Material properties of the cell dictate stress-induced spreading and differentiation in embryonic stem cells

Farhan Chowdhury¹, Sungsoo Na¹,² *, Dong Li³ *, Yeh-Chuin Poh¹, Tetsuya S. Tanaka⁴, Fei Wang³ and Ning Wang¹†

Growing evidence suggests that physical microenvironments and mechanical stresses, in addition to soluble factors, help direct mesenchymal-stem-cell fate. However, biological responses to a local force in embryonic stem cells remain elusive. Here we show that a local cyclic stress through focal adhesions induced spreading in mouse embryonic stem cells but not in mouse embryonic stem-cell-differentiated cells, which were ten times 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 have far more important roles in early development of soft embryos than previously appreciated.

Embryonic stem (ES) cells are one of the principle focuses in biology because of their pluripotency and potential therapeutic applications¹–³. Although it is known that soluble factors are critical in stem-cell differentiation⁴,⁵, recent evidence shows that the physical microenvironment of the cells (for example, shape constraint or substrate stiffness) helps direct the fate of mesenchymal stem cells⁶–⁷. These cells, however, are downstream in cell-lineage specifications, and have limited self-renewal and differentiation capacities in comparison to ES cells. We focus on pluripotent ES cells because 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 understanding mechanisms of lineage determination and development as these cells are derived from the inner cell mass of blastocysts before gastrulation, which 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 was reported that fluid shear stress over whole haematopoietic progenitor cells promotes embryonic haematopoiesis¹¹. However, whether and how ES cells respond to a localized mechanical stress remain elusive.

During the past decade or so, the importance of substrate rigidity in cell functions has become increasingly clear⁷,¹²–¹⁴. The physical and mechanical cues of the extracellular matrix are transduced into intracellular rheological and biochemical changes through unknown mechanisms, but probably through 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 behaviours¹⁶–¹⁸. Therefore, it is suggested that intrinsic intracellular material mechanical properties govern cellular behaviour 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 the 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. As 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 mouse embryonic stem (mES) cells are softer and much more sensitive to a local cyclic stress than their differentiated counterparts. We show that a material property of the cell, the cell softness, dictates the stress-induced spreading response. We reveal the underlying signalling pathways in stress-induced spreading in mES cells. It is known that oct3/4 (pou5f1) expression in mES cells 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 the 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 stiffnesses overnight. As expected from a published report²², the mES cell-differentiated (ESD) cells increased their

¹Department of Mechanical Science and Engineering, University of Illinois at Urbana-Champaign, Illinois 61801, USA. ²Indiana University-Purdue University Indianapolis, Department of Biomedical Engineering, 723 W. Michigan St. SL220, Indianapolis, Indiana 46202, USA. ³Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Illinois 61801, USA. ⁴Department of Animal Sciences, University of Illinois at Urbana-Champaign, Illinois 61801, USA. *These authors contributed equally to this work. †E-mail: nwangrw@illinois.edu.
Figure 1 | Cell softness dictates cell spreading response to stress. a, Stress-induced spreading in mES cells is amplitude dependent. The 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 × 10⁻⁵ or 5.66 × 10⁻⁵ at 3, 5 or 8 min respectively). At 17.5 Pa stress, there was a significant difference in cell area between 3 and 5 min (p < 0.05), but no significant difference in cell area between 5 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 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 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 ± s.e.; at least three independent experiments.) b, Stress-induced cell spreading depends on cell softness. mES, ESD and ASM cells were plated on similar culture conditions (high density of collagen-1, 100 µg ml⁻¹) and on the same substrate stiffness of 0.6 kPa. The change in cell area of ESD and ASM cells is statistically different from that of mES cells at 3 min (p < 0.05). Round ESD and round ASM cells were plated on a low density of collagen-1 (1 ng ml⁻¹) 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 seems to be proportional to cell softness. Cell softness correlates inversely with F-actin density in each cell type (see Supplementary Fig. S5). Mean ± s.e., n = 7, 9, 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 µg ml⁻¹ of type-I collagen and thus was restricted to within an area of ~500 µm². The gel surface outside the islands was uncoated and thus was non-adhesive. 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). Control = cell area at time zero. Mean ± s.e., n = 5, 5 and 9 for µP ESD, µP ASM and mES cells, respectively.

