

Upregulation of miR-197 inhibits cell proliferation by directly targeting IGFBP5 in human uterine leiomyoma cells

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Abstract Uterine leiomyoma (ULM), one of the most common reproductive tract neoplasms in premenopausal women, is a kind of benign tumor with multigene involved. Finding and studying the key gene involved has been a long-needed factor for developing non-surgery therapy and prevention methods. The dysregulated microRNAs were reported to play important roles in ULM pathobiology by regulating tumor growth. Our investigations have revealed that miR-197 is at low expression in ULM. Characterization of the effects of miR-197 in ULM demonstrated that downregulation of miR-197 increased cell growth and induced cell cycle arrest in the G0/G1 phase in vitro, while upregulation of miR-197 expression had the opposite effect on ULM growth and progression. Further research on the mechanism of miR-197 on the proliferation of ULM cells, we showed that miR-197 inhibited cell proliferation of ULM by directly targeting IGFBP5, which was overexpressed in ULM and played an important role in the etiology of ULM. These findings obtained in this study deliver insights and further expand our understanding of the role of miR-197 and its target IGFBP5 in ULM development, which provides a potential novel therapeutic agent to target the proliferation of ULM cells.

Keywords miR-197 · IGFBP5 · Uterine leiomyoma · Proliferation

Introduction

Uterine leiomyoma (ULM) is the most common pelvic tumor in child-bearing women, the incidence of which in women aged 45 is more than 60%, and it can cause menorrhagia, pelvic pain, habitual abortion and infertility, and a series of clinical symptoms and is a serious threat to women's physical and mental health (Feng *et al.* 2013). To date, several pathogenic factors such as estrogens, progesterone, genetic factors, epigenetic factors, growth factors, cytokines, chemokines, and extracellular matrix components have been implicated in ULM development and growth; however, the definite etiology of ULM is still not clear.

MicroRNAs (miRNAs), a family of 19- to 25-nucleotide noncoding small RNAs, have a high degree of conservation, time sequence, and tissue specificity, which play important roles in posttranscriptional gene regulation. Most miRNAs simultaneously target more than one mRNA (Kim and Lim 2014). In addition, several miRNAs target the same mRNA which may cause a synergistic effect (Nam *et al.* 2014). MiRNAs play important roles in the development of cell proliferation, apoptosis, and differentiation and are closely related to tumorigenesis of human disease (Huang *et al.* 2014). Recent studies have shown that miRNAs are involved in cell proliferation, apoptosis, and steroid hormones of ULM cells and regulate the pathogenesis of ULM (Qiang *et al.* 2014). miR-197 was reported to be one of the important miRNAs involved in ULM (Wang *et al.* 2007). In fact, previous studies showed that miR-197 was dysregulated in many tumor tissues and was related with the proliferation and apoptosis of tumor cells. Du *et al.* reported that miR-197 was expressed at higher

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levels in both small cell lung cancer cell lines (SCLC) and non-small cell lung cancer cell lines (NSCLC) than in human bronchial epithelial cells (HBEC), which was a negative regulator of *Fus1* expression in lung cancers (Du *et al.* 2009). Hamada *et al.* found that miR-197 was upregulated specifically in invasive ductal adenocarcinoma (IDA), and overexpression of miR-197 in pancreatic cancer cells resulted in the induction of epithelial-mesenchymal transition (EMT) by targeting p120 catenin (Hamada *et al.* 2013). And anti-miR-197 inhibited migration in hepatocellular carcinoma (HCC) cells by targeting KAI 1/CD82 (Dai *et al.* 2014).

Recently, Wang *et al.* found that there are expression differences of miRNAs including miR-197 between in ULM cells and normal muscle cells (Wang *et al.* 2007). However, the further function and mechanism of miR-197 in ULM were unclear. In this study, our results showed that the expression of miR-197 in ULM was lower than that in the myometrium. And we found that miR-197 functioned as a tumor suppressor gene, the upregulation of which effectively inhibited the proliferation of ULM cells and induced cell cycle arrest in the G0/G1 phase *in vitro*. Further research on the exact molecular mechanism of miR-197 in ULM development and progression showed that insulin-like growth factor-binding protein-5 (IGFBP5), a special nuclear transport domain, heparin-binding motif, and IGF/extracellular matrix/acid-labile subunit-binding site (Lee *et al.* 2013), was the direct target of miR-197. And IGFBP5 has been reported to play important functional roles in different types of cancers (Güllü *et al.* 2012).

