Published in final edited form as:

Nat Mater. 2010 January ; 9(1): 82–88. doi:10.1038/nmat2563.

Cell material property dictates stress-induced spreading and differentiation in embryonic stem cells

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Abstract

Growing evidence suggests that physical microenvironments and mechanical stresses, besides soluble factors, help direct mesenchymal stem cell fate. However, biological responses to a local force in embryonic stem (ES) cells remain elusive. Here we show that a local cyclic stress via focal adhesions induced spreading in mouse ES (mES) cells but not in mES cell-differentiated (ESD) cells that were 10-fold stiffer. This response was dictated by the cell material property (cell softness), suggesting that a threshold cell deformation is the key setpoint for triggering spreading responses. Traction quantification and pharmacological or shRNA intervention revealed that myosin II contractility, F-actin, Src, or Cdc42 were essential in the spreading response. The applied stress led to Oct3/4 gene downregulation in mES cells. Our findings demonstrate that cell softness dictates cellular sensitivity to force, suggesting that local small forces might play far more important roles in early developments of soft embryos than previously appreciated.

Keywords

cell rheology; mechanotransduction; prestress; gene expression; deformation

Embryonic stem (ES) cells are one of the major focuses in biology because of their pluripotency and potential therapeutic applications^{1–3}. While it is known that soluble factors are critical in stem cell differentiation^{4, 5}, recent evidence shows that the physical microenvironment of the cells (e.g., shape constraint or substrate stiffness) helps direct the fate of mesenchymal stem cells^{6, 7}. These cells, however, are downstream in cell lineage

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AUTHOR CONTRIBUTIONS N.W., F.C., T.S.T., F.W. designed the experiments; F.C. S.N., D.L., Y.C.P. performed experiments and analyzed the data. N.W., F.C., T.S.T., F.W. wrote the manuscript. The authors declare no conflict of interest.

Additional methods can be found in Supplementary Information.

specifications, and have limited self-renewal and differentiation capacities in comparison to ES cells. We focus on pluripotent ES cells since little is known about how these cells respond to mechanical forces. Understanding the fundamental processes by which ES cells respond to force is crucial in elucidating mechanisms of lineage determination and development as these cells are derived from the inner cell mass of blastocysts prior to gastrulation that initiates dynamic cellular rearrangements.

It is known that living cells alter their shapes and functions in response to mechanical forces. For example, unidirectional laminar shear flow stresses over a whole endothelial cell facilitate cell spreading and elongation in the direction of the flow⁸. Uniaxial stretching of a vascular smooth muscle cell elongates the cell in the direction of stretching⁹. Cyclic uniaxial stretching of whole mesenchymal stem cells increases cell proliferation and expression of smooth muscle cell markers¹⁰. Recently, it is reported that fluid shear stress over whole hematopoietic progenitor cells promotes embryonic hematopoiesis¹¹. However, whether and how ES cells respond to a localized mechanical stress remain elusive.

During the last decade or so, the importance of substrate rigidity in cell functions is becoming increasingly clear^{7, 12–14}. The physical and mechanical cues of the extracellular matrix are transduced into intracellular rheological and biochemical changes via unknown mechanisms, but likely via conformational changes or unfolding of focal adhesion-based proteins¹⁵ and other proteins. On the other hand, several researchers have proposed that intracellular rheological properties are critical in understanding cellular behaviors^{16–18}. Therefore, it is suggested that *intrinsic* intracellular material mechanical properties govern cellular behaviors and functions. However, no experimental data are available to unequivocally show that intrinsic intracellular rheological properties of living cells are fundamentally important in cellular biological responses to force and in biological functions, despite recent discoveries at the molecular level on the unfolding of focal adhesion protein talin in vitro by force¹⁵, on integrin activation by force in living endothelial cells¹⁹, and on unfolding of spectrin in red blood cells by shear flow stress²⁰. This is *not* a trivial issue. Since in general any individual structural protein under stress is physically connected with the rest of the cytoskeleton network, the overall cell's or cytoskeleton's deformability should dictate how much this protein can be deformed as all forces must be balanced.

