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Contractile responses of engineered human μmyometrium to prostaglandins and inflammatory cytokines

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ABSTRACT

Preterm labor is a prevalent public health problem and occurs when the myometrium, the smooth muscle layer of the uterus, begins contracting before the fetus reaches full term. Abnormal contractions of the myometrium also underlie painful menstrual cramps, known as dysmenorrhea. Both disorders have been associated with increased production of prostaglandins and cytokines, yet the functional impacts of inflammatory mediators on the contractility of human myometrium have not been fully established, in part due to a lack of effective model systems. To address this, we engineered human myometrial microtissues (μmyometrium) on compliant hydrogels designed for traction force microscopy. We then measured μmyometrium contractility in response to a panel of compounds with known contractile effects and inflammatory mediators. We observed that prostaglandin F2α, interleukin 6, and interleukin 8 induced contraction, while prostaglandin E1 and prostaglandin E2 induced relaxation. Our data suggest that inflammation may be a key factor modulating uterine contractility in conditions including, but not limited to, preterm labor or dysmenorrhea. More broadly, our μmyometrium model can be used to systematically identify the functional impact of many small molecules on human myometrium.

INTRODUCTION

Approximately one of every eight infants in the United States is born premature, which is the leading cause of neonatal death.[1] The incidence of premature birth is high in large part because treatment options for spontaneous preterm labor, the most common precursor to premature birth, are extremely limited. For example, a synthetic analog of progesterone is commonly administered as a preventative treatment for patients at risk of spontaneous preterm labor, but is only effective in one-third of recurrent preterm births.[2] Once preterm labor has begun, some drugs, such as the β_2 adrenergic receptor agonists terbutaline and ritodrine, can temporarily delay labor, but rarely to never prevent preterm birth.[3]

Spontaneous preterm labor occurs when the smooth muscle layer of the uterus, known as the myometrium, initiates contractions before the fetus reaches full term.[4,5] Abnormal myometrial contractions also underlie dysmenorrhea in non-pregnant patients, a condition that affects at least 45% of childbearing-aged females and is characterized by painful menstrual cramps.[6,7] Interestingly, patients who experience dysmenorrhea pre-conception are at higher risk of experiencing preterm labor when pregnant,[8,9] suggestive of overlapping etiology of these two conditions. Therefore, establishing the underlying and potentially convergent causes of dysmenorrhea and preterm birth could lead to more successful treatments for these and other disorders caused by abnormal myometrial contractions.

Dysmenorrhea and spontaneous preterm labor are also both associated with increased presence of inflammatory prostaglandins and cytokines.[10,11] Prostaglandins are bioactive lipids with hormone-like effects that are produced by many tissues, including the endometrium,[12] fetal membranes,[13,14] and placenta.[13] Increased production of prostaglandins is considered as one of the primary events preceding preterm labor.[15] Elevated prostaglandins are also observed in patients with dysmenorrhea,[16] which is commonly treated by reducing

prostaglandin production with non-steroid anti-inflammatory drugs (NSAIDs),[17] although these have a failure rate of approximately 20%–25%.[6] Increased levels of cytokines have also been observed in the serum[18] and plasma[19] of patients who deliver preterm and in the plasma of patients with dysmenorrhea.[20] Cytokines are secreted by many cell types, including the endometrium,[21,22] and primarily regulate immune cells. However, select cytokines can also induce hypercontractility in airway smooth muscle.[23–25] Similarly, prostaglandins have been shown to modulate the contraction of bladder smooth muscle[26] and vascular smooth muscle.[27] Studies with uterine tissue explants have shown that distinct types of prostaglandins induce myometrial contraction or relaxation, depending on the prostaglandin receptor subtype.[28–30] Cytokines have also been shown to stimulate myometrial contractions in explanted rabbit uterine tissue,[31] which, according to an *in vitro* study with human cells, may be due to the stimulation of oxytocin production.[32] These studies motivate more investigation into the effects of inflammatory mediators on myometrial contractility, especially given the vast number of prostaglandins, cytokines, receptor subtypes, and cell types involved in processes such as spontaneous preterm labor. These studies also urge the development of new model systems that can be used to systematically evaluate the direct impact of small molecules on the contractility of human myometrium, as existing model systems have many limitations.[33] For example, human uterine tissue strips are difficult to acquire, have very limited survival in culture, and have low reproducibility due to many confounding factors, such as patient-to-patient variability and heterogeneous cellular composition.[34] Animal models are also limited due to the many fundamental differences in pregnancy and parturition between species.[35]

Traction force microscopy (TFM) is a technique that has been widely used to quantify contractile forces generated by many cell types, including epithelial cells,[36] cardiomyocytes,[37] and airway smooth muscle cells.[38] TFM is performed by culturing cells on a hydrogel (or other compliant surface) doped with fluorescent microbeads, which are tracked as the cells generate force and deform the hydrogel.[39] We and others have improved the reproducibility of TFM measurements by micropatterning shape-controlled cells and tissues on the surface, which localizes focal adhesion attachments (and thus force transmission) to relatively consistent locations.[40–42] In this study, we engineered human myometrial microtissues, which we call μmyometrium, and implemented TFM to evaluate their contractile response to contractile agonists, antagonists, and inflammatory mediators. Our results show that oxytocin, prostaglandin F2-alpha (PGF), interleukin 6 (IL-6), and interleukin 8 (IL-8) induced contraction of μmyometrium while prostaglandin E1 (PGE1) and prostaglandin E2 (PGE2) induced relaxation. These data suggest that human myometrium has a direct contractile response to inflammatory mediators, which may play a role in spontaneous preterm labor or other conditions associated with both inflammation and abnormal myometrial contractility, such as dysmenorrhea. More broadly, the μmyometrium assay that we established here can be broadly used to evaluate human myometrial contractility in response to many compounds and help develop more effective therapies for patients experiencing uterine disorders.

RESULTS

Engineering human μmyometrium

As described above, spontaneous preterm labor and dysmenorrhea are two examples of disorders associated with abnormal myometrial contractions that are difficult to investigate due to a lack of human-relevant model systems with functional readouts. To address this, we first engineered myometrial microtissues (μmyometrium) by culturing primary human myometrial cells on an array of fibronectin rectangles ($200 \times 100\,\mu m^2$) microcontact printed on polyacrylamide hydrogels bonded on glass coverslips [Fig. 1(a)], similar to approaches previously developed for cardiomyocytes.[40–42] As expected, the cells adhered to the fibronectin rectangles, forming arrays of shape-controlled aligned μmyometrium with minimal cell adhesion outside of the fibronectin pattern [Fig. 1(b)]. Myometrial smooth muscle cells similarly align into anisotropic tissues *in vivo*.[44] We then stained coverslips for fibronectin, DAPI, and actin to evaluate tissue structure ($n = 25$ tissues). Based on fibronectin immunostaining, tissues had an average length of $215.2 \pm 2.3\,\mu$m, width of $109.5 \pm 1.7\,\mu$m, and aspect ratio of 1.98 ± 0.02 [Fig. 1(c)], indicating that tissue size was relatively consistent. We also found that the average number of nuclei per tissue was 37 ± 2.5 and ranged between 21 and 62, likely due to slight variation in cell seeding and attachment [Fig. 1(c)]. Finally, we quantified the orientation of actin filaments. As expected, the rectangular shape of the fibronectin micropattern induced actin filaments to align uniaxially [Fig. 1(d)], similar to native myometrium.

