# Chapter IV. Substratum compliance and EGF co-regulate spatial patterns in traction forces, cell shape, and proliferation within epithelial multicellular clusters


## Abstract

Cell-generated mechanical forces that act upon cell adhesions play a major role in multicellular morphogenesis. These adhesions undergo dynamic modulations through continuous cellular interactions with the surrounding microenvironment. Elucidating how these microenvironmental cues regulate the biophysical aspects of multicellular structures is pivotal to understanding the emergence of multicellular growth patterns. Here, we demonstrate that substratum compliance and epidermal growth factor (EGF) co-regulate spatial patterns in cell adhesions, traction forces, and cell shape within multicellular clusters. These patterns in adhesions and cell morphology correspond to patterns in proliferation. In the absence of EGF, soft matrices selectively promote the maturation of intercellular contacts and block focal adhesion formation among central cells. In contrast, substratum compliance does not have any apparent effect on focal adhesion formation among peripheral cells. The spatial patterns in cell adhesions correspond to patterns in traction force. Our measurements of traction forces in three-dimensions (3-D) reveal significant spatial gradients in mechanical stresses normal to the substratum as well as in the plane of the substratum. Cells at the interior of a cluster push down upon the matrix, while those in the periphery pull up on the substratum. These pre-established patterns in cell adhesions and traction forces are modified upon

EGF treatment in an EGF dose-dependent manner. Our preliminary results indicate that supra-threshold levels of EGF induce rapid, short-lived traction forces and transient de-compaction of clusters. In contrast, sub-threshold EGF levels do not induce any apparent mechanical or morphological changes. These dose-dependent effects correspond to uniform proliferation versus spatially patterned proliferation in response to supra- and sub-threshold EGF treatment, respectively. These results suggest a model wherein EGF stimulation at supra-threshold levels may eliminate pre-established patterns in adhesions and traction forces en route to driving uniform proliferation. Future studies will test this model by investigating whether the traction forces generated by treatment with supra-threshold EGF act to reduce the pre-established spatial gradient in mechanical stresses and whether this reversal is necessary for the elimination of a spatial pattern in proliferation.

## Introduction

Epithelial cells organize into multicellular structures by establishing highly structured adhesions with their neighbors and the surrounding extracellular matrix (ECM) (1). During morphogenesis, cells continuously sense cues in their microenvironment, such as ECM ligands and soluble growth factors, and respond by modulating their adhesions, cytoskeletal mechanics, and cell shape (2). These biophysical changes in turn affect intracellular signal transduction and control many cellular behaviors including proliferation. Thus, deciphering how these environmental cues control multicellular mechanics and spatial patterns in cell shape and proliferation is central to our understanding of multicellular morphodynamics.

The ECM is dynamic and undergoes continuous remodeling during development (3), aging (4), and disease progression (5). In particular, a physical property of the ECM, compliance, plays a central role in maintaining epithelial organization through tensional homeostasis (6). The stiffening of the matrix is frequently associated with tumor formation (5). Stiffer matrices enable isolated cells to generate larger traction forces on the underlying substratum, leading to bigger and more mature focal adhesions (7). In a multicellular context, these cell-generated forces are also transmitted through cell-cell adhesions, leading to spatial patterns in mechanical stresses and proliferation within multicellular aggregates (8). It is unknown how these spatial patterns in traction forces affect the spatial distribution of focal adhesions in multicellular clusters.

In addition to the mechanical property of matrix, soluble growth factor (GF) may also regulate cellular mechanics and shape by affecting protrusions and actomyosin contractility. EGF stimulates the membrane translocation of Rac1 and its localized activation (9), facilitating lamellipodial extensions. Meanwhile, EGF activates the Rho GTPase effector, ROCK, leading to the phosphorylation of myosin-II regulatory light chains and inactivation of myosin-II phosphatases, which together gives rise to the increased non-muscle myosin-II-mediated contractility (10). In isolated cells, these mechanisms are associated with the formation of new adhesions at the leading edge and the destabilization of focal adhesions in trailing edges, together driving cell migration. Precisely how EGF affects cell mechanics and shape within multicellular clusters remains to be elucidated.