Materials and Methods

Tissue collection. Portions of matched ULM and myometrium were collected from premenopausal women ($n=30$) who were scheduled to undergo hysterectomy for indications related to symptomatic leiomyomas. The age of the patients ranged from 37 to 47 yr (43.5 ± 3.0 yr). The menarche age was 11.3 ± 3.2 yr, and the age at first pregnancy was about 23 ± 6.1 . The body mass index was about 20.7 ± 7.3 . They did not receive any hormone treatment for at least 3 mo before the hysterectomy. Their menstrual cycles were still regular, and diagnoses of leiomyoma were clear. All the leiomyomas used in this study were 3–5 cm in diameter and were collected at the Nanjing Maternity and Child Health Care Hospital. A total of 23 cases have solitary tumors, and other cases have multiple tumors. The surgery was performed 3 to 10 d after cessation of menstruation when the endometrium was under the proliferative phase. The tissues were collected after surgery, and the histopathologic diagnoses were all confirmed as ULM and without degeneration. No tissue was sampled purely for research purposes. Informed consent about the use of these samples was obtained from all patients. Ethical approval was obtained from

the hospital ethics committee. Immediately after collection, the tissues were snapped frozen and kept in liquid nitrogen for further analysis or used for isolation of leiomyoma smooth muscle cells.

Primary culture of uterine leiomyoma cells and characterization. ULM cells were isolated from small portions of leiomyoma and cultured by the digestion and tissue adherence methods as previously described (Güllü *et al.* 2012). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) containing 10% fetal bovine serum (Gibco, Gran Island, NY) and antibiotics at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. Briefly, after being digested with trypsin and cultured for 24 h, the medium was replaced with fresh medium. Afterwards, the medium was replaced every 2–3 d, and the cells were examined under a light microscope. When the cells reached confluence after culturing for 5–6 d, cell passage was carried out. The experiment only included the cells within the sixth generation of the leiomyoma cells.

Oligonucleotide transfection. ULM cells were incubated in a 6-well plate (Greiner, Bahlingen, Germany) at 1×10^5 cells/well in 2 mL DMEM supplemented with 10% fetal bovine serum (Gibco, Gran Island, NY) and antibiotics (Wisent, Quebec City, Canada). The 2'-O-methyl (2'-OMe-) miR-197 mimics and inhibitors were chemically synthesized by Shanghai GenePharma (Shanghai, China). Once the cells were 80% confluent, they were transfected into ULM cells with FuGENE HD6 (Roche) according to the manufacturer's instructions. Cells were also transfected with 25 nM of a stability-enhanced non-targeting small RNA oligonucleotide as a negative control (NC). The transfected lymphatic-sinus mast cells (LSMC) with decreased levels of mature miR-197 were identified by TaqMan-based real-time quantification RT-PCR.

Quantitative real-time PCR (qRT-PCR) for detecting miR-197 expression. Collect human LSMC cells of miR-197 overexpression and control group separately, extract total cellular RNA by TRIzol method, and detect RNA concentration by NanoDrop 2.0 using 500 ng quantitative total RNA for reverse transcription, reversely translated (RT) into cDNA with specific stem-loop primers. The quantitative real-time PCR (qRT-PCR) was used to verify the expression of miR-197.

Cell cycle analysis. After treatment, primary ULM cells were trypsinized and subsequently fixed with ice-cold 70% ethanol for at least 1 h. After extensive washing, the cells were suspended in Hank's balanced salt solution (HBSS) containing 50 µg/L propidium iodide (PI, Sigma-Aldrich, St. Louis, MO) and 50 µg/L RNase A (Boehringer Mannheim, Indianapolis, IN) and incubated for 1 h at room temperature for

subsequent FACScan analysis (Becton-Dickinson, San Jose, CA). Cell cycle analysis was performed by the ModFit LT software.