In this study, we demonstrate that adherent mES cells are softer and much more sensitive to a local cyclic stress than their differentiated counterparts. We show that the material property of the cell, the cell softness, dictates the stress-induced spreading response. We reveal the underlying signaling pathways in stress-induced spreading in mES cells. Oct3/4 (Pou5f1) expression in mES cells²¹ gradually disappears in response to the stress. Our results suggest that a local, small, cyclic stress plays a critical role in inducing strong biological responses in soft mES cells that originate from inner cell mass and in shaping embryogenesis during development.

First we measured the projected areas of mES cells and differentiated cells (derived from these mES cells) on different substrate stiffness overnight. As expected from a published report²², the mES cell-differentiated (ESD) cells increased their projected areas with increasing substrate stiffness (Supplementary Fig. S1). In contrast, mES cell projected areas

were maximal at a substrate stiffness of 0.6 kPa, similar to the “intrinsic” elastic stiffness of these mES cells (Supplementary Fig. S2). These results are consistent with a previous report that cell-substrate stiffness matching is crucial for normal cell functions²³.

Next we explored whether these soft mES cells could respond to a localized external stress. After a mES cell was plated on the substrate of 0.6 kPa overnight, we attached a 4- μ m RGD-coated magnetic bead on the apical surface of the cell and applied a small, oscillatory stress (17.5 Pa at 0.3 Hz) continuously (Supplementary Fig. S3a). Surprisingly, this small local cyclic stress induced time-dependent increases in the spreading of the mES cell. The stress-induced spreading occurred as early as ~30 s after the onset of stress application (Supplementary Fig. S3a). While it is expected that unidirectional stretching or stressing of a whole cell would elongate the cell in the direction of the stretching or the stress^{8,9}, it is not clear whether a small localized oscillatory stress of zero mean magnitude could induce cell protrusion and spreading in many different directions. mES cells on other magnitudes of substrate stiffness also spread in response to the applied stress but the extent of spreading was less, suggesting that the cell-substrate stiffness matching potentiates the optimal spreading response in mES cells to external stress. To quantify changes in cell area, we measured velocity profiles of the cell periphery using an established method²⁴. The mES cell increased normal membrane protrusion velocity and spreading area as a function of stress application time (Supplementary Fig. S3b–d). In sharp contrast, the stiff ESD cell on the same substrate stiffness did not exhibit any changes in normal velocity or cell projected area in response to the same amplitude of the cyclic stress (Supplementary Fig. S3e–h). The lack of stress-induced ESD cell spreading is not due to the limitation of the spreading capacity of these cells, since they continue to spread on stiffer substrates (Supplementary Fig. S1), likely to be driven by much greater myosin-II-dependent endogenous forces. The ESD cells on much stiffer substrates failed to spread in response to the external stress. The summarized data show that mES cells are much more sensitive to a localized cyclic stress than their differentiated counterpart ESD cells (Fig. 1a). The threshold amplitude of stress for mES cell spreading is between 3.5–17.5 Pa (Fig. 1a) and the optimal frequency for spreading is ~0.3–1 Hz (Supplementary Fig. S4), consistent with the published report that the optimal loading frequency for cytoskeletal deformation is ~1 Hz²⁵. Results from stiff human airway smooth muscle (ASM) cells (a well-established differentiated tissue cell type), plated on the same substrate (stiffness) that was coated with the same immobilized amount of collagen-1, showed that they did not spread to the same stress, similar to the stiff ESD cells (Fig. 1a), suggesting that our findings that inversely correlate cell stiffness with spreading responsiveness can be generalized to other cell types.

Cell Softness dictates response to stress

To explore the underlying biophysical mechanism of stress-induced spreading in mES cells, we compared the softness of mES cells with that of ESD cells. Softness is defined as the ratio of strain to stress and is the inverse of stiffness. Softness of mES cells was ~7 times higher than that of ESD cells on the same substrate (Fig. 1b). Since the applied stress was the same for both cell types, this result suggests that the soft mES cells were more responsive because of greater deformation or strains in these mES cells than in ESD cells. To further test this idea, we plated the ESD cells or the ASM cells on sparsely coated matrix

proteins (1 ng/ml collagen-1 on rigid glass overnight) to limit their projected areas and to increase their softness. As predicted, these round intact ESD cells and ASM cells also started to spread in response to the cyclic localized stress (Fig. 1 a, b). The greater the cell softness, the stronger the spreading response (i.e., the more increases in cell area in response to stress) (Fig. 1b). Furthermore, the relative softness of mES cells, round ESD cells, and ESD cells correlated inversely with respective densities of F-actin (Supplementary Fig. S5), consistent with the established evidence that F-actin is a major determinant in cell stiffness²⁶.

