Mechanical homeostasis is altered in uterine leiomyoma

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OBJECTIVE: Uterine leiomyoma produce an extracellular matrix (ECM) that is abnormal in its volume, content, and structure. Alterations in ECM can modify mechanical stress on cells and lead to activation of Rho-dependent signaling and cell growth. Here we sought to determine whether the altered ECM that is produced by leiomyoma was accompanied by an altered state of mechanical homeostasis.

STUDY DESIGN: We measured the mechanical response of paired leiomyoma and myometrial samples and performed immunogold, confocal microscopy, and immunohistochemical analyses.

RESULTS: Leiomyoma were significantly stiffer than matched myometrium. The increased stiffness was accompanied by alteration of the ECM, cell shape, and cytoskeleton in leiomyoma, compared with myometrial samples from the same uterus. Levels of AKAP13, a protein that is known to activate Rho, were increased in leiomyoma compared to myometrium. AKAP13 was associated with cytoskeletal filaments of immortalized leiomyoma cells.

CONCLUSION: Leiomyoma cells are exposed to increased mechanical loading and show structural and biochemical features that are consistent with the activation of solid-state signaling.

Key words: AKAP13, Brx, fibroid, mechanotransduction, RhoA, solid-state signaling

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Leiomyoma are stiff tumors that are characterized by the presence of excessive extracellular matrix (ECM) and fibrosis.[1] Our previous studies have shown not only are genes that encode ECM proteins expressed abnormally in leiomyoma[2,3] but also that the ECM of leiomyoma is altered.[4] Alterations in the structure of the matrix affect its load-bearing properties and directly influence the mechanical load on cells that are growing within the matrix.[5] Studies in other tissue types reveal that changes in mechanical stresses that are applied to cells profoundly affect cell growth and proliferation.[6] Although it is generally accepted that leiomyoma cells exhibit altered growth and apoptosis,[1,7,8] previous studies have concentrated on the involvement of growth factors in leiomyoma development. Despite the striking overproduction of ECM few, if any, studies have characterized the contribution of mechanical signaling to leiomyoma growth.

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Mechanical stress is a well-documented stimulus of muscle hypertrophy, but the few studies of mechanical signaling in uterine smooth muscle cells have focused on pregnancy.[9-11] The cellular process by which mechanical stress is converted to cell signaling is known as *mechanotransduction* or *solid-state signaling*.[12,13] Mechanical stresses that are imposed on the ECM are transmitted through integrins,[14] mechanotransducers, and caveoli[15] to biochemical signals in the cell. Downstream cellular signaling pathways of mechanotransduction include mitogen-activated protein kinases, phosphatidylinositol-3 kinase/Akt, Janus kinase/signal transducer,[16] and nitric oxide.[17] Rho family small GTPases play a critical role in the process. Rho-GTPases function as molecular switches that are on in the GTP-bound state and off in the GDP-bound state.[18] Rho family members are activated by Rho-guanine nucleotide exchange factors (GEFs) to influence cytoskeletal rearrangement, contractility, and cell proliferation.[19] The specific proteins that activate Rho in uterine muscle cells have not been characterized fully.

In addition to physiologic activation, pathologically altered states of mechanical stress can cause fibrosis by

TABLE 1
Patient characteristics

Patient #	Age	Race	Fibroid Size	Location	Time of cycle
1	35	Caucasian	2 x 1.5 x 1.7	Submucosal	Follicular
2	39	African American	7 x 7	Intramural	Periovulatory
3	37	Caucasian	5 x 4	Submucosal	Periovulatory
4	37	African American	1.5 x 1.5 x 1.7	Intramural	Luteal
5	46	African American	2 x 1.3 x 1.4	Submucosal	Follicular
12	39	African American	1 x 1 x 1	Intramural	Luteal
14	37	Caucasian	5 x 4 x 4	Intramural	Ovulatory
16	37	African American	1.5 x 1.2	Intramural	Anovulatory
18	38	African American	1.2 x 1.1 x 1.3	Submucosal	Follicular
19	47	Caucasian	3.5 x 3.5 x 3	Subserosal	NA
20	47	African American	7 x 7 x 6	Subserosal	NA
21	44	African American	4.2 x 4.5 x 5.5	Intramural	NA
23	40	African American	3 x 3 x 3	Intramural	Ovulatory
24	44	Caucasian	7 x 8	Intramural	NA
40	36	Caucasian	5.5 x 4x5	Submucosal	Ovulatory
41	49	Asian	7 x 5 x 8	Subserosal	NA
42	46	Caucasian	5 x 5 x 5	Intramural	Luteal
46	46	African American	4 x 4 x 4	Subserosal	Ovulatory
50	48	African American	5 x 5 x 5	Intramural	Anovulatory
51	44	African American	5 x 3 x 3	Intramural	Luteal
57	45	African American	4 x 4 x 4	Intramural	Luteal

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stimulating fibroblasts to deposit excessive ECM.[6] Very little is known about the pathologic response of uterine smooth muscle cells to mechanical stress, in particular whether altered mechanical stress might lead to leiomyocyte transformation and deposition of excessive ECM, for example as a uterine fibroid tumor. One key feature of uterine fibroid tumors is the production of excessive amounts of ECM.[1,3,4] In fact, growth of large fibroid tumors (>5 cm) was largely due to deposition of ECM,[20] and the composition of this excessive ECM differed from smaller fibroid tumors and myometrium.[21,22] Previous studies of ECM in fibroid tumors have reported increased levels of total glycosaminoglycans and a reduction in hyaluronic acid.[21-24] Several groups have reported that the ECM in leiomyoma is altered in both content[3,21,24] and structure.[4,24] Collectively, these reports suggest that the mechanical properties of the ECM in leiomyoma may differ from normal uterine tissue.

