FKBP51 Contributes to Uterine Leiomyoma Pathogenesis by Inducing Cell Proliferation and Extracellular Matrix Deposition

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Abstract

The FK506-binding protein 51 (FKBP51) binds progesterone receptor (PR), glucocorticoid receptor (GR), and androgen receptor (AR) to coregulate their transcriptional activity. We evaluated FKBP51 expression and function in human leiomyoma vs. myometrial tissues and primary cultures to discover FKBP51 role(s) in the pathogenesis of leiomyomas. Quantification of in situ FKBP51 mRNA and protein levels in paired myometrial vs. leiomyoma tissues from proliferative and secretory phases were analyzed by qPCR (n = 14), immunoblotting (n = 20), and immunohistochemistry (n = 12). Control (scramble) vs. FKBP5 siRNA-transfected leiomyoma cell cultures were assessed for proliferation, apoptosis, and mRNA levels of genes involved in cell survival and extracellular matrix (ECM) formation. Significantly higher FKBP5 mRNA levels were detected in leiomyoma vs. paired myometrium (P < 0.001). Immunoblot (P = 0.001) and immunostaining (P ≤ 0.001) confirmed increased FKBP51 levels in leiomyoma vs. paired myometrium. Compared to control siRNA transfection, FKBP5 silenced leiomyoma cell cultures displayed significantly decreased cell survival factors and reduced proliferation (P < 0.05). Moreover, qPCR analysis revealed significantly lower mRNA levels of ECM, TIPM1, and TIPM3 proteins in FKBP5 silenced leiomyoma cell cultures (P < 0.05). Increased FKBP51 expression in leiomyoma likely involves dysregulation of steroid signaling by blocking GR and PR action and promoting proliferation and ECM production. Evaluating the effect of FKBP51 inhibition in preclinical studies will clarify its significance as a potential therapeutic approach against leiomyoma.

Keywords Uterine leiomyomas · Uterine fibroid · Steroid receptor signaling · Extracellular matrix · Progesterone antagonist

Introduction

Uterine leiomyomas also known as fibroids are benign tumors that affect up to 70% of women [1]. While many women with leiomyomas are asymptomatic, about 30% present with abnormal uterine bleeding, pelvic pressure and bulk-related symptoms, infertility, dyspareunia, or urinary issues [1, 2]. Treatment options include both surgical and medical management depending on the leiomyoma size, location, and patient's symptoms [2]. For patients with uterine leiomyoma who desire fertility, myomectomy, a surgical procedure to remove the leiomyomas, is an option; however, not all patients are surgical candidates nor do all patients feel comfortable undergoing a surgical procedure with an 8–11% complication rate [2]. Even after surgery, leiomyomas may recur and require additional surgery or treatment. Medical options for treatment, including combined oral contraceptive pills, progestin therapy, and gonadotropin-releasing hormone agonists or antagonists, have side effects and limit a woman's ability to conceive [2].

Investigation of precise mechanisms controlling differentiation and growth of uterine leiomyoma cells is required to develop novel effective therapies [1]. Uterine leiomyomas...
arise in the myometrium of the uterus, and while similar to the adjacent normal myometrial tissue, leiomyomas grow at a faster rate and in a whorl-shaped pattern of muscular tissue [3]. Finding functional differences in normal myometrium compared to uterine leiomyoma tissue at the gene or protein level may reveal the etiology of aberrant leiomyomatous growth. Several studies reported that uterine leiomyomas respond to treatment with ovarian steroid hormones [3–5]. Estrogen stimulates the growth of uterine leiomyomas, and it is rare for post-menopausal women, whose estrogen levels have declined, to exhibit sustained growth [3]. While estrogen is considered the major mitogenic factor, progesterone (P4) also plays a key role in uterine leiomyoma pathogenesis [5]. The physiologic actions of these ovarian steroids are mediated by the estrogen receptor and progesterone receptor (PR), and expression of both receptors is elevated in uterine leiomyomas compared to normal myometrium [4, 6]. Glucocorticoids, another steroid hormone, have been found to have the opposite effect on uterine leiomyomas, antagonizing the effects of estrogen [3].