An alternative interpretation to our data is that the smaller the projected cell area, the stronger the spreading response to the externally applied stress. This interpretation is based on the fact that the baseline projected areas of differentiated cells are larger than those of the mES cells on the same substrate (Fig. S2). Thus it is possible that the biochemical responses to stress in these differentiated cells (such as Ca^{2+} influx) might have been similar to those in undifferentiated mES cells, but these biochemical signals were just not potent enough to cause further spreading. To determine whether it is the cell softness or the cell baseline projected area that controls the spreading or protrusion sensitivity to stress, we plated ESD cells or ASM cells on micropatterned adhesive islands (25- μm diameter circles) on the 0.6 kPa substrate coated with high density of collagen-1²⁷. Each ESD cell or each ASM cell on each island had a similar projected area as the mES cell on the 0.6 kPa substrate but was ~8 times stiffer. The ESD cell and the ASM cell failed to extend any protrusions in response to the same applied stress, as the soft mES cell did (Fig. 1c). These data indicate that it is the cell softness, not the projected area, that controls the protrusion and spreading responsiveness to stress. Taken together, these data suggest that the underlying biophysical mechanism for stress-triggered spreading is the deformation of the cytoskeleton and its associated proteins, providing a biological consequence and a functional significance to the recent findings on stress-induced conformational changes and/or unfolding of signaling molecules²⁸ and focal adhesion structural proteins¹⁵.

To further explore the underlying mechanical and biochemical mechanisms of stress-induced spreading in mES cells, we quantified changes in tractional stresses. Traction at the cell periphery increased within the first few minutes of stress application (Fig. 2b), which coincided temporally with the increases in cell areas (Fig. 2a). The ~50% elevation in traction at the cell periphery (Fig. 2c) was preceded by ~40% increases in phosphorylated myosin light chains at the cell periphery by 30 s (Fig. 2e), from the diffusive distribution pattern throughout the cytoplasm prior to the stress application (Fig. 2d), suggesting that myosin II-dependent traction generation at the cell periphery is essential in stress-induced spreading in mES cells.

Consistent with the aforementioned interpretation, pretreatment of the mES cells with myosin II ATPase inhibitor blebbistatin (50 μM for 30 min) or with myosin light chain kinase inhibitor ML-7 (25 μM for 20 min) completely prevented stress-induced ES spreading (Fig. 3a; Supplementary Fig. S6). Furthermore, pretreatment with Rho-associated kinase (ROCK) inhibitor Y27632 (50 μM for 20 min) also prevented spreading of mES cells (Fig. 3a; Supplementary Fig. S6), suggesting that ROCK is also critical in this process. Importantly, pretreatment with PP1 (10 μM for 1 hr), a specific Src tyrosine phosphorylation

inhibitor, blocked stress-induced ES cell spreading (Fig. 3a; Supplementary Fig. S6). This result suggests that Src is critical in the initiation of stress-induced spreading, consistent with a published report on the role of Src in the spontaneous early spreading of adherent cells²⁹. Interestingly, pretreatment with NSC23766 (100 μ M for 1hr), a specific inhibitor of Rac^{30,31}, did not block stress-induced spreading, suggesting that Rac was not important in stress-induced spreading of mES cells (Fig. 3a, Supplementary Fig. S6). The stress-induced cell spreading was specific to integrin-cytoskeleton pathways, since application of the same amplitude of stress via poly-L-lysine coated beads did not induce any changes in cell area in mES cells (Supplementary Fig. S7), consistent with recent findings that rapid Src activation by stress only occurs via activated integrins²⁸ and that an applied stress via integrins induces additional activation of integrins and phosphorylation of focal adhesion kinase¹⁹. Stress-induced spreading in mES cells were completely prevented by pre-treatment with Latrunculin A (0.1 μ g/ml for 30 min), consistent with the role of actin polymerization in cell protrusion and spreading. It should be noted that although these cytoskeletal drugs make the mES cells softer, they interfere with cytoskeletal dynamics and intracellular biochemical processes. Therefore these softer mES cells fail to spread in response to the applied stress, because cell spreading is a complex process that requires dynamic coordination of actin polymerization and myosin II²⁹.