In addition to causing fibrosis, altered mechanical stress can influence cell growth and tumorogenesis.[25] Many tumors have been shown to have an elevated elastic modulus or stiffness,[26] which is an attribute that is used clinically to detect metastatic nodules on peritoneal surfaces at laparotomy and with imaging modalities.[27] Specifically, Rho-GTPases were elevated in stiff tumors[28] and activation of the Rho-dependent kinase has been shown to cause tumor cell dissemination.[29] Activation of Rho-dependent cytoskeletal tension led to increased levels of extracellular signal-regulated kinase (ERK) and cell proliferation[30] and was a requisite step in the acquisition of a malignant phenotype of breast cancers.[25]

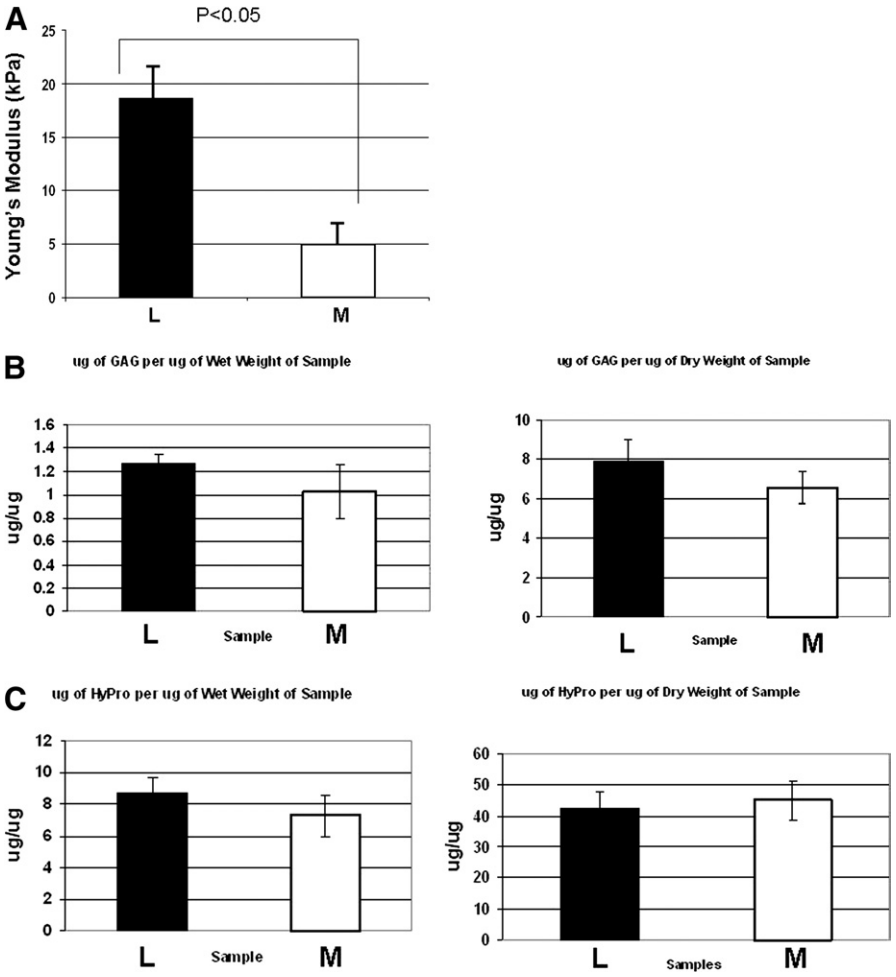
The 2 cardinal features of uterine fibroid tumors, excessive fibrosis and tumor formation, suggest that fibroblasts within a leiomyoma may be exposed to altered mechanical stress. To determine whether altered mechanical stress may play a role in fibroid tumor development, we characterized the state of mechanical homeostasis in matched surgical specimens of leiomyoma and normal myometrium. The findings indicate that the production of an abnormal ECM in fibroid tumors was accompanied by a state of altered mechanical homeostasis within leiomyoma.

Materials and Methods

Tissue collection

Tissues were collected from women who underwent hysterectomy for symptomatic leiomyoma in institutional review board–approved studies at the National Naval Medical Center and the National Institutes

FIGURE 1
Mechanical testing in matched surgical specimens of uterine leiomyoma (*L*) and myometrium (*M*)



A, Measurement of compressive resistance to 0.1 strain (Young's modulus) of 5 matched leiomyoma and myometrial samples. The *Y* axis represents force (Kilopascals [kPa]) at equilibrium required for compressive strain = 0.1. Leiomyoma had significantly greater compressive resistance compared with myometrium ($P = .003$). Similar results were observed in 2 other specimens from patients 23 and 57 that were tested in duplicate. **B,** Measurement of total sulfated glycosaminoglycan content in matched leiomyoma and myometrial 3 paired samples analyzed by compression (**A**) with the use of the blyscan method for wet (**left panel**) and dry weight (**right panel**). The *Y axes* represent the mean ± SEM (*error bars* denote SEM) micrograms of glycosaminoglycan per microgram of sample. The *X axes* represent that sample myometrium or leiomyoma. Averages of 2 tests that were performed on 3 paired samples are shown. Differences were not significant. **C,** Hydroxyproline measurement in the same 3 matched leiomyoma and myometrial samples that were tested in **B**, corrected for wet and dry weight, are shown. The *Y axes* represent the mean ± SEM (*error bars* denote SEM) for hydroxyproline per microgram of sample weight. Averages of 2 tests performed on 3 paired samples are shown. Differences were not significant.

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of Health. For Western blots, tissue was snap-frozen on dry ice and stored at −70°C until processed. For immunohistochemistry, samples were frozen in cryoprotectant (OCT 4583; Tissue-Tek, Elkhart, IN) or were formalin-fixed and embedded in paraffin. For immunogold studies, tissue was fixed in formalin. Characteristics of patients and leiomyoma in the study are summarized in Table 1.

Mechanical testing

From fresh or snap-frozen leiomyoma tissues, 4-5 mm cylindric pieces were cut with an 8-mm biopsy punch (Miltex, Tuttlingen, Germany) and a microtome blade. Height and diameter were recorded, and tissue was subjected to a mechanical testing protocol with the use of displacement control.[31] *Young's modulus* is defined as the longitudinal deformation in terms of strain (fractional change in length) in response to longitudinal stress (force per unit area). Because most tissues exhibit nonlinear viscoelastic changes in physiologic ranges of stress, we used equilibrium assessment of Young's moduli.

