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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 inpaired 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 ($P \leq 0.001$) 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

Capsule: Overexpression of FKBP51 in uterine leiomyoma promotes cellular proliferation and extracellular matrix deposition, thereby contributing to the pathogenesis of these tumors

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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 37^0C 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 37^0C in 5% CO_2. 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 4^0C 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-goat secondary IgG (1:500; 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 4^0C 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 4^0C 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 ß-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 *FN1* (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 $2^{-\Delta\Delta CT}$ was used to calculate relative fold change.

FKBP5 Knockdown by siRNA Transfection

Confluent leiomyoma cell cultures (n = 3) were trypsinized and platted into a 6-well plate (5×10^4 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-FKBP51 antibody to confirm FKBP51 suppression.

Experimental Treatments

Uterine leiomyoma cells were treated with vehicle (control) or 10^{-8} M E_2 (Sigma-Aldrich), medroxyprogesterone acetate (MPA) 10^{-7} M (Sigma-Aldrich), or a combination of 10^{-8} M E_2 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 (1.5×10^3 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 (1.5×10^3 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 confirm the immunohistochemistry results (Fig. 1A), FKBP51 protein levels were further evaluated in proliferative (n = 11) and secretory (n = 9) phases of tissues from leiomyoma and paired myometrium. Immunoblotting results revealed significantly higher FKBP51 protein levels in leiomyoma tissues *vs.* their paired myometrial controls (1.44 ± 0.1 *vs.* 0.82 ± 0.1; $P < 0.001$) (Fig. 1B). Similar to immunohistochemistry results, cycle-dependent analysis revealed that FKBP51 expression is significantly higher in leiomyoma tissue *vs* paired myometrial controls in both proliferative ($P < 0.001$) and secretory phase samples ($P < 0.05$). In leiomyoma tissues, FKBP51 protein levels were elevated in the secretory phase compared to the proliferative phase ($P < 0.05$); however, no phase-related changes were observed in myometrial tissues ($P = 0.240$).

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)

