Mechanobiological Effects of Residual Stress on Epithelial Cell Morphology

By

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Abstract

Cell adhesion and spreading are critical processes in mechanobiology, influenced by extracellular matrix mechanics and substrate properties. While substrate stiffness has been extensively studied, the effects of residual stress on cellular behavior remain largely unexplored. This study examines the impact of residual stress on MCF-10A epithelial cells seeded on prestretched polydimethylsiloxane (PDMS) substrates with stiffness values of 1000 kPa, 100 kPa, and 50 kPa. Cell attachment, spreading, and morphological changes were assessed through fluorescence imaging, image analysis, and statistical evaluation. The findings reveal that stress significantly reduces cell spreading across all stiffness conditions, as evidenced by decreased cell areas on stretched substrates compared to controls. Cells tend to avoid regions of higher residual stress, as cell attachment was markedly lower on stretched substrates. Despite statistical differences in cell aspect ratio, these variations were minor and did not indicate a biologically significant effect of residual stress on cell elongation. These results suggest that residual stress acts as a mechanical regulator of cell adhesion and spreading, which impose constraints on cellular expansion. The study brings a very new insight that needs to be mechanistically understood and incorporated into existing mechanotransduction models that primarily focus on stiffness as the dominant cue. The findings of this work highlight the necessity of incorporating residual stress into experimental and modeling frameworks.

Chapter 1: Introduction

Cells are highly sensitive to their surrounding mechanical environments, and this capability is known as mechanosensing. Mechanosensing allows cells to sense and respond to various cues, such as matrix stiffness, viscoelasticity, and prestrain, by adapting their morphology, mechanical properties, and biological activities. To understand these complex interactions, the motor-clutch model has been widely used to describe the mechanism of mechanotransduction. This model allows researchers to predict how cells generate and regulate forces through dynamic adhesion complexes between the actin cytoskeleton and the extracellular matrix (ECM).

The motor-clutch model (Bangasser et al., 2013) represents the key mechanisms underlying cellular mechanosensitivity. It postulates that myosin motor proteins exert forces on actin filaments, which, in turn, are transmitted to the ECM via molecular clutches such as integrins. These clutches bind to the actin and convey mechanical forces that cells generate during migration, adhesion, and mechanosensation. The model provides insights into the biphasic nature of force transmission, where traction force first increases with substrate stiffness and then declines at higher stiffness levels (Bangasser et al., 2013). This force-stiffness relationship forms the basis for understanding how cells optimize their mechanical interactions with their surroundings. By linking motor forces, clutch binding/unbinding dynamics, and substrate mechanics, the model captures key aspects of mechanotransduction (Bangasser et al., 2013).

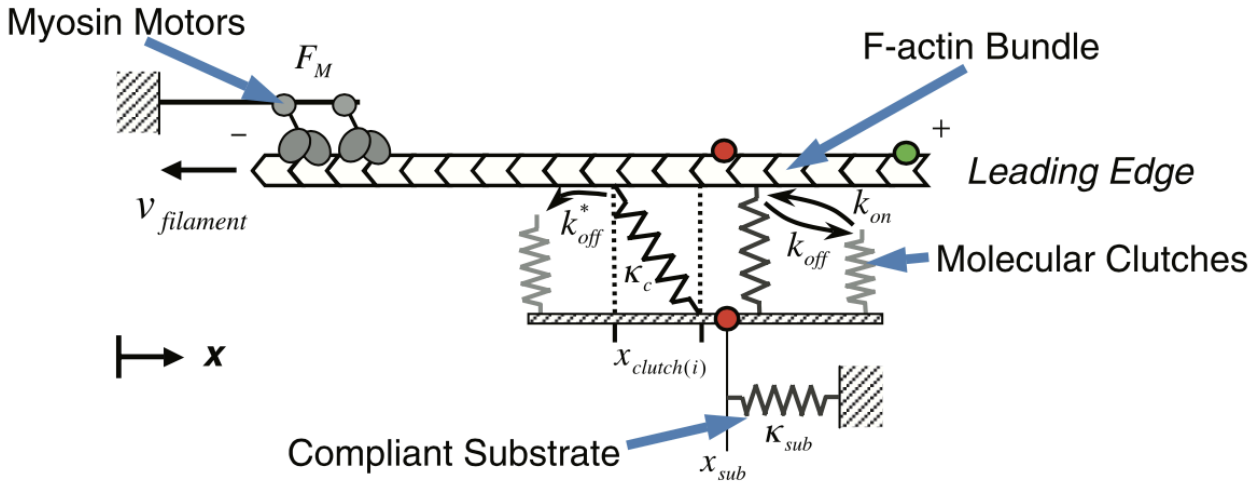


Fig.1. Schematic representation of the mechano-chemical motor-clutch model. Myosin motors generate force (FM) by pulling an F-actin filament bundle leftward at velocity v. Molecular clutches reversibly bind the F-actin with attachment rates k_on and detachment rates k_off to resist retrograde flow. Clutches are stretched to strain x_clutch(i), and disengage at a force-dependent rate, k*_off. The transmitted forces deform the substrate to strain, x_sub, with resistance determined by the clutch stiffness K_c and substrate stiffness k_sub (Chan et al. 2008).

Despite its utility, the classical motor-clutch model often oversimplifies the complexities of the in vivo environment. Cells do not only experience mechanical stiffness but also various types of residual stress, which arise from non-uniform distributions of strain in the ECM. Residual stress can accumulate in tissues due to differential growth, matrix remodeling, or pre-existing tensions (Ambrosi et al., 2019). Studies suggest that residual stress may play a critical role in regulating cellular behaviors, including proliferation, differentiation, and migration (Lanir, 2008). Therefore, a more comprehensive model that includes residual stress is necessary to better understand cell mechanosensitivity in complex environments.

Furthermore, the integration of technological advancements has greatly enhanced the study of cellular mechanosensing. Techniques such as force microscopy, real-time imaging, and computational modeling have provided detailed insights into how cells perceive and respond to mechanical environments. For instance, live-cell imaging techniques allow researchers to observe dynamic cellular behaviors under mechanical stress, while computational tools such as Monte Carlo simulations enable predictions of complex mechanotransduction pathways.

