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Effects of polydimethylsiloxane membrane surface treatments on human uterine smooth muscle cell strain response

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ABSTRACT

In the United States, 1 in 10 infants are born preterm. The majority of neonatal deaths and nearly a third of infant deaths are linked to preterm birth. Preterm birth is initiated when the quiescent state of the uterus ends prematurely, leading to contractions and parturition beginning as early as 32 weeks, though the origins are not well understood. To enable research and discovery of therapeutics with potential to better address preterm birth, the capability to study isolated cell processes of pregnant uterine tissue *in vitro* is needed. Our development of an *in vitro* model of the myometrium utilizing human uterine smooth muscle cells (uSMCs) responsible for contractions provides a methodology to examine cellular mechanisms of late-stage pregnancy potentially involved in preterm birth. We discuss culture of uSMCs on a flexible polydimethylsiloxane (PDMS) substrate functionalized with cationic poly-L-lysine (PLL), followed by extracellular matrix (ECM) protein coating. Previous work exploring uSMC behavior on PDMS substrates have utilized collagen-I coatings, however, we demonstrated the first exploration of human uSMC response to strain on fibronectin-coated flexible membranes, importantly reflecting the significant increase of fibronectin content found in the myometrial ECM during late-stage pregnancy. Using the model we developed, we conducted proof-of-concept studies to investigate the impact of substrate strain on uSMC cell morphology and gene expression. It was found that PLL and varied ECM protein coatings (collagen I, collagen III, and fibronectin) altered cell nuclei morphology and density on PDMS substrates. Additionally, varied strain rates applied to uSMC substrates significantly impacted uSMC gene expression of IL-6, a cytokine associated with instances of preterm labor. These results suggest that both surface and mechanical properties of *in vitro* systems impact primary human uSMC phenotype and offer uSMC culture methodologies that can be utilized to further the understanding of cellular pathways involved in the uterus under mechanical load.

1. Introduction

In 2018, 1 in every 10 infants born in the United States was born preterm [1]. The majority of neonatal deaths and nearly a third of infant deaths are linked to preterm birth [2,3]. During pregnancy, the uterus enters a state of quiescence to carry the fetus to term. Preterm birth is initiated when the quiescent state of the uterus ends prematurely, leading to contractions and parturition beginning as early as 32 weeks, though the underlying cellular mechanisms are not well understood. Tocolytics are a class of clinical compounds currently utilized to postpone preterm birth, but have not proven effective in preventing preterm delivery [4,5]. Animal models investigating pregnancy and preterm birth have limitations for clinical translation due to significant differences in parturition regulation and initiation between humans and other animals [6]. Therefore, development of novel treatment options surrounding preterm birth would benefit from physiologically relevant *in vitro* systems with controlled environments to systematically identify and probe underlying biological pathways and biophysical influences involved in human parturition.

Epidemiological research has found strong clinical comorbidities linking dysfunctional uterine mechanics, such as connective tissue disease and increased uterine load, to preterm birth [7–9]. It is

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hypothesized that changes in collagen fiber alignment and altered uterine wall tension induced by multiple fetuses or dysregulated extracellular matrix (ECM) production disrupt uterine quiescence, leading to premature labor and birth. Uterine wall strain rate has not been specifically studied, but ultrasound measurements indicate that uterine volume increases at a constant rate from 20 to 40 weeks during gestation, and volumetric growth rate of the uterus is doubled during this time frame in twin pregnancies [10]. Notably, the likelihood for preterm birth increases from approximately 10 % in singleton pregnancies to 50 % in twin pregnancies, and up to 95 % in triplet pregnancies, suggesting a relationship between preterm labor and increased uterine load [9]. Though recent efforts to mathematically model uterine mechanics during pregnancy have increased, these studies have focused on the change in mechanical properties of the cervix over the course of pregnancy and largely do not characterize forces on the myometrial wall or the cell strain experienced by smooth muscle cells (SMCs) [11,12].

Both clinical and animal studies indicate that uterine tissue phenotype is impacted by mechanical load *in vivo* [13–15]. For example, uterine SMC (uSMC) hypertrophy driven by mechanical strain is implicated in labor initiation in rats [13], and cervical ripening and labor can be induced through acute mechanical stimulation of the uterus via intra-uterine application of balloon inflation [14]. To better understand how mechanotransduction regulates uterine cells during human pregnancy, the use of *in vitro* strain platforms to model and study the mechanical microenvironment of pregnancy has been adopted by many researchers. Results from these studies demonstrate that *in vitro* strain of uterine cells impacts protein expression, gene regulation, as well as hormone and cytokine production [16–19]. To date, though strain magnitude and duration have been studied in uSMCs [17,20–22], variations in strain rate – which could serve as an *in vitro* proxy of increased uterine growth rates occurring in multi-fetal pregnancies with higher likelihoods of preterm birth – have not yet been reported. Given that clinical data presents multi-fetal pregnancies as a risk factor for preterm labor [9], modeling how mechanical strain rates impact human uSMC phenotype *in vitro* might elucidate strain-based mechanisms by which multi-fetal pregnancies influence preterm labor, particularly by using *in vitro* strain rate as a proxy for increased uterine growth rates [10].

It is important to note that collagen I is most commonly used as a surface treatment for *in vitro* studies, and to date all *in vitro* research published involving mechanically strained uSMCs utilizes collagen I-treated substrates [16–19,23]. Both ECM composition and cell-cell adhesion is important to myometrial tissue function, thus using physiologically relevant ECM proteins *in vitro* is vital to studies focused on mechanotransduction of the uterus. In the non-gravid (non-pregnant) uterus, the main structural proteins of the myometrial extracellular matrix are collagen I, collagen III, and traces of fibronectin surrounding SMC bundles. However, in late-term pregnancy and labor, collagens I and III are significantly downregulated while fibronectin is significantly upregulated in the myometrium [24,25]. To our knowledge, despite the documented changes in ECM expression from early to late-term pregnancy, human uSMC growth on protein-coated substrates other than collagen-I has not been explored under strain conditions. The data presented herein is the first investigation of uSMC response to strain on fibronectin-coated flexible substrates.

Polydimethylsiloxane (PDMS) coating conditions were established based on previous research validating the utility of layer-by-layer (LBL) deposition coating techniques on cell culture platforms by alternating thin-layer coatings of electrolyte and ECM protein solutions [26–28]. Specifically, uSMC growth conditions comprised of different combinations of an electrolyte solution, poly-L-lysine (PLL), with ECM protein coatings physiologically relevant to the myometrial ECM content of early (collagen I/III dominant) and late (fibronectin dominant)-stage pregnancy. After testing the impact of surface coatings on uSMC growth without strain, proof-of-concept experiments were performed to test how uSMCs grown on fibronectin-coated surfaces responded to biaxial strain rates, characterizing cell response through image analysis and gene expression. Increased uterine growth rates in multi-fetal pregnancies (and potential acceleration of myometrial wall strain) was of interest to model due to the associated increased risk of preterm birth [9,10]. Thus, biaxial strain rates were varied in later experiments to study uSMC response to strain rates *in vitro*. This study presents the development of a robust *in vitro* model of the myometrium utilizing primary myocytes – uterine smooth muscle cells responsible for contractions – to provide a platform to examine the cellular mechanisms of uSMCs in response to the myometrial microenvironment during late-stage pregnancy. Our approach provides a foundation for studying pathways potentially involved in human uSMC response to substrate microenvironmental changes using cell morphology and RT-PCR as primary readouts. We demonstrate that both (1) substrate surface treatments and (2) substrate strain mechanics influence uSMC growth, uSMC morphological changes such as nuclei enlargement, and gene expression changes such as IL-6 mRNA upregulation.