MTT assay for cell viability. The MTT assay was used to determine relative cell growth. Human ULM cells were plated at 5×10^3 cells per well in 96-well plates with six replicate wells at the indicated concentrations. After incubation for 24, 48, and 72 h, the cell proliferation assay was performed according to the manufacturer's instructions. The absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay plate reader. Each data point represents the mean of a minimum of six wells. The viability of untreated cells was assumed to be 100%.

Western blot analysis. To determine the levels of protein expression, soluble proteins were isolated by lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl₂, 0.1% Triton X-100, 25 mM MOPS, 100 μ M phenylmethylsulfonyl fluoride, and 20 μ M leupeptin, adjusted to pH 7.2). One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with a corresponding gel concentration using the discontinuous Laemmli buffer system (Bio-Rad Laboratories, Richmond, CA). The electrophoresed proteins were transferred to a polyvinylidene difluoride membrane and subjected to immunoblot analysis with antibodies to purified insulin-like growth factor-binding protein-3 (IGFBP5). The reaction was detected by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). The membranes were reprobbed with a GAPDH antibody (1/2000 dilution, Santa Cruz Biotechnology, Dallas, TX) after washing as a gel loading control.

Luciferase targeting assay. The prediction of miR-197 targets was performed by TargetScan (http://www.targetscan.org/vert_50/). A construct with the fragment of the 3' untranslated region (UTR) of IGFBP5 mRNA containing the putative IGFBP5 binding sequence was cloned into a firefly luciferase reporter construct pGL3 (Promega, Madison, WI, USA) and transfected into human ULM cells using FuGENE HD (Roche, Basel, Switzerland). The construct with a mutated targeting fragment at the 3'UTR of IGFBP5 without the putative miR-197 binding sequence was used as a mutated control. The transfected cells were harvested after 48 h and then assayed with the Dual-Luciferase Reporter Assay System (Promega). The results were obtained from three separate experiments; each one was performed in triplicate.

Statistics. Each experiment was done at least in third duplicate. Numerical data were presented as means and standard errors. Differences between means were analyzed using

Student's *t* test. All statistical analyses were performed using SPSS17.0 software (Chicago, IL).

Results

Evaluation of miR-197 expression in human primary ULM cells. In previous studies, Wang et al. (Wang et al. 2007) collected 55 ULMs, matched myometrium from 41 patients to perform microarray-based global miRNA expression analysis, and found that 45 miRNAs were significantly up- or downregulated in ULMs in comparison to the matched myometrium including downregulated miR-197. To investigate whether miR-197 was at low expression in Chinese ULM tissues, we examined the expression of miR-197 in 30 human ULM specimens compared with the matched myometrium by using TaqMan-based real-time stem-loop RT-PCR analysis, which was normalized to U6 RNA levels. As shown in Fig. 1A, our results confirmed that the expression of miR-197 in ULM was significantly lower than that in the matched myometrium ($P < 0.05$). Our data showed that the levels of miR-197 expression in ULM were only 26.37% of those in the matched myometrium. Meanwhile, the expression of IGFBP5 in the matched myometrium tissues and ULMs was also measured by RT-PCR analysis. As shown in Fig. 1B, the expression levels of IGFBP5 in ULMs were 7.32-folds in comparison to the matched myometrium.

Upregulation of miR-197 suppresses cell proliferation of ULM cells. To explore the function of miR-197 level on the regulation of cell proliferation, cells were transfected with miR-197 mimic and inhibitor, respectively. After 48 h transfection of miR-197 mimic or inhibitor, the expression of miR-197 was determined by RT-PCR. As shown in Fig. 1C, miR-197 expression significantly decreased in miR-197 inhibitor group ($P < 0.05$) and increased in the miR-197 mimic group ($P < 0.05$). MiR-197 inhibitor significantly increased the cell proliferation of ULM cells, while miR-197 mimic significantly inhibited cell proliferation of ULM cells compared with the control groups (Fig. 1D). These results indicate the important role of miR-197 in regulating the proliferation of human ULM cells.

Considering the cell proliferation ability change always accompanying cell cycle change, we analyzed the effects of miR-197 on ULM cell cycle distribution by using a flow cytometric assay. Our results showed that miR-197 inhibitor treatment resulted in a decrease in the number of cells in the G0/G1 phase in comparison to the control groups ($P < 0.05$), while those treated with the miR-197 mimic represented significant increase in G0/G1 phase in comparison to the control groups ($P < 0.05$) (Fig. 1E). These results indicate that the upregulation of miR-197 inhibits the proliferation of ULM

cells, at least in part, by a result of the cell cycle arrest in G0/G1 phase.

