Three-Dimensional Analysis of the Effect of Epidermal Growth Factor on Cell-Cell Adhesion in Epithelial Cell Clusters

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ABSTRACT The effect that growth factors such as epidermal growth factor (EGF) have on cell-cell adhesion is of interest in the study of cellular processes such as epithelial-mesenchymal transition. Because cell-cell adhesions cannot be measured directly, we use three-dimensional traction force microscopy to measure the tractions applied by clusters of MCF-10A cells to a compliant substrate beneath them before and after stimulating the cells with EGF. To better interpret the results, a finite element model, which simulates a cluster of individual cells adhered to one another and to the substrate with linear springs, is developed to better understand the mechanical interaction between the cells in the experiments. The experiments and simulations show that the cluster of cells acts collectively as a single unit, indicating that cell-cell adhesion remains strong before and after stimulation with EGF. In addition, the experiments and model emphasize the importance of three-dimensional measurements and analysis in these experiments.

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 and migration. 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.

Exposure to soluble growth factors can affect cellular protrusions and actomyosin contractility, which in turn affect cellular behavior and mechanics. For example, it was found that clusters of MCF-10A cells maintain contact inhibition of proliferation at a low concentration of epidermal growth factor (EGF) while they undergo contact-independent growth at a higher concentration of EGF (3,4). In addition, clusters of epithelial cells that are exposed to particular growth factors can undergo an epithelial-mesenchymal transition (EMT)-like process (5) with some cell types exhibiting decompaction while still nominally maintaining cell-cell contacts (3) and others undergoing cell scattering. An example is the work by de Rooij et al. (6), who found that the hepatocyte growth factor causes cell-cell adhesion disruption and cell scattering in a process similar to EMT. In addition, it has been demonstrated that EGF can activate EMT in cancer cells (7).

For the processes of cluster decompaction and scattering to occur, the cells must first reduce their adhesions with one another, either partially in the case of decompaction or more completely in the case of cell scattering. The precise mechanism for the reduction in cell-cell adhesion still remains to be elucidated. It is known that EGF stimulates the membrane translocation of Rac1 and its localized activation (8), facilitating lamellipodial extensions. Meanwhile, EGF also activates the Rho GTPase effector, ROCK, leading to the phosphorylation of myosin-II regulatory light chains and inactivation of myosin-II phosphatases, which together give rise to the increased nonmuscle myosin II-mediated contractility (9). In isolated cells, these mechanisms are associated with the formation of new adhesions at the leading edge and the destabilization of focal adhesions at the trailing edge, together driving cell migration. However, in multicellular clusters, the mechanics of the cell-cell bonding adds an extra layer of complexity to the system. A current open question is whether EGF signals cells to downregulate expression of E-cadherin, a membrane protein associated with cell-cell adhesion. In Madin-Darby canine kidney cell clusters, it has been found that E-cadherin expression is not downregulated by EGF (6); however, in cancerous CaSki and SiHa cell clusters, EGF has been shown to reduce E-cadherin expression (7).

Modulating E-cadherin expression is just one way of regulating cell-cell adhesions. Phosphorylation of E-cadherin and β-catenin can modulate E-cadherin/β-catenin binding in addition to affecting the synthesis and turnover of E-cadherin (10,11). Furthermore, the formation and maturation of adherens junctions depends on other proteins, such as Merlin, that associate with α-catenin, an actin-binding protein (12). Finally, E-cadherin-based adherens junctions are not the sole mediators of cell-cell adhesion. Other types of adhesions, such as tight junctions mediated...
by ZO-1, also contribute to cell-cell adhesion (13). The intricate molecular assemblies mediating cell-cell adhesion further motivate the need for a more integrative, quantitative readout of the effect of EGF on cell-cell adhesion mechanics such as a mechanical measurement of the forces associated with cell-cell and cell-substrate adhesion.

To address the response of cell tractions to EGF, the technique of three-dimensional (3D) traction force microscopy (TFM) is applied to clusters of MCF-10A cells before and after stimulation with EGF. A simple mechanical model that makes use of a finite element simulation is then applied to better understand the way in which MCF-10A cells interact in a cluster and adhere to substrate beneath them.

