THE ROLE OF MECHANOSENSITIVE ION CHANNEL PIEZO1 IN DETECTION OF CONFINEMENTS AT ECM

by
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Abstract

Cellular response to confinement at the extracellular matrix (ECM) plays an important role during tissue development. Mishandling these mechanical signals results in a variety of diseases including cardiovascular disease and cancer. In this study, we investigate the role of mechanosensitive Piezo1 channel as a cell sensor for detection of mechanical cues at various substrates. We modified the substrates with various micropatterns, topographies, and stiffness and examined the cell shape in response to ECM modifications. By inhibiting Piezo1 activity with specific pharmacologic inhibitors or by knockout of Piezo1 with siRNA, we have shown for the first time that HEK cells lost their ability to stretch to the full extent on the stripe patterns compared with wild-type cells. This indicates that cells utilize Piezo1 channels for detection of substrate confinements. The location and concentration of Piezo1 channels were visualized using FRET-based fluorescent probes P1-cpstFRET. We show that distribution of Piezo1 is non-uniform in cells on narrow stripes, and Piezo1 clusters exist in areas of high cytoskeletal tension. This work offers a new mechanism of mechanosensing by Piezo1 channels, through which cells utilize ECM cues for remodeling.
1. Introduction

1.1. Mechanotransduction

Mechanotransduction is the mechanism by which cells sense the mechanical stimuli in their microenvironment and convert it to chemical or biological signals [1]. The mechanical properties of the cellular microenvironment have an essential role in cell growth, differentiation, embryonic development, and tissue remodeling [2]. Loss of mechanosensing results in various disorders such as cardiovascular diseases [3], neurological disorders [4], and pulmonary diseases [5, 6].

Mammalian cells are exposed to various topographical and physical confinements while residing in their microenvironments. The mechanical forces imposed by the extracellular matrix (ECM) at the substrate and the surrounding cells, govern cell morphology, function, spreading and volume [5, 7, 8]. It has been shown that substrate stiffness, nanoroughness and micropatterns determine the cell fate in different cell types. For example, in neural stem cells substrate roughness determines neuro-glial lineage [9-12] and in human mesenchymal stem cells, substrate stiffness directs cell differentiation to adipocytes or osteoblasts [13, 14]. The topographical and mechanical characteristics of ECM also affects cancer metastasis [1, 15, 16].

1.2. Micropatterning and 2D cell growth

Cells gain an understanding of their substrate through cell-ECM interactions at adhesion complexes. When adhesive molecules are introduced to a confined region through substrate modification, cell adhesions are limited. It has been shown that cells can recognize the confined areas of the substrate (see Figure 1.1 for more details on confinements) and reshape themselves according to the substrate [17], however, the underlying sensing mechanisms are unknown. Inside the cell, the cytoskeleton actin filaments reorganize following the patterns [18, 19]. This happens by the generation of contractile forces through actomyosin (actin filaments + myosin fibers) [20]. When
the confinement introduces forces that are higher than a certain threshold, the actomyosin will bundle and form stress fibers to strengthen the cells in areas of high traction forces (Figure 1.1.A).

Numerous techniques have been used to modulate the substrates with different patterns, topographies, stiffness, and surface chemical properties. This is accomplished by using emerging engineering techniques to create tailored surface patterns and features or modulate substrate stiffness. Some of the most functional methods for 2D engineered patterns are soft lithography, plasma polymerization, jet patterning, and laser writing [21]. Micropatterns are shown to dictate cell morphology and proliferation during formation of endothelial blood vessels [22] or epithelia in renal tubules [23]. Moreover, surface patterns provide guidance for collective cell migration, which is the coordinated migration of a population of cells while maintaining robust cell-cell, and cell-substrate interactions [24, 25].

The effect of substrate confinements has been widely studied (Figure 1.1). For example, cells grown on T-shape patterns have an overall triangular shape. Such cells developed thick bundles of stress fibers along the non-adhesive edges suggesting a reinforcement of the cytoskeleton in response to the geometrical constraints [26, 27]. Similarly, cells grown on narrow stripes stretch themselves along the orientation of the stripes [28]. Substrate geometry also affects cell polarity and provides guidance for cell expansion and migration direction [29, 30]. Moreover, cells move faster on the narrow patterned stripes or in narrow channels than a broader substrate [31]. These results provide the end-point of cell response to mechanical confinements; however, how the cells detect the ECM mechanical input remains unclear.

These results amply demonstrate the ability of the cells to modulate their shape and movement when subjected to local mechanical constraint of the substrate. However, very little is known about the cellular sensing processes that lead to such remodeling.
Figure 1.1. Effect of substrate patterning on cytoskeleton reorganization and directional growth of cells. (A): Distribution of F-actin on triangular shaped substrate, showing thick bundles of stress fibers along cell periphery [26]. (B): Confined growth of NIH 3T3 fibroblast cell on teardrop pattern, showing the nucleus of the cell has shifted to the blunt end of the teardrop [32]. (C,D): Cells grown on two different sub-cellular microarrays of fibronectin islands, causing elongated growth along the close-packed direction [33]. (E): Elongation of cell on micro-groves [34].

1.3. Mechanosensitive Piezo channels
Among all robust mechanisms of mechanosensing in cells, mechanosensitive ionic channels (MSCs) have a special importance in confinement sensing. Mechanosensitive ion channels are specialized enzymes that make the mechanical signal perceivable by the cell by translating it to the control of ion flux. These channels are different than other ion channels in a sense that they open or close in response to mechanical stimuli [35]. These transmembrane proteins are expressed in all human cells (rendering all cells to be mechanically sensitive), are preserved through evolution as they are functionally expressed in both prokaryotic and eukaryotic cells [35-38]. These channels could be the first responders to mechanical inputs at the ECM that convert them to a variation of ion concentration inside the cells.
Recently, Piezo channels (1 and 2) have emerged as possessing attributes that allow a response to mechanical stimuli [39, 40]. These huge transmembrane proteins (more than 2500 amino acids) form the subunits of Ca\(^{2+}\) permeable non-selective cationic channels for detection of mechanical forces [39, 40]. Piezos have been found in various eukaryotes and in most of the vertebrates. Piezo1 is expressed mainly in non-neuronal cells, whereas Piezo2 is expressed in sensory neurons and in some specialized mechanosensory structures [41].

The Piezo1 protein, initially called Fam38A, responds to mechanical forces in the bilayer [42-44]. It was also called as ‘touch’ sensor because it is involved in integrin activation [45]. In cardiovascular system, Piezo1 is responsible for detecting blood flow enabling endothelial cells alignment along flow direction [46], regulating vascular tone, and enabling blood vessel formation [47, 48]. Moreover, Piezo1-depleted vasculature exhibited defective alignment and elongation of endothelial cells in response to laminar shear stress [3, 49]. In respiratory system, Piezo channels have also proven to be important as knockdown of Piezo2 in sensory neurons in lung causes respiratory distress [41, 50]. Piezo channels also play a critical role in kidney tubule systems, which regulate osmolarity in kidney tubules [23], control lymphatic valve formation [51], and interact with PC-2, a sensory protein crucial in polycystic kidney disease [23, 52, 53]. In brain, these channels determine stem cell fate in differentiation to neuron or glia [11] and regulate neuron-astrocyte interactions through surface nanoroughness perception [12]. Furthermore, Piezo channels also play roles in chondrocytes mechanotransduction [54, 55], adipocyte plasticity [56], osteogenic differentiation [57], and erythrocyte volume homeostasis [58, 59].

