Characterization of tissue biomechanics and mechanical signaling in uterine leiomyoma

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21 A B S T R A C T
22 Leiomyoma are common tumors arising within the uterus that feature excessive deposition of a stiff, disordered extracellular matrix (ECM). Mechanical stress is a critical determinant of excessive ECM deposition and increased mechanical stress has been shown to be involved in tumorigenesis. Here we tested the viscoelastic properties of leiomyoma and characterized dynamic and static mechanical signaling in leiomyoma cells using three approaches, including measurement of active RhoA. We found that the peak strain and pseudo-dynamic modulus of leiomyoma tissue was significantly increased relative to matched myometrium. In addition, leiomyoma cells demonstrated an attenuated response to applied cyclic uniaxial strain and to variation in substrate stiffness, relative to myometrial cells. However, on a flexible pronectin-coated silicone substrate, basal levels and lysophosphatidic acid-stimulated levels of activated RhoA were similar between leiomyoma and myometrial cells. In contrast, leiomyoma cells plated on a rigid polystyrene substrate had elevated levels of active RhoA, compared to myometrial cells. The results indicate that viscoelastic properties of the ECM of leiomyoma contribute significantly to the tumor’s inherent stiffness and that leiomyoma cells have an attenuated sensitivity to mechanical cues. The findings suggest there may be a fundamental alteration in the communication between the external mechanical environment (extracellular forces) and reorganization of the actin cytoskeleton mediated by RhoA in leiomyoma cells. Additional research will be needed to elucidate the mechanism(s) responsible for the attenuated mechanical signaling in leiomyoma cells.

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1. Introduction
2. Uterine leiomyomata are highly prevalent, fibrotic tumors of the uterus that disproportionately affect African American women and are a public health concern (Day Baird et al., 2003; Walker and Stewart, 2005; Lee et al., 2007; Seló-Ojeme et al., 2008; Laughlin et al., 2009). Previously, we (Catherino et al., 2004; Leppert et al., 2004), and others (Wolanska et al., 1998; Mitropoulou et al., 2001; Wolanska et al., 2003; Behera et al., 2007) have shown the ECM of leiomyoma to be increased in amount and altered in composition, compared to the ECM of the uterine myometrium. In addition to a rich glycosaminoglycan (GAG) content (Wolanska et al., 1998; Wolanska et al., 2003), we observed that the ECM was structurally disordered, relative to adjacent myometrium (Catherino et al., 2004; Leppert et al., 2004). Furthermore, leiomyomata displayed an increased stiffness by unconstrained compression in vitro (Rogers et al., 2008) and ultrasound elastography in vivo (Kiss et al., 2006; Stewart et al., 2011). Of note, leiomyomata possess an increased vascularity and fluid content relative to adjacent myometrium (Aleem and Predanic, 1995; Okuda et al., 2008). The increased fluid content is significant because fluid may contribute to the mechanical properties of the tumors and may explain the clinical response of leiomyoma to GnRH agonists and antagonist treatment (Chegini et al., 1996; McCarthy-Keith et al., in press). Despite the remarkable stiffness of leiomyomata, their altered ECM structure and content, and increased water content, little is known about mechanical signaling in leiomyoma.

Mechanical signals are transmitted from the ECM scaffold via transmembrane receptors to the internal cytoskeleton in order to...
maintain an isometric state (for review, Alenghat and Ingber, 2002). Transmembrane receptors, such as the integrins and cadherins (Schwartz and DeSimone, 2008; Wang et al., 2009) respond to stretch (Kaneko et al., 2009), fluid shear stress (Lee et al., 2008), elevated hydrostatic pressure (Riou et al., 2007) and increased osmotic forces (Lunn and Rozengurt, 2004). Although tissues exist under mechanical tension, the resident cells react to, and may be protected from, external loads by the mechanical properties of the surrounding matrix (Tomasek et al., 2002) through secretion of ECM (Brown et al., 1998; Alexopoulos et al., 2005). Notably, increased ECM stiffness may contribute to tumorigenesis (Ingber, 2008; Butcher et al., 2009). For example, Paszek and colleagues demonstrated malignant transformation of mammary epithelial cells (MECs) correlated with increasing ECM stiffness, elevated compression forces, and higher tensional resistance mediated, in part, through increased active RhoA (Paszek and Weaver, 2004; Paszek et al., 2005). RhoA belongs to the Rho family of small GTPases that function as molecular switches to cycle between the inactive GDP-bound and active GTP-bound state (Ridley and Hall, 1992; for review: Wettstreuck and Offermanns, 2002). Rho GTPases are activated by Rho-guanine nucleotide exchange factors (Rho-GEFs) and generate cytoskeletal tension via interaction with cytoskeletal filaments that attach to focal adhesion complexes that lead to activation of downstream effectors, including Rho-associated kinase (ROCK). Thus, Rho signaling might play an important role in leiomyoma stiffness, and possibly growth, but little is known about Rho-signaling in leiomyoma.

