

ABSTRACT

Title of Dissertation: HORMONAL AND MECHANICAL CUES
ON DYNAMIC CELLULAR PROCESSES IN
ENDOMETRIOSIS

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Endometriosis is a chronic inflammatory and fibrotic condition in 1 in 10 women characterized by the abnormal growth of endometrial tissue outside of the uterus. Endometriotic tissues attach to the peritoneal mesothelium lining the pelvic cavity to establish harmful lesions and implants in ectopic sites, leading to pelvic pain, infertility, and reduced quality of life. Dysregulated behaviors such as cell migration, invasion, proliferation, and immune escape drive lesion formation. The endometriotic microenvironment contributing to these cell behaviors is complex, with notable components including high local levels of estradiol (E2), a potent estrogen molecule, fibrotic extracellular matrix stiffening, inflammatory markers, and diverse cellular

interactions. However, dynamic cellular processes in endometriosis are still not well understood. Moreover, the field is understudied and would benefit from more accessible, diverse, and physiologically relevant *in vitro* models.

This dissertation investigates how hormonal and mechanical microenvironmental cues regulate dynamic cellular processes during endometriosis progression using accessible *in vitro* models. Our first focus is on hormonal signaling, as E2 has been widely implicated in endometriosis pathogenesis. Whether commonly used cell culture models faithfully capture E2-driven migratory phenotypes, and how hormonal cues interact with the physical properties of the microenvironment, remain open questions. This work first examines the effects of E2 on actin organization, morphodynamic flexibility, and migratory behavior in an endometriotic epithelial cell line (12Z), with the goal of validating its hormone responsiveness. Unexpectedly, E2 sensitivity was found to be context-dependent, motivating a broader investigation into additional microenvironmental regulators of cell behavior.

Our subsequent studies focus on the role of mechanical tissue stiffening during fibrosis, using polyacrylamide gels to model healthy and pathological matrices. We demonstrate that substrate stiffness regulates epithelial motility, cytoskeletal organization, and collective migration. We then develop an *in vitro* model of endometriotic implantation into the mesothelium and show that endometriotic spheroids preferentially disrupt mesothelial barriers on glass, while fibrotic stiffness produces graded effects.

By characterizing hormone responses and integrating mechanobiology into co-culture *in vitro* systems, this dissertation advances our understanding of early lesion

establishment. This work also informs future *in vitro* model design and provides tools for more physiologically informed endometriosis research.

HORMONAL AND MECHANICAL CUES ON DYNAMIC CELLULAR
PROCESSES IN ENDOMETRIOSIS

by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2026

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2026

Dedication

To all those who taught me how to love

Personal Acknowledgements

I feel so incredibly lucky that I have such wonderful people in my life who have become vital to my overall well-being, especially in this culminating chapter of my academic journey. The past five years would not have been possible without my advisor, Dr. Kimberly Stroka. Dr. Stroka, fondly Kim, has always had the patience to hear out my ideas and encourage me when I showed her data that I thought to be flawed and undesirable. Her steadfastness, responsiveness, and kindness have been a blessing over the last five years, and I am grateful for all of the ways in which she has helped me mature into a scientist and independent thinker.

This work would also not have been possible without my lab mates, Ian, Ken, Nikka, and Li. They have provided me with countless nuggets of scientific wisdom, between helping me identify important research questions, designing experiments, interpreting my results, and editing my written work. My most heartfelt thanks goes to Ian, my best friend in graduate school and also my tormentor. If you read his dissertation, his acknowledgements speak of bubble tea runs and games, but there is no mention of the time he put a piece of dry ice down my lab coat or all the times he took incredibly unflattering pictures of me concentrating and looking idiotic. Seriously though, thank you for always being there for me and making the last few years a joy. Also, a special shoutout to Ken, another steadfast friend. Unlike Ian, you always have such a calming effect on people. I am so grateful for all of your support and all the times you had us in stitches giggling at your witty sense of humor. And to the lab members most crucial to my own research, deepest

gratitude to Nane and Esha, two undergraduates who showed true grit sticking with me in the trenches of experiments and data analysis.

Furthermore, thank you to all of my friends who have supported me over the last few years. Thank you to Emily, Evan, Allison, and Pieper for our regular lunches and happy hours. These people are so accomplished and well put together and I am proud to call them my friends. Thank you to Corey for your friendship and collaboration. Thank you to Ashley for our writing nights. Thank you to Tiffany for our regular boba chats that I always look forward to, and particularly for helping me at a time when my personal life reached a low. Thank you to Roshni, Angelina, and Connor for so many years of cherished friendship. Thank you to all of my dance friends and my Indian dance guru, Lakshmi Aunty, for encouraging me even when the stress of lab work and adulthood threatened our classes.

I would be remiss if I didn't thank my best friend and partner, Ayush. Thank you for lifting my spirits and making each day brighter towards the end even though you had your own stresses to deal with. Given that I met you just two months ago, it's either quite brave or quite foolish for me to immortalize you in these pages, but I hope that if I open this book in ten years from now that you're the one by my side.

And now, I must express my deepest appreciation for those who shaped me in the earliest and most crucial ways. Thank you to my high school geometry teacher, Mr. Neuman, who I believe I have met and kept up with every year since I first took his class in 2014. There are few mentors in my life besides my parents who have taken such interest and care in my development to the extent and length of time you have. You were the first person to teach me how to truly learn and

study, a lifelong skill that I still rely upon. Thank you also to my high school chemistry teacher, Mrs. Hess, for being such a key female role model in STEM and helping me discover my passion for science and research.

Lastly and most importantly, with so much love, I thank my family—Akash, Maa, and Baba. My brother—the glue that holds this family together—has been with me through thick and thin, unfailingly ready to celebrate with me or comfort me with words of wisdom beyond his years. Thank you to my mother, who left her country, language, family, and career behind to establish herself in a new country on the other side of the world. Thank you for the sacrifices you made to give us a life full of love that would leave us wanting for nothing. And thank you to my father—the ultimate lifelong learner—for terrorizing me with math and science concepts that would prepare me for a life pursuing STEM. “Bring me a sheet of paper” will always stick with me and I will probably pass on the generational trauma to my future children. In all seriousness though, thank you for the pivotal role you played in my growth as a scientist and human, and for always loving me unconditionally.

Scientific Acknowledgements

Thank you to the following people, without whom this work would not have been possible:

- Dr. Kimberly Stroka, for her steady mentorship and guidance regarding experimental design, results, data analysis, data interpretation, and all scientific writing.
- My other committee members Dr. Katharina Maisel, Dr. Wolfgang Losert, Dr. Arpita Upadhyaya, and Dr. Erika Moore for their invaluable feedback and support.
- Dr. Asgerally Fazleabas from Michigan State University for key guidance on endometriosis model development.
- My lab mates Dr. Ian Smith, Ken Brandon, Dr. Li Yan, and Nikka Givpoor for their insights and input on my scientific questions, experimental design, and results.
- The staff at the BioWorkshop facility within the Fischell Department of Bioengineering at University of Maryland; particularly Dr. Sijie Hao and Dr. Christian Pick, for providing training and help with instrumentation.
- The staff at the Biosciences Core Imaging Incubator facility at the College of Computer, Mathematical, & Natural Sciences at University of Maryland; particularly Dr. Amy Beaven and Dr. Tieqiao Zhang for allowing us access to the lattice lightsheet microscope.

Chapter 2

- Undergraduate researcher Nane Manukyan for co-authoring several sections of this chapter.

Chapter 3

- Dr. Corey Herr (co-first author on this work) who performed data collection, code development, data analysis, data interpretation, and manuscript development.
- Dr. Wolfgang Losert (co-author), who enabled this collaboration, provided access to the Physics Department's lattice lightsheet microscope, and provided guidance on experiments, data analysis, and interpretation.

Chapter 4

- Undergraduate researcher Esha Chopra (co-author), who performed data collection, data analysis, and manuscript review.
- Dr. Ian Smith (co-author), who performed data collection and critical manuscript review.
- Dr. Asgerally Fazleabas, who generously provided the *ESRI*-upregulated 12Z cell line.

Chapter 5

- Undergraduate researcher Nane Manukyan who performed data collection and data analysis.

Chapter 6

- Undergraduate researcher Nane Manukyan (co-author), who performed data collection, data analysis, and manuscript review.

Chapter 7

- Undergraduate researcher Nane Manukyan (co-author), who performed data collection, data analysis, and manuscript preparation.

Funding sources

- University of Maryland
 - Clark Doctoral Fellowship
 - Summer Research Fellowship
 - Jacob K. Goldhaber Travel Award
 - International Conference Student Support Award
 - Graduate School Center for Writing and Oral Communication Fellow
- External funding
 - NIH National Institute of General Medical Sciences, Maximizing Investigators' Research Award #R35GM142838 to Kimberly Stroka

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List of Abbreviations

AFM – atomic force microscopy

AQP – aquaporin

AZA – acetazolamide

E2 – estradiol

ECF – extracellular fluid

ECM – extracellular matrix

EEC – endometrial epithelial cell

EMT – epithelial-mesenchymal transition

ER – estrogen receptor

ESC – endometrial stromal cell

HBSMC – human bladder smooth muscle cell

HP – hydrostatic pressure

LPA – lysophosphatidic acid

MMT – mesothelial-mesenchymal transition

MPA – medroxyprogesterone

P4 – progesterone

PA – polyacrylamide

PDL – poly-D lysine

PDMS – polydimethylsiloxane

PMC – peritoneal mesothelial cell

PWSS – peristaltic wall shear stress

Chapter 1: Introduction

Endometriosis is a painful gynecological condition occurring in 1 in 10 women globally. Characterized by the abnormal growth of endometrial-like tissue outside of the uterus, endometriosis leads to chronic inflammation and pelvic pain. Despite its prevalence and significant clinical burden, the cause of endometriosis remains unknown and current treatment options are limited to symptom management rather than cure. A defining feature of the disease is the ability of endometriotic cells to migrate, invade, and establish persistent lesions in ectopic locations throughout the pelvic cavity.

Lesion establishment in endometriosis is a multistep process that requires endometriotic epithelial and stromal cells to survive outside their native uterine environment, adhere to ectopic surfaces, penetrate tissue barriers, and proliferate within a chronically inflamed microenvironment. These dysregulated cellular behaviors—cell migration, invasion, proliferation, and immune escape—are likely drivers of disease initiation and progression. Endometriosis cell behaviors and their metastatic-like spread resemble processes observed in fibrosis and cancer, yet endometriosis is a benign disease with distinct regulatory mechanisms. Understanding what drives these aberrant yet non-malignant cellular activities remains a central challenge in endometriosis research.

The surrounding microenvironment that a cell interacts with is known to drive cellular activities in both healthy and disease contexts. The endometriotic microenvironment is complex, comprising of hormonal, mechanical, and biochemical cues that shape cellular processes. Much of

the existing literature has focused on hormonal dysregulation, particularly the role of estradiol (E2), in promoting endometriotic cell proliferation and invasion. Local estrogen synthesis within the peritoneal cavity contributes to inflammation and disease persistence, and estrogen signaling has been shown to regulate cytoskeletal organization and cell migration. However, hormonal signaling alone does not fully explain the variable progression of endometriotic lesions.

Endometriosis is also marked by profound changes to the local tissue microenvironment, including immune dysregulation, altered extracellular matrix composition, and fibrosis. Fibrotic remodeling leads to tissue stiffening and adhesion formation, fundamentally altering the physical context in which cells reside. While these mechanical changes are central to disease pathology, their direct impact on endometriotic cell behavior and tissue interactions has been relatively understudied. Cells actively sense and respond to mechanical cues through mechanotransduction pathways that regulate cytoskeletal tension, adhesion dynamics, and cell–cell interactions. In other disease contexts, mechanical cues are known to strongly influence cell migration, invasion, and barrier integrity. In endometriosis, emerging evidence suggests that altered mechanical properties and force generation may contribute to disease-relevant behaviors, yet the mechanobiology of endometriosis remains poorly defined. In fact, most *in vitro* models of the disease focus on biochemical stimulation while neglecting the mechanical environment. This disconnect limits our ability to fully understand how microenvironmental cues regulate disease initiation.

This dissertation seeks to advance our understanding of endometriosis initiation and progression by investigating the effects of hormonal and mechanical cues on dynamic cellular processes in the disease. This work seeks to do so in a reproducible way that utilizes accessible tools and engineered *in vitro* models that recapitulate key microenvironmental components.

Thus, the research detailed in these pages fills several knowledge gaps regarding endometriosis pathophysiology and how to model it intelligently.

The chapters of this dissertation are outlined in Fig. 1. We first provide an in-depth review of existing endometriosis research, especially as it pertains to our current understanding of hormonally and mechanically driven dynamic cellular processes in the disease (Chapter 2). Given that E2 is understood to increase cell migration and invasion in endometriosis [1–6], we began our experimental work with a study of the effect of E2 on actin and cell shape dynamics in endometriotic epithelial cells (Chapter 3). We used lattice lightsheet microscopy to analyze short-term and long-term effects of E2 on endometriotic cell shape, membrane protrusions, and actin remodeling over time.

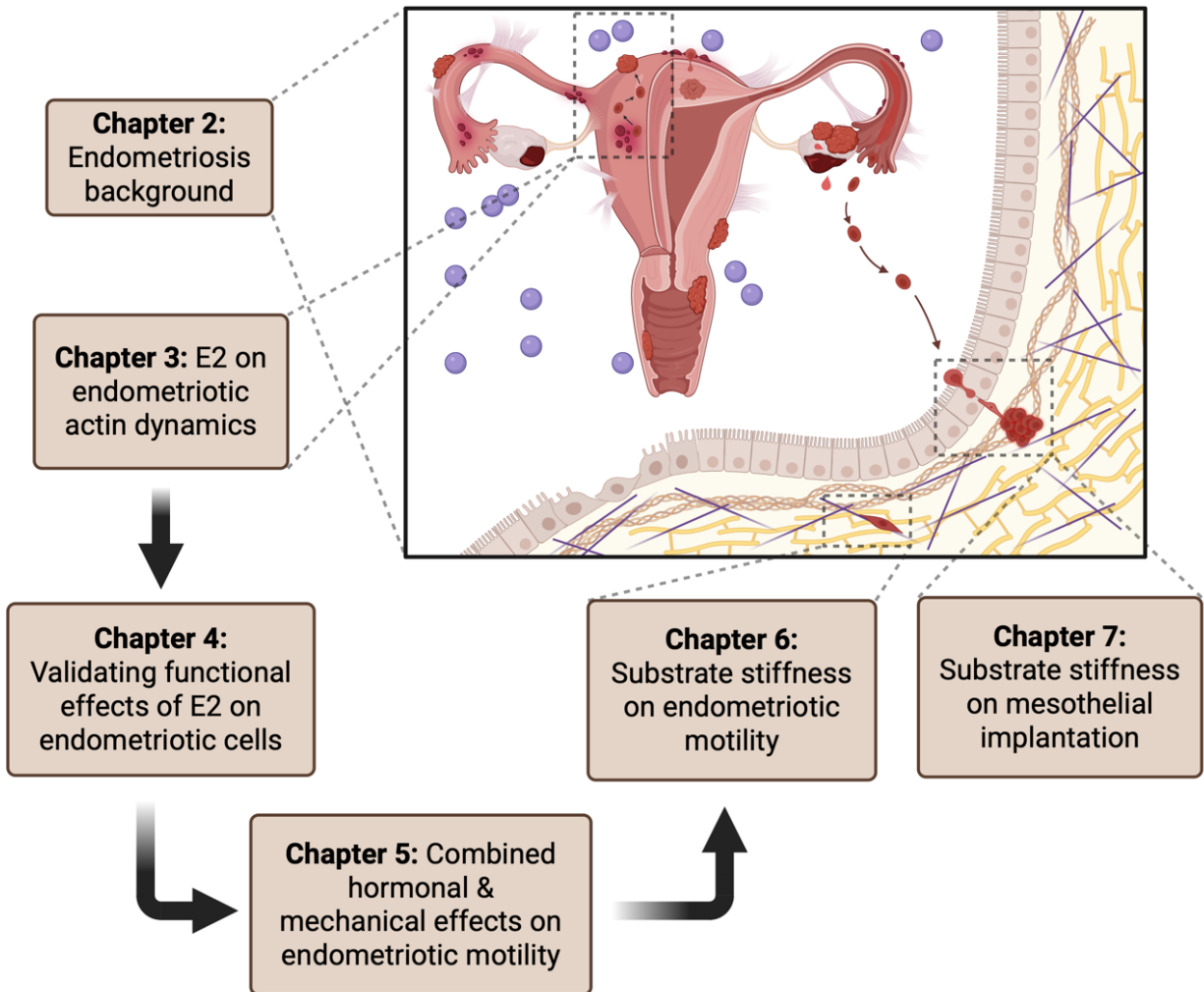


Figure 1: Dissertation overview. Created with Biorender.com.

We then sought to replicate the kind of functional effects of E2 on endometriotic cell behaviors as seen in literature—increased cell proliferation and migration, for example [1–6]. We employed the 12Z human endometriotic epithelial cell line—a cell line that has become widely used as a model for endometriosis—and analyzed its sensitivity to E2 treatment in a variety of proliferation and migration assays (Chapter 4). Surprisingly, the 12Z cell line exhibited only partial sensitivity to E2, with no effects seen in most standard *in vitro* assays. Given the 12Z cells’ context-

dependent response to E2, we hypothesized that adding in physiologically relevant mechanical cues in addition to E2 might alter the 12Zs' hormonal sensitivity. Therefore, we tested the combined effects of substrate stiffness and E2 on 12Z motility (Chapter 5).

To focus on modeling the mechanical environment of endometriosis, a fibrotic disease, we isolated the effects of substrate stiffness on endometriotic cell motility (Chapter 6). We grew 12Z cells on polyacrylamide gels with tunable stiffnesses that mimicked healthy and fibrotic tissue substrates. We measured their motility in 2D and 3D models and probed underlying mechanisms.

In our final project, we developed a model of endometriotic implantation and establishment within the mesothelium in the context of stiff fibrotic tissue. We cultivated monolayers of Met-5A human mesothelial cells on polyacrylamide gels with tunable stiffnesses and seeded 12Z individual cells or spheroids on top. We also investigated tight junction presentation within the mesothelial monolayers as a proxy for barrier integrity. We thus gained new insights on the effects of substrate stiffness on mesothelial barrier function and implantation by endometriotic cells (Chapter 7).

Finally, we summarize the findings in this dissertation, our contributions to science (Chapter 8), and future research directions inspired by this work (Chapter 9).

Chapter 2: Background

2.1 INTRODUCTION TO ENDOMETRIOSIS

2.1.1. The burden of endometriosis

Endometriosis is a chronic inflammatory condition in women characterized by the abnormal growth of the endometrium, the inner uterine lining, outside of the uterus [7]. Endometriosis affects between 6.8% to 13% of reproductive-age women globally, with the true prevalence thought to be higher due to the large number of unreported cases [8]. Endometriotic lesions and tumor-like implants are made up of endometriotic stromal (unique type of fibroblasts) and epithelial cells [9] that attach to and penetrate the peritoneal mesothelium, a layer of epithelial cells that lines all of the surfaces of the pelvic region. Lesions and implants usually appear in ectopic sites such as the ovaries, fallopian tubes, bladder, and rectum. Lesions at the mesothelial surface are termed superficial peritoneal endometriosis; ovarian lesions are termed ovarian endometrioma, and fragments that invade deep into organ walls are termed deep infiltrating endometriosis [10]. While endometriosis is usually located within the pelvic cavity, it can spread to surrounding organs, and in some rarer cases, develop as far as the abdomen, lungs, and brain [7]. The ectopic lesions and implants bleed, causing tissue damage, fibrosis, inflammation, and the resulting formation of adhesions (Fig. 2). These processes are further exacerbated by supraphysiological levels of 17β -estradiol (E2), a potent sex steroid estrogen molecule that gets continuously synthesized *de novo*, in the peritoneal fluid [11,12].

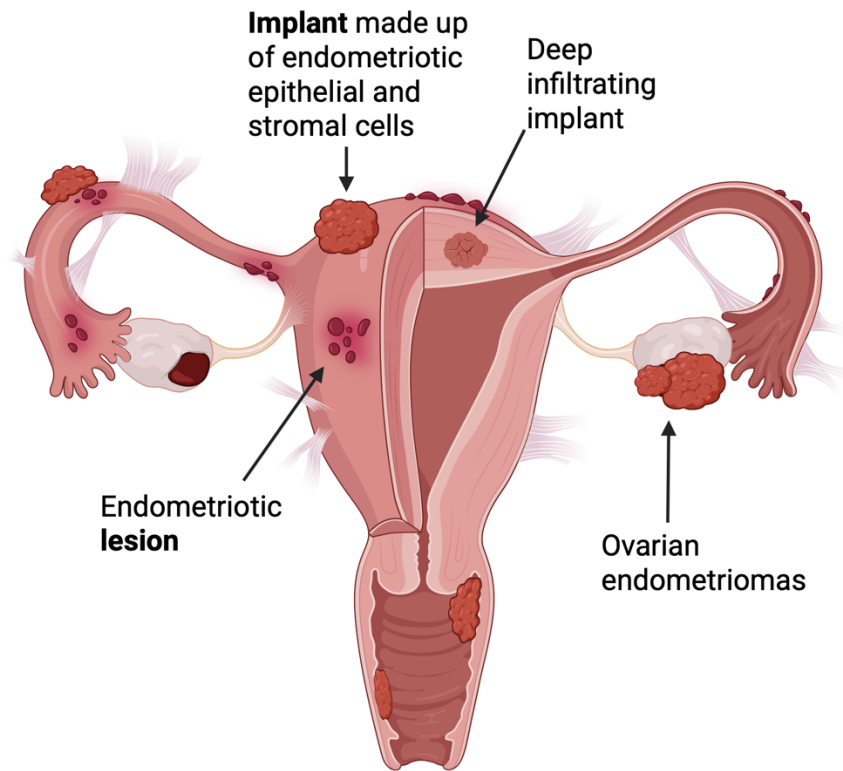


Figure 2: Endometriosis presenting as lesions and implants around the uterus and ovaries causing chronic inflammation and pelvic pain. Created with Biorender.com.

Endometriotic growths lead to symptoms which may include pelvic pain during sex and/or urination, dysmenorrhea, intermenstrual bleeding, and infertility. Beyond the significant impact on patients' quality of life, the disease also carries a hefty economic burden between \$78–119 billion annually in the US [13,14]. Despite the high prevalence, endometriosis is underdiagnosed and remains poorly understood. The lengthy diagnostic delay ranges from 5 to 12 years [15] due to diverse symptom presentation, the need for an invasive laparoscopic procedure, and limited awareness about the condition. Currently, there is no cure, and current treatments—such as hormonal therapies and surgical excision—generally focus on symptom management but do not halt disease progression [7].

2.1.2. Endometriosis pathogenesis

The cause of endometriosis is unknown; while several hypotheses exist, the most widely accepted is Sampson's theory of retrograde menstruation [16]. In this phenomenon, menstrual effluent which contains endometrial tissue can flow backwards through the fallopian tubes and enter the pelvic cavity where they then attach to the peritoneal surface. However, retrograde menstruation occurs in over 90% of women, indicating that diseased cells likely possess unique capabilities to survive and establish harmful lesions and implants [17]. Indeed, the last 40 years have culminated in an ever-growing body of literature demonstrating an array of dysregulated cell behaviors contributing to the pathophysiology of endometriosis, including cell adhesion, proliferation, migration, invasion, immune evasion, angiogenesis, and apoptotic resistance [18]. These behaviors make endometriosis reminiscent of metastatic cancers although it is a benign condition.

All of these abnormal cell behaviors contribute to the complexity of the disease, but cell migration and invasion are particularly crucial pathophysiological processes that go hand-in-hand with disease severity: the disease is initiated by the implantation of endometriotic cells into the peritoneum [19], followed by the metastasis-like spread of endometriosis to ectopic sites. In fact, the establishment of implants and lesions in more locations correlates with disease severity due to the accumulation of scar tissue, according to the endometriosis classification standards set by the American Society for Reproductive Medicine [20]. Depth of infiltration is also positively correlated with pelvic pain [21]. If endometriosis becomes severe enough, doctors may suggest a hysterectomy as a treatment, which not only prevents any future pregnancy but also puts patients

in a menopausal state [22]. Due to the intense consequences of the metastatic-like spread of endometriosis, we chose to focus on dynamic, migratory cell phenotypes in this work.

2.1.3. Cell migration and invasion in endometriosis

A sizable amount of evidence has been obtained indicating that cell migration and invasion are key processes in the development and progression of endometriosis [1,23–26]. Endometriotic cell migration has been shown in mouse models [23,24], and endometriotic cells generally have a higher invasive capacity *in vitro* compared to normal endometrial cells [1,27–33]. Invasion of the peritoneal mesothelium by endometriotic cells has also been observed *in vitro* [26,34–37]. Multiple types of cell migration and invasion, as well as other invasive cell behaviors, could be at play in endometriosis (Fig. 3). For example, individual cells might disseminate from an implant or lesion, or they may migrate collectively [38,39] in addition to their 3D invasion of surrounding tissues [25]. It is possible that endometriotic cells may rely on directed, chemotactic migration to establish growths in new ectopic sites; but like tumor cells [40], they may also be capable of moving randomly with a heightened innate motility. Cells are also likely to experience confinement during invasion, which could arise in multiple instances: A) cells may travel in tissue microtracks [41], B) cells penetrate through the tight cell-cell junctions of the peritoneal mesothelium during the initiation of endometriosis [19], and C) once cells have penetrated the mesothelium, they are capable of squeezing through organ walls during deep infiltration [42]. Individual cell motility, confined migration, and 3D spheroid invasion of endometriotic cell types have not been widely investigated. Moreover, a majority of the migration and invasion studies

focus on endometriotic stromal cells; less is understood about endometriotic epithelial cells even though both cell types are present in ectopic lesions and implants [9].

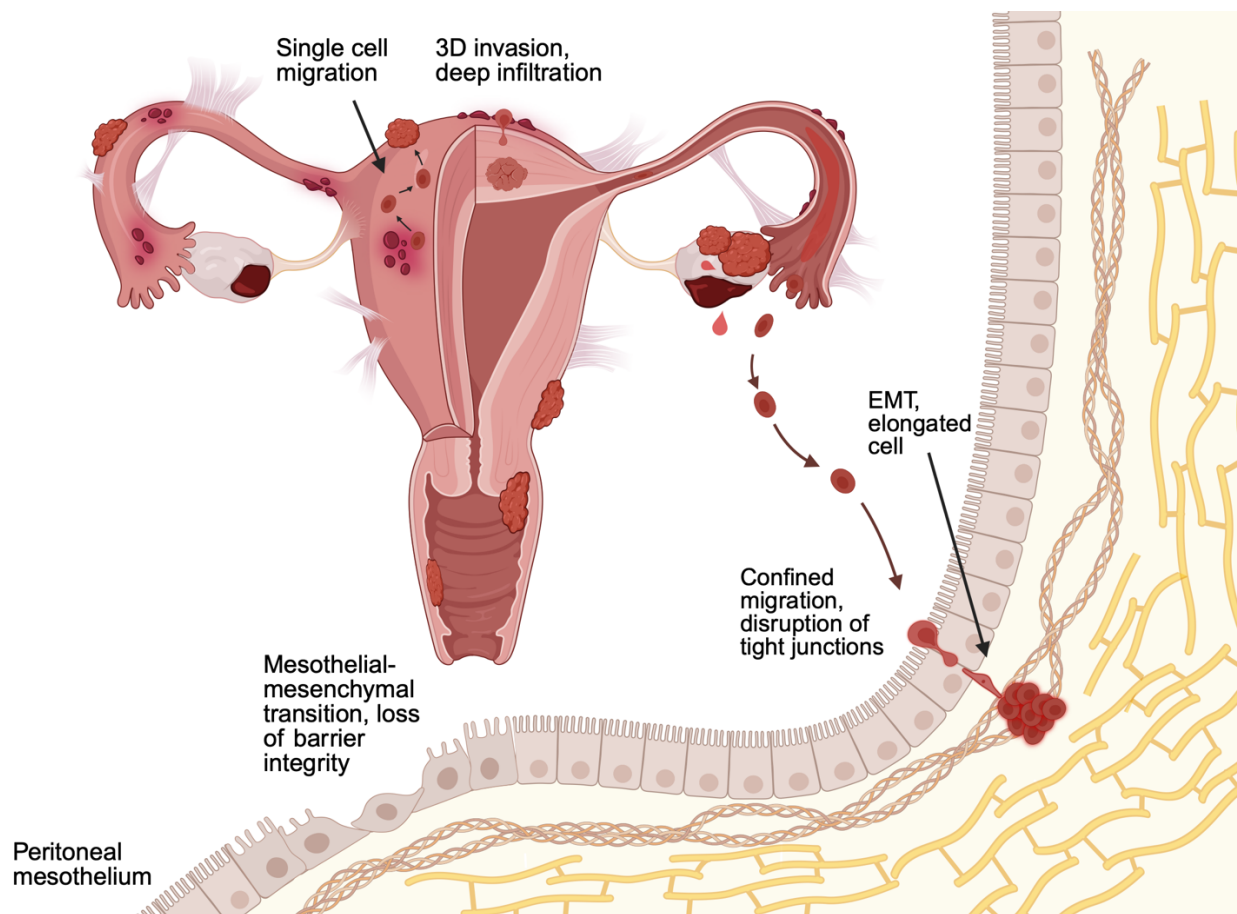


Figure 3: Modes and mechanisms of cell migration and invasion in endometriosis. Created with Biorender.com.

Other indicators of cell invasiveness are also involved in promoting the spread of ectopic lesions and implants as well (Fig. 3), such as epithelial-mesenchymal transition (EMT), cytoskeletal rearrangements, and extracellular matrix (ECM) remodeling: EMT of endometrial epithelial cells is thought to be a prerequisite for initial lesion establishment, as epithelial cells lose their cell-cell junctions and gain the high motility of mesenchymal cells [6,43–46]. Peritoneal

mesothelial cells (PMCs) also undergo EMT, termed mesothelial-mesenchymal transition (MMT). MMT compromises the barrier integrity of the mesothelium via a loss of tight junctions and the exposure of the underlying ECM, facilitating the invasion of endometriotic cells [19,47]. EMT and MMT are characterized by a loss of epithelial markers, such as E-cadherin, laminin, and cytokeratin, and an increase in mesenchymal markers, such as N-cadherin and vimentin. These changes are accompanied by a dynamic rearrangement of the actin cytoskeleton and a more elongated cell shape, which is favorable for invasion. Like in cancer cells [48], cytoskeletal reorganization can endow endometriotic cells with enhanced deformability [32] which may facilitate their navigation through narrow or tortuous spaces. However, the current understanding of cytoskeleton rearrangement in endometriotic cells comes entirely from imaging fixed cells, so their live actin dynamics have not been analyzed to our knowledge. Lastly, the secretion of matrix-metalloproteinases (MMPs) is also suspected to promote cell invasion in endometriosis. MMPs are upregulated in endometriosis, and their expression is affected by a variety of endometriotic microenvironmental components [49,50]. MMPs are capable of enhancing EMT and cell invasiveness in endometriosis [44,51–54], and their inhibition has been shown to limit endometriotic spheroid invasion [25] and lesion formation in a chicken chorioallantoic membrane model [55].

2.1.4. The endometriotic microenvironment

Dysregulated cell activities in endometriosis are united by the fact that they are dependent on their surrounding physical microenvironment. The endometriosis microenvironment is complex, with several notable components: A) enhanced local levels of E2, B) fibrotic ECM with

increased tissue stiffness, C) secreted factors and inflammatory cytokines, D) various cell types including stromal, epithelial, mesothelial, immune, and mesenchymal stem cells, and E) hypoxia and oxidative stress [18,56–59]. Together, these components create an inflammatory environment that is favorable for endometriotic invasion of ectopic sites, mesothelial barrier dysfunction, evasion of immune surveillance, and vascularization of implants [59].

2.2 ESTRADIOL AS A KEY MICROENVIRONMENTAL CUE IN ENDOMETRIOSIS

In an effort to study the regulation of cell migration and invasion in endometriosis, a large portion of the *in vitro* work has tested the impact of various proteins, cytokines, and molecules: TGF β -1 [60–64], HIF-1 α [65,66], TNF- α [67], interleukins [52,68–71], Twist [72], MMPs [28,73–76], aquaporins [77–79], osteopontin [28,80], GLI1 [81], leptin [73], extracellular matrix proteins [25,82], and long non-coding RNA [83–87] are some examples that were found to influence the migration of endometriotic cells. While it is true that cell migration and invasion are complex processes affected by multiple microenvironmental components, E2 plays an especially significant role in promoting invasive cell behavior and the establishment of endometriotic growths. Excess E2, which is continuously synthesized *de novo* by lesions and implants, is a molecular trademark of endometriosis [11].

2.2.1. E2 and ER signaling

E2 promotes cell invasiveness via estrogen receptor (ER) signaling (Figure 4). In the genomic pathway, E2 can diffuse through the cell membrane and bind to an intracellular ER. The ER then dimerizes, enters the nucleus, and acts as a transcription factor that binds to estrogen-response elements (EREs) or promoter regions of genes to directly stimulate invasive cell

behaviors in estrogen-sensitive diseases. In the non-genomic pathway, E2 binds to an ER embedded in the cell membrane. Through interactions with other proteins, the ER-E2 complex indirectly promotes cell invasiveness by activating intracellular signaling cascades such as Ras-Raf-ERK and PI3K/Akt [88]. ERs have two subtypes; ER α and ER β , where ER α generally promotes cell proliferation, especially in breast cancers, and ER β counteracts this hyperproliferation [89]. In normal endometrial tissues, both isoforms are expressed, but ER α expression is significantly greater than ER β expression [90]. In endometriosis, ER β is at least 34-times overexpressed compared to normal endometrium [91–96], though both ER α and ER β are required for lesion growth as seen in mouse models [97,98]. The precise contributions of these two ER subtypes to endometriosis progression is unclear, but their relative expression levels are thought to be indicative of cellular sensitivity to estrogens [99].

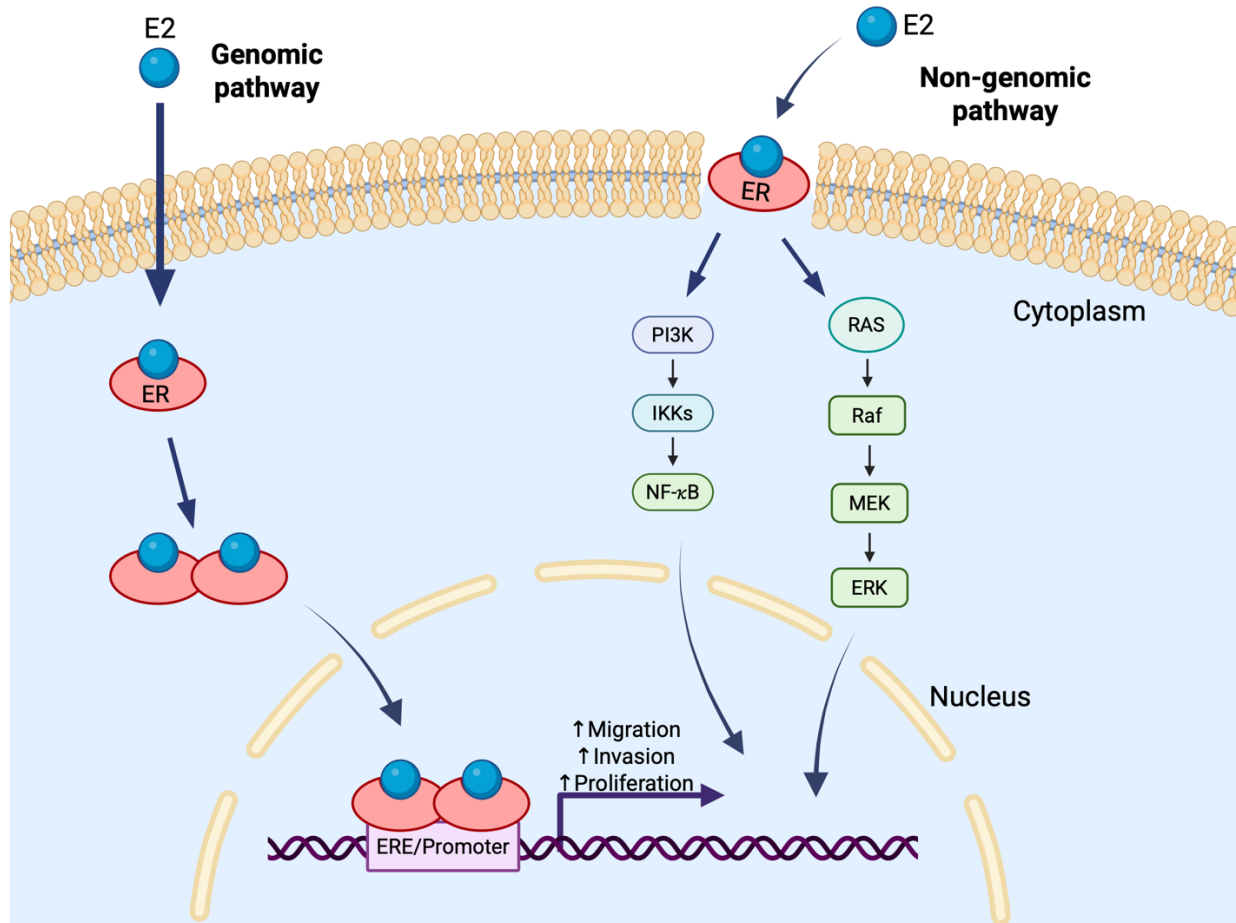


Figure 4: Genomic and non-genomic E2 signaling pathways. Created with Biorender.com.

2.2.2. E2 promotes invasive cellular processes

Extensive prior work *in vitro* and *in vivo* establishes a strong link between E2 and cell invasion [1–6,27,28,45,46,68,100], EMT [6,27,46,100], MMP expression [45,49,53,101], and ectopic lesion establishment in endometriosis [98,102]. E2 also promotes a pro-inflammatory environment [103], activating the NF-κB pathway in endometriotic cells along with other inflammatory pathways [104–106]. Since tight cell-cell junctions are regulated by the NF-κB pathway, the inflammatory milieu may compromise mesothelial barrier integrity [107,108]. E2

may also play a role in promoting endometriotic cell-mediated MMT [100] which would result in a loss of tight junctions as well. Overall, E2 represents a highly relevant microenvironmental cue in endometriosis by influencing dynamic cellular processes such as migration and invasion.

2.3 THE ROLE OF MECHANICAL CUES IN ENDOMETRIOSIS

While E2 levels, immune dysregulation, and cell-cell interactions are established contributors to endometriosis progression, the mechanical component of the endometriosis microenvironment has received comparatively little attention. Given that mechanical cues can activate immune cells [109] and act in conjunction with estrogen signaling [110], they represent an equally important microenvironmental component in endometriosis. Moreover, key endometriotic cell behaviors—such as adhesion, invasion, proliferation, EMT, and fibrotic ECM deposition—are well-documented outcomes of mechanical stimuli in other fibrotic and invasive diseases [111]. Mechanobiology—the study of how cells sense, transduce, and respond to mechanical signals such as stiffness, stretch, and shear stress—offers a powerful framework for studying endometriosis pathophysiology.

2.3.1. Mechanical characteristics of healthy versus endometriotic cells

Endometriotic cells exhibit distinct mechanical properties compared to healthy endometrial cells, which is largely due to the altered endometriotic environment [112]. For example, endometriotic cells are more sensitive to signals from mesothelial cells, causing them to undergo more distinct transcriptional and mechanical changes than healthy cells [113]. Key mechanical properties include cell contractility, stiffness, deformability, adhesion, and migration. A variety of cues and mechanisms influence these properties, ultimately shaping cellular behavior and function

[18,112,114]. Understanding the differences between healthy and diseased endometrial cells is essential for advancing the research of mechanotransduction in endometriosis and identifying potential therapeutic targets for the disease. A summary of these mechanical characteristics is provided in Table 1.

Table 1: Distinguishing mechanical properties of healthy vs endometriotic cells

Characteristic	Up or down (↑↓)	Cell type/model	Likely key contributors	References
Contractility	↑	stromal	α SMA, ROCK-I, ROCK-II, Myosin II, small GTPases	[115,116]
Deformability	↑	stromal	F-actin, vimentin, vinculin, small GTPases	[117]
Stiffness	↓		Filamentous actin network, osmotic swelling	[118]
Adhesion	↑	stromal	CAMs, cadherins, ECM biomolecules, integrins, fibronectin, collagens I/IV, cofilin laminin, vitronectin, vinculin, tenascin-C, talin-1, fascin	[119–125]
	↑	murine endometriotic cysts	FAK	[126]
	↑	epithelial	CAMs, cadherins, ECM biomolecules, talin-1	[119,122,123,127]
	↑	endometrial fibroblasts	TAGLN, ACTA2, ITGAV	[128]
Migration	↑	stromal	RhoA, RhoC, ROCK-I, ROCK-II, MLC, YAP, HIF-1 α , ezrin	[121,129–138]
	↑	epithelial	RhoA, HIF-1 α	[121,129,136–138]

2.3.2. Mechanical cues present in endometriosis

Typical mechanical cues found *in vivo* include tissue stiffness, tensile stretching, fluid shear stress, extracellular fluid (ECF) viscosity, and hydrostatic pressure [111]. Increased matrix stiffness—known to modulate cell behavior [111]—carries the greatest relevance to endometriosis, which is a fibrotic condition in which excessive ECM deposition leads to tissue scarring and stiffening [57]. Tensile stretching, while less studied, could also be of significance because endometriotic lesions can form on ectopic sites such as the bowel [139] and bladder [140]—organs which undergo cyclic mechanical stretching due to peristalsis and expansion/contraction, respectively. Deep infiltrating endometriosis is especially likely to form in these ectopic sites [141] where tensile forces may modulate cell behavior and pain generation. Fluid shear stress is another mechanical cue present in endometriosis, as the pelvic cavity is filled with peritoneal fluid that undergoes cyclic flow during the menstrual cycle. Endometriosis patients have been shown to have a higher peritoneal fluid volume compared to fertile controls [142], which could result in altered flow patterns and increased shear stresses exerted on cells in the peritoneum. Fluid shear stress is also linked to ECF viscosity; peritoneal fluid from endometriosis patients is enriched with cytokines, debris, and immune cells [142–144], which could increase its viscosity and thus the shear stress exerted on the peritoneum. Lastly, the chronic inflammation present in endometriosis leads to painful swelling and fluid retention in the abdomen (called “endo belly”) [145], likely resulting in an increased hydrostatic pressure within the abdominal cavity. A visual summary of these mechanical cues and their effects is provided in Fig. 5.

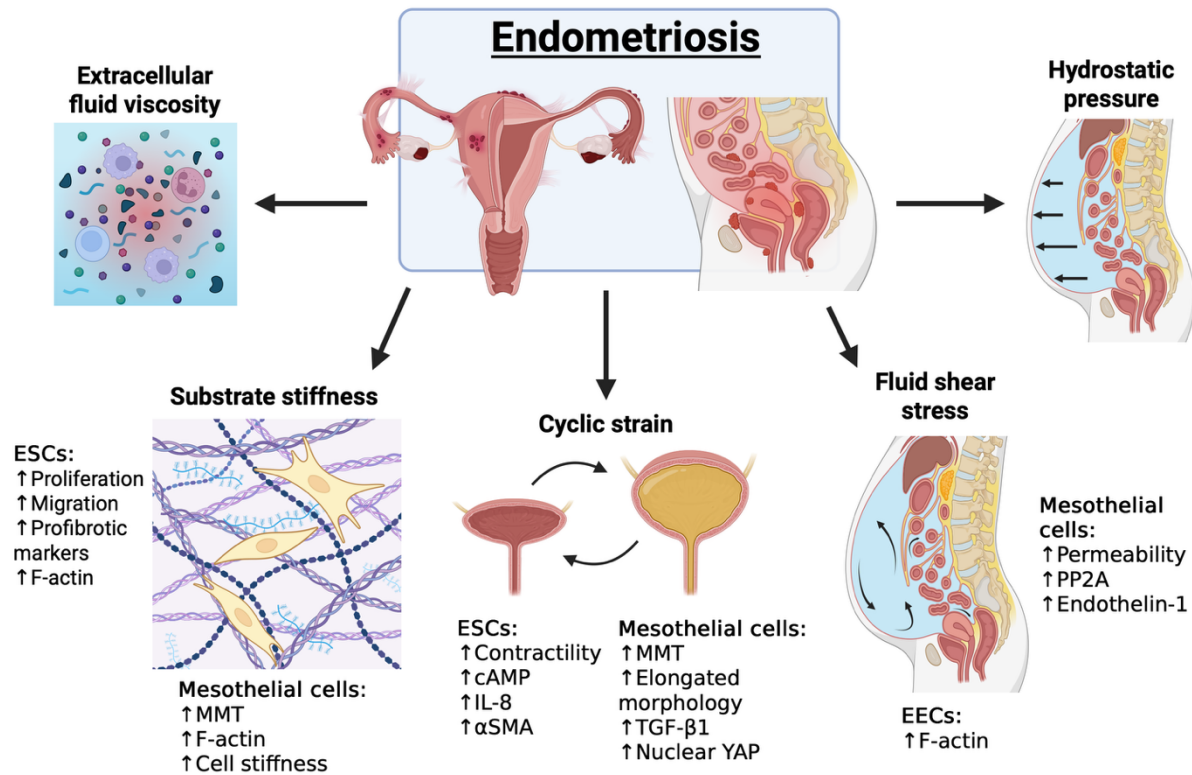


Figure 5: Schematic of mechanical cues present in endometriosis and their effects on cell behaviors. Created with Biorender.com

2.3.3. Cellular response to substrate stiffness

The elasticity of healthy endometrial tissue is reported to be 1.97 kPa in the secretory phase and 3.34 kPa in the proliferative phase [146]. Data on fibrosis of the lung, liver, and bowel indicate that the Young's modulus can increase from 1–3 kPa (normal tissue) to 16.5–20 kPa (fibrotic tissue) [147–151]. Recent work in endometriosis mechanobiology has attempted to characterize the functional response of cells to stiff substrates and identify mechanotransduction pathways involved.

Endometriotic cells. Matsuzaki and colleagues explored the fibrotic phenotype elicited from both endometriotic EECs and ESCs in response to substrate stiffness. On softer substrates, particularly 2 kPa and 4 kPa polyacrylamide (PA) gels, proliferation was inhibited in deep infiltrating ESCs while patient-matched eutopic ESCs were still capable of proliferating. This contrasts with stiffer substrates (16 kPa, 30 kPa, and plastic), where both deep infiltrating ESCs and eutopic ESCs exhibited proliferative activity. These results suggest that fibrotic matrices may promote the growth and persistence of deep infiltrating endometriosis, which cannot survive on softer substrates [152]. Both deep infiltrating ESCs and patient-matched eutopic ESCs exhibited significantly increased F-actin stress fibers and procollagen I expression on stiff (30 kPa) PA gels compared to soft (2kPa) PA gels. Culturing deep infiltrating ESCs on soft substrates significantly diminished their α SMA expression in stress fibers compared to 30 kPa or rigid plastic substrates. Deep infiltrating ESCs also had increased collagen I and cyclin D1 mRNA levels but reduced MMP1 and MMP14 on stiff compared to soft substrates. These results show that patient-derived eutopic ESCs and deep infiltrating ESCs are capable of “feeling” their surrounding ECM stiffness, with softer stiffnesses inactivating the fibrotic phenotype of deep infiltrating ESCs [152]. This may indicate a positive feedback loop, where increased matrix stiffness due to fibrosis enhances the fibrotic phenotype of ESCs, thereby exacerbating the progression of fibrosis.

