Biochemical analysis of force-sensitive responses using a large-scale cell stretch device

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ABSTRACT
Physical force has emerged as a key regulator of tissue homeostasis, and plays an important role in embryogenesis, tissue regeneration, and disease progression. Currently, the details of protein interactions under elevated physical stress are largely missing, therefore, preventing the fundamental, molecular understanding of mechano-transduction. We designed a simple, easy-to-fabricate, large-scale cell stretch device for the analysis of force-sensitive cell responses. Using proximal biotinylation (BioID) analysis or phospho-specific antibodies, we detected force-sensitive biochemical changes in cells exposed to prolonged cyclic substrate stretch. For example, using promiscuous biotin ligase BirA\textsuperscript{+}/C3\textsuperscript{+}tagged \(\alpha\)-catenin, the biotinylation of myosin IIA increased with stretch, suggesting the close proximity of myosin IIA to \(\alpha\)-catenin under a force bearing condition. Furthermore, using phospho-specific antibodies, Akt phosphorylation was reduced upon stretch while Src phosphorylation was unchanged. Interestingly, phosphorylation of GSK3\(\beta\), a downstream effector of Akt pathway, was also reduced with stretch, while the phosphorylation of other Akt effectors was unchanged. These data suggest that the Akt-GSK3\(\beta\) pathway is force-sensitive. This simple cell stretch device enables biochemical analysis of force-sensitive responses and has potential to uncover molecules underlying mechano-transduction.

KEYWORDS
\(\alpha\)-catenin; Akt; biotin identification (BioID); force-dependent protein interactions; GSK3\(\beta\); mechanotransduction; substrate stretch

Introduction
In multi-cellular organisms, individual cells are connected to neighboring cells via cell-extracellular matrix and cell-cell adhesion. This physical integration of cells is an essential feature of epithelial tissues and serves as the molecular basis of tissue architecture. Epithelial tissues, however, are not static structures. For example, epithelial cells migrate collectively during embryogenesis,\textsuperscript{1-3} tissue regeneration,\textsuperscript{4,5} and in some diseases like cancer,\textsuperscript{6,7} in which adhesive contacts play a major role.\textsuperscript{8,9} Therefore, the regulation of cell adhesion dynamics between cells or cell and extracellular matrix is paramount to physiology (and pathology) of epithelial tissues.

One of the key features of all cell junctional complexes is the ability to withstand and transmit physical forces. While the extracellular domain of adhesion proteins binds to the extracellular matrix or neighboring cells, the intracellular domain of adhesion proteins binds to cytoskeletal structures to form a unified structural network across cells and the extracellular matrix. However, this cytoskeletal linkage is not a direct molecular linkage (i.e., adhesion proteins do not directly bind to the actin cytoskeleton). Instead, numerous cytoplasmic proteins interface the intracellular domain of adhesion proteins and the cytoskeletal network, and spatial organization of some proteins in the adhesive complexes has been resolved.\textsuperscript{10,11} These cytoskeletal linkers at cell junctions are thought to regulate the strength of and force-transmission through cell adhesion.

Interestingly, cell junctions respond to externally applied forces. The seminal study on integrin-mediated cell-extracellular matrix adhesion demonstrated that integrin junctions stiffen and recruit talin and vinculin when shear stress is applied.\textsuperscript{12} While integrins have been known to respond to mechanical forces, recent studies have shown that the cadherin complex also responds to external mechanical forces. For example, in live cells, the application of mechanical stress stiffens cadherin junctions\textsuperscript{13} and recruits actin\textsuperscript{14,15} and vinculin.\textsuperscript{15,16} In fact, cadherins appear to be under constant tension.\textsuperscript{17} These
studies suggest both integrin and cadherin junctions are dynamic, force-responsive molecular complexes.