Effect of Piezo1 in cell spreading and migration is shown in various studies and different expression levels and activations of Piezo1 are proven to be determinant in cell mode of migration and speed [1, 16]. Furthermore, cell stretching is shown to induce Piezo1 driven cell division and cell
layer crowding [60], cell extrusion (the process in which other cells in a cell layer will expel a cell out while the cell is still alive) [61], and confinement sensing [1] which is the focus of this article and will be discussed in more detail. Recently, our group has reported that Piezo1 can be activated by fluid shear stress in HEK cells [62]. Piezo1 is able to transmit fluid shear stress to cell nuclei to modulate nuclear size in MDCK cells [63].

These diverse cellular and physiological contributions indicate that mutations and malfunctions in these channels can be the source for mechanosensory-related diseases. The diseases that are found to be associated with Piezos are listed in Table 1. Also for a full list of Piezo mutations is listed in the literature [64].

**Table 1: Human diseases associated with Piezo channels**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Description</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary Xerocytosis</td>
<td>Dehydration of red blood cells and hemolytic anemia</td>
<td>[65-69]</td>
</tr>
<tr>
<td>Congenital lymphatic dysplasia</td>
<td>Lymphedema in all segments of the body (tissue swelling)</td>
<td>[70, 71]</td>
</tr>
<tr>
<td>Arthrogryposis</td>
<td>congenital joint contracture (Mutation in Piezo2 causing slowed deactivation)</td>
<td>[72, 73]</td>
</tr>
<tr>
<td>Cancer</td>
<td>Bladder carcinoma, breast cancer and glioma</td>
<td>[74-76]</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>Inflammation of pancreas (caused by mechanical injury)</td>
<td>[77]</td>
</tr>
</tbody>
</table>

Piezo1 can be activated by membrane stretch. Cox and Bae et al. has shown that ‘force through lipid’ is a robust mechanism of channel activation and disruption of cytoskeleton by cytochalasin
D increases channel sensitivity [78]. However, how the mechanical confinement is converted to membrane tension for channel gating is unknown.

1.4. Recent observation of Piezo1-facilitated cell-ECM interaction

How adherent cells detect mechanical cues from ECM is not clear. Previous studies have shown that integrin, a transmembrane protein that links ECM proteins to actin cytoskeleton, transmits the force from extracellular ECM to the cell directly. During the cell spreading, the traction forces, once above a critical threshold, can be transmitted by integrin-based adhesions to activate Myosin-II pathways that lead to the formation and maturation of new focal adhesions [7]. The distribution of internal forces can be changed by introducing confinement to the cell microenvironment, and this change leads to the remodeling of the cytoskeleton to reduce or homogenize this localized tension [79]. However, while Myosin-II activated cytoskeleton reorganization is a Ca\textsuperscript{2+} dependent process, integrin is not a Ca\textsuperscript{2+} source and other mechanosensors must be involved.

Several recent studies show promises that mechanosensitive Piezo channels can be the primary sensor at the cell basement membrane, that supply Ca\textsuperscript{2+} when opened with mechanical forces.

It has been reported that Piezo mediated Ca\textsuperscript{2+} rise activates Myosin-Rho pathways in MDCK cells, which alter the internal contractility [80]. In MDCK cells, Piezo1 signaling activates the Rho-kinase-dependent myosin pathway resulting in the extrusions of crowded live cells [81]. Piezo1 also contributes to cell division on stretched membranes which is also an actomyosin dependent cellular event [60]. In endothelial cells, a Ca\textsuperscript{2+} elevation via Piezo1 channels is required for remodeling of the cytoskeleton under shear stress; the remodeling was not shown in Piezo1 knockout cells [3, 49].
Hung et al. presented another valuable finding in cells in confined channels or on micro-printed substrates where an increase in intracellular calcium ion level was observed in confined region versus cells on unconfined areas [1]. Further genetic knockdown or inhibition of Piezo1 using the stretch-activated cation channel inhibitor peptide GsMTx4 [82] led to substantial diminishing in these currents suggesting Piezo1 to be responsible for mediating this Ca\(^{2+}\) influx.

Recently, Ellefsen and Chang et al. [83] reported another relevant study of Piezo1 in confinement sensing. They showed that Piezo1 activation (observed by increase in Ca\(^{2+}\)) was co-localized with the areas that had higher traction force, and the Ca\(^{2+}\) flickers were abolished when the actomyosin contractility were disrupted [11, 83]. These studies provide the evidence that Piezo mediated Ca\(^{2+}\) rise alters the internal contractility and cell spreading. However, it still remains unclear how Piezo1 is activated by the substrate confinement. It is also not known how Piezo protein expression and distribution is altered by the confinement, or what would be visualization of the effect of Piezo1 on cytoskeleton remodeling under these conditions. Answering these questions can provide insights on the role of Piezo1 in confinement sensing.

The locations of Piezo1 channels in cells is still under investigation, previous results indicate that the distribution of Piezo1 is force dependent. Two forms of distributions of channels have been observed, first, accumulation as clusters in the cell membrane [3, 12, 60], and second, uniform distribution [11]. Under shear stress, endothelial cells exhibited Piezo1 accumulations at the trailing edge of the cell ([3] in extended data). In epithelial monolayers it was also observed that Piezo1 channels tend to relocate from nuclear envelope (or endoplasmic reticulum, ER) to form aggregates in cell membrane, while they move back to the ER after application of stretch to the monolayer [60]. Although, these studies suggest ‘non-homogenous’ distribution of Piezo1 in cells, no study has yet shown the correlation of their location with traction forces. The only study that has
successfully shown the co-localized activation of Piezo1 with traction forces in the cytoskeleton (as discussed earlier in more detail) indicates the uniform distribution of Piezo1 over cell membrane which is in contradiction with other reported studies [83]. This indicates the need for a controlled study to illustrate the correlation between the location of Piezo1 and tension in the cytoskeleton on confinements.

1.5. Objectives
We summarize the missing information and the unanswered questions from previous studies and outline our approach in the following.

Question set 1. Does Piezo1 have any role on cell sensing mechanical cues at ECM confinements?

Our approach: We have designed and generated specific patterns through microcontact printing to provide mechanical confinements to HEK cells. We will test the role of Piezo1 using specific Piezo1 inhibitor GsMTx4 and more general MSC inhibitor Gd³⁺. We will then test Piezo1 overexpressing and Piezo1 knockout cells. This allows us to identify whether Piezo1 play the role in ECM sensing in these cells.