In another study by the same group, healthy and endometriotic EECs derived from deep infiltrating endometriosis underwent partial EMT on rigid plastic substrates. Softer substrates led to a more rounded morphology, cortical actin, and junction-specific localization of ZO-1 and E-cadherin; conversely, stiffer substrates resulted in a more elongated cell morphology, increased F-actin stress fiber presentation, increased α SMA expression, and a loss of junctional ZO-1 and E-

cadherin [153]. These results are consistent with findings on Ishikawa cells—an endometrial adenocarcinoma cell line—which demonstrate exacerbated malignant phenotypes, altered morphology, and greater traction forces on stiff substrates compared to softer ones [110,154]. Lastly, a study used a polyacrylamide microarray system to demonstrate that disease EECs attachment was significantly increased on 6 kPa regions versus 1 kPa regions [119]. However, substrate stiffness did not influence the attachment of endometriotic ESCs, which is slightly surprising given the earlier findings [152] by Matsuzaki and colleagues. This discrepancy may simply be attributed the use of PA gels with different elastic moduli; 6 kPa is much softer than 30 kPa. Overall, these studies generally suggest that increased matrix stiffness causes EECs and ESCs to adopt more invasive and pathological phenotypes [119,152–154] but more research is needed to corroborate these conclusions.

Mechanisms. Several investigations have focused on identifying molecular mechanisms underlying endometriotic cell responses to increased substrate stiffness. RhoA/ROCK signaling is one of the most well-established mechanotransduction pathways [155]. Stiff substrates have been shown to induce a higher expression of ROCK1 in Ishikawa cells compared to softer substrates. Ishikawa cells stably overexpressing ROCK-I exhibited enhanced proliferative, anti-apoptotic, migratory, and invasive behaviors via phosphorylation of YAP1 [154]. Conversely, inhibiting or knocking down ROCK2 significantly reduced normal and endometriotic ESC proliferation and migration *in vitro*, along with a down-regulation of cyclin D1 and p27 [133]. Expression of RhoA, ezrin, talin, cofilin, and fascin—which are all mechanosensitive proteins—have also been correlated with cell migration in endometriotic cells [122,124,125,129,131]. While insightful, these studies investigated the role of mechanotransduction proteins on cellular behavior in

traditional *in vitro* assays. Their expression or function was not modulated directly on physiologically relevant substrate stiffnesses, which should be explored in future to elucidate molecular mechanisms. Given that numerous focal adhesion molecules and cytoskeletal proteins were implicated in endometriotic cell behavior (See Adhesion and migration section), their role in substrate stiffness mechanotransduction should also be investigated.

RhoA/ROCK signaling may be intertwined with hormonal signaling, as E2 treatment resulted in higher levels of activated RhoA, ROCK1, and ROCK2. This effect is mediated by estrogen receptor- α (ER α), as an ER α knockdown and overexpression led to decreased and increased RhoA, ROCK1, and ROCK2 levels, respectively. The effect of E2 on promoting EMT was also mediated by RhoA expression [129]. In adenomyotic uterine smooth muscle cells too, E2 enhanced RhoA/ROCK signaling [156]. But in another study, hormone treatment—E2 alone and a combination of E2 with medroxyprogesterone (MPA)—did not alter mean traction forces of Ishikawa cells grown on a given stiffness, even though estrogen receptors were detected by Western blot. The number of traction force hotspots—defined as small foci of ~10-fold higher traction forces compared to neighboring areas—was independent of the presence of E2 but was amplified under the E2 and MPA combination treatment. This phenomenon was attributed to the fact that progesterone receptors were only sparsely and heterogeneously expressed in Ishikawa monolayers, so only some single cells and cell clusters responded to MPA treatment [110].

The interplay of E2 signaling and mechanotransduction has not been widely investigated in endometriosis. Across other contexts, such as bone mechanobiology, vascular smooth muscle cells, endothelial cells, and a computational model of fibroblast activation, E2 and estrogen signaling have diverse impacts on mechanical signaling [157–161]. Due to the markedly high local

levels of E2 and the fibrotic microenvironment of endometriotic lesions, further research on this topic would greatly advance our understanding of the disease pathophysiology. Furthermore, excess E2 is coupled with progesterone resistance that contributes to endometriosis progression. Since ROCK1 overexpression induced progesterone resistance in Ishikawa cells both *in vitro* and *in vivo* in a mouse xenograft model [154], the crosstalk between mechanotransduction and progesterone resistance also needs to be further investigated.

Peritoneal mesothelial cells. PMCs make up the epithelial lining of the pelvic cavity and play a crucial role in endometriosis initiation and progression. The peritoneal mesothelial surface serves as a protective barrier that is well-lubricated to allow the smooth sliding of organs within body cavities. While endometriotic cells are known to have invasive capabilities, the partial loss of barrier function in the peritoneal mesothelium likely also facilitates lesion establishment. Menstrual endometriotic tissue fragments [162–164], media conditioned by menstrual effluent [165], and inflammation [166] have all been shown to damage the mesothelium, disrupting its barrier function. This may present as mesothelial-mesenchymal transition (MMT), a type of EMT where PMCs lose their epithelial characteristics and gain mesenchymal characteristics. MMT is associated with cytoskeletal remodeling, a loss of cell contacts, and fibroblast-like morphological changes [167].

Enhanced substrate stiffness is widely known to promote EMT in many types of cancer [168–173]. Liver PMCs cultured on stiff 20 kPa PA hydrogels underwent EMT, showing altered cell morphology and an increased mRNA expression of *Acta2* and *Ctgf* compared to PMCs grown on 0.5 kPa PA gels [174]. In another study, normal lung mesothelial cells (Met-5As) migrated significantly faster on 30 kPa PA gels compared to 0.3 kPa and 4 kPa PA gels. Increasing substrate

stiffness was also positively correlated with cell spreading area, the appearance of assembled bundles of F-actin, and the presence of focal adhesions [175]. These stiffness-induced changes in mesothelial cell morphology, cytoskeletal organization, and motility seem potentially indicative of MMT, but a definitive conclusion cannot be made without further investigation. Moreover, using AFM, Panzetta and colleagues found that the apparent Young's modulus of Met-5As increased significantly with increasing substrate stiffness [175]. This result contrasts with cancer cell behavior; substrate stiffness generally promotes EMT which results in softer cells that can more easily navigate 3D environments [176,177].

For a more holistic understanding of how substrate stiffness impacts mesothelial barrier integrity, cell-cell junctions should be analyzed. The balance between cell-matrix interactions and cell-cell interactions likely dictates mesothelial barrier function. In breast epithelial cells, collective sheet migration was increased on stiff substrates. This motion was coordinated by cell-cell adhesions, as disruption of adherens junctions by manipulating P-cadherin, E-cadherin, and α -catenin disrupted the coordination and directional persistence of the epithelial sheet [178]. However, the Met-5A cell line, which is often used to model normal mesothelial function, did not form mature or specific tight junctions when stained for ZO-1 [179]. Further studies are required to definitively assess the impact of substrate stiffness on mesothelial cell junctions across various cell culture models.

Mechanisms. Increased Snail1 expression and a downregulation of E-cadherin are associated with MMT in PMCs, mediated by TGF- β 1 [180–183]. Specifically, TGF- β 1 led to a decrease in cytokeratin and increase in vimentin expression, indicating a transition to a mesenchymal phenotype [180]. However, Lua and colleagues found that TGF- β 1 failed to fully

induce MMT in mouse liver PMCs cultured at confluence or in serum-free conditions. TGF- β 1 did induce MMT A) at low cell density or B) in the presence of FBS. TAZ mediates MMT in these scenarios: A) cells cultured at high cell density did not exhibit nuclear TAZ, and siRNA knockdown of TAZ in medium density cells suppressed TGF- β 1 induction of MMT. B) Cells at medium density exposed to extracellular lysophosphatidic acid (LPA) in FBS-containing media exhibited nuclear localization of TAZ and enhanced TGF- β 1 induction of MMT via LPA receptor 1. Interestingly, substrate stiffness masks the effects of cell density and LPA—PMCs grown on stiff substrates underwent MMT regardless of cell density or LPA presence, at least partly due to the translocation of TAZ to the nucleus. No additive effect was seen with substrate stiffness and TGF- β 1 treatment. siRNA knockdown of TAZ in PMCs on stiff substrates prevented MMT [174]. Therefore, although TGF- β 1 is known to induce MMT in some *in vitro* contexts [180,181], nuclear translocation of TAZ during mechanotransduction is predominantly responsible for substrate stiffness-mediated MMT [174].

Another group investigated the barrier integrity of human PMCs against ovarian cancer spheroids. Although this study did not involve substrate stiffness, they determined that protein kinase A (PKA) plays a key role in mesothelial barrier function: forskolin activation of PKA reinforced cell-cell junctions through cytoskeletal organization and reduced cell contractility. Jazwinska and colleagues also treated PMCs with Y27632 or calyculin A to decrease or increase their contractility, respectively—cellular contractility was positively correlated with mesothelial clearance and negatively correlated with junctional integrity [119–125]. These results suggest that PKA plays a key role in mechanotransduction and stiffness-mediated mesothelial barrier function.

Other mechanotransduction mechanisms, such as RhoA/ROCK signaling, focal adhesion proteins, and integrins should be further studied for their role in MMT and loss of mesothelial barrier integrity on stiff substrates. Also, given that TGF- β 1 led to an increase in ER α expression in human PMCs [183], the role of hormone-driven MMT should be further investigated, especially if there is crosstalk between E2 and mechanical signaling.

2.3.4. Cyclic stretch, fluid shear stress, extracellular fluid viscosity, and hydrostatic pressure on cell behavior

While far less studied than substrate stiffness, other mechanical cues also have relevance to endometriosis pathophysiology:

Cyclic stretch. Cyclic mechanical stretch of organs that undergo peristaltic muscle movement, such as the bladder and bowel, may affect endometriotic lesion/implant establishment [141]. Endometrial cells are capable of sensing and responding to mechanical strain [184–186]. For example, exposure to cyclic stretch led human ESCs to acquire contractile ability. Cells experienced an increase in α SMA expression, produced higher concentrations of cAMP, and showed a greater degree of contractility in a collagen gel contraction assay compared to non-strained cells [184]. In another study, cyclic stretch significantly enhanced the mRNA expression and protein secretion of interleukin-8 (IL-8) in ESCs [186]. Together, these results demonstrate that cyclic stretch induces a pro-fibrotic, pro-inflammatory, and potentially pro-migratory phenotype in ESCs [184,186] and may play key roles in bladder and bowel endometriosis. More studies are needed to confirm how cyclic stretch impacts lesion establishment and endometriosis progression.

PMCs are also sensitive to stretch. Strippoli and colleagues found that cyclic stretch induced changes to gene expression, morphology, and cell junctions indicating MMT. Cyclic stretch increased TGF- β 1 expression, other profibrotic markers, and nuclear YAP and Snail1 staining in PMCs. Further experiments revealed cooperation between YAP/TAZ and TGF- β 1 signaling to induce MMT in response to mechanical stretch. Caveolin-1 was also identified as a negative regulator of TGF- β 1 signaling during mechanical strain; PMCs from caveolin-1 knockout mice exhibited exacerbated MMT upon stretching. siRNA knockdown of caveolin-1 in human PMCs led to MMT and an increase in expression of TGF- β 1 and its receptor. These data indicate that mechanical stretch induces MMT via caveolin-1-regulated TGF- β 1 signaling and YAP/TAZ activity [187].

Fluid shear stress. Endometriosis patients have a greater volume of peritoneal fluid compared to fertile controls [142] which may lead to increased fluid shear stresses that regulate cell behaviors. In an *in vitro* model of an endometrium exposed to peristaltic wall shear stresses (PWSSs), human EECs and epithelial ovarian cancer cells experienced a significant increase in F-actin in a time-dependent manner [188]. These results indicate that fluid shear stresses may enhance the migratory and invasive potential of endometriotic cells [188,189]. In support, fluid shear stress has been demonstrated to alter cytoskeletal organization, increase migratory behavior, and trigger EMT in other epithelial models [190–192], although mechanisms driving these changes still need to be elucidated [193]. In mesothelial cells, fluid flow increased the release of the growth factor endothelin-1 [194] and expression of PP2A—which partially mediates ERK and p38 MAPK signaling—in a time-dependent manner [195]. The same group found that mesothelial

permeability to small molecules also increased significantly and reversibly upon exposure to fluid flow, suggesting that mesothelial barrier function can be compromised by shear stresses [196]. This could imply that in endometriosis, when high peritoneal fluid shear stresses are present, the mesothelial layer could become more susceptible to secreted factors and extracellular vesicle components released by EECs, ESCs, and immune cells in lesions and implants [59,197,198].

Extracellular fluid viscosity. Peritoneal fluid from endometriosis patients contains cytokines, debris, and immune cells [142–144] that likely increase its viscosity. ECF viscosity, to our knowledge, has not been studied in the context of endometriosis. However, cancer cells exposed to elevated ECF viscosity are known to exhibit enhanced motility, accompanied by actin remodeling. Cell swelling-induced membrane tension activated transient receptor potential cation vanilloid 4 (TRPV4) which triggered an influx of calcium ions, resulting in an activation of RhoA/ROCK signaling. This increase in cellular contractility, driven by ECF viscosity, enhanced the migratory potential of MDA-MB-231 breast cancer cells [199,200]. These data imply a potential role for ECF viscosity in driving invasive cell processes in endometriosis. Interestingly though, one study found that ECF viscosity could only enhance cell spreading in a substrate stiffness-dependent manner. In this instance, MDA-MB-231 cells were more sensitive to ECF viscosity when plated on stiff 30 kPa polyacrylamide hydrogels [201]. Further investigation is needed to tease apart the interplay between concurrent mechanical cues in endometriosis. Other reviews provide more detail on ECF viscosity and mechanisms in other physiological contexts [202,203].

Hydrostatic pressure. Endometriosis patients experience chronic inflammation and fluid retention in the abdomen, bloating that is referred to as “endo belly” [145] and likely results in an

elevated hydrostatic pressure (HP). While HP has not been investigated in endometriosis, human bladder smooth muscle cells (HBSMCs) were exposed to high HP to model bladder outlet obstruction. HP upregulated inflammatory mRNA expression, such as MCP, IL-6, and RANTES, as well as IL-6 production, mediated by NF κ B and ERK1/2 pathways [204]. HP has also been shown to increase apoptosis of retina ganglion precursor-like cells [205], reduce VE-cadherin expression in endothelial cells [206], increase angiogenesis [207], increase proliferation of HBSMCs [208], and decrease chondrocyte viability in a magnitude- and duration-dependent manner [209]. It is difficult to hypothesize the impacts of high HP on cell behavior in endometriosis based on these data, but we speculate that high HP exacerbates chronic inflammation and angiogenic recruitment by endometriotic implants. We direct readers to other reviews for more in-depth discussion of cellular effects and mechanisms driven by HP [210–212].

2.4 MODELING ENDOMETRIOSIS

A broad array of models have been employed to study endometriosis [58,213]. Simplistic single-cell models and 2D *in vitro* models have enabled precise control and isolation of variables. Co-culture models provide additional insight on cellular crosstalk and are more representative of *in vivo* conditions, especially in 3D culture systems. For example, endometriosis-derived organoids, created from both eutopic and ectopic lesions, offer a more accurate representation of the endometriotic environment [214]. However, the labor-intensive protocol and the oversimplification of the organoid models limit their broader application [215]. Spheroids, another three-dimensional model, can be used to investigate the mechanisms underlying endometriotic lesion progression and invasion [216]. However, the variability in lesion characteristics across the

different stages of endometriosis poses as a limitation to the model [120]. *In vivo* models of endometriosis have been developed, primarily using non-human primates and rodents. Although rare, endometriosis can spontaneously develop in menstruating non-human primates, such as monkeys, but can be experimentally induced in both primates and rodents, enabling researchers to investigate lesion progression, pain signaling, and hormonal responses [120]. Nonetheless, biological differences, such as the absence of menstruation in rodents, ethical concerns, and high cost, remain key limitations of animal models. Ultimately, endometriosis models can include many different components (Fig. 6) but considerations such as complexity, dimensionality, cost, potential for high-throughput use, feasibility, and physiological relevance must be made to address the specific scientific question.

2.4.1. The need for *in vitro* models of endometriosis

Although modeling endometriosis has come a long way in the last few decades, there is a continuous need for the development of physiologically relevant and practical *in vitro* models. There are three key points that underscore this need:

- 1) Endometriosis is complex.** Like cancer, dysregulated cell migration, invasion, proliferation, apoptotic resistance, immune evasion, and angiogenesis all orchestrate the pathogenesis of endometriosis. The disease microenvironment is rich with mechanical cues, ECM proteins, various cell types, a multitude of secreted factors and hormonal components, and oxidative stress (Fig. 6).

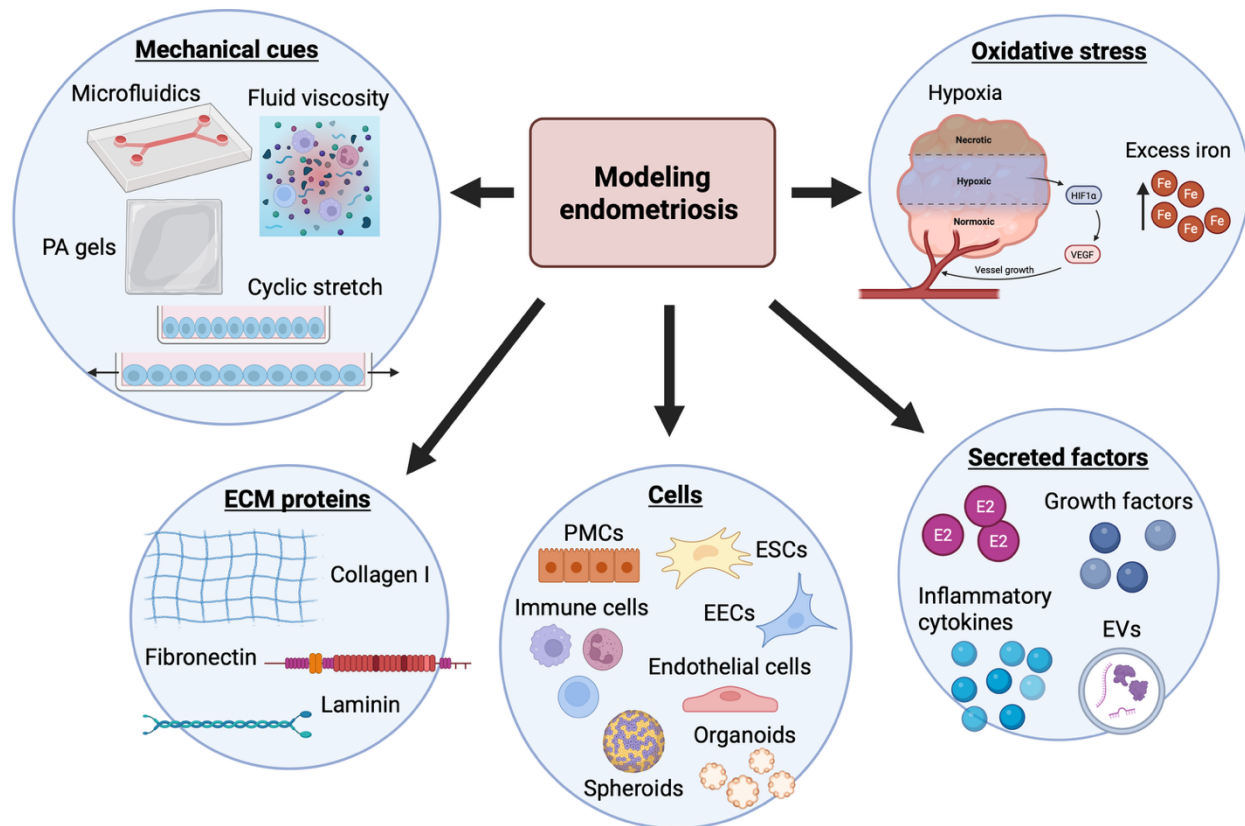


Figure 6: Schematic depicting possible components of an endometriosis *in vitro* model. Models may incorporate one or more mechanical cues, ECM proteins, cell types, secreted factors, and/or sources of oxidative stress. Created with Biorender.com.

- 2) **Endometriosis is poorly understood.** The cause of endometriosis is unknown; the predominant modes and mechanisms of cell dissemination to ectopic sites during endometriosis “metastasis” *in vivo* are still unclear (depicted previously in Fig. 3).
- 3) **The endometriosis research field is not widely accessible.** There is a broad lack of awareness and funding for endometriosis research [219,220]. Moreover, primary cells for endometriosis research are not widely available commercially, and most often, groups that use them have a collaborator with access to patient tissue samples.

Endometriosis is diagnosed with a laparoscopic surgery but finding clinics that routinely perform these surgeries and are willing to share patient samples is not always easy. We speculate that this could at least partially explain why endometriosis is so understudied and research is relatively inaccessible to labs that have not been long established in the field. When tissue samples can be obtained, primary stromal cells are frequently isolated, but according to unpublished observations from researchers at the Feinstein Institutes for Medical Research (New York, US), epithelial cells are harder to isolate and culture. Primary cells are also not ideal for high-throughput or longer-term experiments due to passage number limitations.

Points 1 and 2 above—coupled with the fact that cells cultured in 2D versus 3D may express different levels of ERs, MMPs, cytokeratin, chemokines, hepatocyte growth factor, vascular endothelial growth factor, and E2 biosynthesis genes [25,221,222]—make it crucial that diverse experimental models of migration and invasion are utilized for *in vitro* work. Previous studies largely utilize wound healing and transwell invasion assays which do not capture the complexity of endometriosis or the cell dynamics, as they are generally end-point assays. This has recently motivated the field to prioritize the development of more physiologically relevant *in vitro* models. For example, heterogeneous cell systems have gained traction: normal or disease cells (stromal and/or epithelial) have been cultured with macrophages [223,224], peritoneal cells [26,34,53,64,225–229], and endothelial cells [230]. 3D endometriosis models including organoids [231–233], spheroids [25,229,231,234], chicken chorioallantoic membranes [55,235,236], and organ-on-a-chip/microfluidic models [225] have garnered interest.

Point 3 underscores the need for practical and widely accessible cell culture models of endometriosis. A few cell lines have been used in endometriosis research: many groups have used normal endometrial cell lines, such as human endometrial stromal cells (hESCs) or the Ishikawa adenocarcinoma cell line. Endometriosis disease cell lines, which notably include the 22B stromal cell line and the 12Z epithelial cell line, originate from Dr. Anna Starzinski-Powitz's lab (Frankfurt, Germany) and have not been made widely available in the US. Moreover, Dr. Konrad Lutz from the same lab (Frankfurt, Germany) has noted from unpublished observations that the 22B cell line is very slow-growing and does not represent a better alternative to primary stromal cells. Our group has noted the same slow growth in hESCs (Applied Biological Materials, Canada) from unpublished observations. However, the 12Z cell line has gained traction around the world and has recently become commercially available (Applied Biological Materials, Canada). The 12Z cell line, in our experience, is suitable for use in high-throughput experiments and is easy to culture. 12Zs have been widely accepted by the field as a useful model of endometriosis, as they have been shown to share some phenotypic and molecular similarities with primary cells [237,238]. While it is exciting that 12Zs have the most potential to increase the accessibility of endometriosis research globally, more extensive characterization at various passages is needed to evaluate their physiological similarity to primary cells.

2.4.2. Modeling hormonal-mechanical components

This research in this dissertation, which focuses on hormonal (E2) and mechanical (substrate stiffness) cues on dynamic cellular processes in endometriosis, builds on prior models. Our *in vitro* models incorporating E2 use phenol-free media with charcoal-stripped fetal bovine

serum to remove any trace hormone levels. Our dosage and incubation time with E2 are based on pathophysiological levels and prior literature [3,12,27,46] (Chapters 3–5). To model fibrotic tissue stiffness, polyacrylamide (PA) hydrogels with tunable elastic moduli are widely utilized [146–151]. The research presented in this dissertation utilizes 2 kPa and 30 kPa PA gels coated with collagen I ECM protein to model fibrotic tissue surfaces in endometriosis (Chapters 5–7). Further discussion and details on these modeling decisions are provided in the following chapters.

2.5 CONCLUSION

Endometriosis, a highly prevalent and understudied gynecological condition, poses a significant detriment to women’s health, quality of life, and the economy. Investigating the dynamic cellular processes underlying endometriotic initiation and progression would help advance our understanding of disease pathophysiology, therapeutic strategies, and diagnostic tools. Cell behaviors are dependent on the endometriotic microenvironment, which consists of multiple biological, chemical, and mechanical cues. Endometriosis is particularly marked by supraphysiological local levels of E2 and chronic fibrosis leading to tissue damage and stiffening. We aimed to learn more about how these cues drive endometriotic motility and establishment within the peritoneal mesothelium using a combination of engineered tools and diverse models of cell migration *in vitro*.

2.5.1. Open questions

Given the complexity of endometriosis and that our understanding of it is ridden with gaps, there are virtually endless avenues for investigation. Herein we highlight a key subset of open questions as they pertain to this work:

- How does E2 affect endometriotic cell migration and morphodynamic flexibility? Do E2-mediated cytoskeletal dynamics translate to functional changes in cell migration in the 12Z cell line?
- What are the strengths and limitations of the 12Z cell line as a cell culture model of endometriosis? Do they reflect the behaviors of physiologically relevant primary endometriotic cells?
- How does fibrotic tissue stiffening mediate the initiation and progression of endometriosis? Specifically, how does fibrotic tissue stiffening affect endometriotic invasiveness and mesothelial barrier function?
- How does the combination of hormonal (E2) and mechanical (substrate stiffness) microenvironmental cues affect endometriotic cell motility?
- What kinds of models can we employ to capture the diversity of possible modes of migration in endometriosis (i.e., single cell motility, collective cell migration, 3D dissemination, confined cell migration)?
- How can we model the substrate stiffness-mediated establishment of endometriosis in the mesothelial surface *in vitro*?

2.5.2. Goals of this dissertation

This dissertation aims to provide insight on the open questions above in order to advance the field of endometriosis pathophysiology on a cellular level. We utilized engineered tools and *in vitro* models to answer these questions. As a group that is new to the endometriosis research field, we sought to use easy-to-obtain cell lines to build practical, high-throughput *in vitro* models. We

reasoned that such models would be accessible to other labs like ours that do not have the existing infrastructure to perform endometriosis research and would pave the way for consistency and reproducibility in the field. By isolating key microenvironmental and cellular players and investigating mechanisms underlying the metastasis-like spread of endometriosis, we aimed to 1) advance our knowledge of endometriosis pathogenesis, 2) inform future therapeutic and diagnostic strategies, 3) inform future *in vitro* model design, and 4) inspire broader access to basic endometriosis research.

Chapter 3: Estradiol alters actin and protrusion dynamics in endometriotic epithelial cells¹

3.1 INTRODUCTION

Endometriosis is a hormone dependent disease in which estrogen regulates cellular activities such as proliferation, migration, invasion, and tissue remodeling [6,46,102,239]. These dysregulated cell behaviors contribute to the attachment and infiltration of endometriotic cells into ectopic tissues. Moreover, estrogens have been linked to cytoskeletal remodeling in multiple estrogen-dependent diseases [239–242], which likely influences cell motility and morphodynamic flexibility – a trait which would help cells to migrate through diverse environments such as between peritoneal mesothelial cell junctions, within tissue microtracks, and through vascular and lymphatic barriers in the metastasis-like spread of endometriosis.

Actin dynamics play a key role in directed cell migration [243–245] and the formation of cellular protrusions such as filopodia [246], lamellipodia [247], and invadopodia [248,249]. Upregulating actin polymerization by itself does not create protrusions, however changing actin cytoskeleton dynamics can drive formation and suppression of protrusions [250]. The impact of estrogen on protrusion dynamics and cytoskeletal arrangement in endometriotic cells over time

¹ Adapted from Banerjee, S., Herr, C., Losert, W., and Stroka, K.M. (2026) Estradiol influences epithelial cell actin and shape dynamics imaged with 3D lattice lightsheet microscopy. *Journal of Biological Physics*.

remains unclear. Moreover, most previous cytoskeleton studies fix cells prior to immunofluorescence, which does not allow for the observation of real-time, live-cell dynamics. To overcome this, actin dynamics have been studied in mammalian cells transfected with GFP-tagged actin and imaged using methods such as 2D fluorescence microscopy [251] and the nanoscale precise imaging by rapid beam oscillation (nSPIRO) method [252] for 3D, but these techniques are still limited in terms of image resolution, temporal resolution, and photobleaching.

In the present study, we sought to clarify the effect of 17 β -estradiol (E2), a dominant and potent estrogen molecule, on endometriotic cell cytoskeletal and shape dynamics. We used the 12Z human endometriotic epithelial cell line [237] and 3D lattice light sheet microscopy to capture unprecedented volumetric time-lapses of LifeAct-GFP-labeled actin in live cells. Live-cell imaging with high temporal resolution revealed dynamic cytoskeletal reorganization, 3D membrane protrusions, and membrane ruffling. We quantified the effects of E2 on cell shape, membrane protrusion dynamics, and actin polymerization using custom image analysis pipelines, including optical flow analysis. Our study reveals that 24-hour E2 exposure significantly alters cell morphodynamic behavior, increases protrusiveness, and disrupts actin polymerization coordination in 12Z cells. These findings provide new insight into the role of estrogen in modulating epithelial structural plasticity and promoting migratory phenotypes in endometriosis.

3.2 METHODS

3.2.1. Cell culture

The 12Z cell line was purchased from Applied Biological Materials (Cat. #T0764). Cells were cultured in DMEM/F12 (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-

streptomycin (Gibco). For estradiol studies, about 48 hours prior to imaging, the culture media was replaced with phenol-free DMEM/F12 (Gibco) supplemented with 10% charcoal-stripped FBS (Gibco) and 1% penicillin-streptomycin (Gibco). 17 β -estradiol (E2) was purchased from Sigma Aldrich (Cat. #E8875-1G) in powder form and dissolved in 100% ethanol to create a stock solution. Cells were treated with a vehicle control (0.001% ethanol) or 10 nM E2 for 15 minutes (to visualize short-term effects) or 24 hours (to visualize long-term hormonal effects) prior to imaging. The E2 concentration and incubation times were chosen according to prior literature [253,239,6,254]. The cell line was authenticated by the company prior to shipping and our experiments were conducted at passages 58-68.

3.2.2. Cell transfection and staining

The LifeAct-GFP plasmid, a probe for globular and filamentous actin, was a generous gift from Dr. Denis Wirtz's lab (Johns Hopkins University, MD, US). 12Z cells were transfected with a LifeAct-GFP probe for globular and filamentous actin using electroporation technology. Briefly, cells were suspended in 100 μ L of a supplemented nucleofector solution (Lonza, Cell Line Nucleofector Kit L, cat. #VCA-1005, Germany) with 5 μ g of LifeAct-GFP plasmid, transferred to a cuvette, and subjected to electroporation via the Nucleofector device (Amaxa, Germany). The Nucleofector program X-001 was selected based on viability optimization experiments. Transfected cells were immediately seeded on a chambered coverslip (Ibidi, Germany) coated with 10 μ g/mL collagen I from rat tail (Corning, cat. #354249, NY, US) and incubated overnight prior to imaging. For validation studies, to ensure that the borders of the actin cytoskeleton were

coincident with the plasma membrane, cells were exposed to a plasma membrane stain (CellMask, ThermoFisher, cat. #C56129, MA, US).

3.2.3. Lattice lightsheet microscopy

Fluorescent actin time-lapses were captured using the Zeiss Lattice Light Sheet 7 (LLS7) microscope equipped with the Hamamatsu ORCA-Fusion camera system and the ZEN Microscopy software (Zeiss, Germany). The 30 μm x 1000 nm light sheet was utilized for all imaging. LifeAct-GFP was visualized using the 488 nm laser and for cells with the additional plasma membrane stain, the 560 nm laser was also used. Image acquisition was performed using a time interval of approximately 10 seconds, and image slices were 0.2 μm in thickness. Cells were maintained at 37°C in 90% humidity and 5% CO₂ for the duration of imaging.

3.2.4. Shape dynamics analysis

Cell morphodynamics were analyzed from fluorescence maximum intensity projections obtained from lattice lightsheet microscopy. Time-lapse image stacks were loaded into ImageJ and individually thresholded for optimal object segmentation. Morphology parameters, including circularity, aspect ratio, and solidity, were then obtained from binarized image stacks. Circularity is defined by the formula $\text{circularity} = \frac{4\pi(\text{Area})}{(\text{Perimeter})^2}$, where a value of 1.0 indicates a perfect circle and a value closer to 0 indicates an elongated polygon. Aspect ratio is defined as $\text{aspect ratio} = \frac{\text{Major axis}}{\text{Minor axis}}$. Solidity is defined by $\text{solidity} = \frac{\text{Area}}{\text{Convex hull area}}$, where a cell that is perfectly circular would have a value of 1.0 and a cell that has protrusions and/or indentations

would have a value closer to 0. We refer to the absolute value of the rate of change of morphology – such as the rate of change in circularity – as “morphodynamic speed”.

Protrusions were derived from the binarized image stacks via morphological operations. Each image was first downsized by a factor of 2 in each dimension for the future morphological operations to be computationally feasible. Next, the image was morphologically eroded and dilated with a structuring disk of size 30 pixels in order to remove the protrusions from the cell body. Finally, the binarized protrusion image was found by upsizing the morphologically altered image and subtracting it from the original image.

3.2.5. Optical flow and optical flow alignment

First, the actin fluorescence maximum intensity projections were jitter corrected using phase cross-correlation [255,256]. Actin optical flow was found by applying iterative Lucas-Kanade [257] successively to each 2-frame combination of images in every fluorescence video. Optical flow confidence is calculated using the spatial Hessian matrix [258] in order to only account for the optical flow vectors that accurately track actin. The alignment metric was calculated by finding the dot product of each optical flow vector with its surrounding neighbors [259]. To compute this effectively, the optical flow matrix was convolved with a 2D $N \times N$ gaussian kernel A with elements $A_{ij} = \frac{1}{2\pi\sigma^2} e^{-(j-N/2)^2/(2\sigma^2)} e^{-(i-N/2)^2/(2\sigma^2)}$ with the center $(i,j=N/2)$ set manually to 0. Then, the optical flow alignment was calculated by taking the element-wise inner product of the convolution with the original optical flow matrix. In this study we use $\sigma = 1, N = 13$ in order to capture longer range alignment effects, however the results do not change drastically for values of N between 5 and 31. The protrusion optical flow alignment

(protrusion OF alignment) was found by calculating the average value of the optical flow alignment inside of the protrusion binary image. Previous studies have used different metrics for finding areas of high actin activity that account only for actin fluorescence strength [260], however that does not account for the movement of actin within the cell.

3.2.6. Statistics

Statistical calculations were performed in GraphPad Prism version 10.2 (GraphPad software, CA, US). For comparisons between vehicle control and E2 groups, unpaired Student's *t*-tests were performed. For all tests, P values < 0.05 were considered statistically significant. Data are represented as mean $\pm$ standard error of the mean. Experiments were performed in at least triplicate.

3.3 RESULTS

3.3.1. 3D profiling of cell shape dynamics with lattice lightsheet microscopy

We captured cell shape changes, membrane dynamics, and cytoskeleton organization over time using lattice lightsheet microscopy. 12Z cells were cultured, trypsinized, nucleofected with the LifeAct-GFP plasmid for F-actin, and imaged with lattice lightsheet microscopy (Fig. 7a). Nucleofection is an electroporation-based method in which an electrical field applied to the cells allows nucleic acids to enter the cytoplasm and nucleus. Cellular dynamics were then visualized with a lattice lightsheet time-lapse microscopy, which illuminates planes of the sample with long thin beams to produce a 3D image with high spatial resolution and minimal photobleaching [261]. To clarify whether the boundary actin dynamics visualized with the LifeAct-GFP probe coincided

with the cell membrane, a plasma membrane stain was also added to the cells. Maximum intensity projections of these two channels demonstrate that the boundary of the LifeAct-GFP-expressing cell has a strong overlap with the plasma membrane (Fig. 7b), indicating that the actin cortex covers the whole membrane. We caution that the LifeAct-GFP label would not detect actin cortex-free protrusions, such as initial stages of blebs [262]. An example time series of maximum intensity projections from lattice lightsheet imaging of a LifeAct-GFP-expressing 12Z cell with many active protrusions is shown (Fig. 7c).

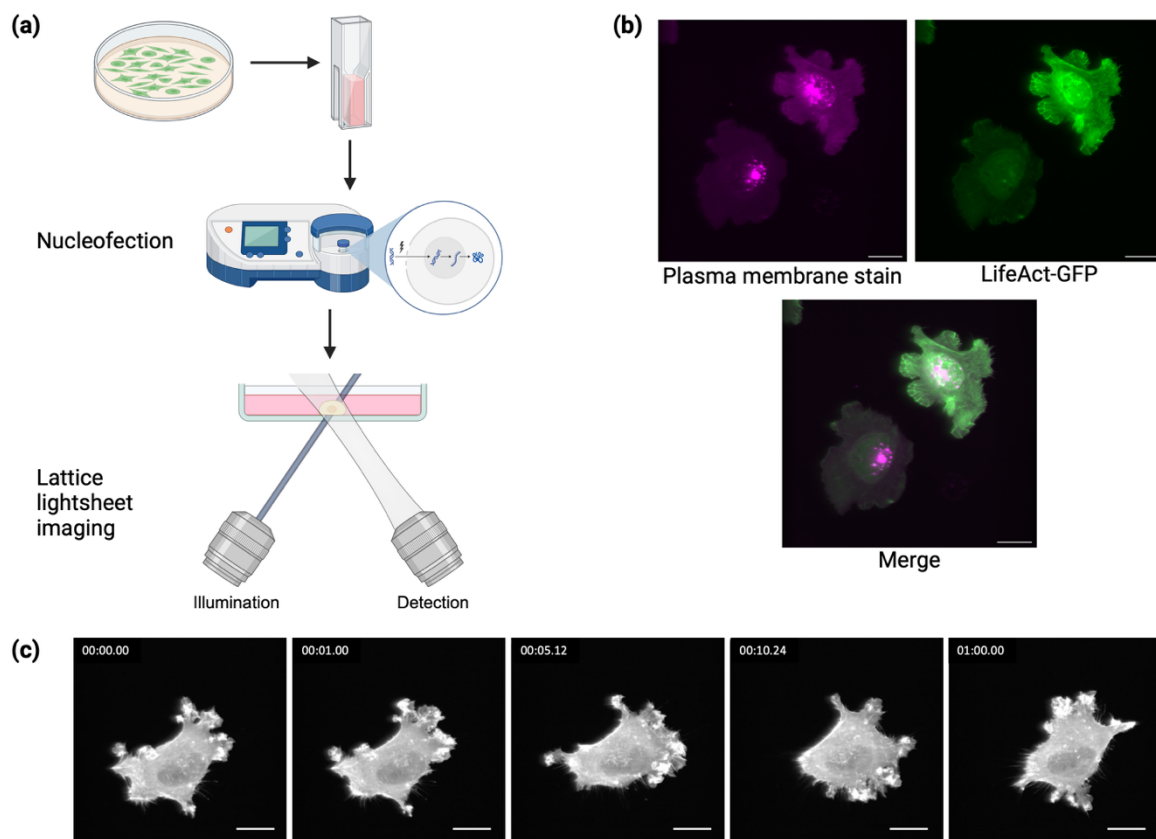


Figure 7: Experimental setup. **(a)** 12Z cells were transfected with a LifeAct-GFP plasmid via nucleofection (an electroporation-based method) and imaged over time using volumetric lattice lightsheet microscopy, which employs long thin beams to illuminate the sample with subcellular resolution. **(b)** A maximum intensity projection showing that the boundary of the actin

cytoskeleton (LifeAct-GFP) coincides with the plasma membrane. **(c)** An example output maximum intensity projection from a lattice lightsheet microscopy time-lapse of a cell transfected with LifeAct-GFP with frames from $t = 0, 1 \text{ min}, 5 \text{ min}, 10 \text{ min},$ and 60 min . Scalebars = $20 \text{ }\mu\text{m}$.

We captured several notable features of the LifeAct-GFP-expressing 12Z cells, indicated with white arrows, at high spatial resolution in 3D from lattice lightsheet microscopy that are visible from isosurfaces (Fig. 8). For example, some cells exhibited actin waves pushing against the membrane at the leading edge of the cell, with hair-like protrusions at the trailing edge of the cell (Fig. 8a). Several cells exhibited 3D membrane ruffles (Fig. 8b and e), filopodia (Fig. 8c and d, Fig. 8f and g), lamellipodia (Fig. 8e and f), and invadopodia-like protrusions (Fig. 8d and g). Time series isosurfaces of 12Z cells show membrane ruffle dynamics (Fig. 8e), lamellipodia growth and retraction (Fig. 8e and f), and protrusion growth and retraction (Fig. 8f and g). Videos featuring these membrane structures in 2D and 3D can be found in Appendix A: Supplementary Material.

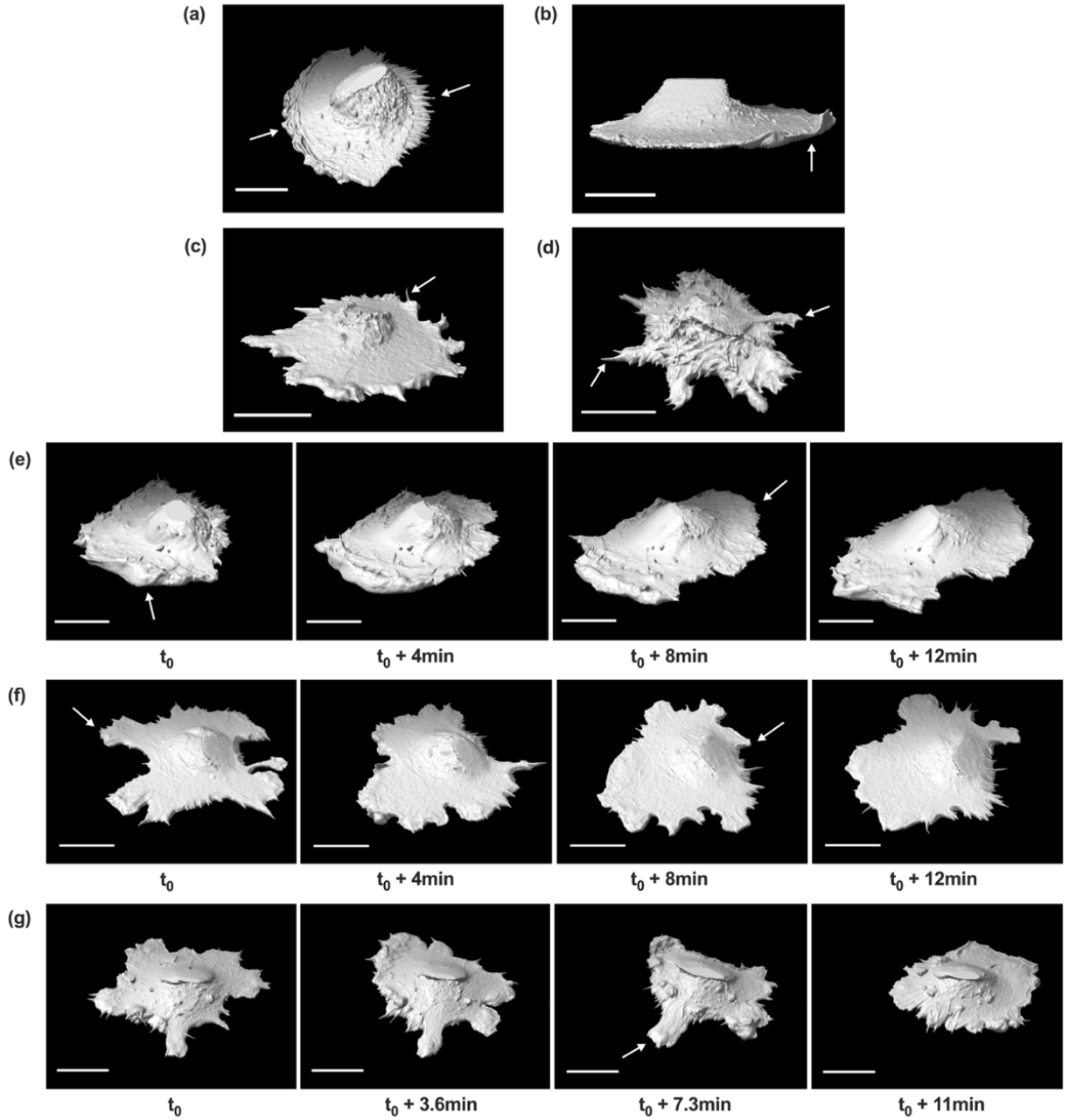


Figure 8: 3D isosurfaces of 12Z cells obtained from lattice lightsheet imaging, with some notable cellular features **(a-d)** and representative time series **(e-g)**. **(a)** Some cells exhibited actin waves at the leading edge of the cell and hair-like protrusions at the lagging edge of the cell (indicated by white arrows). Several cells also exhibited membrane ruffling **(b)**, 3D filopodial extensions **(c, d)**, and active protrusions **(d)**. The dynamics of the membrane ruffling and lamellipodia growth were

captured (e). Protrusion retraction (f, g) and formation (f) were also visualized. All scalebars = 20 μm .

3.3.2. E2 treatment alters 12Z cell shape and morphodynamics

To study 12Z morphology and shape dynamics in response to E2 treatment, we analyzed maximum intensity projections of cells transfected with LifeAct-GFP as acquired from lattice lightsheet time-lapse imaging. Cells were treated with vehicle and E2 treatments for 15 minutes (to visualize short-term effects) or 24 hours (to visualize longer-term hormonal effects) and morphological parameters and morphodynamic speeds were averaged over the duration of each experiment (Fig. 9). Cells treated for 15 minutes did not exhibit any significant E2-induced changes in circularity or solidity (Fig. 9a and c, respectively) but did experience more rapid changes in circularity and solidity upon E2 treatment (Fig. 9b and d, respectively). Of the cells treated for 24 hours, E2 treatment resulted in significantly decreased cell circularity (Fig. 9a), solidity (Fig. 9c), and rate of change of circularity (Fig. 9b) compared to the vehicle control. 24-hr E2 treatment did not significantly alter the rate of change in solidity but a slight downward trend persisted (Fig. 9d). In summary, over a short-term period, E2 treatment did not alter cell morphology but did induce more rapid shape fluctuations; over a long-term period, E2 induced clear morphological changes and reduced fluctuations in shape over time (Fig. 9).