From a physiological perspective, residual stress is also a critical factor influencing cell behavior. Solid tumors, for instance, are characterized by the accumulation of solid stress and increased matrix stiffness due to rapid and unregulated cell proliferation (Stylianopoulos et al., 2013). The residual stress is induced by the interaction between cancer and stromal cells, as well as ECM remodeling. It exerts compressive forces within the tumor, potentially reducing perfusion, inducing hypoxia, and altering cellular behaviors (Jain et al., 2014). Several studies highlight the inhibitory effect of residual stress on tumor cell proliferation. Helmlinger et al. (1997) demonstrated that solid stress inhibits tumor spheroid growth by limiting cell proliferation and inducing apoptosis, while elevated interstitial fluid pressures further exacerbate these effects. Similarly, Kalli and Stylianopoulos (2018) noted that solid stress contributes to vascular collapse, which would impair nutrient delivery and increase hypoxic conditions, potentially leading to increased cancer cell invasiveness.

Residual stress also plays a role in non-cancerous conditions, such as wound healing and fibrosis, where dynamic ECM remodeling influences cellular responses (Junior et al., 2023). Recent studies have shown that cells can actively sense both rigidity and residual stress within substrates. Panzetta et al. (2019) demonstrated that cells are sensitive not only to stiffness but also to strain energy, which can be related to prestrain and residual stress. Similarly, Pajic-Lijakovic et al. (2020) emphasized the importance of residual stress in collective cell migration, suggesting that stress accumulation within the ECM can significantly influence cell-cell interactions and motility. In their work, the role of residual stress accumulation was analyzed in relation to its impact on the viscoelastic behavior of migrating cell clusters (Pajic-Lijakovic et al., 2020).

Based on previous discoveries, researchers have made considerable advancements in theoretical frameworks to address the limitations of classical models by incorporating additional mechanical cues. Panzetta et al. (2023) proposed a modified motor-clutch model that incorporates orthogonal springs to simulate the effects of residual stress, attempting to achieve more accurate predictions of cell behavior under restrained conditions. Similarly, De et al. (2010) developed frameworks linking strain energy to focal adhesion dynamics, which provided a more comprehensive understanding of how cells respond to complex mechanical environments. Other studies have focused on the dynamic nature of stress relaxation and its role in mechanotransduction. Trepat et al. (2008) demonstrated how mechanical responses, such as force generation and viscoelastic relaxation, can be unified across different cellular systems. Adebowale et al. (2021) highlighted how substrate stress relaxation enhances filopodia-mediated cell migration, which emphasized the importance of integrating time-dependent mechanical behaviors into theoretical models.

Research aims to understand the effects of residual stress and lead the pathway to study in the future possible complex interactions between residual stress and substrate stiffness on cell spreading response. While previous models have largely overlooked residual stress as a key mechanical cue, our study seeks to fill this gap by experimentally investigating its effects. Specifically, we formulated two hypotheses: (1) that residual stress in substrates will enhance cell spreading and growth by promoting cytoskeletal tension and facilitating greater substrate engagement; and (2) that residual stress will alter cell shape by modulating cytoskeletal organization, as reflected by changes in aspect ratio and circularity. By examining how cells respond to prestretched substrates, we aim to provide new insights into the role of residual stress in cellular mechanosensing.

To experimentally study the effects of substrate stress and substrate stiffness, we conducted in vitro experiments on cells seeded on prestretched polyacrylamide substrates of varying stiffnesses. The substrates were fabricated through mixing Sylgard 184 and Sylgard 527 elastomers to achieve different stiffnesses, followed by coating with polydopamine and fibronectin to promote cell adhesion. The elastic modulus (E) of the substrate that was tested fell in the range between 50 kPa and 1000 kPa, which covers the stiffness of most abdominal organs and skin (Guimarães et al., 2020). Cell spreading was quantified by fixed cell imaging, and cytoskeletal stiffness was assessed through confocal imaging of actin and paxillin. The results indicate that residual stress induced by prestrain can significantly restrain cell spreading and focal adhesion reinforcement, which contrasts with some of the theoretical predictions.

The current literature supports the value of studying residual stress in relation to cell mechanosensitivity. Studies by Trichet et al. (2012) have shown that focal adhesions and cytoskeletal dynamics are highly sensitive to large-scale mechanical cues, while Trepat et al. (2008) proposed that cell mechanics can be unified under general empirical laws that incorporate various mechanical influences, including prestrain and residual stress. However, most of the existing models fail to incorporate the explicit effects of residual stress, limiting their ability to accurately predict cell behavior in complex microenvironments. Our study addresses this gap by incorporating residual stress into the motor-clutch framework, providing a more complete representation of cell-ECM interactions.

Chapter 2: Materials and Methods

Cell Culture

MCF-10A

The human mammary epithelial cell line MCF-10A was used for all experiments. MCF-10A cells were cultured in media prepared by mixing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 0.5 mg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 10 µg/ml insulin (Sigma), and 1% penicillin-streptomycin (Invitrogen). Cells were maintained in a humidified incubator at 37°C with 5% CO_2 and were given fresh media once every two days. Cells were passaged once they reached approximately 80% confluency and were seeded at a density of 700,000 cells per T25 flask.

Substrate Fabrication

The substrates used for cell culture were fabricated from mixing Sylgard 184 (Ellsworth Adhesives) and Sylgard 527 (Ellsworth Adhesives) elastomers in different ratios to achieve varying stiffness levels. A polypropylene container was used to create PDMS sheets that fit the cell stretching device, MechanoCulture TM (CellScale). Each PDMS sheet included a rectangular well in the middle to hold the cells (**Fig. 2**), preventing them from contacting the metal parts of the machine to minimize the risk of contamination. For control conditions, PDMS substrates were prepared by curing the elastomer mixture in standard polystyrene petri dishes, to ensure an unstretched baseline for comparison. All PDMS substrates were cured at 65°C for 24 hours, then rinsed with deionized water, dried, and autoclaved. The substrates were coated with 2 mg/mL polydopamine (Sigma) solutions prepared in Tris-HCl buffer, followed by a coating of 1 µg/cm^2 fibronectin (Sigma) to promote cell adhesion. For prestretched conditions, the substrates were mounted on the MechanoCulture TM and applied with a 10% strain before the polydopamine and fibronectin coating.

