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ORIGINAL RESEARCH

Mifepristone inhibits IGF-1 signaling pathway in the treatment of uterine leiomyomas

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Purpose: To investigate the role of IGF-1 signaling pathway in the treatment of uterine leiomyomas with mifepristone.

Patients and methods: From October 2015 to December 2018, 50 patients with uterine leiomyoma were included in this study. Overexpression or siRNA of IGF-1 in primary human uterine leiomyoma cells were treated with or without mifepristone. MTT was used to evaluate cell viability in assays of cell proliferation and cytotoxicity. IGF-1 expression in the cells was measured with real-time RT-PCR and Western blotting and manipulated with lentivirus ectopic overexpression or siRNA silencing.

Results: Inhibition of cell viability by mifepristone was found dependent on drug concentration and treatment time. IGF-1 and phosphorylation-ERK1/2 expression were decreased, while phosphorylation-AKT expression was increased after mifepristone treatment. IGF-1 significantly promoted cell growth, while IGF-1 knockdown and mifepristone showed synergistic inhibition effects on cell growth. The overexpression of IGF-1 reversed the inhibition of cell growth and ERK1/2 phosphorylation but showed no effect on AKT phosphorylation.

Conclusion: Our study for the first time demonstrated that IGF-1 signaling via ERK1/2 appears to be an important target of mifepristone in the treatment of uterine leiomyomas, which may provide a new approach to avoid leiomyoma re-growth after cessation of mifepristone.

Keywords: uterine leiomyomas, mifepristone, IGF-1, signal pathway

Introduction

Uterine leiomyoma is the most common benign tumor in the female reproductive system, which can cause abnormal uterine bleeding, dysmenorrhea, infertility, menorrhagia and other complications.[1] Uterine leiomyoma is estrogen- and progesterone-dependent. Besides surgery, medical treatment is widely applied for symptomatic patients, which includes gonadotropin-releasing hormone agonists (GnRHa) and progesterone modulators.[2]

Mifepristone, an anti-progesterone drug, has been widely used for uterine leiomyomas.[3] Studies have demonstrated that treatment with mifepristone results in leiomyomas shrinkage and amelioration of symptoms.[4,5] However, Eisinger and co-workers reported that leiomyoma re-grew slowly after cessation of mifepristone, 5.7 months follow-up after cessation of the drug, with an increase of 20% of baseline volume.[6] Thus, mechanistic insights are needed for more effective utility of mifepristone for the treatment of uterine leiomyoma.

Insulin-like growth factor-1 (IGF-1) is a hormone, similar in structure to insulin, which regulates cell growth and metabolism and acts as a potent inhibitor of

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programmed cell death.[7] Its abnormal expression can induce carcinogenesis, such as ovarian cancer, cervical cancer, endometrial cancer and other cancers.[8,10] Its expression is also often upregulated in uterine leiomyomas.[11] In our previous clinic study, we found that mifepristone treatment led to the inhibition of various gene expression including IGF-1 expression in uterine leiomyomas, and the treatment efficacy of mifepristone was closely related to the level of IGF-1 expression (published in Chinese). It suggested that mifepristone might inhibit uterine leiomyomas through the regulation of IGF-1 expression. However, the role of IGF-1 singling pathway in mifepristone treatment for uterine leiomyomas is not fully understood.

In this study, we sought to directly evaluate whether IGF-1 and its downstream mediators are targeted of mifepristone, as a mechanism of mifepristone-induced cell growth inhibition, by altering the expression IGF-1 in uterine leiomyoma cells with or without mifepristone treatment. Our data experimental evidence indicating that IGF-1 and its downstream mediator ERK1/2 are effectors of mifepristone in uterine leiomyoma.

Materials and methods

Primary uterine leiomyoma cell culture

From October 2015 to January 2018, uterine leiomyoma tissues were obtained from 50 patients (the Han nationality) with age range 42±8.5 years, during the surgery of uterine myomectomy, subtotal or total hysterectomy. Tissues from surgery were identified by pathologist as uterine leiomyomas. The exclusion criteria were patients who used medicines or hormones within 3 months before surgery, or with other complications such as chronic diseases, infections, uterine malignancy and adenomyosis. This study followed the standards of the Declaration of Helsinki and was approved by the Research Ethical Committee of the Second Affiliated Hospital of Wenzhou Medical University (No. KYKT2015-55). Written informed consent was obtained from all patients. The collected tissues from the central localization of uterine leiomyoma were used for isolation of primary uterine leiomyoma cells, and part of cells was then immortalized by ectopic expression of human telomerase reverse transcriptase via lentivirus transduction. Cells were grown in vitro using the methods as previously reported.[12]