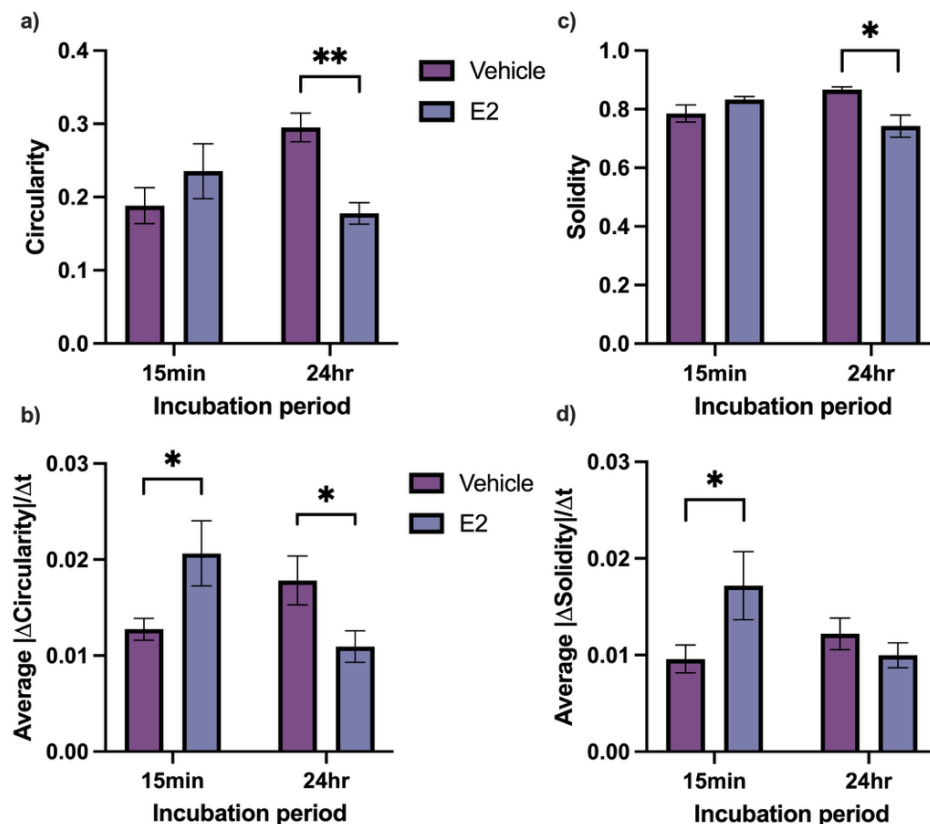


Figure 9: Effect of E2 on 12Z shape and morphodynamics after 15 minutes (N = 7 cells per group) or 24 hours (N = 5 cells per group) of treatment incubation. Circularity (a), rate of change in circularity (b), solidity (c), and rate of change in solidity (d) over the duration of each time-lapse were extracted from binarized maximum intensity projections, and the means $\pm$ SEM are plotted. * $P \leq 0.05$, ** $P \leq 0.01$.

3.3.3. E2 treatment increases membrane protrusions in 12Z cells after 24 hours

To study the effect of E2 on 12Z membrane protrusions, protrusion area and cell area were quantified from fluorescence maximum intensity projections. We automatically segmented the protrusions using morphological operations (see Methods) and two broad categories of cell protrusion dynamics were seen. The first category observed was invadopodia-like dynamics (Fig. 10a) which are characterized by large, non-uniform protrusions with no front of the cell clearly

demarcated. Invadopodia are commonly observed in cancer cells as a means for metastasis and migration [249], however here we use the term due to visual similarities between the protrusions in our cells and true invadopodia. The other category observed was lamellipodia-like protrusions (Fig. 10b), which we classify as cells having broad, flat protrusions focused mainly at the front of the cell. These protrusions tend to be much smaller than the size of the cell and are largely present in unidirectional guidance [263].

To determine how E2 treatment affected protrusion dynamics we found the protrusion area for all cells. E2 treated cells have no change in protrusion area compared to vehicle control cells after 15 minutes (Fig. 10c, left panel), but significantly larger protrusions than vehicle control cells after 24 hours of treatment (Fig. 10c, right panel). We accounted for variability in cell size by looking at the ratio of protrusion area to cell body area. Figure 10d shows that after 15 minutes of E2 treatment (left panel) the ratio of protrusion area:cell area does not change compared to the vehicle control cells. Again, this difference is significant for the cells treated for 24 hours (Fig. 10d, left panel), indicating that the result holds across cell sizes.

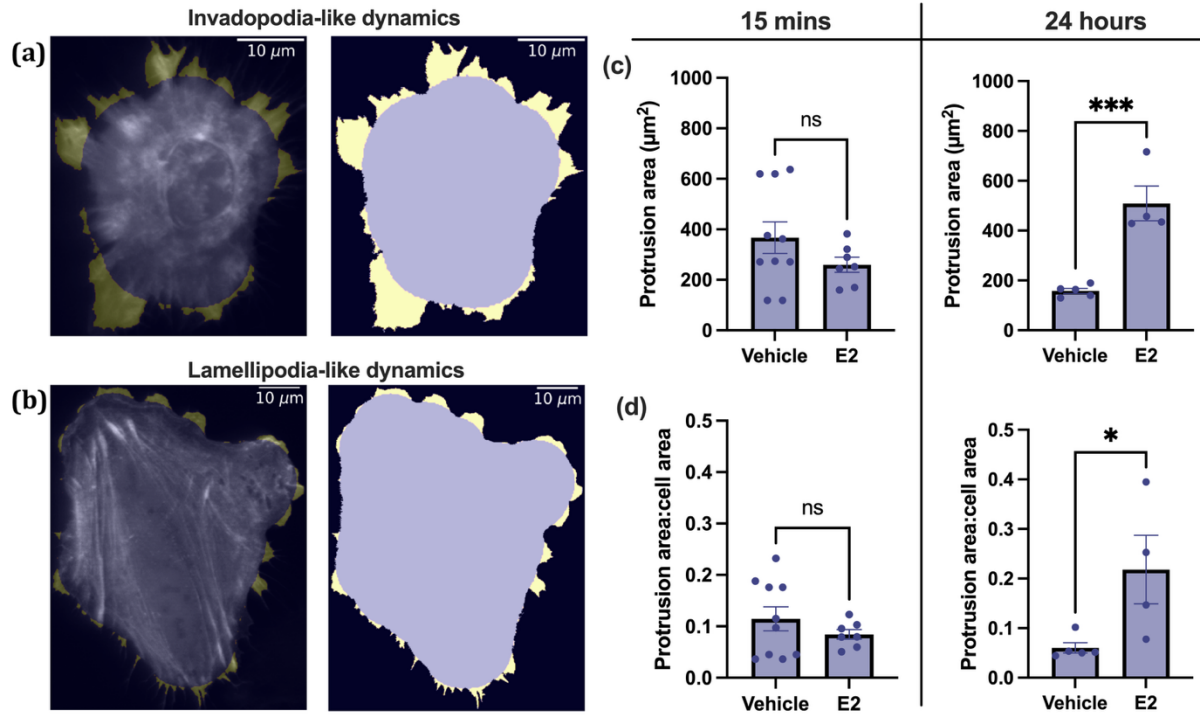


Figure 10: Effect of E2 on cellular protrusions. **(a)** A cell with invadopodia-like dynamics, which are characterized by large protrusions on all sides of the cell. The first image of the series shows cell protrusions in yellow overlaid onto the actin fluorescence image. The second image shows the binarized mask of the cell body (purple) and protrusions (yellow). **(b)** A cell with lamellipodia-like dynamics, which are characterized by broad, short protrusions. **(c)** Protrusion area in μm^2 and **(d)** protrusion area:cell area ratio were extracted from binarized images and the means $\pm$ SEM of individual cell data are plotted. $N = 5$ for Vehicle, $N = 4$ for E2. * $P \leq 0.05$, *** $P \leq 0.001$.

3.3.4. E2 treatment decreases actin optical flow alignment in 12Z cells after 24 hours

To this point we have focused mainly on cell morphodynamics; however, we have fluorescently tagged actin in the cell to investigate the impact of E2 treatment on the dynamic shift in the location of the F-actin scaffolding. Dynamics may be driven by a combination of polymerization, depolymerization, myosin contraction, or cytosolic flow. To quantify actin dynamics we use optical flow [259] to find the speed that the actin is moving within the cells. The optical flow is a 2D vector field (Fig. 11a) that gives the shift in location of the actin scaffolding

as well as an approximation of the speed with which it moves. Areas of the cell with higher actin optical flow have faster change in their scaffolding at that point. However, there was no significant difference in the actin optical flow distributions between the E2-treated cells and the vehicle control cells.

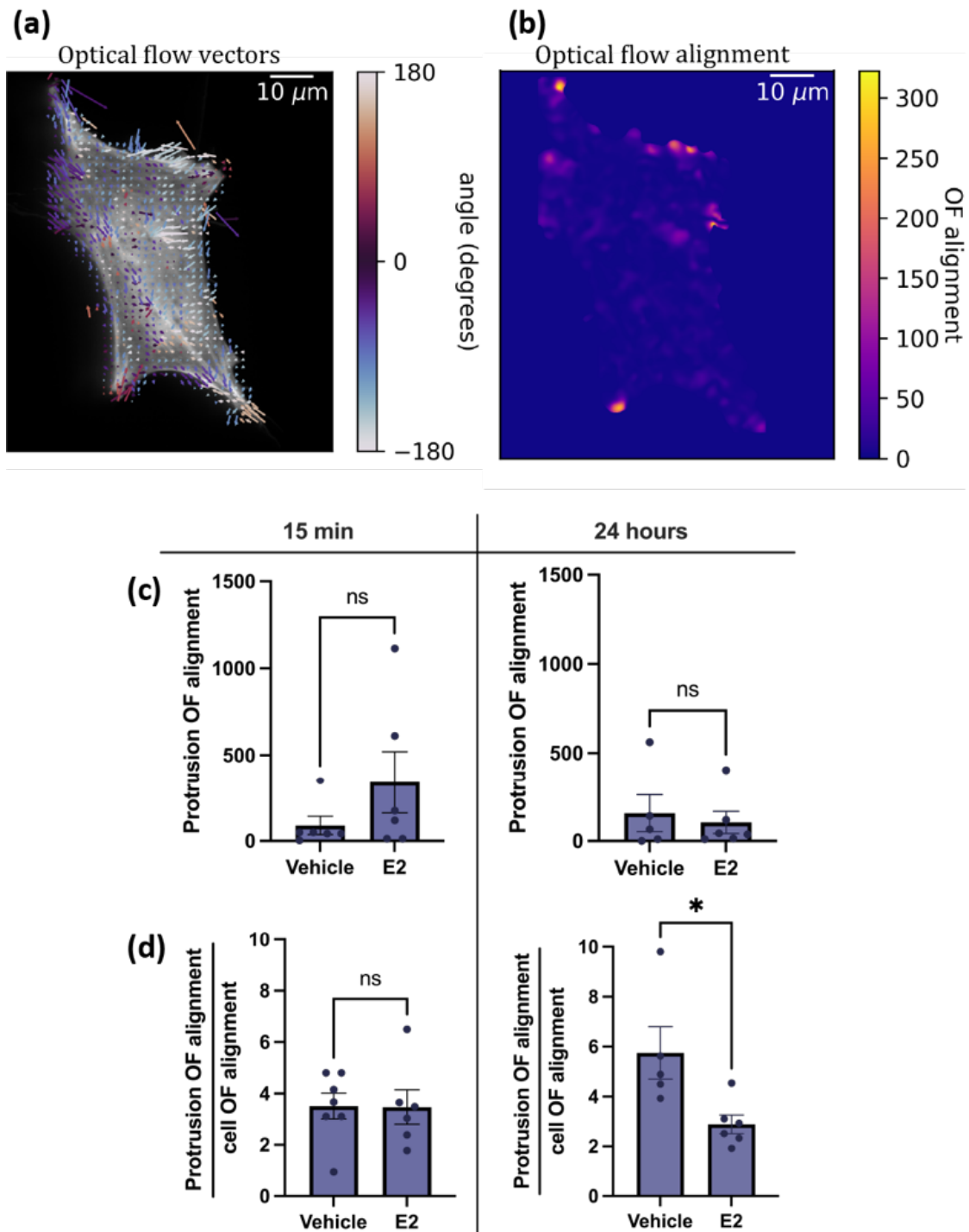


Figure 11: Impact of E2 on actin optical flow alignment in 12Z cells. **(a)** Optical flow (OF) was calculated from actin fluorescence for all timepoints to determine how the actin is moving within the video. The optical flow vectors are displayed on top of one of the corresponding actin

fluorescence images. **(b)** The actin optical flow alignment (OF alignment) is displayed for the cell in **(a)**. Higher values of optical flow alignment mean that the optical flow in that region is pointing in the same direction (see Methods). Using the binarized masks from Fig. 5 the average optical flow alignment was found inside the protrusions and the cell body. The mean protrusion optical flow alignment **(c)** and mean ratio of protrusion optical flow alignment to cell body optical flow alignment **(d)** are plotted $\pm$ SEM, representing individual cell data (N = 5-7 cells per group). * $P \leq 0.05$, ** $P \leq 0.01$.

In order to quantify how the actin was moving collectively within the cell, we calculated the alignment of the optical flow vectors (Fig. 11b). Regions of high alignment indicate regions where coordinated shifts in the cellular scaffolding occur, for example, a wave of actin polymerization that travels in one direction. The optical flow alignment was calculated by taking the spatial average of the dot product of each optical flow vector with its neighbors (see Methods). At any given point, higher alignment means the optical flow vectors around that point have similar directions. Lower values of alignment either mean that the optical flow was very small in that region or that the vectors are unaligned. Since coordinated shifts in the scaffolding are needed to generate protrusions, we expected the alignment to be higher in protrusions than in the cell body. Therefore, we calculated the average alignment in both the cell body and the protrusions separately.

Figure 11c shows no significant difference in the average protrusion optical flow alignment; the protrusions have similar levels of actin alignment in both treatments and incubation times. However, if we consider the ratio of protrusion optical flow alignment:cell body optical flow alignment we see that the ratio is significantly lower for the E2-treated cells compared to the vehicle control in the 24-hour group, but not the 15-minute group (Figure 11d). This means that the E2 treatment, after 24 hours, makes the cell's actin significantly less aligned in the protrusions

compared to the bulk of the cell. With the E2 treatment, the actin in the protrusions becomes more disordered compared to the actin in the bulk.

3.4 DISCUSSION

We used lattice lightsheet microscopy to capture high-resolution cell dynamics, enabling an unprecedented visualization of protrusive activity and actin polymerization dynamics in live endometriotic cells. We showed that this technique can be used to visualize a variety of 3D cytoskeletal structures and fibers, including those associated with ruffles, filopodia, lamellipodia, and invadopodia-like protrusions over time, aligning with previous work [264,265].

This study aimed to elucidate the effect of E2 on epithelial actin and shape dynamics in the context of endometriosis and dysregulated cell invasiveness. We demonstrated that 24-hour E2 treatment significantly reduced average cell circularity and solidity compared to the vehicle control — changes indicative of a transition to a more invasive phenotype as cells adopt a more elongated, protrusive shape. In the short term (15 minutes), E2 did not alter cell morphology but did induce more rapid morphodynamic changes. This contrasts with 24-hour E2 treatment, which resulted in slower morphodynamic changes. These results suggest that E2 may stimulate rapid, transient fluctuations in the short-term—potentially due to rapid, non-classical estrogen signaling pathways—and cause a more stable but invasive morphological state in the long-term—a time scale more on par with classical estrogen signaling that directly regulates transcription [266].

In line with the results in Figure 9, detailed shape analysis over time revealed that although E2-treated cells are highly morphodynamic after 15 minutes, clear, stable morphological shifts are only seen after 24 hours. After 24 hours, vehicle-treated cells generally exhibited larger

fluctuations in circularity compared to E2-treated cells, which settled into a more stable but invasive morphology. Given that E2 is generally known to promote cell invasiveness in endometriosis and reproductive cancers [6,27,239,267–269], this result matches our expectation. Future studies could clarify the mechanisms underlying E2-mediated morphodynamic states in the 12Zs; for example, investigating the distinct, time-dependent contributions of classical and non-classical estrogen signaling pathways to cytoskeletal remodeling would elucidate the impact of E2 on cell shape over time.

We discovered that 24-hour E2 treatment leads to different protrusion dynamics as well as modified actin dynamics within those protrusions compared to vehicle control cells. E2-treated cells generally displayed rapid invadopodia-like dynamics, with high protrusion areas and high ratio of protrusion area:cell body area. These higher area protrusions tended to jut out of the cell in every direction. Vehicle-treated cells tended to display fewer protrusions that were much shorter, lamellipodia-like protrusions with directionality towards the leading edge. Large protrusions in non-polarized cells tend to be a marker of invasive cells [270,271]. This suggests that the E2-treated cells are primed to be more migratory than the vehicle control cells, because they are forming larger, non-uniform protrusions that are reminiscent of invadopodia. This effect appears only after 24 hours of treatment, not after 15 minutes, suggesting that E2-induced invadopodia-like protrusions are not formed as a result of rapid signaling. Classical estrogen signaling pathways affect cells over hours to days and may converge with EGF or TGF- β signaling in the nucleus to initiate invadopodia. Several studies demonstrate E2-induced invasiveness in endometriotic epithelial cells [6,27,28,272], but future work could investigate the expression of invadopodial proteins—such as cortactin or MT1-MMP—in 12Z protrusions and determine

whether E2 treatment enhances their proteolytic activity. This would clarify mechanisms driving E2-mediated invasion in endometriosis.

Within the membrane protrusions, the actin behaved differently in E2-treated cells. After 24 hours, the ratio of protrusion actin alignment:cell body actin alignment was significantly higher in vehicle-treated cells than E2-treated cells, indicating that the actin was more well-aligned in the vehicle control. When E2 was added to the cells, it appeared to make the actin in the protrusions more disordered. This suggests that actin polymerization is less coordinated in the E2-treated cells, which would be beneficial in sensing non-uniform local topography and maintaining shape flexibility. This exploratory, protrusive state could support cell invasion in complex 3D environments where an adaptable cell shape is advantageous, such as peritoneal mesothelial barriers and deep into organ walls. Prior studies show that estradiol activates both RhoA and p21-activated kinase (PAK) [242], which are essential regulators for the formation of lamellipodia and pseudopodia [273,274]. Our results suggest that this change in RhoA and PAK may result in more disordered actin polymerization over a 24-hour time scale.

Our work shows that E2 affects cells most notably after 24 hours of treatment rather than shorter 15-minute timescales. Given that estrogen production occurs continuously within the pelvic cavity of endometriosis patients—not in short doses—we hypothesize that cell cytoskeletal dynamics may be stably intensified in endometriosis, with peaks in actin rearrangement occurring during the estrogen-driven proliferative phase of the menstrual cycle.

Some limitations of this study should be noted. We chose to utilize LifeAct-GFP, a widely used F-actin probe, in our experiments due to its stable signal and low phototoxicity. However, LifeAct-GFP has been previously shown to influence actin dynamics in other cell types [275–277]

and has been unable to stain actin-based filopodia within chick embryo mesenchymal cells [278]. LifeAct-GFP also has a relatively high affinity to G-actin [279] which generates background fluorescence. Although our imaging validates LifeAct-GFP localization with plasma membrane staining, other methods of fluorescently labeling F-actin should be tested and compared with our results. SPY555-actin could be a good alternative which does not require cell transfection but may be more toxic to cells and disrupt actin dynamics at high doses. Additionally, many cells that were imaged experienced a reduction in cell area over time, especially after about one hour of imaging, which could be due to some degree of photosensitivity or sensitivity to electroporation. Future studies that image other cell types using lattice lightsheet microscopy will provide more context on the relative photosensitivity of the 12Z cells. Finally, we were limited in our ability to quantify the 3D dynamics of actin due to computational constraints. However, we demonstrated that the maximum intensity projection of the 3D data can be effectively analyzed for morphodynamics and actin dynamics. Future advances in computational analysis pipelines and hardware will allow us to gain further insight on cytoskeletal remodeling in 3D.

3.5 CONCLUSION

Together, these results suggest that E2 modulates actin polymerization and membrane protrusion dynamics in endometriotic epithelial cells and may prime them for cell invasion. These insights lay the groundwork for future studies exploring the molecular signaling pathways and mechanical feedback loops that underlie hormone-driven motility in endometriosis.

Although this chapter highlights a potential mechanism by which E2 promotes dynamic cell behaviors in endometriosis—namely, enhanced protrusiveness and structural rearrangements—

—we still questioned whether the 12Z cell line was able to recapitulate functional migration induced by E2 in primary cells. The 12Z cell line is the most widely used cell line in endometriosis research; however, cell lines need to be thoroughly characterized and validated to ensure they reflect key disease characteristics. The responsiveness of the 12Z cell line to E2 was not previously well characterized. In Chapter 4, we sought to determine whether the E2-mediated protrusiveness and actin dynamics translated to functional increases in various types of 12Z cell migration.

Chapter 4: Selective estradiol sensitivity in 12Z human endometriotic epithelial cell line

4.1 INTRODUCTION

Endometriosis is an estrogen-dependent disease: estradiol (E2), a potent estrogen, is locally synthesized *de novo* by endometriotic tissues, leading to an excessive accumulation that exacerbates disease progression [95,280]. E2 binds with estrogen receptors (ERs) located in the nucleus and on the plasma membrane to directly and indirectly regulate gene expression. This process impacts downstream cellular activities, such as cell proliferation, morphology, differentiation, migration, and invasion [88]. A deeper understanding of these processes as they contribute to endometriosis pathogenesis requires more widespread research and easier access to *in vitro* cell culture models.

Endometriosis research currently relies predominantly on primary cells. Primary cells have excellent retention of key physiological characteristics, but they are difficult to obtain: primary endometriotic cells are not widely available commercially, and most often, groups that use them have a collaborator that has access to patient tissue samples. Finding clinics that routinely perform laparoscopic surgeries for endometriosis and are willing to share patient samples remains a challenge. When tissue samples are obtained, primary stromal cells are frequently isolated, but according to unpublished observations from researchers at the Feinstein Institutes for Medical

Research (New York, US), epithelial cells are harder to isolate and culture. Primary cells are also not ideal for high-throughput or longer-term experiments due to passage number limitations.

The 12Z human endometriotic epithelial cell line has recently become widely accepted by the field as a useful model of endometriosis, as they have been shown to share phenotypic and molecular similarities with primary cells [237,238]. In our experience, they are suitable for use in high-throughput experiments and are easy to culture. While this advance is advantageous, the physiological relevance of cell lines can be limited by the loss of primary cell traits. The 12Z cell line expresses N-cadherin, P-450 aromatase, MMPs, ER α , and ER β and lacks E-cadherin, matching characteristics of primary endometriotic cells [237,238]. The 12Zs have also demonstrated invasive potential [222,237,238], responsiveness to TGF- β [64], responsiveness to TNF- α in monolayer culture [221], secretion of inflammatory factors [221,222,281], sensitivity to growth hormone-releasing hormone agonists [282], resemblance to uterosacral ligament endometriosis lesions [222], and E2 synthesis [238]. But to our knowledge, no prior investigations have characterized 12Z sensitivity to E2. One study treated 12Z cells with E2: heterogeneous spheroids composed of 12Zs and stromal cells were treated with E2 and demonstrated increased invasion into Matrigel with human peritoneal mesothelial cells (LP9 cell line) embedded within [234]. But it is unclear whether the 12Zs, stromal cells, or both were hormone responsive, especially as the stromal cells were always present at the leading edge of the spheroids [234].

Given that the role of E2 in enhancing endometriotic cell invasiveness and endometriosis progression is so profound, the 12Zs should mirror a similar level of E2 sensitivity as primary cells: approximately a 1.5- to 2.5-fold increase in migration and a 2-fold increase in invasion upon E2 treatment [6,27,28,45,46]. In this chapter, we tested and characterized the E2 sensitivity of the

12Z cell line in a variety of proliferation and migration models. We found 12Zs exhibit selective responsiveness to E2, with altered behaviors in confined migration models but no changes seen in unconfined proliferation and migration systems. Moreover, increasing levels of the gene encoding ER α , a major mediator of estrogenic action [283], did not enhance 12Z responsiveness to E2. We used RNA sequencing to further inform these differences in E2 sensitivity. Given the insufficient responsiveness to E2, we question whether the 12Z cell line is appropriate for use in studying estrogen-dependent endometriotic processes *in vitro*.

4.2 METHODS

4.2.1. Cell culture and treatments

The 12Z cell line was purchased from Applied Biological Materials (Cat. no. T0764). Cells were cultured in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). About 24-48 hr prior to experiments, the media was replaced with phenol-free DMEM/F-12 (Gibco) supplemented with 10% charcoal-stripped FBS (Gibco) and 1% penicillin-streptomycin (Gibco). Passages 55–75 were used for experiments. A modified 12Z cell line with an upregulation of *ESR1* (*ESR1*-12Z) was generously gifted by Dr. Fazleabas's lab and cultured in phenol-free DMEM/F-12 supplemented with 10% charcoal-stripped FBS and 1% penicillin-streptomycin. Passages 6–14 were used for experiments. After recovering from thawing, cell lines were cultured for approximately two weeks at a time, on average. 17 β -estradiol (E2) was purchased from Sigma Aldrich (Cat. # E8875-1G) and dissolved in 100% ethanol to create a stock solution. Another vial of E2 was purchased from MP Biomedicals (Cat. no. 02194565-CF) and dissolved in 100% ethanol to create a stock solution (denoted as MPB

E2). Cells were treated with media containing a vehicle (0.001% ethanol), 10 nM E2, or 10 nM MPB E2, as well as other treatments as specified in the Results section for the dose-dependent experiments. Unless specified as MPB E2, E2 refers to the batch sourced from Sigma Aldrich.

4.2.2. Proliferation and wound healing assays

To quantify cellular proliferation, a Cell Counting Kit-8 assay was employed. Briefly, 10,000 cells were seeded per well in a 96-well plate and cultured for 48 hr with treatments. These include the mitotic inhibitor, mitomycin C (MMC, Sigma Aldrich, cat. #M5353) which was used as a positive control at a concentration of 10 $\mu\text{g/mL}$. 10 μL of CCK-8 solution was then added to each well and incubated for 1-1.5 hr at 37°C. Absorbance measurements were taken in a microplate reader at 450 nm. Plotted absorbance values were normalized to the vehicle group as specified in each figure. To measure collective cell migration, scratch wound healing assays were performed. Approximately 90,000 cells were seeded per well in a 24-well plate coated with 10 $\mu\text{g/mL}$ collagen I from rat tail (Corning, cat. # 354249). Cells were incubated for 48 hr with treatments as they grew into confluent monolayers. Monolayers were manually scratched with a p-200 pipette tip and rinsed with phosphate-buffered saline (PBS) before replacing media with treatments. Wounds were imaged under phase contrast at 10X one hour post-scratch (Initial wound area) and 7.25 hr post-scratch (Final wound area). Wound closure was calculated as (Initial wound area – Final wound area) / Initial wound area, with wound areas determined using ImageJ (National Institutes of Health, Bethesda, MD, USA).

4.2.3. Quantification of cellular morphology

Morphological parameters including cell area, perimeter, solidity, and roundness were extracted using ImageJ to manually trace cells from phase contrast microscope images. Solidity was defined as $solidity = \frac{Cell\ area}{Convex\ hull\ area}$, where a cell that is perfectly circular would have a value of 1.0 and a cell that has protrusions and/or indentations would have a value closer to 0. Roundness was defined as $roundness = \frac{1}{Aspect\ ratio} = \frac{Minor\ axis}{Major\ axis}$, where a highly elongated cell would have a value close to 0.

4.2.4. Single cell random migration analysis

The random migration of single cells was analyzed from time-lapse phase contrast images. Briefly, approximately 5,000 cells were seeded per well in a 24-well plate coated with 10 µg/mL rat tail collagen I, except for the positive control, where the wells were coated with 20 µg/mL poly-D lysine (PDL) to prevent integrin-based cell attachment. Cells were incubated with treatments for 48 hr and then imaged under phase contrast at 10X for 12 hr, with a time step of 10 min. Individual cell trajectories were tracked using the Manual Tracking plugin in ImageJ by clicking on the estimated geometric center of the cell at each time point. Subsequent analysis was performed in MATLAB. Average speed was defined as the cell's total path length divided by the duration of the time-lapse. Directional persistence was defined as the cell's end-to-end displacement divided by its total path length, where a cell that progresses in a perfectly straight line from point A to point B would have a directional persistence of 1; a cell that originates at a point A, moves in a straight line to point B, and directly returns to point A would have a directional persistence of 0. Mean-square displacement (MSD), which is a proxy for how much area (in µm²) a cell covered

during its trajectory, is defined as $MSD = \langle r^2 \rangle = 4D\Delta t$, where Δt is the number of time steps ($\Delta t = 1$ represents one step, or 10 min; $\Delta t = 2$ represents two time steps, or 20 min, etc.) and D represents the diffusivity ($\mu\text{m}^2/\text{time step}$). Diffusivity was derived from the slope m of the linear portion of the MSD curve, where $m = 4D$ and $D = \frac{\langle r^2 \rangle}{4\Delta t}$.

4.2.5. Transwell migration assay

Cell transmigration through a semi-permeable membrane was performed using a transwell migration assay. Transwell inserts for a 24-well plate were coated with 10 $\mu\text{g}/\text{mL}$ collagen I from rat tail. 12Z cells were treated with vehicle or E2 for 48 hr prior to placement in the transwells. 100,000 cells were seeded in the upper chamber in serum-free media containing treatments, with the lower chamber containing media supplemented with 10% FBS. Cells were allowed to transmigrate for 24 hr prior to quantification. Then, media was aspirated from the upper chambers, the top of the transwell inserts were swabbed gently, and the inserts were stained with 5 μM calcein AM for 30 min. Transmigrated cells were imaged under fluorescence. Data are normalized to the vehicle.

4.2.6. Cell migration through confined microfluidic channels and analysis

To analyze 12Z migration in narrow spaces, cells were sent through microfluidic devices containing narrow channels (schematic in Fig. 14C). As previously defined [284], microfluidic devices with confining microchannels 3 μm wide x 10 μm tall x 200 μm long were cast in polydimethylsiloxane (PDMS, Dow) using a positive mold created by photolithography. The inlets and outlets of the PDMS device were made with a 4 mm biopsy punch. The devices were then

irreversibly adhered to a #1 glass coverslip via plasma treatment, followed by 5 min of pressure and an overnight bake at 80°C. One hour before their use, the devices were again plasma treated to create hydrophilic surfaces, and 0.1 mg/mL type I collagen (cat. # C3867, Sigma Aldrich) in PBS was added to the inlets to functionalize the inner walls. Devices with collagen were incubated for 1 hour at room temperature. The devices were then added to a 4-well plate (07-000-207, Fisher Scientific) and UV-treated for 10 min to sterilize them before adding cells. Following initial cell splitting, cells were resuspended in serum-free media, counted, and centrifuged again. Cells were then resuspended to a concentration of 100,000 cells/20 μ L of serum-free media containing vehicle or E2 treatments. 20 μ L of this cell suspension was added to the microchannel cell inlet. After adding cells, the devices were placed in an incubator for 10 min to ensure cell adhesion to the microchannel walls. Afterwards, excess media and adhered cells were removed, and media was added to the remaining wells. Cell inlets and outlets, in addition to 2 media inlets and the media outlet, were filled with serum-free media. The final media inlet was filled with full media to induce a chemokine gradient. Approximately ten regions of interest were selected for each device and imaged at 20X under phase contrast every 15 min for 16 hours.

Individual cell trajectories while in the microchannels were tracked using the Manual Tracking PlugIn in ImageJ by clicking on the nucleus of the cell at each time point. Subsequent analysis was performed in MATLAB. Speed was defined as the cell's total path length divided by the duration of the time-lapse. Data represent speed normalized to the vehicle. Directional persistence was defined as the cell's end-to-end displacement divided by its total path length, where a cell that progresses in a perfectly straight line from point A to point B would have a

directional persistence of 1; a cell that originates at a point A, moves in a straight line to point B, and directly returns to point A would have a directional persistence of 0.

4.2.7. Spheroid outgrowth assay

A spheroid outgrowth experiment was performed to quantify the expansion of 3D cell spheroids on a collagen I gel. 4,000 12Z cells were seeded per well in a round-bottom 96-well ultra-low attachment plate and centrifuged at 150xg for 2 min. Spheroids were harvested at 48 hr and seeded on top of collagen I gels. Phase contrast images were taken at time of seeding (Day 0) and 24 hr (Day 1). The projected area of each spheroid was measured in ImageJ; data represent the area on Day 1 relative to the initial area on Day 0.

4.2.8. Western blotting

Relative levels of estrogen receptor- α (ER α) were measured with western blotting to validate the overexpression of its encoding gene, *ESR1*, in the *ESR1*-12Zs. Cells were cultured for 48 hr and lysed with a protease cocktail inhibitor (1:100; Sigma Aldrich) and phenylmethylsulphonyl fluoride (1:500; Sigma Aldrich, cat. #P7626) in Pierce RIPA buffer (ThermoFisher Scientific). A BCA Protein Assay Kit (ThermoFisher Scientific) was used to determine protein concentration in the lysates. Samples were mixed 1:1 with a buffer containing 20:1 2X Laemmli to β -mercaptoethanol (Bio-Rad, cat. #1610737 and #1610710, respectively) and boiled. Equal amounts of protein were loaded into a Mini-PROTEAN TGX 4-20% precast gel (Bio-Rad) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples were then transferred to a PVDF membrane via the Trans-Blot Turbo machine (Bio-Rad) on the Mixed MW (Turbo) protocol setting (1.3A, up to 25V, 7 min). PVDF membranes were blocked in

an Intercept (PBS) blocking buffer (LI-COR) for one hour and incubated overnight at 4°C with a primary antibody solution consisting of anti-ER α (1:500; Santa Cruz Biotechnology) and anti- α -smooth muscle actin (1:500; Sigma Aldrich, cat. #A5228) as a control in a 1:1 Intercept blocking buffer to 0.1% Tween 20 in PBS mix (PBST). The next day, membranes were washed with PBST and incubated for one hour at RT with a secondary antibody solution consisting of IRDye 680RD Goat anti-Rabbit and Goat anti-Mouse (each at 1:10,000; LI-COR, cat. #926-68071 and #926-68070, respectively) in a 1:1 Intercept blocking buffer to PBST mix. Membranes were then washed with PBS, visualized on an Odyssey CLx imager, and analyzed with the Image Studio software (LI-COR).

4.2.9. RNA-sequencing and data analysis

Vehicle- and E2-treated 12Zs as well as non-treated 12Zs and non-treated *ESR1*-12Z samples were sent for RNA extraction, integrity analysis, library preparation, and sequencing (Novogene Co, Ltd.). Three independent samples for each cell group were pooled before shipping. Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers. Then the second strand cDNA was synthesized using dUTP, instead of dTTP. The directional library was ready after end repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, amplification, and purification. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. After library quality control, libraries were subjected to Illumina Novoseq 6000 sequencing (paired-end 150 bp) with data output of over 20 million read pairs per sample. Raw reads (fastq format) were processed through fastp software.

Clean reads were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data. Full RNA sequencing methods are provided in the Appendix A: Supplementary Material S1.

4.2.10. Microscopy

For all phase contrast images and transmigration fluorescence images, live cells were imaged using the 10X objective (unless otherwise specified) on an Olympus IX-83 inverted microscope (Olympus, Center Valley, PA, USA) and the Olympus cellSens software. Cells were maintained at 37°C, 65% humidity, and 5% CO₂ in an enclosed chamber surrounding the microscope.

4.2.11. Statistical analysis

GraphPad Prism 10 (La Jolla, CA, USA) was utilized to generate graphs and perform statistical analyses. Outliers were identified using the ROUT method with a Q (maximum desired false discovery rate) = 1% only to remove definite outliers. Data were tested for normality. For comparisons between vehicle and E2 groups, unpaired Student's *t*-tests were performed. For comparisons between three or more groups, ordinary one-way ANOVA with a Tukey's multiple comparisons test was performed. Corresponding non-parametric tests were used for non-normally distributed datasets. For all tests, P values < 0.05 were considered to be statistically significant. Data are represented as mean ± standard error of the mean. All experiments were performed in at least triplicates.

4.3 RESULTS

4.3.1. Determination of E2 incubation time and concentration for 12Zs

Multiple functional assays were employed to determine the optimal incubation time and concentration of E2 to apply to 12Z cells. To determine the most effective dose of E2, cells were treated with 10^{-9} M, 10^{-8} M, 10^{-7} M, or 10^{-6} M E2 for 48 hr before testing their migratory and proliferative response. Instead of including all the corresponding vehicle controls, only the lower and upper extremes were tested (0.0001% EtOH corresponding to 10^{-9} M E2 and 0.1% EtOH corresponding to 10^{-6} M E2). None of the E2 concentrations impacted wound closure compared to either of the vehicle controls (Fig. 12A). In a CCK-8 proliferation assay, 10^{-9} M and 10^{-8} M E2 treatments each led to a non-significant increase in absorbance compared to the 0.0001% and 0.1% EtOH control groups (Fig. 12B). This result, combined with support from the literature [2–4,6,12,27], led us to select 10^{-8} M (10 nM) as our concentration for all subsequent experiments involving E2. To determine the most effective incubation time with chosen treatments (0.001% EtOH vehicle control and 10 nM E2) for the 12Z cells, we analyzed cell morphology at the time of adding treatments ($t = 0$), 24 hr, 48 hr, and 72 hr from phase contrast images. Results show that E2 induces slight changes in cell morphology after 48-72 hr (Fig. 12C-G). Cell area (Fig. 12C) and perimeter (Fig. 12D) decreased at 72 and 48 hr, respectively. There were no changes in solidity across the incubation times (Fig. 12E) but a decrease in roundness at 48 hr (Fig. 12F). Representative images of 12Z cells treated for 48 hr are shown (Fig. 12G). Given the decrease in cell perimeter and roundness at 48 hr, along with support from literature [3,6,27,53], we chose 48 hr as the incubation time for all subsequent experiments involving E2.

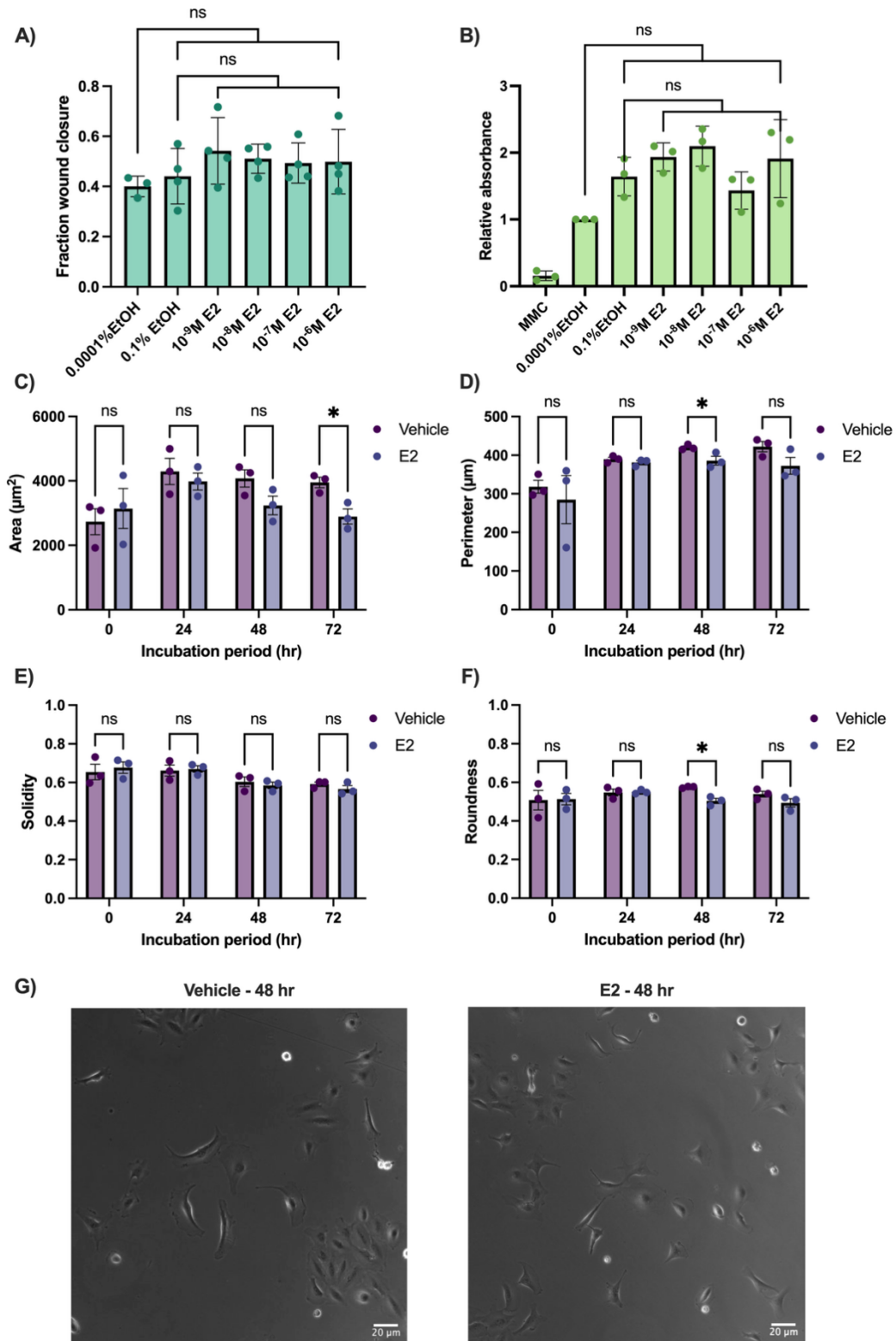


Figure 12: 12Z characterization and determination of E2 dose and incubation time. **A)** Cell migration in a wound healing assay and **B)** absorbance from a CCK-8 assay, reported relative to the 0.0001% EtOH group (with mitomycin C as a positive control), were quantified. From this point on, all subsequent experiments were performed using a concentration of 10 nM E2 and a corresponding 0.001% EtOH vehicle control. We then imaged cells treated with this vehicle control and 10 nM E2 for multiple incubation times and quantified cell morphology parameters including **C)** area, **D)** perimeter, **E)** solidity, and **F)** roundness. **G)** Representative images from the 48hr incubation period are shown. From this point on, 48 hr was selected as the treatment incubation period for all subsequent experiments. Scalebars = 20 μ m. Data points represent biological replicates, error bars represent standard error of the mean. * $P \leq 0.05$, ** $P \leq 0.01$. EtOH; ethanol. E2; estradiol. MMC; mitomycin C.

4.3.2. E2 does not affect 12Z proliferation, collective migration, or single-cell random migration

The responsiveness of 12Zs to 10 nM E2 treatment for 48 hr was assessed *in vitro* with proliferation and migration assays. E2 had no influence on 12Z proliferation in a CCK-8 assay, in which MMC was used as a positive control and absorbance values were plotted relative to the vehicle control group (Fig. 13A). E2 also had no impact on collective migration in a wound closure assay (Fig. 13B,C). We then assessed single-cell random migration in response to E2 (Fig. 13D-H) and used a poly-D lysine (PDL) coating to prevent integrin-based cell-substrate attachment as a positive control. Individual cell trajectories show that E2-treated cells covered slightly more area than vehicle treated cells (Fig. 13D). E2 did not impact cell speed (Fig. 13E), directional persistence (Fig. 13F), or diffusivity (Fig. 13G) compared to the vehicle control group. The mean-square displacement (MSD) graph shows that the E2-treated cells traversed the largest area, but not significantly more than the vehicle-treated cells (Fig. 13H). The 12Z cell line did not appear to be sensitive to E2 treatment in proliferation and 2D migration assays *in vitro*. In case our E2 sourced from Sigma Aldrich was defective, we decided to additionally test an alternative source

of E2 purchased from MP Biomedicals and denoted as MPB E2. However, MPB E2 had no effect on 12Z wound closure migration (Appendix A: Supplementary Material S2 Fig. 39A).

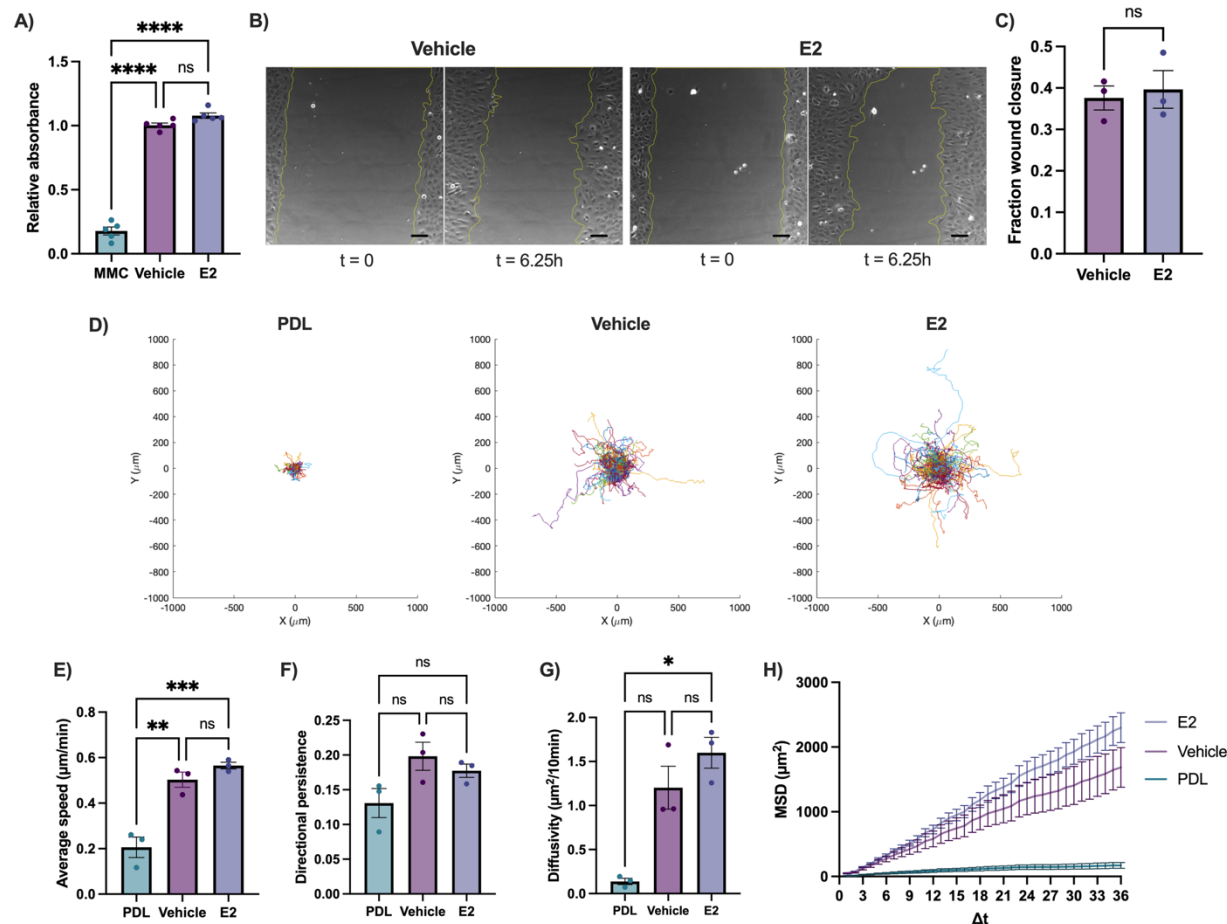


Figure 13: Effect of E2 on proliferative and migratory behaviors of 12Zs. 12Z cells were treated with 10 nM E2 for 48 hr and their functional behaviors were assessed. **A)** Proliferation was measured with a CCK-8 assay (with 10 μ g/mL MMC as a positive control), and absorbance values are plotted relative to the vehicle control group. **B)** Representative pictures of wound closure are shown from time of scratch at t = 0 and midway through the experiment at t = 6.25 hr for both vehicle and E2-treated 12Zs. **C)** Fraction wound closure is calculated. **D–H)** Random migration was measured via single-cell tracking and a poly-D lysine coating, which prevents integrin-based attachment, was used as the positive control. **D)** Single-cell trajectories are shown. **E)** Speed, **F)** directional persistence (defined as the cell's total displacement divided by the cell's total path length), **G)** diffusivity, and **H)** MSD over increasing time steps are plotted. Scalebars = 100 μ m. Data points represent biological replicates, error bars represent standard error of the mean. *P $\leq$

0.05, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. MMC; mitomycin C. E2; estradiol. PDL; poly-D lysine.