FK506-binding protein 51 (FKBP51) is an immunophilin and a common transcription co-regulator of PR, glucocorticoid receptor (GR), and androgen receptor (AR) [7]. These former two steroid receptors induce expression of FKBP5 gene, which contains P4 and glucocorticoid response elements. Because the binding of FKBP51 to both PR and GR inhibits their transcriptional activity [8], increased FKBP51 antagonizes P4 and glucocorticoid effects [7, 9]. In contrast, FKBP51-AR binding induces AR-mediated transcription [10]. Although both uterine myometrial and leiomyoma cells express GR, PR, and AR, steroid-induced proliferation in leiomyoma cells is different than that in adjacent myometrial cells indicating aberrant steroid signaling [3]. While GR signaling represses [3], AR signaling [10] induces proliferation in leiomyoma. Thus, it is unclear what the net effect of FKBP51 expression would be in leiomyoma cells.

Decidualization increases FKBP51 expression in endometrial stromal cells, suggesting a role in fertility [11]. While FKBP51 levels and function have not previously been studied in normal myometrium vs. uterine leiomyomas, both FKBP51 and uterine leiomyoma are regulated or controlled by sex steroids. Thus, we sought to investigate a potential relationship between FKBP51 expression and the pathogenesis of uterine leiomyomas and determine how this regulator of steroid receptors functionally impacts leiomyoma proliferation and extracellular matrix (ECM) production. We hypothesized that there is increased expression of FKBP51 in leiomyoma vs. myometrial tissues, causing dysregulation of steroid receptor signaling, thereby contributing to leiomyoma pathogenesis.

Materials and methods

Collection of Human Specimens

The Institutional Review Board at University of South Florida granted permission to collect tissue specimens and approved all consent forms and protocols (Pro00021684). Paired uterine leiomyoma and adjacent normal myometrial tissues were obtained from women (mean age 36.6 years, range 21–51 years) undergoing hysterectomy or myomectomy for benign indications who were not receiving hormonal medication at the time of surgery. Further details on history of medication use are provided in Supplemental Table 1. The normal myometrial tissues were taken > 1 cm from adjacent leiomyoma tissue. As determined by last menstrual period, only mid-proliferative (cycle day 10–14; n = 11) or mid-secretory (cycle day 22–26; n = 9) samples were chosen to minimize the impact of hormonal variations during the menstrual cycle on results.

Uterine Leiomyoma Cell Culture

Leiomyoma tissue samples were collected within one hour of surgery and placed in Dulbecco Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA). Tissue samples were minced into 1-mm fragments [4, 12] and digested in DMEM containing collagenase type II (5 mg/ml; Gibco) and deoxyribonuclease I DNase I (0.2 mg/ml; Roche Diagnostics, Indianapolis, IN) overnight at 37°C with gentle agitation. The digested tissue samples were separated using a 70 µm strainer [13] and subsequently cultured in 100-mm culture dishes in four different growth conditions to determine the optimal environment for leiomyoma cells using: 1) basal media (DMEM/F12 with 5% bovine calf serum (BCS) and 1% antibiotic and antimycotics (ABAM)); 2) StemECHO mesenchymal stromal cell culture system media (Houston, TX, USA); and 3) basal or StemECHO media with 0.1% Matrigel (1 mL/10 mL) coated plates in DMEM (Corning, Tewksbury, MA), at 37°C in 5% CO₂. Confluent cells collected for RNA isolation to evaluate expression of estrogen receptor (ESR1), progesterone receptor (PGR), glucocorticoid receptor (NR3C1), smooth muscle actin (ACTA2), and vimentin (VIM) were confirmed (data not shown). We confirmed that the combination of StemECHO media on a Matrigel-coated tissue culture plate resulted in optimal growth of the uterine leiomyoma cells. Moreover, we verified the integrity of our cell line using markers for smooth muscle as contamination is common, and if not truly uterine leiomyoma cells, then results may not be generalizable.
to patients [14]. After first passage of leiomyoma cells, we performed mRNA analysis of ACTA2, CNN1, OXTR, GJA1, and VIM to confirm that leiomyoma cell cultures were smooth muscle like cells and the qPCR analysis as well as immunostaining results from leiomyoma tissue sections and cell cultures is available in Supplemental Fig. 1.