Contractile response to oxytocin

Oxytocin is a naturally occurring hormone that induces myometrial contractions and its synthetic analog is the most commonly used drug to induce labor.[45] Thus, we next measured basal and oxytocin-induced forces generated by engineered μmyometrium. We followed the same fabrication steps described above (Fig. 1), with the addition of fluorescent microbeads in the polyacrylamide hydrogels. We then transferred each coverslip to an inverted fluorescence microscope, positioned a single μmyometrium in the field of view, and captured a phase contrast image of the tissue and an image of the embedded fluorescent beads [Fig. 2(a)]. Next, we added synthetic oxytocin (0.5 mM) and captured images each minute for 12 min [Fig. 2(b)]. Finally, we added trypsin to detach cells and continued imaging each minute for six additional minutes [Fig. 2(c)]. Based on the images of the fluorescent beads and the elastic modulus of the hydrogel (1 kPa), we calculated the displacement, stress, and force generated by each tissue at each timepoint relative to its starting timepoint. As shown in Fig. 2(d) and SI Video 1, engineered μmyometrium responded as expected by generating force in response to oxytocin, which plateaued after about 5 min. In response to trypsin, the tissue detached completely from the hydrogel and the beads displaced beyond their initial location, confirming that these smooth muscle cells exerted a basal force on the substrate, as expected. We also observed that force transmission was concentrated at the longitudinal ends of the tissue, similar to previous studies with engineered cardiac myocyte microtissues.[43,46] We expect that, similar to cardiac myocytes, myometrial smooth muscle cells are detaching cell–matrix adhesions in the center of the tissue in favor of cell–cell adhesions and thus function more like a syncytium.

Next, we statistically compared induced forces generated by μmyometrium in response to PBS vehicle or 0.5 mM oxytocin. As shown by the representative images in Fig. 3(a), displacement and traction stress increased when oxytocin was added compared to PBS. When comparing multiple tissues, the amount of force generated by μtissues treated with oxytocin was significantly higher than the PBS vehicle control [Fig. 3(b)]. The amount of basal force generated by

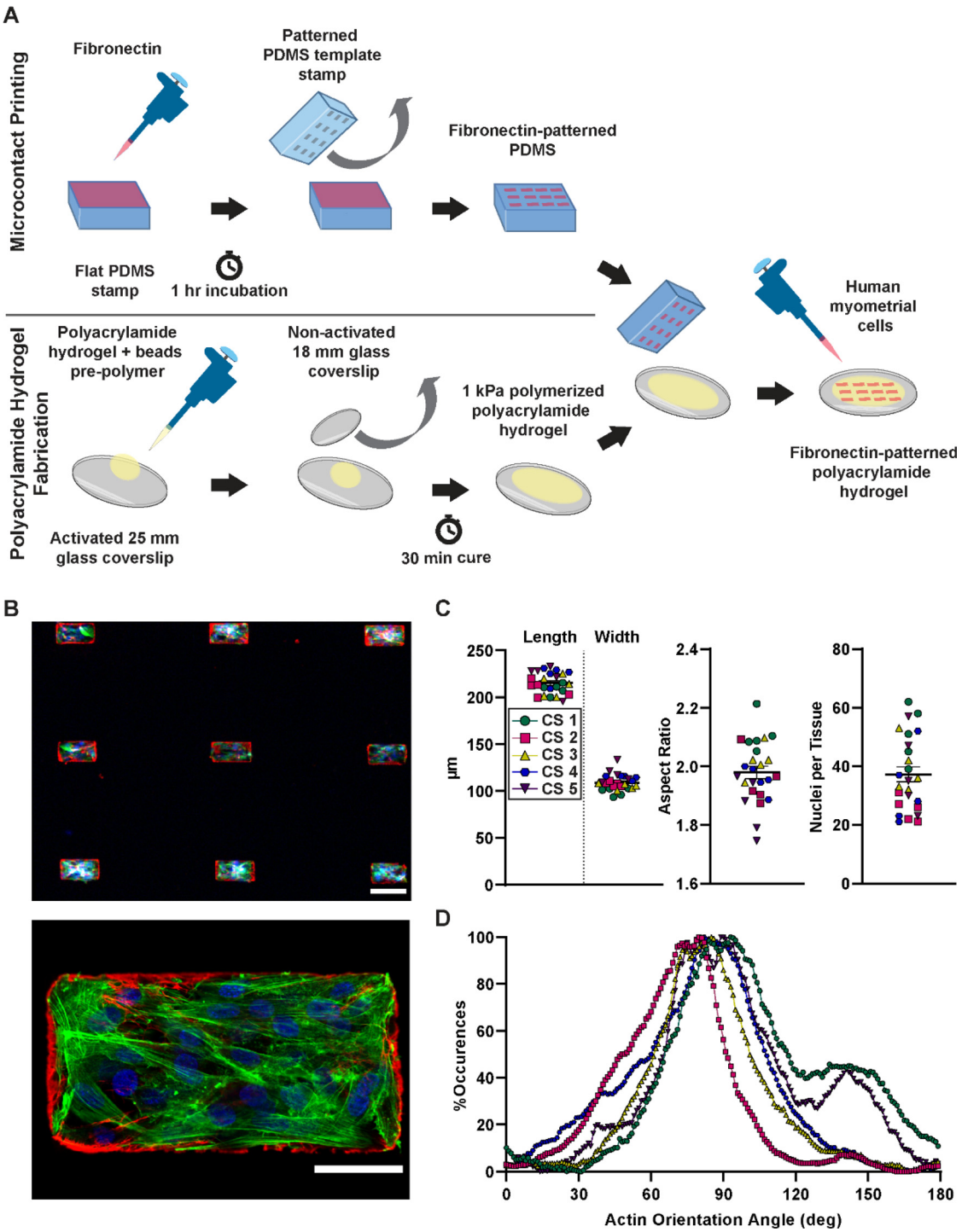

FIG. 1. Engineering and characterizing human μmyometrium. (a) Polyacrylamide hydrogels were fabricated on glass coverslips, micropatterned with an array of fibronectin rectangles, and seeded with primary human myometrial cells to engineer 200 $\times$ 100 μm^2 human myometrial microtissues (μmyometrium). (b) Human μmyometrium stained for fibronectin (red), nuclei (blue), and f-actin (green). Scale bars: 200 μm (top) and 50 μm (bottom). (c) Length, width, and aspect ratio of micropatterned fibronectin rectangles (mean $\pm$ standard error of the mean, n = 25 tissues) distributed amongst five coverslips (CS). Each CS is indicated by a different color and shape. (d) Histogram of actin orientation angles averaged across five μmyometrium per CS. Each CS is indicated by a different color and shape.