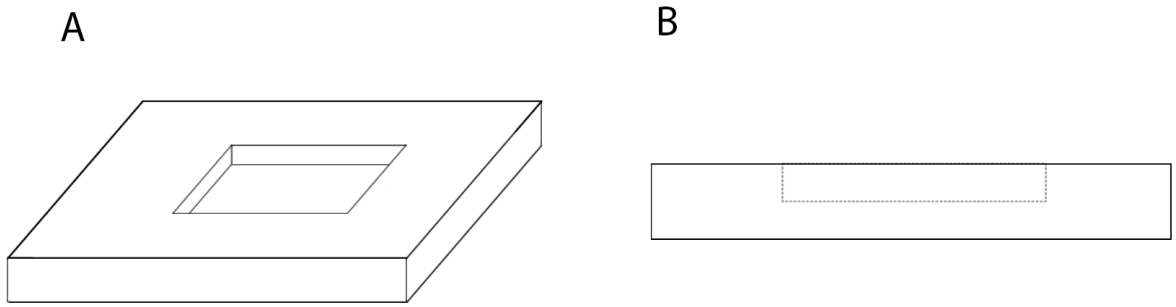


Fig.2. Schematic representation of the PDMS substrate used for cell culture experiments. (A) Isometric view showing the central rectangular well designed to contain cells and culture medium while preventing direct contact with the surrounding metal components. (B) Side view illustrating the positioning of the well within the PDMS substrate.

Substrate Loading and Stretching

The PDMS substrate was mounted onto the stretching device with tapes to secure attachment while preventing substrate tearing (**Fig. 3**). The substrate mounting is followed by surface sterilization with ethanol and ultra violet (UV). All four sizes of the device were exposed to UV for 30 minutes before the polydopamine coating to ensure thorough sterilization. The stretching program was initiated prior to applying the polydopamine solution. The device was programmed with the affiliated software to apply a strain of 0.1, which was maintained consistently for 168 hours.

A 

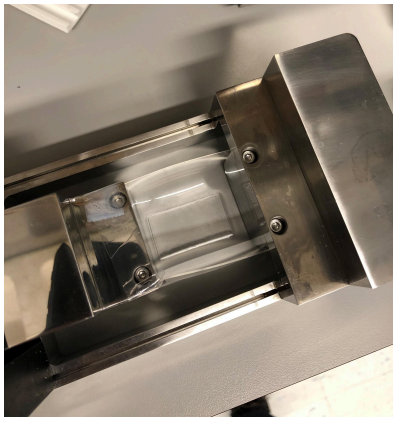
B 

Fig.3. (A) Photograph of the stretching device utilized for applying uniform and controlled strain to PDMS substrates. (B). Close-up view of the PDMS substrate mounted on the stretching device. The chamber in the middle is designed to securely hold seeded cells while maintaining sterility and minimizing contamination risk.

Fluorescence Immunostaining and Image Acquisition

Cells were seeded onto the stretched substrates and controls with a seeding density of 10000 cells every 3 cm^2, and were allowed to spread for 48 hours before immunostaining. Cells were rinsed three times with 1X phosphate-buffered saline (PBS), followed by fixing with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. After fixation, cells were rinsed three times with 1X PBS again to wash off the PFA, and permeabilized with 0.1% Triton X-100 for 15 minutes. Samples were then blocked with 1% bovine serum albumin (BSA) for 30 minutes to prevent non-specific binding. The cells were then stained with Hoechst (5 µg/mL) for nuclei visualization and 2.5 µL/mL Phalloidin for actin filaments. The samples were incubated overnight in the dark environment and subsequently washed three times with PBS before imaging. Imaging was performed with the Olympus FV1000 MPE Multiphoton Microscope.

Image Analysis

Image Analysis Software

The acquired images were processed using CellProfiler, an open-source image analysis software designed to quantify various cellular features. We chose the software for its ability to automate the quantification of cellular features, such as cell size and shape, across multiple images. The software offers a variety of customizable modules for segmentation, feature extraction, and batch processing, making it highly versatile for the study. However, its limitations include the need for manual parameter tuning and potential difficulty in handling very complex or low-quality images, which may lead to inaccurate segmentation.

MCF-10A CellProfiler Pipelines

Custom pipelines were developed in CellProfiler to measure the cell area for each sample. The Smooth module was used to clean the cell boundary. Nuclei were identified with the use of the IdentifyPrimaryObjects module. The IdentifySecondaryObjects module was applied to isolate

cytoplasms based on the presence of nuclei, which meant that only living cells with nuclei would be identified. Next, the MeasureObjectSizeShape module was used to acquire morphology measurements for each cell. The same overall sequence of modules was applied to each set of cell images, with specific parameters adjusted to ensure accurate segmentation for each individual set.