Immunohistochemistry

Double immunostaining was performed to detect FKBP51 and alpha-smooth muscle actin on 4% paraformaldehyde fixed and paraffin-embedded mid-proliferative (n = 6) and mid-secretory (n = 6) phase tissue sections from paired uterine leiomyoma and normal myometrial tissues. In brief, 5-µm serial sections were deparaffinized in xylene and then rehydrated in a descending alcohol series. Slides were treated with 3% hydrogen peroxidase to block endogenous peroxidase activity for 20 min and then followed by antigen retrieval by boiling in citrate buffer solution (10 mmol/L; pH 6.0) for 30 min. After rinsing in Tris-buffered saline (TBS), slides were incubated with 10% normal horse serum (Vector Labs, Burlingame, CA) in a humidified chamber for 30 min at room temperature (RT) and then incubated at 4°C overnight with goat polyclonal antibody against FKBP51 (1:3000, R&D Minneapolis, MN). After rinsing in TBS with 1% Tween 20 (TBS-T), the slides were incubated with biotinylated horse anti-mouse secondary IgG antibody (1:400; Vector Labs) for 30 min at RT. The antigen–antibody complex was visualized by incubating with avidin–biotin–peroxidase complex (Elite ABC kit; Vector Labs) for 30 min and then followed by chromogen 3,3-diaminobenzidine (DAB; Vector Labs) for 30 min at RT to generate a brown stain for anti-FKBP51 antibody.

Double-immunostained slides were incubated with a rabbit polyclonal, anti-alpha smooth muscle actin antibody (1:400; Abcam, Cambridge, MA) overnight at 4°C as a marker of smooth muscle cells. After washing, the slides were incubated in the secondary antibody horse anti-mouse IgG antibody biotinylated (1:400, Vector Labs) in a humidified chamber for 30 min at RT and then with an avidin–biotin kit with phosphatase-based detection (VECTASTAIN ABC AP Kit, Vector Labs) for 30 min. Immunostaining was developed using Vector Red (Vector Labs) to detect alpha-smooth muscle actin as a red stain. For negative controls, an equivalent concentration of nonspecific IgG matched with primary antibody was used. The slides were then covered by an aqueous-based mounting medium. The intensity of immunostaining for FKBP51 was scored by a semiquantitative histologic score (HSCORE) value as previously described [15]. Each slide was evaluated by two investigators blinded to the type of tissue, and an average score of both investigators was used as a final HSCORE for each sample.

Immunoblot Analysis

Immunoblot analysis [4] was performed on protein extracts from mid-proliferative (n = 11) and mid-secretory (n = 9) tissue samples derived from paired myometrium and leiomyoma tissues using RIPA lysis buffer containing protease inhibitor cocktail (Thermo Fisher Scientific). After protein concentration was determined, total (10 μg) protein samples were loaded on 10% Tris–HCl ready gel (Bio-Rad, Hercules, CA), electrophoretically separated and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA). The membrane was blocked with 5% milk in TBS-T for 1 h at RT to reduce nonspecific binding. Samples along with tissue protein lysates, 10−7 M dexamethasone (Sigma-Aldrich, St. Louis, MO)-treated primary cultured decidual cell lysates, were used as a positive control while non-treated decidual cell lysates were accepted as a negative control. The membranes were then incubated overnight at 4°C with a goat polyclonal antibody for FKBP51 (R&D AF4094) in TBS-T containing 5% BSA, followed by incubation with horseradish peroxidase conjugated rabbit anti-goat (VECTOR PI-9500). The proteins were visualized by the LI-COR Odyssey imaging system using chemiluminescence substrate (Thermo Fisher Scientific). Band intensities were quantified using computer densitometry analysis (ImageJ software version 1.52 k; NIH, Bethesda, MD). Immunoblotting results were normalized by stripping and re-probing membranes with β-actin monoclonal antibody (Cell Signaling, Danvers, MA).

RNA Isolation and Real Time Quantitative PCR (qPCR)

Total RNAs from paired myometrial and leiomyoma tissues from mid-proliferative (n = 8) and mid-secretory (n = 6) phases as well as uterine leiomyoma cell cultures were isolated using the RNeasy Mini Kit (Qiagen Inc, Germantown, MD). Reverse transcription was performed using Retroscript kit (Invitrogen) and followed by qPCR using gene-specific TaqMan gene expression assays for FKBP5, PCNA, KI67, CCND1, BCL2, BAX, BAD, COL1A1, COL4A5, COL7A1, LAMC1, COL10A1, TIMP1, TIMP3, MMP1, and FNI (Applied Biosystem, Foster City, CA; Supplemental Table 2) as previously described [16]. All samples were run in duplicate, and expression of the target genes was normalized to β-actin and/or GADPH mRNA levels as an endogenous control and the 2−ΔΔCT was used to calculate relative fold change.