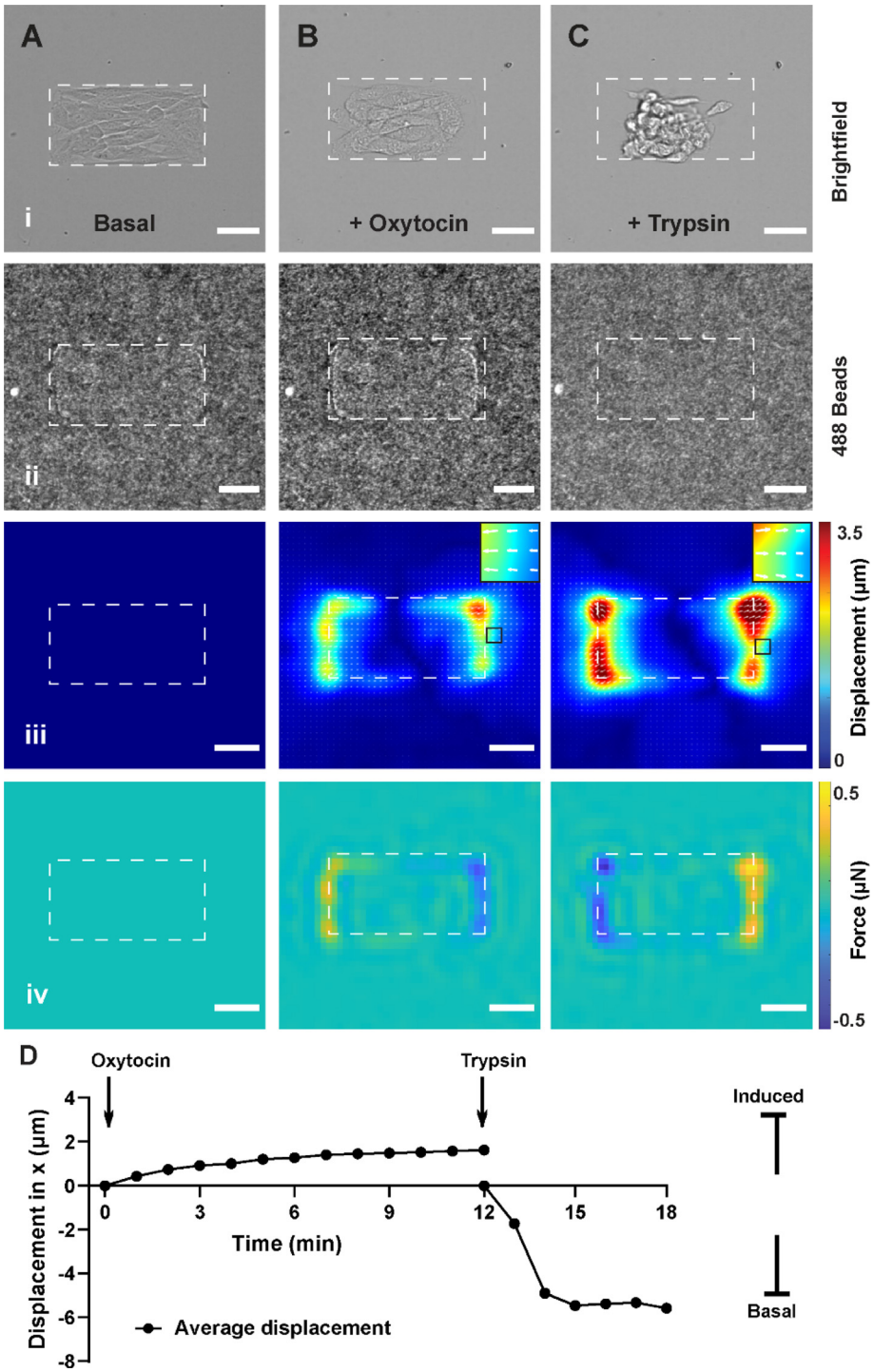

FIG. 2. Measuring basal and oxytocin-induced contractile responses of μmyometrium. (a) Tissues at baseline exert basal tension on the polyacrylamide hydrogel, as shown in the brightfield (i) and fluorescent bead image (ii). Displacement map (iii) and force map (iv) are null because this is the reference timepoint. (b) Oxytocin induces contraction, as shown by brightfield (i) and bead images (ii), displacement vectors pointing inward (iii), and inward force map (iv). (c) Trypsin removes cells (i), resulting in outward bead movement (ii), displacement (iii), and force (iv). Scale bars: 50 μm. (d) Displacement over time for a representative sample. Each dot represents an average of the three maximum displacement vectors at that time point. Positive values indicate contraction and negative values indicate relaxation or release of tension.

tissues (before addition of oxytocin or PBS) was not statistically significant [Fig. 3(b)], but did show some variation, likely due to slight differences in fibronectin patterning, cell seeding, etc. Thus, μmyometrium contracted in response to acute stimulation with oxytocin, as expected.

Response to contractile antagonists

We next asked if μmyometrium would respond to contractile antagonists. Thus, we pretreated μmyometrium with progesterone or caffeine, which are each expected to elicit a weaker contractile

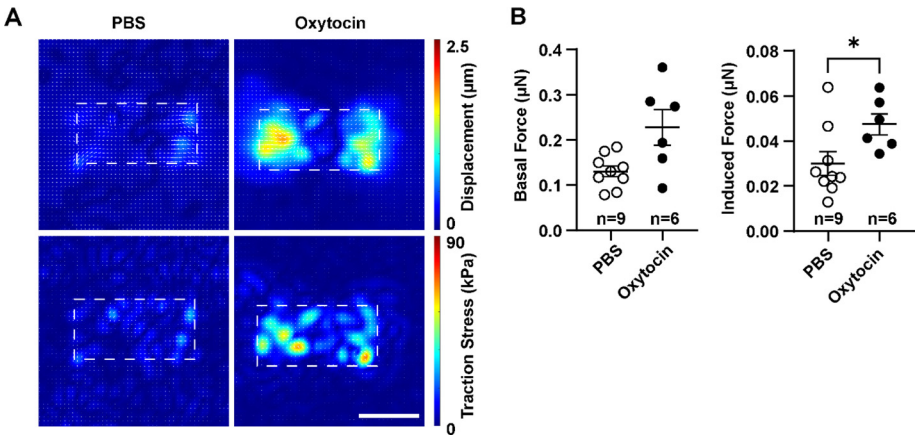

FIG. 3. Displacement and traction stress in response to oxytocin stimulation. (a) Representative displacement (top row) and traction stress (bottom row) maps at the peak frame of contraction induced by PBS or 0.5 mM oxytocin in PBS. Dashed white boxes approximate the tissue borders before oxytocin is added. Scale bar: 100 μm. (b) Quantification of basal and induced forces exerted by μmyometrium (mean ± standard error of the mean). * denotes $p < 0.05$ using unpaired t-test.

