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CONTRIBUTION OF PIEZO1 IN ECM STIFFNESS INCITED EPITHELIAL CELL REMODELING

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ABSTRACT

Cells are sensitive to mechanical cues from the ECM such as, stiffness, topography, and roughness. Tissue stiffening due to ageing or diseases has been shown to affect cell proliferation, differentiation, migration, and apoptosis, but the mechanosensors that respond to ECM stiffness changes have not yet been fully understood. Recent studies on mechanosensitive Piezo1 channels have shown that these channels are sensitive to ECM cues, such as mechanical confinements implemented by micropatterns. We hypothesize that Piezo1 could serve as a force sensor for local ECM stiffness. Using a '2-well PDMS chip' consisting of substrates of different stiffness, namely, hard (~1000 kPa) and soft (~0.1 kPa), we studied the role of Piezo1 on stiffness-dependent morphology changes in epithelial cells. The results show that cells respond to substrate stiffness variations with profound cytoskeletal reorganization and moderate changes in their spreading area and shape. Cells on the hard substrates were only ~20% larger than that on soft substrates within the same culture period of 2.5 hrs and at a similar confluency. However, cells on hard substrates show abundant F-actin bundles that are reorganized to peripheral actin rings on soft substrates. Inhibition of Piezo1 with GsMTx4 or Gd^{3+}, largely reduced the formation of thick actin bundles on hard substrates. Activation of Piezo1 with specific agonist, Yoda1, enhanced the formation of actin bundles. These results indicate that the epithelial cells' response to ECM stiffness is mediated by Piezo1 through its function of permeating Ca^{2+} ions. This study demonstrates that Piezo1 could be an ECM mechanosensor in epithelial cells.

Keywords: Piezo1; ECM stiffness; mechanosensors

1. INTRODUCTION

Adherent cells are subject to mechanical forces from the extracellular matrix (ECM) that regulate their morphology, proliferation and differentiation[1-3]. Cells can sense these forces and adapt accordingly with continuous remodeling. This sensing mechanism forms an integral part of tissue development, regeneration in physiology. Dysfunction of mechanosensors is associated with progression to disease states like cancer[4, 5]. ECM stiffness variations have shown to play a dominant role in tumorigenesis in the recent years[6-8]. Using a tunable 3D collagen hydrogel, Cassereau et. al showed that increasing ECM stiffness increased tumor invasion in mammary epithelial cells[9]. Endothelial cells generated pronounced traction forces on stiff hydrogels compared to softer ones[10]. Engler et. al., showed that varying substrate stiffness can direct the differentiation of mesenchymal stem cells into neurons, bone and muscle cells[11]. Epithelial cells experience a wide range of stiffness from the underlying ECM[12] that regulates wound healing[13], cell-cell junction assembly[14] and homeostasis[15]. Hence, there is a need to further comprehend the ECM sensing and response mechanisms in epithelial cells.

We have previously reported that the mechanosensitive ion channel, Piezo1, is essential for HEK293 cell remodeling on micropatterns on the substrate. Inhibition and knockout of Piezo1, drastically reduced the cell elongation on micropatterns [16]. Thus, we hypothesized that, Piezo1 could also be involved in stiffness dependent cell spreading in epithelial cells.

In this work, we compared the cell spreading area and cytoskeletal organization on two substrate stiffness, namely, hard (1000 kPa) and soft (~0.1 kPa) with and without functional Pieo1 channels. We found that, the epithelial cell spreading area does not show much difference with respect to the stiffness. The cytoskeletal F-actin bundles however, were strongest on hard substrates. Inhibition of Piezo1, inhibited the F-actin formation on hard substrates showing that Piezo1 is required for stiffness sensing in epithelial cells.

2. MATERIALS AND METHODS

2.1 Cell culture

Madin-Darby Canine Kidney (MDCK) cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) in two-well PDMS chips at 70-80% confluence and incubated for

~2.5 hrs. Experiments were conducted in DMEM. For Piezo1 inhibition experiments, we changed the solution to isotone (~320 mOsm) to ensure the potency of the inhibitors.