Question set 2: Where are the Piezo1 channels located in cells? Does the ECM confinement alter Piezo1 distribution or their opening probability? How does Piezo1 density correlate to cytoskeletal forces and cell extrusion?

Our approach: We use a fluorescence-labeled Piezo1 cell line that allows us to visualize Piezo1 distribution, to see where the channels reside and if high concentration of Piezo1 co-localize with high cytoskeletal force
2. Methodology

In this section, detailed procedures for fabrication, printing, cell culture and imaging will be presented.

2.1. Soft lithography and stamp fabrication:

Soft-lithography technology was used to create elastomeric stamps that are finally used to transfer micropatterns to the substrates. In soft lithography, a master consisting of photoresist (SU-8) on silicon wafer is fabricated using photolithography. This master is used to cast an elastomeric polymer, mainly polydimethylsiloxane (PDMS), that serves as a stamp for microcontact printing (µCP) or as featured substrates [8, 17, 21]. In µCP, the PDMS stamp allows stamping ECM molecules on the appropriate substrate which promotes cell adhesion in localized regions. In order to create the stamp, a silicon master was fabricated using soft lithography following pre-established methods [84-86]. Silicon wafers were cleaned by washing with acetone, ethanol, and DI water, and dried under nitrogen stream. To remove the residual water on the surface, wafers were heated on hotplates for 30 min at 130 °C. The negative photoresist, SU-8 2010 (MicroChem), was then poured on the wafer and spin-coated at 500 rpm for 20 seconds followed by 4000 rpm for 1 minute to a final thickness of 8-10 µm. In order to avoid edge bead effect, the photoresist on the edge of the wafer was removed by SU-8 Developer (MicroChem). The wafer was then pre-baked at 60 °C for 1 min and transferred to the aligner for UV exposure. To transfer the patterns onto the coated photoresist, a photomask was designed in AutoCAD and was produced by Front Range Photo Mask (Lake Havasu City, AZ). The exposure dose is a critical variable in the process which determines the feature size, aspect ratio, and profile of the feature walls [87, 88]. Post exposure bake (PEB) was done at 60 °C for one hour. Keller et al. [88], has shown that residual solvent in the photoresist layer at the pre-bake stage might lower the cross-linking temperature of the photoresist.
By conducting PEB at a lower temperature, we avoided the formation of cross-linked photoresist film at the surface of the patterns that limits the curing of the proactive compounds inside the film. This step helps to eliminate the detachment of features during the development. Subsequent to PEB, the wafers were cooled down to room temperature and developed in SU-8 Developer for ~5 min. It is important to know that any change to these steps, specially, pre-bake and exposure dose, will affect all variables of the protocol and they should be optimized again. Silanization was done on the SU-8/Si wafer to produce a passivation of the surfaces to prevent the PDMS form adhering to the master during PDMS release. A few drops of Trichloro (1H,1H,2H,2H-perfluoroctyl) silane (Sigma-Aldrich) were poured in an aluminum dish and were put in a vacuum chamber containing silicon wafer master. Then vacuum was applied for 2 min and chamber was closed to allow the silane vapor reside on the features. PDMS can make chemical bonds with the –OH groups on the SU-8 layer and the addition of a thin silane layer will ‘passivate’ this active contact [89]. Figure 2.1 provides a schematic of the master fabrication procedure. To make PDMS stamps, two-part SYLGARD 184 was mixed in a 10:1 ratio and then degassed in a vacuum chamber. It was then poured on the silicon wafer master, and was baked for 2 hr at 85 °C. The PDMS was then peeled off from the wafer (Figure 2.2) and features were cut into 0.8 mm × 0.8 mm stamps.

2.2. Microcontact printing
We introduced confinement to cells using two methods, 1) created thick PDMS film with groves so that the cells can spread on the top of the groves, and 2) patterned glass substrate with adhesive molecules so that the cells can adhere to the patterned regions. Fibronectin, an ECM protein containing Arg-Gly-Asp (RGD) peptide, was used to form the adhesive areas on the substrate.
To create featured substrates, PDMS stamps were cleaned in ethanol, rinsed with DI water and dried with nitrogen stream. The stamps were left at room temperature to dry out. Then these stamps were transferred to a plasma cleaner (Harrick Plasma) and were treated with air plasma for 3 min.
The fibronectin in an aqueous solution has higher affinity for hydrophilic surfaces, therefore, we treated the glass substrates with air plasma prior to micro-printing. Fibronectin was diluted in phosphate-buffered solution (PBS) containing magnesium and calcium to a final concentration of 50µg/ml. The fibronectin solution was applied to cover the top surface of a PDMS stamp and was allowed to rest for 1 hr. This allows enough fibronectin to homogenously cover the surface. The surface was then rinsed vigorously with PBS and dried carefully with nitrogen stream. The stamps were then brought into contact with the glass coverslip, and were kept in contact for 30 min (weights were added for more homogenous transfer of protein). The quality of patterned fibronectin relies on the plasma treatment; as the surfaces compete, the stamps that are more hydrophilic are much more favorable for the protein and will win this competition of surfaces. The stamps were then rinsed with PBS and were left under the hood to dry. A schematic of this process is depicted in Figure 2.3.

To pattern the adhesive proteins on glass coverslip, the coverslips (25 mm diameter #1.5 (Warner Instruments)) were cleaned in boiling mixture solution of Ammonium hydroxide and hydrogen peroxide for 10 min, and were then rinsed with ethanol and were left to dry under UV exposure. PDMS stamps were then cleaned with ethanol, rinsed with DI water and dried with nitrogen stream.
at room temperature. Fibronectin was reconstituted to 50 µg/ml and was applied over the features on the stamps to reside for 1 hr. After the fibronectin was settled on the surface, the solution was removed from the stamps and they were vigorously rinsed with PBS, dried under nitrogen stream and were stored in the hood. Although, the cleaned coverslips were already hydrophilic, our experiments showed that there is a need for more surface energy to have more protein adsorption. Therefore, we treated the coverslips in the plasma cleaner with air plasma for 3 min to increase the hydrophilicity. The stamp, with features facing down, came into contact with the coverslips and weights were added on top. This resulted in homogenous distribution of proteins on the patterned regions. The patterns were then rinsed with PBS to remove any undesired protein. This process is called microcontact printing (MCP) [93, 94]. In order to prevent cell attachment to the non-patterned areas, we incubated the coverslips in Pluronic F-127 (Sigma-Alrich) solution for 1 hr after microcontact printing. When conducting MCP on a glass substrate, the surface energy becomes a
determinant parameter, as fibronectin requires a hydrophilic surface to adhere, and Pluronic F-127 is more functional on a hydrophobic surface. Thus we optimized our plasma treatment time to meet both these conditions and achieve highly functional microcontact printed surfaces. A schematic of MCP process is shown in Figure 2.4.