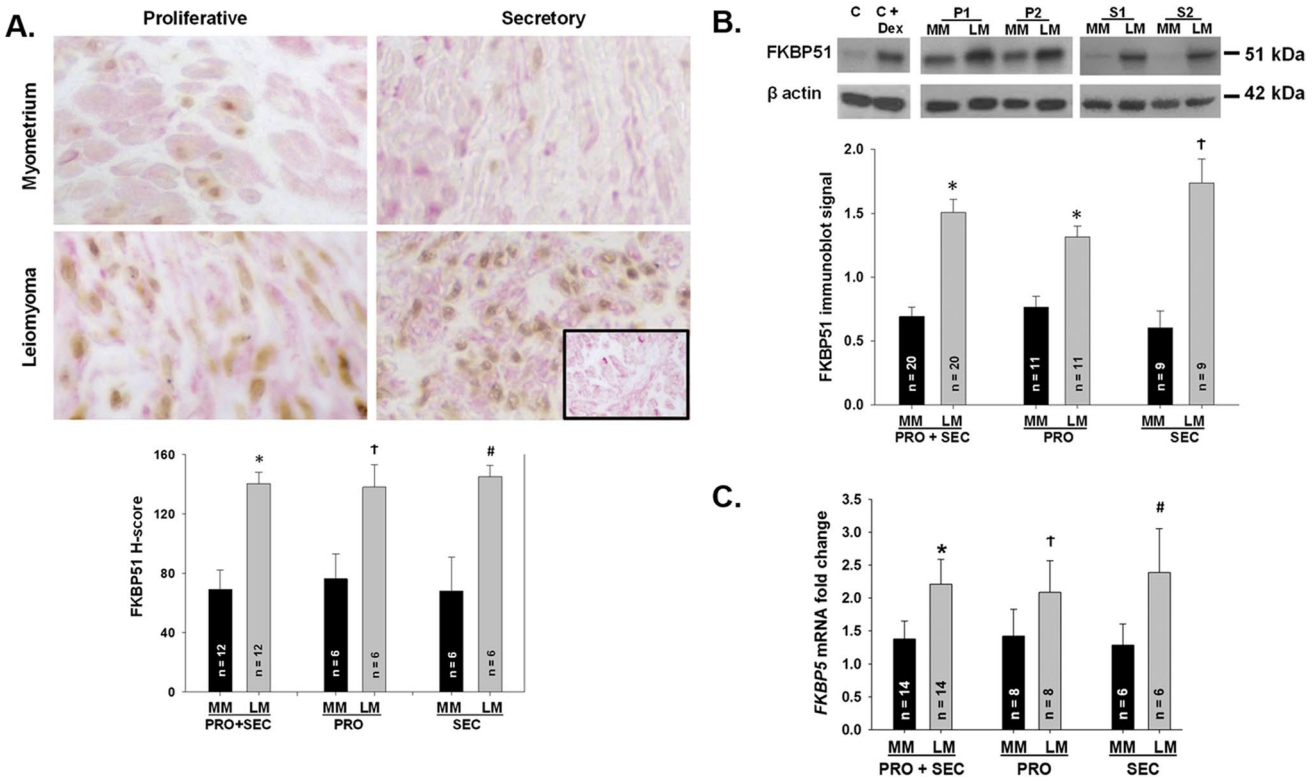

Fig. 1 Greater in situ FKBP51 protein and mRNA levels in leiomyoma *vs.* paired myometrial tissues. (**A**) FKBP51 (brown) and alpha-smooth muscle actin (red) double immunostaining in paired myometrium and adjacent leiomyoma sections. Menstrual cycle phases were proliferative phase (PRO, cycle day 10–14) or secretory phase (SEC, cycle day 22–26). MM: myometrium, LM: uterine leiomyoma. Bars represent Mean ± SEM. *P < 0.001 *vs.* MM; †P = 0.047 *vs.* MM; #P = 0.015 *vs.* MM. Inset image shows negative staining as a control. Original magnification 40x. (**B**) FKBP51 and β actin protein levels detected by immunoblotting in MM and LM tissues. Results were quantified using ImageJ and normalized to β actin. Control is decidual cell lysates without (c) and with dexamethasone treatment (c + Dex). P1, P2 are proliferative phase samples, and S1, S2 are secretory phase samples. Bars represent Mean ± SEM. *P < 0.001 *vs.* MM; †P = 0.003 *vs.* MM. (**C**) FKBP5 mRNA levels in LM compared to MM tissues detected by qPCR. Bars represent Mean ± SEM; *P < 0.001 *vs.* MM; †P = 0.013 *vs.* MM; #P = 0.034 *vs.* MM

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 E_2, 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 E_2 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 ± 0.04 *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).