Here, we set out to quantitatively interrogate how substratum compliance and EGF affect cell mechanics and shape within multicellular clusters and how these effects correspond to spatial patterns in proliferation. We find that softening collagen- or fibronectin-coated elastic substrates promotes spatial patterns in focal adhesions. These spatial patterns in cell-matrix adhesions corresponds to patterns in mechanical stresses in three-dimensions, which include both in-plane and significant out-of-plane traction forces. These pre-established patterns in cell adhesion and mechanics are modified upon EGF treatment in an EGF dose-dependent manner. Treatment with a supra-threshold level of EGF leads to the generation of actomyosin contractility and de-compaction of cell clusters. Our quantitative findings illustrate the integrated role of substratum

compliance and EGF in regulating spatial patterns in traction forces, cell shape, and proliferation within multicellular clusters.

**Results**

*Substratum compliance affects spatial patterns in cell-cell and cell-matrix adhesions.*

To explore the effect of substratum compliance on cell adhesion with its neighbors and with the underlying substratum, we monitored molecular markers associated with the maturation of cell-cell contacts and focal adhesions (FA) – E-cadherin and paxillin, respectively – in normal rat kidney epithelial cells (NRK-52E) cells grown on ColI-coated polyacrylamide (pAc) gels of varying stiffness. On soft substrata, continuous, linear E-cadherin staining was observed (Fig. 1A). In contrast, punctate E-cadherin structures, indicative of immature cell-cell adhesions (11), were found at cell boundaries of NRK-52E cell clusters seeded on stiff substrata. This qualitative observation was corroborated by quantifying the number of discontinuities in cadherin-mediated contacts around the cell perimeter for a range of substratum compliance. These results in NRK-52E cells are consistent with our previous observation in MDCK cells that softer substrata promote the maturation of cell-cell contacts by enhancing the subcellular localization of contact molecules, such as E-cadherin and ZO-1 (Chapter 3).

In addition to its effects on cell-cell adhesions, modulating substratum compliance affected the formation and maturation of FAs in the interior of clusters (Fig.

1B). On soft surfaces, only nascent adhesions remained in the interior of clusters while mature adhesions were concentrated among peripheral cells, establishing a spatial gradient in the extent of cell-matrix interactions. An increase in substratum stiffness diminished this spatial disparity in FA distribution by allowing the formation of mature adhesions in the interior of clusters. In contrast, substratum compliance did not significantly affect the formation of mature FAs at the free edges of the peripheral cells.