Force-dependent protein recruitments at adhesive complexes are thought to be mediated by force-induced protein conformational changes that result in altered biochemical properties. For example, fibronectin, an extracellular matrix protein, assembles into fibrous structures by exposing cryptic bundling sites upon force-induced conformational change. Similarly, both p130cas and talin unfold from applied force which exposes cryptic phosphorylation sites and vinculin binding sites in its rod domain, respectively. Furthermore, the α-catenin-vinculin interaction depends on force-generating myosin II activity, and β-catenin bound α-catenin interacts with an actin filament in a force-dependent manner. Recently, YAP/TAZ proteins were identified as signaling molecules for mechano-transduction. While force-sensitive conformational change is not the only proposed mechanism for force-sensing, the assembly of force-induced protein complexes is the driving mechanism of mechano-transduction. Yet, a list of components in the mechano-sensitive complex at adhesive contacts remains incomplete.

This is in part due to the lack of a large-scale cell culture system that can apply external strain onto cells and promote force sensitive biochemical changes. In some cases, the force-sensitive responses are indirectly inferred from the use of myosin II inhibitors to deplete cellular contractility. Alternatively, external strains are applied to adherent cells using flexible membrane substrates. These flexible substrates are often manufactured with polydimethylsiloxane (PDMS), and stretched by a motor or vacuum. While several types of cell stretch devices are commercially available (e.g., Strex Inc., Flexcell International Corp., Electron Microscopy Sciences), all devices have a limited cell culture area that often optimized for microscopy analysis and are not suited to produce a large quantity of cell lysates for biochemical analysis.

We designed a simple substrate stretch device to exert strain onto adhered cells. This system is based on a thin PDMS membrane as a substrate, which stretches uni-axially and cyclically. The device is easy to fabricate from acrylic sheets and commercially available parts. We characterized the strain profile of the membrane and biochemically tested force-sensitive responses of adherent cells. This device is ideally suited for experiments that require a large quantity of cell lysates to improve the yield of low abundance proteins (e.g., signaling proteins) or for protein identification using mass spectrometry analysis.

Results

Design and fabrication of a large-scale cell stretch device

To analyze biochemical responses of cells experiencing mechanical perturbations, we designed a large-scale cell stretch device to apply strain onto cells. The principle of this cell stretch device is based on a flexible substrate that undergoes cyclic stretch, thereby applying strain onto adherent cells. The membrane is bounded at 2 sides, and clamped onto acrylic mounts (Fig. 1A and S1). The membrane is fabricated from PDMS using a mold (Fig. 1B) and is coated with extracellular matrix to promote cell adhesion. One of the membrane mounts is attached to the carriage and rail system and translated by a servo motor programmed to generate a cyclic stretch pattern (e.g., sine or triangular) with a defined amplitude and frequency (Fig. 1C). To ensure that the commanded stretch pattern is closely replicated by the device, the actual stretch pattern was recorded and analyzed (Fig. 1C). Direct comparison of commanded (line) and actual (circles) membrane stretch pattern demonstrated that the amplitude and frequency of membrane stretch were precisely controlled (Fig. 1C). Additional details of design and fabrication of this device are described in Materials and Methods.

Thickness of the PDMS membrane is a critical design parameter as too thin of a membrane will be easy to rip and too thick of a membrane will be difficult to stretch. Due to the size of the PDMS membrane (typically 19×16 cm with the cell culture area of 14×16 cm), spin coating, often used for micro-fabrication of PDMS film, produces membranes that are too thin, difficult to handle and easy to rip. Instead, we poured hexane-diluted PDMS (to reduce the viscosity and allow for easier handling) in a membrane mold while ensuring the surface was perfectly level (Fig. 1B), and cured the membrane at room temperature. This process ensures consistent thickness and reproducibility. The membrane thickness was routinely checked by weighing small membrane cutouts (see Fig. 2 for membrane characterization).