Glycosaminoglycan assay

Small pieces of frozen fibroid and myometrium specimens were weighed and digested overnight in digestion buffer (0.1 mol/L NaAc, 10 mmol/L cysteine HCl, 50 mmol/L ethylenediaminetetraacetic acid, plus 20 μL papain/mL buffer). Glycosaminoglycan concentration of completely digested samples was determined with the Blyscan Sulfated Glycosaminoglycan Assay (Biocolor, Newtownabbey, UK), according to the manufacturer's instructions. Hydroxyproline content of each sample was determined after proteinase-K digestion, as described elsewhere.[32]

Fluorescein isothiocyanate (FITC) phalloidin stain

Snap-frozen tissue blocks were cut into 5-μm sections and placed on glass slides, fixed for 15 minutes in 3.7% paraformaldehyde, washed 3 × 5 minutes in phosphate-buffered saline solution (PBS), permeabilized in 0.2% Triton X-100 in PBS for 1 hour, washed in PBS with 0.05% Triton, and blocked in 1% bovine serum albumin (BSA) in PBS for 1 hour. Slides were then placed in a dark humidity chamber for 1 hour in 5 μg/mL FITC Phalloidin (Sigma-Aldrich, St. Louis, MO). After being rinsed in PBS/0.05% Triton, slides were mounted in media with DAPI (Vector Laboratories, Burlingame, CA), cover-slipped, and visualized.

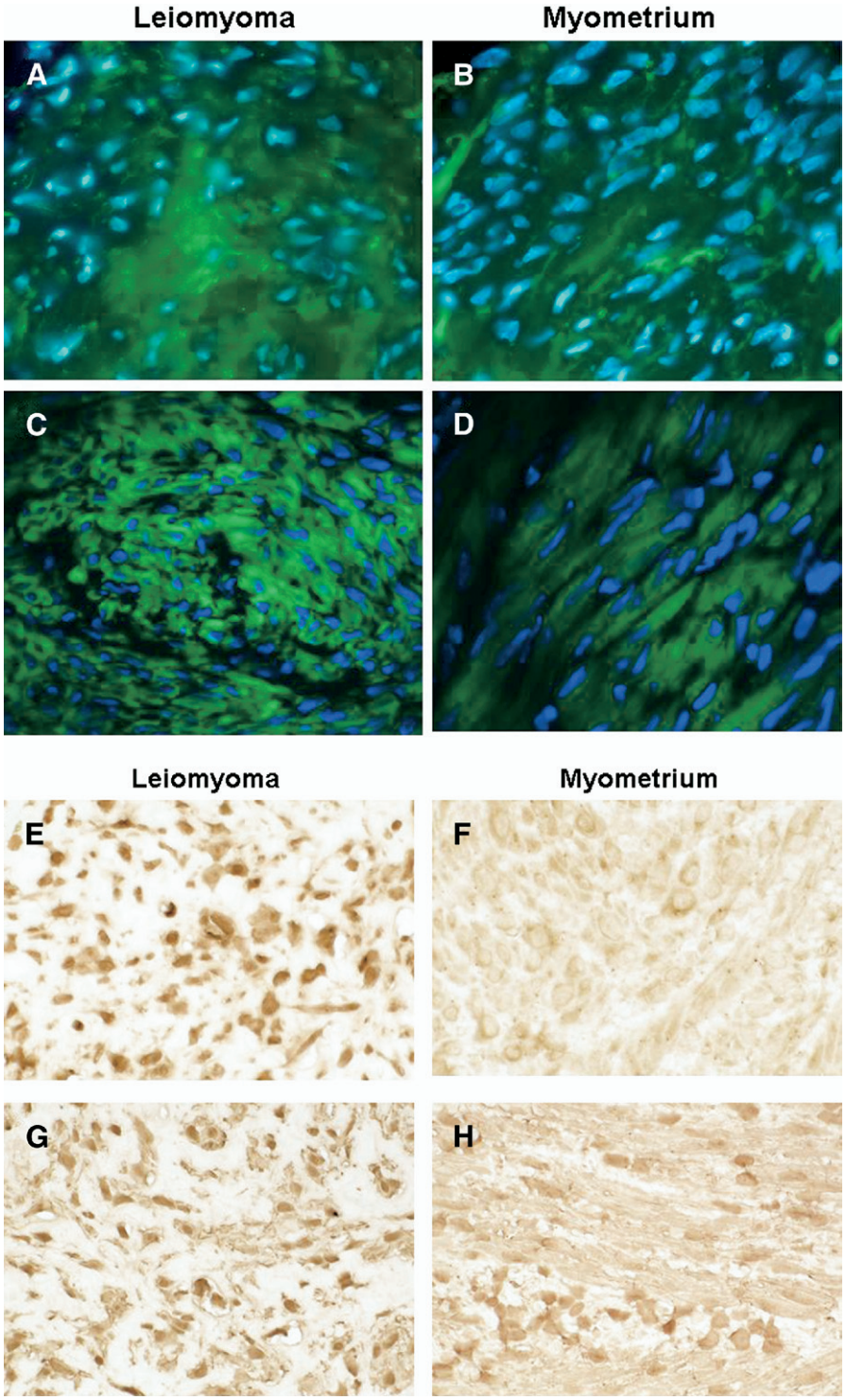
Western blots

Protein was isolated according to a previously described protocol.[33] Samples (50 μg) were resolved in Tris-Glycine gels (Invitrogen, Carlsbad, CA) and transferred to Hybond nitrocellulose membranes (Amersham, Piscataway, NJ). Membranes were blocked for 2-3 hours at 4°C in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST) and 5.0% evaporated milk and incubated overnight at 4°C in TBST with 0.1% BSA and primary antibodies. Membranes were rinsed 3 times in TBST for 5 minutes between all incubations. Secondary antibody was added in TBST with 0.1% BSA at room temperature for 1 hour. Supersignal developing reagents were used according to the manufacturer's directions (Pierce, Rockford, IL), and membranes were exposed to film. For each antibody that was used, horseradish peroxidase–conjugated β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA) was used to confirm equal protein loading. Cos-7 lysates that were transfected with a 170-kd form of AKAP13 (Brx; Upstate, Temecula, CA) was used as a positive control. Recombinant RhoA (Calbiochem, San Diego, CA) was used as a control. Antiphosphorylated p38 mitogen–activated protein kinase (SC 7973; Santa Cruz Biotechnology, Santa Cruz, CA) was used. Extracts were analyzed from specimens of patients 12, 14, 16, 20, 21, 40-42, 46, 50, and 51.