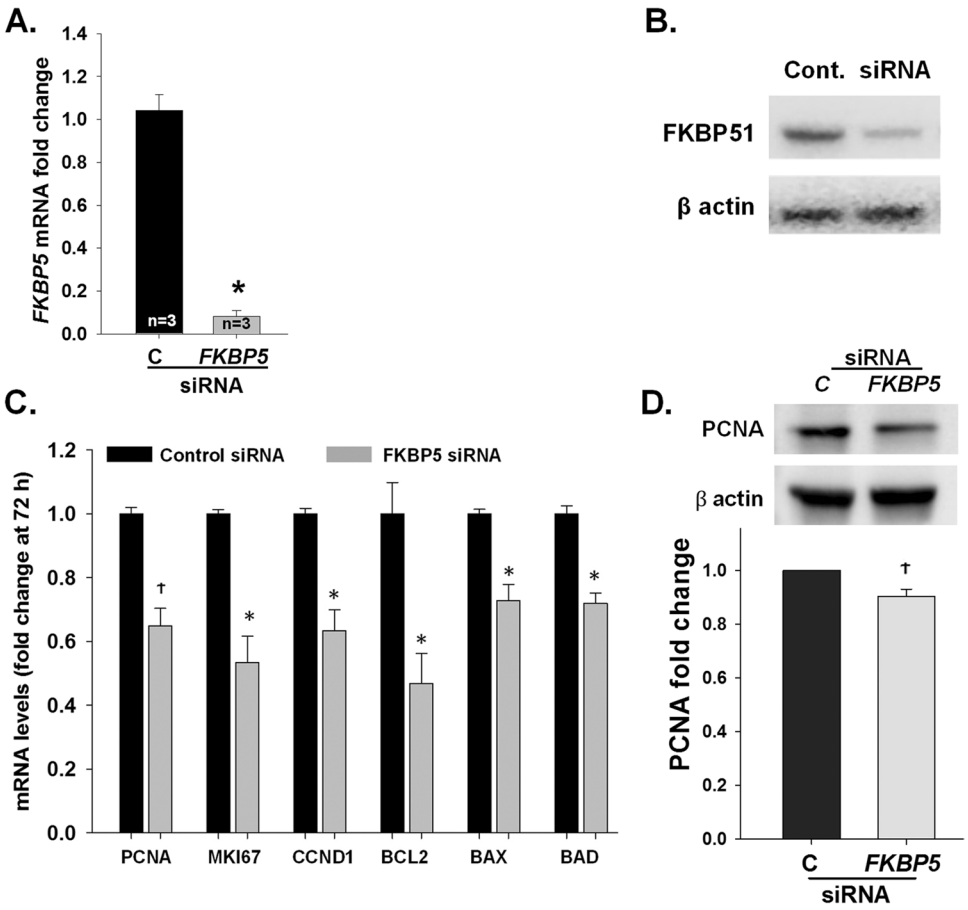

Fig. 2 Silencing *FKBP5* in cultured leiomyoma cells down-regulates cell survival factors. Analyses of FKBP51 mRNA (**A**) and protein (**B**) levels by qPCR and immunoblotting, receptively, in leiomyoma cell cultures at 72 h after transfection with *FKBP5* siRNA. Expression levels of *PCNA, MKI67, CCND1, BCL2, BAX* and *BAD* mRNA (**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 < 0.050 *vs.* corresponding control siRNA

Subsequently, we investigated whether *FKBP5* silencing affects proliferative and/or apoptotic markers. Results from qPCR analysis revelated that *FKBP5* silencing promoted a significant reduction in gene expression levels of cell proliferative/survival factors including *PCNA*, *KI67*, *CCND1* as well as anti-apoptotic *BCL2*, or apoptotic *BAX*, and *BAD* genes (Fig. 2C). This was consistent with a decrease in PCNA protein level when *FKBP5* was silenced (Fig. 2D).

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, E_2 or E_2 + 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; $P \leq 0.005$) (Fig. 3A). Surprisingly, in control siRNA-transfected cells, E_2 resulted in a statistically significant decrease in BrdU incorporation *versus* non-hormone-treated control siRNA group (E_2 treated 0.78 ± 0.03 *vs.* control 1 ± 0; $P \leq 0.001$); however, E_2-mediated reduction was not observed in the *FKBP5* siRNA-transfected cells (E_2-treated 1.18 ± 0.75 *vs.* control 0.79 ± 0.03; $P = 0.481$). In the *FKBP5* siRNA-transfected cells, E_2 + 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 E_2-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 E_2 + MPA-treated FKBP51 siRNA cultures versus E_2 + 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