The cured PDMS membrane was cut to size, plasma cleaned, and collagen coated to promote cell adhesion. The MDCK epithelial cells rapidly adhered to the membrane surface and formed a cell monolayer. Based on our previous study using a commercially available small stretch chamber, the membrane was stretched to 25% strain at a frequency of 0.3 Hz for ~20 hours. Under these conditions, the cells aligned perpendicular to the direction of stretch (Fig. 1D), a morphological response consistent with our previous study using a small stretch chamber.
For biochemical analysis, uniform mechanical exposure minimizes cell phenotype and biochemical variation. Since the strain heterogeneity may be an issue for large substrates, we analyzed strain distribution of the PDMS substrate. To quantify local membrane strains, fiducial markers were printed on the membrane surface, then the membrane was stretched by 25% of its initial length (Fig. 2A). As the membrane stretched, the free sides of membrane bowed inward, and consequently, generating non-zero strain values perpendicular to the stretch direction (Fig. 2A). By tracking the fiducial markers, local displacements and strains were calculated using a custom macro in ImageJ (Fig. 2B). The magnitudes of local strains averaged around 25% along the stretch axis of the membrane (Fig. 2D). This was consistent with a uniform thickness of the membrane (Fig. 2C). While the substrate near the bound edges of the membrane stretched to a lesser extent than the center regions of the membrane (Fig. 2B), only minor deviations from the overall average strain were observed (Fig. 2E), suggesting that the majority of cells will be exposed to similar membrane strains.

**Biochemical analysis of stretch-dependent responses**

Using a commercially available, small cell stretch chamber and proximal biotinylation (BioID) technique, our previous study has shown that the spatial proximity of α-catenin and myosin IIA changes with substrate stretch.\(^{37}\) In this technique, a protein of interest (e.g., α-catenin) is tagged to a biotin ligase (BirA\(^*\), a R118G mutant of BirA) that promiscuously biotinylates surrounding proteins, and the biotinylation profiles of the control and stretch conditions are compared in Western blots analysis. Due to the small scale stretch chambers used in this previous study, the signals of biotinylated proteins in Western blots were often weak despite using a low stringent wash for the isolation of biotinylated proteins.\(^{37}\) The use of low stringent wash (0.2% SDS) preserved strong protein-protein interactions thus isolating non-biotinylated proteins that complicated subsequent analysis.\(^{37}\)

The substrate membrane of our large-scale cell stretch device has a cell culture area equivalent of 4 p100 dishes. MDCK epithelial cells expressing BirA\(^*\)-α-catenin were plated on the membrane and stretched with a triangular cyclic stretch function (25%, 3 second cycle) for 20 hours. Under similar conditions to the small-scale experiments,\(^{35}\) but using a more stringent purification protocol with 2% SDS wash to remove any non-biotinylated proteins from streptavidin beads, we purified biotinylated proteins from BirA\(^*\)-α-catenin expressing cells. Both BirA\(^*\)-α-catenin and β-catenin were biotinylated and the biotinylation levels were similar in the control and stretch conditions (Fig. 3A). As shown previously,\(^{37}\) biotinylation of myosin IIA was significantly increased in
stretch condition relative to control, while biotinylation of myosin IIB was undetectable in both control and stretch conditions (Fig. 3A). These large-scale experiments confirm the previous observation that the spatial proximity of α-catenin and myosin IIA increases in the presence of mechanical perturbation, but without any ambiguity of potential false positives from a low stringent wash condition.

To further demonstrate the utility of the large-scale cell stretch device, phosphorylation status of various signaling proteins was surveyed using commercially available, phospho-specific antibodies. Previous studies have shown that the activity of Src kinase is responsive to external mechanical forces. Src proteins undergo a conformational change that promotes auto-inhibition of its kinase activity, and Y416 or Y527 phosphorylation is a proxy for Src activation or inhibition, respectively. When MDCK epithelial cells were stretched with a triangular cyclic stretch function (25%, 3 second cycle, 20 hour duration), Src protein level was the same in both control and stretch cell lysate (Fig. 3B). Furthermore, the Src phosphorylation at Y416 (activating) or
Y527 (inhibiting) sites was also the similar level in control and stretch conditions, suggesting that Src activity in MDCK cells is not dependent on triangular cyclic stretch under these conditions (Fig. 3B).