FKBP5 Knockdown by siRNA Transfection

Confluent leiomyoma cell cultures (n = 3) were trypsinized and plated into a 6-well plate (5 × 104 cells/well). After
24 h, cells were transfected with either 20 nM FKBP5 specific siRNA (Santa Cruz Biotechnology, Inc., Dallas, TX) or a nonspecific control (scramble) siRNA (Invitrogen) by using Lipofectamine RNAiMax transfection reagent (Invitrogen) as previously described [17]. After 72 h, transfection efficiency was performed by qPCR using Taq-Man gene expression assay for FKBP5 and immunoblotting using anti-FKBP5 antibody to confirm FKBP5 suppression.

Experimental Treatments

Uterine leiomyoma cells were treated with vehicle (control) or 10⁻⁸ M E₂ (Sigma-Aldrich), medroxyprogesterone acetate (MPA) 10⁻⁷ M (Sigma-Aldrich), or a combination of 10⁻⁸ M E₂ and MPA 10⁻⁷ M prior to BrdU proliferation or Cell Death detection assay. Concentrations of agents were based on previous studies [17, 18].

Cell Proliferation by BrdU Incorporation

Confluent leiomyoma cells (n = 3) were passaged into 96-well plates (1.5 × 10⁵ cells/well) for 5-bromo-2′-deoxyuridine (BrdU) Cell Proliferation Assay (Cell Signaling Technology). After 24 h, cells were transfected with FKBP5 or control siRNA and treated with corresponding steroids at 36 h. BrdU solution was added into cells and incubated for 18 h; colorimetric absorbance was then measured at 450 nm at 72 h of siRNA transfection. The absorbance results were normalized by subtracting the blank measurement. The multiple readings for each patient were averaged. Relative colorimetric activity was calculated as a fold change of the control.

Apoptosis Assay

Apoptosis index was determined in parallel experiments using Cell Death Detection ELISA Plus Kit (Sigma-Aldrich). Briefly, cultured leiomyoma cells (1.5 × 10⁵ cells/well) were transfected with either FKBP5 or control siRNAs and treated with steroids. Colorimetric absorbance was measured at 405 nm 48 h after steroid treatment which was 72 h after transfection.

Statistical Analysis

Statistical analysis was performed using SIGMAPLOT version 11.0 (Systat Software, Inc, San Jose, CA). Student t-test was used to compare parametric data between paired myometrial and leiomyoma samples as well as BrdU and apoptotic assay results. For nonparametric data, Mann–Whitney–Wilcoxon Rank Sum Test was performed. Statistical significance was defined a priori as P < 0.05, and data are presented as means ± standard error of the mean.

Results

In Situ Regulation of FKBP51 Immunoreactivity in Uterine Leiomyoma Samples

Use of FKBP51 and alpha smooth muscle actin double-immunostaining in sections of uterine leiomyoma and their adjacent normal myometrial tissues obtained from mid-proliferative (n = 6) and mid-secretory (n = 6) phases confirmed expression of FKBP51 by both leiomyoma and normal smooth muscle cells (Fig. 1A). Immunoreactivity of FKBP51 was exclusively localized in nuclei, whereas alpha smooth muscle actin was in cytoplasm in cells from both leiomyoma and myometrium. HSCORE analysis revealed significantly greater FKBP51 staining intensity in leiomyoma compared to normal myometrial tissue (Mean ± SEM: 140.30 ± 7.6 vs. 69.32 ± 12.8; P < 0.001; Fig. 1A). FKBP51 immunoreactivity was significantly higher in both the proliferative (137.99 ± 15.1 vs. 76.29 ± 16.8; P < 0.05) and secretory (145.18 ± 7.5 vs. 68.08 ± 2; P < 0.05) phases in leiomyoma vs. myometrial tissue. However, FKBP51 immunoreactivity did not differ between proliferative vs. secretory phases in leiomyoma alone or myometrial tissue alone. Specificity of immunostaining was confirmed by incubating control slides in matched normal IgGs instead of FKBP51 primary antibody, which revealed no detectable immunoreactivity (Fig. 1A inset).