Immunocytochemistical verification of uterine leiomyoma cells

The expression of α-smooth muscle actin was used to identify uterine leiomyoma cells. Cells were fixed with 4% paraformaldehyde, washed with PBS and then permeabilized with 0.2% Triton X-100, 15 min. Cells were incubated in a serum-free blocking solution at room temperature for 15 min then incubated with mouse monoclonal anti-α-smooth muscle actin antibody (1:100 dilution; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at overnight. After washing with PBS, cells were incubated with biotinylated goat anti-mouse IgG as second antibody. 3,3′-diaminobenzidine was used to visualize the bound antibodies. Finally, nuclei were stained with hematoxylin. PBS was used as negative control instead of primary antibody.

Construction of recombinant human IGF-1 plasmid

Human IGF-1 (Genebank: NM_000618) gene was synthesized by RT-PCR from cDNA obtained from uterine leiomyoma cells, with the primers (5′-GCCGGAATTCATGGG AAAAATCAGCAGTC-3′; 3′-ATGCGGCCGCGGTCTTC CTACATCC-5′). The IGF-1 fragment and vector plasmid pLVX-IRES-ZsGreen1 (TaKaRa, Japan) were digested by the EcoR1 and Not1 restrictive enzymes and purified using agarose gel electrophoresis IGF-1 gene was cloned into pLVX-IRES-ZsGreen1. The positive recombinant clone, named pLVX-IRES-ZsGreen1-IGF-1, was identified by PCR and sequencing.

Virus preparation, purification, and titer determination

The pLVX-IRES-ZsGreen1-IGF-1 and the lentivirus helper plasmid pMD2.G and psPAX2 were co-transfected into 293T cells using lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. After 48 h post-transfection, virus-containing cells and medium were collected by lysis freezethawing in liquid nitrogen and then through 0.45μM filter. TCID50 standard method was used to detect the viral titer. Finally, the recombinant lentivirus was stored at − until use. The lentivirus was mixed with 8 μg/mL polybrene for infecting target cells. pLVX-IRES-ZsGreen1 lentivirus was used as a negative control.

Preparation of mifepristone and IGF-1 siRNA

Mifepristone (Sigma-Aldrich, St Louis, MO, USA) was diluted to 0.1 M with ethanol, stored at − until use. IGF-1 siRNA (5′-CUGAGCUGGUGGAUGCUCU-3′; 3′-AGAGC AUCCACCAGCUCAG-5′; Sigma-Aldrich, St Louis, MO, USA) was co-transfected into cells with lipofectamine 2000 according to the manufacturer's instructions.

MTT assay

The primary uterine leiomyoma cells were seeded in 96-well plates at 5000 cells/well. MTT (5 mg/mL in PBS; Sigma, USA) was diluted in medium and added to the cells. After additional 3 h, the medium was replaced with DMSO for determining the viability of primary uterine leiomyoma cells by using spectrophotometry at a 490nm wavelength.

RNA extraction and quantitative real-time PCR

RNA extracted from the cells was treated with DNase (Invitrogen, NY, USA) to eliminate genomic DNA contamination. Total RNA (2 μg) was reverse-transcribed into cDNA with random primers and M-MLV reverse transcriptase (Invitrogen, NY, USA). Power SYBR Green PCR Mix (Life Technologies, Carlsbad, CA, USA) was used in quantitative real-time PCR (qRT-PCR). Primers were designed by Primer Blast of NCBI and synthesized by Integrated DNA Technologies (Coralville, IA, USA. IGF-1 primers: 5′-GC CGGAATTCATGGGAAAAATCAGCAGTC-3′; 3′-ATGCG GCCGCGGTCTTCCTACATCC-5′). Amplicon was tested (Sanger sequencing) to confirm the primer specificity. PCR program: initial denaturation (10 min, 1 cycle); denaturation (40); Annealing (55, 40); extension (40); melting curve. GAPDH was used as the internal control, which was not affected by different treatments from previous report.[13]

Western blot

Whole cell extracts were prepared by the lysis of cells in buffer (60 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS) on ice. Proteins were quantified using Pierce BCA (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cellular proteins (50 μg) were solubilized in sample buffer (4% SDS, 0.25 m sucrose, 30 mm , 0.075% bromophenol blue and 0.01 m EDTA-Na_2) and denatured at for 5 min. The proteins were separated by SDS-PAGE using 4% stacking and 12% separating gels and then electro-transferred onto polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 0.05 M Tris-buffered saline with 0.5% triton X-100 (TBS-T, pH 7.4) containing 5% skim milk at room temperature for 2 h and then incubated in 3% skim milk or bovine serum albumin at overnight with primary antibody. The primary antibodies used were IGF-1 (1:1000 dilution; Santa Cruz, CA, USA) p-AKT (1:500 dilution; CST, USA) p-ERK1/2 (1:1000 dilution; Santa Cruz, CA, USA) GAPDH (1:1000 dilution; Santa Cruz, CA, USA). After washing with TBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000 for anti-rabbit-IgG or anti-mouse-IgG) for 1 h at room temperature. Proteins were detected by the Amersham enhanced chemiluminescence Western blotting detection kit (GE Healthcare, Piscataway, NJ).

