Characterizing the mechanics of cultured cell monolayers

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One-cell-thick monolayers are the simplest tissues in multicellular organisms, yet they fulfill critical roles in development and normal physiology. In early development, embryonic morphogenesis results largely from monolayer rearrangement and deformation due to internally generated forces. Later, monolayers act as physical barriers separating the internal environment from the exterior and must withstand externally applied forces. Though resisting and generating mechanical forces is an essential part of monolayer function, simple experimental methods to characterize monolayer mechanical properties are lacking. Here, we describe a system for tensile testing of freely suspended cultured monolayers that enables the examination of their mechanical behavior at multi-, uni-, and subcellular scales. Using this system, we provide measurements of monolayer elasticity and show that this is two orders of magnitude larger than the elasticity of their isolated cellular components. Monolayers could withstand more than a doubling in length before failing through rupture of intercellular junctions. Measurement of stress at fracture enabled a first estimation of the average force needed to separate cells within truly mature monolayers, approximately ninefold larger than measured in pairs of isolated cells. As in single cells, monolayer mechanical properties were strongly dependent on the integrity of the actin cytoskeleton, myosin, and intercellular adhesions interfacing adjacent cells. High magnification imaging revealed that keratin filaments became progressively stretched during extension, suggesting they participate in monolayer mechanics. This multiscale study of monolayer response to deformation enabled by our device provides the first quantitative investigation of the link between monolayer biology and mechanics.

cell mechanics | tissue mechanics | intermediate filaments

Many of the cavities and free surfaces of the human body are lined by a layer of cells one cell thick. Cells within these monolayers are tightly connected to one another by intercellular junctions. Tight junctions form barriers restricting the passage of solutes while others, such as adherens junctions and desmosomes, integrate the cytoskeletons of constituent cells into a mechanical syncytium. Exposure to mechanical stresses is a normal part of physiology for monolayers: intestinal epithelia are stretched during peristaltic movements in the gut, lung alveoli deform during breathing, and endothelia are exposed to pulsatile fluid shear stresses in blood flow (1–3). The mechanical function of monolayers is particularly apparent in disease where mutations or pathogens affecting the cytoskeleton, adherens junctions, or desmosomes result in increased fragility of tissues (4). Development offers perhaps the most vivid illustration of the role of epithelia in withstanding and exerting mechanical stresses. Indeed, embryonic epithelial tissues are under a constant tension generated by spatially restricted cellular actomyosin contractions (5). When cadherin intercellular adhesion is disrupted, embryos fail to properly develop, displaying a disaggregated ectoderm consistent with mechanical failure (6). Early in vertebrate development, the absence of an extracellular matrix (ECM) in the blastopore (7)

together with the lack of effect of inhibition of ECM synthesis on blastopore initiation suggest that the mechanics of monolayers and force generation within monolayers govern blastopore formation (8, 9).

To date, research in cell mechanics has primarily focused on isolated cells, and much is now known about their mechanical properties as well as the underlying biology in normal physiology and disease (10). Comparatively little is known about the mechanics of monolayers, but recently experiments combining traction microscopy with deformation analysis have begun to shed light on this topic. Within monolayers, stresses are propagated over several cell diameters by intercellular adhesion, cells migrate to minimize intercellular shear stress (11), and the collective motion of cells within monolayers displays behaviors reminiscent of a glass transition (12, 13). Despite these advances, our knowledge of monolayer mechanical properties such as stiffness or ultimate strength remains poor due to lack of experimental techniques. Extrapolation of these parameters from single-cell measurements is not possible due to radical differences in cytoskeletal organization associated with the formation of intercellular junctions. Present measurements of intercellular adhesion energy are restricted to durations over which intercellular junctions cannot fully mature (14, 15). Direct experimental measurements on monolayers with mature intercellular junctions would greatly enhance our understanding of the mechanics of epithelial morphogenesis (16, 17) and the effect of pathologies on tissue strength (4).

We have developed a versatile, unique system that allows investigation of the tensile planar mechanical properties of epithelial cell monolayers in isolation from their substrate. We exploit these capabilities to acquire a consistent set of time-resolved quantitative measurements of monolayer mechanics at multi-, uni-, and subcellular scales and provide a detailed characterization of monolayer mechanical properties. Through the spatial organization of their cytoskeleton and interfacing via specialized intercellular adhesions, cells create a tissue that has a much higher elastic modulus than measured in single cells.