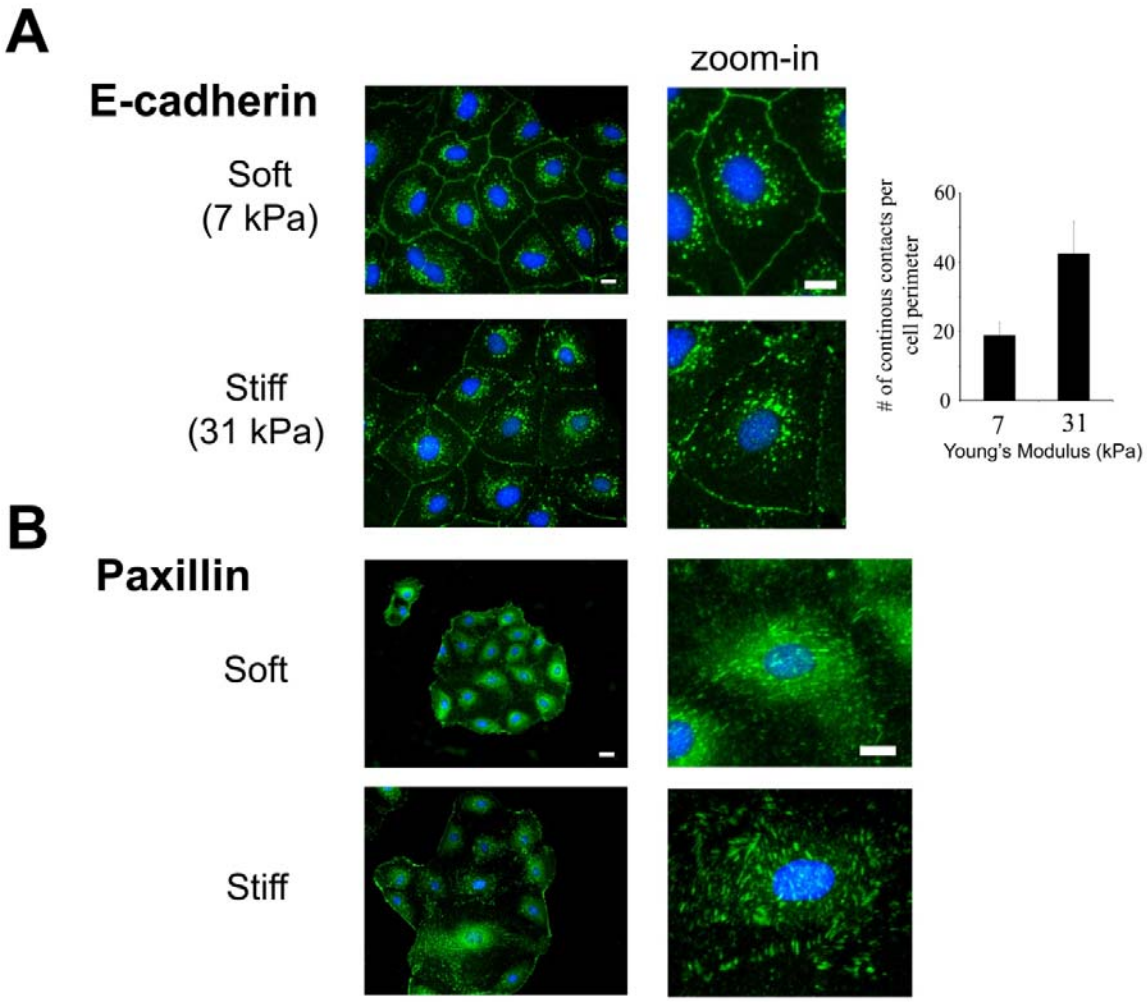


**Fig. 1. The effect of substratum compliance on contact-maturation and spatial pattern in cell-matrix interactions** NRK-52E cells cultured on ColI-coated

polyacrylamide gels of two different stiffnesses (Soft: 7 kPa and Stiff: 31 kPa) were starved and immunostained for (A) E-cadherin and (B) paxillin. Nuclei were co-stained with DAPI. The right panel images represent the magnified views of stained cells located in the interior of clusters. The bar graph shows the immaturity of cell-cell contacts on each substrate. To quantify the immaturity of cell-cell contacts, the number of discontinuities in E-cadherin-mediated contacts was counted and normalized to the cell perimeter. At minimum,10 cells were analyzed per condition. The *error bars* indicate s.d. (n = 3) with duplicates performed in each trial. (Scale bars, (A) 12.5 μm, (B) 25 μm).

*The spatial pattern in cell adhesions corresponds to patterns in traction force in three-dimensions.*

The generation of traction forces influences the formation and stabilization of focal adhesions in isolated cells (12). We asked whether the spatial patterns in cell-matrix adhesions observed in multicellular clusters correspond to spatial differences in traction forces generated by interior versus peripheral cells. Traction force measurements have been conventionally restricted to in-plane stresses (12-13) and have missed the possibility of normal (*z*-directional) forces that cells may exert. Recent data using the three-dimensional digital volume correlation (3D-DVC) method in isolated migrating cells suggests that the magnitude of normal forces may be on par with those of in-plane forces (14). Here, we used the 3D-DVC technique to assess both in-plane and normal traction forces within multicellular clusters.

In MDCK cells grown on soft surfaces, we observed that cells generated in-plane, pulling forces toward the center of clusters overall. The magnitude of these forces markedly decreased toward the center of clusters so that peripheral cells generated approximately 3- to 5-fold higher traction forces than interior cells (Fig. 2A). Intriguingly, the spatial differences in mechanical stresses also existed in normal stresses as peripheral cells pulled on the underlying substrate (out-of-plane) while cells collectively pressed the matrix in the interior of clusters (into-the-plane). These normal stresses were even greater in magnitude than the in-plane forces in some instances.