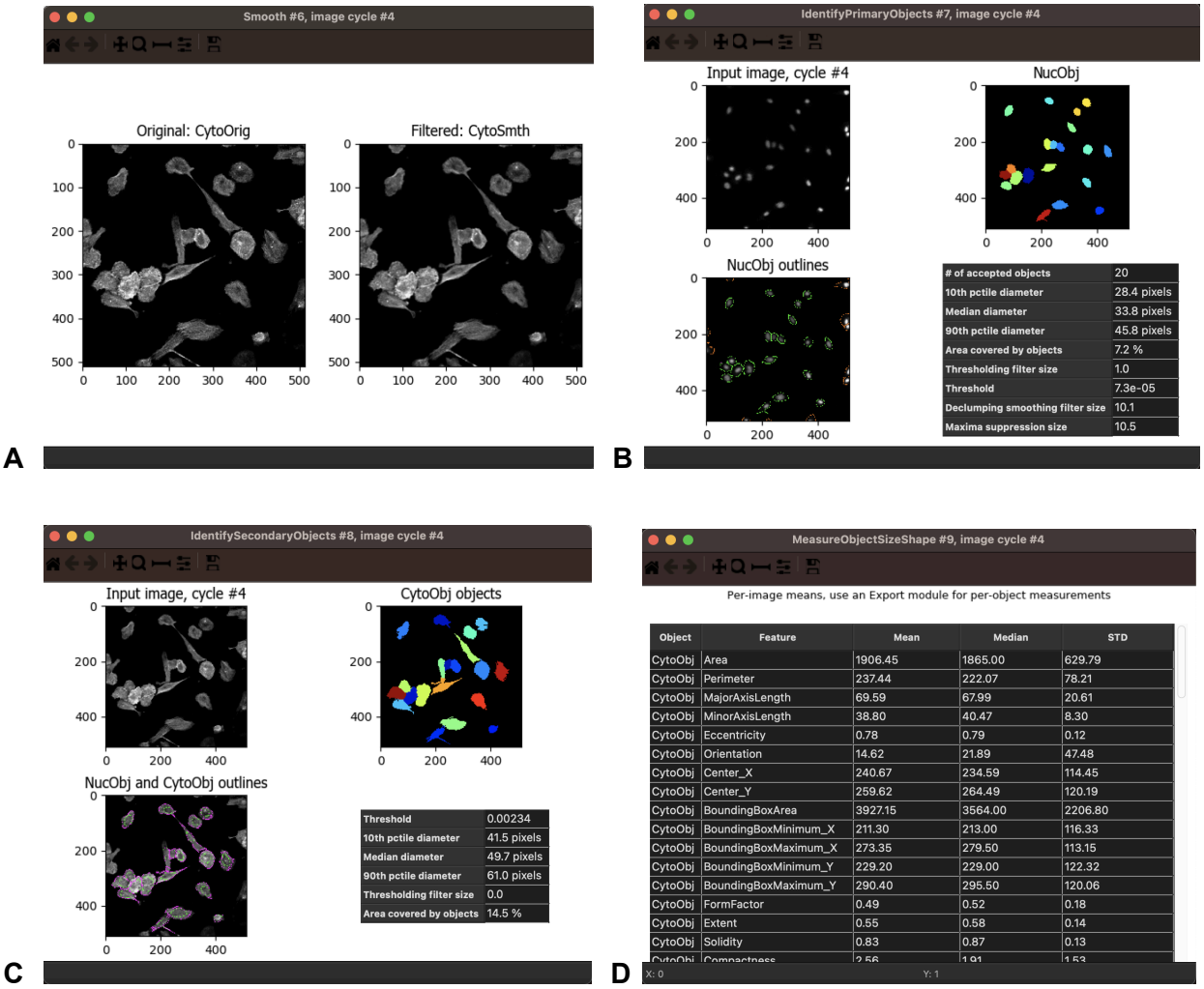


Fig.4. CellProfiler pipeline export interface, displaying processed outputs for quantifying cellular features. (A) Export of the Smooth module in the pipeline, showing pre-processing of images to reduce noise and enhance cellular feature visibility (B). IdentifyPrimaryObjects module export, identifying nuclei as primary objects based on Hoechst staining for defining individual cells in the analysis. (C). IdentifySecondaryObjects module export, illustrating the expansion of nuclear segmentation to identify cell boundaries using actin staining. (D). Export of the Measure module, displaying the extracted quantitative metrics, including cell area and shape descriptions, from the segmented cellular objects.

Statistical Analysis

All data was processed using Google Sheets. The mean cell area and standard deviation were calculated for each experimental condition. The aspect ratio was calculated through dividing the minimum Feret diameter by the maximum Feret diameter and its mean and standard deviation were calculated as well. Statistical comparisons between groups were performed to assess the effects of residual stress on cell spreading and elongation across different substrate stiffness levels.

Chapter 3: Results

Residual Stress in Substrates Significantly Reduces Cell Spreading Areas Across Stiffness Levels

The experimental results indicate that residual stress in the substrate has a pronounced effect on cell spreading. Cells were seeded on both unstretched and prestretched substrates with stiffness levels of 1000 kPa, 100 kPa, and 50 kPa. On prestretched substrates, the average cell spreading areas were notably reduced when compared to those on unstretched substrates of the same stiffness levels. This reduction was consistent across all levels of stiffness, suggesting that residual stress imposes a general inhibitory effect on cell spreading. A two-factor ANOVA revealed that substrate stretch had a highly significant main effect on cell area ($p < 0.0001$), while the effect of stiffness alone was not significant ($p = 0.6795$). Interestingly, the interaction between stretch and stiffness was significant ($p = 0.0002$), indicating that the extent of stretch-induced reduction in cell spreading may depend on the stiffness level, though the overall trend remains inhibitory.

This contradicts our hypothesis, which predicted that residual stress might enhance cell spreading by increasing cytoskeletal tension. Residual stress may alter local substrate properties, such as stiffness and viscoelasticity, or disrupt focal adhesion maturation, both of which are critical for effective cell spreading (Trichet et al., 2012). Mechanical stress has been shown to disrupt focal adhesion dynamics, weakening the ability of cells to establish and maintain strong adhesion sites necessary for spreading (Martino et al., 2018). Additionally, mechanical stress can induce cytoskeletal reorganization, where actin fibers align along the axis of minimal strain, reducing the effective spreading area (Kang et al., 2011). Another potential contributing factor is nuclear deformation caused by mechanical stress, which has been linked to reduced proliferation and spreading through alterations in gene expression and mechanotransduction signaling (Nagayama et al., 2020). Collectively, these effects might help explain how residual stress acts as a mechanical barrier to cell spreading. Without stable focal adhesions, cells cannot generate the necessary traction forces for spreading, leading to smaller growth areas.