To examine the efficacy of our MCP protocol, we used fibronectin conjugated with NHS-Fluorescein (ThermoFisher Scientific) dye to fluorescently label and image the patterns with fluorescent microscope. Conjugation was done based on manufacturer’s protocol. Then the above mentioned microcontact printing protocol was followed but with special care for avoiding the exposure of printed coverslips to light. Final patterns are presented in Figure 2.5.

Figure 2.4. Microcontact printing schematic for preparation of glass substrates [21]. (a) fibronectin solution is added as a drop on top of the PDMS stamp. (b) the coated stamp comes in contact with air plasma treated glass coverslip. (c) patterns are transferred and (d) uncovered areas are treated with Pluronic F-127 to block undesired cell adhesion.
Figure 2.5. Microcontact printed patterns. Fibronectin (red) was printed on the glass substrate. Left shows the fluorescent image of stripe patterns and right shows the T-shapes [79]. Scale bars represent 10 µm.

2.3. Cell culture and Imaging

Human embryonic kidney cells (HEK293T) were used for these experiments. Wild-type HEK293T cells express Piezo1 channels in a normal amount [78]. To study the role of Piezo1 on ECM sensing, Piezo1-overexpressing HEK cell line (hp1-cl), and Piezo1 knockout cell line (P1KO) were used. Both cell lines were received as a gift from Dr. Philip Gottileb (Department of Physiology and Biophysics, University at Buffalo). Briefly, hp1-cl cells are created by stable transfection of a hP1-1591-EGFP-mCherry vector to HEK293T cells. The incorporated EGFP and mCherry proteins allow the use of fluorescence microscopy for spotting the Piezo1 channels in cell architecture, and it has been shown that this incorporation will not change the channel properties (refer to original paper for details [95]). For Piezo1 knockout (P1KO), cells were transfected with Piezo1 siRNA and co-expressed with EGFP to verify transfection. All experiments were done in parallel using all these types of the cells for comparison. The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) complemented with 10% fetal bovine serum (FBS) and
1% penicillin and streptomycin in culture flasks. For experiments, substrates (either the microcontact printed glass substrates or the fibronectin coated PDMS stamps) were placed in a sterile petri dish. Cultured cells were trypsinized and added to each dish. The samples were incubated with humidity and under 5% CO2 in an incubator for ~2 hr. The 2 hr time has shown to be enough to have cells reaching their fully stretched state and longer or shorter times would result in cell detachment or partial stretching, respectively.

2.4. Piezo1 Transfection
To visualize the Piezo1 channels, we transfected the cells with fluorescent probe, P1-cpstFRET, a CFP/YFP fluorophore pair inserted into extracellular domain of Piezo1 protein. For transfection, cells were first cultured for 24 hr to >80% confluency. 1 µg of plasmid DNA, which was received as a gift from Dr. Philip Gottlieb (Department of Physiology and Biophysics, University at Buffalo) was diluted in Opti-MEMI Reduced-Serum Medium. Then the lipid based Mirus TransIT-293 transfection reagent (works as intracellular delivery vehicle) was added and the final solution was added over the cells. Cells were incubated for another 24 hr and were harvested for experiments.

2.5. Imaging and Microscopy
Cells were imaged using an inverted microscope (Axiovert 200M, Zeiss) with a CCD camera (AxioCam, Zeiss) with 20x objective. To remove unattached cells from the solution, media was removed and cells were gently rinsed with fresh media two times. Images were taken in brightfield at 1, 2, 3, and 4 hr and only images at the 2 hr time point are reported.

For fluorescent imaging, a 63x oil immersion objective and a filter set [excitation (ex): 365/40; emission (em): 445/50 nm] were used. In order to avoid the fluorescence interference from the culture media, isotonic solution containing 75 mM NaCl, 5 mM KCl, 2 mM MgCl2, 1 mM CaCl2
and 10 mM HEPES, was used for imaging. The osmolarity was adjusted to 330 mOsm with mannitol. All imaging was conducted in a dark room with only halogen light present to avoid photobleaching of the samples.

### 2.6. Chemicals and Drugs

Gadolinium(III) chloride was used as a non-specific inhibitor of mechanosensitive ionic channels. This chemical cannot be dissolved in cell media as it will precipitate. For these experiments, during the subculture the cells were centrifuged at 2500 rpm for 2 min. Then the supernatant media was removed and cells were reconstituted in isotonic solution and were diluted to the desired concentration. Cells in isotonic solution were then transferred to over the features. After 15 min, cells were examined under microscope to assure cell attachment has initiated. Gadolinium(III) chloride solution (100 µM) was added over the cells after cell seeding. Samples were imaged in 2 hr from the seeding time.

Stretch-activated cation channel inhibitor peptide GsMTx4 [82] was used for Piezo1 specific channel inhibition. For this peptide cells were centrifuged and reconstituted in isotonic solution. Then peptide was added to the solution after 15 min to ensure cell attachment initiation. The samples were then imaged as mentioned earlier.

### 2.7. Image Processing and Statistical Analysis

In order to quantify the changes in the morphology of the cells, we used an image processing and statistical analysis method. Brightfield images processed with several erosions and dilations in MATLAB and the regions of interest were detected using the ‘adaptive threshold’ algorithm built in Image Segmenter app. The output image was then threshold to yield a binary image (Figure 2.6.b). The number of erosions and dilations remained the same for all the images in order to remove bias from processing. This procedure did not include the ‘tail-like’ structures (discussed
in Results and Discussion) and rendered only the body of the cell. These binary images were then transferred to ImageJ for substrate subtractions, and fitting each cell with an ellipse (Figure 2.6.c,d). The ratio of the major axis to the minor axis (L/W) of an ellipse (aspect ratio) was taken and statistically analyzed. This represents the amount of cell stretching over the stripe patterns. Same approach was used for cells on the T-shapes but after acquiring binary images, only cell area was measured using ImageJ.

For statistical analysis, the aspect ratios were averaged over multiple cells in each panel and across multiple experiments. A minimum of four experiments were performed for each condition. Data are shown as the mean ± standard error of the mean (s.e.m.). For statistical analysis we used two Sample t-test after validation of normal distribution using Anderson-Darling test. Values of \( p < 0.01 \) were considered statistically significant.
Figure 2.6. **Image processing for morphological study.** (a): raw images of cells were acquired. (b): Images were transformed to binary using a series of image processing techniques. (c): Cells were fitted with ellipses and (d): demonstrates the major and minor axes on these ellipses.
3. Results and Discussion

In my thesis work, we tested the hypotheses mentioned in 1.5. The results followed by a detailed discussion will be presented in this section.

3.1. Confinement sensing on glass substrate

Cells are capable of detecting and responding to patterned substrates, this effect has been widely reported [1, 12, 28, 96]. To test whether Piezo1 has a role in substrate sensing, we modified the substrates with fibronectin patterns and examined the morphology and spreading rate of HEK cells on the patterns. Two patterns were designed to impose spatial confinement for single HEK cells, parallel stripes of 6 µm wide with 10 µm spacing between adjacent stripes and T-shape pattern with 6 µm linewidth and 40 µm length in each direction (Figure 2.5). Previous studies showed that these dimensions provide significant constrains on HEK cell lateral spreading [28]. We tested the role of Piezo1 on detection of substrate patterns using two methods, 1) genetically knockout or overexpressing Piezo1 protein, and 2) inhibition of Piezo1 channels with specific inhibitors.