**EXPERIMENTAL METHODS**

**Cell culture**

MCF-10A cells were cultured in DME medium/Ham’s F-12 (Invitrogen, Carlsbad, CA) containing HEPES and L-glutamine supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/mL EGF (Peprotech, Rocky Hill, NJ), 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Invitrogen). For experiments, cells were seeded on fibronectin-coated polyacrylamide gels and grown in the complete medium for 24 h. Before imaging, cells were serum starved for an additional 24 h and labeled with 200 nM of Mitotracker Deep Red (Invitrogen) for 15 min.

**Preparation of polyacrylamide gels**

Polyacrylamide gels were made with 10% polyacrylamide (Bio-Rad Laboratories, Hercules, CA) and 0.04% bisacrylamide (Bio-Rad Laboratories). To image the displacements of the polyacrylamide substrates, 1 μm fluorescent polystyrene particles (Invitrogen Yellow-Green Fluorescent Fluorospheres) were added to the gels to a final volume concentration of ~0.2%. The elastic properties of the polyacrylamide substrates were measured by performing a stress-relaxation experiment with a compression device (14) on specimens made separately with an approximate diameter of 10 mm and an approximate height of 5 mm. It was found that the constitutive behavior of the polyacrylamide gels was well-approximated by using the linear, elastic form of Hooke’s law. The Young’s modulus of the material was chosen such that it was low enough for the cells to apply a noticeable displacement to the substrate, but high enough for the cells in each cluster to remain in a monolayer instead of resting on one another. It was found that a value of Young’s modulus of mean ± standard deviation of 7.1 ± 0.4 kPa met this criterion. The polyacrylamide substrates were coated with the heterobifunctional cross-linker sulfo-SANPAH (Pierce, Rockford, IL). Fibronectin (Sigma-Aldrich) was then covalently bound to the polyacrylamide substrates by placing the substrates in a 100 μg/mL fibronectin solution overnight at 4°C.

**Confocal microscopy**

A C1 confocal microscope on a TE2000 stand (Nikon Instruments, Melville, NY) was used with a 60× 1.45 NA oil immersion objective (Nikon Instruments). A 488 nm argon laser and a 633 nm helium-neon laser were used to excite fluorescence of the fluorescent particles and cell dye, respectively. 512 × 512 pixel images were collected in a field of view of 200 × 200 μm². A 20 μm confocal stack was collected with a step size of 0.2 μm. Next, EGF (Peprotech) was introduced into the cell media at a concentration of 100 ng/mL. Additional volume stacks were collected of the cell clusters and underlying substrate 30 min and 90 min after stimulation with EGF. Finally, the cells were lysed with 0.5% Triton X-100 (Invitrogen), and a fourth volume stack was collected. All confocal imaging was performed at 37°C using a custom built enclosed heated with an Air-Therm ATX heater (World Precision Instruments, Sarasota, FL).

**Displacement and traction computation**

Because the confocal imaging resolution in the axial (z) direction was lower than in the in-plane (x-y) directions, the spherical fluorescent particles were imaged as ellipsoids that were elongated in the axial direction. To improve the axial resolution, the Lucy-Richardson deconvolution algorithm (15) was used to deconvolve the image stack from the axial point spread function of the confocal microscope.

To compute the tractions applied by the MCF-10A cell clusters to the polyacrylamide substrate, a digital volume correlation (DVC) algorithm (14) was first used to calculate the full 3D displacement profile within the substrate. This algorithm used the random speckle pattern created by the fluorescent particles within the substrate to compute the displacement of a 64 × 64 × 64 voxel subvolume by comparing how the particles displaced within the subvolume before and after deformation. The correlation was repeated for multiple subvolumes within the substrate at a separation of 8 voxels. The DVC algorithm accounted for both translations and stretches of the subvolume, but not rotations, so it was appropriate for small shear strains of $< 5\%$ (14).

After displacement computation, strains were calculated from the filtered displacement data by fitting a $3 \times 3 \times 3$ window of grid points to a trilinear function to determine the displacement gradients as described previously (14). This method computed the strains directly from the displacement data, and it circumvented the need for an inverse-Boussinesq formulation that would require regularization (16) and additional analysis to account for the finite thickness of the substrate (17). Once the strain tensor was assembled, the stress tensor was computed from the incompressible form of Hook’s law. Because the technique of computing the stress tensor from the displacements through the strain tensor and constitutive law gave the 3D state of stress at any point within the material, the tractions applied by the cell cluster were easily calculated according to the Cauchy relation, $\mathbf{t} = \sigma \cdot \mathbf{n}$. In this equation, $\mathbf{t}$ was the traction vector, $\sigma$ was the 3D stress tensor, and $\mathbf{n}$ was the unit vector normal to the substrate’s surface. To compute the tractions applied by the cells, the traction vector was computed for each point on the top surface of the substrate. Because the traction vector was computed from the 3D stress tensor, both the in-plane (horizontal) and out-of-plane (vertical, or along the axis of the objective lens) components of the traction vector were computed using this technique.