Overall, the presence of residual stress within the prestretched substrates significantly restrain the growth of cells, regardless of substrate stiffness. These findings show that residual stress acts as a mechanical constraint that negatively impacts cell growth and spreading.

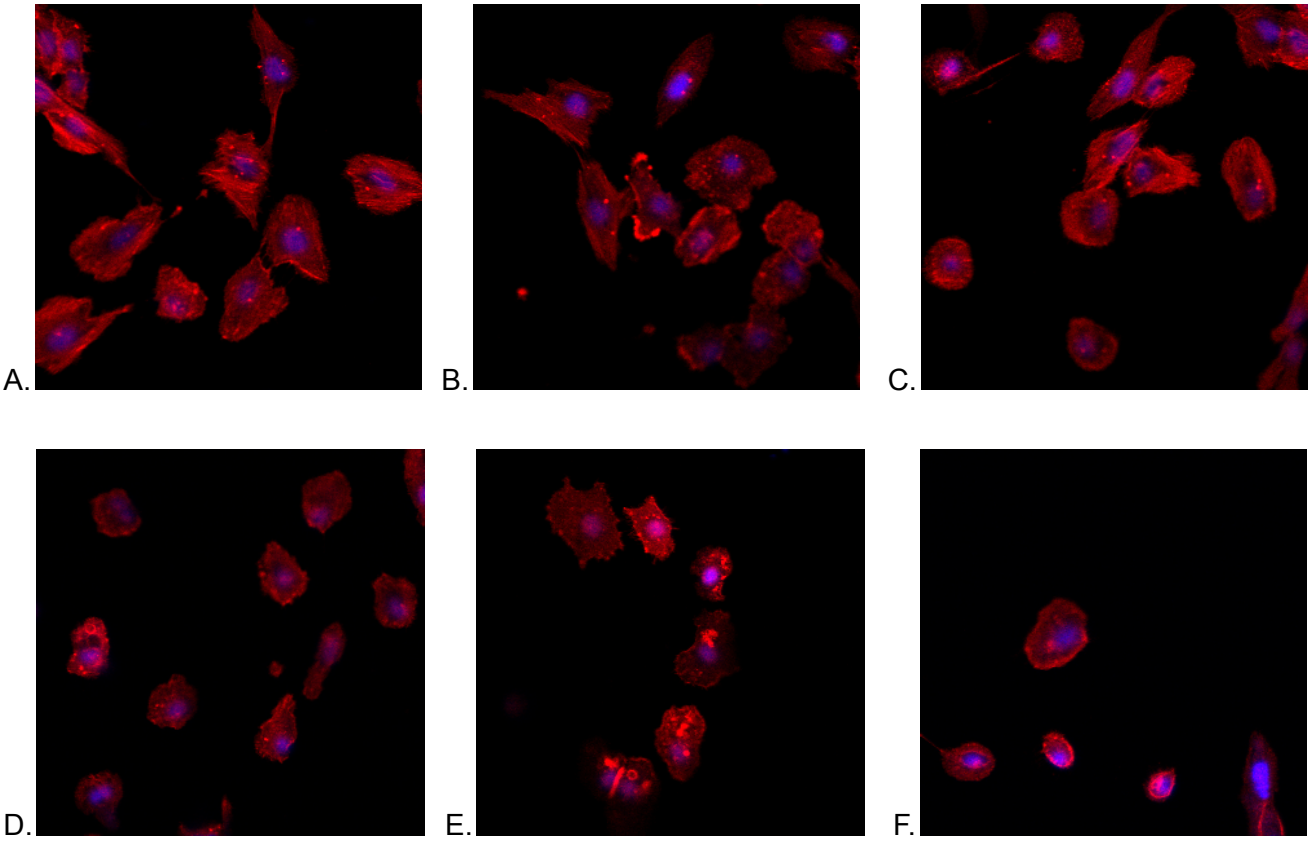
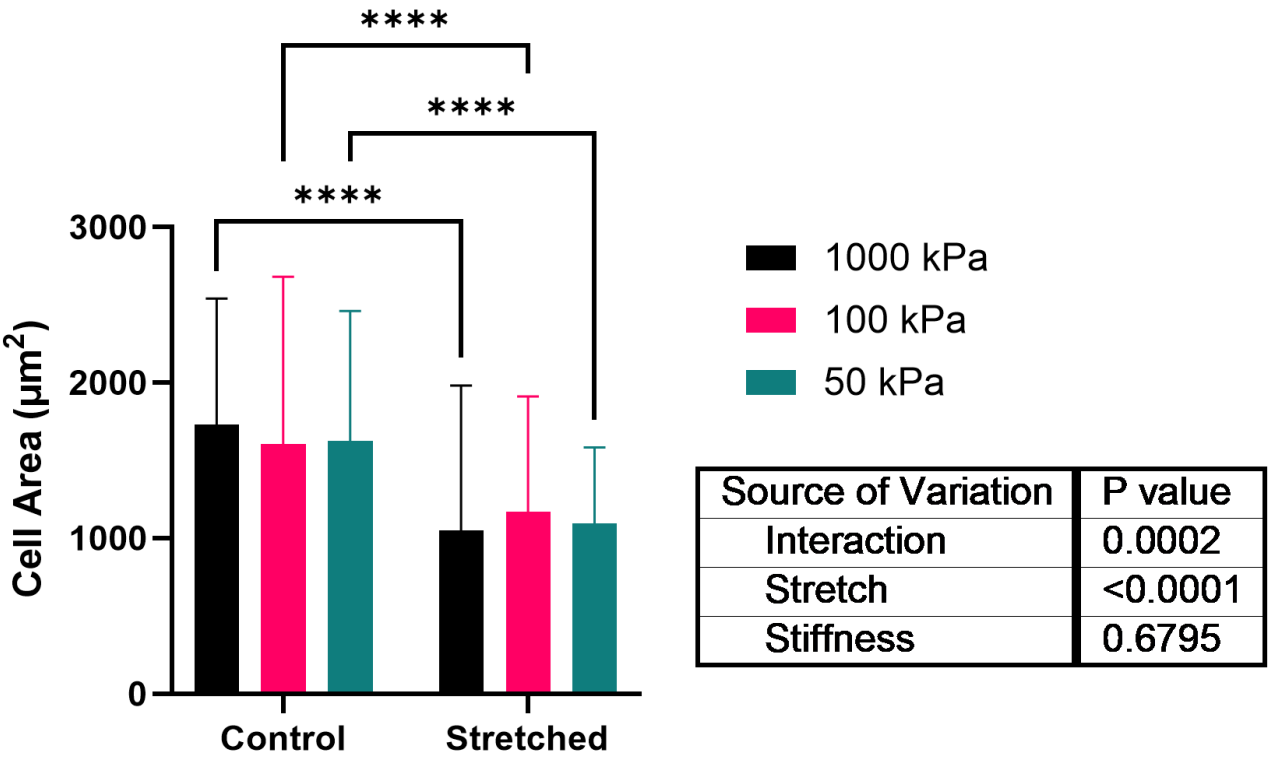