Statistical methods

All statistical analyses were conducted using SPSS version 17 (SPSS Inc, Chicago, IL, USA). All values are expressed as mean and standard deviation. Difference between two groups was analyzed by Student's *t*-test, and differences between three or more groups were analyzed by ANOVA. If variances were homogeneous, then least significance difference method was performed to compare between two groups. If variances were nonhomogeneous, Dunnett's T3 method was used to compare between two groups. A two-tailed *P*-value less than 0.05 was considered statistically significant.

Results

Culture of human uterine leiomyoma cells and infection with IGF-1 lentivirus vectors

To assess the purity of the primary cultured cells, immunocytochemistry staining of the cells with anti-α-smooth muscle actin antibody was performed. As shown in Figure 1A and B, the positive staining of this smooth muscle-specific actin denoted that most of the cells retained their smooth muscle characteristics derived from uterine leiomyomas.

DNA electrophoresis and sequencing showed that the lentivirus vector expressing human IGF-1 gene was correctly

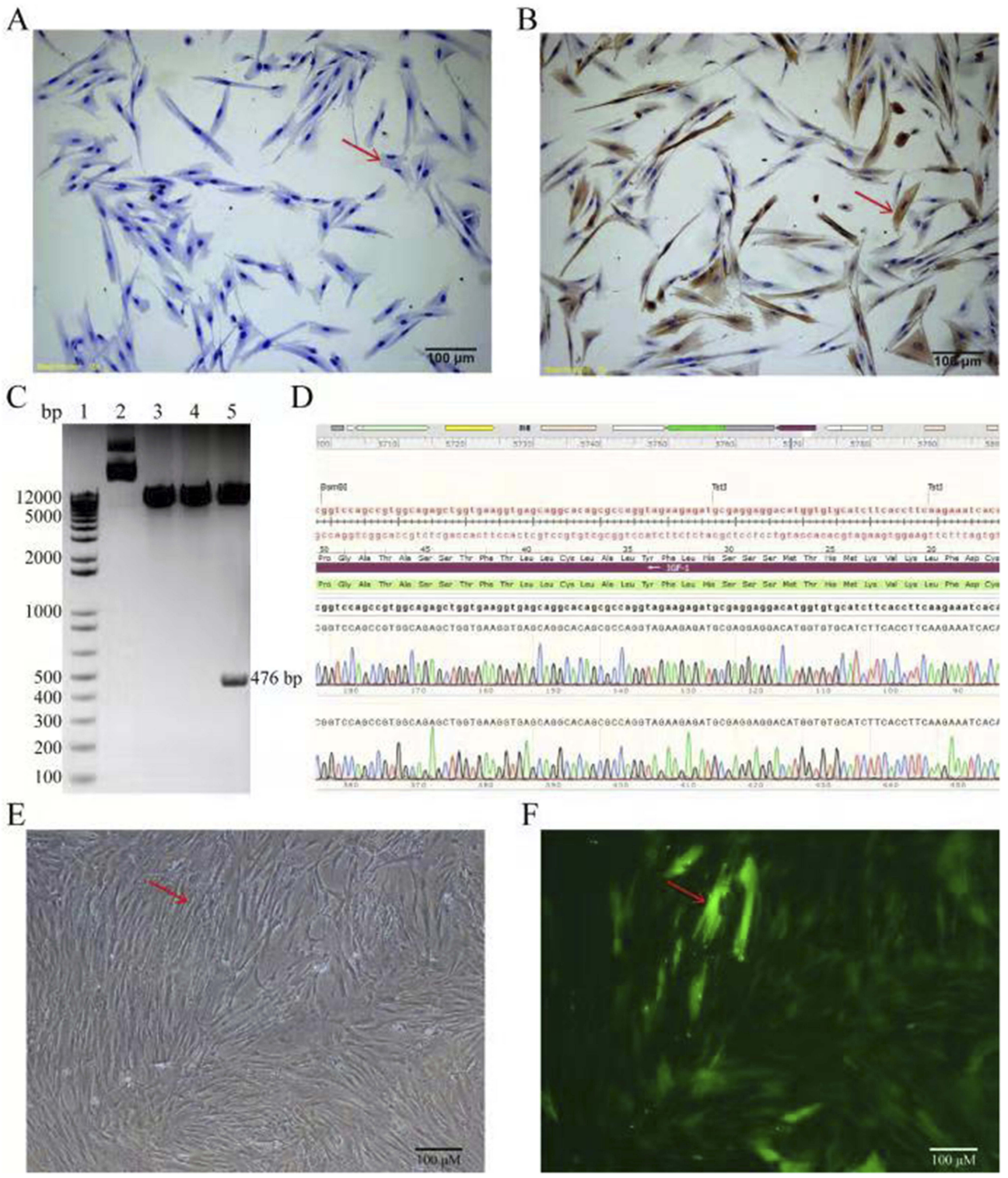

Figure 1 Identification of human uterine leiomyoma cells and infection with IGF-1 lentivirus vectors. (**A**) Negative control of α-actin in human leiomyoma cells (100) (**B**) α-Actin positive staining (100) (**C**) Double digestion of pLVX-IRES-ZsGreen1-IGF-1 (**D**) The sequencing of pLVX-IRES-ZsGreen1-IGF-1 (**E**) The human uterine leiomyoma cells under bright-field microscope (**F**) The GFP transfection efficiency of IGF-1 lentivirus vectors. Red arrows show the details.

constructed (Figure 1C and D). IGF-1 cDNA-contained lentivirus showed high infection efficiency in human uterine leiomyoma cells, with most of the cells showing GFP expression (Figure 1E and F).

Effects of mifepristone on cell viability and IGF-1 expression

To evaluate the effects of mifepristone on viability of primary uterine leiomyoma cells, different concentrations of the drug with different exposure times were used in MTT assay experiments. Figure 2A that cell viability was not significantly different between cells transfected with basal plasmid (control cells) and cells without treatment of mifepristone (WT), indicating that the mifepristone solvent containing low concentration of ethanol (less than 1/1000) did not affect viability of leiomyoma cells in in vitro culture. The inhibition of mifepristone in cell viability was dependent on drug concentrations and treatment times (Figure 2A). Cells treated with both 50 μM and 100 μM of mifepristone for 2, 3, 4 and 6 days had the OD values significantly lower than the control and wild-type cells as well as the cells treated with 10 μM at each corresponding time point (P<0.01) (Figure 2A). Mifepristone at 100 μM appeared to be cytocidal at day 4 and 6 post-treatment (Figure 2A). qRT-PCR was used to detect the change of IGF-1 mRNA expression in cells treated with mifepristone with three concentrations (10 μM, 20 μM, 40 μM) at three time points of treatments (12 h, 24 h,) (Figure 2B). The expression of IGF-1 mRNA in uterine leiomyoma cells treated with 20 μM or 40 μM of mifepristone was significantly decreased at 24 h and 36 h post-treatment (P<0.01) (Figure 2B), although the cell viability was not inhibited at 24 h (1 day) (Figure 2A), suggesting that mifepristone inhibits expression of IGF-1 mRNA leading to inhibit viability of the uterine leiomyoma cells.

Effects of IGF-1 overexpression or on cell viability

Control cells expressed IGF-1 protein at a similar level to the WT cells, indicating that the lentivirus had no effects on the IGF-1 expression (Figure 3A). In contrast, cells infected with IGF-1 cDNA-containing lentivirus showed significantly increased expression of IGF-1 protein, whereas IGF-1 siRNA obviously decreased its protein expression (Figure 3A).

Next, we compared the effects of control siRNA and IGF-1 siRNA at three concentrations (40, 80 and 160 nM) on viability of the uterine leiomyoma cells at day 1, 2, 3, 4 and 6 post-transfection (Figure 3B). Both control siRNA and IGF-1 siRNA at highest concentration (160 nM) significantly inhibited the cell viability over 6 days' time course, this was probably due to the high amounts of lipofectamine 2000 used for transfection resulting in cytotoxicity (Figure 3B). On the other hand, transfection of IGF-1 siRNA at 80 nM significantly inhibited cell viability while the control siRNA showed no effect in comparison to the untreated wild-type cells (Figure 3B).