4.3.3. E2 differentially affects 12Z migration in confined and unconfined systems

The 12Z cell line showed a lack of sensitivity to E2 in proliferation and 2D migration assays, but cells are known to behave in a context-dependent manner: this could include differences between confined vs unconfined systems or between 2D vs 3D [221,285–287]. After treating 12Zs with 10 nM E2 for 48 hr, their migration in confined and unconfined systems was analyzed (Fig. 14). E2 significantly increased transmigration in a transwell assay, with representative images showing the bottom-facing surfaces of the 8 μm -pore transwell inserts (Fig. 14A). Transmigration was quantified relative to the vehicle control (Fig. 14B). The effect of E2 was consistent when we tested the alternative source of E2, MPB E2, on 12Z transmigration (Appendix A: Supplementary Material S2 Fig. 39B). In another confined migration model, we seeded 12Z cells in a previously defined PDMS microfluidic device containing narrow, 3- μm wide by 10- μm tall microchannels (Fig. 14C) [284]. E2 increased cell migration speed through the channels (Fig. 14D) but did not impact chemotactic index, defined as the end-to-end displacement divided by the total cell path length (Fig. 14E). To model unconfined 3D migration, we seeded 12Z spheroids onto collagen I gels (Day 0) and measured their outgrowth 24 hr later (Day 1) (Fig. 14F). E2-treated spheroids did not exhibit differences in outgrowth compared to vehicle-treated spheroids (Fig. 14G).

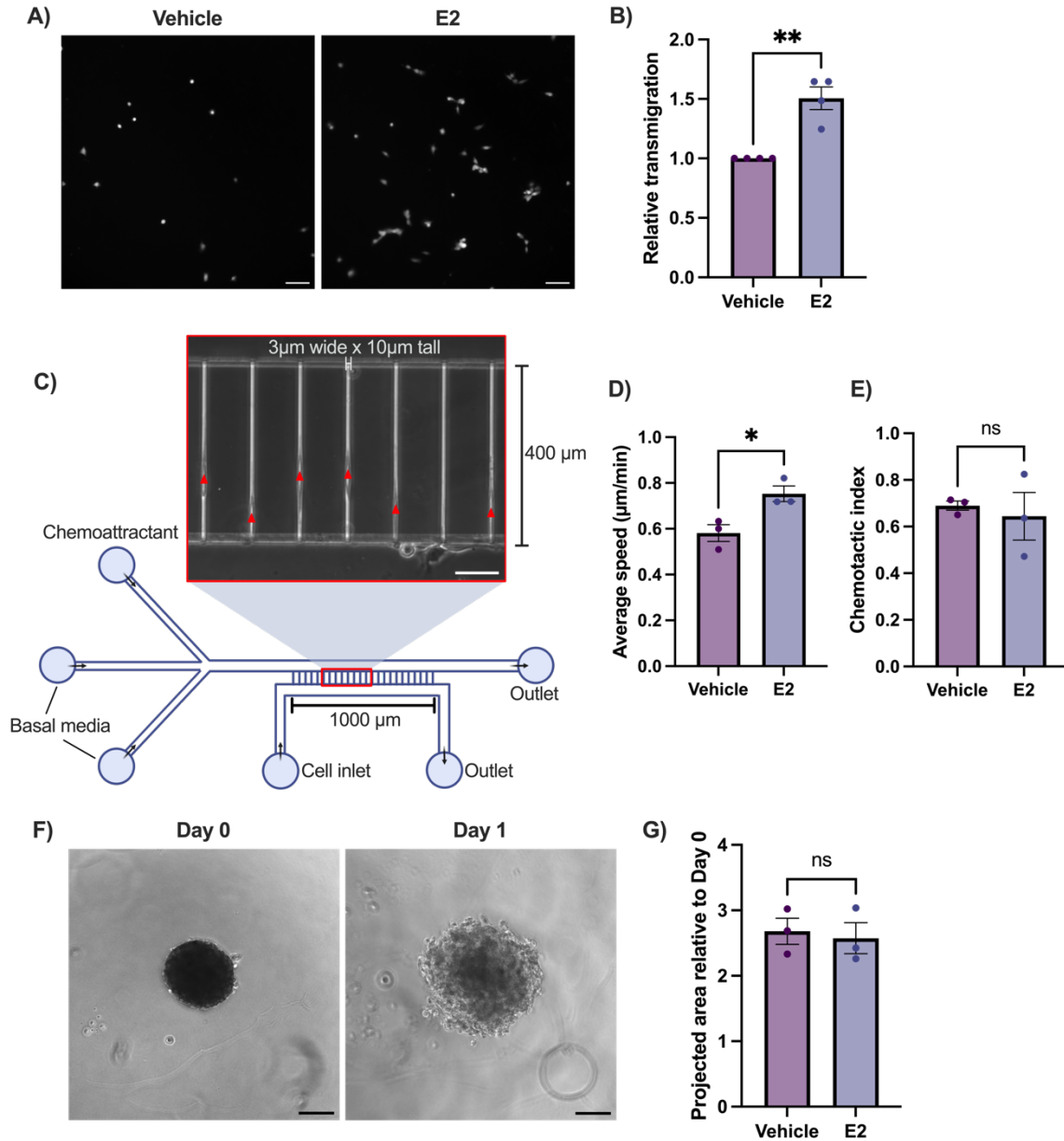


Figure 14: Impact of E2 on 12Zs in confined and unconfined systems. 12Z cells were treated with the vehicle or E2 for 48 hr prior to experiments. **A)** Transmigration in a transwell assay was measured and **B)** quantified relative to the vehicle control. **C-E)** Spontaneous migration of 12Zs through confining microchannels was observed. **C)** The design of the microfluidic device is depicted in the schematic which contains a representative image of cells moving through the microchannels towards the chemoattractant (media with 10% charcoal-stripped FBS); the cell nuclei are marked by red triangles. **D)** Cell speed and **E)** chemotactic index, defined as the end-to-

end displacement divided by the total cell path length, were quantified. **F)** 3D 12Z spheroids were grown and seeded on collagen I gels (Day 0) and their projected area of outgrowth was measured the next day (Day 1). **G)** Projected area is calculated relative to Day 0. Scalebars = 100 μm in panels A and F and 50 μm in panel C. Data points represent biological replicates, error bars represent standard error of the mean. * $P \leq 0.05$, ** $P \leq 0.01$. E2; estradiol.

4.3.4. Upregulation of *ESR1* does not induce functional E2 sensitivity in the 12Zs

Our results show that the 12Z cell line is unresponsive to E2 treatment in most standard *in vitro* models of proliferation and unconfined migration. Estrogenic action is thought to be predominantly mediated by *ESR1*, the gene that encodes ER α [283]. We hypothesized that increasing the levels of ER α would enhance the responsiveness of 12Zs to E2. We obtained a 12Z cell line genetically modified to upregulate *ESR1* [288] from Dr. Fazleabas's lab (Michigan State University), hereafter denoted as *ESR1*-12Z. Upon receiving the *ESR1*-12Z cell line, we verified the increased expression of ER α with a western blot (Fig. 17A). Western blot replicates and the effect of E2 on ER α expression are reported (Fig. 15). E2 had no effect on ER α expression in 12Zs (Fig. 16A) or *ESR1*-12Zs (Fig. 16B).

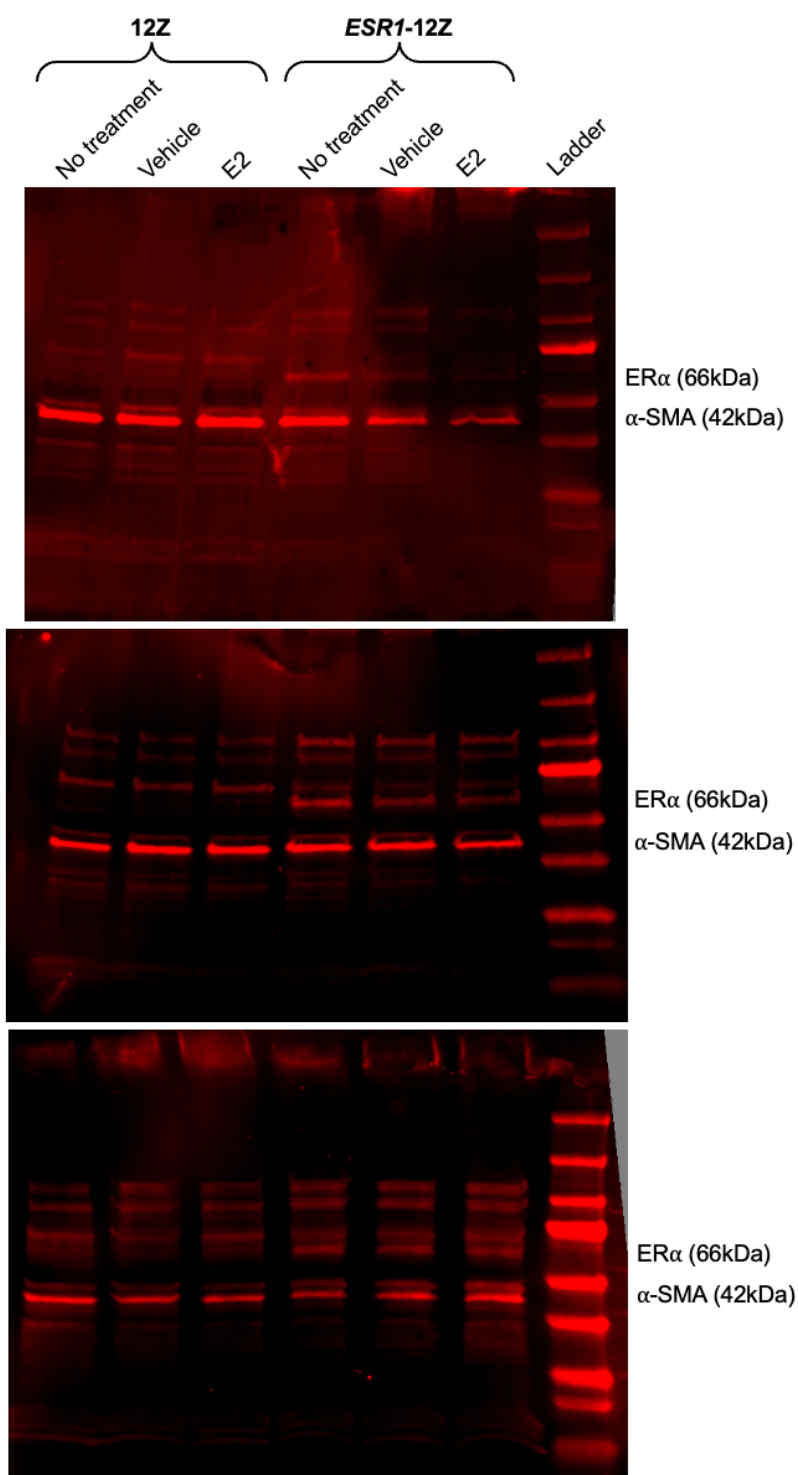


Figure 15: Western blots measuring upregulation of ER α in *ESR1*-12Zs. Western blots were performed using α -SMA (42kDa) as the reference protein and the SeeBlue Plus2 prestained protein ladder. Quantification of ER α expression in *ESR1*-12Zs and 12Zs (no treatments) is shown in the main article in Fig. 4B. Blots are from three independent trials. E2; estradiol, ER α ; estrogen receptor- α , α -SMA; α -smooth muscle actin.

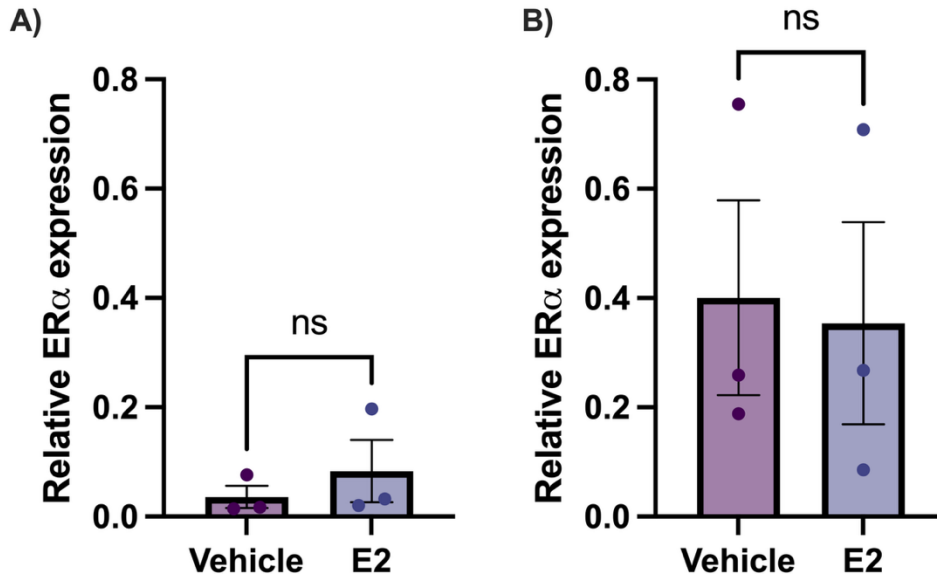


Figure 16: Effect of E2 on ER α expression in 12Zs and *ESR1*-12Zs. Cells were treated with vehicle (0.001% EtOH) and 10nM E2 for 48 hr prior to lysis. ER α expression was quantified relative to α -SMA expression in **A)** 12Zs and **B)** *ESR1*-12Zs. Data points represent biological replicates, error bars represent standard error of the mean. E2; estradiol.

The *ESR1*-12Zs significantly overexpress ER α by about 26-fold compared to the normal 12Zs; in fact, ER α appears to be nearly absent in the 12Zs (Fig. 17B). However, after treating the *ESR1*-12Zs with 10 nM E2 (an alternate source of E2, denoted MPB E2, was also tested) for 48 hr, their functional behaviors showed no changes (Fig. 17C-E) compared to the vehicle control. In a CCK-8 proliferation assay with MMC as a positive control, neither source of E2 led to a change in absorbance compared to the vehicle control (Fig. 17C). Similarly, neither source of E2

significantly impacted wound closure migration (Fig. 17D) or migration through a transwell insert (Fig. 17E).

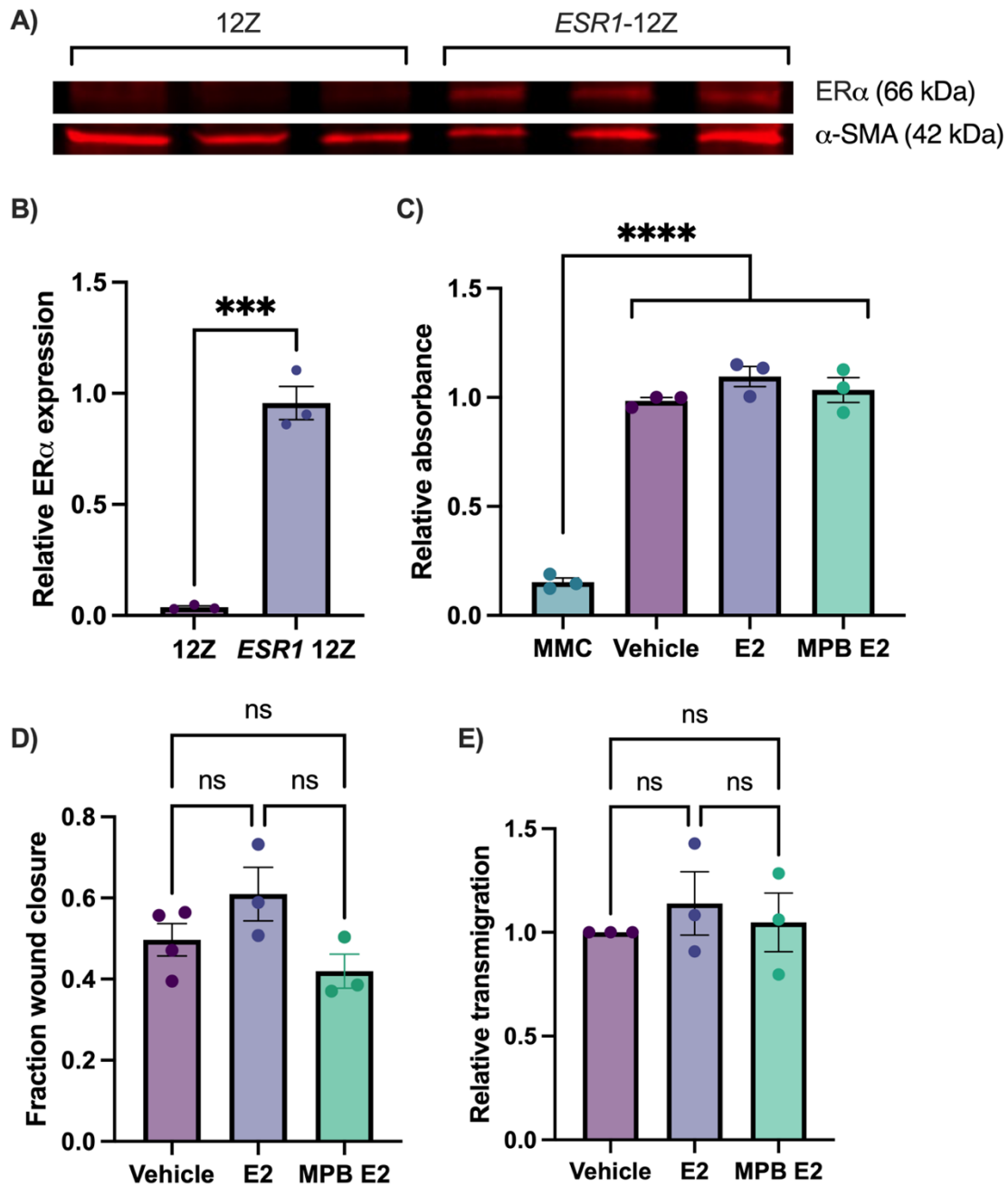


Figure 17: Effect of E2 on proliferative and migratory behaviors of *ESR1*-12Zs. We acquired 12Z cells stably transfected with *ESR1* (*ESR1*-12Zs) [288], the gene encoding the estrogen receptor α

(ER α). **A)** A western blot for ER α expression was performed using α -smooth muscle actin (α -SMA) as the reference protein and **B)** quantified relative to α -SMA to verify the upregulation of *ESR1* in the *ESR1*-12Zs. **C-E)** Then, *ESR1*-12Zs were subject to vehicle, 10 nM E2, or 10 nM MPB E2 for 48 hr and tested for **C)** proliferation in a CCK-8 assay (with 10 μ g/mL MMC as a positive control) reported relative to the vehicle control, **D)** wound closure, and **E)** transwell migration reported relative to the vehicle control. Data points represent biological replicates, error bars represent standard error of the mean. *** $P \leq 0.001$, **** $P \leq 0.0001$. E2; estradiol (original batch purchased from Sigma Aldrich). MPB E2; MP Biomedicals estradiol. MMC; mitomycin C.

4.3.5. Gene expression profiles: effects of E2 treatment or *ESR1* upregulation

Vehicle-treated 12Zs (VE_12Z), E2-treated 12Zs (E2_12Z), normal 12Zs (NT_12Z), and *ESR1*-12Zs (ESR1_12Z) were sent for RNA extraction and sequencing (Novogene Co, Ltd.) to inform trends seen in E2 sensitivity in functional assays. Mainstream hierarchical clustering analysis shows some transcriptomic similarity between VE_12Z and E2_12Z (11,512 genes co-expressed, 451 and 580 genes uniquely expressed, respectively) and relatively less similarity between NT_12Z and ESR1-12Z (10,818 genes co-expressed, 1034 and 1065 genes uniquely expressed, respectively) (Fig. 18A). These intergroup differences are also shown in the 2D principal component analysis (Fig. 18B).

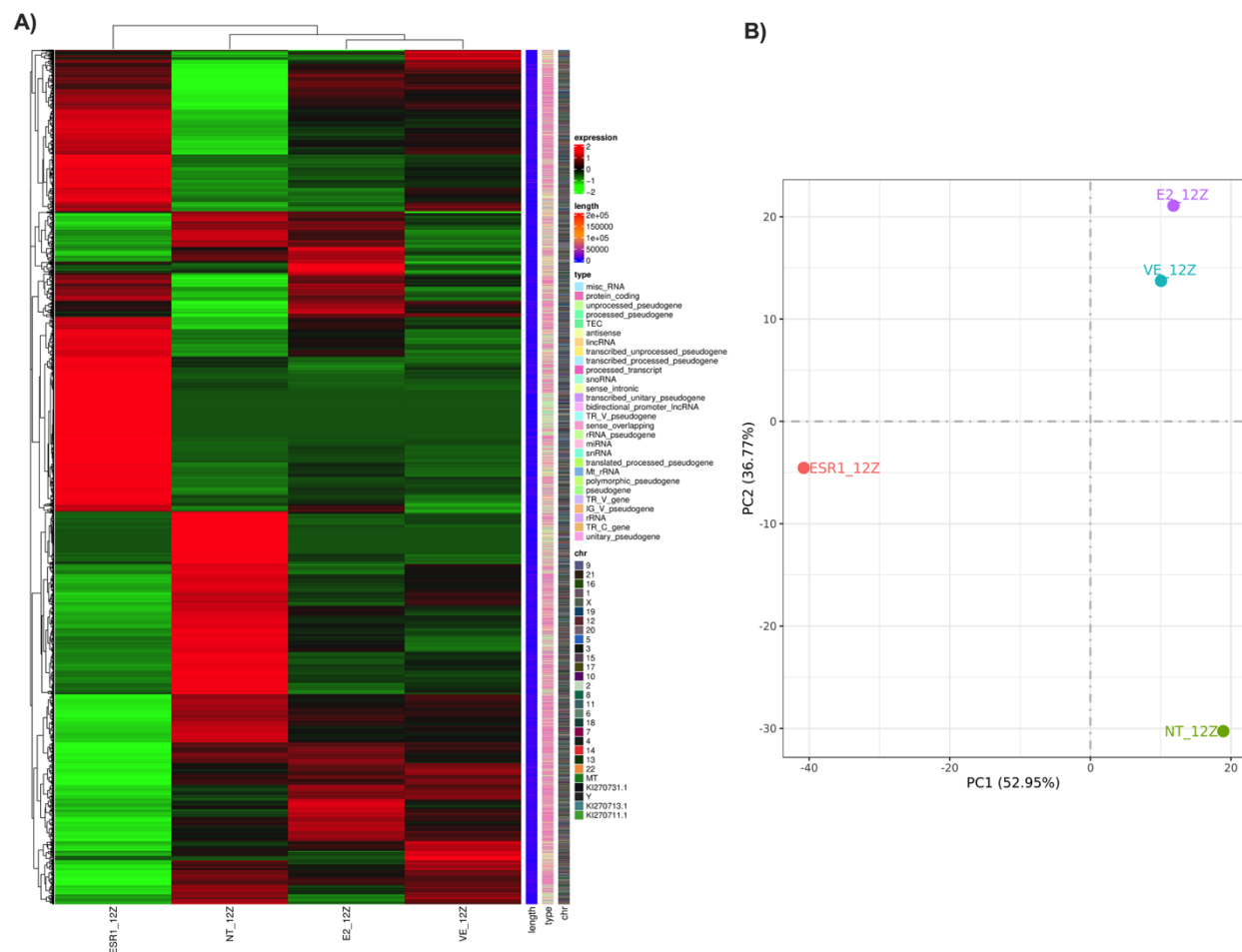


Figure 18: Gene expression profiles of vehicle-treated 12Zs, E2-treated 12Zs, normal 12Zs, and *ESR1*-12Zs. Vehicle-treated 12Zs (VE_12Z), E2-treated 12Zs (E2_12Z), normal 12Zs (NT_12Z), and *ESR1*-12Zs (ESR1_12Z) were sent for RNA extraction and sequencing (Novogene Co, Ltd.). **A)** Gene expression profiles can be visualized in the heatmap of mainstream hierarchical clustering and **B)** intergroup differences can be seen in the 2D principal component analysis plot. Each cell sample contains 3 trials pooled prior to sequencing.

A differential gene expression analysis was carried out using the EdgeR trimmed mean of M values method [289] (adjusted $P < 0.05$) to normalize the data. P-values were adjusted using the Benjamini-Hochberg method to control the error discovery rate [290]. In the E2_12Z group compared to the VE_12Z group, there were 289 differentially expressed genes (DEGs): 185

upregulated and 104 downregulated (Appendix A: Supplementary Material S3). We found that E2 induced a differential expression of many pseudogenes and other non-coding RNAs, as annotated on the volcano plot (Fig. 19A). Out of the top 10 DEGs, 7 were pseudogenes or non-coding RNA. *RN7SL5P*, *PKD1P1*, and *AL591846.1* are examples of significantly upregulated pseudogenes in E2_12Z compared to VE_12Z, while non-coding RNAs *AC126755.2*, *AL391280.1*, *AC008555.8*, and *AC010331.1* were significantly downregulated (Fig. 19B). The roles of these pseudogenes and non-coding RNAs are not well defined, although they are likely capable of indirectly regulating gene expression [291,292].

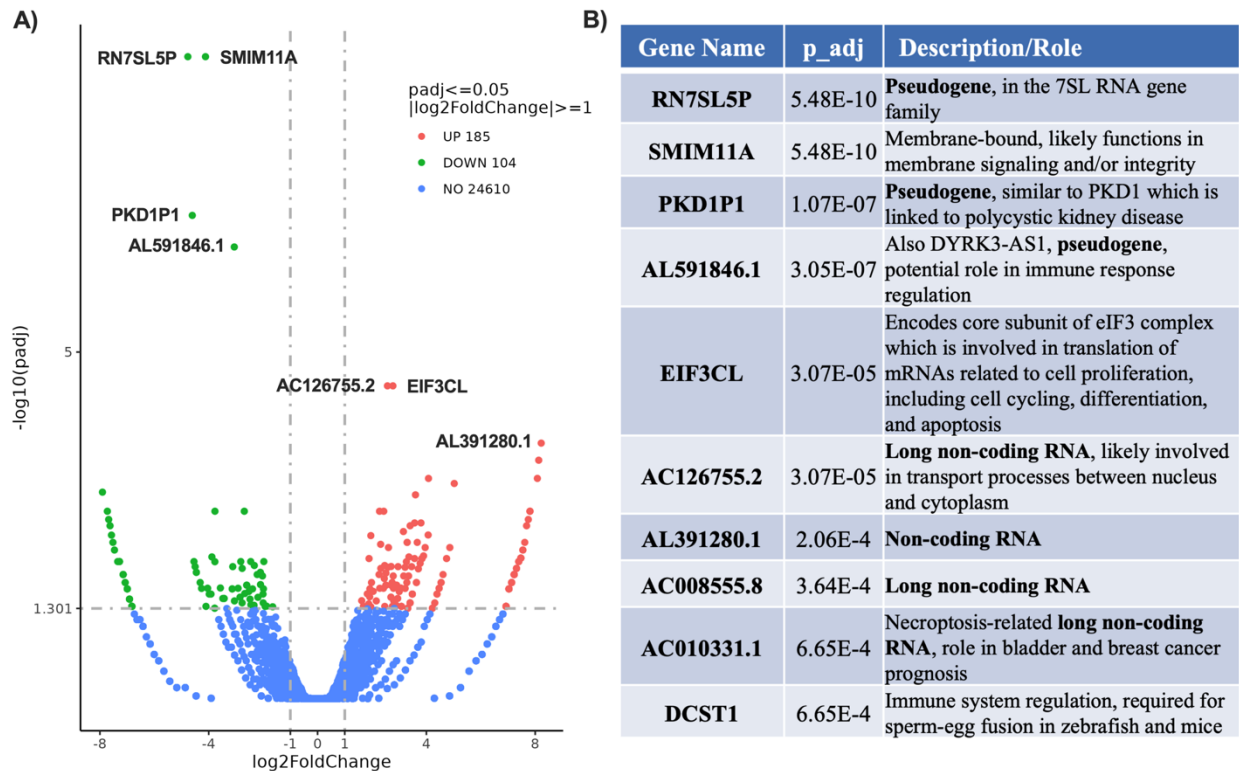


Figure 19: Differentially expressed genes between 12Zs treated with vehicle or E2. Several top DEGs are annotated on the volcano plot. **B)** The top 10 significantly DEGs, including several pseudogenes and non-coding RNAs, are tabulated and described along with their adjusted p-values (p_adj). Differential gene expression analysis was carried out using EdgeR's trimmed mean of M

values with $p_{\text{adj}} < 0.05$ [289]. P-values were adjusted using the Benjamini-Hochberg method to control the error discovery rate [290].

The differential gene expression analysis between NT_12Z and ESR1_12Z revealed much larger transcriptomic differences (Fig. 20). While notable transcriptomic differences likely derive from the difference in *ESR1* expression, some may have occurred due to the effects of stable transfection. There were 6,779 differentially expressed genes (DEGs): 3,229 upregulated and 3,550 downregulated (Appendix A: Supplementary Material S4). The annotated volcano plot shows key DEGs (Fig. 20A): *PKIA*, *PLCBI*, and *EPDRI* were significantly downregulated in ESR1_12Z compared to NT_12Z and are involved in signal transduction and cell invasiveness [293–295]. Unsurprisingly, *ESR1* was the most upregulated gene in the ESR1_12Z. Other notable upregulated genes include *TMPRSS15*, a protease that activates other proteases and cleaves proteins involved in cell signaling [296], and *EPHA3*, which inhibits cell migration [297] (Fig. 20A). To further characterize the DEGs, the KEGG (Kyoto Encyclopedia of Genes and Genomes) [298] dot plot shows the top 20 enriched pathways ordered by adjusted p-value (p_{adj}) with smaller values at the base (Fig. 20B). Out of the 14 pathways that were significantly enriched, several involved cellular signaling and cell-substrate adhesion functions such as cytokine-cytokine receptor interaction ($p_{\text{adj}} = 2.40\text{E-}04$), focal adhesion ($p_{\text{adj}} = 6.69\text{E-}03$), cell adhesion molecules ($p_{\text{adj}} = 0.012$), and ECM-receptor interaction ($p_{\text{adj}} = 0.018$). All of the significantly enriched KEGG pathways are provided for this comparison (Appendix A: Supplementary Material S5).

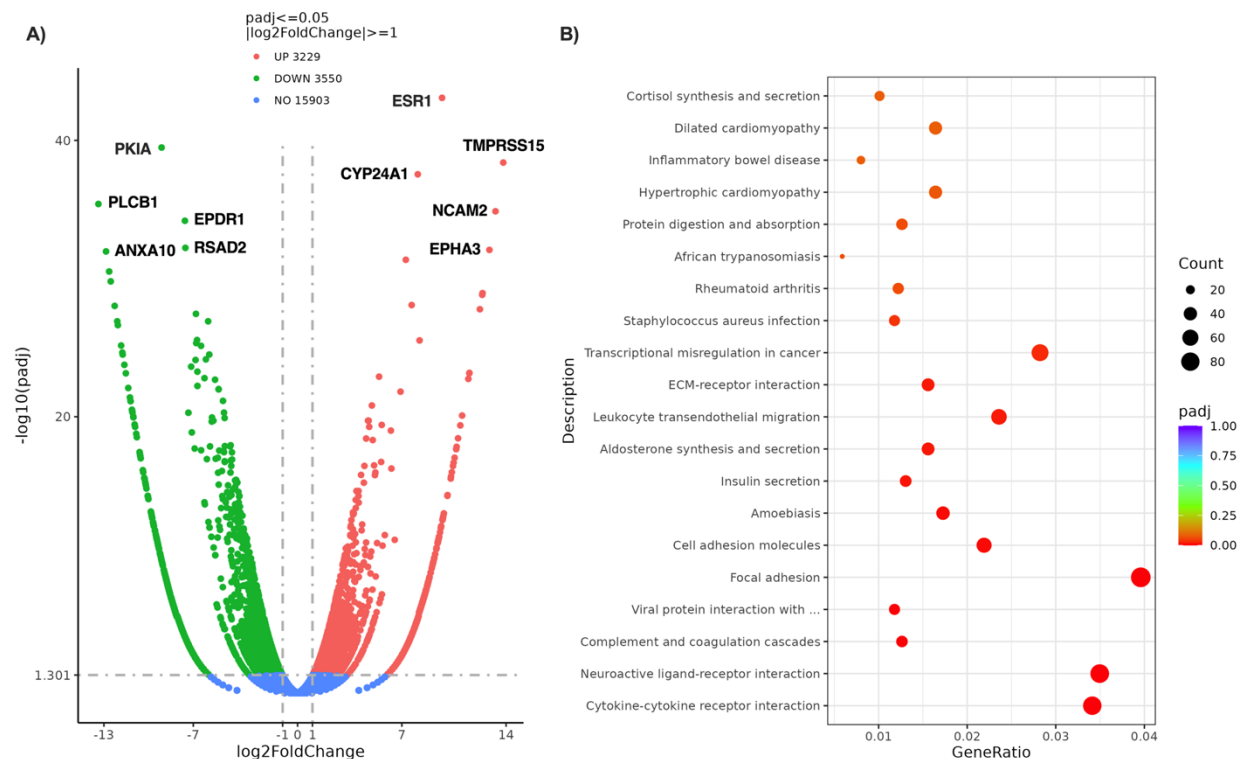


Figure 20: Differentially expressed genes and enriched pathways between 12Zs and *ESR1*-12Zs. **A)** The top 10 DEGs are annotated on the volcano plot, many of which are downregulated proteins involved in signal transduction and membrane trafficking. **B)** The top 20 enriched KEGG pathways are shown in the dot plot (ordered by p_{adj} , smaller values at the bottom), some of which function in cell signaling and cell-substrate adhesion. Differential gene expression analysis was carried out using EdgeR's trimmed mean of M values with $p_{adj} < 0.05$ [289]. KEGG [298] pathways with $p_{adj} < 0.05$ are considered significant. P-values were adjusted using the Benjamini-Hochberg method to control the error discovery rate [290].

4.4 DISCUSSION

E2, a potent estrogen molecule, plays a fundamental role in regulating diverse physiological processes and contributes to the development of both benign and malignant conditions. Despite its broad impact, the precise mechanisms by which E2 modulates cellular activities in different tissue contexts remain incompletely understood. As researchers increasingly

turn to *in vitro* models to study estrogen-dependent processes, it is critical to ensure that the cell lines used retain physiologically relevant responsiveness to E2. Without this validation, conclusions cannot be drawn about estrogenic effects.

In the present study, we sought to characterize the sensitivity of the 12Z human endometriotic epithelial cell line to E2. While this cell line is frequently used in the study of estrogen-mediated conditions like endometriosis, our results challenge its assumed responsiveness to E2 in common *in vitro* models. We observed no significant changes in proliferation, collective migration, or single-cell random migration upon treatment with 10 nM E2 for 48 hours (Fig. 13). Together, these data suggest that the 12Z cell line lacks broad sensitivity to E2 in standard 2D *in vitro* systems, contrary to the sensitivity seen in primary endometriotic epithelial cells [6,27,28,45,46]. A previous study demonstrated that E2 treatment of 12Zs did not enhance their production of pro-inflammatory cytokines [299]. While this is a different experimental system, it reflects a similar theme of a lack of responsiveness to E2 in the 12Z cell line. Another study found that E2 treatment increased the invasion of heterogeneous spheroids composed of 12Z cells and endometriotic stromal cells within Matrigel containing peritoneal mesothelial cells [234]; however, it is unclear whether the 12Zs, the stromal cells, or both were hormone responsive. In fact, the stromal cells might have been more sensitive to E2-induced invasiveness considering they were localized to the leading edges of the spheroids [234].

Interestingly, our results showed that the 12Zs did demonstrate a selective E2 responsiveness in physically confined migration systems (Fig. 14). This suggests that E2 may modulate cell behavior in a context-dependent manner, particularly in environments that mimic mechanical constraints present *in vivo*. It is possible that mechanical cues or confinement activate

signaling pathways or receptor conformations that enable a cellular response to E2 that is otherwise dormant in 2D systems. This phenomenon underscores the importance of considering physical context when evaluating hormone responsiveness *in vitro*. Moreover, others have shown a differential expression of estrogen-related genes in 3D compared to 2D [221]. We observed that E2 treatment modified the gene expression profile in 12Z cells, with a majority of the top 10 DEGs being pseudogenes or non-coding RNAs (Fig. 19). While their exact functions remain unclear, these molecules are increasingly recognized as regulators of gene expression through competing endogenous RNA activity, epigenetic modulation, or chromatin remodeling [291,300]. It is possible that these upregulated molecules play a role in the expression of cytoskeletal regulators, mechanotransducers, cell volume regulators, or nuclear envelope genes. Physical confinement itself may activate E2 sensitivity in the 12Z cells, another question worthy of future investigation. This differential gene expression analysis differs from a previous study that reported an upregulation of some genes related to cell proliferation, apoptosis, motility, and signaling upon E2 treatment of the 12Zs [301]. These contrasting results could be due to the use of different methods (proteomic analysis with mass spectrometry versus transcriptomic analysis with RNA sequencing in this study), different treatment incubation times (24 vs 48 hr), or different passage numbers.

Previously under the hypothesis that cellular sensitivity to E2 depends on *ESR1* expression, it seemed counterintuitive that although the overexpression of *ESR1* substantially increased ER α protein levels, it did not translate to increased functional responsiveness to E2 in proliferation or migration assays (Fig. 17). This implies that E2 insensitivity in the 12Zs is not solely due to low or absent ER α levels but may reflect downstream desensitization of estrogen signaling pathways or epigenetic modifications that uncouple ERs from functional outcomes. RNA sequencing of

ESR1-12Zs revealed a downregulation of several key genes involved in signal transduction and membrane trafficking (e.g., *PKIA*, *PLCB1*, *EPDR1*, *ANXA10*) (Fig. 20), which may contribute to this lack of E2 sensitivity despite *ESR1* overexpression. Enriched KEGG pathways involving cytokine interactions, focal adhesions, cell adhesion molecules, and ECM-receptor signaling further support this hypothesis and suggest an altered regulatory network in *ESR1*-12Zs compared to normal 12Zs. Given the discrepancy between ER α presence and the lack of functional response to E2, future studies should investigate 1) the crosstalk between ER α , ER β , and G protein-coupled estrogen receptor expression and how they determine cellular E2 sensitivity and 2) the balance between classical (genomic) and non-classical (non-genomic) ER signaling pathways in the 12Zs. Classical ER signaling involves the direct binding of ligand-bound ERs to DNA at estrogen response elements (EREs), while non-classical signaling typically occurs through membrane-bound ERs that activate kinase cascades and secondary messengers [266]. It is possible that the 12Z cells rely more heavily on non-genomic ER pathways that are context-dependent or that these signaling routes are impaired altogether. Dissecting the roles and integrity of these signaling pathways could provide insight into why some cellular behaviors, such as confined migration, are selectively responsive to E2 while others are not. Additionally, probing the involvement of G protein-coupled estrogen receptor or cytoplasmic ER pools could help clarify alternative routes of E2 influence.

Overall, these findings prompt a reevaluation of the 12Z cell line's suitability as a model for estrogen-dependent processes. While it may still serve as a tool in endometriosis research, its limited E2 responsiveness in most *in vitro* contexts raises concerns about its utility for studying canonical estrogen signaling pathways. Future work could benefit from comparing the 12Z cell

line to primary cell models with better-maintained hormonal sensitivity. Additionally, combining 12Zs with endometriotic stromal cells in co-culture, or subjecting cells to mechanical cues that mimic the *in vivo* microenvironment, may reveal hidden aspects of hormone signaling not captured in standard assays.

Our study has a few limitations. One limitation is that we only used endometriotic epithelial cells; the disease that the 12Z cell line models, endometriosis, is characterized by the presence of both glandular epithelium and stromal cells. It is unclear whether the presence of stromal cells contributes to the sensitivity of epithelial endometriotic cells to E2. Currently, there is no well-established endometriotic stromal cell line. However, future studies should consider testing E2 sensitivity in a co-culture of 12Zs and primary endometriotic stromal cells compared to each cell type individually. Another limitation of our study is that we obtained our 12Z cell line at passage 55. It is unknown how long the 12Z cell line can be passaged before it starts losing its original phenotype, but some changes have likely occurred: we detected nearly absent levels of ER α in the 12Zs (Fig. 17A,B), contrary to earlier reports of ER α expression in the 12Zs [237,238]. We did not have access to earlier passages of the 12Zs, but it would be beneficial for others who do to study the effects of passaging on the retention of key traits of this particular cell line. Lastly, the *ESR1*-12Z cell line was not accompanied by an empty-vector stable control line; therefore, differences between *ESR1*-12Zs and normal 12Zs could reflect effects of stable transfection/selection and/or passage differences in addition to *ESR1* upregulation.

4.5 CONCLUSION

In conclusion, while 12Z cells are a widely used *in vitro* model of endometriosis, our findings indicate a restricted and context-specific responsiveness to E2. This selective behavior, especially under physical confinement, opens interesting avenues for further exploration into how mechanical and hormonal signaling intersect. However, researchers should exercise caution when using this cell line to model estrogen-dependent processes, particularly in 2D culture systems, and consider verifying hormone sensitivity in their specific experimental context.

Interestingly, our work in the previous chapter demonstrated that E2 induced greater protrusive activity in the 12Z cells. However, this chapter shows that increases in membrane protrusions did not fully lead to an increase in cell migration speed or other malignant behaviors. While protrusions do help with initiating the migration of the leading edge, cells also require contractility, focal adhesions, and front-rear polarity to move—phenomena that E2 may not enhance. Moreover, Chapter 3 suggests that E2 may promote a more exploratory state in the 12Z cells rather than enhancing their directional persistence.

Building from this work, in which the physical surroundings of the cell altered its E2 sensitivity, we hypothesized that culturing the 12Zs on more physiologically relevant matrix stiffnesses might enhance their hormone responsiveness. Therefore, the following chapter explores the combined effects of mechanical cues and E2 on endometriotic cell motility.

Chapter 5: Combined effects of E2 and substrate stiffness on endometriotic cell motility

5.1 INTRODUCTION

Endometriosis is a complex fibrotic disease with hormonal and mechanical microenvironmental cues that shape dysregulated cell behaviors [7]. Given the excessive local buildup of estrogen leading to inflammation and disease progression, the preceding chapters have focused primarily on the role of estradiol (E2), a potent estrogen, on migratory cell phenotypes in the disease. But in addition to estrogen responsiveness, fibrosis is another key hallmark of endometriosis, resulting in tissue scarring and stiffening. The elasticity of healthy endometrial tissue is reported to be 1.97 kPa in the secretory phase and 3.34 kPa in the proliferative phase [146]. While the Young's modulus of endometriosis lesions is not yet defined, data on fibrosis of the lung, liver, and bowel indicate that the Young's modulus can increase from 1–3 kPa (normal tissue) to 16.5–20 kPa (fibrotic tissue) [147–151]. In particular, some prior work indicates potential overlap between E2 signaling and RhoA/ROCK signaling [129,156]. Therefore, it is worth investigating whether hormonal and mechanical cues work in synchrony to drive disease behaviors in endometriosis.

Our work utilizes the 12Z human endometriotic epithelial cell line which has recently gained widespread traction as a useful model for endometriosis research [237]. We previously found that E2 does not influence 12Z proliferation, collective migration, or single-cell migration.

However, 12Zs were selectively responsive to E2 in 3D migration models where the cells were physically confined (Chapter 4). Given that E2 sensitivity in the 12Zs was dependent on their physical environment and the potential interaction between E2 and RhoA/ROCK signaling pathways, we hypothesized that the inclusion of physiologically relevant matrix stiffnesses in our model could increase 12Z hormone responsiveness. This chapter demonstrates that while substrate stiffness does influence functional 12Z behaviors, it does not lend them any further sensitivity to E2 in a random motility assay.

5.2 METHODS

5.2.1. Polyacrylamide hydrogel preparation

Polyacrylamide (PA) hydrogels were formed on glass coverslips as previously described.[302,303] Briefly, 22 x 22 mm glass coverslips were passed through a flame to remove contaminants before being smeared with 0.1M NaOH. Coverslips were then silanized with 3-aminopropyltriethoxysilane (Sigma Aldrich) and activated with 0.5% glutaraldehyde (Sigma Aldrich) in phosphate buffered saline (PBS) for 30 min to enable PA hydrogel attachment. 2 kPa and 30 kPa PA hydrogel precursor solutions contained certain amounts of 40% acrylamide (Bio-Rad), 2% bis-acrylamide (Bio-Rad), 1M HEPES (Gibco), and ultrapure water mixed together. Final concentrations were 4% acrylamide and 0.14% bis-acrylamide for the soft gel; 12% acrylamide and 0.28% bis-acrylamide for the stiff gel. Elastic moduli of PA gels were estimated via submersion compression testing using a Dynamical Mechanical Analyzer 850. Measurements were 1.223 ± 0.201 kPa for the soft gel (called 2 kPa in this manuscript) and 29.81 ± 2.338 kPa for the stiff gel (called 30 kPa in this manuscript). PA hydrogel precursor solutions were syringe-

filtered (0.2 μm filter) and degassed for 20 min. 10% ammonium persulfate (Bio-Rad) in water and N,N,N',N'-Tetramethylethylenediamine (TEMED; Bio-Rad) were added to the solutions to polymerize $\sim 80\text{-}\mu\text{m}$ -thick PA hydrogels on coverslips. PA hydrogels were allowed to polymerize for 30 min before activating with sulfo-SANPAH (Thermo Scientific) to cross-link the extracellular matrix protein to the PA hydrogel surface, as previously described [303]. PA hydrogels were coated with 50 $\mu\text{g/mL}$ collagen I from rat tail (Corning) overnight at 4°C. For experiments on glass, 22 x 22 mm glass coverslips were coated in 50 $\mu\text{g/mL}$ collagen I overnight at 4°C. Validation of equal coating across gels was performed using a biotinylated collagen coating, applying avidin conjugated to Texas Red, and imaging under fluorescence microscopy. All PA hydrogels and coverslips were stored in PBS at 4°C for up to 10 days prior to cell seeding. PA hydrogels and coverslips were equilibrated in full, phenol-free media at 37°C at least 45 min prior to cell seeding.