The resolution of the DVC technique was determined by performing control experiments wherein a polyacrylamide gel with no cells was imaged before and after injecting Triton. Artificial strains in the range of 2% to 6% were applied computationally to one of the volume stacks to compute the resolution under loading. The displacement noise floor was found to range from 0.05 μm (for in-plane displacement components) to 0.10 μm (for out-of-plane displacement components). Upon differentiation, the strain noise floor was found to be ~1% for the out-of-plane (z) components of the strain tensor. Because displacements and strains smaller than the noise floor could not be resolved by DVC, values of displacement and strain that were measured to be smaller than the noise floor were set to zero for plotting and analysis. All experimental data computation and image processing were performed using MATLAB (The MathWorks, Natick, MA).

**Finite element simulation**

A simple finite element (FE) model (Fig. 1) was created in ABAQUS 6.9 (Simulia, Providence, RI) to simulate the contraction of a cell cluster on a compliant substrate. In the model, the substrate properties were chosen such that they matched the properties of the polyacrylamide gels that
were used as substrates in the experiments: the substrate was modeled with linear, elastic 8-node brick elements with a Young’s modulus of 7.1 kPa and a Poisson’s ratio of 0.48. The cells were modeled as linear, elastic 8-node bricks with a Young’s modulus of 1 kPa (19) and a Poisson’s ratio of 0.3 (20). Each cell was composed of 25 brick elements. Following previous models for cellular adhesion (21,22), the cell elements were connected to the substrate by linear springs at each node. Additionally, the exterior nodes of each cell were connected to neighboring nodes with a different set of linear springs. In-plane contraction of the cells was modeled using a thermal strain in the cell elements. Because a cell on a substrate contracts the most in the in-plane (x-y) directions, the thermal contraction was applied only in the in-plane directions. Symmetric boundary conditions were used to model only a quarter of the geometry. In addition, a fixed boundary was applied at the bottom of the substrate.

In the FE model, three parameters required calibration, the spring stiffnesses associated with cell-cell and cell-substrate adhesion, and the amount of thermal strain applied to the cell cluster. The value of spring stiffness to be used can be better understood by dividing the spring stiffness by the distance between the springs, thus creating a quantity with units of force divided by area. This quantity is defined as the contact modulus \( E_{\text{contact}} \). To relate \( E_{\text{contact}} \) to the cell elements’ Young’s modulus, \( E_{\text{cell}} \), a dimensionless parameter that quantifies the adhesion stiffness is defined: \( k = E_{\text{contact}} / E_{\text{cell}} \). Because there are two sets of springs, one set for the cell-cell adhesions and one for the cell-substrate adhesions, two values of \( k \) are used, and they are called \( k_{\text{cell-cell}} \) and \( k_{\text{cell-substrate}} \), respectively. Using the previous definition, the lower bound for both \( k_{\text{cell-cell}} \) and \( k_{\text{cell-substrate}} \) is zero. An approximate upper bound for \( k_{\text{cell-cell}} \) and \( k_{\text{cell-substrate}} \) was set to 1, because increasing the contact stiffness greater than the Young’s modulus of the cell was not expected to have a noticeable effect on the tractions applied by the contractile layer to the substrate. To test this upper bound, a value for \( k_{\text{cell-cell}} \) of 10 was used, and the results were not noticeably different than the approximate upper bound of 1. To determine the lower bound of \( k_{\text{cell-cell}} \), the FE model was solved with values of \( k_{\text{cell-cell}} \) ranging from 0.01 to 1. It was found that the root mean-square (RMS) and maximum tractions changed by only 10% when changing \( k_{\text{cell-cell}} \) from 0.01 to 0.1; therefore, a lower bound of 0.1 was selected for \( k_{\text{cell-cell}} \).