Fig.5. Representative fluorescence images of MCF-10A cells stained for actin (red) and nuclei (blue) cultured on polydimethylsiloxane (PDMS) substrates with different stiffness conditions. Cells on unstretched PDMS substrates with stiffness levels of (A) 1000 kPa, (B) 100 kPa, and (C) 50 kPa, respectively. Cells on stretched PDMS substrates with corresponding stiffness levels of (D) 1000 kPa, (E) 100 kPa, and (F) 50 kPa.



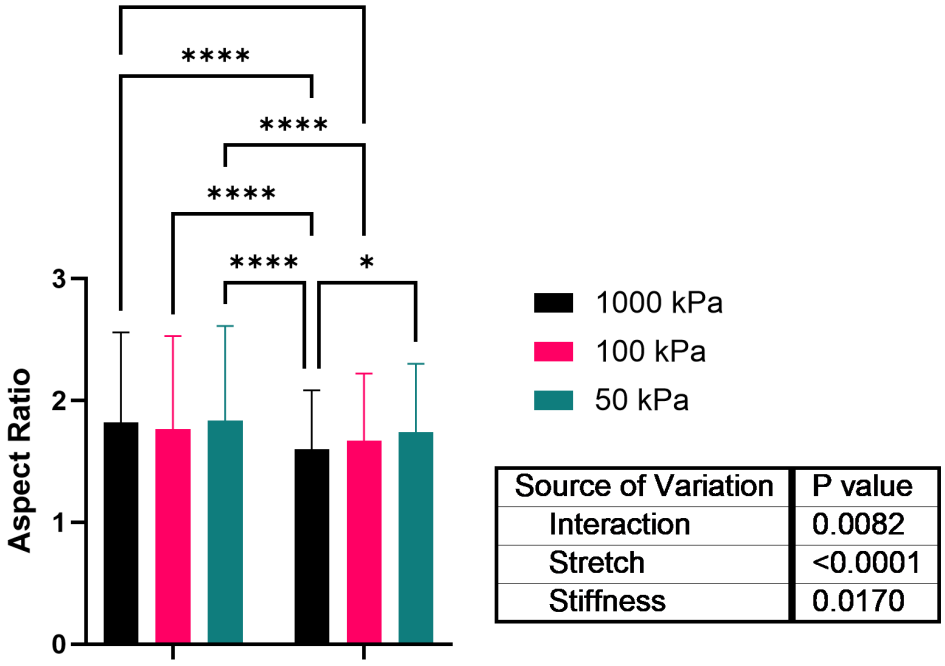
Source of Variation	P value
Interaction	0.0002
Stretch	<0.0001
Stiffness	0.6795

Fig.6. Quantification of average cell areas for MCF-10A cells cultured on unstretched and stretched PDMS substrates of varying stiffness. Bars represent mean cell areas (µm²) ± standard deviation for 1000 kPa (black), 100 kPa (pink), and 50 kPa (cyan) substrates under control (unstretched) and stretched conditions. Statistical analysis indicates significant differences between stretched and unstretched conditions for all stiffness levels ($p < 0.05$).

Changes in Aspect Ratio and Circularity with Substrate Stretching Across Stiffness Levels

The average aspect ratios of cells cultured on control and stretched substrates were analyzed across all stiffness conditions. The results indicate a consistent and statistically significant decrease in aspect ratio for cells cultured on stretched substrates compared to their control counterparts. Specifically, for 1000 kPa, the aspect ratio decreased from 1.82 to 1.60; for 100 kPa, from 1.77 to 1.67; and for 50 kPa, from 1.81 to 1.74. Although the absolute magnitude of change was modest, a two-factor ANOVA revealed a highly significant main effect of substrate stretch ($p < 0.0001$), indicating that residual stress had a robust influence on cell elongation regardless of stiffness level. In contrast, the effect of stiffness alone was smaller ($p = 0.0170$), and the interaction between stiffness and stretch

was not significant. These findings suggest that residual stress systematically limits cellular elongation across different mechanical environments, favoring a more compact morphology over a spindle-like shape. However, the magnitude of these changes is relatively small, suggesting that although residual stress may influence cell morphology by limiting elongation, the practical effect on cell shape is modest.