Recently, we observed that leiomyomata demonstrated increased beta-1 integrin expression and that inhibition of integrin signaling led to a reduction in levels of active RhoA (Malik et al., 2009). Furthermore, we found that the Rho-GEF Brx (AKAP13) was not only expressed at high levels in leiomyoma (Rogers et al., 2008), but AKAP13 was also involved in osmotic signaling (Kino et al., 2009) and osmotic signaling was altered in leiomyoma cells (McCarthy-Keith et al., in press). Taken together, these observations suggest that altered mechanical signaling in leiomyoma involves RhoA and that altered viscoelastic properties contribute significantly to the increased stiffness characteristic of the tumors. Here we examined the biphasic mechanical properties of leiomyomata and characterized the response of leiomyoma cells to dynamic and static mechanical stresses.

2. Results

2.1. Leiomyoma tissue exhibit increased pseudo-dynamic modulus and peak stress

To assess the pseudo-dynamic modulus of myometrial and leiomyoma tissues, a measurement that takes into account the contribution of water and the structure of the ECM to the tissue’s viscoelastic properties, we used a porous stainless steel confined compression chamber (Fig. 1a–c). Surgically obtained leiomyoma tissue samples had mean pseudo-dynamic modulus of 202.7 ± 27.8 megapascals (MPa) per millimeter (mm/mm) (mean ± SEM; Fig. 2a). This was significantly more stiff than myometrial specimens (48.1 ± 25.6 MPa per mm/mm, p < 0.001; Fig. 2a). Furthermore, relative to paired myometrium, leiomyomata held a larger peak strain at 5% displacement (6.96 ± 0.91 versus 1.35 ± 0.70 MPa respectively, p<0.001; Fig. 2b). Comparing these data to our previous assessment of Young’s modulus (Rogers et al., 2008), we noted a much larger pseudo-dynamic modulus when the tissue’s viscoelastic mechanical properties were taken into account. Consistent with prior reports, the leiomyoma samples we analyzed contained more sulfated glycosaminoglycans (sGAG) per DNA content relative to myometrial specimens (Leiomyoma: 0.62 ± 0.080 μg of sGAG per μg of DNA; Myometrium: 0.19 ± 0.012 μg per μg, p < 0.0001; Fig. 2c). A similar difference was noted for collagen (Leiomyoma: 246.7 ± 26.2 μg of collagen per μg of DNA; Myometrium: 97.5 ± 18.7 μg per μg, p < 0.001; Fig. 2d), and the values resembled previously published data obtained with other methods (Berto et al., 2003).

After normalizing the mechanical properties to biologic components and also to tissue sample weights, we performed correlation analyses (Spearman’s correlation for nonparametric data). Both the pseudo-dynamic modulus and the peak strain correlated with one another (p < 0.001). The pseudo-dynamic modulus also correlated with both collagen and sGAG content (p < 0.05). Of note, the peak stress correlated more strongly with the hydrophilic sGAG content (p = 0.003) than with collagen content (p = 0.011). Furthermore, neither mechanical property (pseudo-dynamic modulus or peak stress) correlated with dry weight. In sum, the mechanical properties strongly correlated with components of the matrix that reflect the viscoelastic properties of the tissue, and suggest that leiomyoma cells reside in a mechanically stiff microcellular environment. Furthermore, the results indicate that the molecular rearrangement of
the ECM, including hydration, may play an important role in the stiffness of the tumors. These observations led us to question whether mechanical sensing might be altered in leiomyoma cells.