2.2 Two-well PDMS chip

A PDMS chip was made of two 6-mm diameter PDMS wells on a coverslip. Two elastomers, namely, Sylgard 184 Silicone (elastomer to curing agent, 1:1 ratio, Ellsworth Adhesives) and Sylgard 527 Silicone (Part A to Part B, 1:0.8 ratio, Ellsworth Adhesives) were used as hard (1000 kPa) and soft (~0.1 kPa) substrates. The elastomers were mixed in the desired ratios and ~20 µl of the respective elastomer solution was added in each well of the PDMS chip that formed a thin layer of ~200 µm thickness substrate and cured for 48 hrs. By curing the soft substrate in the PDMS chip, we avoid the problem of transferability and damage to the soft material. The thin substrates allowed high resolution imaging (up to 64x). The devices were then coated with Fibronectin (Sigma Aldrich) and were ready for cell culture.

2.3 Image Acquisition

At 2.5 hrs of cell seeding, the two-well chip was transferred to a stage-top incubator (INUB-ZILCSD-F1-LU, Tokai Hit, Japan) maintained at 37°C and 5% CO_2 for imaging. A Zeiss inverted microscope (Axiovert 200M, Zeiss) with a CCD camera (AxioCam, Zeiss) was used to acquire images. Bright field images were obtained using a 20x objective and immunostaining images were captured using a 64x objective, RFP (Ex: 550/25, Em: 605/70) and DAPI filters (Ex: 365 nm, Em: 445/50 nm).

2.4 Staining

Cells were fixed in 4% paraformaldehyde for 15 min and washed with Phosphate-buffered saline (PBS). Permeabilization was performed using 0.1% Triton X-100 (Sigma Aldrich) for 15 min and followed by washing with PBS. For blocking, 5% goat serum in PBS was incubated in the devices for 1 hr. Phalloidin Alexa Fluor 568 (Invitrogen), at 1:100 dilution was used for staining F-actin and incubated for 60 min. Nuclei were counterstained with Hoechst 33342 dye (Thermofisher Scientific). A drop of anti-fade gold reagent (Invitrogen) was added in each well to sustain the stain.

2.5 Chemicals

GsMTx4 was purified as per previous protocols[17] and used at a working concentration of 5 µM in saline. Gandolinium chloride (Gd^{3+}, Sigma Aldrich) was freshly prepared and diluted to 20 µM in saline. Both drugs were added immediately after cell seeding in PDMS chips at 0 min. Yoda1 (Tocris Bioscience) was dissolved in DMSO to 48 mM, then diluted in saline to a final concentration of 25 µM. Yoda1 was added after 1 hr of seeding.

2.6 Statistical Analysis

The mean cell area and standard error of mean was calculated and plotted using OriginPro. For statistical analysis cell area was averaged over different panels and multiple experiments.

3. RESULTS AND DISCUSSION

3.1 Effect of substrate stiffness on MDCK cells

We placed a hard and a soft substrate in the two-well PDMS chip, and simultaneously measured the cell spreading on the respective stiffness (Fig. 1A). To measure the cell area, gray-scale optical images were thresholded using "Graph cut" in MATLAB Image Segmenter application and binary images were generated (Fig. 1B, C). The binary images were then transferred to ImageJ to calculate the cell area. Using Macros, cells were outlined, numbered and the areas of individual cells were measured (Fig 1D).

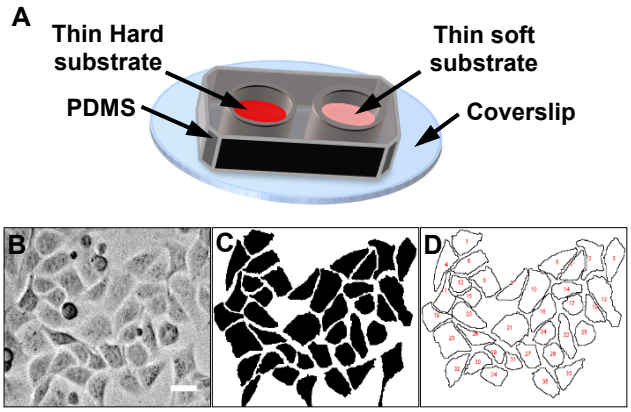

FIGURE 1: TWO-WELL PDMS CHIP AND CELL AREA QUANTIFICATION.