Microarray

Sample collection and microarray analysis were previously described.[3,34] The ex-

FIGURE 2
Structural changes that were associated with uterine leiomyoma



FITC-phalloidin staining of actin (*green*) and cell nuclei (*blue*) with DAPI of sections from matched leiomyoma and myometrial samples. Actin structure was disordered in leiomyoma and differed markedly from normal myometrium. **A,** Leiomyoma and **B,** myometrium were from the same uterus; **C,** leiomyoma and **D,** myometrium were matched specimens from a separate uterus. Note the deformed nuclei (*blue*) and cell structure in **A** and **C**. Representative sections taken from samples obtained from patients 40 and 42. (Original magnification, ×40.) Staining of paired **E,** leiomyoma and **F,** myometrium for RhoA (1:1000) suggested increased staining. (Original magnification, ×40.) Staining of paired **G,** leiomyoma and myometrial **H,** sections for alpha-smooth muscle actin (1:1500). Expression appeared greater in leiomyoma cells. Note the increase in ECM (*clear area*) surrounding the cells in (**G**) compared with (**H**). (Original magnification, ×40.)

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isting database was queried for genes that are known to be involved in mechanotransduction. Results were from 4 paired myometrial and fibroid samples that were collected from patients 2-4.

Immunohistochemistry

Paraffin-embedded tissue samples were cut into 5-μm sections. Sections were stained according to the Vectastain Elite ABC kit protocol (Vector Laboratories), as previously described,[34] with the following modifications: Antigen retrieval was performed with boiling water and unmasking solution in a pressure cooker. Slides were incubated in primary antibody either for 3 hours at room temperature or 4°C overnight. All subsequent steps were carried out according to the manufacturer's instructions (Vector Laboratories). Sections with no primary or no secondary antibodies or incubated with preimmune antisera served as controls. Studies included patients 1-5, 24, 40-42, 46, and 57.

Immunogold

Tissue pieces were removed from a paraffin block and deparaffinized in xylene or taken directly from formalin and placed in absolute ethanol and embedded in LR White (SPI, West Chester, PA). Ultrathin sections were mounted on 150-mesh uncoated nickel grids. Sections were hydrated in blocking buffer (PBS, 0.1% Tween-20, 0.5% cold-water fish gelatin [Ted Pella, Inc., Redding, CA]) for 20 minutes, then incubated in buffer that contained AKAP13 primary antibody (1:125) for an hour. Samples that were not incubated in primary antibody served as a control. Samples were placed in blocking buffer with 2% goat serum for 5 minutes, washed twice in blocking buffer without serum for 2 minutes, incubated in 10-nm gold-conjugated secondary antibody (1:1000; Ted Pella Inc, Redding, CA) for 1 hour, and washed 2 times in PBS and twice in deionized water. Dried grids were stained with uranyl acetate for 3 minutes, rinsed 3 times, and allowed to dry again. Paired samples from patients 18-20, 21, 24, and 40-42 were studied.

Confocal microscopy

Cells were grown on glass chamber slides (Nalge Nunc Int, Rochester, NY), fixed with cold methanol, and permeabilized with 0.2% Triton-X 100 (Sigma-Aldrich, St. Louis, MO). Non-specific sites were blocked using 1% BSA and 10% normal goat serum and slides were incubated either with antibody to alpha smooth muscle actin and 2665 affinity-purified anti-Brx (AKAP13) polyclonal antibody[35] at 1:50 dilution overnight at 4°C. For secondary antibody either FITC (anti-rabbit, 1:100) or Alexa-594 (anti-mouse, 1μg/ml; Invitrogen) was used. DAPI (Sigma-Aldrich) was added at 0.1μg/mL in PBS before the cells were examined with an Axiovert 405 mol/L epifluorescence inverted light microscope (Carl Zeiss, Oberkochen, Germany). Images were acquired with a charge couple device (CCD) camera (Hamamatsu Orca, Shizuoka, Japan).

Statistics

Groups and sample values are reported as mean ± SEM. Groups and values were compared with the use of an unpaired, 2-tailed Student *t* test. Significance was assigned at a probability value of $<.05$. Tests were performed with Stata software (version 9.2; Stata Corporation, College Station, TX).

Results

The Young's modulus, a measure of tissue stiffness, was quantified with average values of 4.9 kPa and 18.6 kPa for myometrial and leiomyoma samples, respectively (Figure 1). Variation was noted between specimens and within specimens, but uterine leiomyoma tissues were typically 2- to 3-fold stiffer than matched myometrium ($P < .05$). Because the presence of an excessive mechanical load can influence ECM formation, we measured 2 components of the ECM, sulfated glycosaminoglycans and hydroxyproline content (reflective of collagen content), in the subset of samples that were exposed to testing. Previous investigators had reported levels of glycosaminoglycan content in leiomyoma,[21-23] and our results also suggested that an increase of glycosaminoglycans and collagen was present in leiomyoma, but the differences were not statistically significant for the number of samples tested.

Cells that are exposed to increased mechanical loading often exhibit structural changes and altered expression of structural support proteins, such as actin; therefore, we examined smooth muscle alpha actin and F-actin in matched surgical specimens of leiomyoma and myometrium. Phalloidin staining (for actin) revealed a striking difference between leiomyoma and myometrial tissues, which was consistent with our previous observation of disordered ECM in leiomyoma.[4] Cell shape within leiomyoma was angular with a compressed appearance in comparison to myometrium

TABLE 2
Microarray results for stress-related genes in leiomyoma and myometrium

Gene	L/M ratio
Alpha-cardiac actin	5.2
Alpha–smooth muscle actin	1.2
Alpha 1 actinin	-2.02
Alpha 1 actinin, skeletal muscle	-1.79
myosin	8.3
Tropomyosin 1 & 4	-3.6
Smooth muscle MHC	-3.3
Alpha-MHC consensus	2.37
Fibrillin 2	3
Vinculin	-1.15
Talin	1.39
ROCK (Rho-kinase)	-1
Rho-GEF FERM	-1.34
Rho-GEF 12	-1.06
Rho-GEF p115	1
Rho-GEF 4	1.2
Rho-GEF AKAP13	4
Endothelin receptor type B	-2.13
Endothelin receptor	-1.57

Samples from patients 2-5 were used for microarray. *L/M*, ratio of leiomyoma to myometrial signal; *MHC*, myosin heavy chain; *GEF*, guanine nucleotide exchange factor.