2.2. Leiomyoma cells have an attenuated response to applied cyclic strain

Previous studies have shown that airway smooth muscle cells (Deng et al., 2009) sense and respond to applied uniaxial cyclic strain in vitro by reorienting their actin cytoskeleton perpendicular to the axis of strain. To determine whether leiomyoma cells exhibit normal sensitivity and response to mechanical strain, we applied 8.9% uniaxial cyclic strain (1 Hz) to leiomyoma cells and compared their reorientation response to myometrial cells. Both cell types responded to mechanical strain (Fig. 3a & b). However, while 70% of myometrial cells reoriented their main axis perpendicular to the direction of strain, only 53% of leiomyoma cells exhibited this response (Fig. 3b). It was possible that the reduced re-orientation was due to constitutively elevated levels of the active Rho-kinase, ROCK. To test this possibility, we added the ROCK inhibitor, Y-27632. Similar to normal endothelial cells (Ghosh et al., 2008), reorganization of normal myometrial cells to mechanical strain was inhibited by Y-27632, consistent with the conclusion that the Y-compound was fully functional in the culture system. In contrast to reports of tumor-derived endothelial cells (Ghosh et al., 2008), treatment with the ROCK inhibitor prior to strain failed to increase the percentage of leiomyoma cells that oriented perpendicularly to applied strain. One interpretation of these findings is that leiomyoma cells have an impaired perception of mechanical strain.

2.3. Response of leiomyoma cells to RhoA activation by mechanical and chemical stimulation

To examine the question of whether leiomyoma cells have impaired mechanical sensing in greater detail, we next quantified levels of active RhoA following either mechanical stimulation or treatment with lysophosphatidic acid (LPA), a known soluble activator of RhoA (Parizi et al., 2000). Basal levels of active RhoA were similar between leiomyoma or myometrial cells when cultured on a pronectin-coated flexible silicone substrate, but levels of RhoA were increased in leiomyoma cells cultured on polystyrene, compared to myometrial cells (Fig. 4a). LPA stimulation led to increased levels of active RhoA in both myometrial cells, and leiomyoma cells within 3 min on a flexible pronectin-coated substrate (Fig. 4b), and there were no significant differences between the cell types. In contrast, LPA stimulation of cells cultured on the stiff polystyrene substrate led to an increase in active RhoA in myometrial cells which also peaked at 3 min, but leiomyoma cells did not exhibit a significant increase over already elevated levels of active RhoA.
2.4. Leiomyoma cells respond abnormally to variation in substrate stiffness

Previous studies have shown that smooth muscle cells sense and respond to increasing substrate stiffness by increased surface area (Engler et al., 2004), and that this response is an indirect measure of how efficiently cells sense and respond to ECM elasticity (Chicurel et al., 1998). As a third approach to assess the responsiveness of leiomyoma cells to mechanical cues, myometrial and leiomyoma cells were cultured for 22 h on polyacrylamide gels that varied in stiffness from 7 kPa (kilopascal) to 140 kPa (Fig. 5). At a baseline substrate of 7 kPa, myometrial cells were more rounded with a reduced surface area, versus leiomyoma cells, respectively (8298 pixels per cell versus 7006 pixels per cell; p = 0.02). On the most rigid substrate, myometrial cell surface area was increased as expected. In contrast, after 22 h leiomyoma cells had an attenuated increase in surface area (19464 pixels per cell versus 710 (SEM) versus 15730 ± 556; p < 0.001). When compared over the four different ECM substrates, myometrial cells responded to a greater degree to more rigid matrices by increasing cell spreading, indicating that leiomyoma cells did not sense, or were unable to respond to a change in their substrate elasticity (Fig. 5a and b; p = 0.0042, two-way ANOVA).

3. Discussion

These studies demonstrate that the ECM microenvironment of leiomyoma cells is characterized by increased mechanical stress. Here we extend the results of our previous study (Rogers et al., 2008) to show that the viscoelastic properties of the ECM contribute substantially to the increased tissue stiffness of leiomyoma. Since the viscoelastic properties of the ECM are complex, it is possible that the interstitial fluid may alter the repulsive forces of the GAGs allowing them to collapse or inflate. Additional studies will be needed to discern how the complex ECM of leiomyoma and its molecular rearrangement contributes to the observed changes in viscoelasticity.