Effects of IGF-1 overexpression on cell viability treated with mifepristone

Ectopic expression of IGF-1 stimulated cell viability, which was not affected by the treatment with mifepristone at 10 μM (Figure 4A). This is consistent with the fact that mifepristone at 10 μM did not affect cell viability (Figure 2A). More interestingly, IGF-1 overexpression attenuated the inhibitory effects of mifepristone at 20 μM (Figure 4B). However, when the uterine leiomyoma cells were treated

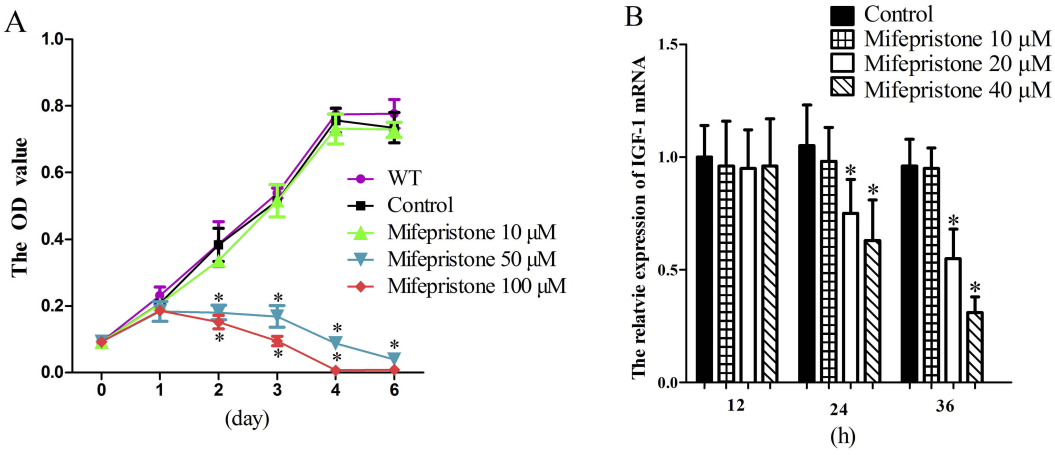

Figure 2 Effects of mifepristone on human uterine leiomyoma cell viability. (**A**) Various concentrations and treatment times of mifepristone on cell viability (**B**) The bar graph of various concentrations and treatment times of mifepristone on cell viability. *Means compared with the control group, P<0.05.

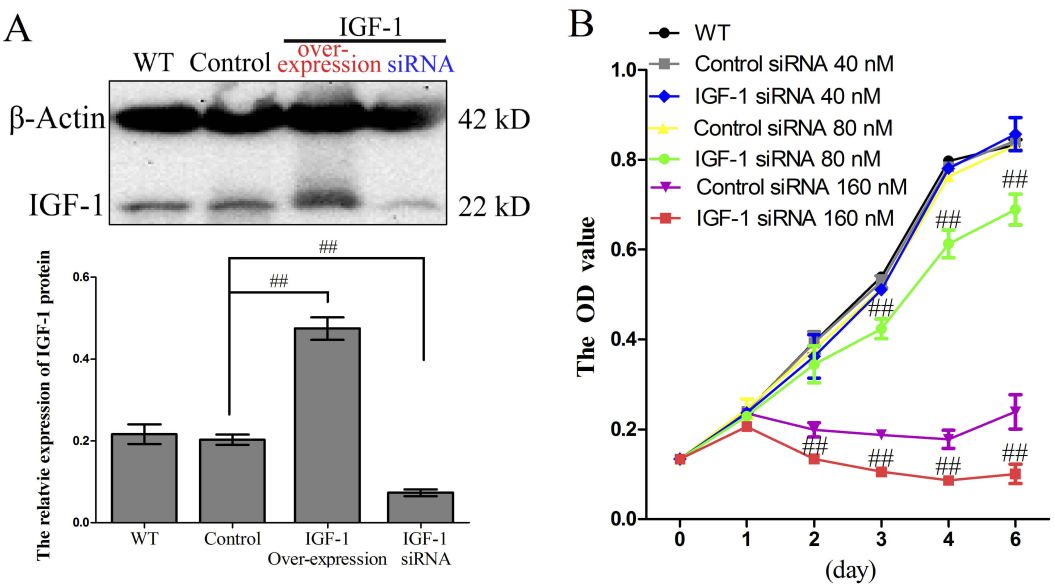

Figure 3 The protein expression of IGF-1 overexpression or knockdown and IGF-1 siRNA on human uterine leiomyoma cell viability. (**A**) The expression of IGF-1 protein in cells transfected with IGF-1 overexpression or siRNA (**B**) The effects of IGF-1 siRNA on cell viability. ##Means compared with the control group, $P<0.01$.

with mifepristone at higher concentrations (40 and 80 μM) that are cytocidal, IGF-1 overexpression could not attenuate the effects of mifepristone (Figure 4C and D). These results suggest that the cytostatic effect of mifepristone is meditated by the downregulation of IGF-1 expression, that overexpression of IGF-1 blocked mifepristone-induced inhibition of cell proliferation, whereas the cytocidal effect of mifepristone is not mediated through IGF-1 inhibition.