IGFBP5 is a predicted target of miR-197. The bioinformatics analysis by TargetScan Release 6.2 software for the target site of miR-197 in IGFBP5 3' UTR is shown in Fig. 2A. The miR-197 seed region and corresponding pairing sites of IGFBP5 mRNA are almost perfectly complementary, a commonly accepted principle in miRNA target prediction. To validate miR-197 regulation of IGFBP5 in human ULM, ULM cells were first transfected with miR-197 mimic, and the results showed that miR-197 mimic efficiently reduced IGFBP5 protein levels, as detected by Western blot analysis (Fig. 2B). And after miR-197 mimic transfection for 48 h, we restored the miR-197 expression by miR-197 inhibitor transfection. The results showed that miR-197 inhibitor transfection rescued IGFBP5 expression, as determined by the protein levels of IGFBP5 (Fig. 2C).

To validate that miR-197 can directly bind to and regulate the levels of IGFBP5 mRNA through the predicted binding sites, we constructed luciferase reporter plasmids (pGL3-WT-IGFBP5-3'UTR plasmids) containing the IGFBP5 3'UTR sequences downstream of firefly luciferase and tested the luciferase activity in human ULM. We also constructed mutant versions of these reporters (pGL3-MUT-IGFBP5-3' UTR plasmids), altering bases in the putative miR-197 binding sites to abolish the predicted miR-197 binding sites. Figure 2D showed the wild-type or mutant IGFBP5 3'UTR construct in the luciferase reporter plasmid used to perform the luciferase targeting assay. All constructs were transfected into cultured human ULM. The reporter assay revealed that transient overexpression of miR-197 efficiently reduced the expression of the luciferase reporter in wild-type group, but did not have an effect on the mutant group. We conclude that miR-197 efficiently controls IGFBP5 expression by directly targeting a sequence motif in the 3'UTR of IGFBP5 in a conserved manner in human ULM.