We noted a four-fold increase in both the pseudo-dynamic modulus and the peak strain in leiomyoma tissue relative to patient-matched myometrium (Fig. 2). Using a confined compression chamber with a porous paten, we observed a much higher modulus than matched myometrium (Fig. 2). Using a confined compression chamber with a porous paten, we observed a much higher modulus than matched myometrium (Fig. 2). Using a confined compression chamber with a porous paten, we observed a much higher modulus than matched myometrium (Fig. 2). Using a confined compression chamber with a porous paten, we observed a much higher modulus than matched myometrium (Fig. 2).
articular cartilage (Cohen et al., 1998; Ateshian et al., 2003; Park et al., 2004), the fluid phase contributes to the viscoelastic properties of fibroids, contributing to large interstitial pressurized forces. For example, after testing bovine cartilage in a confined compression chamber, Soltz and Ateshian (Soltz and Ateshian, 2000) concluded that cartilage dynamic stiffness was derived primarily from flow-dependent viscoelasticity as predicted by the linear biphasic theory and that interstitial fluid pressurization is the fundamental mechanism of cartilage load support. Our findings support the notion that leiomyomata are tumors composed of large amounts of aberrant ECM (Peddada et al., 2008; Malik et al., 2010) and that cells within the tumor continue to grow and proliferate while exposed to increased viscoelastic forces. Changes in the mechanical properties of a tissue and the cellular microenvironment have been shown to contribute to tumor formation in other organ systems and in experimental models (Ingber, 2008; Butcher et al., 2005). The concept that changes in the cellular microenvironment could contribute to tumorigenesis were first suggested by experiments of Bischoff and Bryson (Bischoff and Bryson, 1964) where tumor formation was observed after implanting a rigid piece of metal or plastic, as opposed to the same material as a powder. Alterations to the ECM structure also appear to play a central role in tumor formation and in the tumor cell's ability to sense and respond to the altered physical environment (Weaver et al., 1997; Paszek et al., 2005; Ghosh et al., 2008). The findings reported here, together with our previous data (Rogers et al., 2008), suggest that the mechanical properties of leiomyoma are a key feature of these tumors, and may contribute to their growth, but further studies will be needed to assess whether growth of a specific leiomyoma is correlated to its stiffness. One limitation of the studies presented is that the viscoelastic properties of a tissue are complex, especially in a tissue containing ECM consisting of numerous proteins and glycoproteins all of which may contribute to mechanical behavior. In this report, we have focused on characterization of the differences between leiomyoma and uterine muscle, especially differences in Rho signaling based on our prior report, but a more detailed

Fig. 4. RhoA levels in leiomyoma and myometrial cells at baseline and in response to applied chemical or mechanical strain. a: Assessment of active RhoA in leiomyoma or myometrial cells cultured on flexible pronectin-coated substrate, or uncoated polystyrene. Y axis = relative level of active RhoA. Leiomyoma cells (black bars) demonstrated increased levels of activated RhoA relative to myometrial cells when cultured on polystyrene (p < 0.05). b: Levels of active RhoA in myometrial (gray bars) or leiomyoma cells (black bars) cultured on flexible, pronectin-coated substrate untreated (control) or treated with a chemical activator or RhoA, lysophosphatidic acid (LPA), for minutes as indicated. Y axis = relative level of active RhoA. On the flexible, pronectin-coated substrate levels of activated RhoA in myometrial cells peaked at 3 min. c: Culture of leiomyoma (black bars) or myometrial cells (gray bars) on polystyrene either untreated (control) or treated with LPA for minutes as indicated. Y axis = relative level of active RhoA. Levels of active RhoA were significantly elevated in leiomyoma cells at baseline, and were less affected by LPA treatment. Data in A-C represent the average relative RhoA activation compared to myometrial control from three independent experiments. d: Quantification of active RhoA in myometrial (gray bars) or leiomyoma cells (black bars) to 2 h of applied uniaxial strain. Myometrial cells demonstrated a 2-fold increased active RhoA levels in response to uniaxial strain on pronectin-coated flexible silicone substrate (M Control versus M Strain). Leiomyoma cell active RhoA levels were attenuated and had a muted response (1.3 fold) to mechanical strain (L Control versus L Strain). The cell response was normalized to myometrial control activation of RhoA and reported as the mean ± standard deviation from two independent experiments with 6 wells for each condition.
assessment of the rheological differences between the cells such as reported for other tissue types (Stamenović, 2008) remains to be performed.

Notably, leiomyoma differ from other tumors in that some grow to several centimeters in size. Each uterine leiomyoma represents a monoclonal process, but within a single uterus different tumors arise from different cells, such that within a uterus multiple clones may be represented (Ligon and Morton, 2000). Within one uterus some tumors may grow, while others may undergo a reduction in size (Peddada et al., 2008). Recent reports of assessment of the elastic modulus in vivo (Stewart et al., 2011) may represent a clinical application of our findings to assess the stiffness in vivo and explore a possible correlation with growth or senescence of an individual leiomyoma.