2. Materials and methods

2.1. Membrane fabrication

PDMS membranes were fabricated using Sylgard 184 (Dow Corning, Midland, MI). A ratio of 10:1 w:w of base and curing reagent was evenly mixed to fabricate a substrate previously reported to have a stiffness on the order of 1–2 MPa [29]: this formulation was chosen to match the reported modulus for the non-gravid uterine myometrium (0.5–1 MPa) [29]. Briefly, after mixing the separate base and curing agents, the solution was degassed, cast as a sheet of 0.8 mm thickness by pouring the 10:1 mixed base and curing reagent solution into a flat reservoir mold, then cured at room temperature under vacuum overnight prior to being transferred to a 60 °C oven for 1 h. Membranes were then affixed to acrylic rings with DOWSIL 3140 RTV adhesive (Dow Corning, Midland, MI) and press-fit into a commercial twelve-well plate (Corning, Corning, NY) prior to ethylene oxide sterilization (Andersen, Haw River, North Carolina). After ethylene oxide exposure, PDMS membranes were degassed in a vacuum chamber for one week prior to the start of experiments.

2.2. Membrane functionalization and protein coating

PDMS is the material most used as a substrate in commercial strain systems due to its biocompatibility, tunable stiffness, and elasticity. Despite the advantages of using PDMS as a cell culture substrate in strain systems, untreated PDMS surfaces are highly hydrophobic and must be modified to facilitate cell attachment and growth. A common wet-phase method for PDMS surface functionalization employs electrolyte deposition, sometimes alternating adsorption of polyanion and polycation solutions to form polyelectrolyte layers on surfaces in a process known as LBL deposition [29]. Treatment of PDMS with single layers of cationic solutions used in LBL such as Poly-D-lysine (PDL) and Poly-L-lysine (PLL) has been shown to improve cell attachment, spreading, and growth both alone and in combination with ECM protein coatings [26–28]. Using these surface functionalization methodologies, we aimed to create a surface that supported uSMC growth by mimicking the *in vivo* ECM composition of the uterus by adjusting surface protein coatings. After sterilization, membranes were exposed to oxygen plasma for 2 min. Membranes that received PLL functionalization were incubated with 0.01 % sterile cell culture grade PLL for 5 min at room temperature (Sigma-Aldrich, St. Louis, MO) prior to being aspirated, washed with distilled water three times, and allowed to dry overnight per manufacturer's instructions. Control membranes that did not receive PLL coatings were incubated in PBS to preserve surface wetting properties until membranes underwent protein coating the following day. To coat with protein solutions, human collagen III (Advanced Biomatrix) and human collagen I (Advanced Biomatrix) stocks were further diluted in 0.01 M HCl in PBS to a working concentration of 5 μg/mL. To directly compare

collagen and fibronectin, collagen I and collagen III monomers were maintained for treatment solutions. An acidic environment of pH 2.0 was maintained to preserve monomeric collagen and prevent the formation of collagen fibrils during treatment according to manufacturer's instructions. All ECM proteins in this study were prepared at a concentration of 5 μg/mL, though collagen proteins are normally used at higher concentrations during *in vitro* culture to encourage gelation over coating. PDMS membranes were also treated with human fibronectin (Millipore Sigma) in PBS at a neutral pH per supplier's recommendations, at a working concentration of 5 μg/mL. All ECM solutions were added to membranes and incubated at room temperature for 2 h prior to cell seeding.

2.3. Culture of primary uterine smooth muscle cells

Primary uSMCs were purchased from ATCC and expanded prior to use. For experiments, uSMCs were thawed out of liquid nitrogen and seeded directly onto substrates at a seeding density of ~75,000 cells/cm^2. uSMCs were cultured in an incubator at 37 °C and 5 % CO_2. Cultures were supplemented with Vascular Smooth Muscle Cell Growth Kit (ATCC, Manassas, VA), and media was changed every other day. To investigate the potential impact of PLL and ECM proteins on uSMC phenotype, cells were cultured for 3 days on PDMS substrates coated with fibronectin, collagen I, and collagen III after optional treatment with PLL (Fn + PLL, Fn, Col I + PLL, Col I, Col III + PLL, Col III). Following 3 days of growth on substrates, uSMCs were fixed and nuclei were labeled with Hoechst 33342. Fiji image analysis was performed to quantify and compare nuclei morphologies between treatment groups. Prior to all strain experiments, uSMCs at confluence were switched to a low serum media (1 % FBS) for at least 18 h prior to strain initiation. Cells between passage 3–4 were utilized for all experiments.

2.4. Application of strain

Prior to strain exposure, uSMCs were seeded on Fn + PLL and Fn-treated membranes. Over 2–3 days, uSMCs were cultured until confluent, then switched to a low serum media at least 18 h prior to strain of the substrate. To apply strain, uSMCs were inserted into a biaxial straining apparatus and transferred to a humidified, CO_2-controlled cell culture incubator for the duration of the experiment. Strain parameters were chosen that vary strain rate while holding total strain magnitude and duration of strain time constant. Strain rates were selected to mimic the growth observed in multi-fetal pregnancies. For comparative strain studies, it was decided to select strain rates that double in magnitude to model the approximately doubled uterine volumetric growth that distinguishes twin and singleton pregnancies in late-stage pregnancy [10]. Cells were either strained at a rate of ~3.3 % per hour (1X strain rate) for ~6 h, or 6.7 % per hour (2X strain rate) of active strain until reaching a maximum biaxial strain for ~3 h at which point cell substrates were held at 20 % for an additional ~3 h prior to experiment take-down to match experiment time with the 1X strain rate condition. The maximum final strain for each condition was 20 % biaxial strain. Unstrained controls were cultured adjacent to the strain devices in the same environment for the same duration of time. Upon the conclusion of the strain regimen, devices were removed, media was collected, and cells were either fixed or RNA was extracted for further downstream processing. Cells that were fixed had their nuclei labeled with Hoechst 33342. Nuclei sizes were then quantified and compared between groups. Image analysis of strained membranes was used to confirm percent strain was achieved consistently with PDMS membranes (Fig. S3). Our strain rate parameters were selected to mimic the fold change of growth rate between singleton and twin pregnancies, however it is important to note: (1) *in vitro* strain rates did not scale at the same strain per unit time that is found in the uterine myometrium *in vivo,* and (2) the *in vivo* strain rates previously discussed are approximations based on the data available in literature and could be improved with additional characterization of uterine geometries over the course of pregnancies. The difference between *in vitro* strain rates tested in this study compared to what is estimated as typically seen *in vivo* was primarily due to common constraints of *in vitro* strain models such as cell growth time and the total maximum achievable strain. Rather than growing on the order of 2–3 % per day, the *in vitro* uSMCs were exposed to strain on the order of ~3.3–6.7 % biaxial strain per hour. Despite increased strain rates, these experiments did not have the capacity to reach maximum strain values comparable to the myometrial wall during late-stage pregnancy (given the 100-fold increase in uterine volume over the course of pregnancy) [30].

2.5. Immunoflorescent staining and imaging

Cells were fixed in 4 % PFA for 15 min at room temperature (RT) or with 95 % methanol/5 % acetic acid by volume at 4 °C depending on antibody requirements (Sigma-Aldrich, St. Louis, MO). Tissues were washed with PBS and blocked with 1 % bovine serum albumin for 1 h at RT. Primary antibodies were diluted in blocking buffer and incubated overnight at 4 °C (α-FN raised in rabbit, α-alpha SMA raised in mouse, collagen probe CNA35 binding protein-EGFP conjugate). Secondary antibodies were incubated for 2 h protected from light at RT (Goat α-MS 488 Alexa Fluor®, Goat α-Rb 555 Alexa Fluor®, Thermo Fisher, Waltham, MA). For fibrillar collagen visualization, a CNA35 collagen probe was fluorescently tagged with EGFP (Addgene, Watertown, MA) and incubated with samples overnight at 4 °C. Samples were imaged using a ZEISS LSM700 confocal microscope (ZEISS, Oberkochen, Germany).