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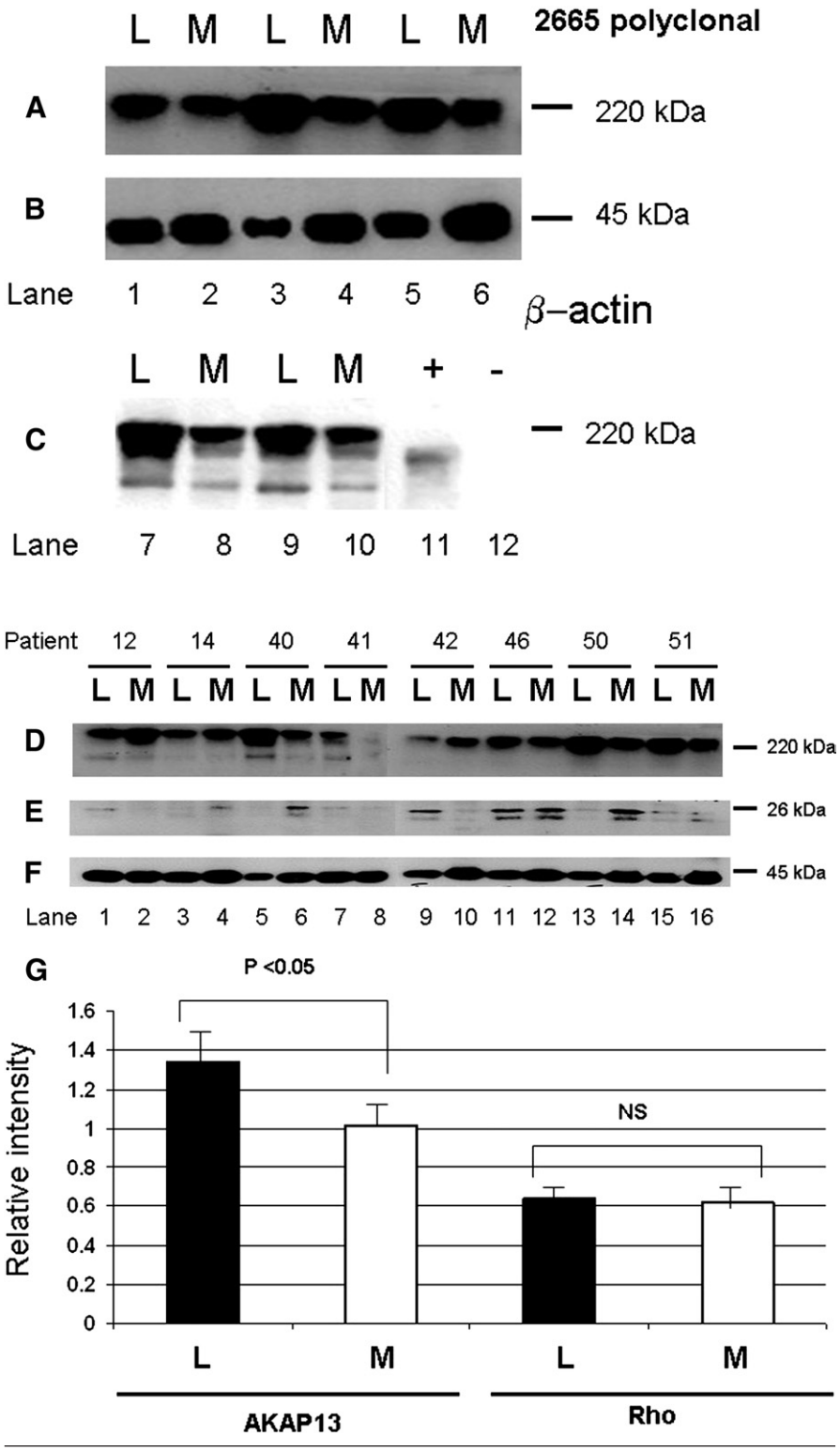
FIGURE 3
Altered expression of factors that were involved in mechanical transduction in matched surgical specimens of uterine leiomyoma (*L*) and myometrium (*M*)



(Figure 2). These findings are consistent with the finding that tissue stiffness was increased in leiomyoma, compared with myometrium.

The finding of increased stiffness led to the question: did leiomyoma exhibit biochemical evidence of activation of solid-state signaling? To characterize the levels of factors that are known to be involved in mechanotransduction, we queried our complementary DNA microarray data of 33,000 transcripts.[3,34] As shown in Table 2, several factors that were involved in solid-state signaling were expressed differentially in leiomyoma. Transcripts of 1 protein, AKAP13 (a Rho-GEF), were over expressed 4-fold based on microarray results. We chose to focus on this factor because (1) RhoA is a

A, Western analysis of leiomyoma and myometrial lysates for expression of the Rho-GEF AKAP13 with the use of 2665 affinity-purified antisera.[36] A 220-kd band was present that was increased in leiomyoma compared with myometrium. **B,** Beta actin control for lysates in **A**. **C,** Western analysis for AKAP13 with the use of monoclonal antibody that was directed against Brx. A 220-kd band is identified. Positive control (+) consisted of lysates prepared from Cos-7 cells that were transfected with a construct that expressed a 170-kd form of AKAP13. The negative control (-), lane 12, were lysates that were prepared from untransfected Cos-7 cells that did not express AKAP13. **D,** Western analysis for AKAP13 in matched leiomyoma and myometrial lysates from 8 patients are shown. In most but not all pairs, expression of AKAP13 was greater in leiomyoma compared with myometrium. Results were repeated in 3 experiments. **E,** Western analysis for RhoA in the same lysates. Readily extractable levels of RhoA were increased in some, but not all, fibroid samples. **F,** Beta actin control for protein loading. **G,** Quantification of Western analysis for AKAP13 and Rho. Signal intensity of Western blots was quantified, corrected for beta-actin, and averaged for 8 paired samples (*error bars* denote SEM). AKAP13 staining was significantly greater ($P < .05$) in leiomyoma than in myometrium. Levels of total Rho were similar (*NS* = not significant).