response. Progesterone is an endogenous hormone that maintains the myometrium in a quiescent state.[47] Natural progesterone and its synthetic analogs are used clinically to mitigate preterm labor.[48] When we pretreated tissues with progesterone for at least 12 h, the basal force trended lower but did not reach statistical significance [Figs. 4(a)–4(c)]. Induced force in response to oxytocin in tissues pretreated with progesterone was unchanged. We also tested the effects of 10 min of pretreatment with caffeine, a compound that is commonly

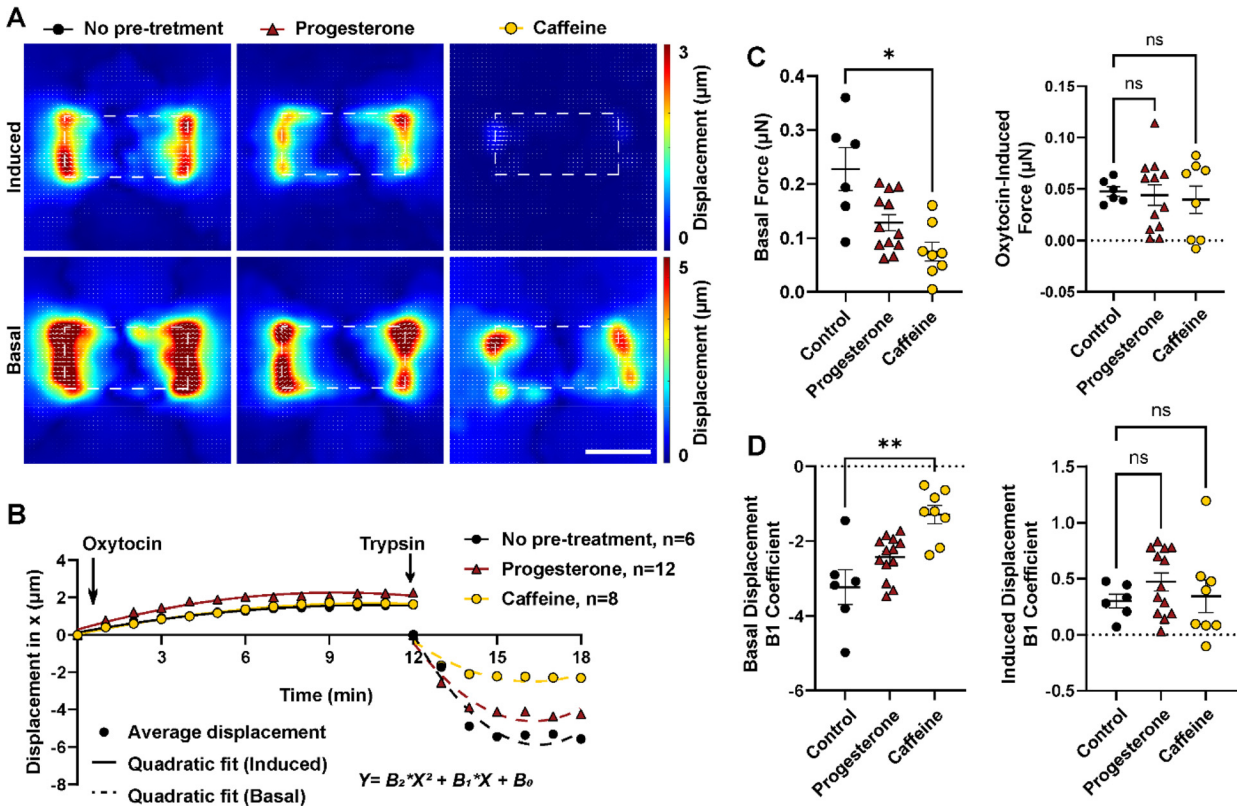

FIG. 4. Responses to contractile antagonists. (a) Representative peak displacement maps for μmyometrium pretreated with indicated contractile antagonist and acutely treated with oxytocin to measure induced force (top row), followed by trypsinization to measure basal force (bottom row). Dashed white boxes approximate the tissue borders before oxytocin is added. (b) Average displacement vs time for μmyometrium with no pretreatment ($n = 6$), pretreatment with progesterone ($n = 12$), or pretreatment with caffeine ($n = 8$) and then acutely treated with oxytocin followed by trypsin. Second order polynomial curves were fit to the oxytocin-induced displacement data (solid line) and basal displacement data (dashed line). (c) Basal and oxytocin-induced forces at maximum displacement. (d) Second coefficient (B1) of second order polynomial fit for basal and induced displacement curves. * denotes $p < 0.05$, ** denotes $p < 0.01$ using Dunnett's multiple comparisons test. Scale bar: 100 μm.

contraindicated during pregnancy. Caffeine pretreatment with a concentration similar to that used in other smooth muscle models[49,50] significantly reduced the basal force but did not impact oxytocin-induced force, aligning with our previous findings that caffeine decreases calcium transient duration in these cells.[51] Rodent studies have also shown that caffeine induces relaxation of uterine smooth muscle cells.[52,53] Thus, both progesterone and caffeine had a greater impact on basal force compared to oxytocin-induced force.

In addition to evaluating force values at peak displacement, we fit a second order polynomial curve to the displacement vs time data after oxytocin addition (induced) to better evaluate the full range of displacement data for each sample. We also fit another second order polynomial curve to the displacement vs time data after trypsin addition (basal). We then compared the second coefficient (B1) of the fit curves because this parameter reflects the tilt and horizontal stretch of the parabola. The B1 parameter showed similar trends to the force data [Fig. 4(d)], indicating that this is also a useful metric of μmyometrium function that accounts for all data points in the timecourse.

Contractile response to inflammatory mediators

We next tested the acute impact of prostaglandins, hormone-like molecules that fluctuate through pregnancy and menstruation, on μmyometrium contraction. For these experiments, cells were not pretreated with any compounds, and prostaglandins were used in place of oxytocin to acutely stimulate contractile responses. PGF, a prostaglandin that has been shown to induce contractions *in vitro* in pregnant and non-pregnant tissue,[54] caused tissues to contract [Figs. 5(a)–5(c)]. Prostaglandin E1 (PGE1) is used clinically in the form of misoprostol to induce uterine contractions.[55] As such, we expected to see the tissues contract in response to a bolus of PGE1, but instead observed relaxation [Figs. 5(a)–5(c)], which may be attributed to regional differences in prostaglandin receptors across the uterus.[56] Similarly, we observed relaxation in response to PGE2 [Figs. 5(a)–5(c)]. We observed similar basal forces for all conditions, as expected [Figs. 5(a)–5(c)]. The second coefficient B1 of a second order polynomial fit to the induced and basal displacement data showed similar trends as the force data [Figs. 5(c) and 5(d)]. Thus, our assay enabled us to distinguish between the contractile or relaxant effects of specific prostaglandins.