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 functions.25

Next we explored whether these soft mES cells could respond to a localized external stress. After a mES cell was plated on a substrate at 0.6 kPa overnight, we attached a 4 µm 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). Although it is expected that unidirectional stretching or stretching of a whole cell would elongate the cell in the direction of the stretching or the stress4,9, 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 potentiate 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 method.24 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 show any changes in normal velocity or cell projected area in response to the same amplitude of the cyclic stress (Supplementary Fig. S3e–b). The lack of stress-induced cell spreading is not due to the limitation of the spreading capacity of these cells, because 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 and 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 Hz (ref.25). Results from stiff human airway smooth muscle (ASM) cells (a well-established differentiated tissue cell type), plated on the same substrate (stiffness), which was coated with the same immobilized amount of collagen-I, 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. The softness of mES cells was about seven times higher than that of ESD cells on the same substrate (Fig. 1b). As 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\(^{-1}\) 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. 1a,b). The greater the cell softness, the stronger the spreading response (that is, the greater the increase in cell area in response to stress) (Fig. 1b).

Furthermore, the relative softnesses 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 chief determinant in cell stiffness\(^{26}\).

An alternative interpretation to our data is that the smaller the projected cell area is, the stronger is 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 (Supplementary 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 a high density of collagen-1 (ref. 27). 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 roughly eight 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 for the recent findings on stress-induced conformational changes and/or unfolding of signalling molecules\(^{28}\) and focal adhesion structural proteins\(^{29}\).

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 tractions 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 before 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).
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 cells\(^\text{35}\). Interestingly, pretreatment with NSC23766 (100 \(\mu\)M for 1 h), a specific inhibitor of Rac\(^\text{30,31}\), 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, because application of the same amplitude of stress through 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 occurs only through activated integrins\(^\text{39}\) and that an applied stress through integrins induces additional activation of integrins and phosphorylation of focal adhesion kinase\(^\text{39}\). Stress-induced spreading in mES cells was completely prevented by pretreatment with Latrunculin A (0.1 \(\mu\)g ml\(^{-1}\) 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 II (ref. 29).

It is known that cdc42 mediates cell filopodium extension and cell spreading\(^\text{35}\). 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,c (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 cells\(^\text{32}\). Our finding that stress-induced spreading in these mES cells depends on cdc42 but not on Rac is interesting, because it is well known that integrin-mediated cell spreading depends on Rac in differentiated cells\(^\text{32,33}\).

**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 green fluorescent protein (GFP) driven by the oct3/4 promoter in undifferentiated cells cultured in the presence of the leukaemia inhibitory factor (+LIF; ref. 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 h, and by ~50% within 72 h, whereas control cells a few micrometres away in the same dish without stress continued to express oct3/4 (Fig. 4, Supplementary Fig. S9). As loss of oct3/4 expression in ES cells is one of the hallmarks for differentiation\(^\text{35}\), our results suggest that a local cyclic stress through a focal adhesion might be sufficient to drive a mES cell to differentiate. If our findings could be extended to early animal embryos, this would provide a new way of locally differentiating a single cell of early lineage while keeping nearby cells undifferentiated.

Accumulating experimental evidence suggests that mechanical contractile forces have 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 the inner-cell-mass cells. In a recent review, the combined effects of growth factors, matrices and mechanical forces in controlling stem cells are discussed\(^\text{37}\). 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 cells\(^\text{7}\). In contrast, we show here that intracellular softness can