FKBP51 Expression Is Upregulated in Uterine Leiomyoma Samples

To further confirm that FKBP51 protein levels reflect parallel changes in mRNA expression, qPCR analysis was performed in paired myometrial vs. leiomyoma tissues from mid-proliferative (n = 8) and mid-secretory (n = 6)
phases. A significantly higher FKBP5 mRNA level was found in leiomyoma compared to paired myometrial tissues (2.21 ± 0.38 vs. 1.38 ± 0.28; P < 0.001; Fig. 1C).

Cycle-dependent analysis revealed that FKBP5 mRNA levels in both proliferative and secretory phases of leiomyomas were higher than that in paired myometrial tissues (PRO 2.10 ± 0.48 vs 1.42 ± 0.40, P = 0.013; and SEC 2.39 ± 0.67 vs 1.29 ± 0.78, P = 0.034, respectively).

MPA, a Mixed Progestin/Glucocorticoid Induces FKBP5 levels in Uterine Leiomyoma Cells

Previous studies showed that FKBP51 is strongly upregulated by glucocorticoid and P4 [7, 9]. Therefore, we first investigated the direct effect of steroids in regulating FKBP5 expression in cultured uterine leiomyoma cells treated with vehicle (control), 10⁻⁸ M E₂, or MPA 10⁻⁷ M for 6 h by using qPCR. Compared to control group, incubation with MPA significantly enhanced FKBP5 mRNA levels (1.00 ± 0.01 vs 6.01 ± 0.79, P = 0.005), while E₂ did not change (1.00 ± 0.01 vs 1.13 ± 0.13, P > 0.05) (Supplemental Fig. 2).

FKBP5 Modulates Genes Involved in Proliferative and Apoptotic Pathways in Uterine Leiomyoma Cells

To identify whether FKBP5 promoted the proliferation and survival of leiomyoma cells through the inhibition of apoptotic pathways, we first silenced FKBP5 expression in uterine leiomyoma cells (n = 3) by transfection with FKBP5 siRNA at a concentration of 20 nM for 72 h. Analysis by using qPCR revealed a 13-fold reduction in FKBP5 mRNA levels following transfection with FKBP5 siRNA compared to control siRNA in transfected cells (0.08 ± 0.001 vs. 1.04 ± 0.14; P < 0.001; Fig. 2A). These findings were also confirmed with immunoblotting analysis indicating decreased FKBP51 protein levels at 72 h in FKBP5 siRNA-transfected cells vs. control (scramble) siRNA-transfected cells (Fig. 2B).
Subsequently, we investigated whether FKBP5 silencing affects proliferative and/or apoptotic markers. Results from qPCR analysis revealed that FKBP5 silencing promoted a significant reduction in gene expression levels of cell proliferative/survival factors including PCNA, KI67, CCND1, BCL2, BAX, and BAD mRNAs (C) and PCNA protein (D) levels in leiomyoma cell cultures at 72 h of transfection with control (scramble) or FKBP5 siRNA. Bars represent Mean ± SEM. *P < 0.001 vs. corresponding control siRNA; †P < 0.05 vs. corresponding control siRNA.