2.6. Image analysis and characterization

As previously discussed, rat uterine myometrial SMCs have been recorded to increase in size in the later phase of pregnancy [31]. In humans, increased nuclei size of myometrial uSMCs has been observed in caesarean tissue samples [32]. For these reasons, image analysis of nuclei morphology was used to assess uSMC morphology in the experiments presented [32,33]. Nuclei size and count were quantified using Fiji image analysis software (Fiji, U.S. National Institutes of Health, Maryland, USA). Images were converted to binary masks, segmented using Fiji's built-in watershed function, and particles were analyzed for pixel area. Areas were converted to micrometers by setting the scale according to image acquisition parameters.

2.7. Gene expression characterization

At the conclusion of strain experiments, cells were lysed and collected for further processing utilizing RLT buffer and a 1 % β-mercaptoethanol suspension (QIAGEN, Hilden, Germany). mRNA was isolated following kit manufacturer's protocols, and a subset of devices were selected to verify quality and quantity of mRNA using the Tapestation 4200 system (Agilent, Santa Clara, CA). All samples had RINe values of 10 and mRNA content on the order of at least 500 pg per sample. cDNA was generated using a SUPERSCRIPT™ IV VILO™ kit, and qRT-PCR was performed using TaqMan™ probes, TaqMan™ Fast Advanced Master Mix, and a Quant Studio 7 (Thermo Fisher Scientific, Waltham, MA). Comparative CT analysis was used to calculate ΔCT, ΔΔCT, and fold change by normalizing to 18S as a housekeeper gene. Fold change was calculated by exponential transformation as $2^{-(\Delta\Delta CT)}$ reported with respect to no strain controls. Standard deviation was calculated using the equation $SD = (s_1^2+s_2^2)^{1/2}$ where s_1 and s_2 represent the standard deviations of CT replicates for the gene of interest and housekeeper 18S, respectively.

2.8. Statistical analysis

All statistical analysis was performed with GraphPad Prism 9.3.1 (GraphPad, San Diego, CA) and R 4.2.1 (R Core Team (2022). R: A

language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). Assuming central limit theorem, a one-way ANOVA with $\alpha = 0.05$ and Tukey's multiple comparisons test was used to assess cell density differences between membrane treatment conditions. To compare nuclei size in strain conditions with and without PLL-treated substrates, data set distributions were assessed with Welch's two sample *t*-test with $\alpha = 0.05$. Unpaired t-tests with $\alpha = 0.05$ were used to compare gene expression differences for each qRT-PCR probe between 1X and 2X strain conditions.

3. Results

3.1. Poly-L-lysine and ECM protein surface treatments impact uSMC adherence and growth over time on PDMS substrates

After PDMS substrates were treated with combinations of PLL and ECM protein coatings (Fn, Fn + PLL, Col I, Col I + PLL, Col III, Col III + PLL), uSMCs were seeded and their growth was monitored over time. PDMS substrates treated with or without PLL presented notable differences in cell aggregation and confluence of uSMCs on both days 1 and 3. Previous research has demonstrated that coating with PLL or PDL enhances cell attachment on hydrophobic surfaces [26–28], and indeed PLL-treated PDMS substrates supported increased confluence and spreading of uSMCs over time compared to non-PLL-treated substrates (Fig. 1). Differences in uSMC culture amongst PLL treatments became more pronounced with an increase in culture time by day 3 (Fig. 1). Col I and Col III -treated substrates yielded cell aggregate formation in non-PLL-treated conditions as early day 1, but by day 3 cell aggregation occurred in all non-PLL-treated ECM conditions (Col I, Col III, and Fn). uSMC confluence and growth was impacted not only by PLL functionalization, but different matrix protein treatments as well. Fn and Fn + PLL -treated PDMS resulted in the highest confluence of uSMCs at both day 1 and day 3 compared to Col I, Col I + PLL, Col III, and Col III + PLL -treated substrates (Fig. 1). Overall, PLL treatment enhanced uSMCs growth on PDMS substrate with fewer occurrences of cell aggregation. Additionally, Fn and Fn + PLL -treated substrates yielded the highest confluence of uSMCs within each treatment group and timepoint compared to other protein treatments, regardless of PLL treatment. Qualitatively, we observed that Fn + PLL-treated PDMS substrates supported the highest confluence and lowest cell aggregation of uSMCs on Days 1 and 3.

3.2. uSMCs stain positive for collagen I and fibronectin after culture on different ECM protein-treated PDMS substrates

To investigate protein expression of uSMCs cultured on ECM protein-treated PDMS substrates, after 3 days of growth, cells were fixed and stained for fibronectin and fibrillar collagen expression. uSMCs expressed fibronectin and fibrillar collagen similarly across Col I + PLL, Col III + PLL, or Fn + PLL substrates. Across all three conditions on day 3, fibronectin staining labeled connections between cell bodies, however, no intracellular staining was observed (Fig. 2). Contrastingly, fibrillar collagen staining with CNA35 labeled cell bodies, but no intercellular collagen staining was observed. PDMS substrates were treated with monomeric collagen, thus collagen-treated substrates were not labeled by the CNA35 probe. These observations were consistent across uSMCs grown on all substrate treatments despite variation in uSMC confluence (Fig. 2), and matched histological descriptions of myometrial SMC expression of collagen I and fibronectin *in vivo* [24].

3.3. Fibronectin-treated PDMS supports significantly higher cell density than collagen I- or collagen III-treated PDMS substrates

To investigate the impact of protein treatment conditions on uSMC confluence, image analysis was used to quantify cell density from the number of nuclei per field of view for Col I + PLL, Col III + PLL and Fn + PLL. Fn + PLL treatments yielded a significantly higher cell density of uSMCs (cell/cm^2) after 3 days of growth compared to Col I + PLL ($p = 0.0015$) and Col III + PLL ($p < 0.0001$) via a one-way ANOVA with

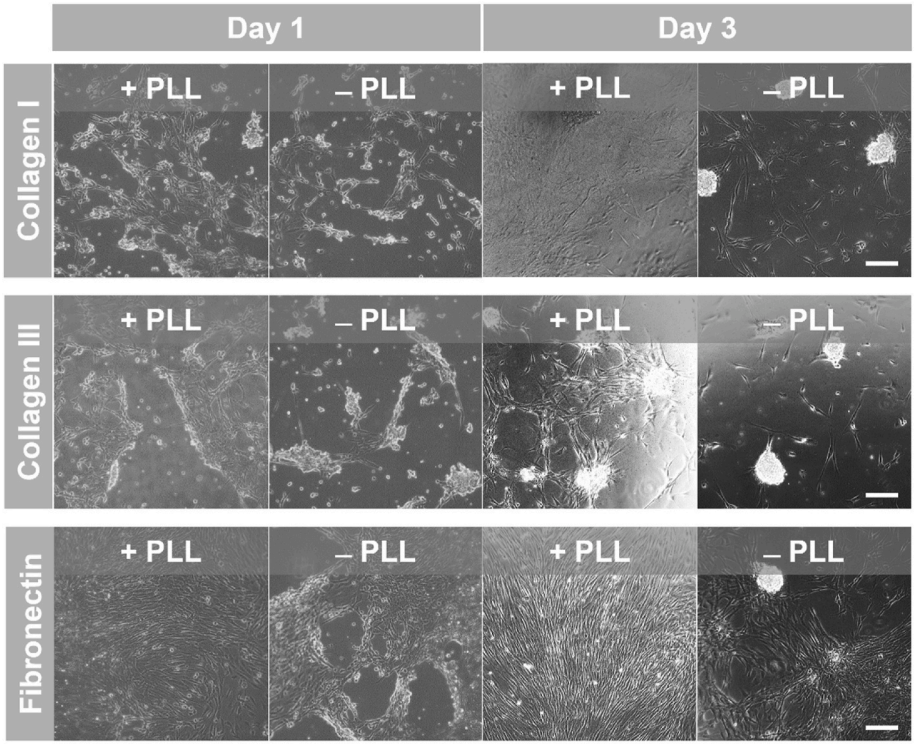

Fig. 1. Phase-contrast images of uSMC growth over time across PLL and ECM treatment conditions. Images of cells were captured days 1 and 3 post-seeding prior to fixation and further processing. Images representative of 3 biological replicates across 3 experiments. Scale bar = 200 μm.