Finally, we tested the acute effects of interleukins IL-6 and IL-8 on contraction of μmyometrium. Both IL-6 and IL-8 caused tissues to contract and exhibit a statistically significant increase in force compared to the PBS vehicle control [Figs. 6(a)–6(c)]. This is contrary to the lack of contraction observed in a rat myometrium model,[57] which may be due to species-specific differences. Prior to cytokine treatment, all tissues exhibited similar basal force [Fig. 6(c)] as expected since tissues were not pretreated. Similar to the previous datasets, the second coefficient B1 of a second order polynomial function fit to the displacement data reflected similar trends as the force data [Figs. 6(c) and 6(d)]. Thus, engineered human μmyometrium contracted in response to acute IL-6 and IL-8 stimulation.

DISCUSSION

Due to the lack of human models to investigate disorders associated with dysfunctional uterine contractility, such as spontaneous preterm labor, we engineered human μmyometrium and validated their

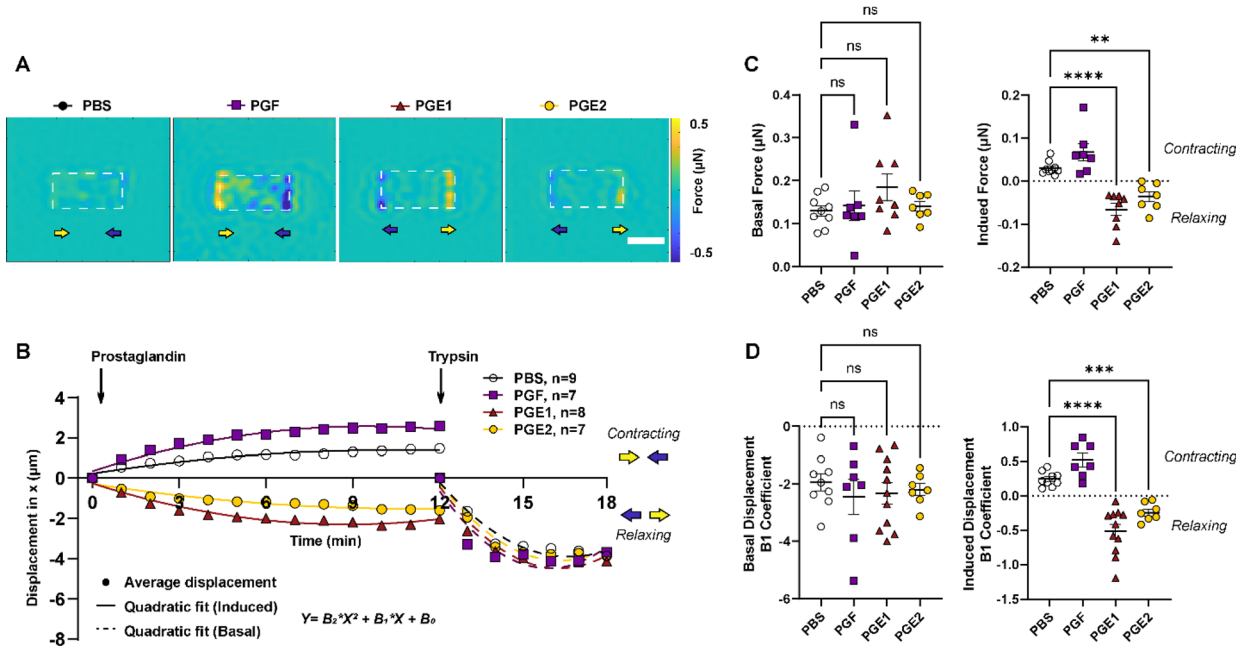

FIG. 5. Contractile responses to prostaglandins. (a) Representative force maps with arrows indicating the direction of bead movement in response to each prostaglandin (PG) or PBS vehicle control. Dashed white boxes outline the tissue borders before PG is added. Scale bar: 100 μm (b) Average displacement vs time after the acute addition of vehicle (phosphate buffered saline, PBS) (n = 9), PGF (n = 7), PGE1 (n = 8), and PGE2 (n = 7), then trypsin. Second order polynomial curves were fit to the induced displacement data (solid line) and basal displacement data (dashed line). (c) Basal and induced forces at maximum displacement. (d) Second coefficient (B1) of second order polynomial fit for basal and induced displacement curves. ** denotes $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, using Dunnett's multiple comparisons test.

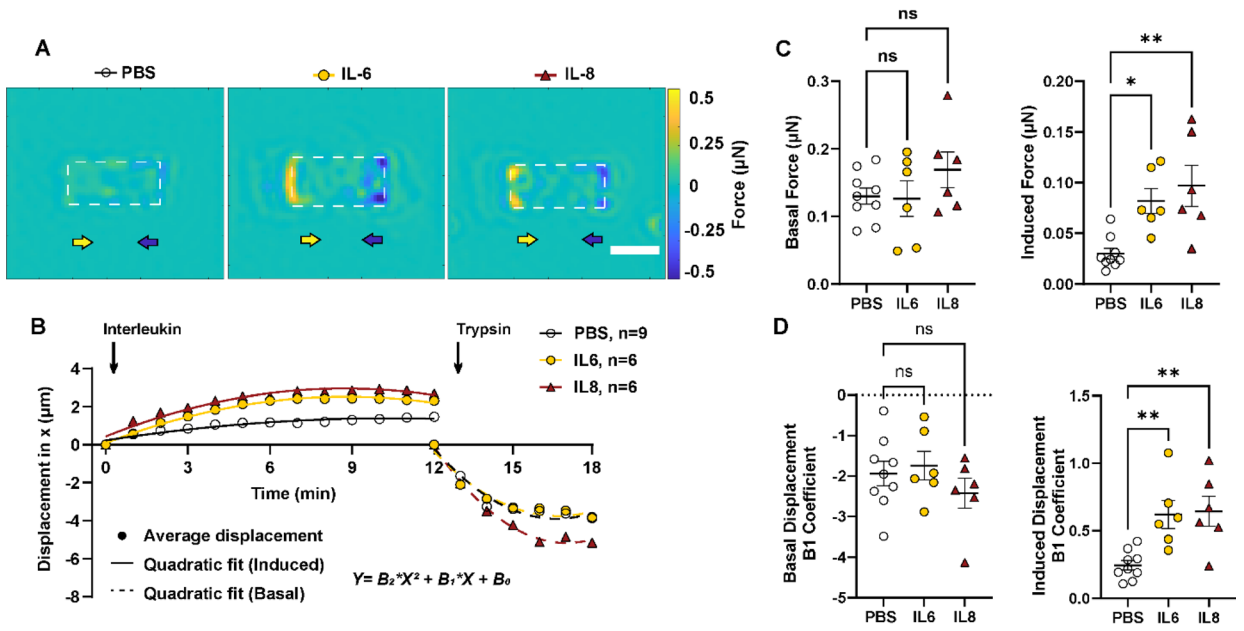

FIG. 6. Contractile responses to interleukins. (a) Representative force plots with arrows indicating the direction of bead movement in response to each interleukin (IL) or PBS vehicle control. Dashed white boxes outline the tissue borders before IL is added. Scale bar: 100 μm. (b) Average displacement vs time after the acute addition of vehicle (phosphate buffered saline, PBS) (n = 9), IL-6 (n = 6), and IL-8 (n = 6), then trypsin. Second order polynomial curves were fit to the induced displacement data (solid line) and basal displacement data (dashed line). (c) Basal and induced forces at maximum displacement. (d) Second coefficient (B1) of second order polynomial fit for basal and induced displacement curves. * denotes $p < 0.05$ and ** $p < 0.01$, using Dunnett's multiple comparisons test.