To determine the amount of thermal strain applied to the cell elements and the cell-substrate contact stiffness, \( k_{\text{cell-substrate}} \), a parametric study was performed by matching the maximum and RMS of the tractions measured computationally to the tractions measured experimentally. It was found that a thermal strain value of \(-2.8\) and a cell-substrate stiffness of \( 0.2 \) matched both the maximum and RMS tractions to within \(-5\%\), which indicated that the model captured the mechanics of cellular contraction on a compliant substrate reliably. A summary of all the model parameters and their values is given in Table 1.

### RESULTS AND DISCUSSION

We use 3D TFM to measure the cell-generated traction stresses transmitted to the underlying substratum during EGF-mediated decompaction of an MCF-10A cell cluster. Representative tractions before and after stimulation with 100 ng/mL EGF are shown in Figs. 2 and 3, respectively. It is found that the cell clusters contract inward with most of the inward tractions applied at the periphery of the cell clusters. In addition, tractions in the out-of-plane direction are present. For all experiments performed, the RMS of the out-of-plane traction component is within a factor of 2 of the RMS of the in-plane traction components. These results emphasize the importance of 3D measurements of the tractions cells apply to a flat substrate. Although Figs. 2 and 3 show results from only one trial, the results discussed here are observed for all of the experiments performed.

By considering the RMS tractions from \( n = 3 \) trials before and after stimulation with 100 ng/mL EGF, it is found that the magnitude of the 3D traction vector increases by a factor of \((\text{mean} \pm \text{standard deviation}) 1.3 \pm 0.2 \) 30 min after stimulation. Ninety minutes after stimulation (data not shown), the relative change in tractions is found to reduce to 1.0 ± 0.2, indicating that the effect of the EGF begins to wear off after 90 min. In contrast, it is found that treatment with 0.1 ng/mL EGF (data not shown) does not

### Table 1 Parameters used in the finite element simulations of a cluster of cells contracting on a linear, elastic substrate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Young’s modulus ( E_{\text{substrate}} )</td>
<td>7.1 kPa</td>
</tr>
<tr>
<td>Substrate Poisson’s ratio ( \nu_{\text{substrate}} )</td>
<td>0.48</td>
</tr>
<tr>
<td>Cell cluster Young’s modulus ( E_{\text{cell}} )</td>
<td>1 kPa</td>
</tr>
<tr>
<td>Cell cluster Poisson’s ratio ( \nu_{\text{cell}} )</td>
<td>0.3</td>
</tr>
<tr>
<td>Cell-cell dimensionless adhesion stiffness ( k_{\text{cell-cell}} )</td>
<td>0.1 and 1</td>
</tr>
<tr>
<td>Cell-substrate dimensionless adhesion stiffness ( k_{\text{cell-substrate}} )</td>
<td>0.2</td>
</tr>
<tr>
<td>Thermal strain</td>
<td>(-2.8)</td>
</tr>
</tbody>
</table>
produce a measureable change in tractions at either the 30 or 90 min time points. It has been shown previously that treatment with a high level of EGF (100 ng/mL) caused contact-inhibited interior cells to enter into the cell cycle, leading to a uniform proliferation pattern within epithelial cell clusters.
Because cellular tractions have been shown to affect cellular proliferation (23), it is possible that high levels of EGF may eliminate pre-EGF patterns of cell-substrate adhesion, thus generating larger cellular tractions to the substrate at the center of the cluster and stimulating uniform cell proliferation. However, the data show that the qualitative patterns of cell-substrate adhesion do not differ drastically before (Fig. 2) and after (Fig. 3) stimulation with EGF; therefore, it is more likely that high concentrations of EGF do not directly affect patterns in cell-substrate adhesion but instead induce uniform proliferation by over-riding downstream inhibitory pathways or providing compensatory activation pathways of proliferation.

In addition to a qualitative comparison of the tractions before and after EGF stimulation, the out-of-plane ($z$) component of the tractions (Figs. 2 $d$ and 3 $d$) provides an important hint as to how the MCF-10A clusters behave mechanically. In addition to the fact that the cell clusters apply tractions in the out-of-plane direction, it appears that the cell clusters apply tractions upward at the periphery of the cluster and downward inside the periphery of the cluster, forming a local traction moment on the substrate. This observation remains the same even after stimulation with EGF. This type of traction moment in the out-of-plane direction has been observed previously for 3D traction with EGF. In addition to the fact that the cell clusters apply tractions upward at the periphery of the cluster and downward just inside the periphery, it appears that the cell clusters apply tractions in the out-of-plane direction, it appears that the cell clusters apply tractions upward at the periphery of the cluster and downward inside the periphery, forming a similar traction moment as in Figs. 2 $d$ and 3 $d$.