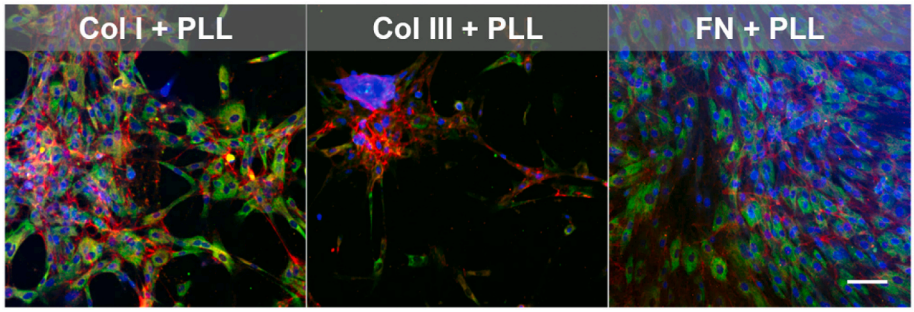

Fig. 2. Protein expression and morphology of uterine SMCs exposed to different membrane surface ECM treatments after 3 days of growth. Cells were fixed and stained with a CNA35 collagen probe (green), α-fibronectin (red), and Hoechst 33342 (blue) to assess cell morphology across conditions. Images representative of conditions with 3–6 biological replicates across 3 experiments. Scale bar = 100 μm.

Tukey's multiple comparisons test (α = 0.05) (Fig. 3). Col I + PLL treatment yielded a significantly higher cell density of uSMCs after 3 days of growth compared to Col III + PLL ($p = 0.0048$) via a one-way ANOVA with Tukey's multiple comparisons test (α = 0.05) (Fig. 3). For PDMS substrates without PLL treatments, Fn-treated substrates yielded significantly higher cell density than Col I and Col III ($p < 0.0001$, $p < 0.0001$), however, there was no significant difference between the two collagen treatments via a one-way ANOVA with Tukey's multiple comparison test (α = 0.05) (Fig. 3). The presence of PLL significantly increased uSMC density on collagen substrates ($p < 0.0001$), but did not significantly impact cell density on collagen III or fibronectin substrates via a Welch two sample *t*-test (α = 0.05) (Fig. S1).

3.4. Poly-L-lysine treated PDMS substrates significantly reduce uSMC nuclei size across all ECM conditions

Enlarged nuclei of myometrial SMCs has been observed in third trimester pregnancies in humans [32]. Given this, investigating uSMC nuclei size change in response to culture conditions was of interest. After 3 days of culture on various substrate treatments, cells were fixed, stained, and imaged to assess nuclei morphology. For all ECM conditions, the presence of PLL on PDMS membranes prior to protein treatments significantly reduced nuclei size of uSMCs on day 3 of culture via a Welch two sample *t*-test ($p < 0.0001$, α = 0.05) (Fig. 4). These results indicated that the presence of PLL significantly impacted nuclei morphology. Additionally, the variation of nuclei size in PLL conditions was lower than uSMCs grown on surfaces not treated with PLL as demonstrated by the standard deviations across conditions: (Col I + PLL: +/− 4.56 μm^2 vs. Col I: +/− 127.2 μm^2, Col III + PLL: + / − 70.45 μm^2 vs. Col III: +/− 112.1 μm^2, Fn + PLL: +/− 56.58 μm^2 vs. Fn: + / − 82.97 μm^2). ECM proteins impacted nuclei area as well, with uSMCs grown on collagen III treated substrates having significantly larger nuclei (μm^2) than those grown on collagen I or fibronectin substrates in PLL or non-PLL conditions when compared via a one-way ANOVA with Tukey's multiple comparisons ($p < 0.0001$, α = 0.05) (Fig. S2). In non-PLL conditions, there was no significant difference in nuclei area, however in PLL conditions, uSMCs grown on Fn + PLL treated substrates had significantly larger nuclei than uSMCs grown on Col I + PLL substrates ($p < 0.0001$, α = 0.05) (Fig. S2).

3.5. uSMCs cultured on poly-l-lysine-treated PDMS substrates undergo significant nuclear morphology changes compared to uSMCs cultured on non-PLL-treated PDMS substrates when exposed to strain

Due to the demonstrated significant impact of PLL treatment on downstream uSMC growth (Figs. 1 and 3), investigating how uSMCs respond to mechanical strain in the presence and absence of PLL treatment was of interest. uSMCs were grown to confluence and serum-starved as previously described, then exposed to strain over 3 h, which ramped to 20 % on membranes either Fn-treated or Fn + PLL-treated. Afterwards, cells were fixed, and nuclei size was quantified. uSMCs grown on Fn + PLL membranes and exposed to strain had significantly larger nuclei compared to unstrained uSMCs grown on Fn + PLL via an unpaired *t*-test ($p = 0.0146$, α = 0.05). uSMCs grown on Fn membranes did not demonstrate significantly different nuclei sizes between strain conditions ($p = 0.2236$, α = 0.05) (Fig. 5).

3.6. Strain rate significantly impacts IL-6 gene expression in uSMCs

It was found that uSMCs on Fn + PLL-treated membranes appeared to undergo morphological changes, such as increased nuclei size, when exposed to strain, thus, additional experiments sought to explore these strain parameters in more depth. Strain rate was of interest due to its hypothesized role in the mechanisms of preterm birth. Preterm birth is significantly increased in twin and triplet pregnancies in comparison to singleton pregnancies [9]. Concurrently, the rate of uterine growth in twin pregnancies has been shown to be approximately double that of singleton pregnancies [10]. Given the correlation between increased growth rates and preterm labor in multi-fetal pregnancies, it was decided to investigate how varied strain rates impact uSMC phenotype *in vitro*. Though strain duration and magnitude has been shown to impact uSMC response *in vitro*, the impact of strain rate on uSMC phenotype has not been previously studied [16–18]. The original strain rate found to induce enlarged uSMC nuclei on Fn + PLL membranes (1X strain rate) (Fig. 5) was compared to a strain rate doubled in magnitude (2X strain rate) to mimic the increased uterine growth experienced in twin pregnancies compared to singleton. To assess how substrate strain rate impacted uSMC gene expression, mRNA was isolated from uSMCs exposed to different strain rates and quantified using qRT-PCR. A qRT-PCR probe set was selected to assess strain impact on uSMC proliferation (KI67), matrix remodeling (ACTA2, COL1A1, FN1), and cytokine activation (IL6) – all processes that change over the course of pregnancy and can be dysregulated during preterm labor [25,31,34,35]. Biaxial strain conditions of ~3.3 % per HR and ~6.7 % per HR to a total maximum strain of 20 % (referred to as 1X strain rate and 2X strain rate, respectively), were normalized to unstrained uSMC controls to evaluate fold change differences in response to strain (Fig. 6).