It is known that Cdc42 mediates cell filopodia extension and cell spreading³². To determine the role of Cdc42 in stress-induced mES cell spreading, we infected the mES cells with small hairpin RNA (shRNA) for Cdc42 using lentiviruses. As shown in Fig. 3b and 3c (Supplementary Fig. S8), Cdc42 knockdown correlated well with the abolishment of stress-induced spreading in these mES cells, consistent with published results in the role of Cdc42 in integrin-mediated spreading of differentiated cells³². Our finding that stress-induced spreading in these mES cells depends on Cdc42 but not on Rac is interesting since it is well known that integrin-mediated cell spreading depends on Rac in differentiated cells^{32,33}.

Stress-induced mES cell differentiation

To further determine the long term effects of a local cyclic stress in mES cell functions, we examined the expression of stably transfected GFP driven by Oct3/4 promoter in undifferentiated cells cultured in the presence of leukaemia inhibitory factor (+LIF)³⁴. After a continuous application of a 17.5-Pa local stress at 0.3 Hz for only 60 min, Oct3/4 expression in these mES cells was downregulated by ~35% within 24 hrs, and by ~50% within 72 hrs, whereas control cells a few micrometers away in the same dish without stress continued to express Oct3/4 (Fig. 4, Supplementary Fig. S9). Since loss of Oct3/4 expression in ES cells is one of the hallmarks for differentiation³⁵, our results suggest that a local cyclic stress via a focal adhesion might be sufficient to drive a mES cell to differentiate. If our findings could be extended to early animal embryos, it would provide a novel way of locally differentiating a single cell of early lineage while keeping nearby cells undifferentiated.

Accumulating experimental evidence suggests that mechanical contractile forces play a role in development (reviewed in ^{ref. 36}). However, inability to access animal embryonic cells during early development makes it difficult to determine how important mechanical forces

are during early development of animals and how sensitive embryonic cells are to force. Cultured ES cells offer an excellent model for studying biological responses to force by inner cell mass cells. In a recent review, Discher et al. discuss the combined effects of growth factors, matrices, and mechanical forces in controlling stem cells³⁷. The importance of substrate stiffness in stem cell differentiation is highlighted. However, the underlying mechanism remains unclear. It has been reported that substrate elasticity modulates intracellular rheology: stiffer matrices result in stiffer cells⁷. In contrast, we show here that intracellular softness can determine cellular biological sensitivity to force at fixed substrate rigidity. Our current work reveals a biophysical mechanism of ES cells in dictating how ES cells respond biologically to a local small force via integrins. Our findings that the softness of mES cells makes them very sensitive to a local cyclic stress of physiologic amplitudes suggest that small local forces (either endogenously generated or from neighboring cells) might play far more important roles in early embryogenesis and development of animals than previously appreciated. Our result that the cytoplasm of mES cells is intrinsically soft is also in accord with a previous finding that the nucleus of human ES cells is intrinsically soft³⁸.

Molecular mechanism of mechanotransduction

Currently it remains elusive what are the intracellular molecular strain sensor(s) in a live cell, although the extracellular domains of integrins have been shown to undergo force-dependent conformational change to enhance adhesion¹⁹, possibly via the catch bond mechanism³⁹. However, accumulating evidence points to the deformation of focal adhesion proteins and possibly other structural proteins as the molecular mechanism of strain sensing. For example, in vitro forcing experiments show that unfolding single talin rods activates vinculin binding¹⁵. It is likely that time-varying, strain-dependent conformational changes and/or unfolding of these protein molecules at focal adhesions⁴⁰ and at other distant sites⁴¹ (e.g., inside the nucleus) are the primary molecular mechanisms of mechanochemical transduction and strain-activated feedback loops⁴². An important test of this hypothesis will be to extend the in vitro work of del Rio et al¹⁵ to a live cell using physiologically relevant amplitudes of time-varying stresses. In addition, we speculate that focal adhesion-based protein opening and/or tyrosine kinase/phosphatase activation not only depends on the modulus of this individual molecule, but also depends on the collective modulus (or its inverse, softness) of the surrounding molecules and nearby cytoskeletal networks that consist of numerous parallel and serial viscoelastic molecular elements. The reason is that force must be balanced everywhere; therefore, the local cell softness near a focal adhesion must be crucial in determining how much a single molecule, such as talin, and other proteins, can be deformed and thus activated.