The effect of mifepristone on signaling pathways related to IGF-1 in uterine leiomyoma cells

To investigate the mechanism that IGF-1 overexpression attenuated the inhibitory effects of mifepristone on cell viability of uterine leiomyomas, expression of phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated protein kinase B (p-AKT) proteins were analyzed by Western blots. As shown in Figure 5A, mifepristone significantly reduced expression of p-ERK1/2 when the cells were treated with either 20 or 40 μM of mifepristone. IGF-1 overexpression could partially recover expression of p-ERK1/2 only when the cells were treated with 20 μM of mifepristone (Figure 5A).

In addition, expression of p-AKT/Akt was significantly upregulated by mifepristone treatment; the increased expression of p-AKT/Akt was obviously associated with increase of the mifepristone concentration from 20 to 40 μM (Figure 5B). IGF-1 overexpression could not prevent expression of p-AKT/Akt induced by the mifepristone treatment (Figure 5B). The results indicated that p-ERK1/2/ERK signals were probably involved in inhibitory role of mifepristone in cell proliferation of uterine leiomyomas cells.

The flow diagram of the effect of mifepristone on signaling pathways related to IGF-1 in uterine leiomyoma cells shown in Figure 6.

Discussion

Uterine leiomyomas, with high prevalence, affects millions of women' health in the world, which real causes are still not clearly understood.[14] In the previous study, we have found that the treatment effects of mifepristone closely related to the level of IGF-1 expression in uterine leiomyomas. We hypothesize that IGF-1 may play an important role in mifepristone in the treatment of uterine leiomyomas. To test this hypothesis, we have up or IGF-1 protein expression in uterine leiomyoma cells then treated with or without mifepristone. In short, IGF-1 plays a crucial part in mifepristone in the treatment of uterine leiomyomas, and ERK1/2 pathway is involved.

Mifepristone can effectively decrease uterine leiomyomas volumes and alleviate symptoms, but leiomyomas regrow after mifepristone withdrawal.[46] Clinical response to mifepristone is variable, with some unresponsive tumors that grow under treatment. This phenomenon also occurs in another hormone replacement drug, GnRHa, and the