Notably, gene expression of KI67, a cell proliferation marker, was downregulated by both strain conditions compared to cells not exposed to strain (1X strain rate: 1.84-fold decrease +/− 0.84, 2X strain rate: 1.18-fold decrease +/− 0.56). ACTA2, the gene coding for α-SMA, a key SMC protein, was also downregulated in both strain conditions (1X strain rate: 1.61-fold decrease +/− 0.35, 2X strain rate: 1.14-fold decrease +/− 0.95). Expression of genes coding for extracellular matrix proteins FN1 (1X strain rate: 1.34-fold increase +/− 0.32, 2X strain rate: 1.04-fold increase +/− 0.76) and COL1A1 (1X strain rate: 1.12-fold decrease +/− 0.35, 2X strain rate: 1.01-fold increase +/− 0.95) were relatively unchanged for both strain conditions, remaining at similar levels to unstrained uSMCs. The gene marker associated with interleukin-6 (IL-6) response was upregulated (3.63-fold increase +/−

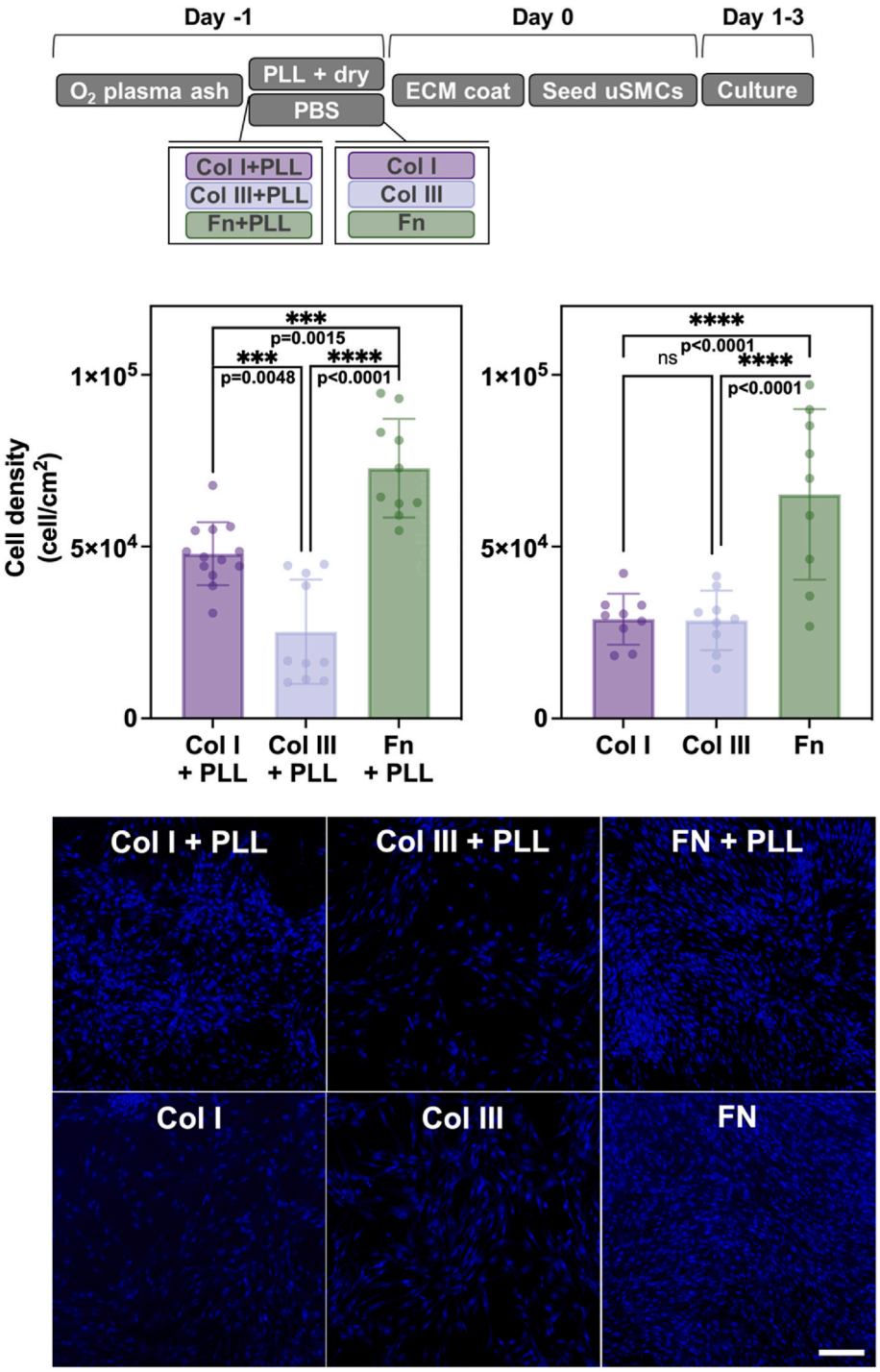

Fig. 3. Fibronectin treatment significantly increased cell density of uSMCs compared to Collagen I and Collagen III. Six images were captured per sample and an automated Fiji script was used to count nuclei within an image and extrapolate cell density values. A one-way ANOVA with Tukey's multiple comparisons test was performed comparing cells grown on membranes treated with different ECMs. Significance values are displayed as (ns, $p > 0.05$, ***, $p < 0.01$, ****, $p < 0.0001$) with an $\alpha = 0.05$. Error bars represent standard deviation from the mean for 3 biological replicates across 3 experiments. Scale bar = 200 μm.

1.56) in 1X strain rate conditions, but downregulated in conditions exposed to 2X strain rate (1.25-fold decrease, +/− 1.37). These IL-6 gene expression changes were significantly different between strain conditions when compared via an unpaired *t*-test ($p = 0.007138$, $\alpha = 0.05$). Gene expression did not significantly vary between strain conditions for KI67, ACTA2, COL1A1, or FN1 (Fig. 6).

4. Discussion

In this study, LBL deposition strategies were utilized to enable investigation of the impact of various ECM proteins on uSMCs grown on PDMS substrates. Using a methodology amenable to modulation of ECM protein coatings, culture conditions were tailored to investigate specific physiologically relevant microenvironments. uSMCs were grown on ECM-treated substrates using several proteins native to the myometrium, namely: collagen I, collagen III, and fibronectin [25]. Notably, it