contractile response to known contractile agonists and antagonists using a traction force microscopy assay. We found that μmyometrium contracted in response to oxytocin and relaxed in response to caffeine, as expected. We then probed the effects of inflammatory mediators on our engineered tissue model and observed contraction in response to PGF, IL-6, and IL-8 and relaxation in response to PGE1 and PGE2. Together, these data indicate novel links between inflammation and uterine contractility and illustrate how our model can be used to systematically investigate the contractile response of human myometrium to small molecules.

We first challenged our μmyometrium model with oxytocin, a hormone naturally produced primarily by the hypothalamus[58] during labor to stimulate uterine contractions. During spontaneous labor, pulses of oxytocin gradually increase in frequency and amplitude as labor progresses. Patient blood samples indicate that oxytocin is present at approximately 20 pM during early labor and up to 200 pM during late stages of labor, although measurements are highly variable.[59,60] Synthetic oxytocin is also the most commonly used drug to induce labor under the brand name Pitocin,[45] which is typically administered at 2–16 mU/min (3.3–26.7 pg/min).[60] In this study, we used 0.5 mM oxytocin, which is much higher than the concentrations measured in pregnant patient blood.[59] However, neighboring reproductive tissues, such as the decidua, also produce oxytocin,[58,61] which may create a higher local concentration within the tissue compared to circulating blood. We also do not know the pregnancy state of the patient who donated the cells used for our study, which is relevant because oxytocin receptor expression is lowest in non-pregnant myometrium and highest in pregnant myometrium at term.[62] Furthermore, we expect that all cells lose some expression of oxytocin receptors during *in vitro* culture and thus become less sensitive to oxytocin. Despite these caveats, we found that oxytocin induced contractions with significance compared to the vehicle control, which can likely be attributed to increased binding to oxytocin receptors that resulted in a greater sensitivity to calcium and a larger influx of intracellular calcium.[63] These results show that our tissues retained contractile function and measurable oxytocin sensitivity *in vitro,* and also align with our previous findings that calcium fluorescence amplitude in human myometrial smooth muscle cells increases in response to oxytocin.[51]

Next, we tested if contractile antagonists would suppress contractions. Progesterone is a steroid hormone synthesized primarily by the corpus luteum in non-pregnant patients and primarily by the placenta in pregnant patients. Progesterone maintains myometrial quiescence and thus is required for pregnancy maintenance.[64] We found that tissues incubated for at least 12 h in progesterone exhibited a slightly lower basal force, yet minimal change to oxytocin-induced force. One explanation for this trend may be that progesterone has both genomic and non-genomic effects that function on different time scales. For example, the decrease in basal force could be caused by a non-genomic mechanism, such as inhibition of G-protein coupled signal transduction, that takes effect more quickly. The genomic effects of progesterone, such as inhibiting translation of the oxytocin receptor gene, may need more time to develop and impact oxytocin-induced contractions.[65] This trend aligns with the clinical use of progesterone as a long-term preventative treatment instead of an acute treatment.[66] Cells likely also lose some sensitivity to progesterone during *in vitro* culture, which we attempted to offset, at least in part, by using concentrations of progesterone in our study (0.127 mM) much higher than those measured in the serum of pregnant women (>10 ng/ml or >32 nM).[67,68]

Of note, exogenous progesterone is applied at relatively high concentrations to try to quell spontaneous preterm birth, such as 200 mg (0.6 mmol) three times a day.[68] Caffeine-treated tissues also exhibited significantly reduced basal forces. Caffeine has consistently been shown to relax smooth muscle,[49,52] but is also discouraged during pregnancy due to its association with preterm labor.[69] However, associations between caffeine and preterm labor have been attributed to caffeine-induced increases in fetal heartrate and movement,[70] not increased myometrial contractility, which is consistent with our findings. These examples highlight that our platform can help dissect the direct effects of compounds on both the basal and induced contractility of human myometrium, independent from other pregnancy-related cells and tissues that may introduce confounding variables.

Many reproductive tissues, including endometrial cells, produce arachidonic acid, which is converted by cyclooxygenase into prostaglandins.[71,72] Patients with dysmenorrhea exhibit elevated plasma prostaglandin concentrations, ranging from 32 to 105 pg/ml, compared to the 20–22 pg/ml observed in patients without dysmenorrhea.[16] NSAIDs are commonly prescribed to patients with dysmenorrhea because they reduce prostaglandin production by inhibiting cyclooxygenase.[12] *In vitro* experiments with non-human myometrial cells have shown that prostaglandins can lead to contraction or relaxation, depending on the prostaglandin subtype and binding receptor.[73] The prevalence of receptor types also varies regionally in the uterus, as the lower uterus usually needs to be more relaxed than the upper uterus such that the fetus can be delivered.[74,75] With our platform, we tested the effects of individual prostaglandins. We found that PGE1 caused μmyometrium to relax, which contrasts with its clinical use in inducing labor.[76] However, our myometrial cells were obtained from a company that does not report the region of origin within the uterus. Thus, it is possible that these cells were retrieved from the lower uterus or are a mixture of cells sourced from multiple regions of the uterus. PGE1 is also known to impact labor by mechanisms that are independent of the myometrium, such as reducing collagen cross-linking in the cervix,[29] and these effects would not be captured in our model. In non-pregnant tissue, PGE2 has been shown to exhibit both contractile and relaxant behavior due to different receptor subtypes, which may explain why it caused relatively minor relaxation in our study and others.[77,78] Future work could account for the region of origin during cell isolation from the uterus such that region-dependent differences in prostaglandin receptors could be identified.