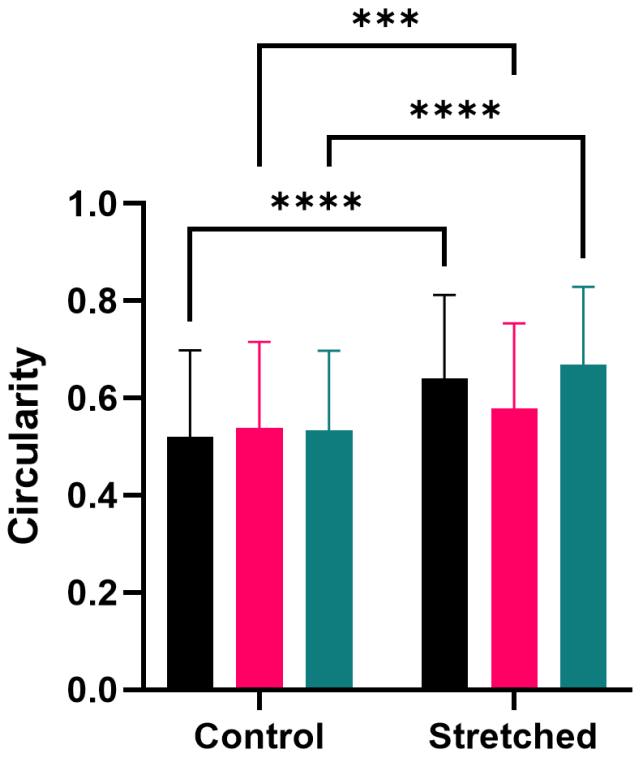
Source of Variation	P value
Interaction	0.0082
Stretch	<0.0001
Stiffness	0.0170

Fig.7. Quantification of the average aspect ratios of MCF-10A cells cultured on unstretched and stretched PDMS substrates of varying stiffness. Bars represent mean aspect ratios ± standard deviation for 1000 kPa (black), 100 kPa (pink), and 50 kPa (cyan) substrates under control (unstretched) and stretched conditions.

One possible explanation for this modest reduction in aspect ratio is that cells possess inherent mechanisms that stabilize their morphology against moderate external mechanical perturbations. Residual stress could subtly alter cytoskeletal tension and actin organization, but not to a degree sufficient to induce major elongation or retraction. Previous studies have indicated that under mechanical stresses, cells often prioritize reorienting their cytoskeleton rather than undergoing substantial elongation (Colombi et al., 2023; Das et al., 2022). Additionally, substrate stress relaxation may dissipate forces applied to the cell over time, reducing the sustained mechanical cues that would otherwise promote larger shape changes (Chaudhuri et al., 2015). Thus, while residual stress

presents an external mechanical cue, cells seem capable of adapting their internal architecture to maintain an overall stable morphology, minimizing pronounced shape changes.

To further assess the influence of residual stress on cell morphology, we analyzed circularity, a parameter that quantifies how closely a cell's shape approximates a perfect circle. Higher circularity values indicate rounder cell shapes, while lower values suggest elongation or irregular morphology. Across all stiffness conditions, cells on stretched substrates exhibited higher circularity compared to their control counterparts. The average circularity values for cells on control substrates were 0.520, 0.539, and 0.533 for 1000 kPa, 100 kPa, and 50 kPa substrates, respectively, whereas cells on stretched substrates had circularity values of 0.640, 0.578, and 0.669 for the same stiffness levels. A two-factor ANOVA revealed highly significant main effects for stretch ($p < 0.0001$), stiffness ($p < 0.0001$), and their interaction ($p < 0.0001$), confirming that residual stress significantly promotes rounder cell morphology and that this effect varies with substrate stiffness.



Source of Variation	P value
Interaction	<0.0001
Stretch	<0.0001
Stiffness	<0.0001

Fig.8. Quantification of average circularity for MCF-10A cells cultured on unstretched and stretched PDMS substrates of varying stiffness. Bars represent mean circularity ± standard deviation for 1000 kPa (black), 100 kPa (pink), and 50 kPa (cyan) substrates under control (unstretched) and stretched conditions.

The mechanism driving these morphological changes might be linked to cytoskeletal stability and mechanical adaptation. Substrate elasticity plays a key role in stabilizing cellular orientation, with stiffer substrates providing structural support that helps maintain shape under mechanical stress (Colombi et al., 2023). Additionally, cells subjected to cyclic mechanical forces tend to adjust their positioning rather than undergo drastic elongation. Cells subjected to cyclic strain reorient along the principal axis of stress rather than deforming significantly (Das et al., 2022). Another factor that might influence this outcome is substrate stress relaxation, where dissipation of mechanical forces prevents extensive cytoskeletal elongation (Chaudhuri et al., 2015). In this context, residual stress does not fully prevent mechanical strain but instead limits elongation, which reinforces a more compact, rounded morphology.

Morphological alterations are not only relevant to mechanosensing but also have broader biological implications. Cell shape has been recognized as an implication for cancer progression. Baskaran et al. (2020) demonstrated that elongated cell morphology is a key predictor of ECM-driven three-dimensional invasion, with cells exhibiting higher aspect ratios displaying greater metastatic behavior. In contrast, rounded cells tend to have lower motility and reduced invasiveness. The increase in circularity observed in our study suggests that residual stress might act as a stabilizing factor that discourages cell elongation, which potentially limits invasive behavior in certain cell types. However, whether this effect holds true for malignant cells remains an open question. Given that cancer cells often override mechanical constraints to maintain invasive potential, further studies are necessary to determine whether residual stress alters tumor cell behavior in a similar manner.

Cell Attachment and Density Significantly Reduced on Stretched PDMS Substrates

Our experimental findings demonstrated a significant difference in the attachment and spreading of cells between stretched and unstretched PDMS substrates. Cells were seeded at a density of 10,000 cells per 3 cm^2 on both stretched and unstretched PDMS substrates and were allowed to rest for 48 hours before being fixed, stained, and imaged. The imaging results showed a noticeably lower number of cells attached to the stretched substrates compared to the unstretched controls. This reduction in cell attachment on stretched substrates was consistent across all stiffness conditions tested.

Cells on stretched PDMS substrates appeared to be much less dense, with significantly fewer cells per unit area compared to those on the unstretched controls. Due to the uneven distribution of cells on the substrates, it was not possible to precisely quantify the cell density. However, the difference in cell density was visually apparent in the images, with cells on the stretched PDMS being considerably less dense. These observations are consistent with prior studies that have indicated that residual stresses or prestrain in substrates can negatively impact cell adhesion (Pajic-Lijakovic et al., 2020). It appears that the presence of residual stress in the prestretched substrates not only restricts cell spreading but also inhibits initial cell attachment, further emphasizing the detrimental effects of mechanical stress on cellular adhesion.