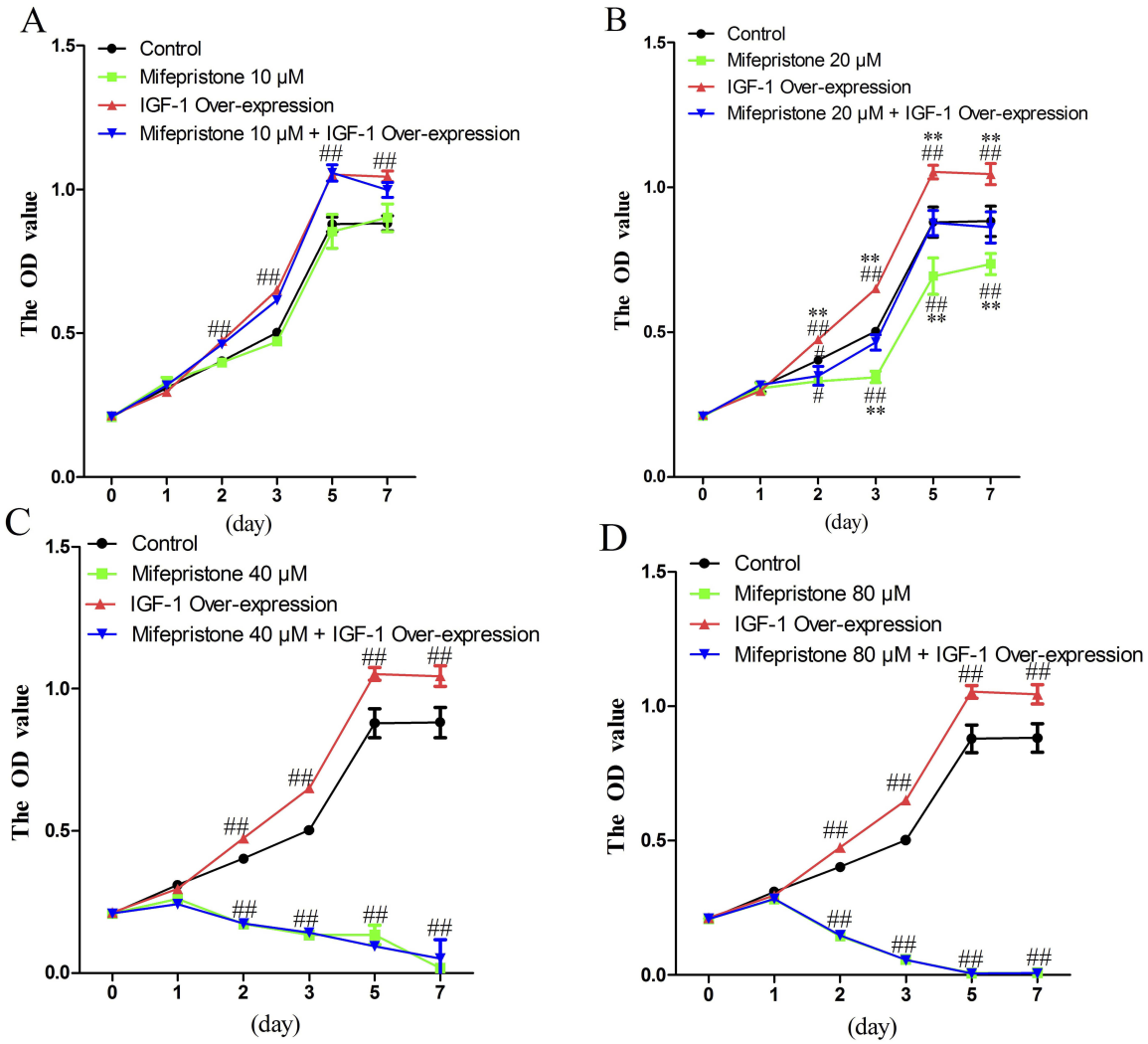

Figure 4 Effects of IGF-1 overexpression on cell viability with or without mifepristone. (**A**) Effects of IGF-1 overexpression on cell viability with or without mifepristone at 10 μM (**B**) Effects of IGF-1 overexpression on cell viability with or without mifepristone at 20 μM (**C**) Effects of IGF-1 overexpression on cell viability with or without mifepristone at 40 μM (**D**) Effects of IGF-1 overexpression on cell viability with or without mifepristone at 80 μM. #Means compared with the control group, $P<0.01$; ##means compared with the control group, $P<0.01$; **means compared with mifepristone 20 μM + IGF-1 overexpression group, $P<0.01$.

exact mechanism remains unclear.[15] Engman et al[16] have proposed glutathione-s transferase mu 1 (GSTM1) as a potential biomarker to predict the treatment effects of on leiomyoma. In this study, we found that mifepristone inhibition on cell proliferation was drug concentration and, and mifepristone inhibition on IGF-1 had similar effects, IGF-1 might be a predicting biomarker similar as GSTM1. Thus, it is very essential to clarify the underlying mechanisms and manage mifepristone effectively for the treatment of uterine leiomyomas, more studies are needed.

IGF-1, as a secreted growth factor, is critical for human multiple biological systems, such as cell growth, development and balance maintenance.[17] In vivo and vitro studies have suggested that IGF-1 is an important factor in the growth process of fibroids.[18,19] In the present study, overexpression or knockdown of IGF-1 had completely opposite action on cell proliferation. Mifepristone could inhibit IGF-I expression, which a time- and dose-dependent in leiomyoma cells. As far as we know, this is the first experimental effort to explore the relationship between mifepristone and IGF-1 in uterine leiomyomas, and related signal pathway.

MAPK signal pathway was considered to be the major pathway responsible for the development of uterine leiomyomas.[20] ERK1/2 is one of the best researched MAPK pathway members, which plays significant roles in cell proliferation and apoptosis.[21,22] Recent works have indicated that selective progesterone receptor modulators (SPRMs) may act

