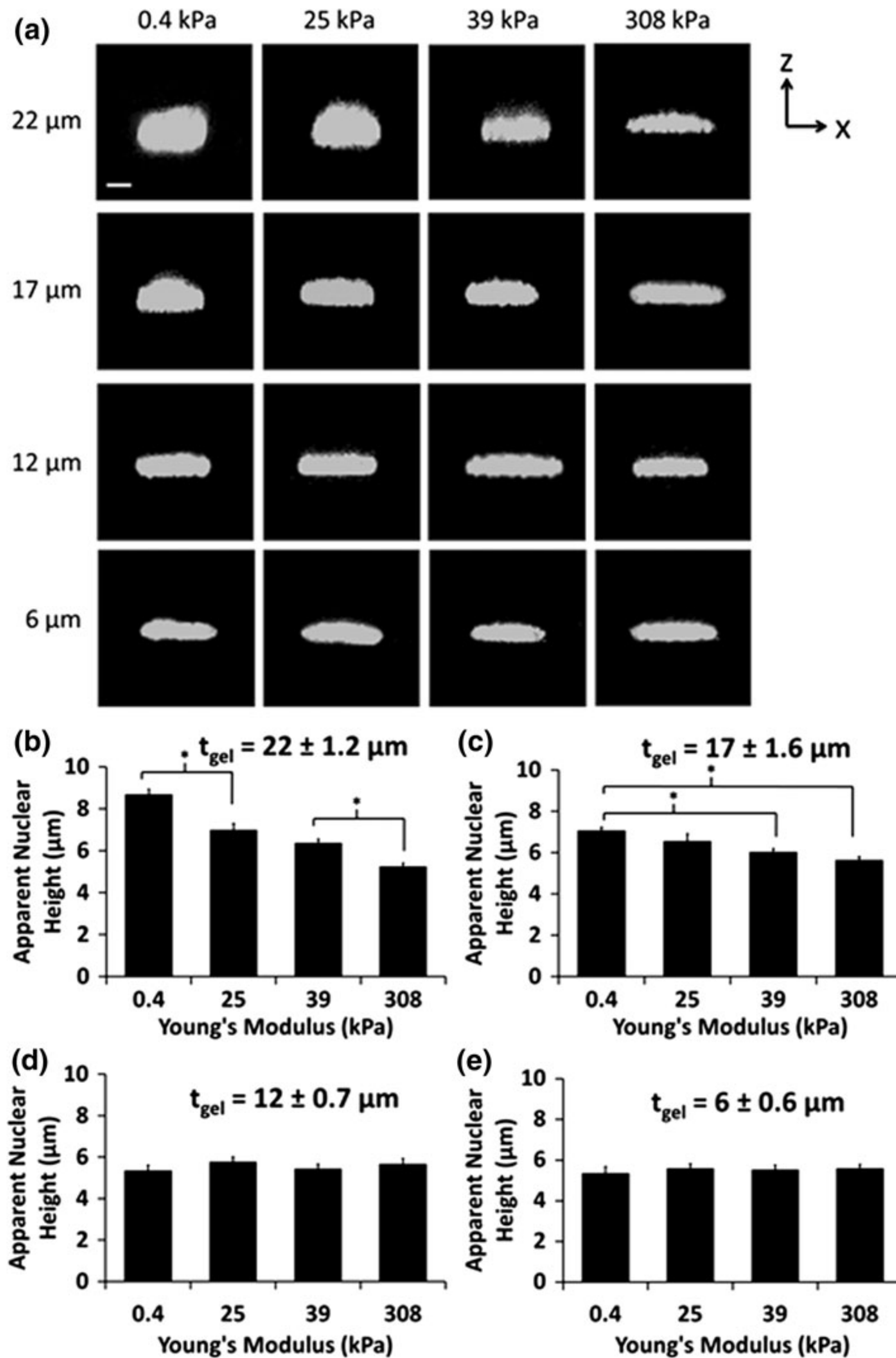

FIGURE 1. Nuclear shape depends on substrate rigidity and gel thickness. (a) Images are representative *x–z* cross-sectional reconstructions of 3T3 fibroblasts stained for DNA. Values on top indicate Young's modulus of PAA gels. Values on the left side indicate thickness of each gel. Nuclei become more flattened with increasing stiffness on 17 and 22 μm thick gels, while all nuclei are flattened on 6 or 12 μm thick gels. Scale bar = 5 μm. (b)–(e) show quantification of apparent nuclear height on gels of controlled thickness and Young's modulus. t_{gel} is gel thickness (mean ± SEM with n = 5 per gel). On thicker gels (b and c), the apparent nuclear height varies with the Young's modulus, but on the thinner gels (d and e), nuclei are flattened and insensitive to Young's modulus. * indicates $p < 0.05$, $n = 30$ for each condition.