Results

Cell Monolayer Culture and Testing Device. The general principle of our system to characterize monolayer mechanics is simple: Monolayers suspended between the extremities of two test rods (one soft and one stiff) are slowly extended by prying the rods apart with a micromanipulator. The applied force can be measured by monitoring the bending of the soft test rod during mono-

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layer extension (Fig. S1). Our mechanical characterization setup addresses four key requirements: (i) monolayers must be free from their substrate such that only the monolayer is load-bearing to allow for simple interpretation of the stress-strain response (Fig. 1), (ii) attachment of the samples to the test rods must require minimal manipulation, (iii) live microscopy imaging at the cellular and subcellular level must be possible during mechanical stimulus, and (iv) measurements must be quantitative to enable comparison between treatments.

To generate cell monolayers free from a substrate with minimal manipulation, we cultured cells on a temporary sacrificial substrate created by polymerizing a drop of collagen between the two rods. Cells were seeded onto this scaffold and cultured until the monolayer extended from one test rod to the other, covering the whole collagen substrate and part of each test rod (Fig. 1A). Prior to mechanical testing, the collagen was removed by enzymatic digestion, leaving the monolayer attached to the test rods by cell-substrate adhesion but devoid of substrate and freely suspended in between (Fig. 1B). In the absence of substrate, monolayers stayed healthy and maintained their characteristic epithelial apico-basal polarization for at least 3 h (Fig. 1C and Fig. S2).

The mechanical testing equipment (Fig. S1A) consisted of two micromanipulators and a top-down microscope to image-test rod positions. A manual micromanipulator kept one rod stationary, while a motorized micromanipulator controlled the displacement of the other. To generate quantitative measurements of monolayer mechanical properties, we developed our culture system to allow for force measurements. Devices consisted of two main components (Fig. S1B): (i) a U-shaped glass capillary with one long arm that acts as a rigid reference rod and a short arm that connects to the flexible test rod, and (ii) a flexible test rod made of NiTi metal wire with a small enough bending rigidity for sub milli-Newton forces to induce a deflection precisely measurable by the microscope. Forces applied onto the monolayer during extension were determined by measuring the deflection d of the wire relative to its predicted unstressed position (dotted line, Fig. S1B) and fitting $d(y)$ with a simple cantilevered beam model (SI Materials and Methods and Fig. S3).

Tissue-Level Mechanics. Using our experimental setup, we characterized the mechanical properties of monolayers of Madine-

Fig. 1. Experimental setup for measuring the mechanical properties of cell monolayers. (A) (Left) Line drawing and (Right) microscopy image. Cell layers (green) were cultured on a sacrificial collagen scaffold gelled between the test rods (red in line drawing and immunostain, scale bar s.b. = 100 μ m). (B) After enzymatic digestion, the collagen layer was completely removed, leaving the monolayer freely suspended between the two test rods as evidenced by the loss of collagen immunostaining (red). (C) ZX profile of a suspended monolayer 3 h after collagen digestion. F-actin is shown in red, gp135 (a classic apical polarity marker) is shown in green, nuclei are shown in blue. Suspended monolayers retained their characteristic polarization despite removal of the substrate. (s.b. = 100 μ m)

Darby Canine Kidney (MDCK-II) cells, a classic epithelial cell model. In the following, unless otherwise noted, we report the engineering strain: $\epsilon = \Delta L/L_0$, with ΔL the monolayer length change and L_0 its original length (Fig. S4).

Living tissues are intrinsically viscoelastic with both physical and biological phenomena contributing to their time-dependent mechanical properties. Measured physiological strain rates in monolayer covered tissues range from approximately $0.04\% \cdot s^{-1}$ in developing drosophila embryos (18) and tens to hundreds of $\% \cdot s^{-1}$ in alveolar epithelium and mitral valve tissue (19, 20). To investigate the time-dependent mechanical properties of monolayers, we characterized their creep response to two distinct step increases in stress, respectively with high (3 kPa) and low stress (0.7 kPa). When the monolayers were subjected to low stress loading, strain increased rapidly in response to stress application before reaching a plateau that subsisted over 200 s (Fig. 2A, grey line). In contrast, when high stress loading was applied, no plateau was reached and strain increased continually with time (Fig. 2A, black line). Plotting these response curves in log-log scale revealed that monolayer creep followed a power law in response to high-stress step loading but not following low-stress step loading (Fig. 2A, inset), suggesting that monolayers behave as viscoelastic solids below a certain critical stress and as complex fluids above. Power law creep responses had an exponent $\beta = 0.15 \pm 0.03$, slightly less than generally reported for single cells [$\beta \sim 0.3$ – 0.5 (21)]. Consistent with the observations upon low-stress loading, stress relaxation of monolayers also reached a plateau after approximately 50 s (Fig. S5E), suggesting a limit for elastic behavior. Estimates of relaxation rates for computational models and comparative studies could be obtained by fitting stress relaxations with appropriate rheological models (SI Materials and Methods and Fig. S5 G–H). The time scales needed