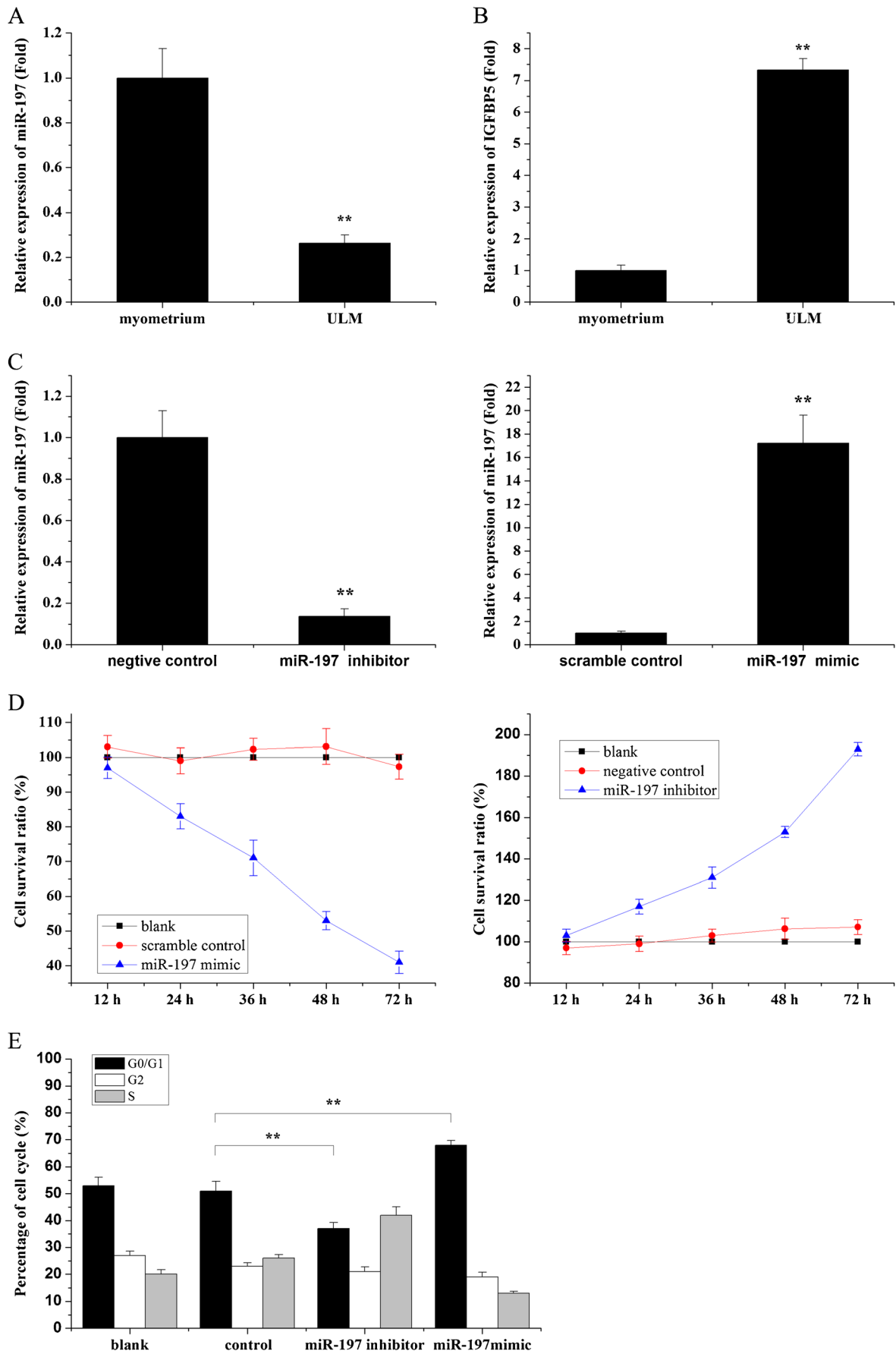
Knockdown of IGFBP5 reverses the antitumor effects of miR-197 inhibitor in primary human ULM cells. To examine whether IGFBP5 is the key factor regulated by miR-197 in primary human ULM cells, we detected the antitumor effects of miR-197 inhibitor after knockdown of IGFBP5. As shown in Fig. 3A, IGFBP5 siRNA (siIGFBP5) effectively abolished the expression of IGFBP5 in ULM cells. In present study, we showed that knockdown of IGFBP5 expression by siIGFBP5 could effectively inhibit the cell growth of ULM cells (Fig. 3B). Further, after ULM cells were co-transfected with miR-197 inhibitor and siIGFBP5, our results showed that knockdown of IGFBP5 levels could reverse the increased cell proliferation induced by miR-197 inhibitor (Fig. 3C).

Discussion

Recent researches have showed that microRNAs regulated gene expression through mRNA degradation and repression. MiRNAs play important roles in the regulation of cell proliferation, apoptosis, morphogenesis, and differentiation. And studies have shown that several microRNAs, including miR-197, miR-21, miR-23b, miR-29b and let-7, and their predicted target genes are significantly dysregulated in uterine leiomyoma compared to normal myometrium. In this study, our results showed that the expression of miR-197 in leiomyoma was lower than that in the myometrium. Here, we further characterized the function and the potential mechanism of miR-197 in human ULM. And we found that miR-197 functioned as a potential anti-oncogene. Upregulation of miR-197 in human ULM cells resulted in cell proliferation inhibition and G0/G1 arrest. Additionally, we found that miR-197 targeted the proapoptotic IGFBP5, which played an important role in cell growth and transformation of normal (myometrium) and tumorous smooth muscle cell (SMC) tissues (Chivukula *et al.* 2014). Thus, it is reasonable to consider miR-197 as an evolutionarily conserved anti-oncomiRNA and a potential therapeutic target for ULM.