To better understand whether cells act cooperatively as a single unit, a finite element simulation is used to investigate how cell-cell adhesion affects the tractions that clusters of cells apply to a flat substrate. The FE model used here extends previous finite element studies on cell clusters (23) by using linear springs at the locations of cellular adhesions to study adhesion strength. Like the experimental data, the simulation computes tractions that tend to pull the substrate inward in the in-plane directions (see Figs. 4 $a$ and 5 $a$). In addition, the simulation predicts large tractions in the out-of-plane direction (Figs. 4 $b$ and 5 $b$), which highlights the importance of 3D TFM measurements for a full understanding of cellular mechanical behavior. These out-of-plane tractions result from a bending deformation of the contractile layer (i.e., the cell cluster), which pulls upward at the periphery of the cluster. To maintain force equilibrium in the cluster, the cells push downward inside the periphery.

By changing the stiffness of the springs, it is possible to investigate the effect that changing the adhesion strength has on the tractions applied by the cell cluster to the substrate. As shown in Fig. 4 $b$, it is found that using the upper bound of the dimensionless cell-cell contact stiffness, $k_{\text{cell-cell}} = 1$, (indicating a high level of cell-cell adhesion) produces an upward traction at the periphery of the cluster and a downward traction just inside of the upward traction, which forms a local moment similar to what is observed experimentally. This matching of the traction moment indicates that the model captures the mechanics involved in the contraction of a cluster of cells on a flat, compliant substrate. Together with the experimental data, this simple finite element simulation demonstrates that the cells in a cluster act cooperatively.

To further understand how the cells adhere to each other, the FE model is used to simulate the effect that reducing the cell-cell contact stiffness from the upper bound of $k_{\text{cell-cell}} = 1$ to the lower bound of $k_{\text{cell-cell}} = 0.1$ has on the tractions applied by the cell cluster to the polyacrylamide substrate. The results for the reduced contact stiffness ($k_{\text{cell-cell}} = 0.1$) are shown in Fig. 5. Although the in-plane traction components (Fig. 5 $a$) do not differ from the model with a higher contact stiffness (Fig. 4 $a$), a comparison of Figs. 4 $b$ and 5 $b$ shows that the out-of-plane tractions do differ. The large fluctuations in out-of-plane traction magnitudes in Fig. 5 $b$ are not observed experimentally either before (Fig. 2 $d$) or after stimulation with EGF (Fig. 3 $d$); therefore, we conclude that the MCF-10A cells maintain...
To verify that this conclusion is independent of the strong cell-cell adhesions before and after stimulation with EGF. To better understand the reason that a 2D TFM measurement cannot resolve the difference between cells adhering tightly and loosely to one another, consider the hypothetical system of two cells interacting with each other as shown in Fig. 6. In Fig. 6a, the cells are tightly adhered to each other; therefore, at the location of adhesion, they apply minimal tractions to the substrate beneath them. In contrast, Fig. 6b shows a pair of cells that do not adhere to each other, but instead they adhere to the substrate beneath them. Here, the cells apply tractions to the substrate in the in-plane (horizontal) direction and out-of-plane (vertical) direction. However, at the location between the cells, the displacements caused by the in-plane tractions that the cells apply cancel each other out by the mechanical principle of superposition, so no displacements can be observed by a 2D TFM experiment. This issue of traction superposition causing zero net displacement should be considered in any TFM experiment involving multiple cells or cell clusters, especially the experiments that use a traction imbalance within the cell cluster to measure cell-cell tractions (25,26). On the basis of this analysis, we conclude that the best way to verify that cells are well-adhered to each other is by observing the out-of-plane traction component in addition to the in-plane traction components. If an experimenter
and the interest in multicellular systems, 3D traction measurement techniques should be considered for future studies.

SUPPORTING MATERIAL

A figure is available at http://www.biophys.org/biophys-supplemental/S0006-3495(12)00218-4.

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REFERENCES

18. Reference deleted in proof.