Fig. 2. Mechanical properties of cell monolayers. (A) Creep response following step application of low (0.7 kPa, grey) and high (3 kPa, black) stress. The plotted responses are averaged over at least six experiments each. At low stress, following a rapid increase in strain, monolayers reached a plateau that lasted for the remainder of the experiment. At high stress, no plateau was reached and strain increased continually with time. (Inset) Creep response curves plotted in log-log scales. The creep response at high stress (black) was well fitted by a linear function with slope $\beta = 0.15 \pm 0.03$; whereas at low stress the creep response was not linear. (B) Stress-extension curves shown for 12 different monolayers. All curves displayed three distinct regimes of loading: (i) an initial "toe" region (blue box) as the monolayer becomes loaded under tension, (ii) a linear extension regime (green box) from which an elastic modulus can be calculated, and (iii) a plateau (red box) which corresponds to plastic deformation and eventual failure. (C) Deformation of a monolayer under stretch. Images acquired by bright-field microscopy for a monolayer at 0 and >80% extension. At >80% extension, the monolayer delaminated from the test rods (arrows) suggesting that cell-cell adhesion is stronger than cell-substrate adhesion for this geometry. (s.b. = 1 mm)

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Fig. 4. Monolayer mechanics at the cellular level. (A) Three-dimensional isosurface reconstruction of cells within a monolayer before (red) and after (green) extension. Cell height decreased with extension (side view: black arrowheads: 0% strain, white arrowheads: 50% strain) but cellular projected area increased (top view). (B) Cell volume was conserved during extension, suggesting that the constituent cells are incompressible. (C–E) Segmented images of cells expressing E-cadherin GFP were used to calculate the cellular deformations before and during stretch. (F) The calculated cellular strain matched the monolayer strain near perfectly, indicating that no intercalation takes place during extension. (G) The orientation of cell-long axes prior to stretch was widely distributed with a small bias along the x -axis but, during stretch, cellular orientations were nearly exclusively aligned with the direction of extension. The anisotropy of alignment calculated as $A = 1 - (\% \text{ aligned perpendicular}) / (\% \text{ aligned parallel})$ almost doubled when the cells were subjected to 39% strain.

properties and, in tissues, loss of function mutations affecting cytoskeletal and adhesive proteins lead to increased fragility. We examined cytoskeletal organization during monolayer extension paying particular attention to protein constituents of adherens junctions and desmosomes, key structures in intercellular junctions.

In adherens junctions, adjacent cell membranes are tethered to one another by classical cadherins (E-cadherin in epithelial cells) that are linked intracellularly to the actomyosin cytoskeleton, thus integrating neighboring cells into a mechanical syncytium (25). Removal of the collagen substrate led to a general rounding of the cells and a reduction in projected surface area due to loss of basal adhesion (Fig. 5A and B and Fig. S4) but no change in the localization of E-cadherin was observed. E-cadherin distribution was not affected by extension (Fig. 5B and C). F-actin remained localized to intercellular junctions (Fig. 5D and E) but had a somewhat less uniform distribution under strain displaying some enrichment at tricellular junctions (Fig. 5F). Myosin regulatory light chain (MRLC), a component of myosin II whose phosphorylation controls contractility, displayed a dramatic change in localization upon removal of the substrate and under strain. Localization changed from being cytoplasmic (Fig. 5G) to punctate and junctional (Fig. 5H and I), reminiscent of myosin localization in embryonic epithelial tissues (5) and suggesting a role for myosin contractility in monolayer mechanics.

Fig. 5. Subcellular organization in stretched monolayers. OC: On collagen, PS: pre-stretch, % indicates the percentage strain ($s.b = 10 \mu\text{m}$). (A–C) E-cadherin GFP remained localized to cell junctions in all conditions. (D–F) Life-act GFP, an F-actin marker, remained localized at cell junctions throughout extension but appeared less uniform at low stretch. (G–I) The regulatory light chain of myosin was primarily cytoplasmic in cells on collagen (G), but underwent a dramatic relocalization to cell junctions pre-stretch (H) and at high extension (I). (J–L) The keratin 18 filament network spanned the entire monolayer on collagen (J) and looked largely bundled prior to extension (K). Application of stretch induced rearrangement of the filaments, suggesting that they served to transmit stress across cell boundaries (arrowhead). (M) Keratin filaments (green) formed an intercellular network with nodes at the cell centres (white arrows) that linked cells to one another across cell boundaries (blue) in stretched monolayers. (N) Keratin filaments (green) appeared tensed across cell junctions (blue) perpendicular to the direction of extension (white arrows) and bundled (grey arrows) at cell junctions parallel to the direction of extension.