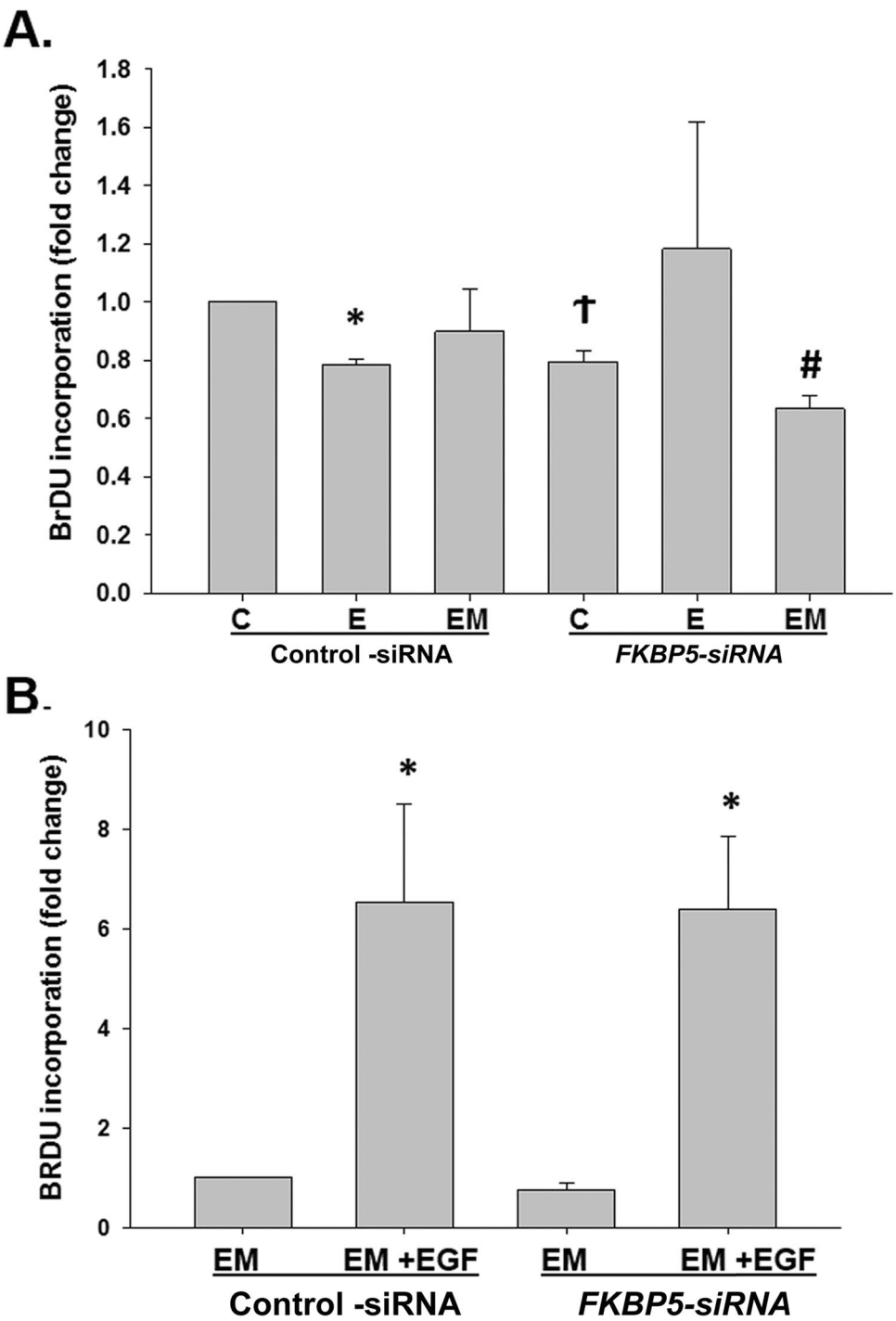

Fig. 3 Reduced BrdU incorporation in leiomyoma cell cultures after transfection with *FKBP5* siRNA. (**A**) Levels of BrdU incorporation in control (C), estradiol (E); estradiol + medroxyprogesterone (EM)-treated leiomyoma cell cultures at 72 h of transfection with control- (scramble) or *FKBP5* siRNA. Bars represent Mean ± SEM. *P < 0.001 vs. corresponding control siRNA; #P < 0.05 vs. control *FKBP5* siRNA; †P ≤ 0.005 vs. non-hormonal control siRNA; n = 3. (**B**) BrdU incorporation after treating cells with epidermal growth factor (EGF) both in leiomyoma cell cultures at 72 h of transfection with control or *FKBP5* siRNA. Bars represent Mean ± SEM. *P < 0.050 *vs.* EM control siRNA; n = 3

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

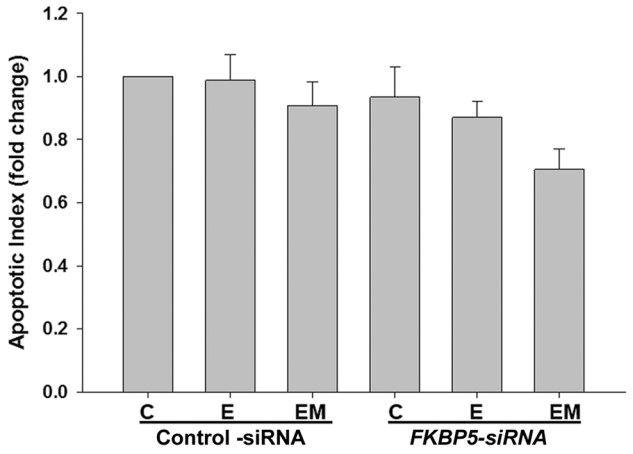

Fig. 4 Apoptotic index in leiomyoma cell cultures transfection with control or *FKBP5* siRNA. Levels of apoptosis in control (C) or estradiol (E) or estradiol + medroxyprogesterone (EM)-treated leiomyoma cell cultures following transfection with control or *FKBP5* siRNA for 72 h. Bars represent Mean ± SEM; n = 3

treatment with *FKBP5* siRNA *vs.* control siRNA transfection of leiomyoma cell cultures, qPCR measured mRNA levels of several ECM proteins involving 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 mice 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 E_2 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 progestin/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 FKPB51 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