The establishment of a tumor microenvironment by leiomyoma cells characterized by increased viscoelastic forces begs the question: is mechanical signaling altered in leiomyoma cells? The results indicate that myometrial cells responded to perturbation of the extracellular mechanical stresses as expected; but by three different measures of mechanosensing, leiomyoma cells appeared to have an attenuated response relative to myometrial cells. Specifically, leiomyoma cells failed to reorient perpendicularly to the applied uniaxial strain direction, had an attenuated RhoA activation response to uniaxial strain, and showed a diminished ability to change morphology in response to altered substrate stiffness. In contrast to these three observations which suggest an impaired response to extracellular mechanical cues, on the extremely rigid polystyrene plates with an estimated stiffness of 2–4 GPa (Paszek et al., 2005), leiomyoma cells demonstrated increased basal levels of active RhoA relative to myometrial cells. These observations could be considered contradictory. We interpret the increase in the basal levels of RhoA on the polystyrene substrate may reflect prior adaptation of the leiomyoma cells to a very stiff microenvironment. However, with each dynamic mechanical challenge, leiomyoma cells were not as adroit in their response, suggesting a fundamental alteration exists in communication between the external mechanical forces and the ability of the actin cytoskeleton to reorganize via RhoA. The findings suggest that mechanical signaling in leiomyoma cells is fundamentally altered, because in all 4 assays involving external mechanical cues, leiomyoma cells responded abnormally.

One plausible explanation for the seemingly contradictory results is that leiomyoma cells have become fundamentally adapted to their very stiff microenvironment, and are insensitive to more moderate and subtle mechanical cues. Stated differently, the cell response to mechanical stimulation could be down-regulated through feedback mechanisms, although the mechanisms responsible remain unknown. In support of this explanation, and contrary to the findings of Ghosh and colleagues (Ghosh et al., 2008) for capillary endothelial cells, the fundamental alteration in leiomyoma cells was not ROCK-dependent, as demonstrated by the finding that leiomyoma cells pretreated Y27632 prior to uniaxial straining remained largely impaired.

Fig. 5. Response of myometrial or leiomyoma cells to substrates of varied stiffness. a: Leiomyoma and myometrial cells were cultured on collagen-coated polyacrylamide gels of varying stiffness, then treated with calcein AM and fluorescent images were obtained 22 h after plating for assessment of cell spreading. Stiffness as indicated. b: Mean surface area per cell was determined using ImageJ software as indicated for myometrial cells (gray line) or leiomyoma cells (black line). Myometrial cell spreading responded to the increased substrate stiffness more than leiomyoma cells. Values represent a mean of four independent experiments with a minimum of 45 cells measured per condition (* trend comparison: p < 0.05).
unchanged (Fig. 3). In further support of this explanation, leiomyoma cells contain increased levels of the Rho-GEF AKAP13 (Rogers et al., 2008), and knockdown of AKAP13 differentially affected leiomyoma cells, compared to myometrial cells (Owen et al., 2010). Thus, the results are consistent with the notion that leiomyoma cells have undergone a specific adaptation to their stiff microenvironment that is not ROCK-dependent, is associated with increased levels of Rho-GEF, and this adaptation persists in tissue culture. Additional experiments will be needed to unravel the specific changes associated with the mechanotransduction response of leiomyoma cells.

In conclusion, these results reveal that the increased stiffness and elastic moduli demonstrated in leiomyomata is accompanied by an altered mechanosensory response characterized by an attenuated levels of active RhoA. A further understanding of mechanotransduction as it relates to leiomyomata may explain why some leiomyoma grow and others do not, and could help to guide future treatments for this very prevalent pelvic tumor.