FKBP51 is Responsible for Leiomyoma Cell Proliferation but Not Apoptosis

To determine the direct effects of FKBP51 on cell survival of uterine leiomyoma cells, we evaluated cell proliferation and apoptosis by BrdU incorporation and ELISA-based apoptosis assays, respectively, at 72 h in FKBP5 siRNA or control siRNA-transfected cells treated with non-hormonal control, E2, or E2 + MPA. Under hormone-free conditions, proliferation rate was significantly decreased in cultures where expression of FKBP5 was knocked down compared to control siRNA (0.79 ± 0.03 vs. 1 ± 0; P ≤ 0.005) (Fig. 3A). Surprisingly, in control siRNA-transfected cells, E2 resulted in a statistically significant decrease in BrdU incorporation versus non-hormone-treated control siRNA group (E2 treated 0.78 ± 0.03 vs. control 1 ± 0; P ≤ 0.001); however, E2-mediated reduction was not observed in the FKBP5 siRNA-transfected cells (E2-treated 1.18 ± 0.75 vs. control 0.79 ± 0.03; P = 0.481). In the FKBP5 siRNA-transfected cells, E2 + MPA treatment resulted in a significant decrease in BrdU incorporation compared to non-hormonal-treated FKBP5 siRNA-transfected cells (0.63 ± 0.07 vs. 0.79 ± 0.06; P = 0.047). The difference in BrdU incorporation level between the E2-treated control siRNA cultures versus E2-treated FKBP51 siRNA cultures was not statistically significant (P < 0.41). Similar to control siRNA-treated cultures versus FKBP5 siRNA-treated cultures, there was a decreased BrdU incorporation in E2 + MPA-treated FKBP51 siRNA cultures versus E2 + MPA-treated control siRNA cultures, but the difference did not attain statistical significance (P = 0.16).

Epidermal growth factor (EGF) is a well-known inducer of proliferation and is found in both myometrial and leiomyoma cells [19]. Conversely, an EGF receptor blocker reduces cellular growth [19]. To better understand the role of FKBP5 on EGF-mediated cell proliferation, E + MPA-treated cells after transfection with either control or FKBP5 siRNAs were treated with 10 ng/ml
recombinant EGF. EGF significantly induced cell proliferation in both control (6.1-fold) and FKBP5 (6.3-fold) siRNA-transfected cells; however, no significant difference was observed between FKBP5 and control siRNA-transfected groups (Fig. 3B). In parallel experiments, similar levels of apoptotic index were found between the control siRNA and FKBP5 siRNA with or without steroid treatment (P = 0.536; Fig. 4).

**FKBP5 Silencing in Uterine Leiomyoma Cell Cultures Inhibits ECM Production.**

To better understand the role of increased FKBP5 levels in uterine leiomyoma pathophysiology, we also evaluated the mRNA levels of genes involved in ECM synthesis and deposition in control or FKBP5 siRNA-transfected cells since excessive ECM deposition plays a major role in uterine leiomyoma growth and pathogenesis (19, 20). Following 72-h
treatment with FKBP5 siRNA vs. control siRNA transfection of leiomyoma cell cultures. qPCR measured mRNA levels of several ECM proteins involved in leiomyoma pathogenesis, including collagen type1 A1 (COL1A1), COL4A5, COL7A1, COL10A1, fibronectin (FN1), and laminin c1 (LAMC1) as well as tissue inhibitors of metalloproteinases (TIMP1 and TIMP3) and matrix metalloproteinase 1 (MMP1) mRNA levels. These specific collagen genes were selected after literature review of which ECM proteins are found in uterine leiomyoma [19–21]. These analyses revealed a 1.68-fold reduction in COL4A5, 1.76-fold reduction in COL7A1, 1.54-fold reduction in FN1, and 1.49-fold reduction in LAMC1 levels in FKBP5 knockdown vs. control siRNA-transfected leiomyoma cells (P < 0.05; Fig. 5A). FKBP5 knockdown also resulted in a significant decrease in TIMP1 and TIMP3 mRNA levels (TIMP1 in FKBP5 siRNA vs. control siRNA: 1.25 ± 0.30 vs. 2.34 ± 0.56, P < 0.01; TIMP3 in FKBP5 siRNA vs. control siRNA: 1.1 ± 0.18 vs. 1.69 ± 0.20; P < 0.01), and a significant increase in MMP1 mRNA levels (FKBP5 siRNA vs. control siRNA: 1.71 ± 0.47 vs. 1.29 ± 0.43; P < 0.05; Fig. 5B). When immunoblotting was performed for LAMC1 and TIMP1 (Fig. 5C), results also showed a decrease in protein level in FKBP5 knockdown vs. control siRNA.