In contrast, the activity of Akt, an essential kinase for cell survival, proliferation, and cell migration pathway, is stretch-dependent. Active Akt is phosphorylated at S473.\(^{40}\) While the total Akt level remained constant...
regardless of substrate stretch, the phosphorylation at S473 significantly decreased with the stretch application (Fig. 3C), suggesting that Akt activity decreases with prolonged exposure to a force-bearing condition. Interestingly, the total level and the phosphorylation status of Akt upstream effectors, PTEN (S380) and PDK1 (S241) remained constant (Fig. 3D and E). The total level and phosphorylation status of Akt downstream effector cRaf (S259) also remained constant (Fig. 3F), while the phosphorylation of another Akt downstream effector, GSK3β (S9), decreased without any changes to the total level of GSK3β (Fig. 3G). Since GSK3β is an Akt substrate and GSK3β S9 phosphorylation inhibits its activity, these results suggest that stretch-dependent cell signaling deactivates Akt, and as a consequence, activates GSK3β.

Interestingly, the lower level of substrate stretch (14%) also decreased the phosphorylation of Akt at S473 and GSK3β at S9 similar to the level of 25% stretch (Fig. 3C and G). Thus, between 14 and 25% stretch, stretch-dependent Akt or GSK3β phosphorylation was not strongly dependent on the magnitude of stretch. Note that all samples were loaded equally by quantifying the cell lysate concentration and as indicated by the tubulin blot (4 representative experiments from panel B-G were blotted with a tubulin antibody, Fig. 3H). These results demonstrate the large-scale cell stretch device as an ideal experimental platform to test biochemical responses as a result of mechanical perturbations.

Discussion

Force-sensitive protein interactions are a key biochemical step in mechano-transduction. One method for analyzing force-sensitive protein interactions is promiscuous biotinylation by a mutant BirA+ biotin ligase, which labels proximal proteins in live cells. A stretch device can be used to apply force-bearing conditions to the BirA+ expressing cells and the difference in biotinylation patterns of control and force-bearing conditions identifies potential force-sensitive interactions. Unfortunately, biotinylated proteins are often low-abundance. Due to the limited quantity of cell lysates obtained from small-scale, stretch devices, force-dependent biochemical analysis using proximal biotinylation has been inherently difficult despite a highly-efficient streptavidin purification protocol.

We designed an easy-to-fabricate, large-scale cell stretch device to probe force-sensitive biochemical responses of an epithelial cell monolayer. This device yields a quantity of cell lysates similar to traditional biochemical preparations, thus providing opportunities to analyze force-sensitive, low abundance proteins. This large-scale stretch device overcomes the sample limitation in proximal biotinylation analysis, as demonstrated by the detection of force-sensitive interaction between α-catenin and myosin IIA (Fig. 3A). Furthermore, to identify a comprehensive list of proximal proteins, purified biotinylated proteins can be analyzed using mass spectrometry, which often requires a micro-gram quantity of cell lysates. This large-scale stretch chamber will aid future proteomic studies on force-sensitive protein interactions using proximal biotinylation analysis.

An additional application includes the detection of force-sensitive post-translational modifications. Previous studies have shown that the Src protein is activated with the application of external forces. The application of tagging force via a ligand-coated particle on the cell surface, for example, promotes Src activation. In this case, Src activation is detected by its phosphorylation status or an activation sensitive Fluorescence Resonance Energy Transfer (FRET) probe. However, in our biochemical analysis, Src phosphorylation status remained constant regardless of substrate stretch (Fig. 3B), suggesting that mechanical perturbation by substrate stretch is not sufficient for Src activation in our model system. This stretch independent Src phosphorylation is consistent with a similar substrate stretch experiment using a small scale device, suggesting that sub-cellular stimulation, and not substrate stretch that strains a whole cell body, may be responsible for Src activation.