5.2.2. Cell culture, seeding, and treatments

The 12Z human endometriotic epithelial cell line was purchased from Applied Biological Materials (Cat. # T0764). Cells were cultured in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). The cell line was mycoplasma free and authenticated by the company prior to shipping. Cell line authentication and mycoplasma testing were performed again at least 6 months afterwards. Cells from passages 55-80 were used for experiments. About 28-48 hr prior to experiments, the media was replaced with phenol-free DMEM/F-12 (Gibco) supplemented with 10% charcoal-stripped FBS (Gibco) and 1%

penicillin-streptomycin (Gibco). Approximately 250,000 cells were seeded per well onto PA hydrogels in full phenol-free media.

17 β -estradiol (E2) was purchased from MP Biomedicals (Cat. no. 02194565-CF) and dissolved in 100% ethanol to create a stock solution. Cells were treated with phenol-free media containing a vehicle (0.001% ethanol) or 10 nM E2 at the time of seeding onto PA hydrogels (24 hr prior to the start of the timelapse microscopy). Treatments were kept on the cells for the duration of the timelapses (12 hr).

5.2.3. Quantification of cellular morphology

Morphological parameters including cell area, perimeter, solidity, and roundness were extracted using ImageJ to manually trace cells from phase contrast microscope images. Solidity was defined as $solidity = \frac{Cell\ area}{Convex\ hull\ area}$, where a cell that is perfectly circular would have a value of 1.0 and a cell that has protrusions and/or indentations would have a value closer to 0. Roundness was defined as $roundness = \frac{1}{Aspect\ ratio} = \frac{Minor\ axis}{Major\ axis}$, where a highly elongated cell would have a value close to 0.

5.2.4. Single cell random migration analysis

The random migration of single cells was analyzed from time-lapse phase contrast images. Cells were incubated with treatments for 24 hr and then imaged under phase contrast at 10X for 12 hr, with a time step of 15 min. Individual cell trajectories were tracked using the Manual Tracking plugin in ImageJ by clicking on the estimated geometric center of the cell at each time point. Subsequent analysis was performed in MATLAB. Average speed was defined as the cell's

total path length divided by the duration of the time-lapse. Directional persistence was defined as the cell's end-to-end displacement divided by its total path length, where a cell that progresses in a perfectly straight line from point A to point B would have a directional persistence of 1; a cell that originates at a point A, moves in a straight line to point B, and directly returns to point A would have a directional persistence of 0.

5.2.5. Microscopy

For all phase contrast images, live cells were imaged using the 10X objective on an Olympus IX-83 inverted microscope (Olympus, Center Valley, PA, USA) and the Olympus cellSens software. Cells were maintained at 37°C, 65% humidity, and 5% CO₂ in an enclosed chamber surrounding the microscope.

5.2.6. Statistical analysis

GraphPad Prism 10 (La Jolla, CA, USA) was utilized to generate graphs and perform statistical analyses. For comparisons between vehicle and E2 and stiffness groups, Ordinary Two-way ANOVA was performed with a Tukey's multiple comparisons test. For all tests, P values < 0.05 were considered statistically significant. Data are represented as mean $\pm$ standard error of the mean. All experiments were performed in at least triplicates.

5.3 RESULTS

5.3.1. Substrate stiffness and E2 treatment on endometriotic cell morphology

To investigate the combined effects of substrate stiffness and E2 treatment, we seeded 12Z cells on 2 kPa, 30 kPa, and glass substrates and treated them with a vehicle control (0.001%

ethanol) or 10 nM E2 24 hr prior to imaging. Morphological parameters were obtained by tracing cells in phase contrast images. Irrespective of treatment, cells seeded on the softest gel and the glass appeared smaller and not as well-adhered compared to cells on the 30 kPa gel (Fig. 21A). However, E2 had no effect on cell area (Fig. 21B), circularity (Fig. 21C), solidity (Fig. 21D), or minor axis:major axis ratio (Fig. 21E) on any of the substrates compared to its vehicle control. Therefore, the morphological sensitivity of 12Zs to E2 treatment does not depend on substrate stiffness.

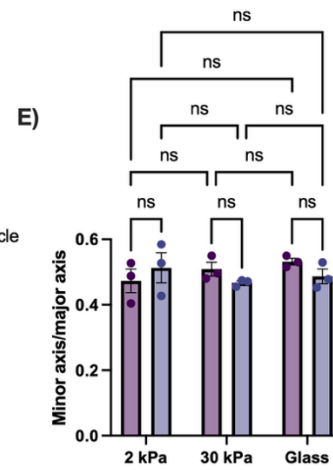
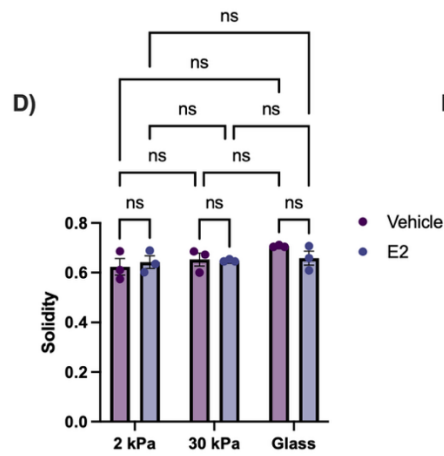
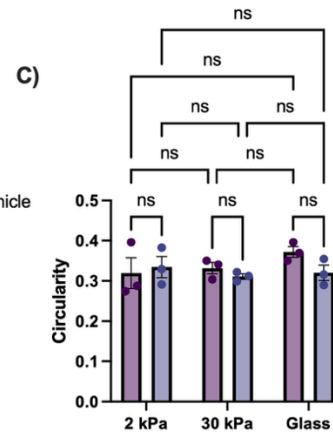
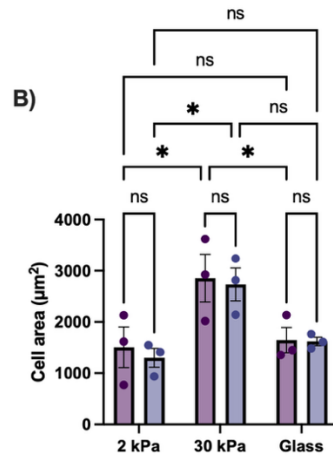
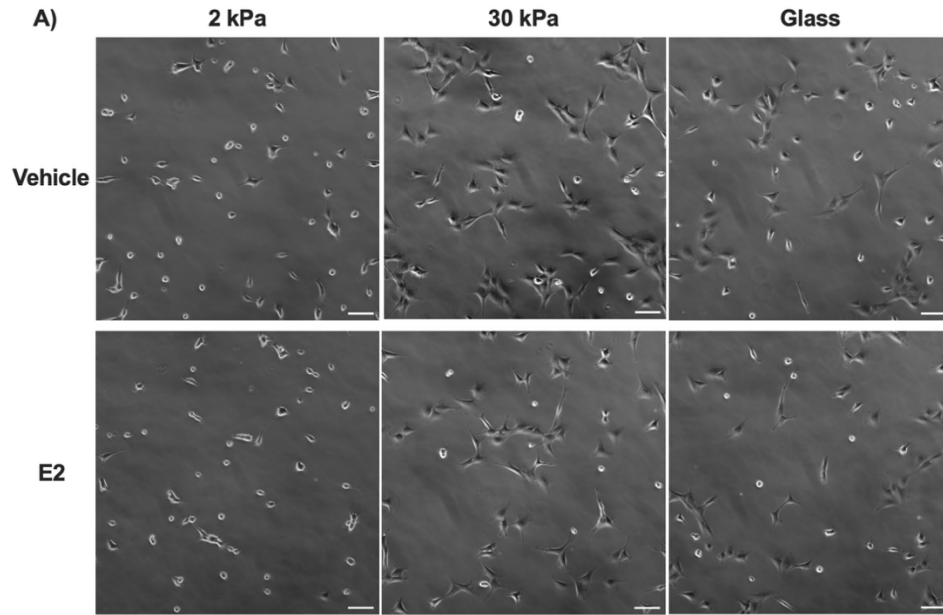


Figure 21: Substrate stiffness and E2 treatment on 12Z morphology. 12Z cells were cultured on substrates of varying stiffness overnight before assessing their morphology. Vehicle (0.001% ethanol) and E2 (10 nM) treatments were added to the cells 24 hr prior to imaging. **A)** Phase contrast images show small, rounded cells on the 2 kPa and glass substrates and more adhered cells on the 30 kPa substrate, regardless of treatment. We quantified the cell **B)** area, **C)** circularity, **D)** solidity, and **E)** ratio of the minor to major axis. Scalebars = 100 μ m. Data points represent trials; error bars represent standard error of the mean. 30-40 cells were analyzed per stiffness per trial. * $P \leq 0.05$.

5.3.2. Substrate stiffness and E2 treatment on endometriotic cell motility

To further probe the combined effects of substrate stiffness and E2 treatment, we seeded 12Z cells on 2 kPa, 30 kPa, and glass substrates and treated them with a vehicle control (0.001% ethanol) or 10 nM E2 24 hr prior to imaging in a random motility assay. Treatments were maintained on the cells for the 12-hr duration of phase contrast timelapses. Individual cells were manually tracked, and their trajectories show stiffness-dependent—but not E2-dependent—migration (Fig. 22A–C). There were also clear stiffness effects, but not hormonal effects, on cell speed (Fig. 22D). Lastly, neither hormonal nor mechanical cues affected the directional persistence of the cells (Fig. 22E).

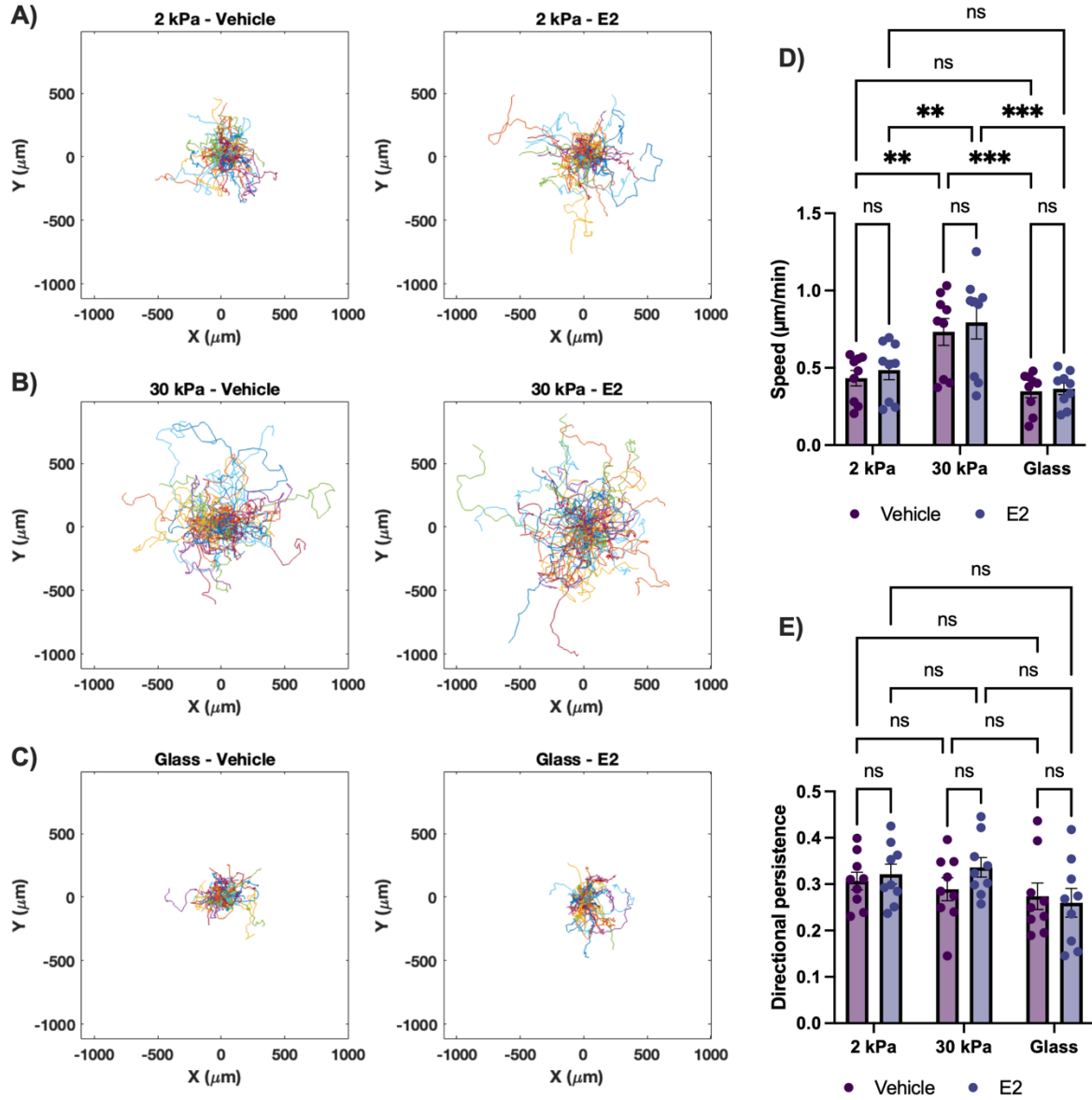


Figure 22: Substrate stiffness and E2 treatment on 12Z motility. 12Z cells were cultured on substrates of varying stiffness overnight before imaging with phase contrast timelapse microscopy. Vehicle (0.001% ethanol) and E2 (10 nM) treatments were added to the cells 24 hr prior to the start of imaging and maintained during the 12-hr timelapse. Individual cell trajectories are shown for control and E2-treated cells on **A)** 2 kPa, **B)** 30 kPa, and **C)** glass substrates. We quantified **D)** average cell speed and **E)** directional persistence. Each data points represents an average of 10 cells within a field of view; error bars represent standard error of the mean. 30 cells were analyzed per experimental group per trial, and data from three trials are pooled. ** $P \leq 0.01$, *** $P \leq 0.001$.

5.4 DISCUSSION

Our previous results showed that the 12Z cell line demonstrated a lack of sensitivity to E2 in standard 2D functional assays, but that certain physical cues do lead to E2-mediated 12Z cell behaviors (Chapter 4). Given that endometriosis is a fibrotic disease, increased substrate stiffness is a physiologically relevant mechanical cue that we hypothesized could affect the response of 12Zs to E2. However, using PA gels with varying elastic moduli and E2 treatments, we found that neither the morphological nor motile response of 12Zs depend on E2 on any of the substrates.

Our study has a few limitations. One limitation is that we only used endometriotic epithelial cells; endometriosis, is characterized by the presence of both glandular epithelium and stromal cells. It is unclear whether the presence of stromal cells contributes to the sensitivity of epithelial endometriotic cells to E2. Currently, there is no well-established endometriotic stromal cell line. However, future studies should consider testing E2 sensitivity in a co-culture of 12Zs and primary endometriotic stromal cells compared to each cell type individually. Furthermore, the mechanomemory of 12Z cells is not well defined. We cultured cells (previously grown on plastic) on substrates overnight before performing assays which appeared to be enough time to visualize cellular changes, but future studies should clarify the duration of mechanical memory in endometriotic epithelial cells. Another limitation of our study is that we obtained our 12Z cell line at passage 55; an earlier passage of the cell line was not available. It is unknown how long the 12Z cell line can be passaged before it starts losing its original phenotype.

5.5 CONCLUSION

This chapter demonstrates that healthy and fibrotic substrate stiffnesses are insufficient to induce E2 sensitivity within the 12Z cell line, as assessed by cell morphology and motility assays. In light of this and preceding chapters, the 12Z cells cannot reliably recapitulate functional responses to E2 seen in primary cells that retain key disease characteristics. Although the 12Z cell line fails to model the estrogen-dependence of endometriosis, it does exhibit mechanical sensitivity. Given that all types of endometriosis involve fibrosis [57]—which leads to tissue damage and stiffening—we sought to further explore how fibrosis contributes to the disease pathogenesis. Working with the strengths and limitations of the tools available to us, we decided to shift our focus from hormonal cues to mechanical cues in endometriosis. In Chapter 6, we begin by examining the effects of fibrotic substrate stiffness on the motility of endometriotic cells and probing underlying mechanisms.

Chapter 6: Fibrotic substrate stiffness enhances endometriotic epithelial cell motility

6.1 INTRODUCTION

Endometriosis is a fibrotic condition in which lesions and implants in ectopic sites cause inflammation, tissue damage, and scarring. Fibrosis, tissue scarring and stiffening resulting from inflammation, occurs when fibroblasts deposit excessive amounts of extracellular matrix (ECM) components. Fibrosis is a key pathological feature of all types of endometriosis [57]. As an excess of ECM accumulates—primarily collagen—tissues experience a significant increase in stiffness. While the Young's modulus of endometriosis lesions is not yet defined, healthy tissues such as lung, liver, and bowel have a Young's modulus of 1–3 kPa which increases to 16.5–20 kPa during fibrosis [147–151]. Cells have the ability to sense and respond to mechanical stimuli such as substrate stiffness. This process of mechanotransduction results in downstream changes to cell behavior, including proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) [111,304]. These mechanically-driven cell behaviors can become dysregulated and contribute to disease severity and progression [111,305]. For example, increased ECM stiffness promotes malignant endometrial cancer cell phenotypes via an elevated expression of ROCK1, a mechanosensitive kinase [154]. Stiff substrates also promote actin stress fiber formation and proliferation of deep infiltrating endometriotic stromal cells [152]. Broadly in the pelvic region, substrate stiffness has been shown to enhance the migratory and invasive phenotypes of ovarian

cancer cells [306], cervical cancer cells [307,308], bladder cancer cells [309], and colorectal cancer cells [310,311].

The effects of substrate stiffness are well established in cancer and stromal cells but are underexplored in endometriosis. Moreover, previous studies on stiffness-mediated cell migration primarily rely on 2D cell culture models, leaving open the question of how fibrotic stiffness influences multicellular collective expansion. Thus, the mechanobiological principles governing how fibrotic tissue stiffness regulates endometriotic cell motility, both at the single-cell and collective levels, remain poorly defined.

In this chapter, we cultured 12Z human endometriotic epithelial cells on polyacrylamide (PA) gels with tunable stiffness (2 kPa as healthy; 30 kPa as fibrotic) as well as glass as an ultra-stiff extreme (~ 70 GPa), to model mechanically driven cell motility in endometriosis, including both 2D and 3D cell culture. Our results demonstrate a positive correlation between substrate stiffness and cell migration, mediated by cytoskeletal arrangement and Rho/ROCK signaling activity. Broadly, this work contributes to the understanding of how fibrosis as a biomechanical state regulates multiscale cell migration during the pathogenesis of endometriosis.

6.2 METHODS

6.2.1. Cell culture

The 12Z human endometriotic epithelial cell line was purchased from Applied Biological Materials (Cat. # T0764). Cells were cultured in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). The cell line was mycoplasma free and authenticated by the company prior to shipping. Cell line authentication and

mycoplasma testing were performed again at least 6 months afterwards. Cells from passages 55-80 were used for experiments.

6.2.2. Polyacrylamide hydrogel preparation

Polyacrylamide (PA) hydrogels were formed on glass coverslips as previously described [302,303]. Briefly, 22 x 22 mm glass coverslips were passed through a flame to remove contaminants before being smeared with 0.1M NaOH. Coverslips were then silanized with 3-aminopropyltriethoxysilane (Sigma Aldrich) and activated with 0.5% glutaraldehyde (Sigma Aldrich) in phosphate buffered saline (PBS) for 30 min to enable PA hydrogel attachment. 2 kPa and 30 kPa PA hydrogel precursor solutions contained certain amounts of 40% acrylamide (Bio-Rad), 2% bis-acrylamide (Bio-Rad), 1M HEPES (Gibco), and ultrapure water mixed together. Final concentrations were 4% acrylamide and 0.14% bis-acrylamide for the soft gel; 12% acrylamide and 0.28% bis-acrylamide for the stiff gel.

Elastic moduli of PA gels were estimated via submersion compression testing using a Dynamical Mechanical Analyzer 850. Measurements were 1.223 ± 0.201 kPa for the soft gel (called 2 kPa in this dissertation) and 29.81 ± 2.338 kPa for the stiff gel (called 30 kPa in this dissertation). PA hydrogel precursor solutions were syringe-filtered (0.2 μ m filter) and degassed for 20 min. 10% ammonium persulfate (Bio-Rad) in water and N,N,N',N'-Tetramethylethylenediamine (TEMED; Bio-Rad) were added to the solutions to polymerize ~80- μ m-thick PA hydrogels on coverslips. PA hydrogels were allowed to polymerize for 30 min before activating with sulfo-SANPAH (Thermo Scientific) to cross-link the extracellular matrix protein to the PA hydrogel surface, as previously described [303]. PA hydrogels were coated with 50

μg/mL collagen I from rat tail (Corning) over night at 4°C. For experiments on glass, 22 x 22 mm glass coverslips were coated in 50 μg/mL collagen I overnight at 4°C. Validation of equal coating across gels and coverslips was performed using a biotinylated collagen coating, applying avidin conjugated to Texas Red, and imaging under fluorescence microscopy. All PA hydrogels and coverslips were stored in PBS at 4°C for up to 10 days prior to cell seeding. PA hydrogels and coverslips were equilibrated in full media at 37°C at least 45 min prior to cell seeding.

6.2.3. Quantification of cellular morphology

Morphological parameters including cell area, circularity, solidity, and roundness were extracted using ImageJ to manually trace cells from phase contrast microscope images. Circularity was defined as $circularity = \frac{4\pi * Cell\ area}{Perimeter^2}$, where a value of 1 represents a perfect circle. Solidity was defined as $solidity = \frac{Cell\ area}{Convex\ hull\ area}$, where a cell that is perfectly circular would have a value of 1.0 and a cell that has protrusions and/or indentations would have a value less than 1.0. The minor axis to major axis ratio represents the inverse of aspect ratio, where a highly elongated cell would have a value closer to 0.

6.2.4. Single cell random migration

The random migration of single cells was analyzed from time-lapse phase contrast images. Briefly, approximately 80,000 cells were seeded per substrate in a 6-well plate and allowed to adhere overnight. Cells were imaged under phase contrast at 10X for 12 hr, with a time step of 15 min. Individual cell trajectories were tracked using the Manual Tracking plugin in ImageJ by clicking on the estimated geometric center of the cell at each time point. Subsequent analysis was

performed in MATLAB. Average speed was defined as the cell's total path length divided by the duration of the time-lapse. Cells were classified as fast ($\text{speed} > 1 \text{ } \mu\text{m}/\text{min}$), medium ($0.5 \text{ } \mu\text{m}/\text{min} < \text{speed} < 1 \text{ } \mu\text{m}/\text{min}$), or slow ($\text{speed} < 0.5 \text{ } \mu\text{m}/\text{min}$). Directional persistence was defined as the cell's end-to-end displacement divided by its total path length, where a cell that progresses in a perfectly straight line from point A to point B would have a directional persistence of 1; a cell that originates at a point A, moves in a straight line to point B, and directly returns to point A would have a directional persistence of 0.

6.2.5. Spheroid spreading assay

Approximately 2,000-2,500 12Z cells were seeded per well in an ultra-low attachment, round-bottom 96-well plate (Corning), which was then centrifuged at 150 xg for 2 min. Cells self-assembled into spheroids over the next ~48 hr of incubation. Resulting spheroids were approximately 200-250 μm in diameter. 8-10 spheroids were seeded per substrate within a 6-well plate and allowed to settle for 1 hr prior to imaging. Phase contrast imaging was then performed at 10X for 20 hr, with a time step of 20 min. The projected area of the spheroids at multiple time points was measured using ImageJ. As spheroids adhered and spread onto the substrates, the speed of the leading edge was measured using the Manual Tracking plugin in ImageJ. The effect of proliferation was negligible compared to migration, as dividing cells were tracked and represented less than 2% of the spheroid population. Specifically, each spheroid resulted in one leading edge speed which was the average of the instantaneous velocities of three circumferential points on a given spheroid: usually west, north, and east.

6.2.6. Immunocytochemistry

Immunocytochemistry (ICC) was utilized to visualize cell cytoskeletal components. Briefly, cells were then fixed in 4% paraformaldehyde for 20 min on ice with gentle agitation, washed with PBS, and permeabilized in 0.2% TritonX-100 (Sigma-Aldrich) for 5 min. Samples were washed with PBS again and blocked in 2.5% goat serum (Abcam) for 1 hr at room temperature (RT). The primary antibody for paxillin (1:200, Santa Cruz Biotechnology), vimentin (1:100, Cell Signaling Technology), or phosphorylated myosin light chain (pMLC) (1:200, ThermoFisher Scientific) was diluted in 2.5% goat serum, added to the samples, and incubated overnight at 4°C. The following day, samples were washed with PBS and incubated for 1-1.5 hr with Hoechst (1:10,000; ThermoFisher Scientific), Alexa Fluor 488 phalloidin (1:200; ThermoFisher Scientific), and the secondary antibody Alexa Fluor 488 goat anti-mouse IgG (1:200; ThermoFisher Scientific) diluted in 2.5% goat serum at RT. Samples were then washed with PBS and imaged at 40X under fluorescence using an FV3000 Laser Scanning Confocal Microscope. Fluorescence intensity across the cells was measured from segmented images in ImageJ.

6.2.7. Pharmacological modulation of contractility

Y27632, an inhibitor of Rho-associated protein kinase (ROCK), was chosen to decrease cell contractility. Oleoyl-L-alpha-lysophosphatidic acid sodium salt (LPA), an activator of G protein-coupled receptors (GPCRs) that regulate cellular processes such as cell migration and mechanosensing, was chosen to indirectly increase cell contractility. Y27632 was purchased from Sigma Aldrich and dissolved in ultrapure water. 10 µM Y27632 and the vehicle, 0.1% water, were

added to 12Z cells on 30 kPa substrates 30 min prior to imaging. LPA was purchased from Thermo Scientific and dissolved in 0.1% fatty acid-free bovine serum albumin (FAF-BSA; Sigma Aldrich). Final concentrations of 5 μ M LPA and the vehicle, 0.0001% FAF-BSA, were added to the 12Z cells on 2 kPa substrates at the start of imaging.

6.2.8. Microscopy

For all phase contrast images, live cells were imaged using the 10X objective on an Olympus IX-83 inverted microscope (Olympus, Center Valley, PA, USA) and the Olympus cellSens software. Cells were maintained at 37°C, 65% humidity, and 5% CO₂ in an enclosed chamber surrounding the microscope.

6.2.9. Statistical analysis

GraphPad Prism 10 (La Jolla, CA, USA) was utilized to generate graphs and perform statistical analyses. Outliers were identified using the ROUT method with a Q (maximum desired false discovery rate) = 1% only to remove definite outliers. Data were tested for normality. For comparisons between three or more groups, ordinary one-way ANOVA with a Tukey's multiple comparisons test was performed. Corresponding non-parametric tests were used for non-normally distributed datasets. For all tests, P values < 0.05 were considered statistically significant. Data are represented as mean $\pm$ standard error of the mean. All experiments were performed in at least triplicates.

6.3 RESULTS

6.3.1. Fibrotic substrate stiffness encourages cell spreading

12Z cells were seeded on 2 kPa, 30 kPa, or glass substrates and imaged with phase contrast microscopy the following day. Cells seeded on the softest gel and the glass appeared smaller and not as well-adhered compared to cells on the 30 kPa gel (Fig. 23A). Indeed, cells on the 30 kPa gel were significantly more spread out compared to both 2 kPa and glass substrates (Fig. 23B). However, we found no other differences in cell morphology parameters between the stiffness groups, including circularity (Fig. 23C), solidity (Fig. 23D), and minor axis:major axis ratio (Fig. 23E). Full frequency distributions for these morphological parameters are also provided (Appendix A: Supplementary Material S1, Fig. 40).

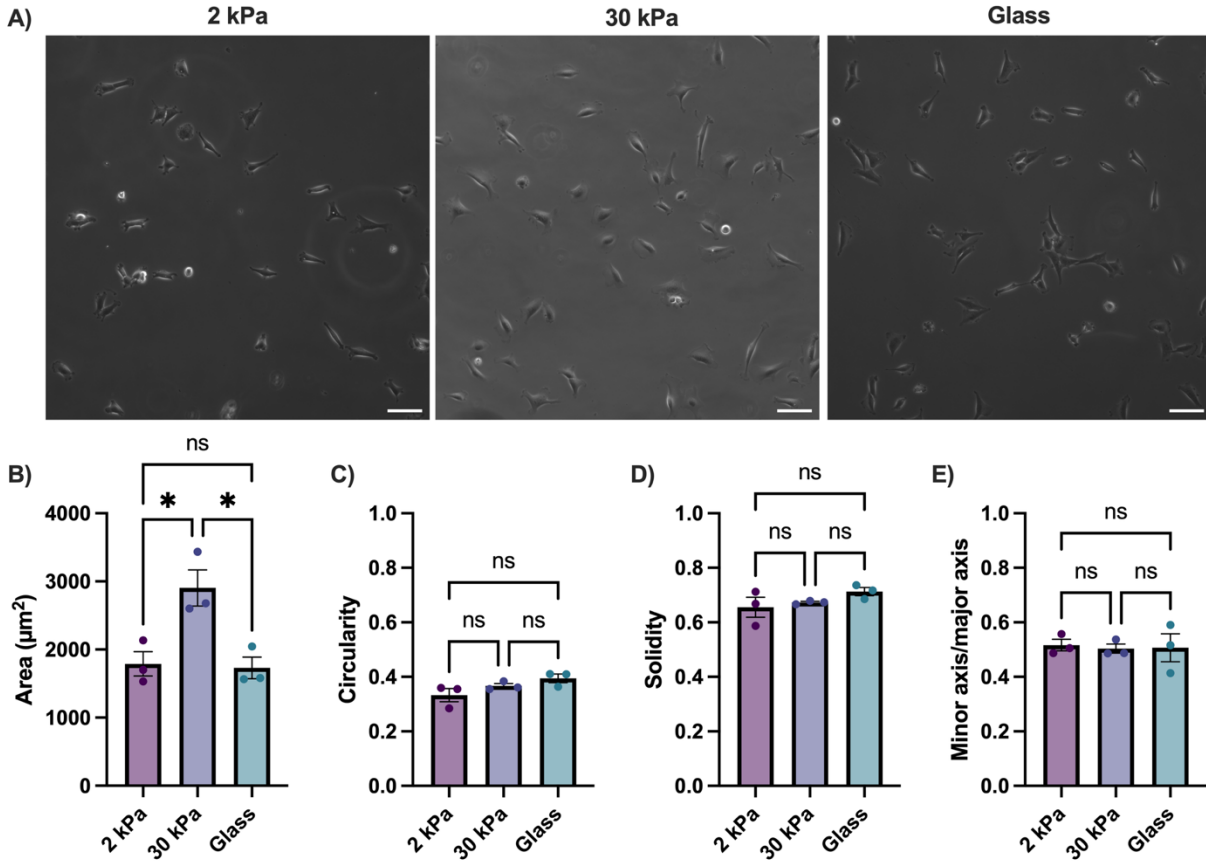


Figure 23: Effect of substrate stiffness on 12Z cell morphology. 12Z cells were cultured on substrates of varying stiffness overnight before assessing their morphology. **A)** Phase contrast images were taken of 12Zs on 2 kPa, 30 kPa, and glass substrates. We quantified the cell **B)** area, **C)** circularity, **D)** solidity, and **E)** ratio of the minor to major axis. Scalebars = 100 μm. Data points represent trials; error bars represent standard error of the mean. 30-40 cells were analyzed per stiffness per trial. * $P \leq 0.05$.

6.3.2. Stiff substrates enhance single and collective cell motility

We aimed to assess the effect of substrate stiffness on endometriotic cell motility. 12Z cells were seeded on 2 kPa, 30 kPa, and glass substrates and their motility was measured the following day using timelapse microscopy. Single cell random migration was measured using Manual Tracking in ImageJ. Individual cell trajectories from the entire 12-hr timelapse show that 12Zs

plated on the 30 kPa gel migrated the most extensively (Fig. 24A). The 30 kPa group contained a significantly greater proportion of fast-moving cells and a significantly reduced proportion of slow-moving cells compared to the 2 kPa and glass stiffnesses (Fig. 24B). Accordingly, cells migrated significantly faster on the 30 kPa gel compared to the other stiffnesses (Fig. 24C). Substrate stiffness did not impact cell directional persistence, defined as the end-to-end displacement divided by the total path length (Fig. 24D). For a representative subset of the cell trajectories, we plotted average cell speed over time and found that while overall speed stayed relatively steady from the beginning to the end of the 12-hr timelapse, cell speed tended to fluctuate with a period of 30-60 min across all stiffness groups (Fig. 24E). Individual cell speeds plotted over time also reflect this 30-60 min fluctuation timescale (Appendix A: Supplementary Material S2, Fig. 41).

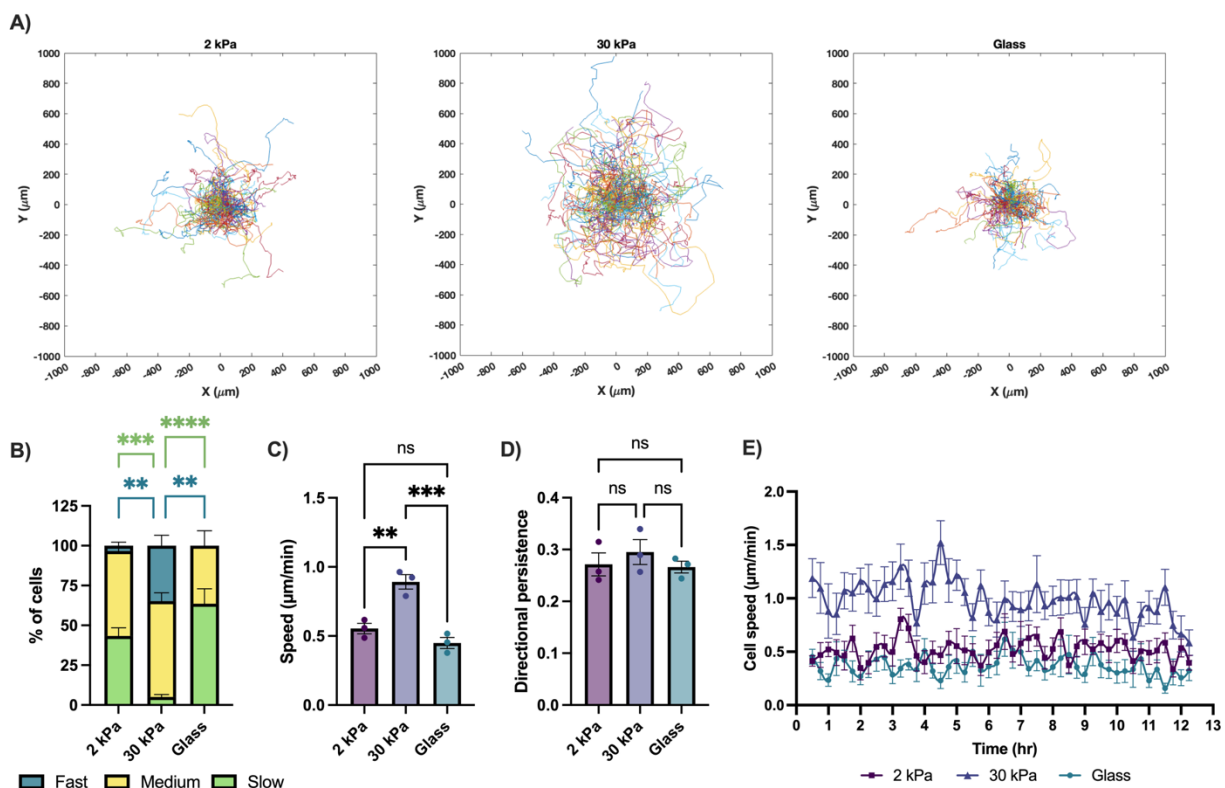


Figure 24: Single cell motility on different substrate stiffnesses. 12Z cells were cultured on substrates of varying stiffness overnight before measuring their random motility using timelapse microscopy for 12 hr. **A)** Trajectories of individual cells are plotted across all three trials. We quantified **B)** percentage of cells categorized as fast (speed > 1 μm/min), medium (0.5 μm/min < speed < 1 μm/min), or slow (speed < 0.5 μm/min), **C)** average cell speed, **D)** directional persistence, and **E)** cell speed over time, where each data point represents an average from a subset of 12 cells (4 cells per trial). Unless specified, data points represent trials; error bars represent standard error of the mean. 30-40 cells were analyzed per stiffness per trial. **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

We then aimed to study collective cell motility in a more physiologically relevant 3D model of endometriotic establishment. 12Z spheroids, approximately 250 μm in diameter, were seeded onto 2 kPa, 30 kPa, and glass substrates and allowed to settle for 1 hr prior to timelapse imaging. As cells disseminated from spheroids onto the substrate (Fig. 25A), the projected area was measured at multiple time points up to 20 hr (Fig. 25B). Spheroid outgrowth was significantly

increased on the 30 kPa and glass substrates compared to the 2 kPa at 2.5 hr (Fig. 25C). A similar trend persisted at 5 hr (Fig. 25D) but the differences in outgrowth began to level out thereafter (Fig. 25B). We also measured the speed of the leading edge of the spheroids. When averaged across the entire 20-hr timelapse, there were no differences in leading edge speed (Fig. 25E); however, leading edge speed increased with substrate stiffness during early (Fig. 25F) and early-mid establishment (Fig. 25G). In this context, “early” refers to the first 2.5 hr of spheroid spreading and “early-mid” refers to the first 5 hr of spheroid spreading.

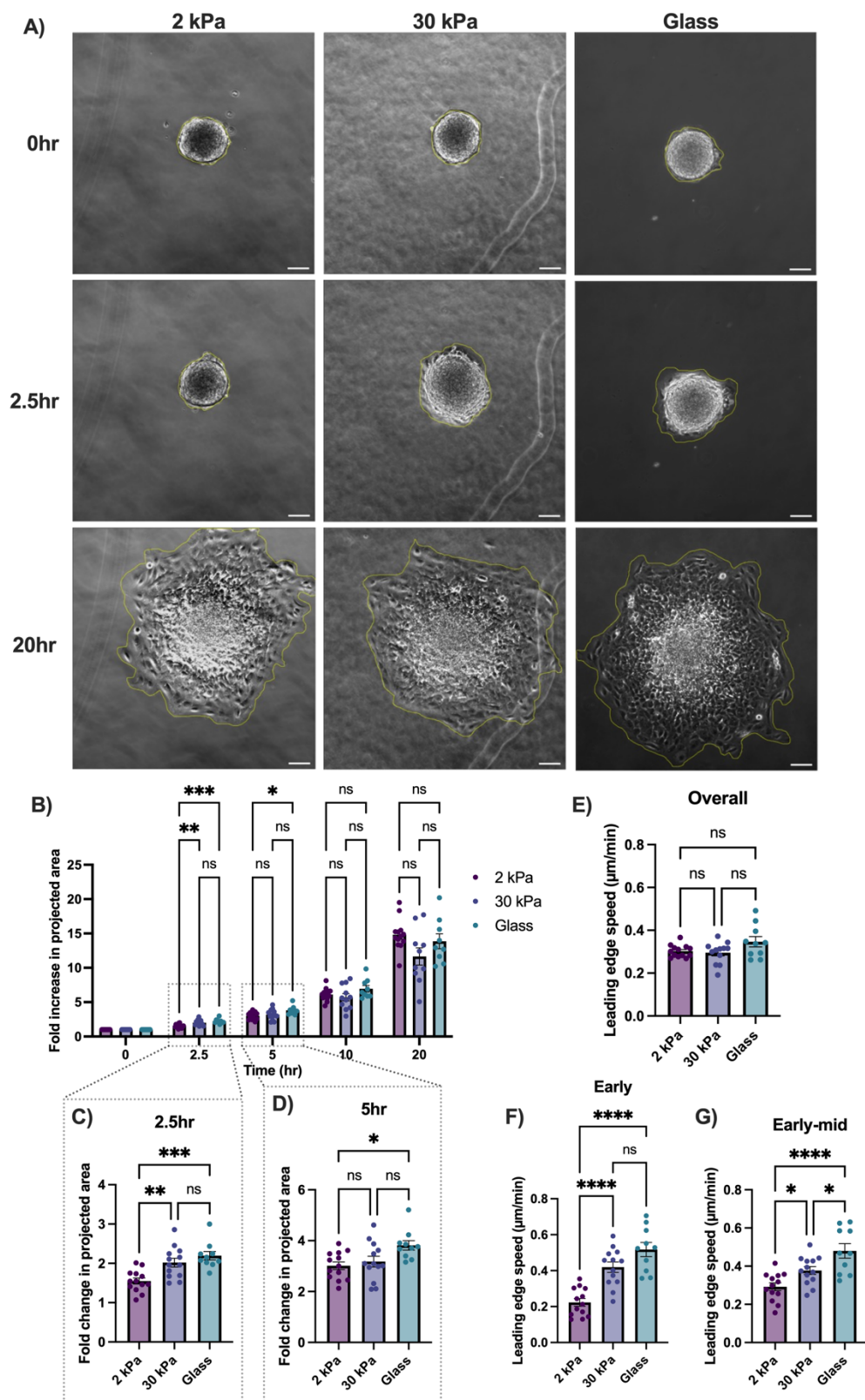


Figure 25: Effect of substrate stiffness on 12Z spheroid outgrowth. 12Z spheroids were seeded on substrates of varying stiffness and their outgrowth was measured using timelapse microscopy for 20 hr. **A)** Phase contrast images show spheroids on 2 kPa, 30 kPa, or glass substrates at $t = 0$ h, $t = 2.5$ hr, and $t = 20$ hr. We quantified **B)** the fold increase in projected spheroid area at multiple time points. We provide a zoomed in view of the fold change in projected area at **C)** $t = 2.5$ hr and **D)** $t = 5$ hr. We also measured the speed of the leading edge of the spheroid, averaging across **E)** the whole 20-hr timelapse, **F)** the first 2.5 hr, and **G)** the first 5 hr. Scalebars = 100 μ m. Data points represent spheroids pooled across three trials; error bars represent standard error of the mean. 10-15 spheroids were analyzed per stiffness. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

6.3.3. Substrate stiffness alters actin stress fiber presentation, membrane structures, and focal adhesions

To probe the mechanism(s) underlying differences in cell motility on different substrate stiffnesses, we analyzed cytoskeletal components in the 12Zs. Cells were plated on 2 kPa, 30 kPa, and glass substrates and fixed the following day. A fluorescent stain and ICC were used to visualize F-actin and the focal adhesion protein paxillin, respectively, in the 12Zs. Confocal images show altered actin stress fiber presentation, membrane structures, and focal adhesions across the three stiffnesses (Fig. 26A). Actin stress fibers were rarely present in 12Zs cultured on 2 kPa and glass substrates, while the majority of 12Zs on the 30 kPa substrate exhibited clear stress fibers (Fig. 26B). Of the cells that had any actin stress fibers, we observed that those on the 2 kPa gels usually had only 1-3 visible fibers, while the 30 kPa and glass exhibited more. We therefore quantified the fraction of stress fiber-presenting cells that exhibited more than 5 clear stress fibers to confirm this observation (Fig. 26C). We also observed bright spots of actin towards the periphery of cells on the 2 kPa substrates—which we call “actin puncta”—that were much less prominent on the 30 kPa (Fig. 26D). Additionally, paxillin images show that focal adhesion density was significantly

higher in cells on the 30 kPa gels than 2 kPa and glass (Fig. 26E). These focal adhesions were also larger in area (Fig. 26F).

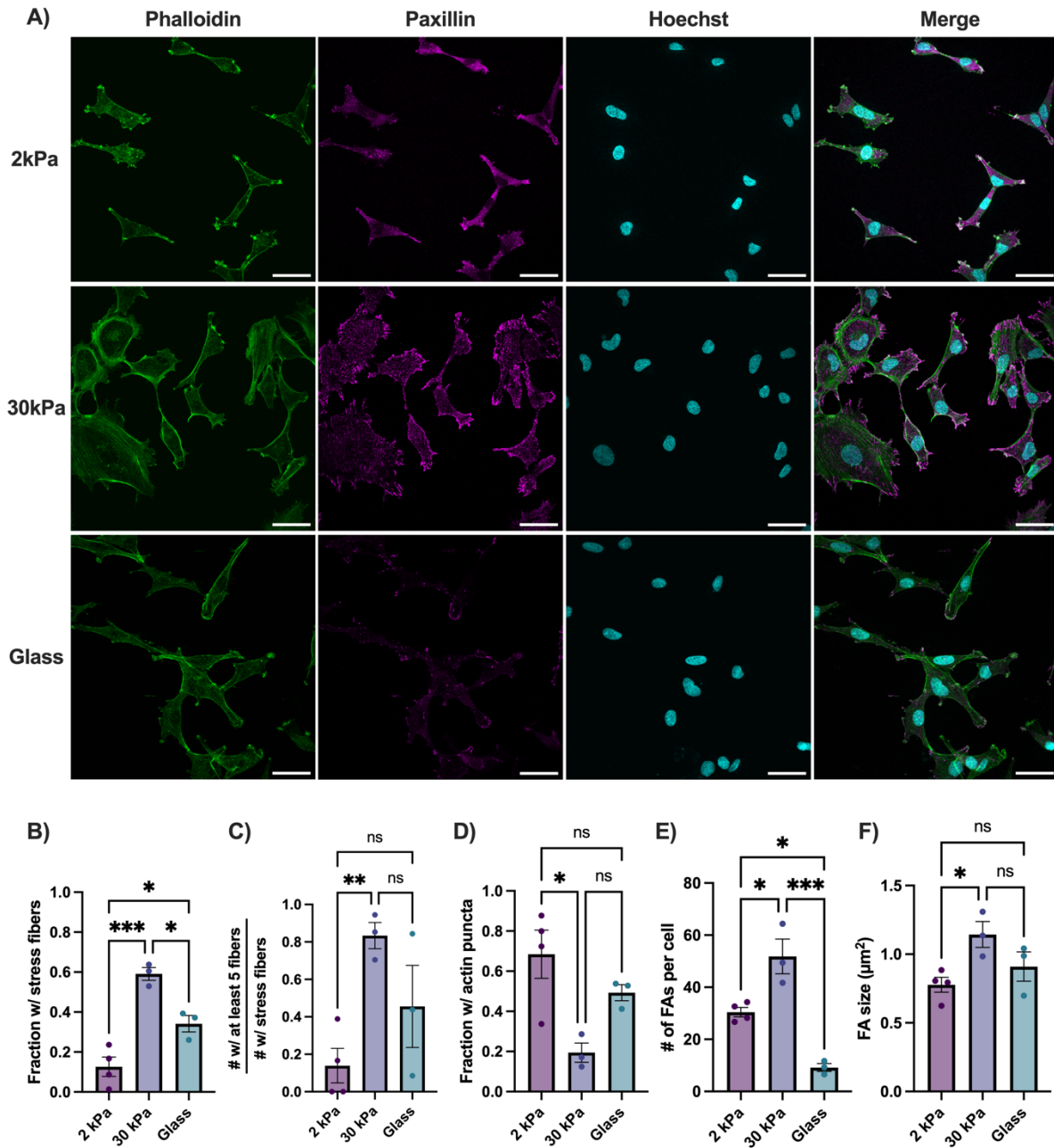


Figure 26: Impact of substrate stiffness on F-actin organization, membrane structures, and focal adhesions. 12Z cells were cultured on substrates overnight prior to fixation and fluorescence

microscopy. **A)** Confocal images show F-actin (Phalloidin), focal adhesion proteins (paxillin), and nuclei (Hoechst) for cells on 2 kPa, 30 kPa, and glass substrates. We quantified **B)** the fraction of cells exhibiting actin stress fibers, and **C)** the fraction of stress fibered cells exhibiting more than 5 stress fibers, **D)** the fraction of cells exhibiting punctate actin membrane structures, **E)** the average number of focal adhesions per cell, and **F)** the focal adhesion size. Scalebars = 50 μm . Data points represent trials; error bars represent standard error of the mean. 4-5 images were analyzed per stiffness per trial. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. FA; focal adhesion.