4. Experimental procedures

4.1. Mechanical testing of leiomyoma and myometrial tissue

Specimens of leiomyoma and paired myometrium were collected from women undergoing hysterectomy for symptomatic leiomyoma in institutional review board-approved studies. Patient characteristics are described in Table 1. Surgical specimens were snap-frozen. Cylindrical specimens were precisely cut using a 5-mm punch biopsy (Miltex Inc., York, PA) and a 5-mm height cutting apparatus. Tissue was re-hydrated with normal saline approximately 2 min, weighed and then placed into the confined compression chamber attached to the Enduratec ElectroForce 3200 (Bose Corporation, Eden Prairie, MN) (Fig. 1a & b). Control experiments with varied re-hydrated times and frozen versus fresh tissue for both human fibroid tissue as well as beef muscle revealed no significant differences in tissue behavior for the tests conducted within the time frame used for the experiments. The saline filled stainless steel piston with a porous (40 micron pore size) stainless steel membrane (Small Parts Inc., Miramar, FL) was then placed adjacent to tissue (Fig. 1a). An initial 15 second ramp to 0.5 N was applied to the specimens to ensure proper contact between the tissue and the piston. After a 60 second relaxation cycle, a 5% displacement force was applied. Because the force generated under strain rate used in a conventional dynamic test was too large (exceeding 200 Newtons) we performed a pseudo-dynamic modulus test using a slow ramp (5% displacement in 4 s) which measured Young's modulus (stress (MPa) per displacement [mm]) per tissue cross sectional area (mm²) (Fig. 1c). The peak strain and the relaxation modulus (Young's modulus) at 5% displacement were measured. The pseudo-dynamic modulus and the peak strain at 5% displacement were measured during a 1200 second cycle (Fig. 1c).

The tissue was then re-weighed, dried using a SpeedVac and vapor trap device (Thermo Savant, Waltham, MA) and then digested (0.56 U/ml papain, 2 mM L-cysteine, 2 mM EDTA, 55 mM NaCitrate, and 150 mM NaCl) at 60 °C overnight.

4.2. Biologic assays for tissue samples

The digested surgical specimens were then analyzed for DNA content, sulfated glycosaminoglycan (GAG) and collagen content. DNA content was determined using a Picogreen assay kit (Picogreen; Invitrogen, Carlsbad, CA). Sulfated GAG content was determined with the 1,9-dimethylmethylene blue (DMMB) method (Farndale et al., 1986) and was normalized to a known quantity of chondroitin-4-sulphate. Collagen content was determined using a basic hydroxyproline assay described by Reddy and Enwemeka (Reddy and Enwemeka, 1996). PureCol (Sigma-Aldrich, St. Louis, MO) was used to generate standard curves.

4.3. Cell culture

Immortalized leiomyoma and myometrial cells which have been previously described and which retain features of the respective tissues (Malik et al., 2008) were cultured on polystyrene in culture medium composed of DMEM F12 (Invitrogen, Carlsbad, CA), 10% FBS, 1% glutamate, and 1% antibiotic mixture. Immortalized cells were used because primary cultures of leiomyoma cells do not retain features of the tumor in passage, and the immortalized cells strongly resemble in vivo tumors when compared using microarray analysis of ECM gene expression and other characteristics (Malik et al., 2008).

4.4. Mechanical strain application to leiomyoma and myometrial cells

Immortalized leiomyoma and myometrial cells were cultured on pronectin coated, flexible silicone substrates (Uniflex culture plates, Flex Cell International Hillsborough, NC) for 2 days to ~70–80% confluence. Both cell types were then exposed to a maximum of 8.9% uni-axial cyclic strain at a 1 Hertz sinusoidal waveform for 18 h using a custom manufactured loading device that uses commercial BioFlex plates and a computer-controlled vacuum stretch apparatus. The choice of 8.9% strain was empirically chosen based on prior data that suggested leiomyoma most strongly resemble tendon (Rogers et al., 2008) and prior reports using a similar strategy of comparison with tumor cells (Ghosh et al., 2008). Since the focus of application of mechanical strain was to examine fundamental differences in RhoA signaling between cell types, and not a rheological assessment of cell characteristics, relaxation and recovery were not independently tested with the system. Control cells were cultured on the same pronectin-coated substrates placed in the same incubator and were positioned in the same strain apparatus, but did not receive applied strain. In some cyclic strain experiments, both cell types were treated with or without Y-27632, ROCK inhibitor, (10 μM) (Calbiochem EMD, San Diego, CA) for 30 min prior to application of strain.