It might not be a coincidence that an unfertilized egg has a stiffness of ~ 10 Pa⁴³, an ES cell has a stiffness of ~ 500 Pa (Supplementary Fig. S2), a brain neural cell has a stiffness of ~ 100 – 500 Pa¹², a typical differentiated tissue cell (e.g., a smooth muscle cell) has a stiffness of 1 – 5 kPa⁴⁴, a skeletal muscle cell has a stiffness of ~ 12 kPa²³. The respective softness of various types of cells might manifest their different physiological functions and sensitivities to force in a multi-cellular organism. An evolutionary advantage for an early lineage cell to become stiffer as the cell divides and differentiates into a more differentiated tissue cell

might be to protect the organism from injuries by force, since the ability to respond to touch and to resist mechanical stress is postulated to be one of the most primitive features of metazoans that had evolved millions of years ago. Matching cell material property with that of its substrate is known to be critical in forming striation in skeletal muscle cells²³ and optimizing cardiomyocyte beating⁴⁵, but stiffness matching may have broader implications. As proposed recently⁴⁶, nutrient-rich uncompacted soft ocean sediments ~2 billion years ago provided a selective evolutionary pressure favoring those very earliest eukaryotes that were better able to perform mechanical functions of invasion, crawling and forage, which are optimized when material properties of the cell match those of their very soft paste-like microenvironment. We perhaps see here in the ES cell the echo of those early evolutionary events.

It is established that stress can regulate gene expression, but those previous studies are generally performed by stretching or fluid flow shearing whole cell surfaces, followed by analyses of average biological responses from millions of cells. Hence, it is difficult to elucidate mechanisms of mechanosensing and mechanotransduction. To our knowledge, our current study reveals for the first time that a small cyclic stress over a focal adhesion can downregulate Oct3/4 gene expression in single mES cells, likely due to the soft material property of these cells. It is known that germ layer cells migrate greatly during gastrulation to initiate cellular rearrangements that are tension-dependent. Therefore it is not clear if the rotational shear stresses applied via the magnetic twisting cytometry technology could mimic that physiological process. Future studies are needed to elucidate the specific mechanisms of stress-induced inhibition of Oct3/4 expression in these mES cells, to determine if these findings can be extended to human ES cells and induced pluripotent stem (iPS) cells, and to find out whether stress-induced signals inhibit known pluripotency-supporting pathways mediated by molecules such as mTOR⁴⁷. It will also be interesting to determine what type of germ layer cells (endoderm, mesoderm, or ectoderm) can be derived from these soft ES cells by what mode of mechanical perturbations.

METHODS

Cell culture and differentiation assay

Cells were thawed and cultured as described previously⁴⁸. In short, undifferentiated mouse embryonic stem (mES) cells (W4, 129/SvEv) were maintained in the standard culture condition in the presence of Leukaemia Inhibitory Factor (LIF; Chemicon). mES cells at passage 11 were thawed onto a feeder layer of mitotically-inactivated primary murine embryonic fibroblasts (mEF). mES cells were passaged onto culture dishes coated with 0.1% gelatin for several times in every 2 days to remove feeders. For the differentiation assay, trypsinized mES cells, at passage 15–16, were plated on gelatin-coated dishes at a low density of 100 cells/cm². Following day, the mES cells were fed with the medium without LIF and with 1 μ M Retinoic Acid (all-trans, Sigma) (–LIF/+RA). The mES cells in these conditions were fed with fresh medium every day for 4–5 days before experiment. In –LIF/+RA culture condition mES cells became differentiated to a heterogeneous population of differentiated cells (ESD) cells which were cultured with the complete medium.