Interestingly, Akt phosphorylation, and thus its activation, was significantly changed with the exposure to substrate stretch. The Akt signaling pathway regulates cell survival, proliferation, cell migration. Akt phosphorylation at S473 decreased in stretch condition (Fig. 3C), suggesting that Akt-mediated signaling is inhibited upon cell stretch. Interestingly, previous studies have shown that cell stretch can lead to increase in Akt phosphorylation. For example, Akt phosphorylation increased with the applications of cyclic substrate stretch in smooth muscle cells, cardiomyocytes, mesangial cells and fibroblasts. While Akt activation likely depends on cell types and its context, all previous studies have used a shorter stretch time than current work. This is consistent with an observation that substrate stretch increases the insoluble pool of Akt within 10 minutes, indicating that Akt is membrane bound and activated. In smooth muscle cells, the initial increase in Akt phosphorylation, however, peaked at 6 hours and started to decline, consistent with this study (Fig. 3C). These studies highlight the diverse roles of the Akt signaling pathway and demonstrate force-induced signaling regulation strongly depends on time scale.

Furthermore, PTEN, PDK1 and cRaf phosphorylation status did not change while their total protein levels also remained constant (Fig. 3D-F). In contrast, GSK3β phosphorylation at S9 decreased (Fig. 3G), suggesting that...
GSK3β is a down-stream effector of the force-sensitive Akt signaling pathway. Interestingly, in air-way smooth muscle cells, the application of stretch decreases the level of GSK3β by microRNA-26a, demonstrating an alternative pathway to regulate GSK3β in a force-dependent manner. Furthermore, substrate stretch has been shown to activate cell proliferation mediated by Yap1 and β-catenin. While GSK3β phosphorylates β-catenin in epithelial cells for degradation, how GSK3β activity changes β-catenin’s nuclear translocation upon stretch is unclear and may be a critical force-sensitive function of GSK3β.

Force-sensitive regulation of signaling pathways emerged as a key regulator of cell behavior due, in part, to innovative tools to probe single cell force responses (using optical tweezers, atomic force microscopy, and FRET-based protein activity/conformation sensors). These tools are indispensable for detecting rapid force-initiated responses. In contrast, the use of cell stretch devices with in situ proximal biotinylation can detect spatial proximity of proteins under force-bearing conditions. Furthermore, biochemical analysis of cell lysates obtained from stretch devices detects long-lasting, force-sensitive responses that alter cell phenotype. In addition to signaling molecules described here, previous studies have shown that the activation of ERK signaling is responsive to mechanical stretch. There are undoubtedly other forcesensitive signaling molecules to be discovered. With the ease of collecting concentrated cell lysates exposed to cyclic mechanical strain, this large-scale stretch device is an ideal platform to identify other force-sensitive signaling pathways. Therefore, the device outlined in this study has a wide range of applications in force-sensitive responses, and the potential to identify the molecules responsible for mechanotransduction.

Materials and methods

Design and construction of cell stretch device

The cell stretch device is based on an acrylic-based platform, a thin polydimethylsiloxane (PDMS) membrane as a substrate and a BioAssay dish (24.5 × 24.5 cm, Corning, Inc.) as a tissue culture dish (Figure S1A). This easy-to-fabricate cell stretch device requires minimal fabrication as acrylic sheets can be easily and precisely cut using a laser cutter (readily accessible through university facilities or private companies), constructed with a minimal number of screws and a fast-setting acrylic cement (TAP Plastics). The servo motor is mounted on the platform using an acrylic motor mount glued to the platform (Figure S1B). The membrane is bounded at opposing sides by a sliding and a fixed mount using a simple clamp system (Figure S1C and D). While the stationary membrane clamp is glued to the base acrylic frame, the sliding clamp is mounted to a carriage and rail system (McMaster-Carr) and is connected to a servo motor (Hitec RCD, Inc.) via a threaded shaft (Tamiya, Inc.). A cover was similarly assembled using acrylic sheets. All acrylic sheets were purchased from McMaster Carr and were laser cut at the in-house prototyping facility using Kern Micro 24 system (Kern Lasers Systems).