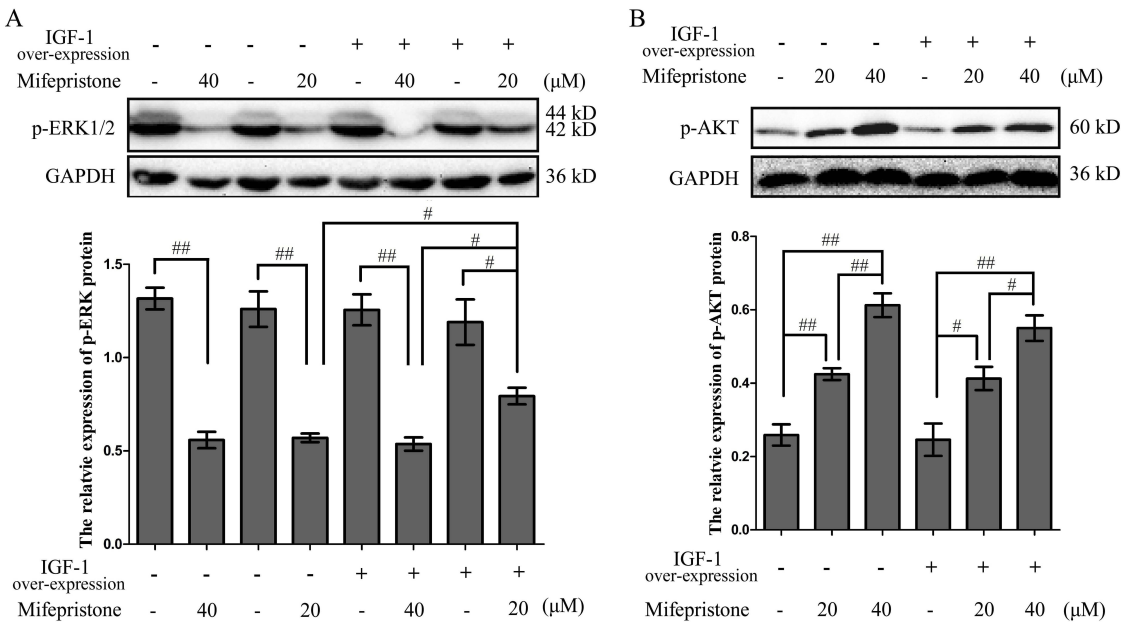

Figure 5 The effect of mifepristone on signaling pathways related to IGF-1 in uterine leiomyoma cells. (**A**) The phosphorylated ERK1/2 expression of IGF-1 on mifepristone in the treatment of uterine leiomyomas (**B**) The phosphorylated AKT expression of IGF-1 on mifepristone in the treatment of uterine leiomyomas. #Means $P<0.05$, ##means $P<0.01$.

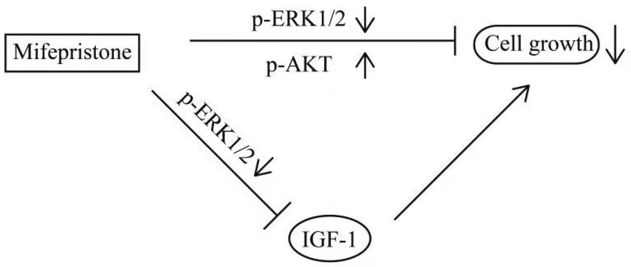

Figure 6 The flow diagram of the effect of mifepristone on signaling pathways related to IGF-1 in uterine leiomyoma cells.

at genomic and nongenomic levels. Segar's group has proposed that AKAP13 modulates ERK pathway in uterine leiomyomas cells.[23] Integrins may also be involved in SPRM signaling, as reported by Dolmans's team.[24] ERK1/2 protein was increased in uterine leiomyomas when its expression inhibited the uterine leiomyoma growth significantly reduced, indicating that ERK1/2 probably plays a causal role in the proliferation of uterine leiomyoma cells.[25,26] However, the exact mechanism for the upregulation of ERK1/2 or its phosphorylation is still not well understood. In the present study, we have found that the overexpression of IGF-1 played a role in the increase of p-ERK1/2 in uterine leiomyoma cells, and mifepristone significantly reduced the p-ERK1/2. At some special concentrations, IGF-1 overexpression could reverse the function of cell growth inhibition by mifepristone. Such effects may come from the up or downstream of the ERK1/2 signaling pathways. These results may explain Eisinger's observation (some leiomyoma re-grew slowly after cessation of mifepristone),[6] and IGF-1 antagonists are a potential area of exploration for uterine leiomyoma. However, currently, it is not clear how IGF-1 the ERK1/2 phosphorylations in uterine leiomyomas cells. Overall, the results suggest that inhibition of ERK1/2 pathway could be one of the mechanisms by mifepristone, and IGF-1 could reverse these effects t a certain extent.

In addition to MAPK, AKT is another signaling pathway that plays a critical role in the progression of uterine leiomyoma. And p-AKT expression was significantly increased in uterine leiomyomas, and the cell proliferation was obviously inhibited when p-AKT blocked.[27] In our present study, we found that mifepristone upregulated p-AKT expression in uterine leiomyoma cells, and AKT signaling pathway was related with mifepristone inhibition on cell proliferation, but it seems that AKT pathway was not related with mifepristone inhibition on IGF-1 expression. Although the AKT signaling pathway involved in the progression of uterine leiomyoma seems clear, the mechanism that mifepristone p-AKT expression is still unknown, more studies are needed.

Conclusion

In summary, our study firstly demonstrated that IGF-1 plays a crucial role in mifepristone in the treatment of uterine leiomyomas, and ERK1/2 pathway is involved. Advances in understanding over the complex interplay

between the IGF-1 and mifepristone in uterine leiomyomas will benefit for development of novel agents and therapeutic strategies.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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