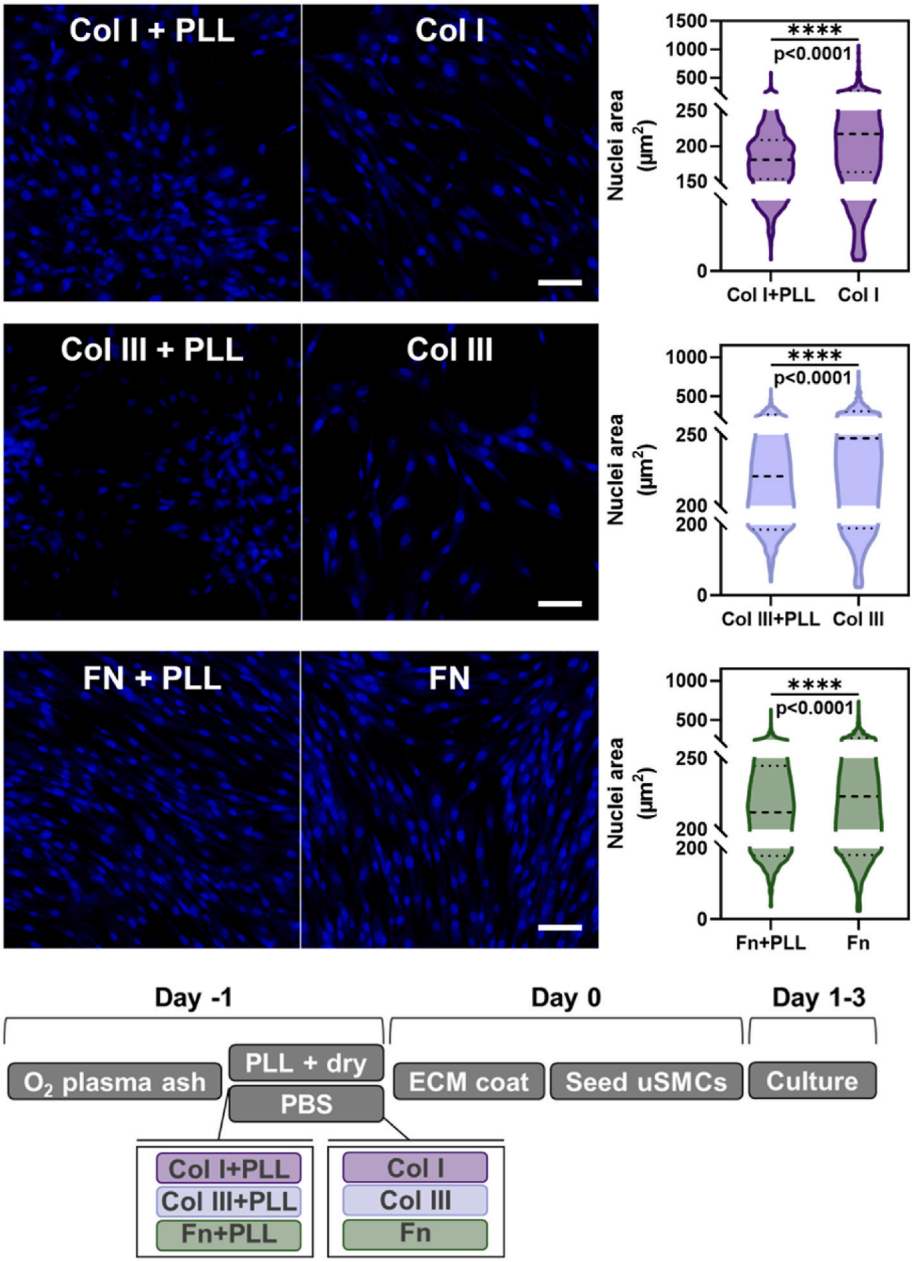

Fig. 4. Nuclei morphologies of uSMCs exposed to different membrane surface treatments. Six images were captured per sample and an automated Fiji script then analyzed nuclei area in square micrometers across groups. A Welch two sample *t*-test was performed comparing cells grown on membranes treated with PLL or PBS control prior to each ECM treatment. Significance values are displayed as (****, $p < 0.0001$) with an $\alpha = 0.05$. Violin plots are shown in which thick dashed lines represent median of 3–6 biological replicates across 3 experiments, comprising of thousands of analyzed nuclei. Thin dotted lines represent the upper and lower quartiles of the sample pool. Experimental timeline is displayed at the bottom of the figure. Scale bar = 100 μm.

was found that exposing PDMS membranes to PLL prior to protein coating enhanced uSMC growth and impacted nuclei morphology. After optimizing growth and surface coating conditions, uSMCs grown on Fn + PLL-treated PDMS membranes were then exposed to strain, resulting in enlarged nuclei, a morphology associated with uSMC morphology in late-stage pregnancy [32]. The presence of enlarged uSMC nuclei in response to strain was dependent on treatment with PLL. Previous work looking at the impact of strain on uSMCs only utilized collagen I-treated substrates; thus this was the first time to our knowledge that the strain response of human uSMCs cultured on fibronectin-treated PDMS has been reported [16–18,23]. Additionally, strain experiments using uSMCs previously were focused on strain parameters such as duration, frequency, and magnitude, yet strain rate had not yet been explored prior to this study. This study's focus on strain rate was based on the underlying hypothesis that increased rates of preterm birth in multi-fetal pregnancies might be due to an increased uterine growth rate that triggers premature labor. In conjunction with nuclear size analysis as a morphological indicator of uSMC strain response, gene expression changes in response to changes *in vitro* strain rates were analyzed by RT-PCR to assess the regulation of genes identified in other strain studies and genes significantly impacted by pregnancy *in vivo* [36]. It was found that strain rate can directly impact IL-6 (a cytokine dysregulated in certain cases of preterm labor [15] and a regulator of oxytocin secretion [37]) gene expression in uSMCs.

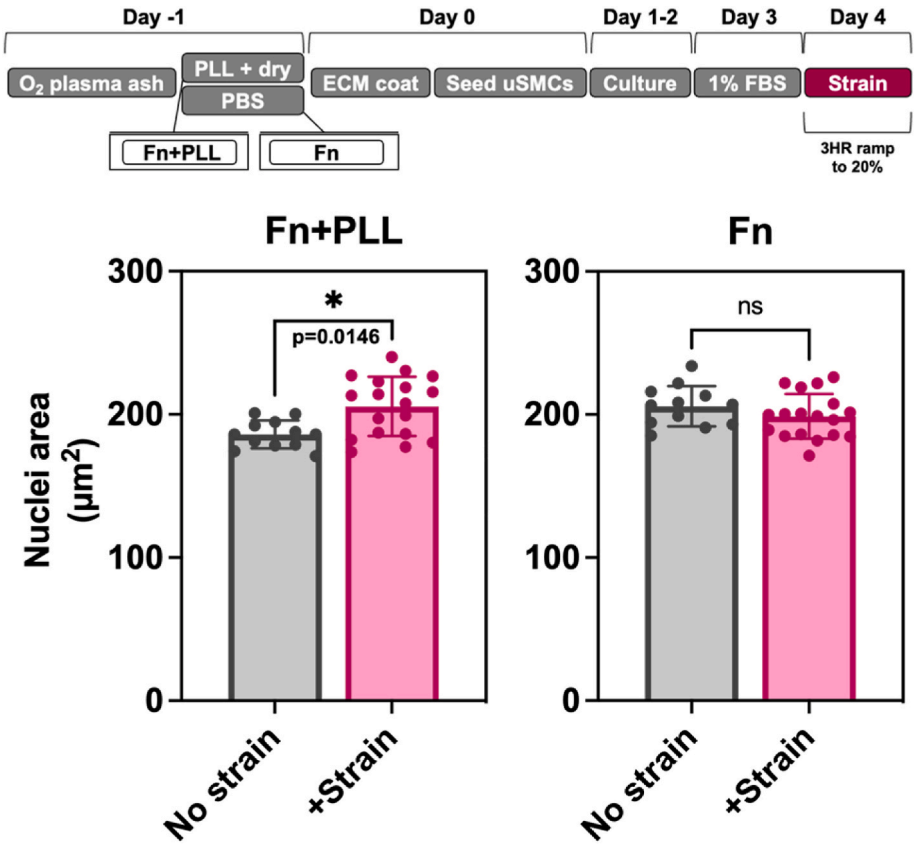

Fig. 5. Nuclei morphology of uterine SMCs exposed to strain with and without PLL treatment was assessed with image analysis. Six images were captured per device and an automated Fiji script was used to analyze nuclei area in square micrometers across groups. An unpaired *t*-test was performed comparing strain to the no strain control condition for + PLL and -PLL conditions with significance values displayed as (ns, $p > 0.05$, *, $p < 0.05$) with an $\alpha = 0.05$. Error bars represent standard deviation from the mean across 3 biological replicates.

4.1. The use of positively charged electrolytes on hydrophobic surfaces enhances uSMC growth

It has been previously demonstrated that the use of positively charged electrolytes combined with protein surface coatings can enhance cell attachment and spreading on PDMS substrates in multiple cell types [27,28]. The data presented in this study demonstrated that PLL treatment of PDMS enhanced cell attachment and growth of uSMCs over time (Fig. 1). It was found that treating PDMS with PLL prior to treating with ECM proteins collagen I, collagen III, or fibronectin resulted in higher cell confluence on both days 1 and 3 of the culture timeline (Fig. 1). The finding that higher instances of cell clumping occurred on membranes not treated with PLL suggests that cell-substrate interactions were less favorable than cell-cell interactions, resulting in the 3-dimensional cell clumps that were observed in the absence of PLL functionalization prior to ECM coating (Fig. 1). PLL treatment indeed led to lower occurrences of cell clumping, however, the degree of cell-substrate interactions and resulting growth that occurred was also impacted by ECM protein coating. uSMCs were more confluent on fibronectin-coated substrates, compared to collagen I and III, demonstrating that both PLL presence and ECM protein were impactful substrate parameters for uSMC growth (Figs. 1–3).