Interleukin levels fluctuate throughout a normal menstrual cycle and pregnancy. Interleukin levels generally peak at times of heightened contractility, including the early follicular phase of the menstrual cycle, when the endometrium is sloughed off, and during the last trimester of pregnancy, when the fetus is pushed out. Serum levels of interleukins IL-6 and IL-8 are also increased in patients who experience dysmenorrhea and preterm labor.[18–20] For example, one study observed that IL-6 levels in cervical fluid were significantly elevated in patients experiencing preterm labor, with a concentration of approximately 600 pg/L compared to just 60 pg/L in patients experiencing term labor.[79] Thus, excessively high levels of interleukins may contribute to abnormal contractile behavior during menstruation and/or pregnancy. Our observation that IL-6 and IL-8 stimulated contractions of engineered myometrium supports the theory that interleukins impact the contractile behavior of the uterus. Given this potential causative relationship, IL-6 and IL-8 may be considered as predictive biomarkers of contractile pathologies, and establishing more robust reference intervals may aid clinicians in providing more effective treatments. In addition, interleukins or their receptors may be promising targets for therapeutic intervention. For example, Tocilizumab is an IL-6 receptor inhibitor that has been approved for treatment of rheumatoid arthritis[80] and may also be considered as a treatment of uterine contractile disorders.

As with oxytocin and progesterone, an important caveat for our experiments with prostaglandins and interleukins is that our *in vitro* concentrations greatly exceeded the measured concentrations in patient fluid samples. However, also similar to oxytocin, we cannot clearly measure or predict the local concentration of these factors within the myometrium, and we expect cells *in vitro* to lose some sensitivity to these molecules. Furthermore, in this study, we focused on the acute contractile effects of these factors. However, long-term exposure to these inflammatory factors likely occurs *in vivo* and may cause remodeling of the cells at the transcriptional level. Thus, evaluating both contractility and cell remodeling after longer incubations with these factors is an important topic for follow-up studies.

Our model is a highly simplified system that is advantageous for systematically measuring the direct impact of small molecules on the contractility of human myometrium without other confounding factors. Due to its simplicity, our model is also relatively easy to scale-up for medium-throughput applications. However, our model is lacking any form of vascularization, which is an important source of paracrine signaling factors and also controls the delivery of drugs and other small molecules from the blood to myometrial smooth muscle cells. The vasculature in the myometrium also undergoes extensive remodeling during pregnancy.[44] Thus, vascularization is an important feature to consider for future iterations of our model, especially to improve its suitability for applications in drug screening. Many immune cells are also embedded in human myometrium,[81] which are key regulators of cytokine signaling and should also be integrated in future models of the myometrium, similar to recent work in engineered cardiac tissue models.[82] We also anticipate that 3-D engineered myometrium models,[83] which can also be selectively doped with supporting cell types, may demonstrate enhanced maturity and sensitivity to small molecules, as observed in other engineered muscle systems.[84,85] Other tissues in the female reproductive system, such as the placenta, fetal membrane, and decidua, also produce and are impacted by cytokines[86–88] and prostaglandins.[13] Thus, our model could be further advanced by pairing it via microfluidics to other engineered models of female reproductive tissues, such as the endometrium,[89,90] placenta,[91,92] or EVATAR, a multi-compartment microfluidic system with ovary, fallopian tube, cervix, liver, and endometrium.[93]

Collectively, due to the many cell and tissue types present in the female reproductive system, models that can systematically incorporate crosstalk between distinct cell and tissue types and provide quantitative functional readouts will be invaluable for establishing mechanisms of human uterine contractility in physiological and pathological contexts. This knowledge will likely reveal opportunities for new therapies to address any number of conditions associated with abnormal uterine contractility. Furthermore, these systems can also serve as testbeds for evaluating the safety and efficacy of drugs and other small molecules on uterine function.

LIMITATIONS OF THE STUDY

A major limitation of our approach is that cells are in a 2-dimensional format and thus lack many of the complex cell–cell and

cell–matrix interactions that are present in native myometrium or 3-dimensional tissue models. We selected polyacrylamide hydrogels because they enable the deformability required to measure force generation and better mimic the rigidity of soft tissues. While the elastic modulus of our hydrogel is slightly lower than what may be found in native myometrium,[94] it is within the same order of magnitude and is soft enough to exhibit measurable deformation caused by these cells. Another limitation is the use of purified fibronectin, which does not represent the diversity of native myometrial ECM.[95] Future modifications to the platform may incorporate additional matrix proteins, such as collagen, elastin, or proteoglycans. Our model also lacks physiological cell–cell interactions, as the tissues comprise purified myometrial smooth muscle cells without any vasculature or immune cells.[81] These supporting cells likely play a key role in regulating myometrial contractility. We also used primary cells isolated from only one patient, and thus our data may not be representative of the general population. We are also unaware of the pregnancy state of the patient or the region of origin of the cells within the uterus, which are critical variables that can be investigated in future studies. The concentrations of our small molecules are also vastly higher than *in vivo* measurements, likely due to a loss of sensitivity caused by *in vitro* culture.

METHODS

Fabrication of PDMS stamps

A chrome photomask with 200 × 100 μm^2 rectangular voids separated by a minimum of 500 μm was designed in AutoCAD (AutoDesk) and manufactured by the UCLA Nanolab mask shop. The photomask was positioned in a mask aligner (Suss MicroTec) above a silicon wafer spin-coated with SU-8 2005 negative photoresist (MicroChem Corp), which was then exposed to UV light based on manufacturer recommendations. The exposed photoresist was then developed, resulting in wafers with raised rectangular features. Sylgard 184 polydimethylsiloxane (PDMS) (Dow Corning) was cast and cured on the master molds to create template stamps with rectangular voids. PDMS was also cast and cured onto featureless wafers to create flat stamps.

Fabrication of polyacrylamide hydrogels

Similar to previous studies,[41,96] 25 mm glass coverslips were submerged in sodium hydroxide (0.1 M) for 5 min, (3-Aminopropyl)triethoxysilane (APTES) (0.5% v/v in 95% ethanol) for 5 min, fresh 95% ethanol 3 times for 5 min each, and glutaraldehyde (0.5% v/v in ultrapure water) for 30 min, then rinsed with water. Polyacrylamide hydrogels were prepared as previously described.[96] Briefly, 40% acrylamide (Bio-Rad), 2% N,N′-methylenebisacrylamide (Sigma-Aldrich), and ultrapure water were combined in a ratio of 5:2:5.[41] 9 μl of TEMED (Thermo Fisher Scientific) was added to 2.501 ml water. 180 μl of the acrylamide mixture was combined with 60 μl of 10× PBS, 6 μl of fluorescent microparticles (Invitrogen, 0.2 μm, yellow-green 505/515), 251 μl of the TEMED solution, and 3 μl of 10% ammonium persulfate. 50 μl of this solution was supplemented with 10 μl streptavidin acrylamide (Invitrogen). 20 μl of the final solution was pipetted onto an activated 25 mm coverslip, flattened with a non-activated 18 mm coverslip, then left undisturbed for 45 min for polymerization. Once fully cured, the non-activated coverslip was removed from the hydrogel using a razor blade and the remaining glass coverslip with hydrogel was rinsed and submerged in PBS for storage at 4 °C.