To the best of our knowledge, this is the first report analyzing the function and mechanism of miR-197 in human ULM. Differential expression of miR-197 has been reported in several cancers with upregulation seen in SCLC, NSCLC, squamous cell carcinoma of the tongue, and male breast carcinoma and with downregulation in gastric cancer (Wong *et al.* 2008; Du *et al.* 2009; Lehmann *et al.* 2010; Li *et al.* 2011). The level of miR-197 was also found to be higher in the plasma from lung cancer patients with metastasis than in those without metastasis and significantly decreased in responsive patients during chemotherapy (Zheng *et al.* 2011; Güllü *et al.* 2012). In this study, we have examined the differential expression of miR-197 in ULM and in the myometrium and found that miR-197 has lower expression in ULM. Upregulating the expression of miR-197 in ULM effectively suppressed cell proliferation of ULM cells, while downregulating the expression of miR-197 significantly enhanced the cell growth of ULM cells, suggesting miR-197 played an important role during tumorigenesis of ULM. Considering the cell growth often

Fig. 1 Effects of miR-197 on cell proliferation and G0/G1 arrest of human ULMs. (A) Levels of miR-197 expression in human myometrium tissues and ULMs were detected by real-time RT-PCR. (B) Levels of IGFBP5 expression in human myometrium tissues and ULMs were analyzed by real-time RT-PCR. (C) Expression levels of miR-197 in ULMs were measured at 48 h after transfection of miR-197 inhibitors or mimics. (D) The viability of ULMs after transfection with miR-197 inhibitors or mimics was measured by the MTT assay. (E) The population of ULMs in each cell cycle phase in a 48-h exposure to miR-197 inhibitors or mimics transfection was analyzed by flow cytometry analysis. ** $P < 0.01$.