4.2. uSMCs can be grown on fibronectin-treated surfaces in vitro for strain studies

Given the prevalence of Col I-treated surfaces in the literature containing uSMC strain experiments and collagen I's presence in nonpregnant myometrium ECM, it was originally hypothesized that Col I-treated membranes would be most conducive to uSMC growth [16–18]. Contrary to the presented hypothesis, Fn-treated substrates supported the highest confluence of uSMCs over time compared to Col I and Col III -treated PDMS substrates (Figs. 1–3). This was surprising, given that fibronectin is not as prevalent in nonpregnant and early-stage pregnancy myometrium to the degree collagens I and III are. Despite this, both Fn + PLL and Fn-treated substrates had significantly higher uSMC cells per square centimeter than Col I or Col III-treated substrates after 3 days of growth (Fig. 3). There are several hypotheses as to why uSMCs appeared to grow better *in vitro* on a less prevalent ECM protein than what is found in the nonpregnant myometrium *in vivo*. It is possible that the protein coating concentration of collagen I was suboptimal. Indeed, a lower concentration was used as compared to previous studies growing uSMCs on collagen I (collagen I coating concentrations used in the referenced studies were not reported, but the listed commercially available membranes from FlexCell are coated with 200 μg/mL of bovine bone collagen I). As previously mentioned, the treatment methodology exposed membranes to monomeric collagen to better compare to fibronectin, rather than coating with parameters to yield collagen fibril or gel formation. The formation of monomeric collagen requires an acidic pH, a parameter that has been demonstrated to impact SMC growth *in vitro* [38]. Electrostatic interactions might have also impacted the coating of ECM proteins on the PDMS membrane surface. Collagen I and collagen III have isoelectric points ranging from a pH of 7.2–3.1, thus coating in acidic conditions below the isoelectric point likely resulted in collagen monomers holding a net positive charge compared to fibronectin treatments at a neutral pH, which likely resulted in fibronectin having a net negative charge [39,40]. It is possible that PLL treating PDMS surfaces increased fibronectin adsorption due to favorable electrostatic interactions but was less beneficial for collagen proteins that held net positive charges during substrate coating. However, a study by Wang

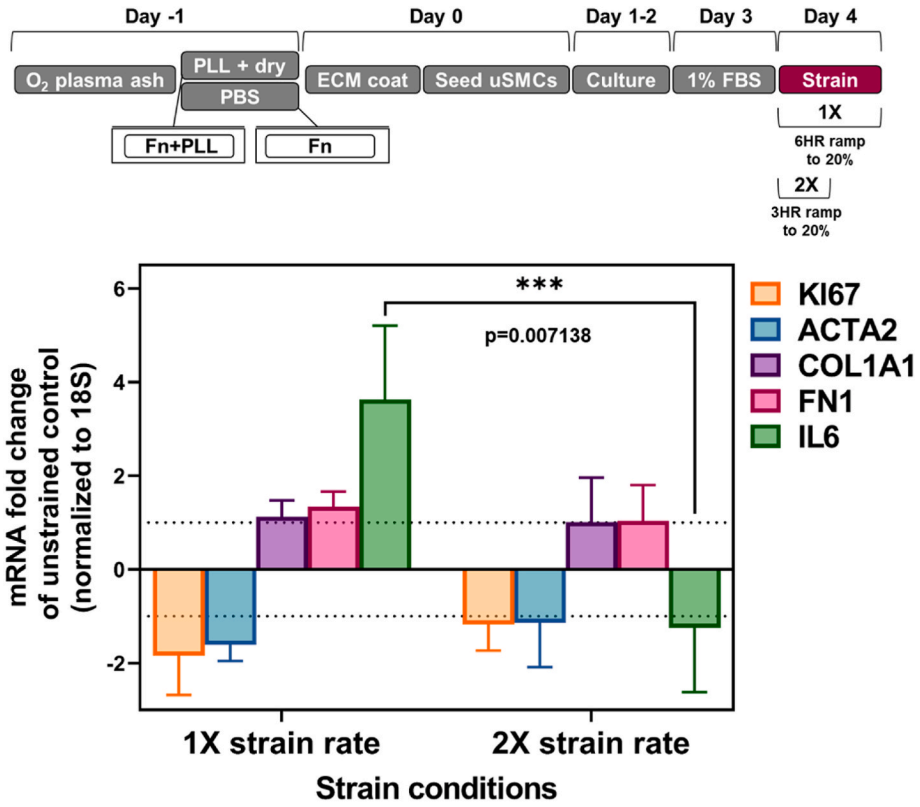

Fig. 6. Gene expression changes of uterine SMCs exposed to strain conditions normalized to unstrained cells. The fold change of genes KI67, ACTA2, COL1A1, FN1, and IL6 in strained conditions compared to unstrained conditions were calculated using the comparative CT method. An unpaired *t*-test was performed to compare strain conditions. Significance values are displayed as (***, $p < 0.01$, $\alpha = 0.05$). Groups that do not display statistics labels were found to be non-significant. Error bars represent±error across 3 biological replicates.

et al. found that Col I + PLL-treated PDMS coated in acidic conditions resulted in higher levels of collagen I protein adsorption compared to fibronectin protein adsorption levels on PDMS treated with Fn + PLL with a neutral buffer [27]. The authors used higher concentrations of 50 μg/mL for their protein coatings compared to the 5 μg/mL used herein, suggesting that both isoelectric point and protein concentration impact coating conditions [27,28]. Future studies equilibrating protein adsorption between ECM proteins in conjunction with PLL coating would elucidate what parameters were directly responsible for enhanced uSMC growth on Fn + PLL-treated PDMS. Given this, we hypothesize that PLL treatments increased the likelihood of cell-substrate interactions through a combination of: (1) direct electrostatic interactions between the negatively charged cell membranes and positively charged surface and (2) increased ECM protein adsorption, both which have been shown previously to improve cell adhesion and spreading on PDMS substrates [26,27]. While the electrostatic interactions of fibronectin and collagen monomers with the PDMS surface may have been assisted by PLL, it should be noted that fibronectin-treated surfaces improved uSMC growth in treatments lacking PLL as well. Of the treatment groups tested, Fn + PLL was the best at supporting growth of uSMCs over time (Fig. 1). In this study, the presented data supports that PLL enhanced cell attachment regardless of protein coating, however, uSMCs also had increased cell density on fibronectin coatings compared to collagen I and collagen III coating regardless of PLL treatments (Figs. 1–3).

4.3. Surface treatments impact confluence and nuclear morphology, however uSMC actin and collagen protein expression do not change across PDMS substrate treatments

Despite the differences between uSMCs grown on differently treated substrates, immunofluorescent staining indicated relatively similar protein expression regardless of ECM coating condition (Fig. 2). Cell density varied on substrates and Fn + PLL treated PDMS yielded the highest confluence of uSMCs, however all uSMC bodies stained positive for intracellular collagen I and connective fibronectin networks regardless of ECM coating (Fig. 2). This protein expression validated previous histology reports of mature, non-pregnant myometrial tissue that observed SMCs staining positive for collagen I in the cytoplasm, with fibronectin staining close to cell bodies and interconnecting SMC bundles [24].

4.4. Treating PDMS surfaces with positively charged electrolytes prior to cell seeding impacts uSMC nuclear morphology

uSMCs grown on fibronectin-treated substrates in unstrained conditions exhibited significantly altered nuclear morphology compared to other ECM protein treatments (Fig. S2). Despite this, there was a significant difference in nuclei sizes between PLL and control conditions, regardless of the ECM protein treatment (Fig. 3). Though the relationship between surface charges and cell attachment has been routinely studied, the role of surface charge distribution and its influence on nuclei morphology has not been thoroughly explored [41]. We found that across all ECM coating conditions, PLL-treated PDMS prior to ECM treatments and cell seeding resulted in significantly smaller uSMC nuclei compared to cells cultured on non-PLL treated substrates (Fig. 3). Given the role of surface charge in cell-substrate interactions, it is not surprising that the presence of PLL impacted uSMC nuclei morphology, though the direct cellular mechanisms are not currently understood. Factors influencing nuclear morphology span the entirety of the cell, ranging from the nuclear lamina and envelope to extranuclear features such as cytoskeletal components [42]. External cues from cytoskeletal

components are relayed through the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex on the nuclear envelope, which in turn can alter nuclei morphology and in some cases, gene expression [43,44]. Previous research suggests that signaling through the LINC complex impacts changes in both nuclei morphology and cell response to strain [45]. Reduced cell spreading can also result from processes outlined in the receptor saturation model [46]. Gaudet et al. has previously demonstrated that the density of collagen I available for integrin binding directly impacts cell spreading [46,47]. If the presence of PLL directly impacted ECM protein binding to the PDMS surface, then a reduction in the amount of ECM protein available for integrin binding could have consequently increased uSMC spreading and directly impacted nuclei morphology through the LINC complex, which has the potential to impact cell migration, differentiation, and genome stability [43]. The mechanism through which PLL significantly reduces nuclei size of uSMCs is not understood, thus further probing as to why this occurs and how it might influence the downstream behavior of uSMCs is warranted.