Thus, our measurements of traction forces in three-dimensions reveal significant gradients in normal forces in addition to the previously observed spatial patterns in in-plane forces within multicellular clusters (8). In particular, spatial patterns in normal forces involve the directional changes (i.e., out-of-plane at the periphery versus into-the-plane at the interior), revealing that most tugging forces against the underlying substratum are exerted at the periphery of clusters. This is consistent with our observations that matured focal adhesions are predominantly found along the free edges of peripheral cells. Thus, the spatial patterns in traction forces correspond to spatial patterns in the extent of focal adhesion maturation in multicellular clusters.

Finally, we examined the role of these 3-D mechanics developed within multicellular aggregates on spatial patterns in proliferation. We assessed cell cycle activity within clusters whose myosin-mediated contractility was abrogated by applying a myosin-II ATPase inhibitor, blebbistatin. In contrast to a vehicle control wherein cell

aggregates exhibited a spatially patterned growth at 100 ng/ml EGF stimulation, cells pretreated with blebbistatin displayed uniform growth (Fig. 2B). These effects of blebbistatin treatment confirm the previous observation that cell-generated traction forces play a key role in regulating growth patterns (8). Here, we show that these traction forces are patterned not only in the plane of the substratum but also normal to the substratum. Furthermore, we demonstrate that these spatial patterns in traction forces correspond to patterns in the maturity of cell-matrix and cell-cell adhesions (Fig. 1).