Genetically knockout Piezo1. We tested Piezo1 knockout HEK cells (P1KO), Piezo1 overexpressing cell line (hp1-cl) and compared with control cells (HEK wild type) on parallel stripe patterns, and found that both control and hp1-cl cells were stretched significantly along the patterns within 2 hours (Figure 3.1.a,c), however, P1KO cells were not able to stretch to the full extent on the stripes (Figure 3.1.b). A detailed examination of the cell images shows that Piezo1 knockout cells (P1KO) have narrow ‘tail-like’ shapes along the stripes and exhibit overall smaller stretched areas ($\frac{A_{WT}}{A_{P1KO}} = 1.7$, $p < 0.05$). These results indicate that Piezo1 plays a role in cell’s detection of substrate pattern. Without Piezo1, cells lost the ability to stretch themselves.
Figure 3.1. Morphology of cells on stripe micropattern. (a) Wild-type HEK293T cells; (b) P1KO cell line; (c) hp1-cl (overexpressed cell line). All images are taken at ~2 hr after seeding on patterns Red boxes show the approximate location of patterns on which the cell resides. Scale bar shows 10 µm.

As controls, we examined the three cell types on fibronectin coated substrate without pattern, and found that all cells spread on the substrate in random orientations (Figure 3.2). This indicates that the changes in cell morphology on patterned substrate is due to the ability of cells’ detection of substrate confinements.

To assess the time required for maximal cell stretching, we imaged the cells at 0.5, 1, 2, and 3 hr after seeding, and found that cells achieved full stretch in two hours. Shorter periods led to immature spreading, while extended culture time (> 4 hr) on the patterns caused gradual retraction from the patterns and finally detachment and apoptosis.

A statistical analysis of more than 4 experiments for each conditions shows that the mean length to width ratio (aspect ratio) of individual control cells is 3.96 times larger than P1KO cells (Figure 3.3). To evaluate the degree of cell stretching, we fit each cell with an ellipsoidal as shown in Figure 2.6, and the ratio of major axis to minor axis represents cell stretching, with larger values for more
Figure 3.2. Cells grown on the unconfined regions of the glass substrate. HEK293T cells (left panel) P1KO cell line (middle panel) and hp1-cl (right panel), showing random growth. All images were taken at ~2 hr after seeding on patterns. Scale bar shows 10 µm.

Adaption to the pattern. The statistical analysis shows that the difference of cell stretching between Piezo knockout and control cells are significant (Figure 3.3, \( p < 0.05 \)).

Note that the fitting is based on the body of the cell, the tail-like feature is not included. These results show that Piezo1 channels are required for cell stretching.

T-shapes were used to verify that the observed role of Piezo1 in confinement sensing is not specific to the stripe patterns we have used for these experiments. As we expected, P1KO cells on the T-shapes did not stretch to full extent (Figure 3.4). This is while HEK-WT cells happened to stretch to cover all over the feature and result in a complete triangle (Figure 3.4.a). Statistical results indicate a significant difference in area of spreading for these two experiments with P1KO spreading average 60% of what control cell can spread (Figure 3.5).
Figure 3.3. Aspect ratio comparison for cells on glass patterned substrate. Figures show quantification of image analysis results for HEK-WT (*n* = 53), HEK-P1KO (*n* = 62), and Hp1-cl (*n* = 76). * represent *p* < 0.05.

Figure 3.4. Morphology of cells on T-shape micropattern. (a) Wild-type HEK293T cells; (b) P1KO cell line. All images are taken at ~2 hr after seeding on patterns. Red boxes show the approximate location of patterns on which the cell resides. Scale bar shows 10 µm.
Figure 3.5. Spreading area comparison for cells on T-shape micropatterns. Figures show quantification of image analysis results for HEK-WT (n = 15), HEK-P1KO (n = 24). * represent p < 0.05.

Inhibition of Piezo1 channels with inhibitors. To further attest the role of Piezo1 channels in the cell response to substrate patterns, we inhibited the Piezo1 channels with GsMTx4 that is a known inhibitor for Piezo-type ion channels [40, 97], and a general MSC inhibitor Gd$^{3+}$. For these experiments, we tested hp1-cl and HEK wild-type cells on the stripe patterns. For this purpose, GsMTx4 (10 µM) was added to the solution after 15 min of cell seeding on the patterns and the cells were incubated for 2 hr. Figure 3.6 shows the typical images of cell spreading on the stripes with and without the presence of GsMTx4. As expected, the cells treated with GsMTx4 did not stretch significantly on the patterns, showing that GsMTx4 inhibited the cell response to the substrate patterns. In fact, they demonstrated a similar morphology as Piezo1 knockout cells, a blowing cell body with long tails (Figure 3.1.b). These tails are much narrower than the patterned area, making a
**Figure 3.6. Effect of GsMTx4 on the cell’s response to patterned substrate.** Images show Hp1-cl cells without (a) and with (b) the presence of GsMTx4 (10 µM). Peptide was added after 15 min and all images are taken at ~2 hr after seeding on patterns. Red boxes show the approximate location of patterns on which the cell resides. Scale bar shows 10 µm.

A clear distinction from the expansion seen in control cells (**Figure 3.6.a**). Morphological analysis in **Figure 3.7** show that the mean aspect ratio for control is 1.34 times higher than GsMTx4 treated cells. This confirms less deviation from original spherical (aspect ratio = 1) cell morphology for GsMTx4 inhibited cells compared to controls.

These experiments were conducted in isotonic solution because the peptide is insoluble in media. The cells have overall less expansion in saline compared with culture media. Therefore, the effect of the peptide is compared with control cells in saline solution.

A detailed analysis shows that the inhibitor did not completely eliminate cell spreading compared with P1KO cells (compare **Figure 3.6.b** with **Figure 3.1.b**). As discussed in previous sections, Piezo1 can
Figure 3.7. Effect of GsMTx4 on cell stretching on patterned substrates. Major/minor axis ratio was measured for Piezo1 expressing cells (hp1-cl) with (n = 37) and without GsMTx4 treatment (n = 61), showing inhibiting Piezo1 reduced cell stretching. The effect is statistically significant (* $p < 0.05$).

be located on the cell membrane as well as intracellular space, depending on various factors including the extracellular mechanical environment [60, 98]. GsMTx4 inhibits the membrane bound channels more efficiently, while its effect on the intracellular Piezo1 proteins could be much less [78]. The intracellular unblocked channels, can contribute to activation of signaling pathways and thus this can explain why GsMTx4 treated cells have much larger tail-like structures (Figure 3.6).