4.5. Treating PDMS substrates with positively charged electrolytes prior to cell seeding and strain experiments impacts uSMC strain response

After showing that uSMCs grew differently on + PLL substrates (Figs. 1–3), it was also found that uSMCs cultured on Fn + PLL PDMS substrates had nuclei morphologies significantly larger when exposed to strain than those of unstrained controls. Contrastingly, uSMCs cultured on Fn PDMS substrates did not demonstrate any significant morphological differences when exposed to strain (Fig. 5). These findings indicate that treatment with PLL prior to fibronectin coating played an important role in uSMCs detecting and responding to substrate strain. It is hypothesized that upon strain of the PDMS substrate, tension on the nuclear envelope through these cell-substrate interactions significantly increased nuclei size. On PDMS substrates not coated with PLL, fibronectin adsorption, and thus cell-substrate interactions, were potentially less prevalent, resulting in a less detectable impact on nuclei morphology under strain conditions (Fig. 5). Future work would benefit from exploring how PLL surface treatments impact gene and protein expression of structures involved in cell-substrate interactions of the myometrium, such as integrins and focal adhesions. These uSMC-substrate interactions might impact how the myometrium responds to uterine wall tension and the maintenance of uterine quiescence in pregnancy. This area of research could also inform why ECM irregularities caused by pathologies such as fibroids and Hypermobile Ehlers-Danlos Syndrome lead to an increased likelihood of preterm birth [7,48].

4.6. In vitro strain impacts gene expression changes, however, does not induce changes in ECM protein gene expression in uSMCs

Genes COL1A1 and FN1, coding for ECM proteins collagen I and fibronectin, respectively, were unchanged in response to 1X and 2X strain rates (Fig. 6). Previous characterizations of SMC response to strain *in vitro* demonstrate that FN1 and COL1A1 upregulation occurs in renal SMCs, occurring as early as 1 h, maximizing gene expression 12 h post strain initiation, and persisting until 48 h [36]. Previous studies have demonstrated that uterine cells can demonstrate physiological changes under strain conditions in as little as 15 min [19]. There was no increase in FN1 or COL1A1 after 6 h of uSMC exposure to strain conditions, therefore the strain rates examined failed to significantly alter gene expression of ECM proteins previously impacted in other SMC strain studies [36].

In vivo, KI67 gene expression is upregulated as an indicator of proliferation when myometrial uSMCs are in a state of hyperplasia during early-stage pregnancy [31], however uSMCs downregulated gene expression of KI67 in the presence of strain, mimicking a late-stage pregnancy phenotype (Fig. 6). ACTA2, the gene that codes for α-smooth muscle actin (α-SMA), was downregulated in both strain conditions compared to unstrained controls (Fig. 6). This finding was surprising, given that α-SMA gene expression has previously been shown to be upregulated in pregnant myometrial tissue [34]. This might indicate that α-SMA upregulation in pregnancy occurs via a pathway not linked to uterine tissue mechanics, or that mechanical factors alone are insufficient to increase α-SMA expression without hormonal changes or other external signaling factors.

4.7. In vitro strain rate impacts uSMC mRNA expression of IL-6, a marker for pre-term birth

There was a significant difference in IL-6 gene expression between strain rate conditions (Fig. 6). IL-6 is a pleiotropic cytokine that, when secreted, regulates innate immune cell activity, is upregulated during labor, and can increase oxytocin secretion in uSMCs [37]. Interestingly, IL-6 is increased both in the myometrium of twin pregnancies experiencing pre-term labor as well as spontaneous labor at term, thus the impact of strain rate on IL-6 mRNA expression is physiologically relevant with regard to the pregnant uSMC phenotype [15]. Despite an increase in IL-6 mRNA expression of uSMCs exposed to 1X strain rate, there was a significant decrease in gene expression for uSMCs exposed to a higher strain rate (2X strain). The difference between IL-6 gene expression across strain conditions in this study is hypothesized to be due to uSMC desensitization to strain over time. There are verified instances of uterine cells becoming “desensitized” to strain over a 7 day time course [19], thus it is possible that the 2X strain rate condition initially triggered uSMC upregulation of IL-6, but the response equilibrated over the second half of the experiment when the PDMS membrane was held statically at the strain maximum. Future work could benefit from investigating strain profiles at various timepoints to determine how gene expression changes over time in uSMCs on dynamically strained substrates. Other studies investigating strain in uSMCs followed a similar growth timeline to this study, growing cells on collagen I-treated substrates to confluence by days 3–4, exposing to low serum conditions, then performing strain experiments in timeframes as short as 1 h and as long as 24 h [16–18]. Expanding time frames to track gene expression, ECM remodeling, and cytokine release over longer timelines, particularly when comparing to a multi-month physiological process such as pregnancy, would greatly improve the physiological relevance of future strain studies.

4.8. uSMCs can be studied under in vitro strain across different substrates to recapitulate the dynamic microenvironment of the myometrium during pregnancy

To date, a majority of studies investigating strain of uSMCs *in vitro* have been performed on commercially available silicone elastomer bottomed plates pre-coated with collagen I (FlexCell®, Burlington, NC) [16–19]. uSMC response to strain on other ECM protein coatings such as fibronectin have not yet been explored. This is the first report of uSMC response to strain *in vitro* on a fibronectin-treated substrate. It was shown that strain only significantly impacted nuclei morphology compared to unstrained controls when uSMCs were grown on Fn + PLL-treated substrates compared to Fn-treated substrates. Varied strain rate parameters were also explored and, to emulate the increase in uterine growth rate that occurs in twin versus singleton pregnancies, two different strain rates were used. The strain rates tested were doubled in magnitude based on pregnant patient ultrasound data [10]. It was found that different strain rates significantly impacted gene expression of IL-6, a marker for the inflammatory cytokine upregulated in certain cases of preterm labor [35,37,49] (Fig. 6). Comprehensively, the surface treatment methodologies presented in this study and their demonstrated application with the *in vitro* myometrial model herein highlights a new approach in which uSMCs can be stimulated in different microenvironments to better understand implicated pathways and potential treatments of preterm labor.

5. Conclusions

By advancing learnings and methodology in substrate preparation for the purpose of straining uSMCs *in vitro*, this study has the potential to assist in further understanding of how the uterine phenotype is influenced by tissue mechanics throughout pregnancy and labor in multiple chemical microenvironments. Previous work has utilized collagen I-treated membranes to investigate mechanically stimulated cellular pathways in uSMCs. This study aimed to explore how varying parameters such as the functionalization of PDMS and coating protein identity impacted uSMC growth. It was found that electrostatically functionalizing the PDMS surface with a charged solution such as PLL prior to ECM coating supported cell-substrate interactions and Fn + PLL coating yielded the highest confluence of uSMCs on PDMS substrates. Fn + PLL coated PDMS membranes were utilized for strain experiments and PLL treatments proved to be imperative in observing uSMC response to strain. Additionally, strain rates selected to mimic differing uterine growth rates based on twin and singleton pregnancies significantly impacted uSMC gene expression of IL-6, a key inflammatory cytokine implicated in preterm labor and a demonstrated regulator of hormone secretion in uSMCs. Overall, the data presented herein supports the utility of a new surface treatment methodology for understanding the role of strain in uterine biology and preterm birth.

Informed consent statement

Not applicable.

CRediT authorship contribution statement

Elizabeth E. Marr: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Brett C. Isenberg:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Joyce Y. Wong:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2023.10.006.

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