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**Figure 3 | Stress-induced ES cell spreading depends on myosin II activity, Src and cdc42, but not on Rac activity.** a, Summarized data after drug treatments were compared with those of untreated cells (\(n = 5\) cells). Control = cell area before stress application. Inhibiting myosin II ATPase with Blebbistatin (50 \(\mu\)M for 30 min; \(n = 7\) cells), inhibiting myosin light chain kinase with ML7 (25 \(\mu\)M for 20 min; \(n = 5\) cells), inhibiting ROCK with Y27632 (50 \(\mu\)M for 20 min; \(n = 5\) cells) or inhibiting Src activity with PP1 (10 \(\mu\)M for 1 h; \(n = 5\) cells) all prevented stress-induced cell spreading; that is, there were 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 \(\mu\)M for 1 h; \(n = 5\) 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 \(\mu\)g ml\(^{-1}\) for 30 min) (\(n = 10\) cells) to disrupt F-actin also prevented stress-induced spreading. Mean ± 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; Lanes 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). \(n = 9, 8, 9\) and 8 cells for Lanes 1–4 respectively; mean ± 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 Lanes 2–4). Note that cdc42 knockdown correlated strongly with abolishment of stress-induced spreading response, suggesting that cdc42 is critical in stress-induced protrusion and spreading.

Supplementary Fig. S6), suggesting that ROCK is also critical in this process. Importantly, pretreatment with PP1 (10 \(\mu\)M for 1 h), a specific Src tyrosine phosphorylation inhibitor, blocked stress-induced ES cell spreading (Fig. 3a; Supplementary Fig. S6). This
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 through 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 neighbouring cells) might play far more important parts 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 soft

Figure 4 | A local cyclic stress substantially diminishes oct3/4 expression in mES cells. a. Bright-field (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 h (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 μg ml⁻¹) coated 0.6 kPa substrate. (Scale bar, 10 μm.) b. Summarized data for the cells in mES cell culture medium that were exposed to stress (+stress, +LIF/−RA; n = 5), the cells in the same dish but that were not stressed (−stress, +LIF/−RA; n = 9), the cells in mES cell culture medium in separate dishes (+LIF/−RA; n = 9) and the cells in the differentiation medium (−LIF/+RA; n = 10) are shown here. oct3/4 expression is normalized with respect to time zero (control). Mean ± S.E.; two independent experiments.

Molecular mechanism of mechanotransduction

At present 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 adhesion, possibly through the catch bond mechanism. 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 binding. It is likely that time-varying, strain-dependent conformational changes and/or unfolding of these protein molecules at focal adhesions and at other distant sites (for example, inside the nucleus) are the primary molecular mechanisms of mechanochemical transduction and strain-activated feedback loops. An important test of this hypothesis will be to extend the in vitro work of del Rio et al. 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 Pa (ref. 43), an ES cell has a stiffness of ~500 Pa (Supplementary Fig. S2), a brain neural cell has a stiffness of ~100–500 Pa (ref. 12), a typical differentiated tissue cell (for example, a smooth-muscle cell) has a stiffness of 1–5 kPa (ref. 44) and a skeletal-muscle cell has a stiffness of ~12 kPa (ref. 23). The respective softness of various types of cell might manifest their different physiological functions and sensitivities to force in a multicellular organism. An evolutionary advantage for an early embryonic or progenitor cell to become stiffer as the cell divides and differentiates into a more differentiated support tissue cell might be to protect the organism from injuries by force, because the ability to respond to touch and to resist mechanical stress is postulated to be one of the most primitive features of metazoa that evolved millions of years ago. Matching a cell material property with that of its substrate is known to be critical in forming striation in skeletal muscle cells23 and optimizing cardiomyocyte beating, but stiffness matching may have broader implications. As proposed recently, nutrient-rich uncompacted soft ocean sediments about two billion years ago provided a selective evolutionary pressure favouring those very earliest eukaryotes that were better able to carry out 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 these early evolutionary events.

It is established that stress can regulate gene expression, but these previous studies were generally carried out 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 explain mechanisms of mechanosensing and mechanotransduction. To our knowledge, our present 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, probably owing 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 whether the rotational shear stresses applied through the magnetic twisting cytometry technology could mimic this physiological process. Future studies are needed to understand the specific mechanisms of stress-induced inhibition of oct3/4 expression in these mES cells, to determine whether these findings can be extended to human ES cells and induced pluripotent stem cells, and to find out whether stress-induced signals inhibit known
pluripotency-supporting pathways mediated by molecules such as mTOR (ref. 47). It will also be interesting to determine what type of germ-layer cell (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 previously44. In brief, undifferentiated mES cells (W4, 129/SvEv) were maintained in the standard culture condition in the presence of LIF (Chemicon). mES cells at passage 11 were thawed onto a feeder layer of mitotically inactivated primary murine embryonic fibroblasts. mES cells were passaged onto culture dishes coated with 10% gelatin several times every two to three days to remove feeders. For the differentiation assay, triplicated mES cells, at passage 15–16, were plated on gelatin-coated dishes at a low density of 100 cells cm⁻². The following day, the mES cells were fed with the medium without LIF and with 1 μM 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 the −LIF/+RA condition, mES cells became differentiated to a heterogeneous population of differentiated (ESD) cells, which were cultured with the complete medium.