During EMT, cells lose their epithelial characteristics and adopt mesenchymal phenotypes such as elongated morphology, increased migration, and upregulation of markers such as vimentin. Figure 23 demonstrated that cell morphology, other than area, did not change on the different stiffnesses. However, differences in migration were revealed on the three substrates (Fig. 24, 25). To further assess any potential EMT phenotypic changes, we cultured 12Z cells on 2 kPa, 30 kPa, and glass substrates and performed ICC to stain for vimentin, a mesenchymal marker. No significant change in vimentin expression was observed between cells on the 2 kPa compared to the 30 kPa. This suggests that the increase in cell motility on the 30 kPa gel is not driven by EMT-associated phenotypic changes. (Fig. 27).

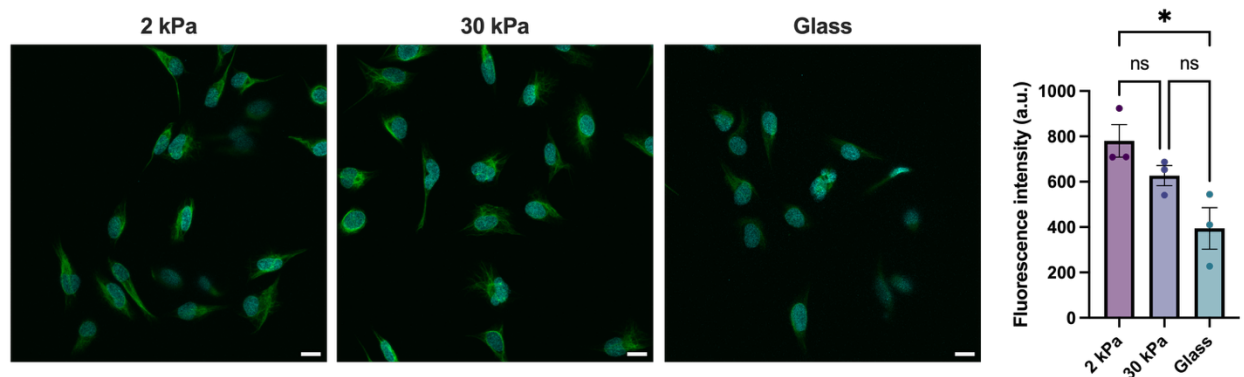


Figure 27: Vimentin expression in 12Zs cultured on varying substrate stiffnesses. 12Z cells were cultured on substrates of varying stiffness overnight before assessing their vimentin (green) expression with ICC. Nuclei are stained cyan. We quantified average fluorescence intensity of vimentin in the images. Scalebars = 20 μm . Data points represent independent trials; error bars represent standard error of the mean. * $P \leq 0.05$.

6.3.4. Pharmacological modulation of contractility alters cell motility on soft and stiff substrates

To further validate our earlier finding that substrate stiffness impacts cell motility due to alterations in cell cytoskeletal components (Fig. 26) we reassessed 12Z motility with pharmacological modulation of cellular contractility. Cells were seeded on 2 kPa and 30 kPa gels and treated and imaged the following day. LPA—an activator of GPCRs that activate pathways such as Rho/ROCK, PI3K/Akt, and MAPK/ERK—was applied to cells on the 2 kPa gels at a 5 μ M dose to increase contractility. The selective ROCK inhibitor Y27632 was applied to cells on the 30 kPa gels at a concentration of 10 μ M to decrease contractility. Alteration of contractility by LPA or Y27632 was verified by analysis of phosphorylated myosin light chain (pMLC) expression (Appendix A: Supplementary Material S3, Fig. 42). Samples were only imaged for 4 hr due to the propensity of LPA to bind plastic and thus get depleted in the well. Phase contrast images of cells treated for ~2 hr revealed that 30 kPa-grown cells were more spread out on the substrate compared to 2 kPa, consistent with our earlier finding (Fig. 23B), and that 30 kPa cells treated with Y27632 had a distinctly shrunken appearance (Fig. 28A). LPA increased the random migration of 12Zs on the 2 kPa gels while Y27632 decreased migration on the 30 kPa gels, as seen in the single-cell trajectories (Fig. 28B). Accordingly, 2 kPa cell speed was significantly increased with LPA treatment while 30 kPa cell speed was significantly reduced with Y27632 treatment (Fig. 28C). The contractility modulators had no effect on directional persistence of cells (Fig. 28D). Similar to our result in Fig. 24E, cell speed fluctuated over time with a period of 30-60 min across all groups (Fig. 28E).

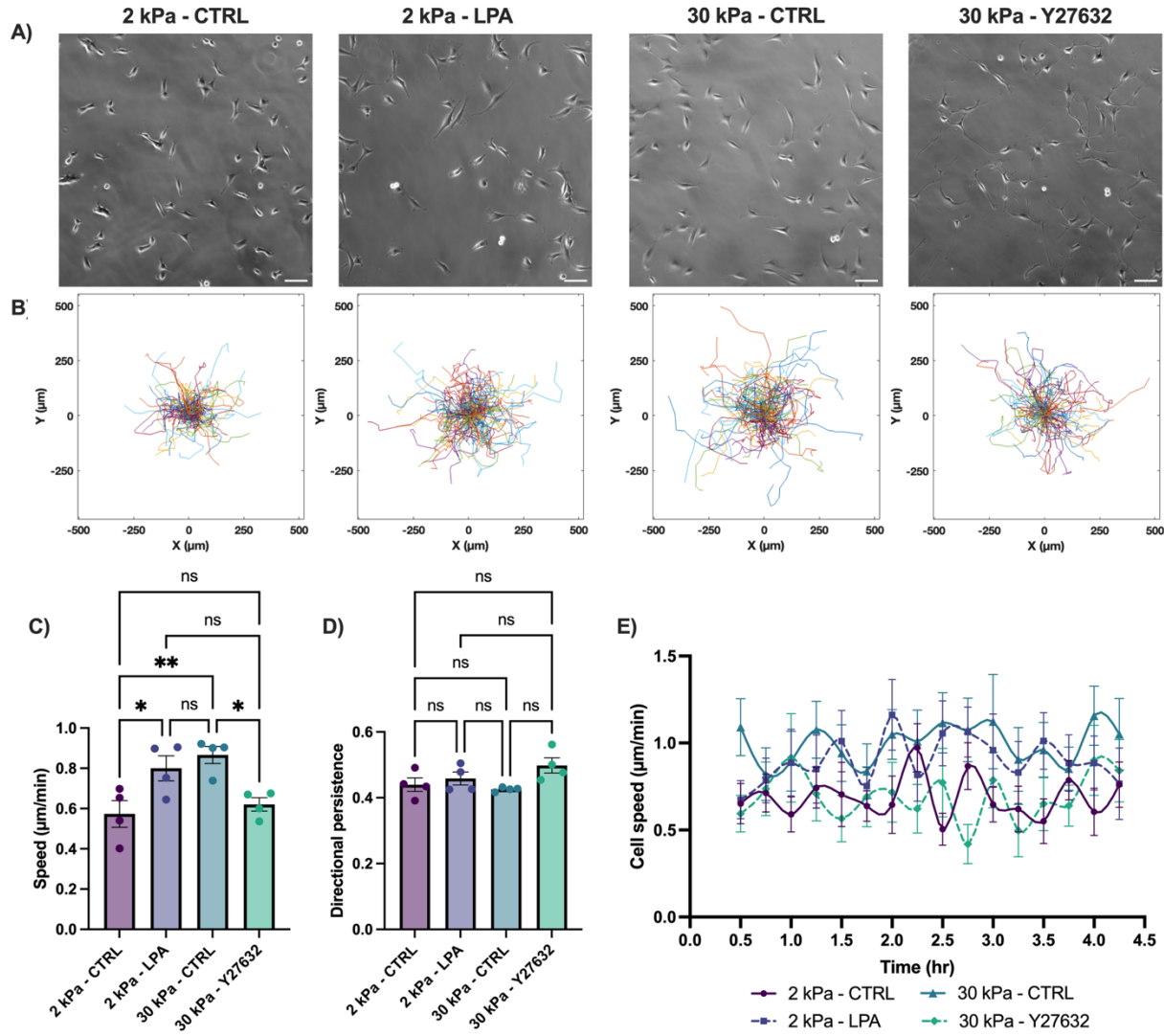


Figure 28: Role of cell contractility in substrate stiffness-mediated cell motility. 12Z cells were cultured on 2 kPa and 30 kPa substrates overnight before contractility modulators—LPA and Y27632, respectively—were added and cell motility was measured via timelapse microscopy for 4 hr. **A)** Phase contrast images show 12Zs cultured on 2 kPa and 30 kPa after ~2 hr of treatment with the vehicle or drug. **B)** Trajectories of individual cells are plotted across all trials. We quantified **C)** average cell speed, **D)** directional persistence, and **E)** cell speed over time, where each data point represents an average from a subset of 12 cells (3 cells per trial). Scalebars = 100 μm . Unless specified, data points represent trials; error bars represent standard error of the mean. 30-40 cells were analyzed per stiffness per trial. * $P \leq 0.05$, ** $P \leq 0.01$. CTRL; vehicle control. LPA; lysophosphatidic acid.

6.4 DISCUSSION

In this study, we aimed to study the role of fibrotic tissue stiffening in endometriotic epithelial cell motility. Our data show that fibrotic substrate stiffness increases the migratory behavior of endometriotic epithelial cells for both single-cell and multicellular spheroids. 12Zs cultured on the 30 kPa PA gel demonstrated greater cell spreading area, increased speed, prominent actin stress fibers, enhanced focal adhesions, and greater early spheroid spreading compared to cells on the soft 2 kPa gel. These data suggest that fibrotic tissues may enhance cell migration by optimizing cytoskeletal architecture to generate contractile forces. These findings are particularly relevant for endometriosis, where epithelial cells encounter fibrotic microenvironments yet may not undergo full EMT, suggesting that mechanical regulation of migration may play a dominant role.

However, it is worth noting that for the most part, substrate stiffness did not enhance migratory cell phenotypes without limit. While cells migrated faster and had larger area on 30 kPa gel compared to those on the soft gel, cell speed and area was significantly reduced on glass compared to 30 kPa. This non-monotonic trend persisted with cytoskeletal arrangement as well: actin stress fibers and focal adhesions were most pronounced on the 30 kPa gel, with reduced paxillin and stress fibers on the 2 kPa and glass substrates. These findings are similar to other key mechanobiological studies that show that migratory cell phenotypes are impaired on extremely soft and extremely stiff substrates, and that optimal migration occurs on an intermediate stiffness or adhesion strength [303,312–315]. Mechanistically, cell-substrate interactions and migration are regulated by actomyosin contractility, focal adhesion dynamics, and substrate stiffness. On soft substrates, cells are unable to generate sufficient traction forces due to substrate deformation,

resulting in limited spreading and motility. On substrates with intermediate stiffness, such as 30 kPa, traction forces can be effectively transmitted and adhesions dynamically remodeled, enabling maximal migration and cell spreading. However, on extremely stiff substrates such as glass, adhesion dynamics may become impaired due to excessive stabilization of focal adhesions and reduced turnover. This results in suboptimal migration despite increased stiffness. Future work should investigate integrin availability and focal adhesion turnover within the cells on the soft, stiff, and glass substrates.

Our only finding that did not reflect this biphasic phenomenon was that both 30 kPa and glass promoted early (2.5-5 hr) 12Z spheroid expansion, with glass exhibiting the greatest outgrowth and leading edge speed. In fact, spheroids on glass exhibited a significantly faster leading edge compared to both 30 kPa and 2 kPa during the first 5 hr of expansion. This early outgrowth on glass suggests that collective migration may rely on different force-generation mechanisms than single-cell migration, potentially involving population-level tension or cooperative adhesion dynamics. Future work could shed light on the interplay between cell-cell tension versus cell-matrix adhesive forces during spheroid expansion on different substrate stiffnesses.

Our results suggest that stiffness-dependent increases in epithelial migration can occur without evidence of EMT, as vimentin expression was unchanged between soft and intermediate stiffness conditions. This suggests that enhanced migration arises from cytoskeletal and contractility-based mechanisms rather than transcriptional phenotypic switching.

Pharmacological modulation of contractility further supported a mechanistic role for cytoskeletal force generation in stiffness-driven cell migration. Increasing contractility and

migration pathways in cells on the 2 kPa gel led to an increase in speed, approximately equal to the speed of cells cultured on the 30 kPa gel. Conversely, cells on the 30 kPa gel experienced a reduction in speed, approximately equal to the speed of cells on the 2 kPa gel, when treated with a contractility inhibitor. These results indicate that substrate stiffness tunes the level of intracellular tension required for efficient epithelial migration and are consistent with findings in other mechanobiological studies that modulated contractility [313,316–319].

Limitations and caveats of this work must be addressed. First, the mechanomemory of 12Z cells is not well defined. We cultured cells (previously grown on plastic) on substrates overnight before performing assays which appeared to be enough time to visualize cellular changes, but future studies should clarify the duration of mechanical memory in endometriotic epithelial cells. Spheroid outgrowth in 3D could not be assessed due to the cytotoxic nature of PA gels; future work could embed 12Z spheroid in collagen gels of increasing stiffnesses to study mechanically driven invasion in 3D. Third, the use of LPA to enhance contractility, while informative, affects multiple signaling pathways and therefore does not isolate a mechanotransduction pathway. Future studies could focus on dissecting the role of specific signaling pathways in stiffness-mediated cell motility. This work creates another avenue for future research; the small actin puncta we observed frequently in cells on the 2 kPa and glass substrates could be further characterized. We speculate that they may represent points of adhesion to the substrate, since cells cannot spread fully and have minimal contact with the substrate.

6.5 CONCLUSION

The data in this chapter identify robust and reproducible mechanobiological principles governing how endometriotic cells reorganize their cytoskeleton and migrate within fibrotic mechanical microenvironments. Understanding how substrate stiffness regulates multiscale cell motility may shed light on the pathogenesis of endometriosis and potential therapeutic targets.

However, the establishment of ectopic lesions and implants in endometriosis is not only dependent on endometriotic cell motility, but also on the receptivity of the peritoneal mesothelium that lines the pelvic cavity. The mesothelium is a layer of epithelial cells that acts as a protective barrier but can be compromised in endometriosis. The effects of fibrotic environments on mesothelial barrier function is the subject of investigation in the next chapter.

Chapter 7: Substrate stiffness enhances endometriotic epithelial implantation into the mesothelium

7.1 INTRODUCTION

Endometriosis is a prevalent gynecological condition characterized by the abnormal growth of endometriotic tissue outside of the uterus, leading to inflammation and fibrosis [7,57]. Endometriotic tissue fragments attach to the peritoneal mesothelium lining the pelvic cavity and form ectopic lesions and implants that cause chronic inflammation and tissue damage. Successful lesion formation requires endometriotic cells to breach the mesothelium, an epithelial cell layer that serves as a protective barrier in healthy contexts. While hormonal and inflammatory factors contributing to this process have been extensively studied, the role of the mechanical microenvironment in regulating mesothelial barrier integrity and susceptibility to endometriotic implantation remains poorly understood.

Fibrosis is a key hallmark of endometriosis, resulting in tissue scarring and stiffening. The elasticity of healthy endometrial tissue is reported to be 1.97 kPa in the secretory phase and 3.34 kPa in the proliferative phase [146]. Data on fibrosis of the lung, liver, and bowel indicate that the Young's modulus can increase from 1–3 kPa (normal tissue) to 16.5–20 kPa (fibrotic tissue) [147–151]. In other epithelial and endothelial systems, increased substrate stiffness has been shown to alter cytoskeletal tension, junctional organization, and barrier function [178,320–322]. However, whether fibrotic matrix stiffness directly modulates the ability of the mesothelium to resist

endometriotic epithelial implantation has not been systematically investigated. Moreover, it is unclear whether endometriotic establishment *in vivo* occurs only through multicellular aggregates or also via single cell invasion—both modes of endometriotic establishment remain to be tested against mesothelial barrier function in fibrotic environments.

In preceding chapters, we demonstrated that substrate stiffness enhances endometriotic epithelial motility and contractility, particularly under fibrotic-range mechanical conditions. These findings raise the possibility that stiffened tissues may promote endometriosis progression not only by increasing the migratory capacity of endometriotic cells, but also by altering the susceptibility of the mesothelium itself. In this chapter, we developed an *in vitro* co-culture model to examine how substrate stiffness holistically influences endometriotic epithelial implantation into mesothelial monolayers. Human Met-5A mesothelial monolayers were cultivated on soft (healthy) and stiff (fibrotic) polyacrylamide (PA) gels and glass as an extreme stiffness, then challenged with either individual 12Z endometriotic epithelial cells or multicellular spheroids. In parallel, we assessed changes in the morphology of incorporated endometriotic cells and examined mesothelial tight junction organization—both in isolation and after co-culture with endometriotic cells—as a proxy of barrier integrity.

This chapter seeks to define how mechanical stiffening of the microenvironment modulates the early stages of endometriotic establishment within the peritoneal mesothelium. By focusing on barrier-level mechanics and collective cell interactions, this work extends prior mechanobiological studies of endometriosis beyond endometriotic cell motility to include mesothelial susceptibility during lesion initiation.

7.2 METHODS

7.2.1. Polyacrylamide hydrogel preparation

Polyacrylamide (PA) hydrogels were formed on glass coverslips as previously described.[302,303] Briefly, 22 x 22 mm glass coverslips were passed through a flame to remove contaminants before being smeared with 0.1M NaOH. Coverslips were then silanized with 3-aminopropyltriethoxysilane (Sigma Aldrich) and activated with 0.5% glutaraldehyde (Sigma Aldrich) in phosphate buffered saline (PBS) for 30 min to enable PA hydrogel attachment. 2 kPa and 30 kPa PA hydrogel precursor solutions contained certain amounts of 40% acrylamide (Bio-Rad), 2% bis-acrylamide (Bio-Rad), 1M HEPES (Gibco), and ultrapure water mixed together. Final concentrations were 4% acrylamide and 0.14% bis-acrylamide for the soft gel; 12% acrylamide and 0.28% bis-acrylamide for the stiff gel. Elastic moduli of PA gels were estimated via submersion compression testing using a Dynamical Mechanical Analyzer 850. Measurements were 1.223 ± 0.201 kPa for the soft gel (called 2 kPa in this manuscript) and 29.81 ± 2.338 kPa for the stiff gel (called 30 kPa in this manuscript). PA hydrogel precursor solutions were syringe-filtered (0.2 μ m filter) and degassed for 20 min. 10% ammonium persulfate (Bio-Rad) in water and N,N,N',N'-Tetramethylethylenediamine (TEMED; Bio-Rad) were added to the solutions to polymerize ~80- μ m-thick PA hydrogels on coverslips. PA hydrogels were allowed to polymerize for 30 min before activating with sulfo-SANPAH (Thermo Scientific) to cross-link the extracellular matrix protein to the PA hydrogel surface, as previously described [303]. PA hydrogels were coated with 50 μ g/mL collagen I from rat tail (Corning) over night at 4°C. For experiments on glass, 22 x 22 mm glass coverslips were coated in 50 μ g/mL collagen I overnight

at 4°C. Validation of equal coating across gels and coverslips was performed using a biotinylated collagen coating, applying avidin conjugated to Texas Red, and imaging under fluorescence microscopy. All PA hydrogels and coverslips were stored in PBS at 4°C for up to 10 days prior to cell seeding. PA hydrogels and coverslips were equilibrated in full growth medium at 37°C for at least 45 min prior to cell seeding. For all experiments, Met-5A monolayers were grown by seeding approximately 250,000 cells per gel and allowing monolayers to form over 72 hr.

7.2.2. Cell culture and seeding

The 12Z human endometriotic epithelial cell line was purchased from Applied Biological Materials (Cat. # T0764). Cells were cultured in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). The cell line was mycoplasma free and authenticated by the company prior to shipping. Cell line authentication and mycoplasma testing were performed again at least 6 months afterwards. Cells from passages 55-80 were used for experiments.

7.2.3. Single-cell incorporation assay

12Z cells were stained with 25 µg/mL CellTracker Green CMFDA (ThermoFisher, Cat. # C7025) for 30 min in serum-free medium. 12Zs were then trypsinized and approximately 40,000 cells were seeded per well on top of Met-5A monolayers grown on 2 kPa, 30 kPa, and glass substrates at the start of imaging. Phase contrast and fluorescent channels were acquired every 10 min for 12 hr.

7.2.4. Quantification of cellular morphology

Morphological parameters including cell area, perimeter, solidity, and roundness were extracted using ImageJ to manually trace cells from phase contrast microscope images. Solidity was defined as $solidity = \frac{Cell\ area}{Convex\ hull\ area}$, where a cell that is perfectly circular would have a value of 1.0 and a cell that has protrusions and/or indentations would have a value closer to 0. The minor axis to major axis ratio represents the inverse of aspect ratio, where a highly elongated cell would have a value close to 0.

7.2.5. Spheroid incorporation assay

Approximately 48 hr prior to the start of the experiment, 12Z cells were stained with 25 µg/mL CellTracker Green CMFDA (ThermoFisher, Cat. # C7025) for 30 min in serum-free medium, unless otherwise specified. Cells were then trypsinized and 2,000-2,500 12Zs were seeded per well in an ultra-low attachment, round-bottom 96-well plate (Corning), which was then centrifuged at 150 xg for 2 min. Cells self-assembled into spheroids over the next ~48 hr of incubation. Resulting spheroids were approximately 200-250 µm in diameter. Then, Met-5A monolayers on substrates were stained with CellTracker Orange CMRA (ThermoFisher, Cat. # C34551) for 30 min in serum-free medium, unless otherwise specified. 8-10 spheroids were seeded per well within a 6-well plate and allowed to settle on mesothelial monolayers for 1 hr prior to imaging. Phase contrast, green fluorescent, and red fluorescent channels were then obtained for 20 hr, with a time step of 20 min. The projected area of the spheroids at multiple time points was measured using ImageJ.

7.2.6. Immunocytochemistry

Immunocytochemistry (ICC) was utilized to visualize tight junction localization. Briefly, cells were then fixed in 4% paraformaldehyde for 20 min on ice with gentle agitation, washed with PBS, and permeabilized in 0.2% TritonX-100 (Sigma-Aldrich) for 5 min. Samples were washed with PBS again and blocked in 2.5% goat serum (Abcam) for 1 hr at room temperature (RT). The primary antibody for ZO-1 (ThermoFisher Scientific) was diluted in 2.5% goat serum at 1:100, added to the samples, and incubated overnight at 4°C. The following day, samples were washed with PBS and incubated for 1-1.5 hr with Hoechst (1:10,000; ThermoFisher Scientific) and the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:500; ThermoFisher Scientific) diluted in 2.5% goat serum at RT. Samples were then washed with PBS and imaged with confocal microscopy. Junctional fluorescence for each cell was measured from segmented images in ImageJ.

7.2.7. Microscopy

For all timelapse imaging, live cells were imaged under phase contrast and/or fluorescence using the 10X objective on an Olympus IX-83 inverted microscope (Olympus, Center Valley, PA, USA) and the Olympus cellSens software. Cells were maintained at 37°C, 65% humidity, and 5% CO₂ in an enclosed chamber surrounding the microscope. For confocal microscopy, fixed samples were imaged at 40X (unless otherwise specified) under fluorescence using an FV3000 Laser Scanning Confocal Microscope.

7.2.8. Statistical analysis

GraphPad Prism 10 (La Jolla, CA, USA) was utilized to generate graphs and perform statistical analyses. Outliers were identified using the ROUT method with a Q (maximum desired false discovery rate) = 1% only to remove definite outliers. Data were tested for normality. For comparisons between three or more groups, ordinary one-way ANOVA with a Tukey's multiple comparisons test was performed. For comparisons between stiffness groups and time points, Ordinary Two-way ANOVA was performed with a Tukey's multiple comparisons test. Corresponding non-parametric tests were used for non-normally distributed datasets. For all tests, P values < 0.05 were considered statistically significant. Data are represented as mean $\pm$ standard error of the mean. All experiments were performed in at least triplicates.

7.3 RESULTS

7.3.1. Met-5As form confluent monolayers on substrates by 72 hours irrespective of substrate stiffness

We first sought to reproducibly grow and characterize mesothelial monolayers on PA gels with tunable stiffness and glass as an extreme stiffness. Mesothelial Met-5A monolayers were grown on 2kPa, 30kPa, and glass substrates for 72 hr and imaged with phase contrast microscopy. Despite seeding equal numbers of cells across substrates, early (24-hr) monolayers were relatively sparse on the soft gel and glass compared to 30 kPa. The 2 kPa and glass substrates more frequently exhibited small, rounded, non-attached cells compared to the stiff gel at 24 hr, but these differences resolved by 72 hr, at which point all substrates supported confluent or nearly confluent monolayers (Fig. 29A). Quantification of coverage, or the fraction of the image taken up by cells, also

demonstrates that 2 kPa and glass substrate monolayers were significantly less developed at 24 and 48 hr—but not 72 hr—compared to 30 kPa monolayers (Fig. 29B). To approximate the degree of cell spreading on the substrates, we divided the total coverage by the number of cells in each image. At 24 hr post-seeding, cells took up significantly less area on the 2 kPa and glass substrates compared to 30 kPa, but no differences in coverage per cell were found at 48 or 72 hr post-seeding (Fig. 29C). Together, these data indicate that a fibrotic substrate stiffness influences early mesothelial monolayer formation, but by 72 hr, Met-5A monolayers are confluent and contain approximately equal cell sizes irrespective of substrate stiffness (Fig. 29). Therefore, for all subsequent experiments, we chose to cultivate Met-5As for 72 hr to achieve equal monolayers across stiffness conditions.

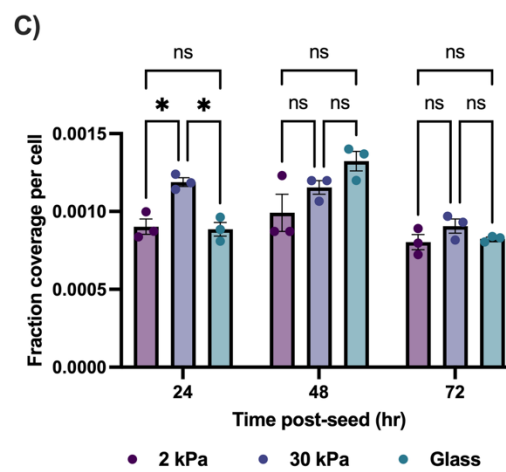
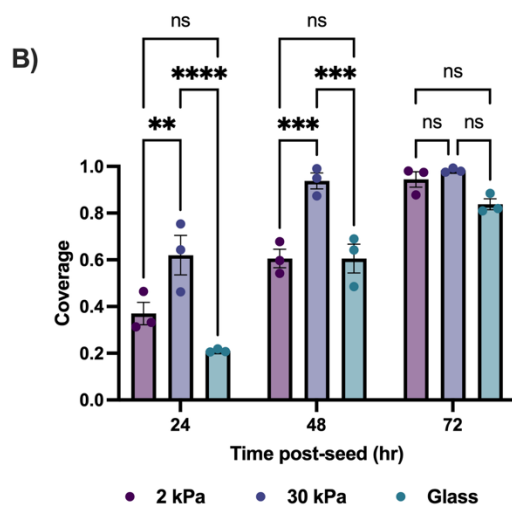
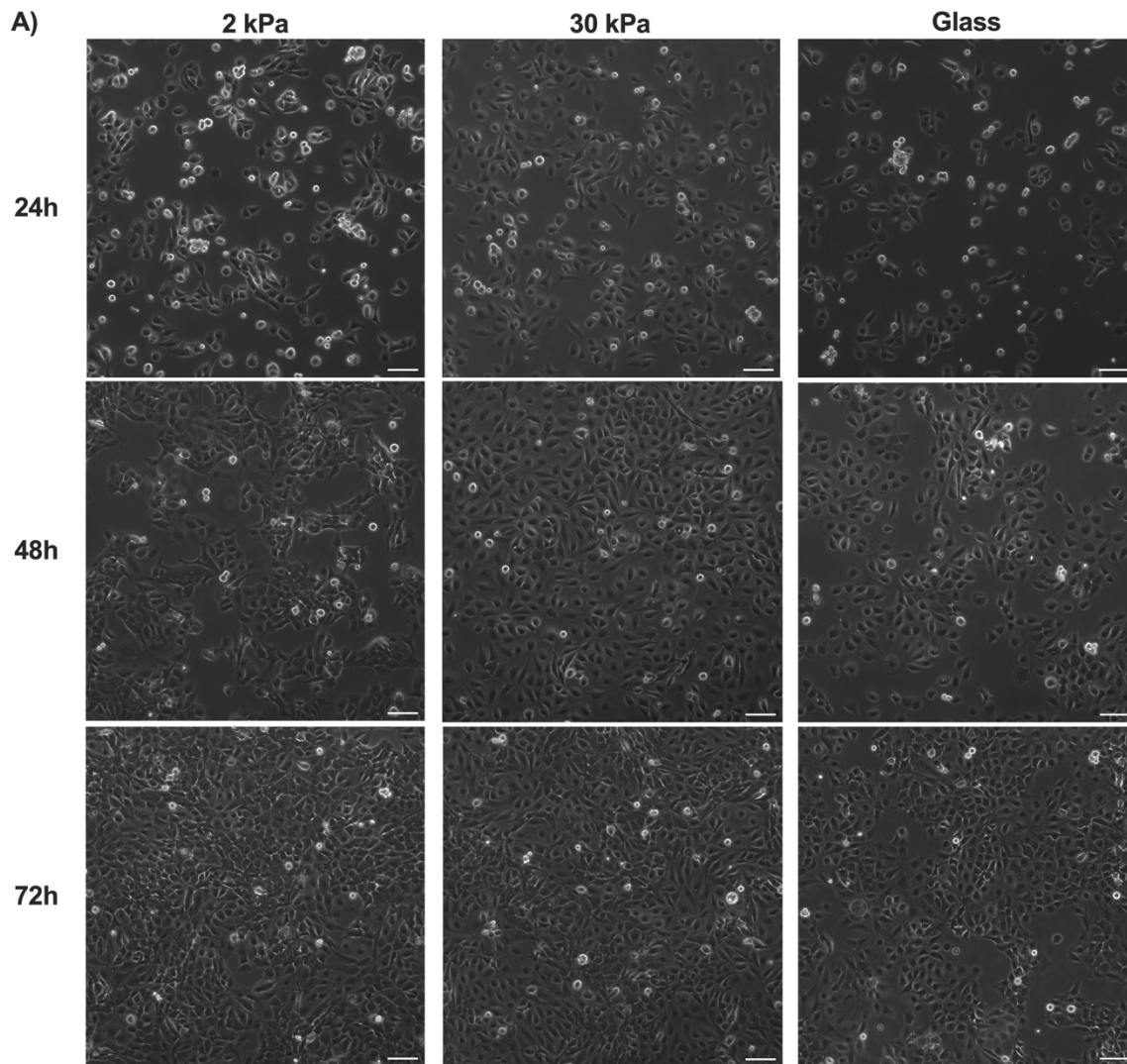


Figure 29: Mesothelial monolayer development over time on 2 kPa, 30 kPa, and glass substrates was assessed. Met-5As were seeded onto substrates and imaged at 24, 48, and 72 hr post-seeding. **A)** Representative phase contrast images showing Met-5A monolayers growing to confluence over three days. **B)** Coverage, or percent image area taken up by cells, was quantified across stiffness groups and time points. **C)** Coverage was divided by the number of cells in the image to obtain fraction coverage per cell. Scalebars = 100 μ m. Data points represent trials; error bars represent standard error of the mean. For each time point, 3-5 images were analyzed per stiffness per trial. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

7.3.2. Substrate stiffness does not impact mesothelial barrier function against single endometriotic epithelial cells

To test the effect of substrate stiffness on barrier function of mesothelial monolayers, we first seeded 12Z human endometriotic epithelial cells stained with a green cell tracker on top of Met-5A monolayers on substrates and performed timelapse microscopy. Phase contrast and fluorescence overlaid images show that 12Zs are rounded and not incorporated at 0 hr (the time of seeding) but are generally more adhered and incorporated into the mesothelium at 6 hr (Fig. 30A). Zoomed-in images show examples of non-incorporated 12Z cells (red arrows) and fully incorporated 12Z cells (white arrows) (Fig. 30B). For each timelapse, we defined the fraction incorporated as the number of 12Z cells attached within the mesothelium divided by the total number of 12Zs in the initial frame of the timelapse. We quantified incorporation at 6 hr post-seeding and did not find any differences between substrate stiffnesses (Fig. 30C).

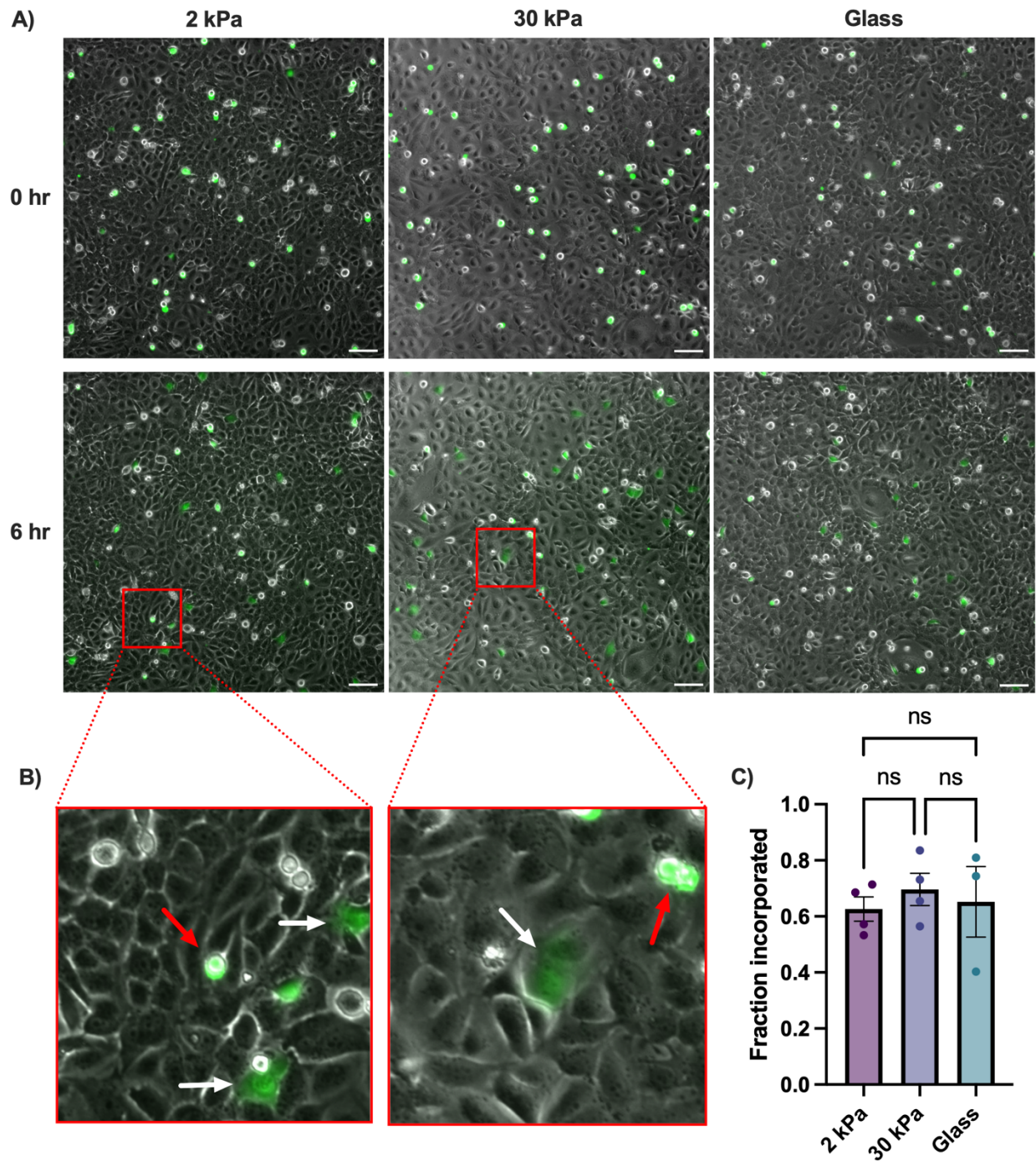


Figure 30: Effect of substrate stiffness on incorporation of single 12Z cells into mesothelial monolayers. Mesothelial monolayers were grown on 2 kPa, 30 kPa, and glass substrates and 12Z cells (stained green) were seeded on top at the start of timelapse imaging. **A)** Overlaid phase

contrast and fluorescence images show 12Zs (green) on top of the mesothelial monolayers at 0 hr post-seed and 6 hr post seed. **B)** Enlarged images show non-incorporated 12Zs (red arrows) and incorporated 12Zs (white arrows). **C)** We quantified fraction of 12Zs incorporated into the monolayer by 6 hr post-seed. Scalebars = 100 μm . Data points represent trials; error bars represent standard error of the mean. 3-6 images were analyzed per stiffness per trial. ns, $P > 0.05$.

7.3.3. Substrate stiffness modestly impacts the morphology of incorporated endometriotic epithelial cells

We sought to characterize the effect of substrate stiffness on the morphology of 12Z cells that successfully incorporated into the mesothelial monolayers at 6 hr post-seeding. We previously demonstrated that 12Z cell area is increased on 30 kPa substrates compared to 2 kPa and glass, while no other morphological differences were found (Chapter 6). Although all monolayers were confluent during endometriotic incorporation, 12Z cells incorporated into glass-grown monolayers were significantly smaller in area compared to 30 kPa. While not statistically significant, we also observed a similar trend to our result in Chapter 6—an increase in cell area on 30 kPa compared to the soft gel (Fig. 31A). No other differences in cell morphology, including circularity (Fig. 31B), solidity (Fig. 31C), and minor axis to major axis ratio (Fig. 31D), were observed between 12Zs incorporated into the different stiffness-bound mesothelia.

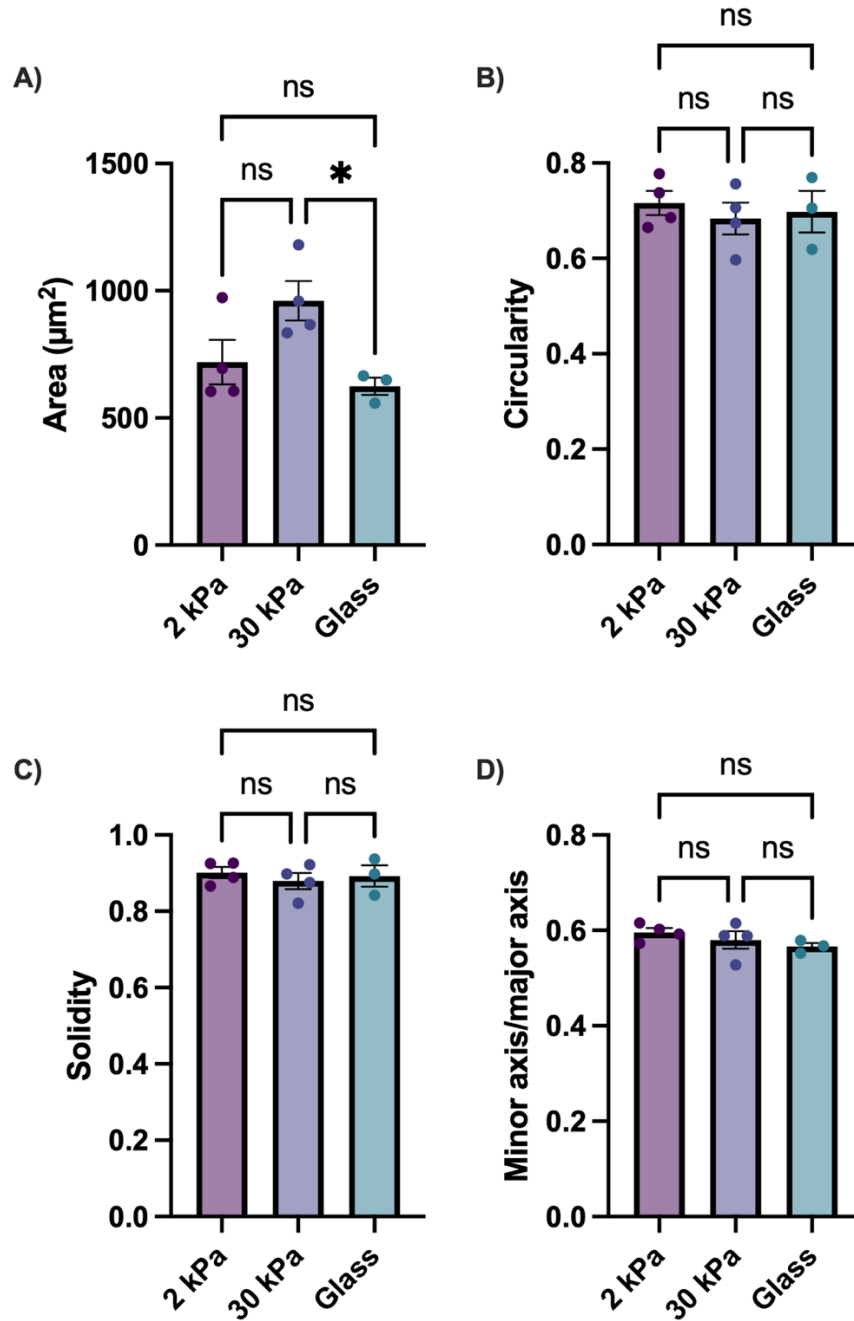


Figure 31: Morphology of endometriotic cells incorporated within the mesothelium. Individual 12Z cells were allowed to incorporate into Met-5A monolayers grown on 2 kPa, 30 kPa, and glass substrates for 6 hr post-seeding. Cell **A)** area, **B)** circularity, **C)** solidity, and **D)** ratio of minor axis to major axis were quantified. Data points represent trials; error bars represent standard error of the mean. Approximately 25-30 cells were analyzed per stiffness per trial. * $P \leq 0.05$.

7.3.4. Substrate stiffness impacts mesothelial barrier function against endometriotic epithelial spheroids

While single endometriotic cell invasion is possible in endometriosis, it is equally or more likely that multicellular endometriotic fragments actively penetrate the mesothelium. In a physiologically relevant model, we initiated the self-assembly of 12Z spheroids (stained with a green cell dye) and seeded them on top of substrate-bound Met-5A monolayers (stained with an orange cell dye). We then performed fluorescence imaging of spheroids incorporating into the mesothelia for 20 hr. Images of spheroids at 20 hr post-seed compared to 0 hr show an increase in spheroid dissemination on 30 kPa and glass compared to 2 kPa substrates (Fig. 32A). Fold change in projected spheroid area was approximately equal for the first 5 hr post-seed, but significantly greater on glass at 10 and 20 hr (Fig. 32B). We also quantified spheroid-hole overlap at 20 hr, defined as the difference between the projected area of the spheroid and the size of the hole it created in the mesothelium divided by the projected area of the spheroid, and found no differences between stiffness groups (Fig. 32C).

as (Spheroid projected area – hole area in mesothelium)/spheroid projected area, represents the overlap between 12Zs and Met-5As. Scalebars = 100 μ m. Data points represent spheroids pooled from at least three trials; error bars represent standard error of the mean. *P $\leq$ 0.05.

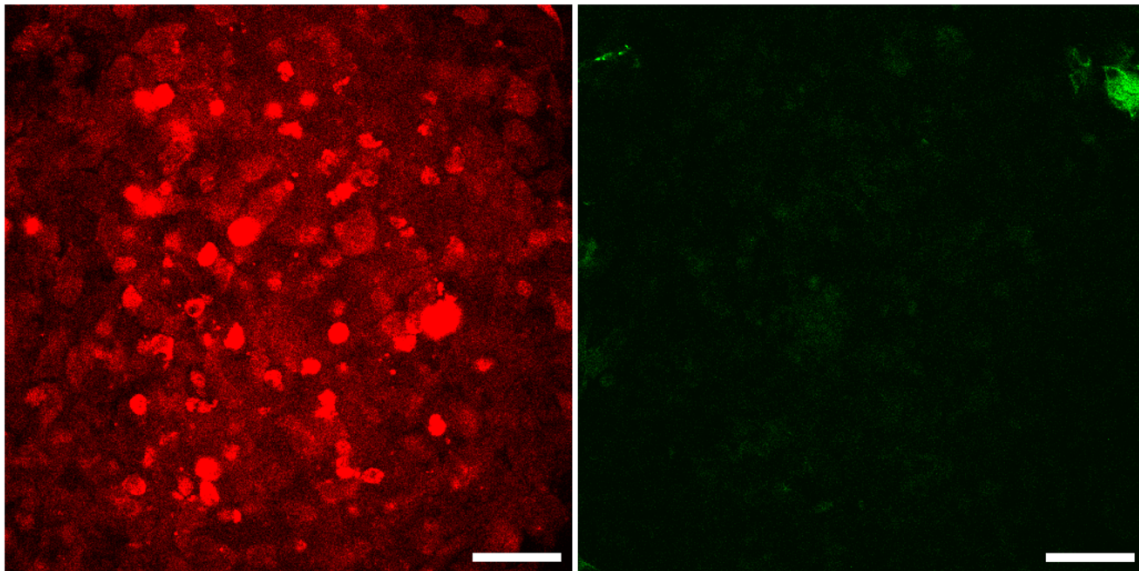
To better understand the physical result of spheroid implantation into the mesothelium in 3D, we took Z-stack confocal images of incorporated spheroids. Met-5A monolayers were grown on substrates and a GFP ZO-1 tension sensor (ZO-1 TS) was applied in order to distinguish them from 12Z cells. 12Zs were stained with an orange cell tracker prior to spheroid formation. 20 hr after seeding spheroids onto monolayers, samples were fixed and imaged with fluorescence microscopy. Our preliminary data show the 3D physical arrangement of endometriotic cells (red) and mesothelial cells (green) after spheroid incorporation (Fig. 33). Spheroids displaced mesothelial cells, as a lack of Met-5As was found underneath the center of 12Z spheroids (Fig. 33A). At the leading edge of the spheroids, we observed some areas where 12Z cells that were disseminating from the spheroid were settling on top of Met-5As, as demonstrated in the YZ and XZ orthogonal views (Fig. 33B).