GsMTx4 treated HEK-WT was also compared with P1KO cells and statistical analysis shows a difference in the aspect ratio of these cells after 2 hr culture time (Figure 3.8). Although GsMTx4 treated cells showed a significant reduction in aspect ratio, the remaining stretch is slightly larger
Figure 3.8. Comparison of stretching in Piezo1 inhibited and knockout cells. Figures show quantification of image analysis results for HEK-WT + GsMTx4 ($n = 63$), and HEK-WT treated with GsMTx4 ($n = 49$). * represent $p < 0.05$.

than Piezo1 knockout cells. This is probably due to the existence of other MSCs as we discussed earlier. These results confirm the role of intracellular Piezo1 channels in ECM sensing.

As control, we inhibited Piezo1 using non-specific MSC inhibitor Gd$^{3+}$. Gd$^{3+}$ (100 µM) was added to the solution after 15 min of cell seeding, and the cells were cultured for 2 hr before imaging. The result shows that Gd$^{3+}$ has more pronounced effect on Piezo1 overexpressing cells (hp1-cl) compared to GsMTx4. As shown in Figure 3.9, Gd$^{3+}$ treated cells remain un-stretched on the patterns, in fact, they are rounded up at the end of experiments. In comparison, the control cells have remodeled to a full stretch on the stipes in the same period in a parallel experiment (Figure 3.9.a). Statistical analysis of 3 experiments under each condition shows that Gd$^{3+}$ treated cells have an average lower aspect ratio (aspect ratio = 1.4) compared to HEK-P1KO cells (aspect ratio = 2.3).
Figure 3.9. Morphology of cells on stripe micropatterns after Gd$^{3+}$ treatment. For the drug treatment, Gd$^{3+}$ (100 uM) was added at 15 min after seeding and images were taken at ~2 hr. Images of Hp1-cl cells grown on patterned substrate with (b) and without drug treatment (a), show that Gd$^{3+}$ totally eliminated the cell spreading on the patterns. Red boxes show the approximate location of patterns on which the cell resides. Scale bar shows 10 µm.

This also shows how close these cells are to the non-adherent spherical cell morphology (aspect ratio =1, Figure 3.10). These results confirm that Piezo1 has a main role in cell ECM sensing while other MSCs may also participate to the cell remodeling since inhibiting Piezo1 channel alone did not fully eliminate the spreading (Figure 3.8). Two previous studies have suggested TRPV4 could be a potential candidate mechanosensor [52, 54]. In urothelial cells, stretching induced calcium increase can be abolished by reducing both Piezo1 and TRPV4 expression, indicating they both contribute to the process, maybe through a coupled mechanism [52]. Importantly, Miyamoto et al. has tested various amounts of membrane stretch on urothelial cells and found that at lower stretches, it is the Piezo1 but not the TRPV4 that induces the calcium ion influx [52]. Thus, although these channels could contribute to the cells sensing, Piezo1 functions as the primary sensor.
**Figure 3.10. Effect of Gd\(^{3+}\) on cell stretching on stripe patterns.** Piezo1 expressing cells (hp1-cl) were treated with Gd\(^{3+}\)(100µM, n = 52) and compared with non-treated (n = 61) and Piezo1-knockout cells (P1KO). It shows Gd\(^{3+}\) is the most potent inhibitor for cell stretching. * represent \(p < 0.05\).

**Discussion.** Using Piezo1 knockout and inhibition methods, we demonstrated Piezo1 channels mediate cell sensing on the substrates. These findings, for the first time, provide proof that Piezo1 is a primary cell sensor for detecting substrate confinement.

We noticed that both Piezo1 knockout (P1KO) cells and the inhibitor treated cells showed a unique ‘tail-like’ feature along the stripes. The tails are much narrower than the stripes and do not stretch to fully cover the patterned areas (**Figure 3.1.b** and **Figure 3.6.b**). We think that the tails represent the lamellipodium extrusions due to the initial cell approach to surrounding substrates. The lamellipodium activity leads to the contact formations on adhesive surfaces [28]. In migrating cells,
the initial lamellidpodium extrusions will be followed by formation and maturation of focal adhesions that require an increase in contractile forces [99-101]. The lamellipodium activity could dynamically alter local membrane tension and activate membrane-bound Piezo1 channels at the cell leading edge, this in turn, increases intracellular Ca\(^{2+}\) and promote further increase in actomyosin forces. Cells lacking Piezo1 channels are unable to provide substantial actomyosin forces that are required for forming stable focal adhesions, therefore, they are unable to reach full extension on the patterns.

Since Piezo1 functions as a non-selective cationic channel, they could activate Ca\(^{2+}\) dependent actomyosin forces that is required for cell expansion and migration. A recent study using Chinese hamster ovary (CHO) cells showed that Ca\(^{2+}\) elevation occurred in migrating cells that are mechanically constrained but not in the cells on uniform substrates [102]. Furthermore, inhibiting Piezo1 mediated Ca\(^{2+}\) influx decreased migration velocity [102]. This supports the idea that local mechanical stress is transmitted through Ca\(^{2+}\) signaling mediated by Piezo1 channels. We have started to look into Piezo1 triggered Ca\(^{2+}\) signaling in the later sections.

Overexpressed cells also showed a different morphology from the wild-type HEK293T cells on the stripes. These cells had shorter length of spreading on the confinements, however, no sign of ‘tail-like’ structures. This means that the remodeling has taken place in full extent. The cells are also more likely to intrude into adjacent patterns. This phenomenon happens most likely because of the high expression levels of Piezo1 at the cell edges that enable Ca\(^{2+}\) influx to reach their threshold earlier than control cells.
3.2. Cell response to featured PDMS substrate

It has been widely studied and documented in the literature that substrate stiffness is a key determinant in cellular events such as adhesion and spreading [103]. Cells spread to larger areas on stiffer substrates, while spreading to a less extent on softer substrates [103]. We further tested cells on PDMS substrate with groves that is also softer than glass substrates. A PDMS film with 6 µm wide stripes, and 10 µm wide and 8 µm deep trenches was used as substrate. The top surface was coated with fibronectin (Figure 2.3). Wild-type HEK293 and P1KO cell lines were grown on top of the stripes. Figure 3.11 shows the typical images of cells on the stripes for Piezo1 knockout (P1KO) and control (HEK wild-type) cells. As expected, the control cells stretched on the stripes, but to a less extent, compared with the patterned glass substrate (Figure 3.1). P1KO cells, on the other hand, showed minimal stretch and remained rounded at their original site of attachment.

Figure 3.11. Morphology of cells on stripe featured PDMS substrate. (a): HEK293T cells; (b): P1KO cell line, showing Piezo1 knockout cells lost their ability to respond to substrate cures. All images are taken at ~2 hr after seeding on patterns Red boxes show the approximate location of patterns on which the cell resides. Scale bar shows 10 µm.
For control, cells were grown on PDMS substrate without trenches. Figure 3.12 shows typical images of cells on unconfined regions on PDMS substrate. As shown in the figures, both cells are able to attach and spread on PDMS. These two images show no detectable difference between P1KO and HEK293T cells. Therefore, the significant difference in morphology between P1KO and HEK293T cells should be attributed to the presence of Piezo1.