The reduction in cell density on stretched substrates highlights the inhibitory role of residual stress in the cell attachment process. Residual stress has been shown to interfere with focal adhesion formation, weakening integrin clustering and impairing the cell's ability to anchor to the substrate (Boccafoschi et al., 2009). Additionally, it has been demonstrated that cells tend to avoid areas of high residual stress and cluster in regions with lower mechanical strain (Lee et al., 2008). This reduction in adhesion corresponds with the observed decline in cell growth, which reinforces the notion that stable attachment is necessary for proper cell expansion.

Chapter 4: Discussion

This study investigated the impact of residual stress on cell growth, adhesion, and morphology using MCF-10A cells cultured on stretched and unstretched PDMS substrates across three stiffness levels. The results revealed that residual stress significantly inhibits cell spreading, as cells on stretched substrates exhibited smaller growth areas than those on unstretched controls. Furthermore, cell attachment was markedly lower on stretched substrates, with fewer cells adhering despite identical seeding densities. Statistical analysis confirmed that these differences were highly significant, emphasizing the inhibitory role of residual stress. Additionally, aspect ratio analysis demonstrated that cells on stretched substrates exhibited higher elongation compared to controls, with two-factor ANOVA confirming statistical significance for all stiffness levels. The findings demonstrate that residual stress exerts a complex influence on cellular behavior, with distinct effects on spreading, morphology, and attachment. This dual effect suggests that residual stress modifies the mechanical cues perceived by cells, potentially altering substrate prestrain and stiffness in ways that disrupt normal cellular responses. The decreased aspect ratio observed under residual stress conditions suggests that cells attempt to adapt to mechanical strain by reorganizing their cytoskeleton to align with the direction of applied stress. However, the reduced spreading indicates that this adaptation does not translate to improved adhesion or growth, potentially due to disruptions in integrin-ECM interactions and focal adhesion stability.

These findings challenge the initial hypothesis, which stated that residual stress would promote cell spreading by enhancing cytoskeletal tension and focal adhesion reinforcement. Instead, residual stress appears to act as a disruptive cue, hindering cellular mechanosensitivity. Residual stress may alter the substrate's mechanical properties, such as stiffness and prestrain, which in turn affect how cells perceive and respond to their environment. Reduced spreading suggests that cells struggle to reorganize their cytoskeleton and form stable focal adhesions under residual stress conditions. Similarly, lower attachment densities indicate that residual stress disrupts integrin-ECM interactions, which are critical for cell adhesion (Wozniak et al., 2004).

Although contradicting our hypothesis, the inhibitory effects of residual stress observed in this study align with some of the previous research that emphasize the importance of substrate mechanics in regulating cellular behaviors. Pajic-Lijakovic et al. (2020) reported that residual stress negatively affects collective cell migration, consistent with our findings on reduced cell attachment and spreading. Similarly, Trichet et al. (2012) demonstrated that focal adhesions and cytoskeletal

dynamics are highly sensitive to substrate mechanics, supporting the notion that mechanical disruptions, such as residual stress, impair cellular functions.

Several limitations of this study should be noted. The smaller sample size for cells on stretched substrates may have introduced biases or reduced statistical power. Additionally, the use of a single cell line (MCF-10A) limits the generalizability of the findings to other cell types. Moreover, while residual stress was modeled using prestretched substrates, the exact magnitude and spatial distribution of stress were not directly measured, which might affect reproducibility. Finally, the imaging and quantification methods, while robust, relied on fixed samples, which do not capture dynamic cell behaviors over time.

Chapter 5: Future Directions

This study has provided considerable understanding into the influence of substrate stiffness and residual stress on cellular behavior, but certain limitations point to opportunities for further investigation. One key area for improvement involves refining the experimental controls. In this study, control samples were cultured on PDMS cured in petri dishes, while stretched samples were seeded on PDMS sheets mounted on a mechanical stretching device, due to its limited availability, which restricted the ability to perform experiments simultaneously under identical conditions. This discrepancy in culture conditions may introduce unintended variables that influence cellular behavior. Future studies should aim to standardize control conditions by using an unstretched PDMS substrate within the same device to ensure a more direct comparison of mechanical effects. Additionally, the stiffness and prestrain conditions used in this study represent only a limited range of mechanical environments. Expanding these parameters in future experiments could capture a wider variety of cellular responses and provide deeper insights into how cells adapt to mechanical cues such as stiffness and residual stress.

A broader exploration of cell types is also necessary to enhance the generalizability of these findings. While this study focused on epithelial cells, other cell types, such as fibroblasts, endothelial cells, and immune cells, might exhibit distinct mechanosensitive behaviors in response to similar mechanical cues. Incorporating such variations would contribute to a more comprehensive understanding of how mechanical environments influence different cellular populations. Furthermore, future studies can consider examining tissues with varying ECM compositions to better represent the diversity of mechanical environments found in vivo.

In addition, future study may explore new variables and methodologies to refine our understanding of mechanosensing. Real-time imaging techniques, for instance, could capture the dynamic changes in cell morphology and adhesion during mechanical stimulation. Similarly, analyzing molecular markers of cytoskeletal organization and focal adhesion dynamics would provide deeper mechanistic insights.

Further exploration of residual stress in computational models could refine current frameworks like the motor-clutch model to more accurately reflect the mechanical complexity of cellular environments. Although this study did not directly modify the model, the results highlight the influence of residual stress on cellular behavior, which suggests that its incorporation into theoretical models could yield deeper insights. Future work could involve developing simulations that explicitly account

for residual stress within the substrate, which would allow for a more precise understanding of how prestrain affects cellular traction forces and cytoskeletal adaptation.

In tissue engineering, findings from this study could guide the design of scaffolds that replicate both stiffness and residual stress, optimizing them for tissue regeneration and repair. In cancer therapy, understanding how mechanical cues influence tumor progression could guide the development of strategies to modulate the tumor microenvironment. Similarly, the application of these findings to chronic conditions such as fibrosis and cardiovascular disease could lead to novel interventions that restore mechanical homeostasis in affected tissues.

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