**Discussion**

As demonstrated in situ using mRNA, protein, and histological analyses, there is increased FKBP51 expression in uterine leiomyoma cells compared to normal myometrial cells. This increase in FKBP51 expression in uterine leiomyoma appears to dysregulate steroid receptor signaling. Mehine et al. [14] found that uterine leiomyomas can be classified into four subtypes according to mutation in MED12, HMGA2, FH or COL4A5/6 genes, and that FKBP5 levels increase commonly in leiomyoma harboring these genetic alterations. Concerning the PR, FKBP51-PR binding inhibits transcriptional activity [3, 8]; therefore, increased FKBP51 antagonizes effect of P4. While estrogen stimulates the growth of uterine leiomyoma [3], growth is also affected by P4, but in a less straightforward manner. Results of P4 on leiomyoma growth vary among animal models and in vitro studies; guinea pig leiomyoma growth is inhibited by P4, but in contrasting mouse cell culture experiments, P4 causes increased growth and proliferation [5]. Stimulatory effects of P4 are supported by findings that PCNA expression is greatest in the secretory phase of the menstrual cycle, a hormonal milieu dominated by P4 [5].

FKBP5 mRNA and protein levels are increased in proliferative and secretory phase leiomyoma vs. myometrial specimens, whereas E2 treatment unaltered FKBP5 levels in leiomyoma cultures. While the proliferative phase is an estrogen-dominant environment, as we previously reported for decidual and endometrial stromal cells [7, 9], the glucocorticoid effect is likely responsible for the increased FKBP51 level in leiomyoma cells during proliferative phase, which corresponds to the significant increase in FKBP5 mRNA expression by the treatment with MPA that displays mixed progesterin/glucocorticoid effects (Supplemental Fig. 2).

In clinical practice, many women with uterine leiomyoma are treated with combined oral contraceptive pills containing estradiol and progestin which control symptoms of abnormal uterine bleeding [2]. However, some studies show that leiomyoma may increase in size on P4 treatment perhaps due to an increase in ECM components [2, 21]. In contrast, P4 antagonists, such as mifepristone, have been shown to decrease the size of uterine leiomyoma [2, 21]. Of note, not all leiomyomas respond to P4 antagonists; therefore, different genetic background of uterine leiomyoma, such as FKBP51 level, might explain why these medications are more effective in some patients compared to others [4].

In cell culture, after silencing FKBP5 in uterine leiomyoma, several cell survival factors such as PCNA, MKI67, and CCND1 were decreased (Fig. 2C). PCNA, MKI67, and CCND1 are markers of cell proliferation and survival; therefore, lower levels suggest that there is less DNA replication and cell proliferation when FKBP5 is silenced. These findings are consistent with BrdU proliferation assay results that show decreased proliferation when the FKBP5 gene is silenced and even further reduction linked to treatment with E2 + MPA. When FKBP5 is silenced, BCL2 mRNA, which promotes cell survival as an anti-apoptotic agent, was also decreased. This decrease in BCL2 as well as decrease in the previously mentioned markers of cell proliferation suggests...
Fig. 5 FKBP5 silencing decreased mRNA and protein levels of ECM proteins and TIMPs in leiomyoma cells. (A) Expression levels of collagen type I alpha 1 chain (COL1A1), collagen type IV alpha 5 chain (COL4A5), collagen type VII alpha 1 chain (COL7A1), collagen type X alpha 1 chain (COL10A1), fibronectin 1 (FN1); Laminin Subunit Gamma 1 (LAMC1) mRNAs in leiomyoma cells after treatment with control siRNA or FKBP5 siRNA for 72 h. (B) Tissue inhibitor of metalloproteinases TIMP1 and TIMP3 and matrix metalloproteinase 1 (MMP1) levels in control or FKBP5 siRNA-treated leiomyoma cell cultures for 72 h. (C) Protein levels for LAMC1 and TIMP1 detected by immunoblotting in control or FKBP5 siRNA-treated leiomyoma cell cultures for 72 h were also compared. Bars represent Mean ± SEM; n = 4. *P < 0.010 vs. corresponding control. **P < 0.050 vs. corresponding control siRNA.
that silencing FKBP5 would reduce uterine leiomyoma growth and proliferation.

In contrast, the apoptotic index was not significantly different between the control group and the FKBP5 siRNA group. One potential explanation is that besides reducing anti-apoptotic (BCL2) gene level, silencing FKBP5 also induces a significant decrease in the pro-apoptotic genes, BAX and BAD. These anti- and pro-apoptotic signals balance apoptotic cell death in leiomyoma cell following silencing FKBP5. Overall, there is a moderate inhibitory effect on cell proliferation following FKBP5 silencing. Thus, increased FKBP51 promotes leiomyoma formation.