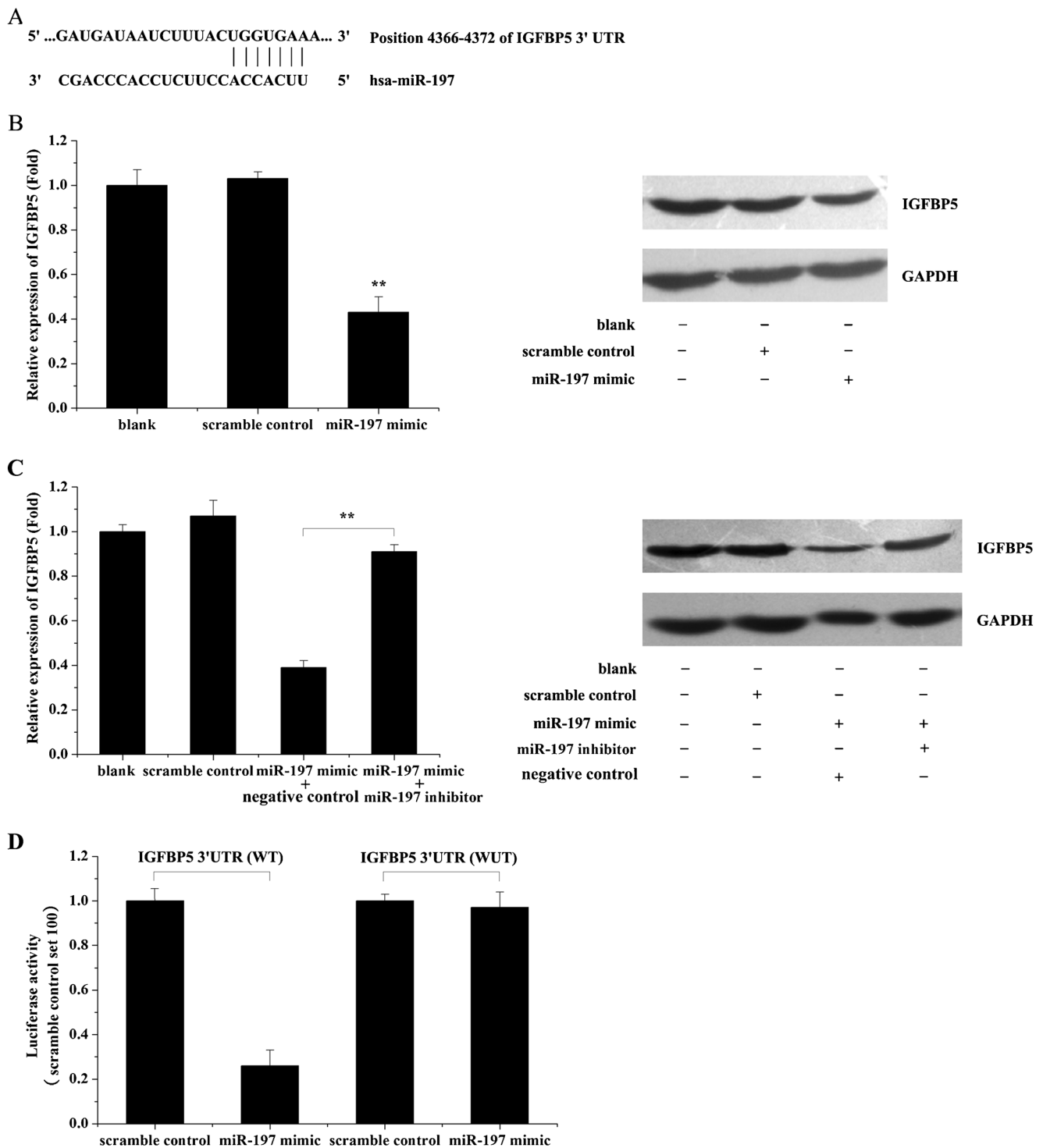


Fig. 2 Identification of IGFBP5 as a direct target gene of miR-197. (A) The miRNA target pairing sequences were analyzed by the TargetScan software. Seven binding sites were predicted on the 3' UTR region of the IGFBP5 mRNA. (B) Cells were transfected with the miR-197 mimic, and the expression of IGFBP5 was analyzed by Western blot. (C) Cells were

co-transfected with the miR-197 mimic and miR-197 inhibitor, and the expression of IGFBP5 was analyzed by Western blot. (D) Luciferase constructs were transfected into cells with the miR-197 mimic. Luciferase activity was assayed 48 h after transient cotransfection. The ratio of the normalized response to control luciferase activity is shown.

involved in cell cycle progression, cell cycle profile changes were analyzed after miR-197 upregulation or downregulation, and our data showed that the miR-197 level was positively

correlated with the accumulation of cells in G0-G1 phase, which demonstrated that miR-197 potentially regulated cell cycle to affect the cell growth of ULM.