rigidity, we over-expressed GFP-KASH4 which disrupts nuclear-cytoskeletal linkages through competitive binding to SUN proteins (Roux *et al.*[43]; Fig. S4 shows GFP-KASH4 localization to the nuclear envelope). On KASH4 over-expression, the shape of nuclear *x–z* cross-sections was found to be insensitive to substrate rigidity (Fig. 2a). On more rigid substrates, the mean spreading area of cells was high and

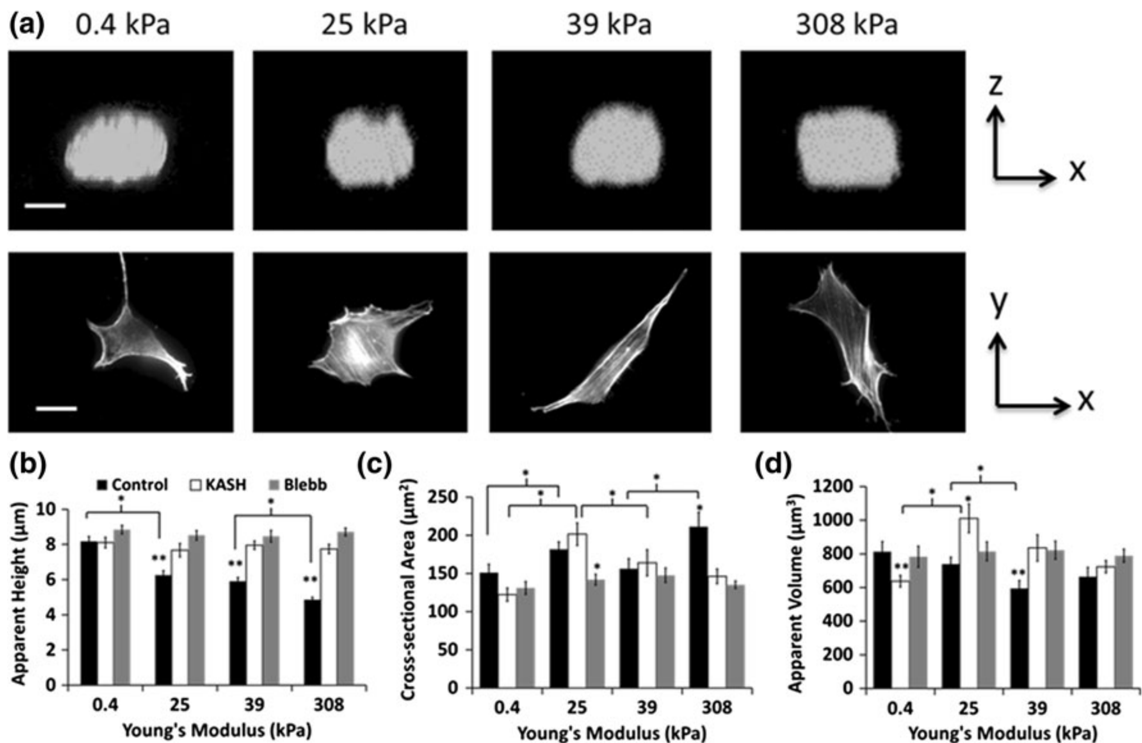

FIGURE 2. Rigidity dependence of nuclear shapes is eliminated on KASH4 over-expression or myosin inhibition. (a) Panels show representative cross-sections of nuclei generated from confocal images and images of the F-actin cytoskeleton in cells expressing GFP-KASH4 and cultured on substrates of controlled rigidity. Nuclei appear rounded on all substrates (top panel, scale bar = 5 μm), and cells do not spread well or assemble many stress fibers (bottom panel, scale bar = 20 μm). Values on top indicate Young's modulus of PAA gels. (b) Rigidity dependent trends are observed in nuclear height for control cells but not for KASH4-expressing or blebbistatin-treated cells. Nuclear cross-sectional area (c) and volume (d) do not show significant dependence on substrate rigidity in control, KASH4-expressing and blebbistatin-treated cells. Error bars indicate SEM, *n* = 30 cells per condition, * $p < 0.05$, ** $p < 0.05$ between control and both KASH4 cells and blebbistatin-treated cells.