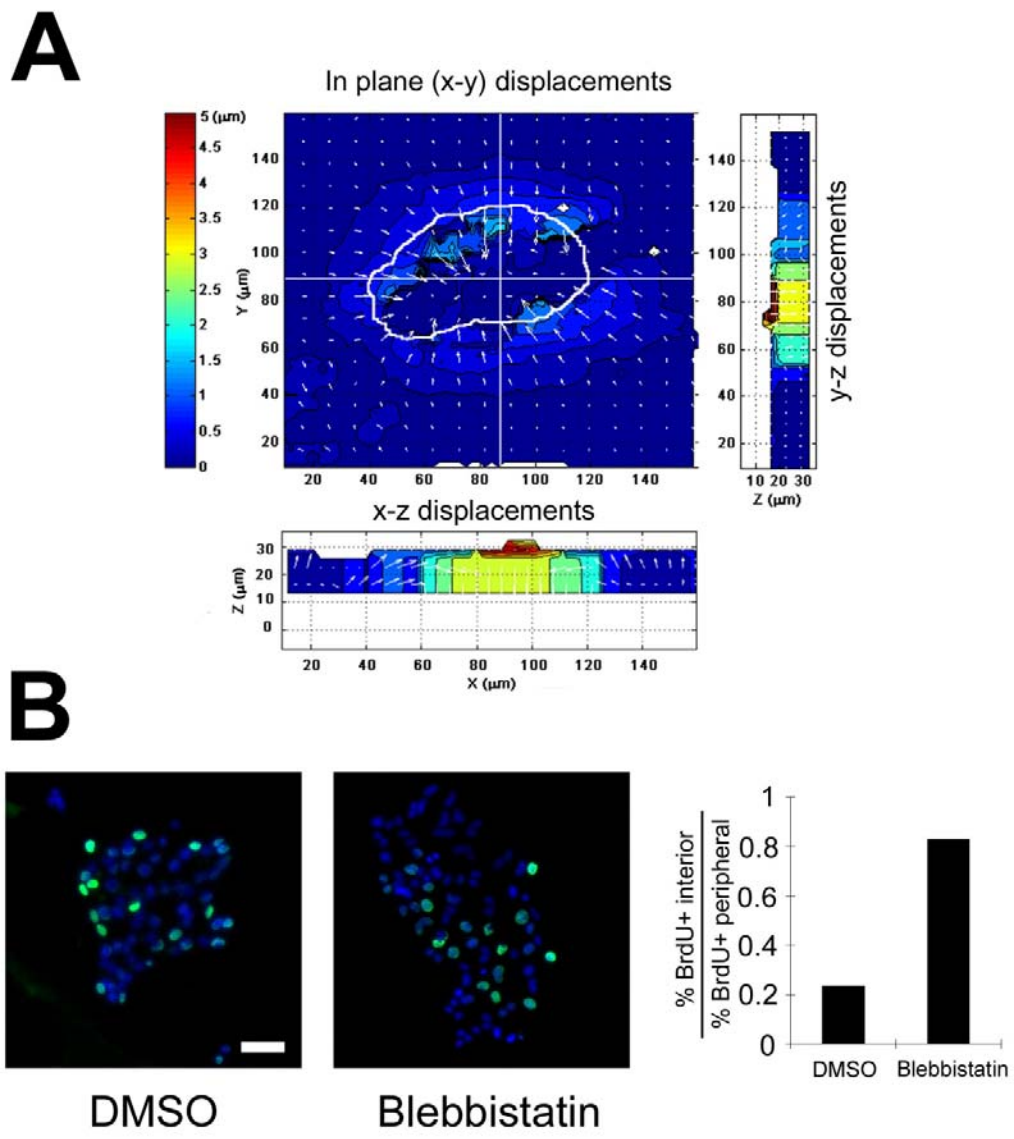


**Fig. 2. The spatial patterns in cell adhesions correspond to spatial gradient in mechanical stresses within multicellular aggregates.** (A) Traction forces within a multicellular cluster seeded on a soft gel. MDCK cells grown on bead-containing pAc gels (7 kPa) were serum starved and live-stained with mitotracker for fluorescence imaging of clusters. Bead displacements induced by cell-generated traction forces were

measured by comparing the bead distribution before and after lysing cells with 0.5 % Triton. White lines in parallel and vertical directions indicate the planes for which *x-z* and *x-y* directional displacements were measured, respectively. Total of two independent trials were performed. At least, three samples were examined per condition in each trial. (B) The effect of inhibiting traction forces on the spatial pattern in proliferation. Serum-starved MDCK cells on soft surfaces were pretreated for 2 h with blebbistatin or the solvent DMSO, and then stimulated with 100 ng/ml EGF. The effect of the pharmacological inhibition of non-muscle myosin-II activity on BrdU uptake (green) was determined by immunofluorescence. Nuclei were co-stained with DAPI (blue). The fractions of interior and peripheral cells incorporating BrdU were quantified. The graph shows the ratio of these two fractions for cells pretreated with DMSO or blebbistatin. (Scale bar, 50 μm)

*Treatment with supra-threshold amount of EGF induces traction forces and transient de-compaction of cell clusters*

Our results reveal that cells seeded on soft matrices establish spatial gradients in cell-cell and cell-matrix adhesions and in traction forces. These spatial patterns in adhesive events are established in the absence of serum or exogenous growth factors. Yet, these pre-established patterns clearly correspond to a pattern in proliferation following EGF stimulation. Furthermore, we have shown previously that if clusters are treated with a supra-threshold level of EGF, the cell clusters will exhibit uniform proliferation (Chapter 3). These observations raise the possibility that supra-threshold

levels of EGF may eliminate pre-established patterns in adhesions and traction forces en route to stimulating uniform cell proliferation (Fig. 3A). Alternatively, supra-threshold EGF may not affect spatial patterns related to adhesion but yet induce uniform proliferation by overriding downstream inhibitory pathways or providing compensatory activation pathways.

To resolve between these alternative scenarios, we sought to evaluate the effect of EGF stimulation on the spatial pattern in adhesive events. As an initial study, we focused on quantifying changes in cell shape as a gross phenotypic measure that would lump together any effect on cell-matrix and cell-cell adhesions and traction forces. We treated MCF-10A clusters grown on soft surfaces (7 kPa) with 0.1 and 100 ng/ml EGF, which are sub- and supra-threshold amounts of EGF in this system. Treatment with 100 ng/ml EGF induced a transient de-condensation of MCF-10A clusters so that the projected area of the clusters increased up to ~40% by 100 min after stimulation (Fig. 3B, left panel). In contrast, treatment with 0.1 ng/ml EGF did not induce any apparent changes in cluster size. Thus, supra-threshold EGF levels that eliminate the spatial pattern in proliferation also trigger a transient decompaction of the cell cluster. We hypothesize that such de-compaction involves an increase in cell-matrix adhesions and a diminishment of cell-cell adhesions. Future studies will examine this more closely using the aforementioned tools to probe cell-matrix and cell-adhesions.