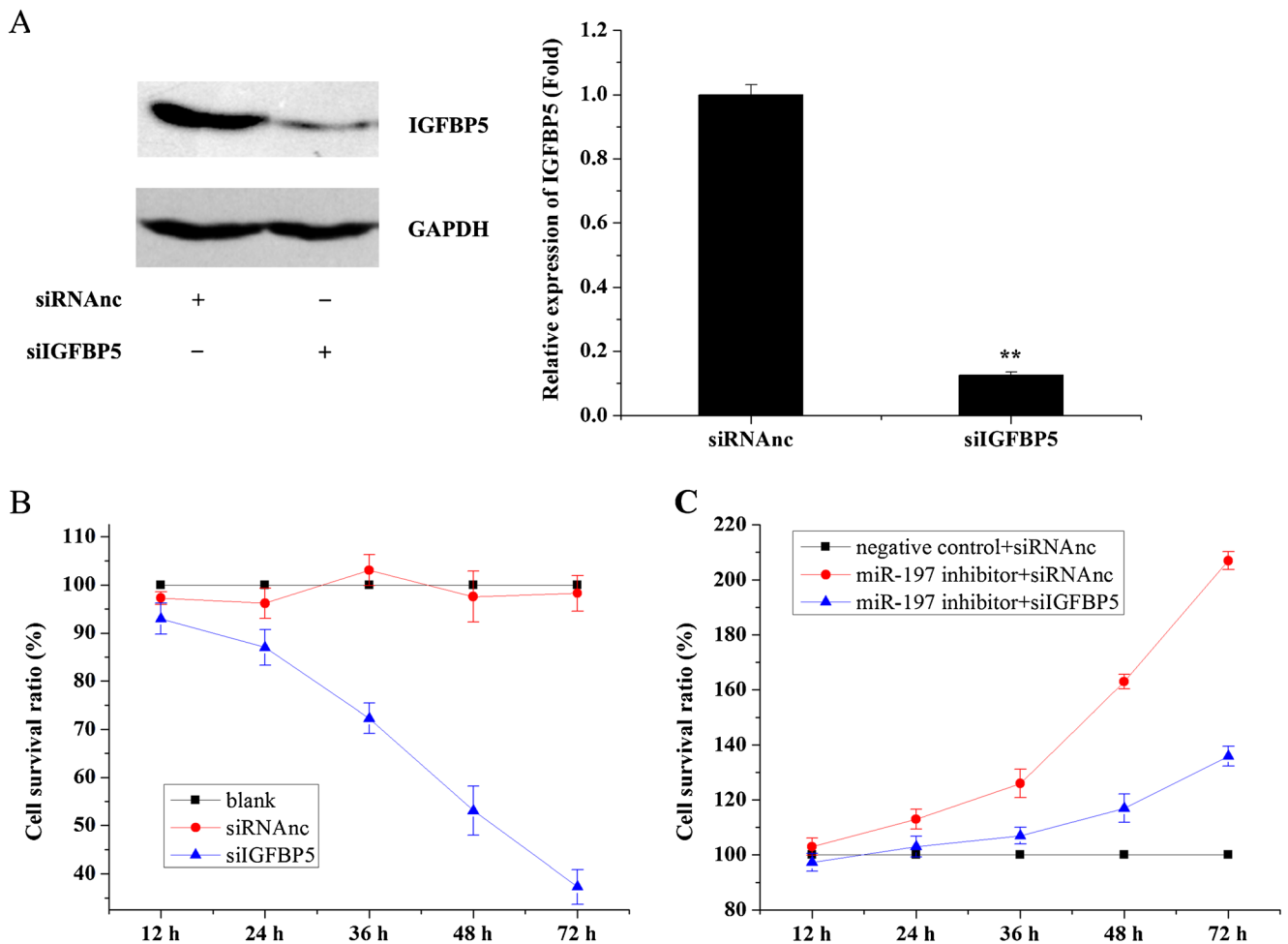


Fig. 3 siIGFBP5 reverses the antitumor effects of miR-197 inhibitor in ULM cells. (A) Cells were transfected with siIGFBP5 or siRNAnc and then the expression of IGFBP5 was detected by Western blot. (B) The viability of ULM cells after transfection with siIGFBP5 or siRNAnc was

measured by the MTT assay. (C) The viability of ULM cells after transfection with miR-197 inhibitor+siIGFBP5 or miR-197 inhibitor+siRNAnc was measured by the MTT assay.

In mammals, insulin-like growth factor-binding proteins (IGFBPs), which consist of six structurally related proteins including IGFBP1, 2, 3, 4, 5, and 6, have been confirmed to be critical regulators of the mitogenic activity of insulin-like growth factors (IGFs). IGFBP5, one of these IGFBPs, has been shown to regulate cell growth, determine cell fate, and play a role in the metastatic process in cancer development. Clinical findings show that IGFBP5 has the potential to be a useful clinical biomarker for predicting response to therapy and clinical outcome of patients with cancers such as retinoblastoma, glioblastoma, adenocarcinoma, breast cancer, non functioning pituitary adenoma, prostate cancer, and estrogen receptor positive breast cancer, and so on (Güllü *et al.* 2012). Simon *et al.* reported that IGFBP5 was involved in regulating the growth of gastrointestinal stromal tumors (Simon *et al.* 2013). In urothelial carcinoma (UC), IGFBP5 overexpression was significantly associated with advanced tumor stage, conferred poorer clinical outcome, and played an important role in tumor progression (Liang *et al.* 2013). And Cirilo *et al.* found that IGFBP5 was upregulated in

the ULMs when compared with the adjacent normal myometrium by transcriptional and protein analyses and confirmed it played an important role in the etiology of ULMs (Cirilo *et al.* 2013). In our study, we also found that the expression of IGFBP5 in ULM was significantly higher than that in the myometrium. Our data showed that knockdown of IGFBP5 by siRNA effectively inhibited the cell growth of ULM, and overexpression of miR-197 decreased the amount of IGFBP5 protein; however, co-transfection of miR-197 inhibitor rescued the downregulated level of IGFBP-5 protein by miR-197 mimic. Moreover, luciferase reporter studies showed that miR-197 regulated the level of IGFBP5 protein by directly targeting a conserved region in the 3'UTR of IGFBP5, which suggested that IGFBP5 was a direct target of miR-197 in ULM.

In this investigation, we demonstrate that low expressed miR-197 plays an important role in the cell proliferation of ULM and functions as an anti-oncogenic gene. And miR-197 regulates the progression of ULM, at least in part, by targeting IGFBP5.

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