Human airway smooth muscle (ASM) cells were isolated at autopsy within 8 hrs of death from tracheal muscle of lung transplant donors (approved by the University of Pennsylvania Committee on studies involving human beings) at University of Pennsylvania in Dr. Panettieri's laboratory⁴⁹. We used de-identified HASM cells supplied by Dr. Panettieri who obtained the tissue through NDRI (National Disease Research Interchange) in a manner that excludes all unique identifying information. All our procedures were approved by the Institutional Review Board of University of Illinois at Urbana-Champaign. The ASM cells were cultured following published protocols²⁸.

Quantification of membrane protrusion velocity profiles

Edge velocity profiles display the edge dynamics during cell spreading. This technique is described before in details²⁴. We utilized their approach (CellMAP) where input was a high contrast time lapse sequence (5 sec interval) of a single cell and the outputs were the normal cell edge velocity as a function of space (over entire arclength) and time, mean normal velocity over time, and change in cell area.

Applying a local stress

Magnetic twisting cytometry (MTC) is a well established method for applying controlled and precise local mechanical stresses of physiologic magnitudes to a living cell^{18, 26, 44, 48, 50}.

Cell softness quantification

Cell stiffness measurement technique is described before^{18, 25, 26, 44, 48, 50}. The cell complex softness is defined as the ratio of strain to the applied stress (i.e., the applied specific torque) and thus is the inverse of the cell complex stiffness. Cell softness (unit=kPa⁻¹) is a useful parameter here because molecular motors (e.g., myosin II) are force (the independent variable) generators and because strain-dependent opening of proteins are likely to be important in changing protein activities and cell functions¹⁵.

Cell area and traction measurements

Cell spreading area was measured by ImageJ (NIH) using active contours algorithm. Cell traction measurements have been described in details elsewhere⁴⁶. Generated traction maps were used for further quantification. Based on the gray scale traction field we took a one micron thick annular section at the cell boundary at different time points and measured the intensity. Mean intensity within the annular section, representing the tractional stress developed at the cell boundary, at time zero (before twisting of magnetic bead) was set to 1 arbitrary unit (A.U.). Traction profiles were plotted over time around the boundary. Micropatterned adhesive islands on soft polyacrylamide gels were produced following published methods²⁷.

Lentivirus production and mES cell infection

For shRNA-mediated knockdown of Cdc42, the pLKO.1-puro Vector (Sigma-Aldrich) was used. We used Viralpower Lentivirus Packaging System (Invitrogen) to package lentivirus for Cdc42 knockdown, following the manufacturer's instructions.

Western Blot

To quantify Cdc42, infected mES cells were lysed directly with 200 µl laemmli sample buffer (BIO-RAD). 20 µl of each sample were analyzed by Western Blotting. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

EGFP and DsRed expressions in mES cells driven by Oct3/4 and CAGGS promoter

A mouse ES cell line, namely OGR1, that expresses EGFP under the promoter of Oct3/4 (Oct3/4::EGFP)⁵¹ was transfected with 0.5 µg of pCAGGSDsRedT3_T2A_Puro (T. S. Tanaka et al., unpublished results) with FuGene (Roche) according to the manufacturer. Then, OGR1 that expresses DsRed.T3 stably was selected by puromycin (2 µg/ml; Invitrogen). DsRed.T3 has no toxicity in mouse ES cells⁵².

Stress-mediated differentiation

To investigate if local application of a local cyclic stress is capable of down-regulating Oct3/4 expression in the long term, we used OGR1 cell line that simultaneously expresses EGFP and DsRed driven by Oct3/4 and CAGGS respectively. mES cells were plated sparsely on 0.6 kPa substrates on top of grid dishes to track particular cells of interest over a long period. Ferromagnetic magnetic beads were attached to the apical surface of the cells via integrins and incubated for 15 min. A 17.5-Pa local stress at 0.3 Hz was applied for ~1 hr, which increased mES cell spreading area by ~65% (Supplementary Fig. S11). EGFP and DsRed expressions driven by Oct3/4 and by CAGGS promoter respectively were monitored every few hours. Continued expression of DsRed, under the promoter CAGGS, indicates the cell to be in an active state of translation. Loss of EGFP expression indicates down-regulation of Oct3/4, one of the hallmarks for differentiation. The stressed cells were labeled as '+stress, +LIF/-RA' condition. The cells without beads (i.e., no stress) in the same dish were also monitored (-stress, +LIF/-RA). Other dishes were monitored and EGFP-Oct3/4 were quantified as negative (+LIF/-RA) or positive (-LIF/+RA) controls. 1 µM retinoic acid (RA) was used in the -LIF/+RA condition. Student's t-test was used for all statistical analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Michael Hong and Kevin Moore for their assistance. We thank R. Panettieri for providing human airway smooth muscle cells. This work was supported by NIH grants GM072744 (to N.W.), GM083812 (to F.W.), the USDA Cooperative State Research, Education and Extension Service, Hatch project ILLU-538-323 (to T.S.T.), and by University of Illinois (to N.W.).