Desmosomes link the keratin intermediate filaments of neighboring cells to one another, forming a second supracellular cytoskeletal network. Genetic mutations of keratins or desmosomal proteins result in fragile epithelia (4). On collagen, keratin localized perinuclearly with short wavy segments linking cells to one another (Fig. 5J). After removal of collagen, keratin filaments remained perinuclear (Fig. 5K), and at high strain, filaments became aligned in the direction of stretch (Fig. 5L). When imaged at higher magnification, a keratin supracellular network was clearly visible (Fig. 5M) and filaments straddling intercellular junctions appeared taut and aligned parallel to the direction of extension (Fig. 5N, arrows). This change in conformation from wavy at low strain to taut at high strain suggested that keratins are involved in a nonlinear mechanical response of the monolayer to stretch, as proposed by others (26, 27) and consistent with the mechanical properties of isolated keratins and keratin networks in cells (27, 28).

Subcellular Perturbations Lead to Changes in Tissue-Level Mechanics. We examined how perturbations at the molecular scale affected mechanics at the tissue scale. The filamentous actin network is a

paint a picture of monolayer mechanics where cells adhere strongly to one another and therefore can pull strongly on one another through myosin contractility, leading to the development of a tissue-level tension and as a result higher stiffness. Furthermore, such a process may be self-reinforcing with higher tensions leading to myosin recruitment (30) and increased myosin duty ratio (31). Imaging of intermediate filaments revealed that their aspect changed from wavy to taut with increasing monolayer extension, suggesting that at high strains they become load-bearing and therefore may be involved in a nonlinear mechanical response of the monolayer as previously proposed (26, 27). Such an interpretation would be consistent with the fragile epithelium symptoms observed in patients with mutations in keratins or desmosomal proteins (4) but will necessitate further study. Together our experimental methods pave the way for quantitative investigations of monolayer mechanics at the subcellular, cellular, and tissue level and they should be widely applicable to any cell type forming strong intercellular junctions.

During development, embryonic morphogenesis is in large part due to changes in the organization and mechanics of epithelial monolayers. Over the past decade, researchers have devised many experimental and computational techniques to study the mechanics of morphogenetic events, but a detailed understanding has been hindered by a lack of tools to directly characterize monolayer mechanics. For example, measurements of embryonic epithelial tension by laser cutting rely on monitoring tissue recoil, something that depends both on monolayer tension and stiffness. Though laser cutting successfully allows for comparative measurements of tension to be effected, combining it with our techniques would allow for deconvolution of tension and stiffness and hence absolute measurements. In computational models of epithelia, the contribution of cytoskeletal components (in particular actomyosin contractility) to monolayer mechanics is often accounted for by spring networks and line tensions acting in bulk or at intercellular junctions (16, 17). However, estimating the value of the corresponding parameters has proven challenging.

Based on our measurements of monolayer stiffness for a range of biological and chemical perturbations, suitable estimates of these parameters can be obtained. Direct experimental measurements of monolayer mechanics combined with computational models will therefore allow for a better understanding of multicellular aggregate mechanics.

Materials and Methods

Additional information can be found in the *SI Text*.

Force Measurement Device Fabrication. Borosilicate capillaries were bent into a "U" shape and cut to size. One arm was left long to act as a rigid reference rod and the other cut short to serve as a connection for the flexible test rod (Fig. S1B). NiTi alloy wire was cut to length, dipped into UV-curing glue and threaded into the static rod. Another wire of similar length made up the flexible rod. Two pieces of Tygon tubing were attached to each wire to act as a substrate for collagen polymerization and cell culture. The test rods were glued to the bottom of 50-mm plastic-bottomed Petri dishes.

Cell Culture on Devices. Collagen type 1A was reconstituted on ice and a droplet was deposited between the device test rods. Devices were placed at 37 °C for 90–120 min and allowed to dry, giving a thin layer of collagen between the test rods. The collagen support was rehydrated by depositing a 10 μ l droplet of culture medium on it. Approximately 25,000 cells were placed onto the collagen support and incubated at 37 °C for 30 min. Finally, culture medium was added such that the test rods were completely submerged.

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