Microcontact printing

Lyophilized fibronectin (Corning) was incubated with aqueous sodium carbonate and sulfo-NHS-LC-biotin (ThermoFisher) overnight, then dialyzed to remove unconjugated biotin. The remaining biotinylated fibronectin was collected and stored at 4 °C. To microcontact print the hydrogel, flat PDMS stamps were incubated with diluted biotinylated fibronectin (200 μg/ml in PBS) for an hour, then dried with compressed air. Similar to previous studies,[97] template stamps with rectangular voids were placed in a UVO Cleaner model 342 (Jelight Company) for 20 min then pressed against fibronectin-coated flat stamps. The template stamp was then removed and the flat stamp was pressed onto dried polyacrylamide hydrogels. The substrate was rinsed then submerged in PBS, exposed to low levels of UV light in a biosafety cabinet for sterilization, and stored at 4 °C until cell seeding.

Myometrial smooth muscle cell culture

Cell culture media was prepared by combining vascular cell basal medium (ATCC PCS-100-030), vascular smooth muscle cell growth kit (ATCC PCS-100–042), including fetal bovine serum, and penicillin–streptomycin (0.1%) and sterilizing with a 0.22 μm vacuum filter. Human myometrial smooth muscle cells (ATCC) were obtained at passage two, expanded, cryopreserved, and thawed as needed for experiments at passage four. T75 tissue culture flasks were incubated with 0.1% gelatin (Stem Cell Technologies) for 30 min, followed by incubation with cell culture media for 30 min. A cryopreserved cell aliquot was then thawed for 2 min at 37 C, resuspended in 3 ml media, and divided amongst four T75 flasks. Cells attached for a minimum of one day, then were detached using 0.5% trypsin-EDTA and seeded onto prepared coverslips at a density of 150 000 cells per 3 ml media in a 6-well plate. After 24–36 h, cells were observed for attachment to the micropatterned fibronectin rectangles, then serum starved for 12 h prior to live imaging.

Immunostaining and characterization of tissue structure

For immunostaining, cells were cultured on hydrogel substrates prepared without fluorescent beads. Seeded coverslips were fixed by rinsing with PBS, incubating for 10 min with 4% paraformaldehyde and 0.2% TritonX-100, then rinsing again in PBS prior to storing for up to one week at 4 C. To stain, coverslips were incubated for 1.5 h with rabbit anti-fibronectin antibody (Sigma Aldrich, 1:200), rinsed three times in PBS, then incubated again for 1.5 h with DAPI (Invitrogen, 1:200), Phalloidin (Life Technologies, 1:200), and Alexa Fluor 546 goat anti-rabbit antibody (Invitrogen 1:200). Coverslips were again rinsed three times in PBS before mounting on microscope glass slides with prolong Gold antifade (Invitrogen) and sealed with nail polish.

Stained coverslips were imaged using a 20× objective on a Nikon Ti inverted fluorescence microscope with Andor Zyla scientific CMOS camera. Nuclei per island were counted using DAPI signal and ImageJ Analyze Particles. Island size was calculated using fibronectin signal and ImageJ. Cytoskeletal alignment was calculated using actin signal and ImageJ OrientationJ. Data are displayed as mean ± standard error of the mean.

Traction force microscopy

Tissues were serum starved for a minimum of 12 h before imaging. 15 min before imaging, coverslips were rinsed three times with Tyrode's solution (5.0 mM HEPES, 1.0 mM magnesium chloride, 5.4 mM potassium chloride, 135.0 mM sodium chloride, 0.33 mM potassium phosphate, 1.8 mM calcium chloride, 5.0 mM D-glucose, and pH 7.4 at 37 °C), then transferred in 300 μl Tyrode's solution to a temperature-controlled imaging chamber mounted on a Nikon Ti inverted fluorescence microscope with Andor Zyla scientific CMOS camera. Movement of fluorescent beads was captured using the 10× objective, 1.5× zoom, at a frame rate of one image per minute for 12 min. Trypsin-EDTA (0.05%) was then added at a ratio of 1:1 for the purpose of identifying the original or force-free location of fluorescent beads, and images were collected for an additional 6 min. The treatment durations were decided empirically. Brightfield images of tissues were taken before the contraction, before the addition of trypsin, and after the trypsin exposure.

To measure response to oxytocin, one fluorescent bead image was captured before the addition of 300 μl phosphate buffered saline (PBS) or 300 μl synthetic oxytocin (1 mM) (Sigma Aldrich) in PBS for a final concentration of 0.5 mM. For drug response experiments, cells were pre-incubated with progesterone or caffeine prior to imaging, then 1 mM of oxytocin was added after the first bead image was captured. Progesterone (Millipore Sigma, 0.127 mM) was added to serum-free media and incubated for a minimum of 12 h before imaging. Caffeine (Sigma Aldrich) was combined with Tyrode's for a concentration of 10.3 mM and added to coverslips during adjustment time on the microscope for a total of 10 min of incubation. To measure responses to inflammatory mediators, 300 μl of prostaglandins [2 μM for prostaglandin E1 (PGE1), prostaglandin E2 (PGE2), and prostaglandin F2α (PGF), Sigma Aldrich][98] or cytokines [2 μg/ml in PBS for interleukin-6 (IL-6) and interleukin-8 (IL-8), R&D systems][32,99] were added immediately after the first reference image was collected.

Matlab was used to quantify bead displacement and force generation, as previously described.[41,100] Briefly, an image sequence of the beads from a given tissue was uploaded. Each frame was divided into a grid with 4096 squares, each 6.88 × 6.88 μm^2. Each element of each frame was compared to the corresponding element in the initial frame to develop a displacement vector field. The three maximum vectors of each frame were averaged, then all tissues within one condition were averaged to develop a displacement vs time graph. A second order polynomial equation was fit to the displacement curves and the second coefficient (B1) of the equation was compared for each curve. For induced force responses, the frame used to calculate the peak of the contraction or relaxation was visually selected. The frame used to calculate the basal (unstimulated) force response was consistently identified as the last frame of the trypsin incubation. Using Fourier transform traction cytometry on the displacement vector field, traction stress vectors at the peak of contraction, relaxation, or detachment were calculated. These traction stress vectors were multiplied by their x-coordinate, summed, and then divided by the length of the tissue to calculate either basal or induced force.[41]

SUPPLEMENTARY MATERIAL

See the supplementary material for Video S1, contractile response to oxytocin, related to Fig. 2. (A) Static brightfield image of μmyometrium indicating tissue outline. (B) As μmyometrium contracts in response to oxytocin, underlying beads displace toward the center of the tissue. (C) Displacement and (D) traction stress heat maps are calculated from bead displacement.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Ethics Approval

Ethics approval is not required.

Author Contributions

Antonina P. Maxey: Conceptualization (equal); Formal analysis (lead); Investigation (lead); Methodology (equal); Software (lead); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal). **Sage J. Wheeler:** Formal analysis (supporting); Investigation (supporting). **Jaya M. Travis:** Investigation (supporting). **Megan L. McCain:** Conceptualization (lead); Funding acquisition (lead); Methodology (equal); Resources (lead); Supervision (lead); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal).

Data AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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