MDCK cells were seeded on fibronectin treated substrates and allowed to spread. At ~2.5 hrs, the cells were imaged and analyzed for differences in morphology and cell spreading area. The morphology of MDCK cells on both substrates was similar (Fig. 2A), which showed typical hexagonal/polygonal packing of epithelial cells. Interestingly, we found that the cell spreading area on hard substrates was only slightly larger (~20%) than the soft substrate cells (Fig. 2B). The percentile of cells attached per frame was comparable for both substrates, which showed more cells attached to the hard substrate than the soft for the same culture time (Fig. 2C). Note that this observation was restricted to a time window of 2.5 hrs after seeding, most cells spread to their maximum extent within this time. It has been reported that epithelial cells on polyacrylamide gels of varying stiffness from 6 to 60 kPa showed minimal changes in morphology and migration velocity[18], consistent with our observation.

As the cells interact with ECM via focal adhesions that are linked to the cytoskeleton, we investigated if the stiffness has any effect on the cytoskeleton. After cell spreading for 2.5 hrs, the cells were fixed and stained with phalloidin. We found that the cells on hard substrates showcased thick F-actin bundles whereas, on soft substrates, no bundles were present (Fig. 3). The F-actin on soft substrates, however, reorganized to the periphery forming a ring (Fig. 3B).

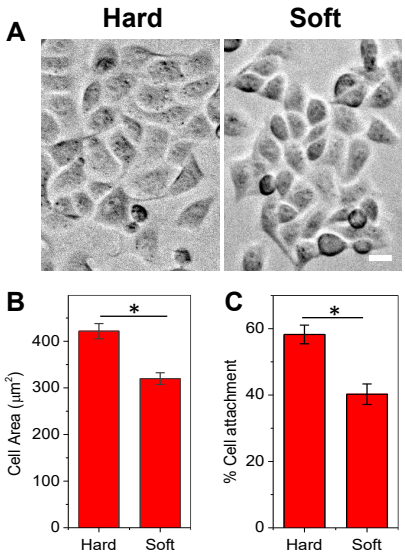

FIGURE 2: EPITHELIAL CELL MORPHOLOGY ON HARD AND SOFT SUBSTRATES. **A:** Cell spreading on hard and soft substrates after 2.5 hrs. **B:** Statistical mean of cell spreading area on hard and soft (n = 90, $*p < 0.001$). **C:** Percentile of cell attachment on hard and soft substrates after 2.5 hrs of seeding (n=30 panels, from more than 4 experiments, $*p < 0.005$). Scale bar represents 20 μm**.**

A similar reorganization of parallel F-actin bundles from the cell interior to the periphery was seen in shear stress response of epithelial cells[19]. We have previously reported that Piezo1 is a flow sensor that transmits shear stress to the intracellular cytoskeleton causing cytoskeletal reorganization, thus, it is possible that the cells use the same sensor for detection of both flow and ECM mechanical signals.

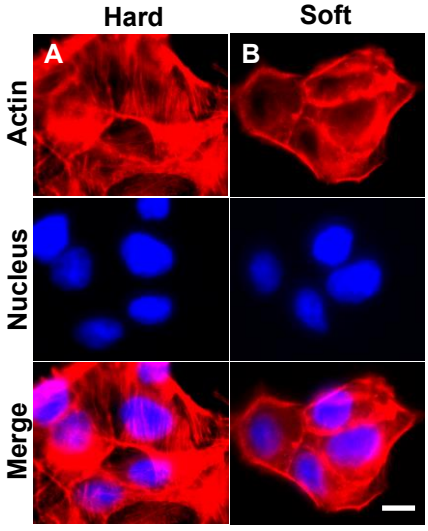

FIGURE 3: STIFFNESS DEPENDENT REORGANIZATION OF F-ACTIN. F-actin, nucleus stained MDCK cells on hard and soft substrates, showing that thick F-actin bundles on the hard substrate were reorganized to the periphery on soft substrate. Scale bar represents 10 μm.

3.2 Piezo1 is required for cytoskeleton reorganization

To assess the role of Piezo1 in ECM stiffness incited cell remodeling, we inhibited Piezo1 with GsMTx4, a known mechanosensitive channel inhibitor. The inhibitor was added during cell seeding. We found that, with an inhibited Piezo1, cells did not form F-actin bundles on hard substrates. Cells appeared similar on hard and soft substrates (Fig. 4, middle panel). Piezo1 inhibition did not show any effect on soft substrate cells i.e., both control and piezo1 inhibited cells have shown ring shape F-actin along cell periphery (Fig. 4, lower panel). As another control, we used Gd^{3+}, a non-specific mechanosensitive channel inhibitor that had a similar effect as GsMTx4 on hard and soft substrate cells, further validating the Piezo1 effect on cytoskeletal reorganization. This result indicates that Piezo1 plays an essential role in cell detection and response to the stiffness of the substrates.