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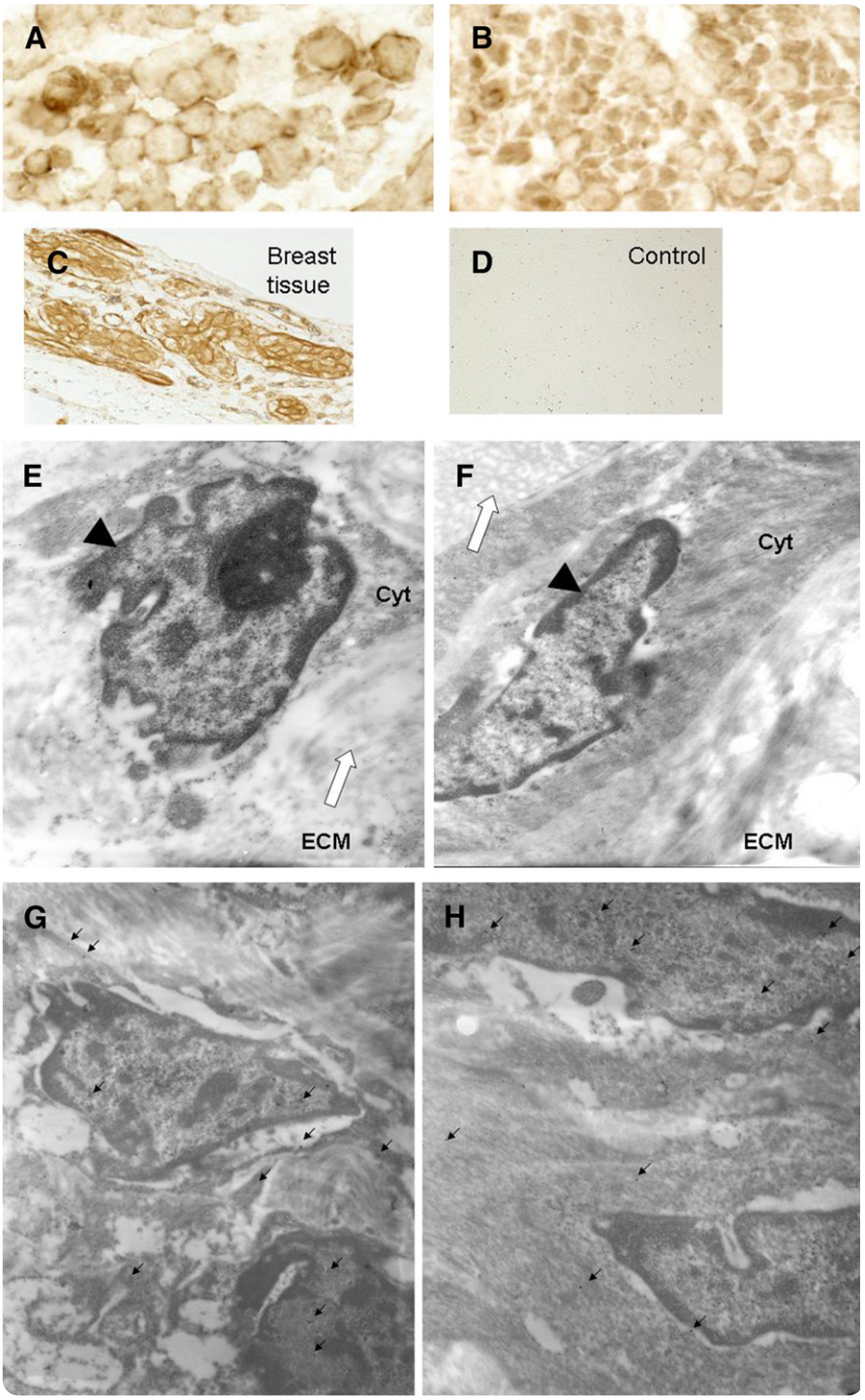
known target of AKAP13,[36] and RhoA is involved in the cellular response to mechanical stress[19,25,28-30]; (2) it was the only Rho-GEF that is expressed differentially, based on the microarray results; and (3) we had cloned this factor on the basis of its ability to augment estrogen action,[35,37] and leiomyoma are known to be estrogen-responsive.

To confirm the microarray data for AKAP13, we performed Western analyses on paired fibroid and myometrial samples. Western analysis with the use of both a rabbit polyclonal antisera directed against AKAP13 and a monoclonal antibody to AKAP13 revealed the presence of a 220-kd band (Figure 3). Quantification of Western results indicated that AKAP13 was over significantly over-expressed in leiomyoma ($P < .05$), in support of the microarray (messenger RNA) results. Levels of nonmembrane-associated RhoA have been noted to correlate with activation of RhoA.[15] We performed immunohistochemical staining for Rho of matched fibroid and myometrial lysates to determine whether increases in AKAP13 were coupled with increased levels of RhoA. There were no significant differences in total cytoplasmic RhoA (Figure 3, **G**).

Because mechanical homeostasis appeared to be altered on the basis of direct

FIGURE 4
Subcellular distribution of AKAP13 in matched leiomyoma and myometrial specimens



Immunohistochemical localization of AKAP13 (1:500) in **A,** leiomyoma and **B,** myometrium tissues. (Original magnification, ×63.) **C,** Positive control, breast tissue; **D,** negative control equals fibroid tissue that is stained with preimmune antisera. Staining for AKAP13 often appeared increased with a perinuclear appearance in fibroid tumors. Immunogold study of matched **E,** leiomyoma and **F,** myometrium specimens from patient 20 with the use of 2665 antisera that was directed against AKAP13. Note the angular cell shape, reduced cytoplasm (*Cyt*), and notched nucleus in the leiomyoma (**E**) compared with myometrium (**F**). The *round black dots* indicate localization of protein; the *black triangle* points to nucleus. The *white arrow* (**E**) points to the irregular bundles of collagen fibers in leiomyoma, compared with the regular bundles of collagen fibers seen en-face noted by the *white arrow* in **F**. (Original magnification, ×21,000.) Immunogold staining from another matched pair of **G,** leiomyoma and **H,** myometrial tissues from patient 24 that were stained with antisera that was directed against AKAP13. In this view, the *arrows* point to AKAP13 expression in the nucleus, nuclear envelope, and cytoplasm. (Original magnification, ×15,500.) Results were repeated in matched samples from 4 patients. Immunogold studies were conducted on specimens from patients 18-21.