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Figure 1. Cell softness dictates cell spreading response to stress

a, Stress-induced spreading in mES cells is amplitude-dependent. Amplitude is the magnitude of change in a sinusoidal oscillatory forcing system where the mean magnitude is zero. ES cells did not spread at 0 or 3.5-Pa stress but started to protrude and spread at 17.5-Pa stress ($n=7, 5$, or 9 cells for $0, 3.5$, or 17.5 Pa stress, respectively). There were no significant differences in cell area change between 0 and 3.5 -Pa stress ($p>0.58, 0.23$, or 0.68 at $3, 5$, or 8 min). In contrast, there were significant differences between 3.5 and 17.5 Pa stress ($p<0.0007, 4.92\times 10^{-5}$, or 5.66×10^{-5} at $3, 5$ or 8 min respectively). At 17.5 Pa stress, there was significant difference in cell area between 3 min and 5 min ($p<0.05$), but no significant difference in cell area between 5 min and 8 min ($p>0.23$). In sharp contrast, for ESD cells and ASM cells there were no stress-induced changes in cell area even at 17.5 -Pa applied stress ($n=7$ cells for both cell types). There were no significant differences in cell area change between 3 min and 5 min ($p>0.30$ for ESD and $p>0.09$ for ASM) or 5 min and 8 min ($p>0.47$ for ESD and $p>0.37$ for ASM). Round ESD cells and round ASM cells spread but to a lesser degree than mES cells (Supplementary Fig. S10). (Means $\pm$ s.e.; at least 3 independent experiments) **b**, Stress-induced cell spreading depends on cell softness. mES cells, ESD, and ASM cells were plated on similar culture conditions (high density of collagen-1, 100 $\mu\text{g/ml}$) and on the same substrate stiffness of 0.6 kPa. The change in cell area of ESD and ASM cells is statistically different from mES cells at 3 min ($p<0.05$). Round ESD and round ASM cells were plated on low density of collagen-1 (1 ng/ml) coated on the rigid glass. Changes in cell area (spreading) after 3 min of stress application (17.5 Pa at 0.3 Hz) were plotted. Note that stress-induced cell spreading appears to be proportional to cell softness. Cell softness correlates inversely with F-actin density in each cell type (see Supplementary Fig. S5). Mean $\pm$ s.e., $n=7, 9, 7, 7$, and 9 for ESD, round ESD, ASM, round

ASM, and mES cells respectively. **c**, Cell softness, rather than cell projected area, dictates spreading or protrusion responses to stress. Each ESD cell or ASM cell was plated on a micropatterned adhesive island (25- μm diameter circles) on 0.6 kPa substrate stiffness coated with 100 $\mu\text{g/ml}$ of type-1 collagen and thus was restricted to within an area of $\sim 500 \mu\text{m}^2$. The gel surface outside the islands was uncoated and thus was nonadhesive. No visible protrusion on the micropatterned ESD and ASM cells (μP ESD and μP ASM) was observed when stressed for 5 min. The data of μP ESD and μP ASM cells are significantly different from those of mES cells at 5 min ($p < 0.006$ and $p < 0.007$ respectively). Mean $\pm$ s.e., $n=5$, 5 and 9 for μP ESD, μP ASM and mES cells respectively.