4.5. Modulation of substrate stiffness

Porous polyacrylamide gels of increasing stiffness coated with type 1 Collagen (Invitrogen, Carlsbad, CA) were prepared as previously described (Wang and Pelham, 1998) with minor modifications as follows. Coverslips were treated with dichlorodimethylsilane (Sigma-Aldrich, St. Louis, MO) before using them to cover the 20 μL of gel solution that was applied to an activated bottom coverslip. During activation of the polyacrylamide surface and conjugation with type 1 Collagen, 400 μL of sulpho-SANPAH was used. Stiffness measurements of the gels were estimated based on the final acrylamide to Bis ratio as previously studied (Engler et al., 2004; Tse and Engler, 2010). Polyacrylamide gels were allowed to equilibrate for 30–45 min in culture medium at 37 °C. To analyze the effects of varying substrate stiffness on cell spreading, cells were cultured on collagen-coated polyacrylamide gels of varying stiffness at a low density (20,000 to 40,000 cells/9.5 cm²) to minimize cell–cell interactions. Cells were treated with 2 μM calcein AM (Invitrogen, Carlsbad, CA) 22 h after plating and 30 min prior to obtaining fluorescent images for assessment of cell spreading.

4.6. RhoA activation assay

RhoA activity was determined by using the absorbance based G-LISA RhoA activation assay per manufacturer instructions (Cytoskeleton, Denver, CO). Immortalized leiomyoma and myometrial cells were cultured to ~50–60% confluence on either polystyrene or on the same pronectin coated culture dishes used in the reorientation experiments (Flex Cell International). Cells were serum starved for approximately 17 h and then treated with 10 μM Lyso PA (LPA or 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate, Avanti Lipids, Alabaster, AL) for increasing time periods. Cells were lysed in G-LISA cell lysis buffer.
buffer at 4 °C and lysates were snap frozen in liquid nitrogen for subsequent assay. Immortalized leiomyoma and myometrial cells cultured on pronectin-coated flexible silicone substrates were serum starved for approximately 17 h (0.5% PBS media) and then exposed to 8.9% uniaxial cyclic strain for 2 h. Control cells of both cell types were under the same conditions, but did not receive strain. Immediately following the completion of strain, both strained and control cells were lysed in G-LISA cell lysis buffer at 4 °C and lysates were snap frozen in liquid nitrogen. All lysates were assayed for RhoA-GTP per the G-LISA RhoA activation assay. The signal indicating the level of RhoA-GTP was determined by a microplate spectrophotometer measuring absorbance at 490 nm. The absorbance was normalized to unstrained myometrial baseline (control) samples and all samples were reported as fold-increase over myometrial control. Data are representative replications of three separate experiments.

4.7. Microscopy and image analysis

Mechanically strained cells fixed with 4% paraformaldehyde and live cells spreading on polyacrylamide gels were visualized and all images were taken with a Leica microscope at a 10X magnification and DFC320 camera at the same magnification for all conditions (Leica Microsystems, Bannockburn, IL). For mechanical strain experiments, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS 1X, blocked with 5% normal goat serum and 1% bovine serum albumin in PBS 1X, stained with Alexa Fluor 546 Phalloidin and DAPI (Invitrogen, Carlsbad, CA). Image analyses were performed using ImageJ software (National Institutes of Health Bethesda, MD). For cyclic strain experiments, fluorescent images were analyzed to determine the angle between the longest axis of the cell and the direction of the applied uni-axial cyclic strain. These results are reported as the percentage of cells aligned at 90°+/− 30° relative to the direction of the applied strain and also as angular distribution profiles for cell populations obtained by the grouping of angles of individual cells into 20° intervals. For cell spreading studies, live cells cultured on polyacrylamide gels were treated with calcein AM and fluorescent images were obtained 22 h after plating for assessment of cell spreading. Fluorescent images were converted to 32-bit images and cell areas were measured using threshold imaging within ImageJ software. Results are reported as the mean area surface per cell.

4.8. Statistical tests

All data were obtained from replica experiments and are expressed as the mean (error bars = SEM). Statistical significance was determined by using Student’s t test two-sample assuming equal variance and assumed at p < 0.05. An ANOVA was used to compare how the myometrial and leiomyoma cells respond to different substrate stiffness (Graph Pad Software Inc., La Jolla, CA). Spearman rank correlation analyses were performed for mechanically tested tissue samples to determine statistical dependence between the non-parametrically distributed results including: dry weight, DNA, sGAG, and collagen content, pseudo-dynamic modulus, and peak stress.

Conflict of interest

None.

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Disclosure

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of Health and Human Services, the Department of Defense or the U.S. Government.

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Appendix A. Supplementary data

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