Human ASM cells were isolated at autopsy within 8 h of death from tracheal muscle of lung transplant donors (approved by the University of Pennsylvania Committee on studies involving human beings) at the University of Pennsylvania in Dr. Panettieri’s laboratory98. We used de-identified HASM cells supplied by Dr. Panettieri, who obtained the tissue through the National Disease Research Interchange in a manner that excludes all unique identifying information. All our procedures were approved by the Institutional Review Board of the University of Illinois at Urbana-Champaign. The ASM cells were cultured following published protocols99.

Quantification of membrane protrusion-velocity profiles. Edge-velocity profiles show the edge dynamics during cell spreading. This technique has been described before in detail100. We used that approach (CellMAP), where input was a high-contrast time-lapse sequence (5 s interval) of a single cell and the outputs were the normal cell edge velocity as a function of space (over entire arc length) and time, mean normal velocity over time and change in cell area.

Applying a local stress. Magnetic twisting cytometry is a well-established method for applying controlled and precise local mechanical stresses of physiologic magnitudes to a living cell19,43,44,45.

Cell-softness quantification. The cell-softness measurement technique has been described before16,21,22,44,45,98. The cell complex softness is defined as the ratio of strain to the applied stress (that is, 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 (for example, myosin II) are force (the independent variable) generators and because strain-dependent opening of proteins is likely to be important in changing protein activities and cell functions46.

Cell area and traction measurements. Cell spreading area was measured by ImageJ (NIH) using the active contours algorithm. Cell traction measurements have been described in detail elsewhere47. Generated traction maps were used for further quantification. On the basis of the grey-scale traction field we took a 1-μm-thick annular section at the cell boundary at different time points and measured the intensity. The mean intensity within the annular section, representing the tractional stress developed at the cell boundary, at time zero (before twisting of the magnetic bead), was set to one arbitrary unit (a.u.). Traction profiles were plotted over time around the boundary. Micropatterned adhesive islands on soft polyacrylamide gels were produced following published methods22.

Lentivirus production and mES cell infection. For shRNA-mediated knockdown of cdk4, the plKO.1-puro vector (Sigma-Aldrich) was used. We used the Viralpower lentivirus packaging system (Invitrogen) to package lentivirus for transduction. cdk4 knockdown, following the manufacturer’s instructions.

Western blot. To quantify cdk4, infected mES cells were lysed directly with 200 μl Laemmli sample buffer (Bio-Rad). 20 μl of each sample were analysed by Western blotting. The blots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce).

Enhanced GFP and DsRed expressions in mES cells driven by oct3/4 and CAGGS promoters. A mES cell line, namely OGR1, that expresses enhanced GFP (EGFP) under the promoter of oct3/4 (oct3/4::EGFP; ref. 51) was transfected with 0.3 Hz was applied for 30 s. The cell–stiffness measurement technique has been described before23,24,25,97. EGFP expression indicates downregulation of oct3/4, one of the hallmarks for differentiation. The stressed cells were labelled as +stress, +LIF/+RA condition. The cells without beads (that is, no stress) in the same dish were also monitored (−stress, +LIF/+RA). Other dishes were monitored and EGFP-oct3/4 was 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. Additional methods can be found in Supplementary Information.

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Author contributions

N.W., T.S.T. and F.W. designed the experiments; F.C., S.N., D.L. and Y.C.P. carried out experiments and analysed the data. N.W., F.C., T.S.T. and F.W. wrote the manuscript.

Additional information

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