Figure 2. Stress-induced spreading in mES cells correlates with accumulation of phosphorylated myosin light chain and elevation of tractions at the cell edge

a, A brightfield image shows the time course of a mES cell spreading in response to the applied stress (17.5 Pa at 0.3 Hz). **b**, Corresponding traction in the same ES cell in response to the applied stress. **c**, Average tractions at 1-μm annulus around the cell boundary as a function of time after stress application. A.U.=arbitrary unit, tractions normalized by the traction at zero applied stress. $n=8$ cells, mean $\pm$ s.e. **d**, Phosphorylated myosin light chain (MLC Phosph) was accumulated to the cell periphery (white arrow) 30 s after stress application in comparison to a diffuse cytoplasmic distribution at time zero. **e**, Phosphorylated myosin light chain at 1-μm annulus around the cell boundary. A.U.=arbitrary unit, normalized by the values at zero applied stress. $n=23$ and 11 cells for 0 and 30 sec respectively; mean $\pm$ s.e. (Scale bar, 15μm.)

Figure 3. Stress-induced ES cell spreading depends on myosin II activity, Src, Cdc42, but not on Rac activity

a, Summarized data after drug treatments were compared with those of untreated cells ($n=5$ cells). Control = cell areas before stress application. Inhibiting myosin II ATPase with Blebbistatin ($50 \mu\text{M}$ for 30min; $n=7$ cells), inhibiting myosin light chain kinase with ML7 ($25 \mu\text{M}$ for 20min; $n=5$ cells), inhibiting ROCK with Y27632 ($50 \mu\text{M}$ for 20min; $n=5$ cells), or inhibiting Src activity with PP1 ($10 \mu\text{M}$ for 1hr; $n=5$ cells), all prevented stress-induced cell spreading, i.e., no significant changes in cell areas between 0 and 10 min and between 0 and 20 min ($p>0.05$). For inhibiting Rac with NSC23766 ($100 \mu\text{M}$ for 1hr; $n=5$ cells), there were significant changes in cell areas ($p<0.006$ and $p<0.0009$) between 0 and 10 min and between 0 and 20 min. Latrunculin A ($0.1 \mu\text{g/ml}$ for 30 min) ($n=10$ cells) to disrupt F-actin also prevented stress-induced spreading. Mean $\pm$ s.e. **b**, Cdc42 is necessary for stress-induced spreading in mES cells. Western blots of Cdc42 in mES cells under different conditions. Lane 1, non-target shRNA control; Lane 2–4, different constructs to knockdown Cdc42. An independent experiment showed similar results. **c**, Corresponding changes in cell areas after stress application after Cdc42 knockdown (17.5 Pa at 0.3 Hz). $n=9, 8, 9, 8$ cells for Lane 1–4 respectively; mean $\pm$ s.e. (for Lane 1, $p<8.68\times 10^{-7}$ and $p<2.66\times 10^{-6}$ comparing between 0 and 5 min, 0 and 10 min; there were no significant changes ($p>0.05$) for Lane 2 through Lane 4). Note that cdc42 knockdown correlated strongly with abolishment of stress-induced spreading response, suggesting that Cdc42 is critical in stress-induced protrusion and spreading.

Figure 4. A local cyclic stress substantially diminishes Oct3/4 expression in mES cells
a, Brightfield (BF) images (top), corresponding GFP images of Oct3/4 expression (middle), and corresponding DsRed images of a constitutive promoter (CAGGS) expression (bottom), all from the same cell(s), are shown over time. Cells attached to RGD-coated beads (black dots) were continuously stressed for ~1 hr (17.5 Pa at 0.3 Hz) and Oct3/4 expression or CAGGS expression was measured over time in the homogeneous pluripotent mES cells (assessed by the uniform high GFP fluorescent intensity in all mES cells, unique cell shapes, and colony forming capability) plated on high density collagen-1 (100 μ g/ml) coated 0.6 kPa substrate. (Scale bar, 10 μ m.) **b**, Summarized data for the cells in mES cell culture medium that were exposed to stress (+stress, +LIF/-RA; closed circles, n=5), the cells in the same dish but were not stressed (-stress, +LIF/-RA; open circles, n=9), the cells in mES cell culture medium in separate dishes (+LIF/-RA; open squares, n=9), and the cells in the differentiation medium (-LIF/+RA; closed squares, n=10) are shown here. Oct3/4 expression is normalized with respect to time zero (control). Mean $\pm$ s.e.; two independent experiments.