In addition to measurements of morphology, we conducted preliminary experiments to measure the cell-generated forces transmitted to the underlying

substratum during the transient EGF-mediated de-compaction. We measured bead displacements in 20 min time windows following stimulation with 100 ng/ml EGF (Fig. 3B, right panel). Initial observations suggest measurable traction force responses to treatment with 100 ng/ml EGF. In contrast, treatment with 0.1 ng/ml EGF did not produce measureable bead displacements. Future studies will test the hypothesis that the traction forces generated by treatment with supra-threshold EGF act to diminish the spatial disparity in pre-established traction forces.

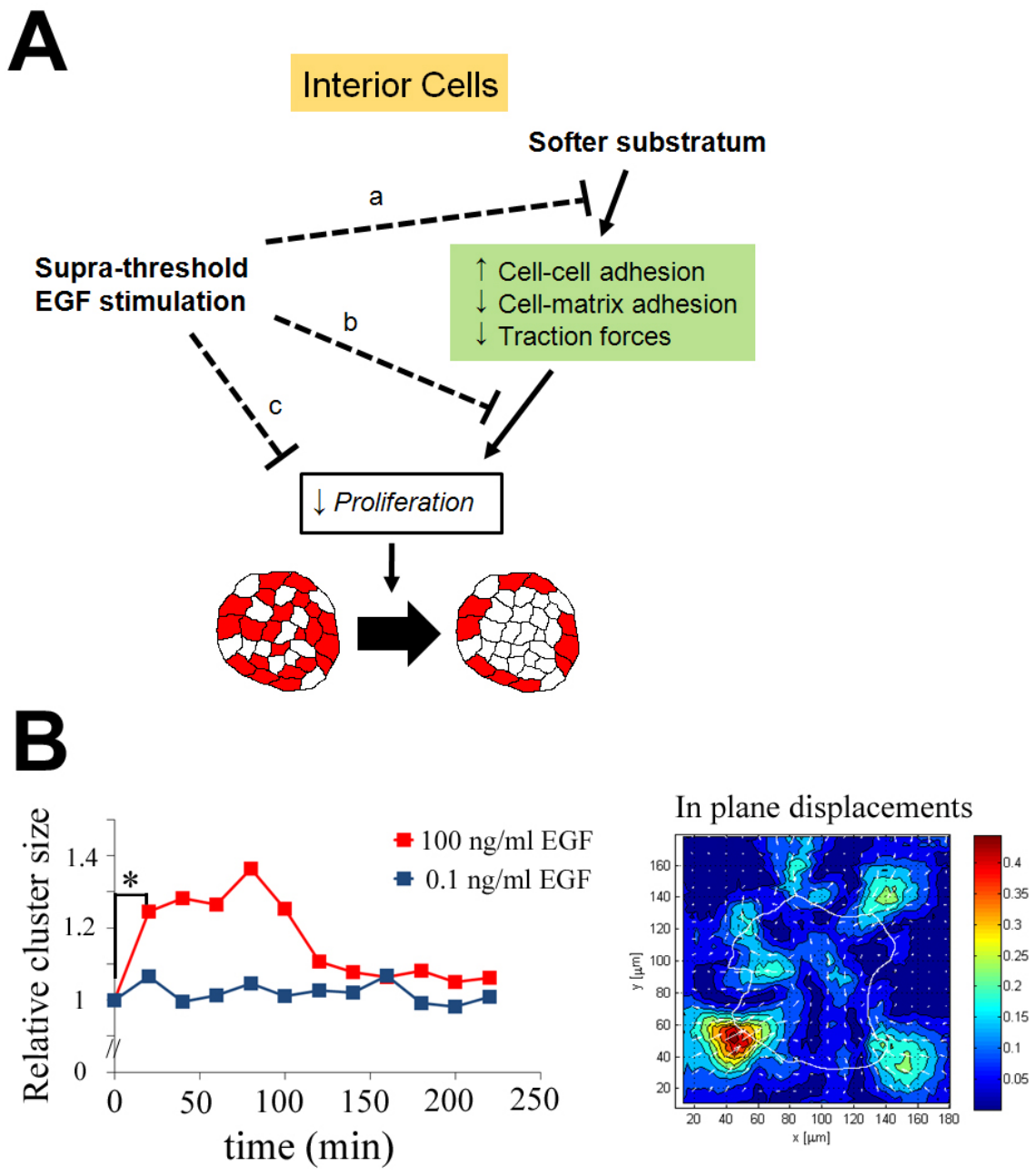


**Fig. 3. Treatment with supra-threshold levels of EGF induces rapid, short-lived traction forces and transient de-condensation of clusters.** (A) The emerging model from our results is that soft substratum promotes cell-cell adhesions and blocks the formation of focal adhesions and generation of traction forces among interior cells.