the cells assembled clearly visible stress fibers (Fig. S3). On GFP-KASH4 over-expression, cells did not spread as well, even on the stiff substrates. KASH4 expressing cells assembled basal stress fibers, but fewer than control cells on all substrates (Fig. 2a). Thus, disrupting the nucleus-cytoskeleton linkage not only alters nuclear shape by dissipating nuclear tension, but also alters cytoskeletal organization.

Shown in Fig. 2b are comparisons of nuclear heights measured on the different substrates under different conditions. The clear trend in the nuclear height as a function of substrate rigidity observed in control cells is not present in KASH4 expressing and blebbistatin treated cells (blebbistatin inhibits non-muscle myosin II ATPase activity,[23,30]). While the nuclear cross-sectional area (*x*–*y* plane) differed modestly between soft and rigid substrates (Fig. 2c), nuclear volume did not differ significantly on the different substrates in control, KASH4 expressing and blebbistatin treated cells (Fig. 2d). Similarly, no significant trends were observed in the *x*–*y* aspect ratio—the minor axis divided by the major axis of the nuclear *x*–*y* cross-section (Fig. S5C); nuclear shape remained roughly elliptical on the different substrates. In summary, nuclear volume was insensitive to substrate rigidity while the height of the nuclei was smaller on rigid substrates compared to soft substrates. These effects of substrate rigidity on nuclear shape were absent in cells where the LINC complex was disrupted or myosin II was inhibited. The results can be explained with a model in which actomyosin generated forces are high on the nuclear surface on rigid substrates; the fact that actomyosin generated traction stresses in the cell are higher on rigid substrates supports such a model.[19,44,62,63]

Effect of the Perinuclear F-actin Cap on Nuclear Shape

In NIH 3T3 fibroblasts, Khatau *et al.*[29] showed the presence of a perinuclear F-actin cap which forms a dome-like network above and to the side of the nucleus in cultured cells. Apical F-actin cables may transfer mechanical forces to the nuclear surface.[35,39] We therefore asked if the rigidity dependence of nuclear shape was due to rigidity dependence of the F-actin cap. We first quantified the presence of the F-actin cap on different substrates by performing confocal microscopy of actin stress fibers at different confocal planes in the cell