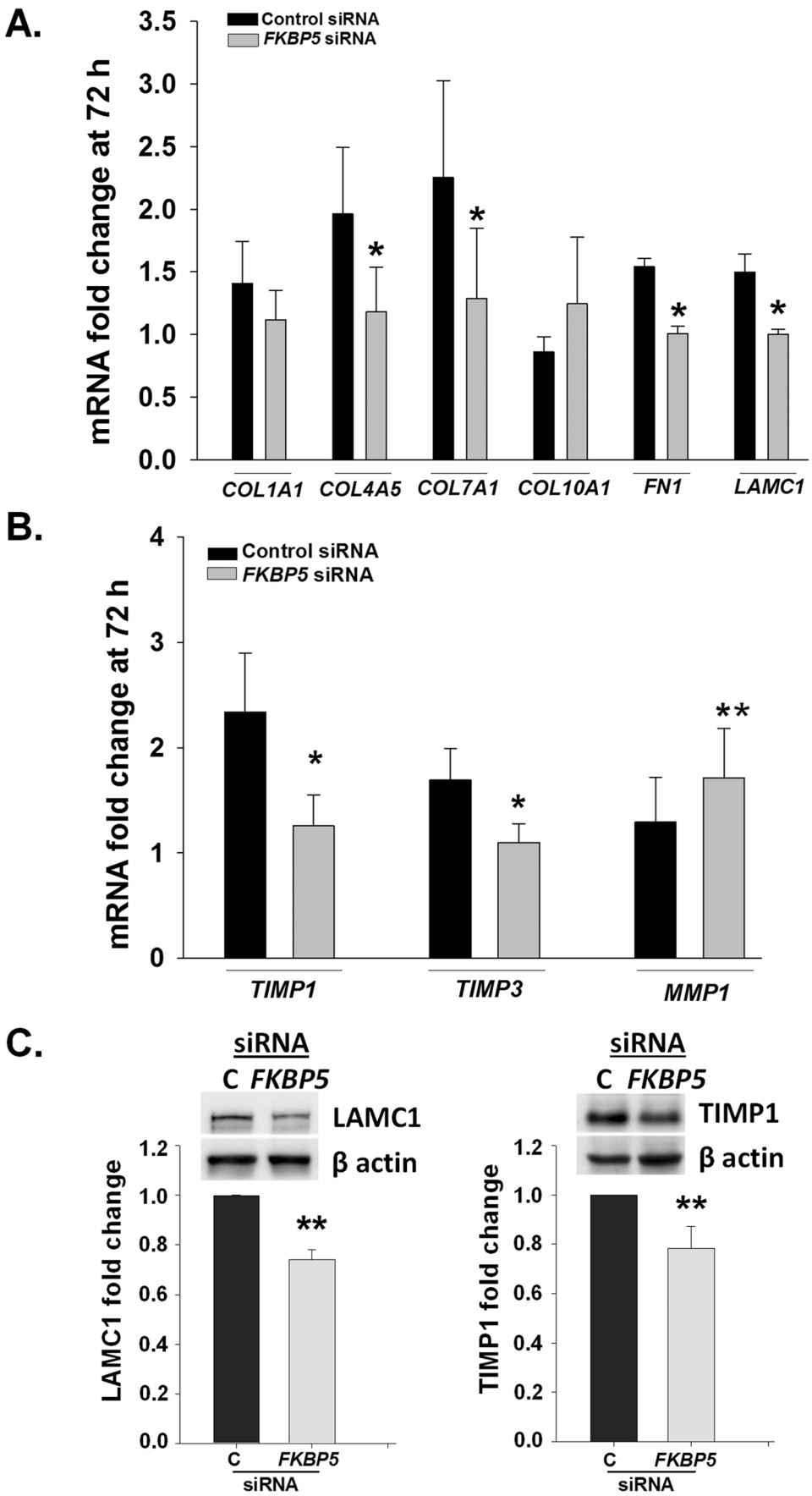

Fig. 5 *FKBP5* silencing decreased mRNA and protein levels of ECM proteins and TIMPs in leiomyoma cells. (**A**) Expression levels of collagen type 1 alpha 1 chain (*COL1A1*), collagen type IV alpha 5 chain (*COL4A5*), collagen type VII alpha 1 chain (*COL7A1*), collagen type X alpha 1 chain (*COL10A1*), 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. Protein levels for LAMC1 and TIMP1 (**C**) 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 FKPB5 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 FKPB5. Overall, there is a moderate inhibitory effect on cell proliferation following FKBP51 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 FKBP51 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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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 (Pro00021684).

Presentation 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.

Conflicts of Interest The authors report no conflicts of interest.

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