These events correspond to the cell cycle arrest of interior cells and the onset of spatial pattern in proliferation. We hypothesized three possible scenarios wherein treatment with supra-threshold levels of EGF acts to rescue interior cells from their cell cycle arrest en route to eliminating the spatial pattern in proliferation. Supra-threshold levels of EGF may (a) reverse the effect of soft matrices on cell-cell and cell-matrix adhesions and traction forces, (b) override downstream inhibitory pathways, or (c) activate compensatory activation pathways. (B) Serum-starved MCF-10A clusters on soft surface were stimulated with 0.1 and 100 ng/ml EGF. The relative changes in cluster area and bead displacements caused by cell-generated forces were quantified over time at 20 min intervals. The *inset* shows a representative displacement map between 0 and 20 min for clusters treated 100 ng/ml EGF (indicated by *asterisk*). Cluster sizes are reported relative to the cluster area at the time of EGF stimulation (i.e., 0 min).

**Discussion**

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Here, we demonstrate that substratum compliance and EGF co-regulate spatial patterns in cell adhesions, traction forces, and cell shape within multicellular clusters. The patterns observed in these adhesive and morphological processes correspond to spatial patterns in proliferation within multicellular clusters.

In the absence of EGF, softening the adhesive matrix promotes the maturation of intercellular contacts and diminishes focal adhesions among central cells. Meanwhile, cells at the periphery remain strongly anchored to the underlying substratum exhibiting

mature focal adhesions along the free edge. This spatial pattern in cell-matrix adhesions corresponds to spatial disparities in traction forces. Our measurements of mechanical stresses in three-dimensions reveal that in addition to the previously reported spatial gradients in *in-plane* forces (8), there exists a significant spatial pattern in *normal* forces. These normal forces are predominantly pulling up on the matrix at the periphery of clusters and push into the matrix toward the center of clusters.

Treatment with EGF modulates the spatial patterns in cell shape and traction forces that were pre-established under serum- and growth factor-free conditions. Importantly, the effect depends on the dose of EGF used. When the level of EGF is below a threshold amount needed to maintain patterned proliferation, stimulation with EGF has no effect on the area of the cell cluster and traction forces. In contrast, treatment with a supra-threshold amount of EGF induces rapid, short-lived decompaction of clusters and traction forces. These results suggest a model wherein treatment with supra-threshold levels of EGF attenuates pre-established spatial disparities in cell adhesivity and shape en route to eliminating the spatial pattern in proliferation.

During single-cell migration, acute EGF stimulation rapidly promotes protrusion and forms new adhesions at a leading edge, which are followed by myosin-II-mediated retraction at a trailing edge, together pushing a cell body forward (10). In a mature multicellular cluster, however, interior cells that are surrounded by neighboring cells cannot form protrusions and may not readily form additional adhesions with the substratum, thus failing to generate traction forces on their own. Instead, cells at the

periphery of the cluster may extend protrusions toward their free edges, strengthen their adhesions, and generate traction forces. These contractile forces are further transmitted toward the interior of clusters through cell-cell adhesions. The transmitted forces may contribute to pulling interior cells toward the periphery and disrupting their cortical contractility, consequently leading to the re-arrangements of the actin cytoskeleton and spreading of interior cells. Finally, these series of events are likely to allow them to eliminate spatial patterns in cell shape during a critical window of time for cell cycle decision, thus initiating cell cycle activity among interior cells.