The servo motor uni-axially and cyclically displaced the sliding membrane mount to stretch the PDMS membrane. The PDMS membrane with the initial length of 14 cm was stretched to 17.5 cm (25%) or 16 cm (14%) at a frequency of 3 seconds per cycle. The servo motor was controlled by a Micro Maestro RC servo controller (Pololu Corp) and an Arduino Uno micro-controller, which were programmed using the Maestro servo software and Arduino software. The servo motor was powered by a variable power supply (9316PS, Marlin P. Jones & Assoc. Inc.).

PDMS membrane fabrication and characterization

To fabricate consistently uniform PDMS membranes, we designed a membrane mold using a borosilicate glass plate (McMaster-Carr) as a base and a laser cut acrylic as a frame. A 2-part epoxy (JB Weld) or PDMS was used to adhere the acrylic frame to the glass base. To ensure that the mold is perfectly level, a leveling platform was constructed with 3 fine-adjustment thumb screws (McMaster-Carr) as adjustable feet for the platform. The platform was leveled using a high-precision level (Geier and Bluhm) and the fine-adjustment thumb screws. Two parts PDMS is mixed at 1:10 ratio (total 15 g) then diluted in an equal volume of hexane. The diluted PDMS solution was poured onto a membrane mold on the leveling surface, and was cured at room temperature.

To monitor the movement of sliding mount, time-lapse movies were taken with a cell phone camera, and the movement was analyzed using ImageJ (see Fig. 1C). To quantify the strain profile of the PDMS membrane, fiducial markers were painted onto the membrane using a black spray paint and a transparency mask with a grid pattern consisting of 2 mm diameter circular holes that are 4 mm apart. ImageJ and a custom macro were used to quantify the strain distribution of the membrane by tracking the movement of the dots as the membrane was stretched from 0% to 25%. The strain was calculated based on the initial (ΔL0) and final (ΔL) spacing between the markers: ΔL/ΔL0−1.

Purification of biotinylated proteins and western blot analysis

The purification of biotinylated proteins was described previously. Briefly, 30 × 10^6 cells were seeded on
large-scale cell stretch chambers and cultured for 24 hours in a DMEM media. Subsequently, biotin was added resulting in a biotin concentration of 50 μM and the membrane was stretched overnight. The control, non-stretch condition was treated identically except the motor was not powered. After 20–24 hours, the cells were washed 3 times with PBS buffer. Next, cells were lysed in BioID lysis buffer supplemented with cComplete™ Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche), then sonicated and centrifuged to isolate the supernatant. The protein concentration of the supernatant was measured with a RC/DC assay kit (Bio-Rad). Equal concentrations of lysate were added to BioMag streptavidin beads (Qiagen) and incubated overnight. Lysates were then washed with 2% SDS buffer. Finally, biotinylated proteins were eluted with a sample buffer containing 3 mM biotin and boiled for 15 minutes.

The cell lysates from control and stretch conditions were also used in the phosphorylation analysis of Src and Akt. Based on the cell lysate concentrations quantified by the RC/DC assay, the same amount of cell lysates was loaded in each lane. The equal sample loading was also verified using Ponceau staining and anti-α-tubulin blot (Fig. 3H).

For western blot applications, primary antibodies used for proximal biotinylation include: αE-catenin (15D9, Alexis Biochemical), β-catenin (Clone 14, BD Biosciences), non-muscle myosin IIA (Rabbit Polyclonal, Sigma), and non-muscle myosin IIB (Rabbit Polyclonal, Sigma). Primary antibodies used in Src signaling pathway analysis include: Src (32G6), Phospho-Src Tyr416 (mAb 5558), total PTEN (D4.3), Phospho-PTEN Ser380 (Ab 9551), total PDK1 and Phospho-PDK1 Ser241 (mAb 3438). All Akt and Src signaling antibodies were purchased from Cell Signaling. As a loading control, the samples were analyzed using α-tubulin (DM1α, Sigma). The signals on the nitrocellulose membrane were detected using a WesternBright Quantum Chemiluminescence Kit (Advansta).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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