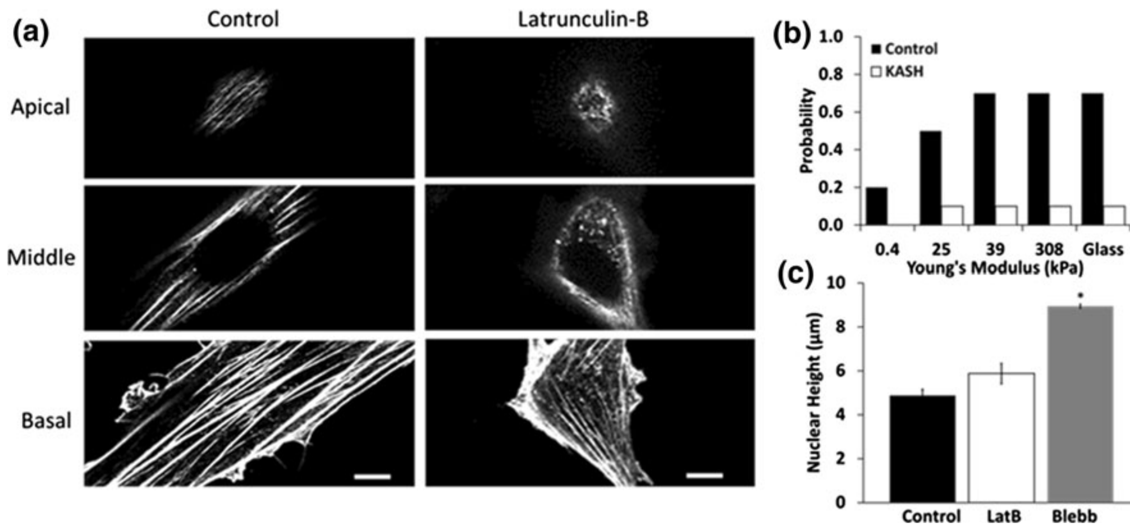

FIGURE 3. The perinuclear F-actin cap is not the dominant contributor to nuclear force. (a) Representative images of F-actin stress fibers on the apical, middle and basal planes relative to the nucleus. Treatment of cells with latrunculin-B at low dosages disrupts a majority of apical stress fibers, while leaving basal stress fibers intact. (b) Plots show the probability of observing distinct apical stress fibers in fibroblasts on different substrates. The F-actin cap increases in probability with rigidity; GFP-KASH4 over-expression eliminates this dependence. (c) Nuclear height increases only modestly on disrupting the actin cap in comparison with the increase in nuclear height due to blebbistatin treatment. Error bars indicate SEM, $n = 10$ cells per condition,* indicates $p < 0.05$.

(Fig. 3a).The F-actin cap can be selectively disrupted by treatment with latrunculin-B (Invitrogen) as demonstrated previously.[29] We found that on stiff glass substrates, treating cells with latrunculin-B (80nM for 30 min.) preserved basal stress fibers (Fig. S6B-D) but eliminated actin bundles on the apical surface of the nucleus consistent with the results in Khatau *et al.*[29] (Figs. 3a and S7A). To confirm that actin cap presence was not altered by the latrunculin B solvent, DMSO, we cultured cells with the same amount of DMSO (Fisher) for 30 min prior to fixation at 24 h. We saw no significant change in actin cap presence between control and DMSO-treated cells (Fig. S8).

Interestingly, cells containing apical stress fibers (on top of the nucleus) were more frequent on stiff substrates (Fig. 3b), suggesting that the F-actin cap is more probable on stiff than soft substrates (similar effects were observed for basal stress fibers, Fig. S6). We next disrupted the F-actin cap selectively with latrunculin-B on the most rigid substrate (glass) where the effects on the nuclear height are most pronounced. However, the nuclear height changed only by a modest amount on disruption of the F-actin cap compared with changes in the nuclear height on myosin inhibition with blebbistatin (Fig. 3c; also compare with differences in nuclear height on the different substrates in Fig. 2b in control cells). These results suggest that the perinuclear F-actin cap is not the dominant determinant of nuclear shape in NIH 3T3 fibroblasts, even on the stiffest substrate studied here where intracellular actomyosin forces are expected to be maximal.[19,21,22,41,45,49,57,62,63]

An Intact LINC Complex is Required for Rigidity Sensing

We next tested the hypothesis that an intact nuclear-cytoskeletal complex is required for rigidity sensing. Single cells were cultured on substrates and their random crawling imaged over several hours. Cell speed was computed from the measured cell trajectories. The speed of cell motility and the spreading area were observed to depend on substrate rigidity; however, over-expression of GFP-KASH4 eliminated the rigidity dependence of both speed and cell spreading (Figs. 4a, 4b; Fig. S9). The spreading area and mean cell speed both were significantly reduced in KASH4 overexpressing cells.

DISCUSSION

An important finding in this paper is that the nucleus is rounded on soft substrates and is flattened on stiff substrates. The rounded cross-section of the nucleus on soft substrates is likely due to the low actomyosin forces generated in cells on soft substrates; on more rigid substrates, actomyosin forces increase and hence the nucleus flattens in shape. This is consistent with observations by others[27] that the nucleus flattens and changes orientation as the cell spreads more.[6] Given the diversity of actomyosin tension generating structures in the cell, we now discuss some possibilities. First, the differences in nuclear shape between soft and rigid substrates appears not to be due