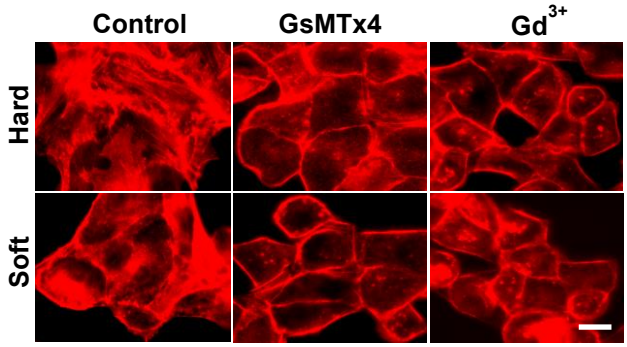

FIGURE 4: EFFECT of PIEZO1 INHIBITION ON F-ACTIN REORGANIZATION ON HARD AND SOFT SUBSTRATES. The inhibitors were added during cell seeding, and the cells were fixed and stained after 2.5 hrs. Scale bar represents 10 μm.

3.3 Piezo1 activation increased actin fibers

We then tested the cell response in the presence of Piezo1 agonist, Yoda1, that activates the channels. As expected, application of Yoda1 did not alter the mean cell attachment. However, the cells developed more abundant and thicker actin bundles in the presence of Yoda1 on the hard substrates, while there was no observable difference on the soft substrates with and without Yoda1 (Fig. 5). Figure 5 shows phalloidin staining after 2.5 hrs of seeding.

It is known that Piezo1 functions as a Ca^{2+} permeable channel that mediates Ca^{2+} influx in the presence of mechanical signals. The effect of Yoda1 on cells' response to ECM stiffness suggests that MDCK cell reorganization utilizes Piezo1 mediated Ca^{2+} influx. We and others have previously shown that Ca^{2+} influx is responsible for cell expansion on micropatterns that activate Rho-ROCK activated Myosin-II contractile forces[16]. Our result is consistent with previous findings, and it further suggests that cells spreading could occur without significant traction forces.

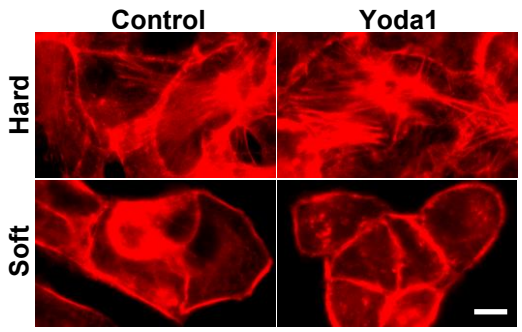

FIGURE 5: EFFECT of PIEZO1 AGONIST ON F-ACTIN REORGANIZATION RESPONSE TO ECM STIFFNESS. Yoda1 (25 μm) was added 1 hr after seeding, and the cells were fixed and stained after 2.5 hrs. Scale bar represents 10 μm.

3.4 Discussion

By modifying the stiffness of substrates, we have shown that epithelial cells spread on both hard and soft substrates. The substrate stiffness mainly affects the F-actin reorganization, showing thick stress fibers across the cells on the hard and peripheral F-actin on soft substrates. Inhibition of Piezo1 channels eliminated the difference, suggesting that Piezo1 plays a critical role on substrate sensing. It is well known that Piezo1 functions as a mechanosensitive cation channel that mediate Ca^{2+} influx. We and other groups have previously reported that Piezo1s also have additional role in the substrate sensing, possibly via their interactions with cell adhesion proteins. Our results using Piezo1 inhibitors and agonist, respectively, eliminated and enhanced cytoskeleton remodeling according to the substrates. It shows that Piezo1 channels could be a general sensor for the detection of ECM mechanical cues, leading to cell remodeling. The function of permeating Ca^{2+} mainly contributes to the cytoskeleton reorganization and traction forces.

4. CONCLUSION

Using a two-well PDMS chip with hard and soft substrates, we showed that epithelial cells respond to substrate stiffness with distinct F-actin organization. Treatment of cells with Piezo1 inhibitor eliminated the F-actin organization on the hard substrates. Activation of Piezo1 channels with agonist enhanced F-actin bundles. These results show that Piezo1s mediate the cell response to substrate stiffness via their function as Ca^{2+} permeable channels.

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