**Conclusions**

In conclusion, our findings demonstrate that substratum compliance and EGF jointly control spatial patterns in cell adhesions, traction force and cell shape, and proliferation within multicellular aggregates. *In vivo*, maintaining proper cellular mechanics and shapes is the central model of tissue homeostasis (4). Events that perturb tensional balances among multicellular structures lead to malignant transformation (6). Meanwhile, our results reveal that EGF signaling, a canonical mitogenic pathway, modulates the spatial dynamics of multicellular mechanics and organization. This property may play a pivotal role in several physiological processes such as branching and wound healing by spatially imparting advantageous phenotypes for tissue outgrowth. However, during cancer progression, it may provide a pathway for genetic mutations in oncogenes to affect morphological evolution. Finally, quantitative insights on controlling multicellular patterns in mechanics, shapes, and proliferation using a

combination of soluble growth factors and mechanically compliant materials may provide design strategies for constructing synthetic microenvironments for applications such as tissue engineering and regenerative medicine.

## Materials and Methods

*Preparation and Characterization of Adhesion Ligand-coated Polyacrylamide Substrates*

Polyacrylamide substrates were prepared using techniques described by Wang and colleagues (15). Substrate stiffness was manipulated by varying bis-acrylamide concentrations while keeping the acrylamide concentration constant (10%). Type I collagen (Sigma-Aldrich) and fibronectin (Sigma-Aldrich) were covalently bound to the substrates by using a heterobifunctional cross-linker, sulfo-SANPAH (Pierce). The surface density of adhesion ligands on the substrates were examined as described in Fig. S1 of Chapter 3. Finally, Young's modulus of polyacrylamide substrates were measured by performing compression testing (16).

*Cell Culture and Reagents*

MCF-10A cells were cultured in growth medium as described previously (17). MDCK cells were cultured in Dulbecco's modified Eagle's medium containing HEPES and L-glutamine (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen). NRK-52E were cultured in Dulbecco's modified Eagle's medium containing HEPES and L-glutamine (Invitrogen) supplemented with 5 % (v/v) calf serum (Invitrogen). Experiments were conducted following the procedures dscribed by Chapter 3.

The following antibodies were used: anti-BrdU (Roche Applied Science), anti-E-cadherin (BD Transduction laboratory), anti-paxillin, anti-vinculin (Sigma-Aldrich), anti-ZO1 (Zymed), DECMA-1 (Sigma-Aldrich), and Alexa dye-labeled secondary antibodies (Invitrogen). DAPI and phalloidin were obtained from Sigma-Aldrich and Invitrogen, respectively. The pharmacological inhibitor, blebbistatin, was purchased from Calbiochem.

*Immunofluorescence and Image Acquisition*

Fixed cells were permeablized, blocked, and sequentially incubated with primary and secondary antibodies. The cells were co-stained with DAP and mounted using ProLong Gold Antifade (Invitrogen). Fluorescence images were acquired using the Zeiss Axiovert 200M microscope.

*Traction force microscopy*

Displacements of fluorescence beads (Invitrogen) embedded in pAc gels were tracked using DVC algorithm described by Ravichandran and colleagues (14).

*Subcloning membrane-mCherry construct into a retroviral vector*

The membrane-mCherry construct was kindly provided by S. Fraser (California Institute of Technology). The construct was subcloned from its original parent vector (pCS) into the 5' *Hpa*I and 3' *Cla*I sites of the retroviral vector pLHCX (Clontech). Briefly, pCS-membrane-mCherry was used as the PCR template to amplify the complete membrane-

mCherry coding sequence, with *Hpa*I and *Cla*I sites added to the 5' and 3' ends, respectively. The PCR product was digested with *Hpa*I and *Cla*I, and subcloned into the multiple cloning site (MCS) of pLHCX vector through these two sites, generating pLHCX-membrane-mCherry. The coding sequence of pLHCX-membrane-mCherry was verified by DNA sequencing (Laragne) with 5' and 3' pLHCX primers and alignment with the original membrane-mCherry sequence provided by Scott Fraser.

*Retrovirus production and usage*

Retroviral infection was used for the stable expression of mCherry in MCF-10A cells. Retrovirus was produced by triple transfection of HEK 293T cells with 5 μg each of VSV-G, gag-pol, and pLHCX-membrane-mCherry using Lipofectamine (Invitrogen). Virus-containing supernatant was collected and used to infect MCF-10A cells in the presence of essential growth factors and 8 μg/ml polybrene.

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