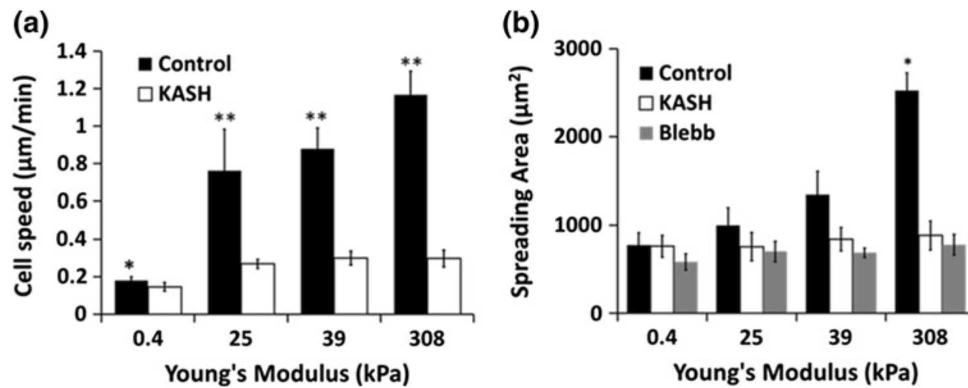

FIGURE 4. KASH4 over-expression disrupts rigidity sensing. (a) Mean cell speed increases with rigidity, but this rigidity dependence is abolished on KASH4 over-expression. Cell speed in KASH4 expressing cells also decreases significantly compared to control. * indicates $p<0.05$ between control cells on the softest gel and on any other stiffness. ** indicates $p<0.05$ between control and KASH4 cells on the same gel. (b) KASH4 expression or blebbistatin treatment similarly abolishes the dependence of cell spreading area on substrate rigidity. Cells expressing GFP-KASH4 are spread less than control cells. Error bars indicate SEM, $n = 10$ cells per condition, * indicates $p<0.05$ between 308 kPa and all other stiffnesses for control cells.

to a modulation of the perinuclear F-actin cap. We found only modest changes in nuclear height on disrupting the F-actin cap (which may compress the nucleus in the *z*-direction) compared with changes due to myosin inhibition. This suggests that actomyosin cables on the apical surface of the nucleus are not significant contributors to determining the vertical height of the nucleus at least in NIH 3T3 fibroblasts.

A second possibility is the presence of vertically downward compressive forces owing to the tensed, apical actomyosin cortex (which is distinct from actomyosin bundles that form the F-actin cap). In rounded cells on soft substrates, this downward compressive force is likely to be low, and as the cell spreads and flattens, the force is expected to be high. Physically, one would expect that such a compressive force would not require linkage between the nucleus and the cytoskeleton. A force that requires linkage would resemble more of a sideways pulling force on either side that flattens the nucleus. We tried to address this question by over-expressing GFP-KASH4 in the cell which delinks the nucleus from the cytoskeleton. While we did observe vertical rounding of the nucleus, there was also a concomitant decrease in the degree of cell spreading; therefore we are unable to discriminate between the two types of scenarios. We favor the pulling hypothesis where linkages with the tensed actomyosin cytoskeleton (or linkages with other nuclear-linked cytoskeletal elements that are pulled on by the actomyosin cytoskeleton) primarily pull on the nuclear surface and flatten it out. This is consistent with observations by others that pulling on integrin receptors distorts the nucleus[37]; it is difficult to envisage how this could happen with only a downward compressive force on the apical nuclear surface.

An important result of this study is also that KASH4 over-expression alters cytoskeletal organization and decrease in cell spreading. This supports the concept that the linkage between the nucleus and the cytoskeleton establishes not only nuclear shape and position,[20,36,38] but also organizes the cytoskeleton and hence stabilizes cell shape.[26] This is not unreasonable if one considers that the nucleus occupies significant space in the cell (10–15 μm in a cell of the order of 40 μm) and is very rigid. In this sense, it appears that an important nuclear function is to act like a scaffold inside the cell, functioning to balance and propagate cytoskeletal forces. Tuning of nuclear shape (through modulation of cytoskeletal forces) may in part explain why DNA, rRNA, mRNA and protein synthesis and ultimately cell fate are profoundly altered by cell shape.[1,12,18,42,53,59]

CONCLUSIONS

We have shown that in NIH 3T3 fibroblasts, nuclear shape is sensitive to substrate rigidity in a nuclear-cytoskeletal linkage dependent manner. Rigidity sensing in NIH 3T3 fibroblasts is disrupted on interfering with nuclear-cytoskeletal linkages. The results are consistent with a model in which substrate rigidity modulates nuclear tension.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi:10.1007/s12195-013-0270-2) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

The authors have no conflicts of interest related to this paper.

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