A)

Spheroids displace Met-5As

12Z spheroid

Met-5A ZO-1 TS underneath



B)

12Zs overlap with Met-5As

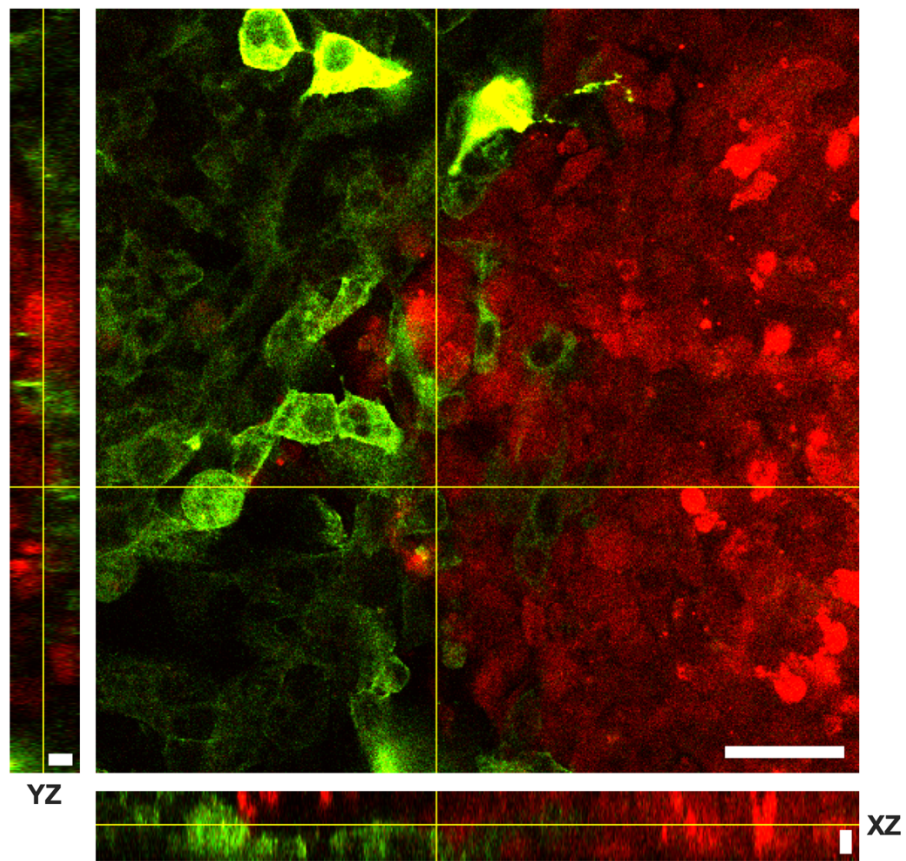


Figure 33: Spheroid implantation within the mesothelium *in vitro*. A GFP ZO-1 tension sensor was expressed in Met-5A monolayers on substrates; then 12Z spheroids stained with an orange cell dye were seeded on top and allowed to incorporate for 20 hr before fixing and imaging. **A)** 12Z spheroids displaced the mesothelial cells beneath them. **B)** 12Zs at the leading edge settled on top of Met-5As, as shown in the YZ (left) and XZ (bottom) orthogonal views. Scalebars = 50 μm except for YZ and XZ slice projections, where scalebars = 10 μm .

7.3.5. Substrate stiffness induces tight junction remodeling in mesothelial barriers

Cell-cell junctions are critical for normal barrier function in endothelial and epithelial tissues. To probe the mechanism underlying the differences in substrate stiffness-mediated spheroid incorporation, we sought to characterize cell-cell junctions within the mesothelium. We chose to analyze the expression and distribution of tight junction protein ZO-1 in Met-5A monolayers grown on 2 kPa, 30 kPa, and glass substrates. Met-5A monolayers generally did not exhibit strong junctional ZO-1 expression, with mostly nuclear and cytoplasmic nonspecific staining across all substrates (Fig. 34A). Despite that, we compared the total area of junction-specific ZO-1 on a per-cell basis that we did observe on each substrate. While there was no significant difference between 2 kPa and 30 kPa junctional area, Met-5As on the glass substrate demonstrated a significant reduction in junctional ZO-1 (Fig. 34B). Therefore, extremely stiff substrates play a role in the loss of tight junctions in mesothelial barriers while softer substrates can support at least modest tight junction presentation *in vitro*.

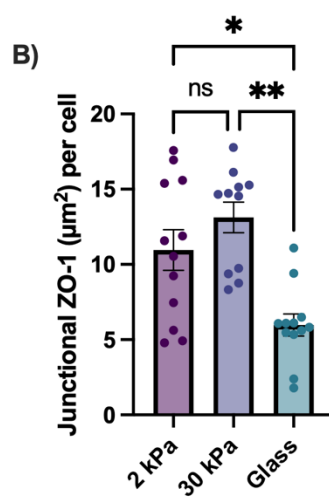
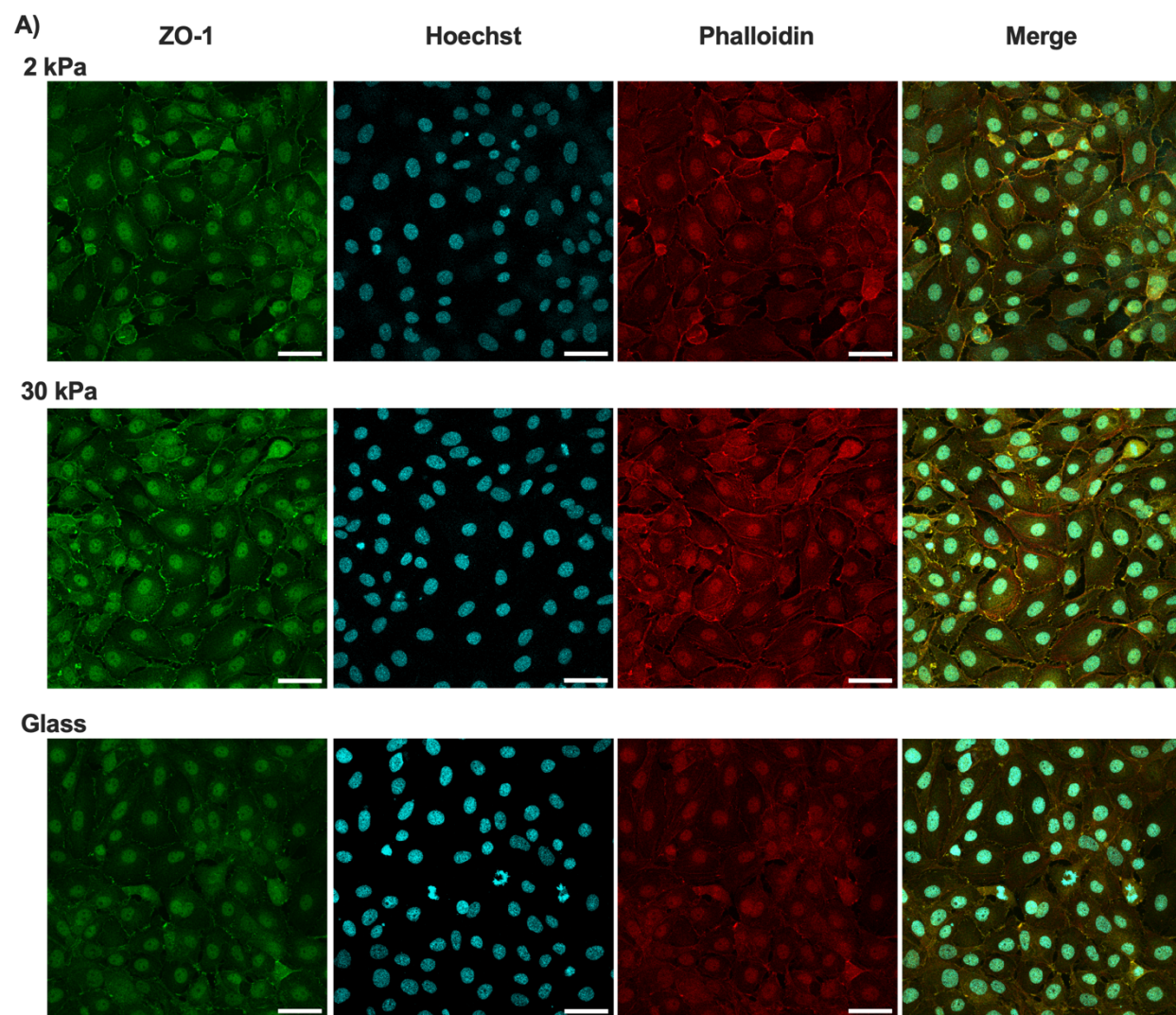


Figure 34: ZO-1 presentation in mesothelial monolayers cultivated on 2 kPa, 30 kPa, and glass substrates. Met-5A monolayers were grown on substrates for 72 hr, fixed, and stained for tight junction protein ZO-1. **A)** ZO-1, nuclei (Hoechst), actin (phalloidin), and merged confocal images are shown for each stiffness. **B)** Junctional ZO-1 area was quantified for each stiffness. Scalebars = 50 μ m. Data points represent images pooled from at least three trials; error bars represent standard error of the mean. * $P \leq 0.05$, ** $P \leq 0.01$.

We then aimed to elucidate tight junction distribution in mesothelial monolayers after co-culture with endometriotic epithelial cells. We seeded 12Z spheroids stained with an orange cell dye on top of Met-5A monolayers on substrates, allowed them to incorporate for 20 hr, and fixed and performed ICC for ZO-1 presentation. We observed very diffuse and nonspecific ZO-1 distribution (green) around the site of spheroid incorporation within mesothelial monolayers, especially on glass substrates, where mesothelial cell borders cannot be distinguished (Fig. 35). Compared with mesothelial ZO-1 presentation without 12Z cells present (Fig. 34), the presence of 12Z spheroids led to even greater disruption of tight junctions (Fig. 35).

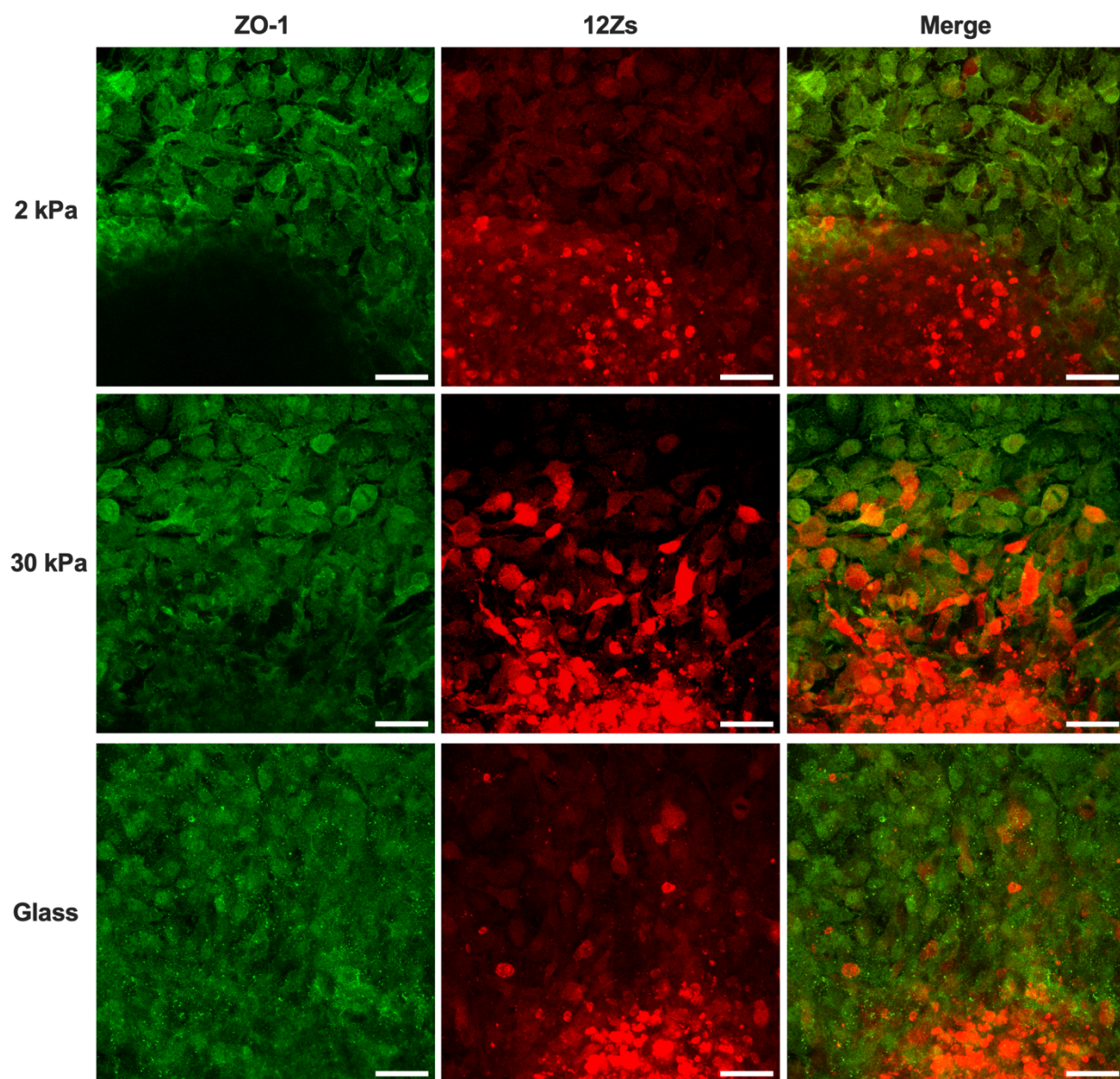


Figure 35: Tight junction presentation in the mesothelium at the site of spheroid incorporation. After 12Z spheroids (red) were incorporated into Met-5A monolayers grown on 2 kPa, 30 kPa, and glass substrates, samples were fixed and immunostained for ZO-1. Around the site of spheroid incorporation, ZO-1 is broadly cytoplasmic, diffuse, and nonspecific within the mesothelial monolayers, especially on glass, where cell borders are poorly defined. Images represent data from at least three independent trials. Scalebars = 50 μ m.

We also imaged mesothelial ZO-1 at least 500 μm away from the site of spheroid incorporation within the same samples. ZO-1 expression in Met-5A monolayers away from 12Z spheroids mostly resembled ZO-1 in Met-5As cultured alone (Fig. 34A), with mostly cytoplasmic and nuclear localization and a noticeable decrease in expression on glass compared to the PA gels (Fig. 36); the main difference was a loss of even the modest junction-specific ZO-1 seen in mesothelial monolayers on 2 kPa and 30 kPa substrates without 12Z presence. However, ZO-1 presentation in monolayer areas away from the spheroids (Fig. 36) still appeared more defined and strongly expressed compared to mesothelial ZO-1 at the site of spheroid incorporation (Fig. 35). Additionally, mesothelial cells away from spheroids tended to exhibit stronger nuclear localization of ZO-1 on 30 kPa substrates compared to 2 kPa and glass (Fig. 36). Overall, extremely stiff substrates result in a loss of mesothelial tight junctions, with junctions also being disrupted by endometriotic cell presence in a proximity-dependent manner (Figs. 34–36).

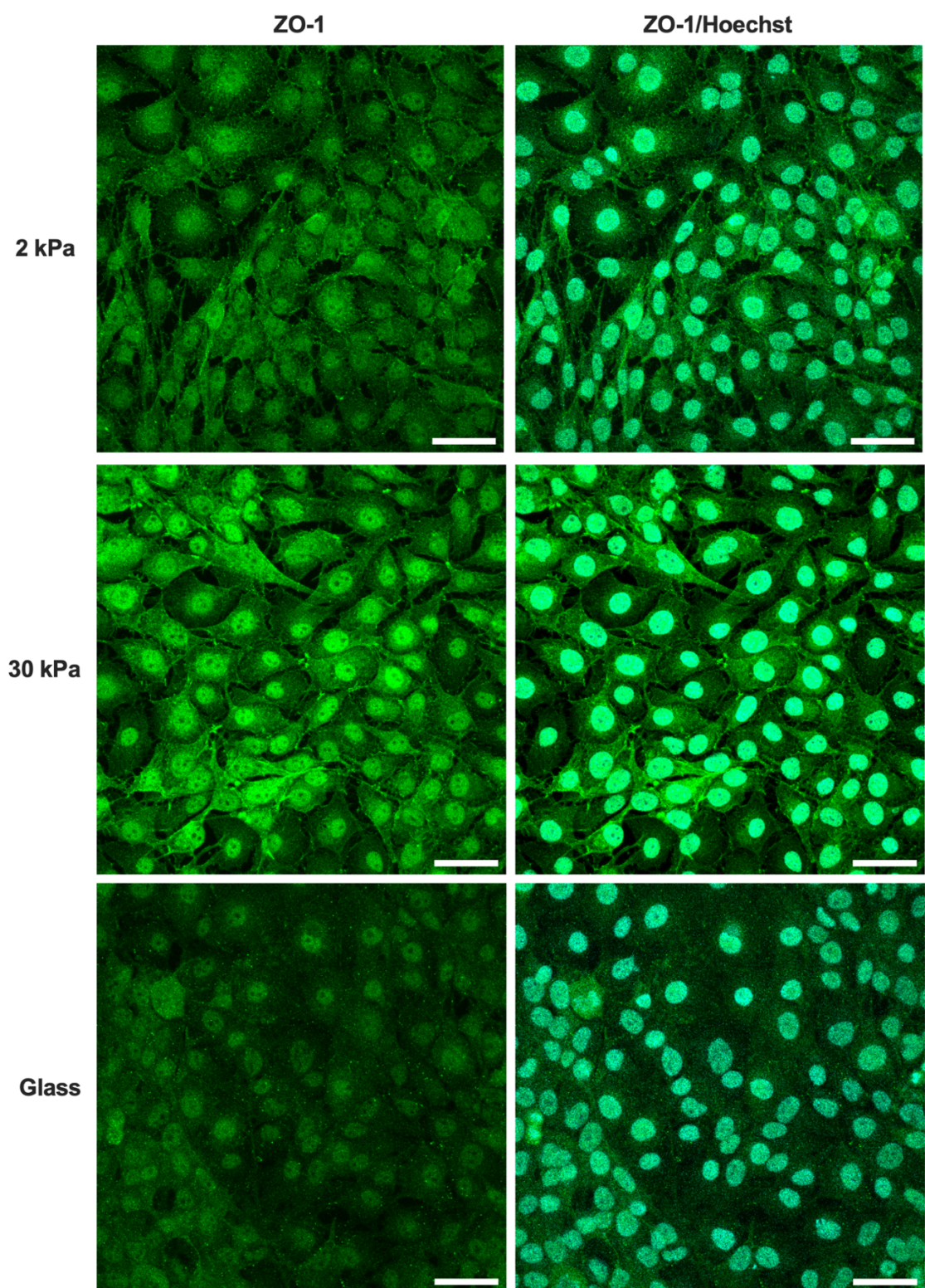


Figure 36: Tight junction presentation in the mesothelium away from the site of spheroid incorporation. After 12Z spheroids were incorporated into Met-5A monolayers grown on 2 kPa, 30 kPa, and glass substrates, samples were fixed and immunostained for ZO-1. Images were taken more than 500 μm away from any site of spheroid incorporation. Scalebars = 50 μm . Images represent data from at least three independent trials.

7.4 DISCUSSION

In this study, we investigated how fibrotic substrate stiffness influences endometriotic epithelial implantation into the mesothelium by modulating mesothelial barrier function and cell–cell interactions. We developed an *in vitro* model of endometriotic establishment and used it to demonstrate that substrate stiffness plays a critical role in regulating the susceptibility of mesothelial barriers to disruption by multicellular endometriotic spheroids, while having minimal impact on resistance to single endometriotic epithelial cells. Together, these findings suggest that mechanical stiffening modulates mesothelial susceptibility to collective endometriotic disruption, with extreme stiffness revealing the upper bound of this effect.

We first confirmed that Met-5A mesothelial cells form confluent monolayers on 2 kPa (modeling healthy tissue), 30 kPa (modeling fibrotic tissue), and glass (extreme stiffness) substrates. While early monolayer development was slower on soft and glass substrates with differences in cell spreading, all monolayers were confluent by 72 hr post-seeding independent of substrate stiffness. This finding indicates that final monolayer formation and coverage are not limited by mechanical compliance, consistent with prior reports that epithelial cells can form monolayers on a range of substrate rigidities [323,324]. However, confluence alone does not equate to functional barrier integrity, particularly in epithelial systems where junctional organization and cytoskeletal tension are tightly coupled to mechanical cues.

Indeed, while substrate stiffness did not significantly alter mesothelial resistance to incorporation by single endometriotic epithelial cells, it markedly impacted barrier function against endometriotic spheroids, which we used to model endometriotic fragments that enter the pelvic cavity during retrograde menstruation. Mesothelial monolayers cultured on stiffer substrates exhibited increased susceptibility to spheroid-induced disruption, resulting in greater spheroid incorporation and barrier compromise. Despite this trend, statistically significant increases were observed only on the extremely stiff glass substrates compared to the soft 2 kPa gels. Spheroid incorporation on fibrotic (30 kPa) substrates was intermediate and did not reach statistical significance relative to the soft condition. These findings suggest that extreme mechanical stiffening is sufficient to compromise mesothelial barrier resistance to collective endometriotic invasion, while more physiologically relevant fibrotic stiffness may sensitize—but not fully overcome—the mesothelial barrier under the conditions tested here. Additional biochemical and inflammatory cues present *in vivo* may be required for fibrotic stiffness to robustly destabilize the mesothelium *in vitro* [325,326].

The fact that the stiffness-dependent effect was not observed in the single-cell incorporation assay underscores the importance of multicellular forces and collective interactions during early stages of endometriotic establishment. Cell spheroids can generate coordinated traction forces and clear the mesothelial surface [229,327,328]. In contrast, isolated endometriotic cells may be insufficient to perturb the mesothelium regardless of substrate stiffness. Clinically, endometriotic multicellular fragments resulting from retrograde menstruation may be more likely to attach to the mesothelium rather than single cells. The distinction we observed between single cell and collective cell activities mirrors broader principles in mechanobiology, where collective

cell behaviors may emerge only above force or density thresholds [329]. We also discovered that incorporated 12Z cells within the monolayers share similar morphological trends on the different substrates when compared to 12Zs sparsely seeded on substrates lacking Met-5As (Chapter 6.3.1), indicating that mesothelial presence and monolayer confluence do not mask the effects of substrate stiffness on 12Z cell shape.

Consistent with the stiffness-dependent spheroid incorporation, we observed pronounced remodeling of mesothelial tight junctions, as assessed by ZO-1 localization, in response to both substrate stiffness and spheroid proximity. On glass substrates, mesothelial ZO-1 expression was diminished and poorly organized even in the absence of endometriotic cells, indicating an inherent loss of junctional integrity in extremely rigid mechanical environments. The presence of endometriotic spheroids further exacerbated this effect in a proximity-dependent manner, leading to diffuse, nonspecific ZO-1 distribution at sites of incorporation. Notably, mesothelial regions distant from spheroids retained more defined ZO-1 localization, suggesting that barrier disruption may be spatially coordinated and driven by local mechanical and cellular interactions rather than global monolayer failure. These findings align with evidence that excessive substrate stiffness can destabilize epithelial and endothelial junctions by altering cytoskeletal tension, mechanosensitive signaling pathways, and junctional protein trafficking [178,320–322]. In the context of endometriosis, fibrotic stiffening of the peritoneal environment—perhaps in combination with inflammatory cytokines, secreted factors, and other mechanical cues—may sensitize the mesothelium to collective endometriotic disruption.

This work complements and extends the findings of Chapter 6 by demonstrating that fibrotic stiffness not only enhances endometriotic epithelial motility but may also play a role in

priming the mesothelium for endometriotic implantation. Together, these chapters support a model in which fibrosis promotes endometriosis progression through dual, mechanically mediated mechanisms: enhancing the invasive potential of endometriotic epithelial cells while simultaneously rendering the mesothelial lining more vulnerable to disruption.

Several limitations of this study should be acknowledged. The use of an immortalized mesothelial cell line may not fully recapitulate the heterogeneity or mechanical responsiveness of primary peritoneal mesothelial cells. We also did not include endometriotic stromal cells in the spheroids; endometriotic implants *in vivo* are composed of both glandular epithelial cells and stromal cells. Additionally, while ZO-1 provides insight into tight junction organization, mesothelial barrier function is governed by a broader network of junctional and cytoskeletal components that warrant further investigation. Others have also demonstrated nonspecific ZO-1 localization in Met-5A monolayers [179], so adherens junctions could be of greater relevance to barrier function. Future studies could utilize live-cell imaging of junctional dynamics or more physiologically relevant co-culture models to more directly link mechanical forces to mesothelial barrier integrity in endometriosis.

7.5 CONCLUSION

This work establishes substrate stiffness as a potential regulator of mesothelial barrier susceptibility during endometriotic establishment. By highlighting the mechanical priming of the mesothelium in fibrotic environments and significant failure on extremely stiff substrates, these findings provide a mechanistic framework for understanding why endometriotic lesion initiation may be facilitated within stiffened, inflamed pelvic tissues. More broadly, this chapter underscores

the importance of incorporating barrier mechanics and collective cell behavior into *in vitro* models of endometriosis pathogenesis.

Chapter 8: Summary and contributions to science

Endometriosis is a complex condition marked by aberrant cellular proliferation, migration, invasion, angiogenesis, and immune evasion. While all of these behaviors synchronize to drive endometriosis, this dissertation focused on migratory and invasive cell behaviors due to their prominent roles in disease severity, pain, and determining patient outcomes. In particular, this work explored the influence of hormonal and mechanical microenvironmental stimuli on dynamics of endometriotic cells.

8.1 Estradiol alters actin and protrusion dynamics in endometriotic epithelial cells

In Chapter 3, we characterized the effects of estradiol (E2) on endometriotic epithelial cell shape and actin dynamics using high resolution volumetric lattice lightsheet microscopy. Short and long timescales were investigated to parse rapid signaling effects versus longer transcriptional activity. On short timescales (15 min), E2 induced more rapid fluctuations in morphology compared to the vehicle control. On longer timescales (24 hr), E2 induced clear morphological changes and reduced fluctuation in circularity. Most notably, after 24 hr of treatment, E2 induced large invadopodia-like protrusion dynamics and reduced actin optical flow alignment compared to the vehicle control. Treated cells had high protrusion areas and high ratios of protrusion area:cell body area, with protrusions extending out of the cell in every direction. This protrusive phenotype in non-polarized cells—reminiscent of invadopodia—is typically indicative of invasive behavior. The actin within the protrusions was also more disordered upon 24-hr E2 treatment, which could

facilitate cell environment sensing in a more exploratory state. However, these effects were not seen after 15 minutes of E2 treatment, suggesting that longer-term signaling is required to drive these structural changes. Given that E2 is continuously synthesized locally in endometriosis—not in short bursts—we hypothesize that cell cytoskeletal dynamics may be stably intensified rather than being dependent on the menstrual cycle. This chapter elucidated the role of E2 in altering cytoskeletal plasticity and shape dynamics in endometriotic epithelial cells, potentially priming them for increased invasiveness.

8.2 The 12Z human endometriotic epithelial cell line exhibits selective estradiol sensitivity

Chapter 4 built upon the work in Chapter 3 by validating the functional effects of E2 on the 12Z human endometriotic epithelial cell line, the most widely accepted cell line for endometriosis research. We found that E2 does not influence 12Z proliferation, collective migration, or single-cell migration. However, 12Zs were selectively responsive to E2 in 3D migration models where the cells were physically confined. Upregulating ER α , the primary mediator of estrogenic action, in the 12Zs did not enhance their functional E2 sensitivity. To delineate the underpinning of these behaviors, RNA sequencing was performed and revealed a differential expression of pseudogenes and non-coding RNAs in E2-treated 12Zs compared to vehicle control-treated 12Zs. Given the 12Zs' context-dependent E2 sensitivity, we hypothesized that situating the cells in a more physiologically relevant environment could increase their response to E2. However, culturing 12Zs on soft, stiff, and extremely stiff substrates did not affect their motility in response to E2 (Chapter 5).

Given these results, and the fact that endometriosis is an estrogen-dependent condition, we advise researchers to exercise caution when using the 12Z cell line. Although the 12Z cell line demonstrates many similarities to primary endometriotic cells, they mostly lack functional E2 responsiveness, and therefore their use *in vitro* should be limited to estrogen-independent models. This work prompts researchers to question whether the 12Z cell line, which mostly lacks functional E2 responsiveness, should be used *in vitro* as a model of endometriosis, which is an estrogen-dependent condition.

8.3 Fibrotic substrate stiffness enhances endometriotic epithelial cell motility

The persistent lack of hormone responsiveness in the endometriotic epithelial cells led us to focus on relevant mechanical cues and their effects on dynamic cellular processes in endometriosis. In Chapter 6, we sought to determine the impact of fibrotic tissue stiffening on endometriotic cell motility, since endometriosis is a fibrotic disease. We used polyacrylamide (PA) hydrogels with tunable stiffness—with glass as an extreme stiffness reference—to model mechanically driven single-cell and multicellular migration. We found that stiff substrates promoted cell speed, actin stress fiber formation, focal adhesion presentation, and spheroid expansion compared to the soft substrate. Increasing cellular contractility on the soft substrate and decreasing contractility on the stiff substrate led to an increase and decrease in cell speed, respectively. These findings provide mechanistic insight on how fibrosis regulates endometriotic epithelial cell migration in the pathogenesis of endometriosis, which is a benign yet invasive, metastatic-like condition.

8.4 Substrate stiffness enhances endometriotic epithelial implantation into the mesothelium

In Chapter 7, we aimed to understand the effects of substrate stiffness more holistically on the barrier function of the mesothelium against endometriotic cells. To this end, we developed an *in vitro* model of endometriotic implantation within the mesothelium on a range of stiffnesses. Increased substrate stiffness enhanced the incorporation of endometriotic multicellular spheroids—but not single cells—into the mesothelium, with extremely stiff (glass) substrates demonstrating the most significant effect. In line with this result, mesothelial monolayers on glass experienced a marked loss of tight junction protein ZO-1 compared to the soft substrate. The presence of endometriotic spheroids further exacerbated this effect in a proximity-dependent manner; mesothelial regions distant from sites of spheroid incorporation retained more defined ZO-1 localization, suggesting spatial coordination of barrier disruption rather than bulk monolayer failure. While extreme mechanical stiffening is sufficient to significantly reduce mesothelial barrier integrity against collective endometriotic invasion *in vitro*, a more physiologically relevant fibrotic stiffness may sensitize the mesothelium but not significantly destabilize it without additional inflammatory cytokines. These data provide insight on how mechanical cues shape peritoneal lesion establishment in endometriosis.

8.5 CONTRIBUTIONS

8.5.1. Contributions to science

- Determined the effect of E2 on endometriotic epithelial cell morphology over short and long time scales (Chapter 3)

- Demonstrated that E2 promotes invadopodia-like protrusion dynamics and reduces actin alignment within the protrusions (Chapter 3)
- Provided evidence that the 12Z human endometriotic epithelial cell line does not functionally respond to E2 except in confinement and therefore should be used with caution in estrogenic endometriosis models *in vitro* (Chapter 4)
- Demonstrated persistent lack of hormone sensitivity in 12Zs upon upregulation of estrogen receptor α (Chapter 4)
- Characterized gene expression profiles in 12Zs (E2-treated compared to control and *ESR1*-12Zs compared to normal 12Zs) (Chapter 4)
- Determined combined effects of hormonal and mechanical cues on endometriotic cell motility (Chapter 5)
- Developed an *in vitro* model of matrix stiffness-mediated endometriotic implantation for both single cells and multicellular spheroids (Chapter 7)
- Investigated the impact of substrate stiffness on single endometriotic epithelial cell incorporation within the mesothelium, and their resulting morphology (Chapter 7)
- Investigated the impact of substrate stiffness on multicellular endometriotic spheroid incorporation within the mesothelium (Chapter 7)
- Probed the effect of substrate stiffness on tight junction distribution within mesothelial monolayers—in isolation and after spheroid co-culture (near and away from sites of incorporation) (Chapter 7)

- Characterized the 3D physical arrangement of endometriotic cells and mesothelial cells during spheroid incorporation (Chapter 7)

8.5.2. Contributions to the Cell and Microenvironment Engineering Lab (PI Dr. Kimberly Stroka), University of Maryland

- Updated protocols for western blots, immunocytochemistry, cell proliferation
- Developed aquaporin-based cell swelling protocol
- Developed stable aquaporin knockdown 12Z cell line
- Acquired materials and expanded cell lines for endometriosis research, a new direction of the lab
- Wrote new MATLAB script to analyze single cell random migration
- Expanded LifeAct plasmid
- Established protocol for nucleofection, an electroporation technique
- Trained lab mates on lattice lightsheet microscope Zeiss LLS7
- Established collaboration with Dr. Wolfgang Losert's lab (Dept. of Physics, University of Maryland)
- Initiated relationship with Dr. Asgerally Fazleabas (Michigan State University) and acquired *ESR1*-12Z cell line
- Developed protocols for endometriotic single cell incorporation and spheroid incorporation assays
- Completed multiple research publications and conference poster presentations

- Trained and mentored two undergraduate researchers: Esha Chopra (awarded ASPIRE, NSF GRFP) and Nane Manukyan (awarded ASPIRE, BS-MD program)
- Trained Gemstone undergraduate team

8.5.3. Publications

- **Banerjee, S.**, Manukyan, N., Stroka, K.M. (*In preparation*). Fibrotic substrate stiffness on mesothelial barrier function against endometriotic establishment.
- **Banerjee, S.**, Manukyan, N., Stroka, K.M. (*Submitted*). Endometriosis mechanobiology. *Reproductive Sciences*.
- **Banerjee, S.**, Manukyan, N., Stroka, K.M. (*Revisions submitted*). Fibrotic substrate stiffness enhances endometriotic epithelial cell motility. *Tissue Engineering Part A*.
- **Banerjee, S.**, Chopra, E., Smith, I.M., Stroka, K.M. (*In revision*). Selective estradiol sensitivity in the 12Z human endometriotic epithelial cell line.
- **Banerjee, S.**, Herr, C., Losert, W., Stroka, K.M. (2026). Estradiol influences epithelial cell actin and shape dynamics imaged with 3D lattice lightsheet microscopy. *Journal of Biological Physics*.
- Smith, I.M., Hengen, A.C., Abraham, A.W., et al. (2025). Aquaporin 5 expression regulates MDA-MB-231 spheroid multicellular invasion. *American Journal of Physiology-Cell Physiology*, 329(4).
- Smith, I.M., Ursitti, J.A., Majeti Venkata, S.P. et al. (2024). High throughput cell mechanotyping of cell response to cytoskeletal modulations using a microfluidic cell deformation system. *Microfluidics and Nanofluidics*, 28(78).

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8.5.4. Presentations

- **Banerjee, S.**, Manukyan, N., Stroka, K.M., (2026, March, upcoming). *Fibrotic substrate stiffness enhances endometriotic epithelial cell motility*. Society for Reproductive Investigation Annual Meeting, San Juan, PR
- **Banerjee, S.**, Chopra, E., Smith, I.M., Stroka, K.M., (2025, April). *Selective estradiol sensitivity in the 12Z endometriotic epithelial cell line*. Society for Reproductive Investigation Annual Meeting, Charlotte, NC
- **Banerjee, S.**, et al. (2024, October). *Estradiol influences epithelial cell actin and shape dynamics imaged with 3D lattice lightsheet microscopy*. Biomedical Engineering Society Annual Meeting, Baltimore, MD
- **Banerjee, S.**, Chopra, E., Smith, I.M., Stroka, K.M., (2024, April). *Migration and morphodynamics studies on 12Z human endometriotic epithelial cells reveal weak estradiol sensitivity*. Society for Endometriosis and Uterine Disorders Congress, Geneva, Switzerland
- **Banerjee, S.** (2024, August). *Estradiol sensitivity in a cell culture model of endometriosis*. Fischell Department of Bioengineering Enhancing and Promoting Interactions and Conversations Retreat

- **Banerjee, S.** (2023, August). *Characterizing migratory cell behavior in endometriosis*.
Fischell Department of Bioengineering Enhancing and Promoting Interactions and
Conversations Retreat
- Three-Minute Thesis Competition 2022, First Place in Clark School of Engineering

Chapter 9: Future work and outlook

This dissertation presents novel findings that advance our understanding of how hormonal and mechanical cues dictate dynamic cellular behaviors relevant to endometriosis *in vitro*. Herein, we detail avenues for future research building from this work.

9.1 Estradiol (E2)-induced cytoskeletal dynamics

In Chapter 3, we demonstrated that E2 treatment for 24 hr induces large, non-uniform membrane protrusions in 12Z endometriotic epithelial cells. These protrusions bore some resemblance to invadopodia, but the cells were situated on a 2D surface while invadopodia extend into the extracellular matrix (ECM) in 3D. The function of these protrusions should be investigated in future work. Invadopodia could be ruled out or confirmed by analyzing the expression of invadopodia markers, cortactin or MT1-MMP. A gelatin degradation assay would also help determine the effect of E2 on the proteolytic activity of the protrusions.

Our experiments investigating actin dynamics in the 12Z cells were performing with 2D cell culture, but cells *in vivo* experience more complex 3D environments. For example, imaging E2-mediated actin dynamics in cells embedded within a collagen I gel to mimic the native ECM would carry more physiological relevance. Cells under physical confinement would also lead to interesting 3D findings that could shed light on disease processes such as deep infiltration in organ walls or squeezing between intact cell-cell junctions in the peritoneal mesothelium. Future

advances in computational tools will be able to support optical flow and morphodynamics analyses in 3D.

9.2 Functional E2 response in the 12Z cell line

We demonstrated in Chapter 4 that the 12Z cell line, a widely used cell culture model for endometriosis research, is only selectively responsive to E2 *in vitro*. Future research should work towards identifying another cell line that can more accurately recapitulate endometriosis pathophysiology; or, perhaps other additional microenvironmental cues are needed to constitute a more reliable *in vitro* model of endometriosis.

The only context in which 12Z cells responded to E2 treatment was when cells experienced some degree of physical confinement (8 μm -wide transwell pores and 3 μm -wide microchannels in a microfluidic device). We plan to further explore mechanisms enabling E2-mediated cell migration in confinement. Previous research has highlighted a pivotal role for aquaporins (AQPs), which are transmembrane water channel proteins, in confined cell migration. The “Osmotic Engine Model” describes how AQPs coordinate with ion pumps to facilitate an influx of water at the leading edge of the cell and water efflux at the trailing edge, propelling the cell forwards [41]. Moreover, endometriotic cells have been shown to overexpress AQPs [77–79,330–332]; this occurs perhaps in part due to the high levels of E2 present in endometriosis, as several AQPs isoforms contain estrogen response elements in their promoter regions [77,330,331,333,334]. In addition to AQPs, the RhoA/ROCK pathway has also been identified as a potential regulator of E2-mediated invasiveness in endometriosis [129].

To verify these findings, we performed one trial of a transwell migration assay in which we treated 12Z cells with 0.025% dimethyl sulfoxide (DMSO), 100 μ M acetazolamide (AZA), an AQP1 and AQP4 inhibitor [335,336], or 10 μ M Y27632, a ROCK inhibitor, for 24 hr prior to the assay. AZA and Y27632 each reduced transmigration compared to the vehicle control (Fig. 37), although more trials are needed to confirm this trend. We then plan to test whether these inhibitors can reverse the effects of E2 on 12Z transwell migration.

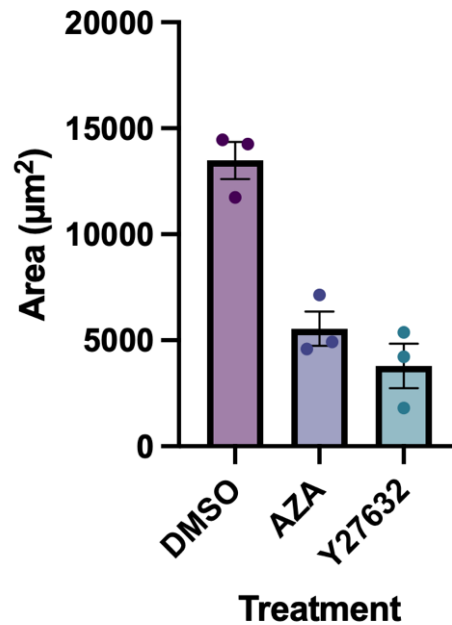


Figure 37: Preliminary data showing the effects of AQP and ROCK inhibitors on 12Z transwell migration. After treatment, 12Zs were allowed to migrate for 24 hr through 8- μ m pores of a transwell towards the bottom chamber which contained serum. The area of cells that migrated to the bottom surface of the transwell insert was plotted. Data points represent technical replicates within one trial; error bars represent standard error of the mean. DMSO; dimethyl sulfoxide. AZA; acetazolamide.

We also measured the expression of estrogen receptor (ER)- α in the 12Z cells. We hypothesized that the expression of ER α would dictate cellular responsiveness to E2, but that was

not that case (Chapter 4). In future, the levels of ER β and G-protein coupled estrogen receptor, which participate in classical and non-classical estrogen signaling, respectively, should be tested and modulated in the 12Z cell line since they may drive E2 sensitivity more than ER α . The effect of passage number on the cell line's ability to model E2 sensitivity *in vitro* should also be characterized; this was a limitation for us as we could only obtain the 12Zs at a passage of 55.

9.3 Role of substrate stiffness on endometriotic cell dynamics

In Chapter 6, we showed that the migratory behavior of endometriotic epithelial 12Z cells is strongly regulated by substrate stiffness. In future, intracellular actin dynamics, especially using lattice lightsheet microscopy, could be studied with respect to substrate stiffness to better understand how the cytoskeleton dynamically responds to mechanical stimuli. We also analyzed both single cell migration and collective spheroid expansion on varied substrate stiffnesses. Although spheroids are 3D cell culture models, we still seeded them onto 2D substrates—while polyacrylamide (PA) gels are useful tools for tuning matrix stiffness, they would be inherently cytotoxic to cells if cells were embedded in them. In future, spheroid outgrowth in 3D could be assessed by embedding them in collagen gels of increasing stiffnesses to study mechanically driven invasion in 3D. Moreover, heterogeneous spheroids self-assembled with epithelial and stromal endometriotic cells would model endometriotic implant dissemination in a more physiologically relevant manner. Spheroids composed of 12Z cells and endometriotic stromal cells invading within Matrigel had stromal cells localized at the leading edges [234], so whether this distribution is maintained in the presence of normal and fibrotic mechanical cues should be tested.

Secondly, we observed small actin puncta at the periphery of 12Z cells, predominantly on 2 kPa and glass substrates (Chapter 6). It is unclear what these membrane protrusions are. Their identity could be clarified by staining for phospho-FAK, Arp2/3, fascin, phospho-myosin light chain, or cortactin to determine whether they are nascent adhesions, lamellipodia nucleation, filopodia tips, blebs, or invadopodia.

Lastly, protein upregulation/downregulation studies would help solidify the mechanistic role of specific mechanotransduction pathways in regulating endometriotic epithelial cell motility on various matrix stiffnesses. We hypothesize that a ROCK1 knockdown would decrease cell speed and contractility on the 30 kPa substrate, and that a ROCK1 upregulation would increase speed and contractility on the 2 kPa substrate.

9.4 Role of substrate stiffness on mesothelial barrier integrity

In Chapter 7, we characterized the effects of substrate stiffness on mesothelial barrier function via two particularly notable readouts: the extent of endometriotic epithelial spheroid incorporation and tight junction presentation in the mesothelium.

Our spheroid incorporation results showed that fibrotic (30 kPa) substrates increased incorporation into the mesothelium but not in a statistically significant manner. This may be because inflammatory cues interact with stiffness to regulate mesothelial barrier function. We plan to test spheroid incorporation on 2 kPa and 30 kPa PA gels with the addition of a physiologically relevant biochemical cue—TNF- α , which may result in larger junctional disruption on the stiff substrate if fibrotic stiffness primes the mesothelium into a more mechanosensitive state.

Our ZO-1 readouts provide multiple avenues for future research. After imaging ZO-1 in mesothelial monolayers cultured alone on the different substrates, we quantified the junctional area (Chapter 7). However, a more interesting analysis could be performed using the Stroka Lab's Junction Analyzer Program to phenotype the junctions and classify them as continuous or discontinuous [337]. We also analyzed ZO-1 presentation after co-culture with endometriotic spheroids, both at the site of incorporation and at least 500 μm away. We found that the presence of endometriotic cells further disrupted cell junctions in a proximity-dependent manner (Chapter 7). Collecting conditioned media from 12Z cells, analyzing its composition, and testing the effects of key biochemical cues on mesothelia on soft and stiff substrates would elucidate the mechanisms and microenvironmental crosstalk leading to a loss of mesothelial cell contacts. In the same vein, investigating how matrix stiffness mediates the secretome of endometriotic epithelial cells could also shed light on how fibrosis feeds into the inflammatory environment in endometriosis.

Given the protective role of tight junctions in epithelial and endothelial barriers, we hypothesized that rescuing mesothelial tight junctions on extremely stiff substrates could mitigate spheroid incorporation. We collected data showing that forskolin appears to increase junctional ZO-1 expression in Met-5A monolayers grown on glass, although their localization is still not exclusive to cell borders (Fig. 38). Further quantification will be performed to confirm the effect of forskolin on mesothelial tight junctions and resistance to spheroid implantation.

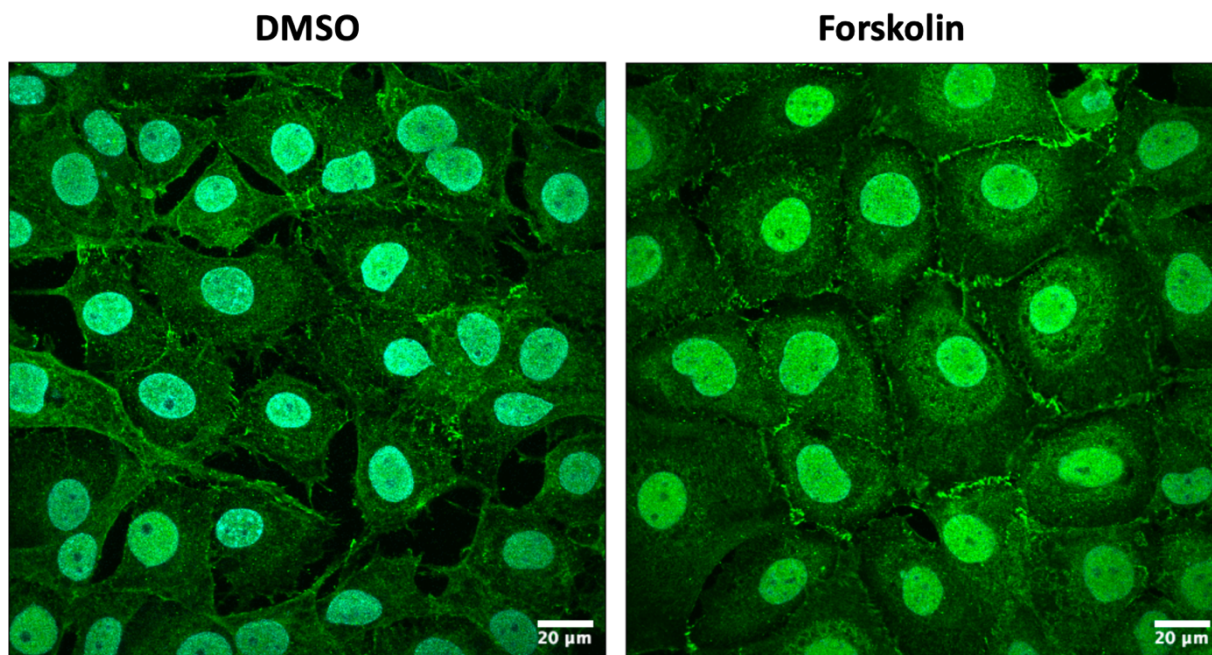


Figure 38: Effect of forskolin on mesothelial tight junction presentation. Met-5A monolayers were grown on glass and treated with 20 μ M forskolin or 0.001% DMSO for 6 hr prior to fixation and immunostaining for ZO-1 (green). Nuclei were stained with Hoechst (cyan). Scalebars = 20 μ m. Images are representative of three trials.