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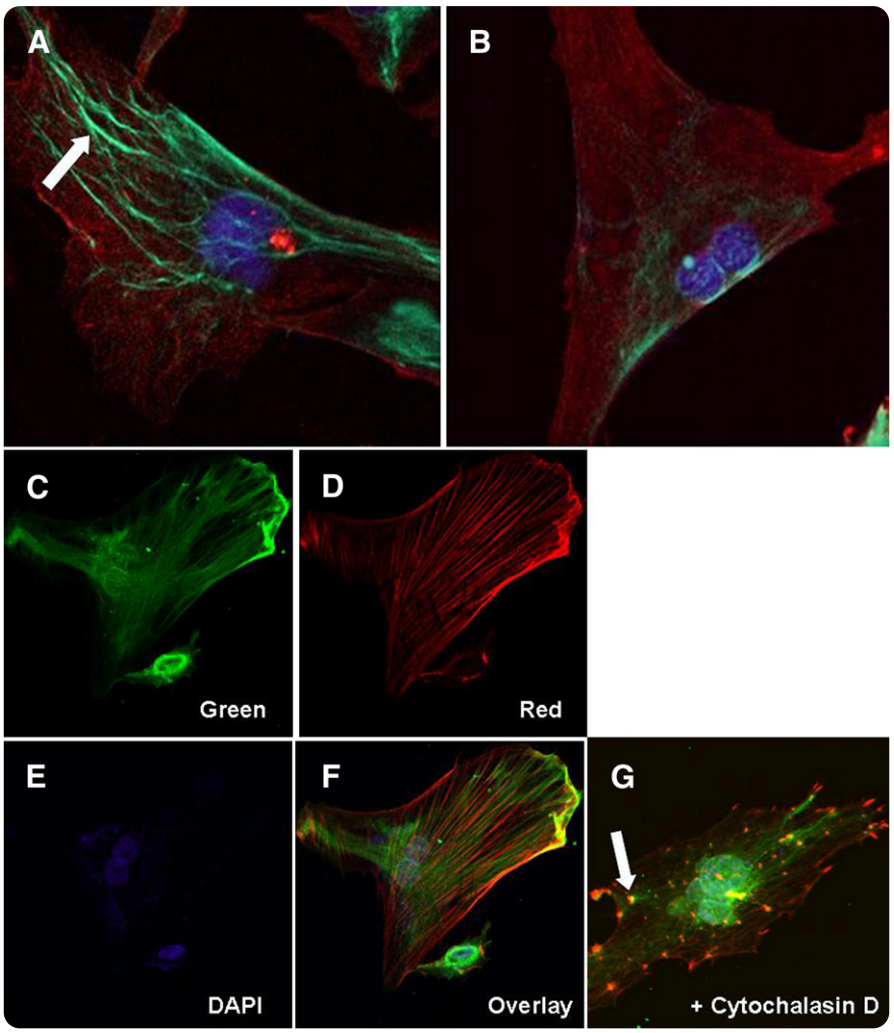
FIGURE 5
Altered focalization of AKAP13 to cytoskeletal structures in immortalized cultured leiomyoma and myometrial cell lines



A, Confocal laser microscopy of leiomyoma cell that was stained with antisera that were directed against AKAP13 (*green*) and alpha smooth muscle actin (*red*). AKAP13 protein appeared to localize to cytoskeletal filaments. DAPI-stained nucleus appears *blue* (original magnification, ×40, oil). **B,** Confocal laser microscope image of myometrial cell that was stained for AKAP13 (*green*) and alpha smooth muscle actin (*red*). Staining for AKAP13 protein was less robust. DAPI-stained nucleus appears *blue*. Results were repeated in 3 independent experiments. **C,** Confocal image of leiomyoma cell simultaneously stained for AKAP13 (*green*) and F-actin (*red*). Note green staining localized to cytoskeletal filaments. **D,** Same cell, stained for actin (*red*) filaments. E, Same cell, stained with DAPI that shows the nucleus (*blue*). **F,** Same cell with overlay of AKAP13 (*green*) and actin (*red*), which suggests close association of AKAP13 with actin structure. **G,** Overlay of leiomyoma cell stained for AKAP13 (*green*) and actin (*red*), but treated with cytochalasin D to dissolve actin filaments. Staining for AKAP13 localized to regions in the periphery of the cell (*white arrow*) in the absence of the actin cytoskeleton.

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measurement of tissue, and levels of AKAP13 were elevated in leiomyoma, we questioned whether the subcellular localization of AKAP13 might be altered in leiomyoma relative to myometrium. To examine the subcellular localization in native tissues, we performed immunohistochemical analysis of matched fibroid and myometrial samples (Figure 4). These studies suggested that the pattern of staining for AKAP13 was altered in fibroid tumors, with increased staining often near the cell membrane or nuclear envelope, but resolution was not sufficient to define the subcellular localization accurately. To more precisely identify the subcellular localization of AKAP13 in uterine fibroid tumors, we performed immunogold studies. Electron microscopy substantiated the increased localization of AKAP13 in perinuclear region and further suggested that the protein may associate with filaments in the cytoplasm.

To examine this possibility, we performed confocal microscopy on immortalized uterine fibroid and myometrial cells (Figure 5). These immortalized cells have been characterized and closely resembled the native leiomyoma and myometrium from which they were derived.[38] In cells that were derived from leiomyoma, the staining for AKAP13 was present robustly and localized primarily to cytofilaments (Figure 5, **A**) in comparison with cells that were derived from myometrium (Figure 5, **B**). The possible association with cytoskeletal filaments led us to perform dual-labeling experiments on the immortalized leiomyoma cells. Overlay experiments with confocal microscopy suggested that the pattern of AKAP13 staining and actin were strikingly similar and often overlapped (Figure 5, **F**). Disruption of the actin cytoskeleton with cytochalasin D also disrupted and altered localization of staining for AKAP13 (Figure 5, **G**), which suggested that a significant proportion of AKAP13 was associated with structural actin filaments in leiomyoma cells.