It is likely that a complex interaction exists between both estrogen and P4 to cause leiomyoma proliferation [5]. Estrogen itself causes an increase in the PR, and therefore might amplify the effects of P4 on leiomyoma [5]. Glucocorticoids, by comparison, have been found to antagonize the effects of estrogen and inhibit the growth of uterine leiomyoma [3]. Because FKBP51-GR binding inhibits GR transcriptional activity [3], increased leiomyoma FKBP51 expression likely antagonizes these glucocorticoid effects [7], increasing uterine leiomyoma growth and proliferation. This hypothesis is supported by our cell culture results showing that once FKBP51 is reduced in uterine leiomyoma, proliferation also decreased in the absence of exogenous steroid treatment.

Another important hormonal relationship to consider is FKBP51-AR binding which induces AR-mediated transcription [10]. Since AR signaling induces proliferation in leiomyoma [10], increased FKBP51 in uterine leiomyoma likely increases AR signaling to promote the growth of uterine leiomyoma compared to normal myometrium. Thus, collectively, increased in FKBP51 expression in uterine leiomyomas dysregulates steroid signaling by blocking GR action and stimulating AR action to promote proliferation of leiomyoma cells. The impact on PR is less clear, however, as FKBP51 also blocks PR action, involving a more complex relationship with the ER.

Increased ECM protein is a histopathologic hallmark of uterine leiomyomas and plays a particularly important role in the growth of these tumors to large sizes [20]. Our analysis for ECM genes revealed that FKBP5 knockdown is associated with downregulation of several ECM genes. These results indicate that FKBP51 expression enhances over-production of collagen, fibronectin, and laminin in uterine leiomyoma cells. This suggests that increased FKBP51 in leiomyoma promotes cell differentiation toward a secretory type of smooth muscle cells, resulting in increased ECM deposition in leiomyoma tissues. Our results showing significant reduction in mRNA levels of ECM proteins in leiomyoma cells following silencing of FKBP5 indicate that increased FKBP5 levels enhance ECM protein production in leiomyoma cells. Similarly, the decreased levels of TIMP1 and TIMP3 in FKBP5 knockdown leiomyoma cells suggest that higher FKBP51 levels prevent ECM protein degradation by inhibiting activity of metalloproteinases via TIMPs in leiomyoma tissues. In addition, increased MMP1 mRNA levels in FKBP5 knockdown leiomyoma cells support that FKBP51 also reduces ECM degradation by inhibiting expression levels of MMP1.

Proteomics research has shown that there are other proteins, such as secreted Frizzled-related protein 4 (sFRP4), that are over-expressed in uterine leiomyoma and thought to promote P4 mediated leiomyoma growth [4]. One study on leiomyoma growth found that P4 increases production of ECM proteins, and along this same pathway, using a P4 antagonist, mifepristone, resulted in reduced ECM protein levels [21]. Since FKBP51 also functions as a P4 antagonist like mifepristone, this suggests that it is FKBP51’s interaction with GR and AR promotes the pathogenesis of leiomyomas by promoting proliferation and ECM deposition.

**Conclusions**

Although PR, GR, and AR are each expressed in both uterine myometrial and leiomyoma cells, steroid-induced proliferative response of leiomyoma is different than that in adjacent myometrial cells, indicating aberrant steroid signaling in uterine leiomyoma [3]. Our results suggest that enhanced FKBP51 may contribute to this aberrant steroid signaling in uterine leiomyoma cells. Moreover, these results indicate that increased FKBP51 expression induces cell proliferation and over-production of collagen, fibronectin, and laminin in uterine leiomyoma cells promoting a secretory type of smooth muscle like cells leading to increased ECM deposition.

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**Availability of Data and Material** Data will be made available with any reasonable requests.

**Code Availability** Not applicable.

**Declarations**

**Ethics Approval** The Institutional Review Board at University of South Florida granted permission to collect tissue specimens and approved all consent forms and protocols (Pro00021664).
These findings were accepted for presentation at the Society for Reproductive Investigation’s 67th Annual Scientific Meeting on March 10–14, 2020, in Vancouver, British Columbia; however, this conference was cancelled due to the COVID-19 pandemic and the abstract was withdrawn.

The authors report no conflicts of interest.

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