Besides tight junctions, adherens junctions may play more of a role in Met-5A monolayer integrity and should be investigated on different substrate stiffnesses. Another proxy for stiffness-mediated barrier integrity, mesothelial-mesenchymal transition (MMT), should be assessed by measuring the expression of N-cadherin, α -smooth muscle actin, and E-cadherin.

Lastly, impacts of healthy and fibrotic substrate stiffnesses should be tested on primary mesothelial cells. If possible, samples should be obtained from the pelvic cavities of women, as the Met-5A cell line was derived from pleural fluids and is not exclusively female-derived—sex and tissue origin may affect cellular function. Primary mesothelial cells may exhibit more physiologically accurate junction presentation and mechanosensitivity.

9.5 Other hormonal and mechanical aspects of endometriosis

Estrogen dependence and increased tissue stiffness during fibrosis are two key hallmarks of endometriosis. However, there are other similar aspects worth exploring; for example, E2 responsiveness in endometriosis is accompanied by marked progesterone (P4) resistance. P4 is another steroid hormone that regulates normal reproductive function and has anti-estrogenic effects, but endometriotic lesions tend to experience a loss of progesterone receptor (PR) expression and attenuated P4 signaling [338]. The 12Z cell line has previously been shown to express PR [237], but its functional response to P4 is not well characterized. Moreover, increased ECM stiffness was shown to enhance PR-B activation via mechanosensitive pathways in uterine fibroid cells [339]. Whether this mechanical-hormonal crosstalk exists in the 12Z cell line and its implications for modeling endometriosis deserves further exploration.

Lastly, there are other mechanical cues worth studying in endometriosis. For example, cyclic stretch of organs that undergo peristaltic movement, such as the bladder and bowel, may affect endometriotic lesion/implant establishment [141]. While the effects of cyclic strain have been investigated on human ESCs [184,186], they have not been tested on endometriotic epithelial cells, to our knowledge. Given that cyclic strain has increased contractility in ESCs [184] and induced MMT in mesothelial cells [187], cyclic strain should be directly tested in a model of lesion establishment. Our *in vitro* model of endometriotic spheroid establishment in the mesothelium could easily be adapted to uniaxial or equiaxial tension device systems to mimic *in vivo* tissue strain. These experiments could be especially insightful for understanding deep infiltrating endometriosis—which tends to affect the bladder and bowel—and developing future therapeutic strategies.

9.6 Outlook

This dissertation highlights the importance of considering biochemical and mechanical microenvironmental cues when studying endometriosis-relevant cellular behaviors. While hormonal signaling—particularly estradiol—has long been recognized as a central driver of disease progression, the findings presented here demonstrate that hormone responsiveness alone does not fully account for the dynamic behaviors underlying lesion establishment. Instead, cellular responses to E2 were found to be context dependent, while mechanical substrate stiffness clearly modulated cytoskeletal organization, migratory phenotypes, and interactions with tissue barriers. These results suggest that future studies of endometriosis will benefit from experimental frameworks that move beyond single-factor perturbations and instead interrogate how hormonal, mechanical, and inflammatory signals act in concert to regulate disease processes.

Building on this work, several directions emerge for future investigation. Incorporating inflammatory cues alongside controlled mechanical environments may help clarify how fibrosis and inflammation jointly influence mesothelial barrier susceptibility and lesion establishment. Expanding these models to include additional cell types—such as stromal cells, immune cells, or primary mesothelial cultures—would further improve physiological relevance and enable exploration of multicellular and paracrine interactions. More broadly, the *in vitro* platforms developed in this dissertation provide a foundation for systematically dissecting early, mechanically mediated steps of endometriotic establishment that are difficult to capture *in vivo*. By enabling controlled manipulation of physical context while preserving key aspects of cellular behavior, these approaches may ultimately inform more comprehensive models of endometriosis—and potentially even other estrogen-dependent and/or fibrotic conditions—and guide future

efforts to identify therapeutic strategies that address both the biochemical and biomechanical facets of the disease.

Appendices

Appendix A: Supplementary Material

Chapter 3

Movie Supplemental_2D.avi. Maximum intensity projection of a 12Z cell's actin dynamics. 12Z cells were transfected with a LifeAct-GFP plasmid via nucleofection and imaged over time using lattice lightsheet microscopy. The movie depicts the maximum intensity projection of a 12Z cell exhibiting rapid protrusion dynamics. Scalebar = 20 μm , total time elapsed = 1 hour.

Movie Supplemental_3D.mp4. 3D rendering of a 12Z cell's actin dynamics. 12Z cells were transfected with a LifeAct-GFP plasmid via nucleofection and imaged over time using lattice lightsheet microscopy. The movie depicts a 3D rendering of a 12Z cell (the same cells as in Movie Supplemental_2D.avi) exhibiting rapid protrusion dynamics. Total time elapsed = 1 hour.

Chapter 4

S1. RNA Sequencing Methods

Library preparation for Transcriptome sequencing

Non strand specific library: Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers followed by the second strand cDNA synthesis. The library was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification. The

library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection.

Strand specific library: Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers. Then the second strand cDNA was synthesized using dUTP, instead of dTTP. The directional library was ready after end repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, amplification, and purification. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection.

Clustering and sequencing

After library quality control, different libraries were pooled based on the effective concentration and targeted data amount, then subjected to Illumina sequencing. The basic principle of sequencing is "Sequencing by Synthesis", where fluorescently labeled dNTPs, DNA polymerase, and adapter primers are added to the sequencing flow cell for amplification. As each sequencing cluster extends its complementary strand, the addition of each fluorescently labeled dNTP releases a corresponding fluorescence signal. The sequencer captures these fluorescence signals and converts them into sequencing peaks through computer software, thereby obtaining the sequence information of the target fragment.

Data quality control

Raw data (raw reads) of fastq format were firstly processed through fastp software. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing poly-N and low quality reads from raw data. At the same time, Q20, Q30 and GC

content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Reads mapping to the reference genome

Reference genome and gene model annotation files were downloaded from genome website. Use HISAT2 (2.2.1) to build the index of the reference genome, and use HISAT2 to align paired-end clean reads to the reference genome. HISAT2 can use the gene model annotation file to create splice-aware alignments, providing better alignment accuracy compared to other non-splice alignment tools.

Quantification of gene expression level

featureCounts (2.0.6) was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels.

Differential expression analysis

For DESeq2 with biological replicates: Differential expression analysis for two conditions/groups was performed using the DESeq2 R package (1.42.0). DESeq2 provides statistical programs for determining differential expression in digital gene expression data using models based on negative binomial distribution. The resulting P-value is adjusted using the Benjamini and Hochberg's methods to control the error discovery rate. The threshold of significant differential expression: $\text{padj} \leq 0.05$ & $|\log_2(\text{foldchange})| \geq 1$. For edgeR without biological

replicates: Prior to differential gene expression analysis, for each sequencing library, read counts were adjusted using the edgeR R package (4.0.16) by scaling normalization factors to eliminate differences in sequencing depth between samples, followed by differential expression analysis. The resulting P value is adjusted using the Benjamini and Hochberg's methods to control the error discovery rate. The threshold of significant differential expression: $\text{padj} \leq 0.05$ & $|\log_2(\text{foldchange})| \geq 1$.

GO and KEGG enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler (4.8.1), in which gene length bias was corrected. GO terms with corrected P-value less than 0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (<http://www.genome.jp/kegg/>). We used clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) is a computational approach to determine if a predefined Gene Set can show a significant consistent difference between two biological states. The genes were ranked according to the degree of differential expression in the two samples, and then the predefined Gene Set were tested to see if they were enriched at the top or bottom of the list. Gene set enrichment analysis can include subtle expression changes. We use the local version

of the GSEA analysis tool <http://www.broadinstitute.org/gsea/index.jsp>, GO, KEGG data set were used for GSEA independently.

PPI analysis of differentially expressed genes

PPI analysis of differentially expressed genes was based on the STRING database, which contains known and predicted protein-protein interactions. For species present in the database, we construct the network by extracting the target gene list from the database. Otherwise, we use diamond (version 0.9.13) to align the target gene sequences with selected reference protein sequences, and then establish the network based on the known interactions of the selected reference species.

AS analysis

Alternative Splicing is an important mechanism for regulating gene expression and protein variability. The rMATS (4.3.0) software was used to analyze alternative splicing (AS) events, which mainly include five types of variable splicing events: SE (skipped exon), RI (retained intron), MXE (mutually exclusive exons), A5SS (5' alternative splice site), and A3SS (3' alternative splice site).

SNP analysis

The GATK (v4.1.1.0) software was used to perform SNP calling.

Fusion Analysis

Fusion gene refers to the chimeric gene formed by the fusion of all or part of the sequences of two genes, which is generally caused by chromosome translocation, deletion and other reasons. The STAR_fusion (version 1.12) software was utilized for the detection of fusion genes. SATR_fusion is a software package that uses fusion output results of STAR alignment to detect

fusion transcripts, including SATR alignment and SATRfusion.predict, and SATRfusion.filter was used to correct the predicted results of SATR_fusion to ensure the accuracy of the results.

S2. We found that the 12Z cell line demonstrated a lack of responsiveness to E2 treatment in proliferation and migration assays (Fig. 13) which prompted us to question the reliability of our source of E2. We obtained a new vial of 17 β -estradiol (MP Biomedicals), denoted MPB E2. We treated 12Zs with 10 nM MPB E2 or the vehicle control (0.001% EtOH) for 48 hr and tested their migratory response. MPB E2 had no effect on wound closure (Fig. 39A) but did significantly increase transmigration in a transwell assay (Fig. 39B). These trends matched our results using the original source of E2 (Sigma Aldrich, cat. #E8875-1G), as seen in Fig. 13B–C and Fig. 14A–B. Therefore, we found no reason to question the integrity of the E2 from Sigma Aldrich, whose Certificate of Analysis reports a purity greater than 98% by high-performance liquid chromatography.

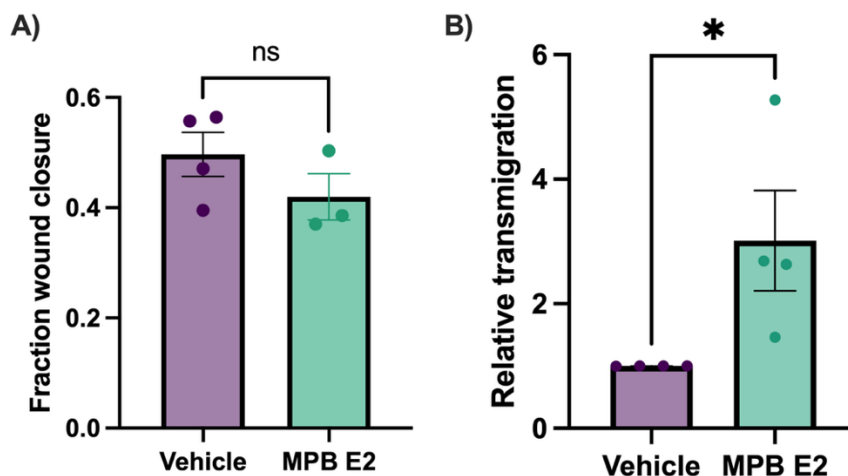


Figure 39: Functional assessment of alternative source of E2 on 12Zs. E2 purchased from MP Biomedicals (MPB E2) was applied to 12Zs and their migratory response was determined. **A)**

Fraction of wound closed is reported and **B**) transwell migration is reported relative to the vehicle control. Data points represent biological replicates, error bars represent standard error of the mean. * $P \leq 0.05$. MPB E2; MP Biomedicals estradiol.

S3. E2_12Z vs VE_12Z Differentially expressed genes (.xls)

S4. NT_12Z vs ESR1_12Z Differentially expressed genes (.xls)

S5. ESR1_12Z vs NT_12Z Significantly enriched KEGG pathways (.xls)

Chapter 6

S1. To assess the effect of substrate stiffness on cell morphology, we seeded 12Z cells on 2 kPa, 30 kPa, and glass substrates and allowed them to adhere overnight before imaging. Morphology parameters were derived from tracing cells in ImageJ and distributions of cell area (Fig. 40A), circularity (Fig. 40B), minor axis/major axis ratio (Fig. 40C), and solidity (Fig. 40D) are shown.

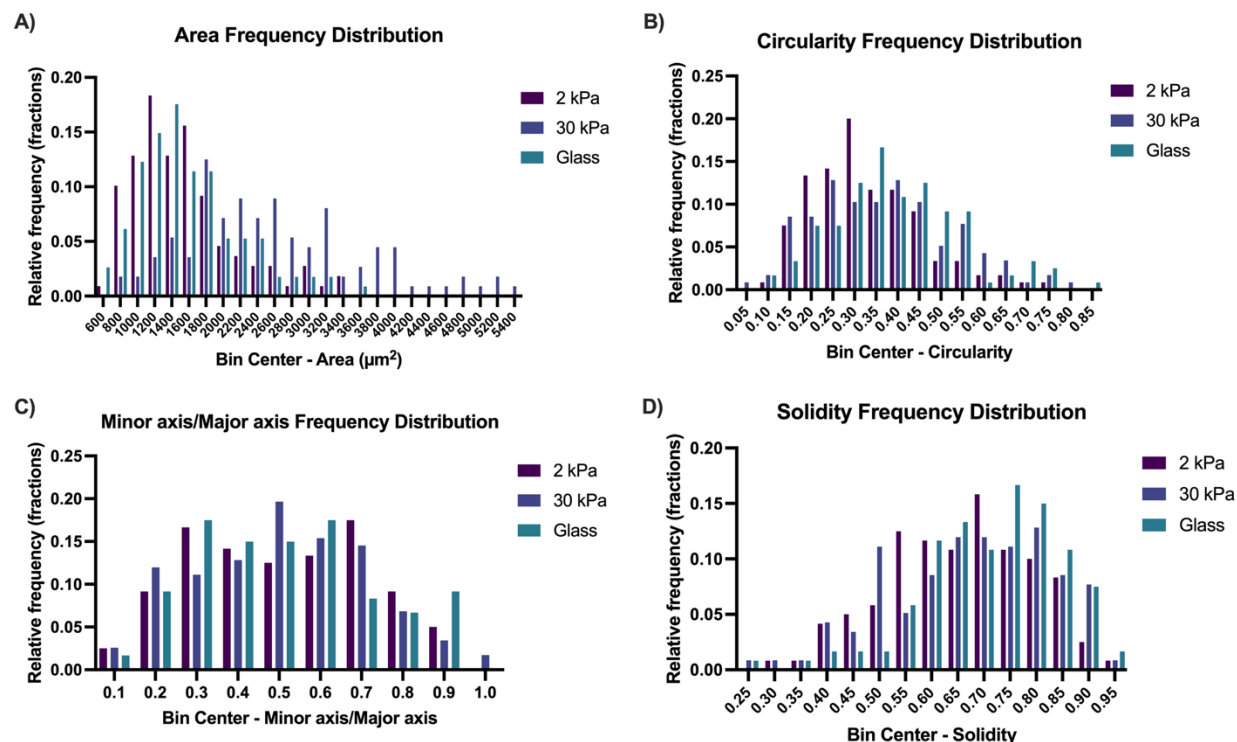


Figure 40: Frequency distributions of 12Z morphological parameters on varying substrate stiffnesses. 12Z cells were cultured on substrates of varying stiffness overnight before assessing their morphology from phase contrast images. Relative frequency distributions of cell morphological parameters, including **A)** area, **B)** circularity, **C)** minor to major axis ratio, and **D)** solidity were plotted and include data from all three trials. 30-40 cells were analyzed per stiffness per trial.

S2. To assess the effect of substrate stiffness on endometriotic cell migration, 12Z cells were seeded on 2 kPa, 30 kPa, and glass substrates and their motility was measured the following day using timelapse microscopy. Single cell random migration was measured using Manual Tracking in ImageJ. For a representative subset of the cells analyzed, we plotted individual cell speed over time and found that while overall speeds stayed relatively steady from the beginning to the end of the 12-hr timelapse, they tended to fluctuate with a period of 30-60 min across all stiffness groups (Fig. 41A–C).

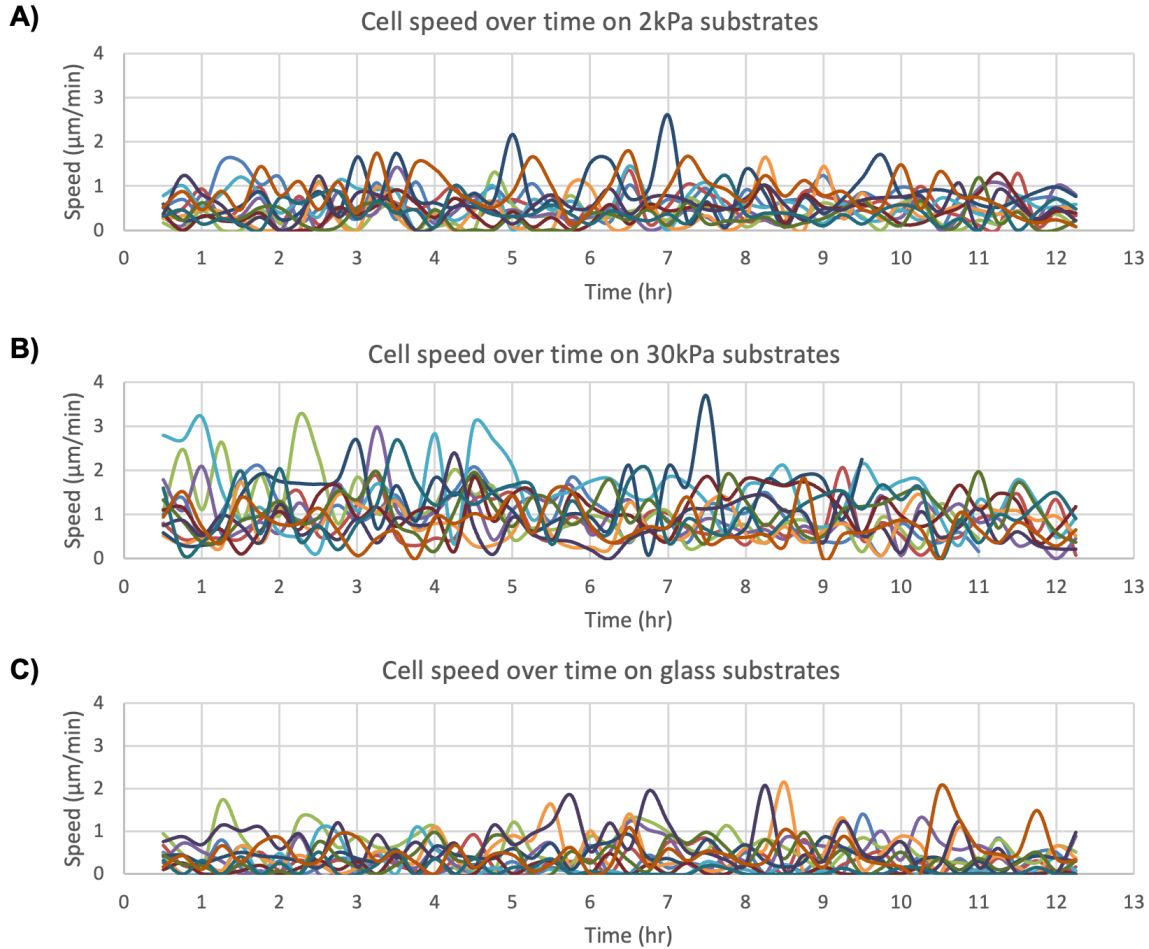


Figure 41: Instantaneous cell speed over time on varying substrate stiffnesses. 12Z cells were cultured on substrates of varying stiffness overnight before assessing their motility from 12-hr phase contrast timelapses. Cell speed over time is plotted for **A)** 2 kPa, **B)** 30 kPa, and **C)** glass substrates, where each curve represents a cell and each plot contains a subset of 12 cells (4 cells per trial). Instantaneous cell speed was calculated at every 15 mins.

S3. We sought to verify whether pharmacological agents modulated cell contractility. Lysophosphatidic acid (LPA) was expected to increase contractility on the soft 2 kPa substrate while Y27632 was expected to decrease contractility on the 30 kPa substrate. 12Z cells were seeded on 2 kPa, 30 kPa, and glass substrates, treated for 30 min the following day, fixed, and stained for phosphorylated myosin light chain (pMLC). ICC results show that LPA significantly

increased pMLC expression compared to the control while Y27632 significantly decreased pMLC expression compared to the control (Fig. 42).

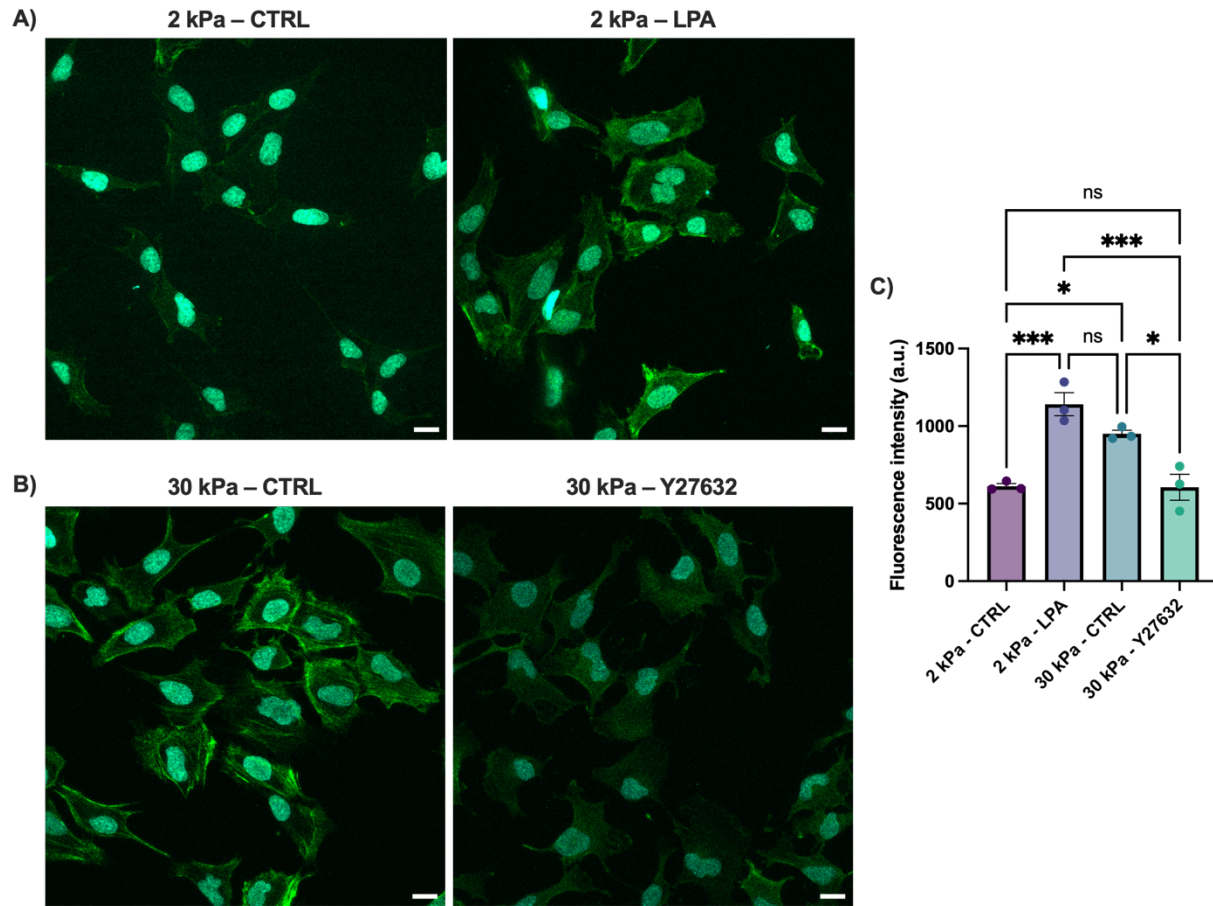


Figure 42: pMLC expression in 12Zs upon treatment with pharmacological modulators of contractility. 12Z cells were cultured on 2 kPa and 30 kPa overnight before being subject to treatment with LPA or Y27632, respectively. The expression of pMLC (green) was then assessed with ICC (nuclei are cyan). **A)** Enhancement of contractility via LPA on soft gel, **B)** reduction of contractility via Y27632 on stiff gel, and **C)** quantification of pMLC fluorescence intensity. Scalebars = 20 μ m. Data points represent independent trials, error bars represent standard error of the mean. * $P \leq 0.05$, *** $P \leq 0.001$. CTRL; vehicle control. LPA; lysophosphatidic acid.

Appendix B: Single-cell random migration analysis MATLAB script

The following MATLAB script was written to analyze data collected from a random migration experiment where cells are tracked manually in ImageJ. The code is commented and inputs are Excel spreadsheets with cell trajectories. Columns from left to right in each spreadsheet should include Track, Slice, X position, Y position, Distance, Velocity, and Pixel Value. Code outputs are shown in Fig. 43–45.

```
% Analyze 2D random migration from Manual Tracking in ImageJ
% This example is set up for analyzing motility of cells on 3
% stiffness groups, 4 videos per stiffness, ~10 cells per video.
% Note, all cells must be tracked for the same amount of time

cf = 0.5416667; % Enter conversion factor from ImageJ in microns
% per pixel

step = 15;      % Enter time step (min)

% Replace this path with your own path to the folder with your
% xls files. Select the files in the order you want them to be
% analyzed.

[file, path] = uigetfile({'*.xlsx'},...
```

```

    '/Users/shohini/Library/CloudStorage/OneDrive-
UniversityofMaryland/Data/SUBSTRATE STIFFNESS/SS & 12Zs/random
mig/25-8-12 random mig','MultiSelect','on');
cd(path)

%% Setup and initialization

numofsheets = zeros(1,width(file));
for k = 1:width(file)
    numofsheets(k) = length(sheetnames(file{1,k}));
end
len = min(numofsheets);

% Initialize final result cell arrays
CavgVel = cell(1,width(file));
CCI = cell(1,width(file));
CmeanVel = cell(1,width(file));
CpathLength = cell(1,width(file));
CtotalDisp = cell(1,width(file));
CMSD = cell(1,width(file));
avgMSD = width(file);
counter = 1;

```

```

%% Read & analyze data from excel files and sheets.

% Each number in results spreadsheet represents a cell, and each
% column represents an xls file (a position)

for i = 1:width(file)
    sheets = sheetnames(file{1,i});
    % Initialize vectors containing results from positions
    totalDisp = zeros(len,1);
    pathLength = zeros(len,1);
    CI = zeros(len,1);
    avgVel = zeros(len,1);
    meanVel = zeros(len,1);
    MSD = zeros(len,1);

    % Set up plot for cell trajectories, edit labels
    subplot(1,3,1); % First plot in a 1x3 matrix of plots
    title('2 kPa')
    xlabel('X (\mum)')
    ylabel('Y (\mum)')

    % Change values in the if-statements and/or add if

```

```
% statements based on positions in each condition/number of  
% experimental groups
```

```
if i>4 & i<9  
    subplot(1,3,2)  
    title('30 kPa')  
    xlabel('X (\mum)')  
    ylabel('Y (\mum)')  
end
```

```
if i>8 & i<13  
    subplot(1,3,3)  
    title('Glass')  
    xlabel('X (\mum)')  
    ylabel('Y (\mum)')  
end
```

```
% Edit/add if statements based on experimental groups. Each  
% number i is set to represents the start of a new experimental  
% group after the first. (Ex. 12 positions, 4 for each of three  
% stiffnesses. 1–4 are 2kPa, 5–8 are 30kPa, 9–12 are glass.
```

```
if i == 5  
    counter = 1;  
end
```

```

if i == 9
    counter = 1;
end

for j = 1:length(sheets)
    T = readtable(file{1,i},
'Sheet',sheets(j),'PreserveVariableNames',true);
    x = T.X;
    y = T.Y;
    duration = step*height(T); % Total duration of time-
% lapse (min)
    totalDisp(j,1) = cf*sqrt((x(1)-x(end))^2 + (y(1)-
y(end))^2); % Distance between start and end points
    pathLength(j,1) = sum(T.Distance) + 1; % Total path
% length traveled
    CI(j,1) = totalDisp(j,1)/pathLength(j,1); % Chemotactic
% index (unitless)
    avgVel(j,1) = pathLength(j,1)/duration; % Path length
% over duration (um/min)
    meanVel(j,1) = mean(T.Velocity); % Average of
% instantaneous velocities (um/min)

```

```

% Plot trajectories

trajx = x-x(1);    % Normalize to origin
trajy = y-y(1);    % Normalize to origin

hold on

axis([-1200 1200 -1200 1200])
plot(trajx,trajy)

% Change values in the if-statements and/or add if
% statements based on positions in each condition/number of
% experimental groups

if i<5
    Traj_X_2kpa(:,counter) = trajx;
    Traj_Y_2kpa(:,counter) = trajy;
end

if i>4 & i<9
    Traj_X_30kpa(:,counter) = trajx;
    Traj_Y_30kpa(:,counter) = trajy;
end

if i>8 & i<13
    Traj_X_glass(:,counter) = trajx;
    Traj_Y_glass(:,counter) = trajy;

```

```

end

counter = counter+1;

% MSD calculations
r_sq = zeros(height(T),1); % Initialize r_sq list
MSD(1,j) = (sum(T.Distance.^2)-1)/height(T);
for k = 2:height(T) % Says what step size we're at
    for m = 1:k:height(T)-k % Loop through positions at
% that step size
        r_sq(m) = (cf*sqrt((x(m)-x(m+k))^2 + (y(m)-
y(m+k))^2))^2;
    end
    MSD(k,j)= sum(r_sq)/height(T);
end
end

CavgVel{1,i} = avgVel;
CCI{1,i} = CI;
CmeanVel{1,i} = meanVel;
CpathLength{1,i} = pathLength;
CtotalDisp{1,i} = totalDisp;

```

```

% MSD

delta_t = zeros(height(T),1);

for l = 1:length(delta_t)
    delta_t(l) = step*l;
    avgMSD(l,i) = mean(MSD(l,:));
end

CMSD{1,i} = [delta_t,MSD];

end

%% Process the cell arrays to print in columns and export to
% Excel. Each column in the results output represents all the
% cells in the corresponding input spreadsheet

n = cellfun(@numel,CavgVel);
n_max = max(n);
n_vectors = numel(CavgVel);
C_AV = cell(n_max,n_vectors);

for ii = 1:n_vectors
    C_AV(1:n(ii),ii) = num2cell(CavgVel{ii}(:));
end

n = cellfun(@numel,CCI);

```



```

n_max = max(n);
n_vectors = numel(CCI);
C_CI = cell(n_max,n_vectors);

for ii = 1:n_vectors
    C_CI(1:n(ii),ii) = num2cell(CCI{ii}(:));
end

n = cellfun(@numel,CmeanVel);
n_max = max(n);
n_vectors = numel(CmeanVel);
C_meanVel = cell(n_max,n_vectors);

for ii = 1:n_vectors
    C_meanVel(1:n(ii),ii) = num2cell(CmeanVel{ii}(:));
end

n = cellfun(@numel,CpathLength);
n_max = max(n);
n_vectors = numel(CpathLength);
C_pathLength = cell(n_max,n_vectors);

for ii = 1:n_vectors
    C_pathLength(1:n(ii),ii) = num2cell(CpathLength{ii}(:));

```

```

end

n = cellfun(@numel,CtotalDisp);
n_max = max(n);
n_vectors = numel(CtotalDisp);
C_totalDisp = cell(n_max,n_vectors);

for ii = 1:n_vectors
    C_totalDisp(1:n(ii),ii) = num2cell(CtotalDisp{ii}(:));
end

n = cellfun(@numel,CMSD);
n_max = max(n);

n_vectors = numel(CMSD);
C_MSD = cell(length(CMSD{1}),n_vectors);

col = 1;
for ii = 1:n_vectors
    s = size(CMSD{ii});
    C_MSD(1:s(1),col:col+s(2)-1) = num2cell(CMSD{ii});
    col = col+s(2);
end

```

```

% Write and export results to a spreadsheet

writecell(C_AV, 'results.xlsx', 'Sheet', 'Average velocity');
writecell(C_CI, 'results.xlsx', 'WriteMode',
'append', 'Sheet', 'Directional persistence');
writecell(C_meanVel, 'results.xlsx', 'WriteMode',
'append', 'Sheet', 'Mean instantaneous velocity');
writecell(C_pathLength, 'results.xlsx', 'WriteMode',
'append', 'Sheet', 'Path length');
writecell(C_totalDisp, 'results.xlsx', 'WriteMode',
'append', 'Sheet', 'Total displacement');
writecell(C_MSD, 'results.xlsx', 'WriteMode',
'append', 'Sheet', 'MSD');

%% Plot average MSDs (mean square displacement)

halfway = floor(height(T)/2);
err2kpa = zeros(halfway,1);
err30kpa = zeros(halfway,1);
errglass = zeros(halfway,1);
dt = delta_t(1:halfway);

```

```

for r = 1:halfway
    twokpa(r,1) = mean(avgMSD(r,1:4));
    err2kpa(r,1) = std(avgMSD(r,1:4)/sqrt(4));
    thkpa(r,1) = mean(avgMSD(r,5:8));
    err30kpa(r,1) = std(avgMSD(r,5:8)/sqrt(4));
    glass(r,1) = mean(avgMSD(r,9:12));
    errglass(r,1) = std(avgMSD(r,9:12)/sqrt(4));
end

% Produces a plot with one MSD curve for each experimental group
% Change the labels according to your experimental groups
figure
plot(dt,[twokpa thkpa glass])
legend('2 kPa','30 kPa','Glass','Location','Best')
xlabel('\Delta t (min)')
ylabel('MSD (\mu m^2)')

% Produces the same plot but with error bars and best fit lines
figure
errorbar([dt dt dt],[twokpa thkpa glass],[err2kpa err30kpa
errglass])
xlabel('\Delta t (min)')

```

```

ylabel('MSD (\mu m^2)')

% Best fit lines and their slopes (forcing zero intercept);
% slope = diffusivity
slope2kpa = dt\twokpa;
slope30kpa = dt\thkpa;
slopeglass = dt\glass;
hold on
plot(dt, [polyval([slope2kpa 0],dt) polyval([slope30kpa 0],dt)
polyval([slopeglass 0],dt)])

% Change the labels according to your experimental groups
legend('2 kPa', '30 kPa', 'Glass', '2kPa best fit', '30kPa best
fit', 'Glass best fit', 'Location', 'Best')

```

OUTPUT:

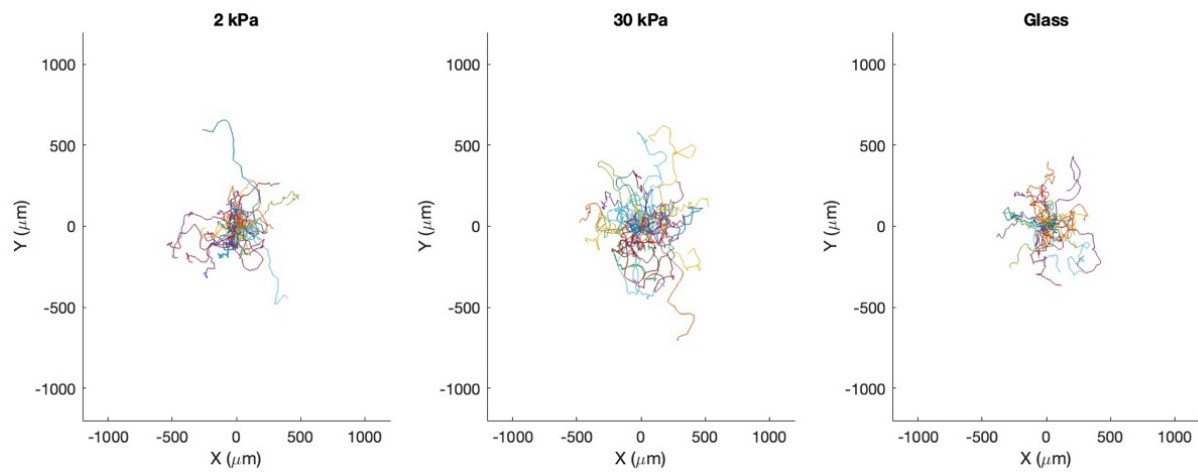


Figure 43: Cell trajectories output from MATLAB script.

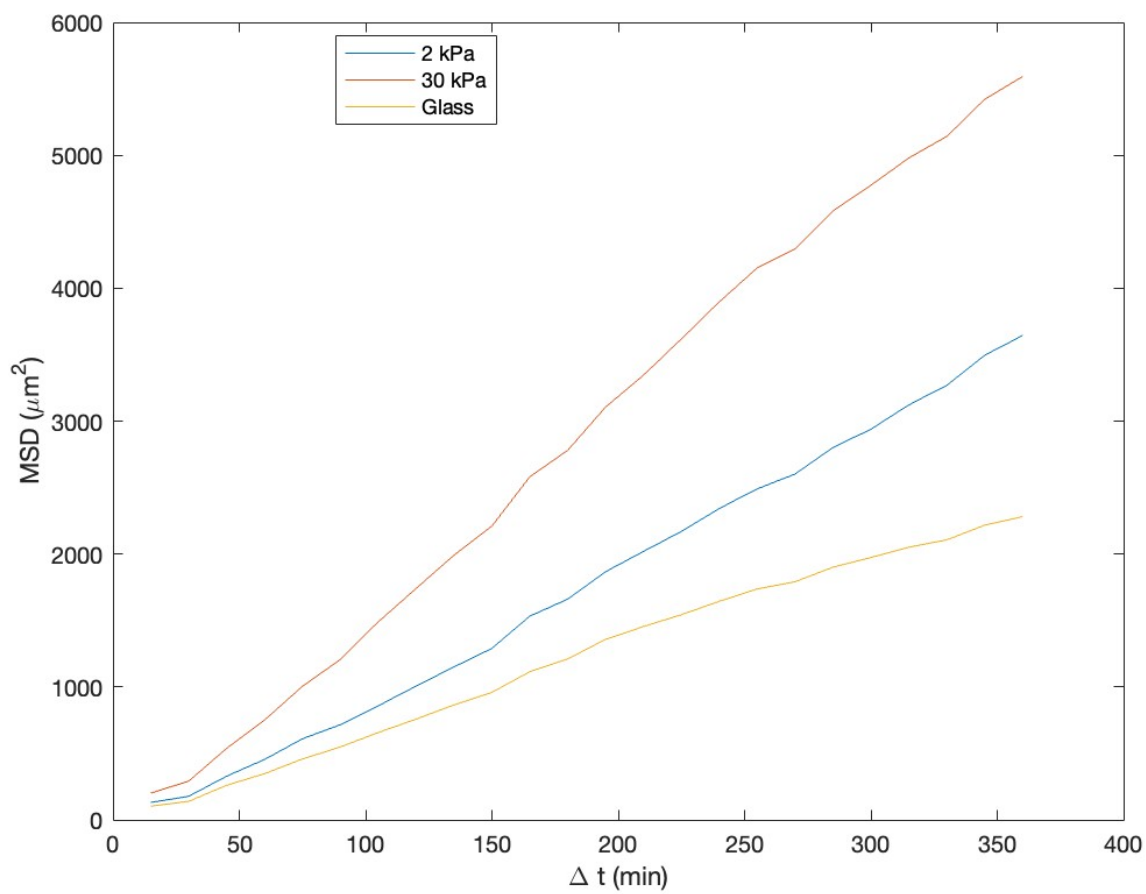


Figure 44: MSD curves output from MATLAB script.

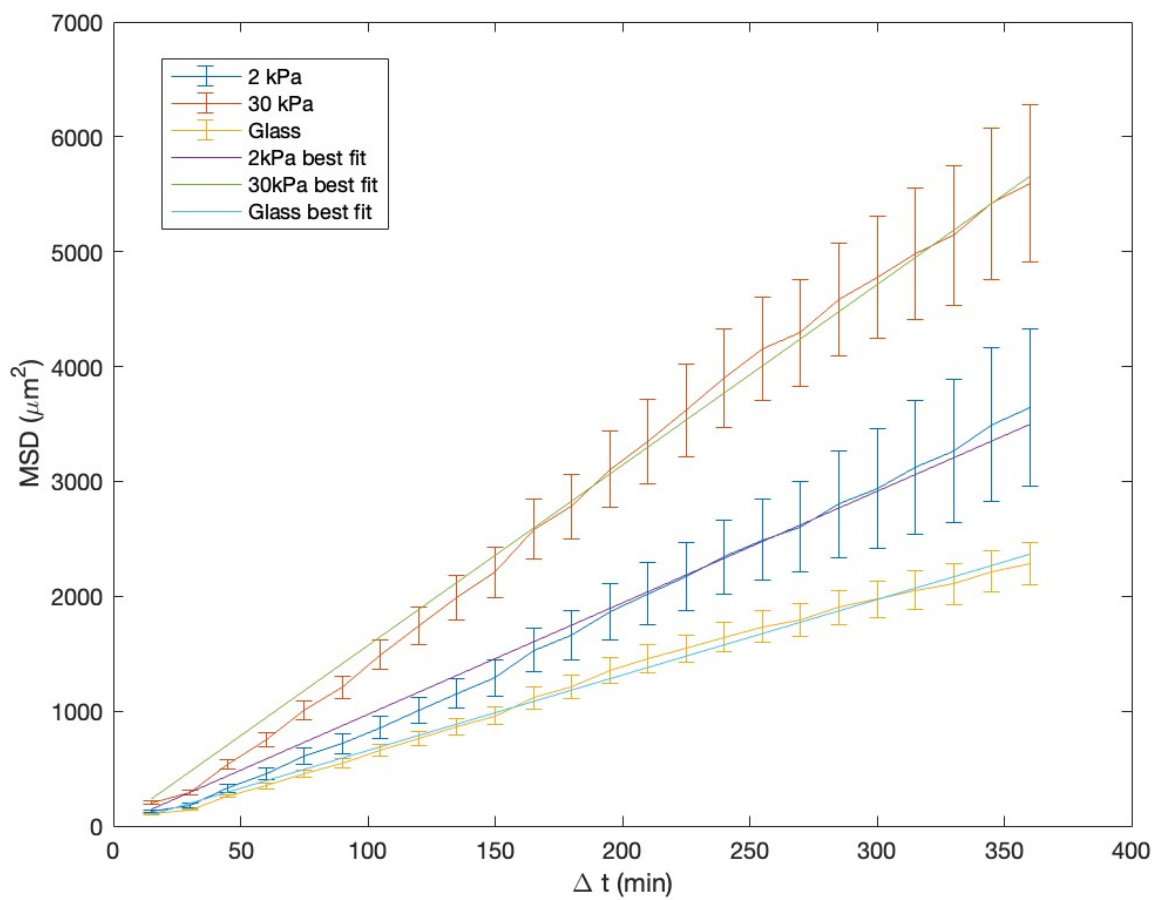


Figure 45: MSD curves with standard deviation error bars and lines of best fit, output from MATLAB script.

Glossary

Barrier function or barrier integrity – the ability of an epithelial layer (or endothelial) to keep out substances and cells

Contractility – ability of cells to generate force to change shape via actomyosin protein complexes

Cyclic strain – repeated mechanical deformation of a material or tissue

Diffusivity – The slope m of the linear portion of the mean-square displacement curve, where $m = 4D$ and $D = \frac{\langle r^2 \rangle}{4\Delta t}$.

Directional persistence – Migrating cell's end-to-end displacement divided by its total path length

Ectopic – located in an abnormal place; outside the uterus

Elastic modulus or Young's modulus – ratio of stress to strain, measuring a material's resistance to elastic deformation (in Pascals)

Endometriosis – prevalent gynecological condition consisting of extrauterine growth of endometriotic tissues leading to inflammation, fibrosis, and pelvic pain

Endometriotic – pertaining to the disease of endometriosis

Endometrium – inner lining of the uterus

Estradiol – potent estrogen molecule (E2)

Estradiol sensitivity – the ability of a cell to functionally respond to estradiol stimulation

Eutopic – located in its true place; inside the uterus

Extracellular matrix – the 3D network of proteins and polysaccharides that supports tissue structure

Fibrosis – tissue damage, scarring, and stiffening due to inflammation

Filopodia – thin, finger-like cellular protrusions made of F-actin that probe the environment to guide cell movement

Focal adhesion – multi-protein complex that anchors the cell cytoskeleton to its extracellular matrix

Incorporation – the attachment and integration of a cell or group of cells into a confluent cell monolayer

Lamellipodia – broad, flat projections at the leading edge of migrating cells

Lattice lightsheet microscopy –advanced microscopy imaging method in which planes of the sample are illuminated with long thin beams to produce a 3D image with high spatial resolution and minimal photobleaching

Mean-square displacement – Approximation for how much area a migrating cell covers during its trajectory, defined as $MSD = \langle r^2 \rangle = 4D\Delta t$, where Δt is the number of time steps ($\Delta t = 1$ represents one step, or 10 min; $\Delta t = 2$ represents two time steps, or 20 min, etc.) and D represents the diffusivity.

Morphodynamics – how a cell changes shape over time

Optical flow – pattern of apparent motion of image objects, quantification of actin dynamics

Peritoneal mesothelium – epithelial layer of cells lining all surfaces of the pelvic cavity

Protrusions – extension of the cell membrane involved in cell environment sensing, migration, and invasion

Solidity – $solidity = \frac{Cell\ area}{Convex\ hull\ area}$, where a cell that is perfectly circular would have a value of 1.0 and a cell that has protrusions and/or indentations would have a value closer to 0.

Spheroid – 3D spherical tumor-like assembly of cells

Tight junction – multi-protein complexes that seal adjacent epithelial (or endothelial) cells to create a selectively permeable barrier

ZO-1 – Zonula occludens-1, a tight junction protein anchored to the cell cytoskeleton which regulates tissue barrier properties

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