**Figure 3.12. Cells on the unconfined regions on PDMS substrate.** Left: HEK293T cells; Right: P1KO cell line. All images are taken at ~2 hr after seeding on patterns. Scale bar shows 10 µm.

The statistical data (Figure 3.13) shows that cells on the featured PDMS substrate had less extension compared with the glass substrate. As discussed earlier, Piezo1 expression is elevated in the stiffer environments [104]. With lower stiffness, the cytoskeletal forces could also be reduced, and thus there will be less stretching conveyed to the channel. Additionally, on a softer substrate, there is
Figure 3.13. Comparison of aspect ratio of HEK and P1KO control (glass substrate) with HEK (n = 39) and P1KO (n = 40) cells on PDMS stripes. Images were taken at 2 hr after seeding. The data shows that P1KO cells lost their ability to stretch on the PDMS stripes. * represent $p < 0.05$.

less chance for the activation of the auxiliary channels which is previously shown to be less sensitive [52]. On stiffer substrate, both the Piezo1 and the other contributing channel(s) will be engaged, yielding in larger Ca$^{2+}$ influx. Therefore, this observation is expected and it confirms the role of Piezo1 in substrate sensing.

Interestingly, P1KO cells on the PDMS substrates did not show the tail-like feature. This suggests that lamellipodium extrusions may not be able to attach to the soft substrate due to lower binding affinity [16]. It has been reported that cells on the softer substrates are softer and have fewer adhesions [105]. These protrusions are necessary for cell substrate probing and when the lamellipodium reaches out, focal adhesions can form. This also explains that cells generally expend longer on a stiffer stripe substrate (or spread to larger area on stiffer uniform substrate). P1KO cells on
the soft substrate lack both mechanisms of confinement sensing and protrusion capability, therefore, no local tension-dense regions could emerge and the subsequent remodeling could not occur.

By comparing the remodeling of P1KO with Gd$^{3+}$ treated cells (Figure 3.14), we can see that Gd$^{3+}$ treated cells show a slightly larger elongation (aspect ratio = 1.2) compared with P1KO cells (aspect ratio = 1.1). As discussed earlier, the inhibition efficiency by Gd$^{3+}$ is not 100%. The difference in these two cases can also indicate that the Piezo1 channels that are located inside the cell might contribute to the process.

![Histogram](image)

**Figure 3.14. Comparison of cells treated with Gd$^{3+}$ and P1KO on soft substrate.** Piezo expressing cells were treated with Gd$^{3+}$ (100µM) and compared with P1KO cells, showing Gd$^{3+}$ did not block the cell expansion completely. * represent $p < 0.05$.

### 3.3. Piezo1 functions through Ca$^{2+}$ signaling

It has been reported that intracellular Ca$^{2+}$ level is higher in cells on confinements compared to unconfined cells [1]. This Ca$^{2+}$ increase was suppressed with Piezo1 channel inhibitor GsMTx4 or
by knockdown of Piezo1 proteins [1]. To test that Piezo1 mediates cell response to ECM confinements through Ca\(^{2+}\) signaling, we designed an experiment to chemically induce intracellular Ca\(^{2+}\) release while keep Piezo1 channels blocked. This allows us to discriminate the role of Piezo1-mediated Ca\(^{2+}\) influx from other Ca\(^{2+}\) sources. In this experiment, HEK cells were cultured on the glass substrate with stripe patterns in the presence of GsMTx4 (10 µM) (added at 15 min after seeding). An additional thapsigargin (Tg, 5 µM) was added at the time of seeding that can release the Ca\(^{2+}\) from ER stores [106]. Cells treated with only GsMTx4 showed a spherical body with long tails (Figure 3.15.a), similar to previous observations under same condition. In contrast, cells treated with both drugs showed an elongated body, demonstrating a significant stretching on the stripes (Figure 3.15.b). Our statistical analysis confirms these results (Figure 3.16). Since Piezo1 channels are blocked, elevation of intracellular Ca\(^{2+}\) from Ca\(^{2+}\) stores enabled cell remodeling on the patterns. This result suggests that Ca\(^{2+}\) signaling is involved in cell response and Piezo1 mediates cell remodeling via its functions as a Ca\(^{2+}\) permeable channel. Loss of Piezo1 mediated Ca\(^{2+}\) influx can be replenished with other Ca\(^{2+}\) sources.

Introducing a large portion of intracellular calcium to the cytosol could trigger other remodeling pathways. It is also likely that while the cell is under GsMTx4 treatment, the tail like structures show up as a result of remaining activity of Piezo1 channels on ER membrane. However, since the force applied to the cell is conveyed through the focal adhesions, it should travel through the complex network of cytoskeleton elements to be transferred to the ER. Therefore, probably the Piezo1 channels in the ER do not sense enough force to release all the content of calcium ion stores and
Figure 3.15. Morphology of HEK cells on stripe micropatterns after treatment with GsMTx4 (a) and GsMTx4 + Thapsigargin (b). Thapsigargin was added to cell solution at time zero and GsMTx4 after 15 min of cell seeding. All images are taken at ~2 hr after seeding on patterns. Red boxes show the approximate location of patterns on which the cell resides. Scale bar shows 10 µm.

Figure 3.16. Evaluating the role of intracellular Ca^{2+} in cell remodeling. Aspect ratio of HEK cells treated with GsMTx4 (n = 37), both GsMTx4 and Thapsigargin (Tg) (n = 47), and without drugs as control. The results show that intracellular calcium plays a major role in cell remodeling. * represent $p < 0.05$. 
remodeling with the case of GsMTx4 alone does not happen to full extent. Future studies are required to show the exact contribution of Piezo1 channels on the ER in cell remodeling.

3.4. Distribution of Piezo1 in confined cells

In epithelial cells, it is has been shown that Piezo1 can locate at multiple cellular domains including plasma membrane, nuclear envelope, and ER [81]. Piezo1 relocates from the ER to the cell membrane by application of substrate stretch and vice versa [81], therefore, the distribution of Piezo1 is stress dependent. Cells on the confined substrate show irregular shapes and non-uniform distribution of cytoskeletal forces [28]. We hypothesize that the localization of forces could lead to a unique distribution pattern of Piezo1s, where higher Peizo1 expression coincides with the higher sensitivity of the cell. We examined the piezo1 distribution in HEK cells using a fluorescent probe, P1-cpstFRET, a CFP/YFP fluorophore pair that are genetically inserted into extracellular domain of Piezo1 channel. This allows us to assess the location and expression level of Piezo1 in live cells. By comparing this image with our previously measured force distribution in patterned HEK cells [28], we can see whether the localization of Piezo1 happens due to localized traction forces.