Comment

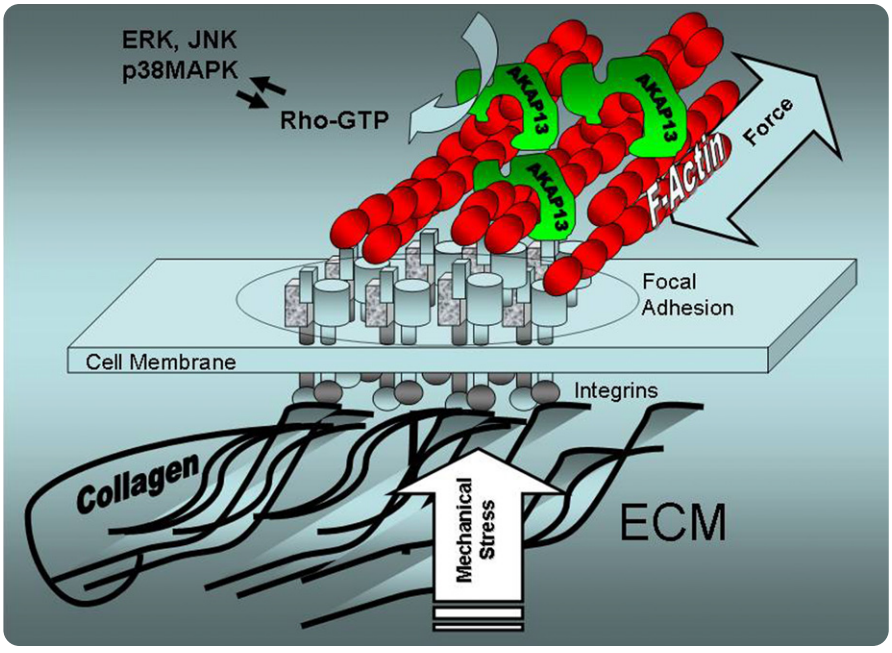
The findings reveal that cells that comprise uterine leiomyoma are exposed to increased mechanical loading. Consistent with the increased physical force, cells in leiomyomata exhibited structural changes that were associated with mechanical stress, such as an angular cell shape, altered actin organization, distor-

tion of the nuclear envelope, and altered expression of the Rho-GEF, AKAP13.

The results imply that leiomyoma cells have defective perception of mechanical stress. It is reasonable to assume that the leiomyoma cells themselves have a tensile elastic modulus of approximately 0.52 kPa[39] (the value for chondrocytes). In contrast, normal myometrium had an elastic modulus of 5 kPa, which indicates that 90% of the stiffness of myometrium could be attributed to structural aspects of the tissue. For leiomyoma, 97% of the measured stiffness would be attributable to structure of the ECM if the cell modulus was similar to chondrocytes. We interpret the findings to suggest that either (1) leiomyoma cells do not sense applied mechanical stress properly (have in appropriately low signaling) and, as a result, produce excessive ECM; or (2) leiomyoma cells produce excessive amounts of load-bearing ECMs to stress-shield themselves, because the cells are overly sensitive to mechanical stress. In either scenario, mechanical sensing by leiomyoma cells appears to be abnormal; however, additional studies will be needed to confirm and characterize the nature of the abnormality.

Our previous studies of uterine fibroid tumors with the use of microarrays have served to focus attention on the ECM of uterine fibroid tumors and its similarity to tissues that undergo remodeling and repair.[40] We noted that fibroid tumors were composed of an ECM with altered organization of structural elements, such as collagen fibers.[4] In addition, we[3] and others[21,24,41] have noted that fibroid tumors express altered levels of key components of the ECM, such as the proteoglycans dermatopontin and versican. The current study builds on the role of an altered ECM in leiomyoma formation and begins to address the following question: Why do leiomyoma cells produce an excessive and altered ECM? The data suggest that mechanical signaling is altered in leiomyoma cells. It is worth noting that excessive mechanical stress of muscle cells and abnormal repair have been shown to induce fibrosis in other muscular tissues, for example in the heart.[42]

FIGURE 6
Working model of mechanical stress signaling in leiomyoma cells



Leiomyoma cells are under increased mechanical loading compared with myometrial cells. Structure of the ECM is altered in leiomyoma. The increase in mechanical load is associated with alteration in cell structure, nuclear shape, and actin organization as shown in Figures 2 and 4. Expression of the Rho-GEF AKAP13 appeared to be increased in leiomyoma cells and was associated with the cytoskeleton. Future studies are needed to determine whether increased levels of Rho-GEF activity might alter the balance of growth/apoptosis through stress-activated kinases.

Rogers. Mechanical homeostasis is altered in uterine leiomyoma. Am J Obstet Gynecol 2008.

Activated states of solid-state signaling have also been shown to affect cell growth. Proliferation of breast cancer cells was increased significantly when the tension reached 1.2 kPa. However, breast cells with chromosomal anomalies that are consistent with metastasis did not metastasize until mechanical force was >5 kPa.[25] Breast tumors exhibited an average elastic modulus of 4.0 kPa, but can differ from surrounding tissue by 90-fold.[27] Myometrium stiffness averaged 5 kPa, although fibroid tumors from the same uterus typically exhibited a 2- to 3-fold increase in stiffness. The 2- to 3-fold increase in stiffness that was observed relative to surrounding muscle may be sufficient to increase proliferation or reduce apoptosis of the leiomyoma cells.

We were somewhat surprised that microarray results revealed increased levels of the Rho-GEF, AKAP13 (Brx) because our previous studies of AKAP13 had focused on its ability to augment estrogen[35,37] and glucocorticoid action.[43] We were intrigued by the fact that sex steroids influence leiomyoma growth, but conversely excessive glucocorticoids (such as Cushing's syndrome) cause muscle atrophy. Because the microarray results focused attention on this transcript, we proceeded to characterize the expression of AKAP13 in leiomyoma. Altered expression of AKAP13 in leiomyoma was supported by Western analysis, immunohistochemical stains, confocal microscopy, and immunogold studies. We postulate that the increased expression of AKAP13 may result, in part, from the altered state of mechanical loading (Figure 6) and, through altered Rho signaling, may contribute to the altered state of growth and apoptosis characteristic of leiomyoma.[7,8]

Any physical measurement of tissues is limited by the fact that physiologic tissues are nonhomogeneous in viscoelastic properties and anisotropic. Although we

are not aware of any previous mechanical assessment in fibroid tissue, skeletal muscle was softer along fibers than across fibers.[27] Nonetheless the 3-fold difference that was observed was greater than differences the were attributable to specimen orientation alone. The changes in glycosaminoglycans and collagen that we measured were consistent with previous reports[21-23,44]; however, because our focus was on the biochemical intracellular mechanisms that accompany the increased mechanical loading, we did not expand the number of samples that were tested and were satisfied that the direction of changes resembled published values, although our results for glycosaminoglycan and hydroxyproline were not statistically significant.

In summary, these results are consistent with the activation of mechanical signaling in leiomyoma. The results are in agreement with the general understanding that transforming growth factor beta signaling is augmented in leiomyoma that leads to the secretion of excessive amounts of ECM. These observations indicate that cells within a leiomyoma exist in a state of altered mechanical homeostasis that may contribute to the production of excessive amounts of ECM. ■

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