HEK cells were transfected with P1-cpstFRET and cultured on the stripe patterns on glass and imaged using CFP or RFP channel. Figure 3.17 shows a representative cell on confined areas. It clearly shows that Piezo1 channels accumulate in the stretched areas during cell expansion on the pattern (Figure 3.17, indicated by red arrows). The image was taken at the earlier stage of the cell stretching, so that the functional channel plaques were present. A redistribution may occur after cells are fully reorganized and cytoskeletal forces are released. Ridone and Pandzic et al.[98] and other groups [3, 60] have previously shown that Piezo1 channels form clusters on the cell membrane and the regional accumulation of Piezo1 is force dependent. Here we show, for the first time,
that the Piezo1 location is altered by ECM cues. The localized accumulation of Piezo1 channels could be responsible for cell response to ECM confinements.

As controls, we imaged the cells in unconfined regions and over-sized cells (probably before division) that grown on multiple stripe patterns (Figure 3.18). In both cases, cells showed no localized concentrations of Piezo1. Generally, cells had uniform distribution of Piezo1 on their plasma membrane in both cases.

The distribution of Piezo1 varies drastically at different stages of cell spreading (Figure 3.19). In Figure 3.19.a,b, cell shows no remodeling as overall lower amounts of Piezo1 is expressed in the cell, which shows Piezo1 is essential for remodeling. Figure 3.19.c,d shows a cell at the early stage
Figure 3.18. Fluorescent image of P1-cpSTFRET expressing HEK cells on uniform glass substrate (a) and over-sized on stripe patterns (b). Images are taken at ~2hr after seeding using a GFP filter set. The over-sized cell, probably prior to dividing, could cover more than one stripe and ignore the gap (b). Red boxes show the approximate location of patterns on which the cell resides. Scale bar shows 10 µm.

of remodeling, in which Piezo1 translocated to the tip area of the tails (indicated by red arrows) and probably helps remodeling and cell growth in those areas. As mentioned earlier, these tail-like regions can function as a probe to examine the substrate, hence, the Piezo1 channels can be located to these areas, enabling Ca^{2+} influx that facilitates cell stretching. Figure 3.19.e,f shows a cell at a fully stretched and relaxed state. At this stage, the Piezo1 channels are spread evenly throughout the plasma membrane showing no accumulation.
Figure 3.19. **Localization of Piezo1 at different stages of cell remodeling.** Left column: fluorescent live cell images; Left column: intensity maps. (a,b) shows low expression of Piezo1 yields less cell spreading and narrower tails. (c,d) shows that high density of Piezo1 clusters exhibit at the tips of the cell extrusions at the early stage of remodeling. (e,f) shows that the cell achieves full stretch and Piezo1 spreads uniformly over cell membrane. Images are taken at ~2hr after seeding using a GFP filter set. Red boxes and arrows show the regions of interest. Scale bar shows 10 µm.
To better understand the correlation between cytoskeletal forces and Piezo1 relocation in a cell under confinements, we compared the Piezo1 map (Figure 3.17) with previously published cytoskeletal force distribution measured in the same type cells on the same patterns. Figure 3.20.a shows the distribution of actin filaments in cells under specific confinements (the triangular shape)[107]. It clearly shows straight actin bundles along the cell edges, which suggests higher cytoskeletal tension. On the stripes (Figure 3.20.b,c), the cytoskeletal tension was directly measured using force sensitive FRET probe, actinin-sstFRET, that can report the forces in actin filaments in live cells [79]. Blue regions indicate the areas with higher tension in the cytoskeleton. As expected, we observed that Piezo1 accumulation (indicated by a red arrow in Figure 3.17) occurs in the areas with higher tension. Noticeably, in Figure 3.20.b, the side that has a shorter tail (indicated by blue arrow), has already undergone extensive remodeling and tension is relaxed. Therefore, the tension in this side has been transferred to areas closer to the cell body. Considering this correlation between tension in the cytoskeleton and the location of Piezo1 channels in cell membrane, it is now evident that Piezo1 channels could be recruited in sites of induced higher traction forces.

These two experiments can also be used to resolve a previous discrepancy in the literature where some studies observed only uniform distribution [11, 83], while others reported cluster formation under mechanical stimuli [3, 60]. The studies by Ellefsen and Chang et al. [83] showed that Piezo1 channels are distributed uniformly over the membrane of the cell. The smallest pattern used in their study is a 17.3 \( \mu \text{m} \times 17.3 \mu \text{m} \) square, but it hardly can impose any constrains to cells compared with our stripe patterns (6 \( \mu \text{m} \) stripe, 10 \( \mu \text{m} \) gap). That could be treated as unconstrained substrate, and that explains why they did not observe Piezo1 accumulation [83]. It is also possible
Figure 3.20. Distribution of force inside cell cytoskeleton. (a) Thick actin bundles at cell periphery and large population in cell probe (lamellipodium) [107]. (b,c): distribution of force on stripe patterns [79]. White arrow shows the area of high tension in long tail while blue arrow shows the short tail that has already undergone remodeling and force is redistributed.

that localization of Piezo1, or localized overexpression of this channel, happen due to an overwhelming (more than a certain threshold) mechanical stimuli for which the cell needs to urgently recruit more Piezo1 channel leading to more flux of ions and rapid remodeling. This threshold, as
we have visualized in the earlier section, causes the Piezo1 channels to accumulate in the stretched regions.

Further studies are required to understand the casualty of local tension and Piezo1 expression, and how the Piezo1 channels are opened by the substrate constrains. Live cell imaging can be a powerful technique to show the real-time movement of the channels on the plasma membrane (a previously was described in other studies as well [83, 98]). With our force sensitive FRET sensor, P1-cpstFRET, we can directly measure the conformational changes of the piezo1 channels during cell stretching on confinements.

Based on our results we propose the following model. the initial cell approach on the patterns activates local Piezo1 channels that allow the elevation of intracellular Ca^{2+} level and trigger Ca^{2+} dependent actomyosin forces, enabling focal adhesion maturation and cell expansion. This cell expansion further increases the membrane tension and causes more Piezo1 mediated Ca^{2+} influx. This established a feedforward loop. A similar feedback mechanism has been reported in brain tumor studies, where Chen et al [104] has shown that Piezo1 is overexpressed in human gliomas and its expression was correlated to aggressive tumor migration and increased tissue stiffness via integrin-FAK signaling. The stiffened tissue, in a feedback loop, further promoted the expression of Piezo1 [104].
4. Conclusion

In this work, we showed strong evidence that Piezo1 functions as a cell sensor for ECM sensing on substrate confinement. This is proved by genetically knockout Piezo1 in HEK293 cells and by inhibition of Piezo1 channels with specific inhibitor GsMTx4. By growing cells on the soft features, we show that the activation of Piezo1 is lower than other mechanosensitive channels, making it a primary sensor for ECM forces. Piezo1 channels function via Ca\(^{2+}\) signaling. Using fluorescence probe, P1-cpstFRET, we show that Piezo1 distributed non-uniformly in patterned cells. High density of Piezo1 clusters was present in highly stretched regions on the pattern which co-localized with higher cytoskeletal tension. These results demonstrate, for the first time, the role of Piezo1 channels as a sensor for cell’s detection of substrate mechanical cues.
5. References

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