Metapristone (RU486 derivative) inhibits endometrial cancer cell progress through regulating miR-492/Klf5/Nrf1 axis

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Abstract

**Background:** Endometrial cancer is the prevalent invasive gynecological cancer in the world. The pathogenesis of endometrial cancer involves many signaling pathways which are related with transcription factors or microRNAs. Metapristone is a hormone related drug and widely used in endometrial cancer clinical therapeutics. However, the deep regulatory mechanism of metapristone is not clear. In this research, we aimed to figure out the specific molecular mechanism during the treatment of endometrial cancer with metapristone.

**Methods:** In this study, RL95-2 cells and Ishikawa cells were used as the endometrial cancer cell models. miR-492 was transfected into RL95-2 cells and Ishikawa cells. The miRNA expression was measured by qRT-PCR. Moreover, the mice tumor model was used to confirm the function of metapristone and the regulating process by miR-492/Klf5/Nrf1 axis *in vivo*. The protein expression was measured by western blot. Cell proliferation and apoptosis was monitored using the MTT assay, cell colony formation assay and EdU assay.

**Results:** Firstly, the results indicated that metapristone as a kind of hormone-related drugs could significantly inhibit the endometrial cancer cell growth through regulating cell apoptosis-related gene expression. Meanwhile, miR-492 was detected to be highly expressed in the endometrial cancer cell lines. Overexpression of miR-492 could promote the cell proliferation and inhibit the cell apoptosis. Furthermore, the results demonstrated that the downstream target genes of miR-492 were *Klf5* and *Nrf1*, which were inhibited by metapristone. At the animal level, metapristone also inhibited the endometrial cancer cell growth through down-regulating the expression of miR-492 and decreasing the protein level of Klf5 and Nrf1.

**Conclusion:** Taken together, this study indicated that metapristone inhibited the endometrial cancer cell growth through regulating the cell apoptosis related signaling pathway and the expression of miR-492 and its downstream target genes (*Klf5* and *Nrf1*), which provided the theoretical basis of endometrial cancer in clinical treatment.

Introduction

Endometrial cancer is the most aggressive cancers in female, after breast, lung, and colorectal cancers (CRC)[1]. In female-specific cancers, the mortality of endometrial cancer is much higher than others including ovarian and cervical cancers[2]. According to pathology, endometrial cancer is classified into two types: Type I is the most common endometrioid adenocarcinoma (80–90%) and Type II includes non-endometrioid subtypes such as serous, clear cell and undifferentiated carcinomas, as well as carcinosarcoma/malignant-mixed Müllerian tumor (10–20%)[3]. There are many risk factors that causes endometrial cancer. First, one kind of risk factors is related with hormone. Unopposed exposure of the endometrium to estrogen is the main risk factor for type I endometrial cancer, including polycystic ovary
syndrome. Other risk factors that are not involved with hormone, include family, old age, thyroid disease, hypertension, Lynch syndrome, diabetes mellitus and obesity[1, 4].

In China, many patients are diagnosed with advanced stage of endometrial cancer and have rapid progress of disease in one year[5]. To date, surgery remains the primary option for the early-stage patients. Advanced and recurrent patients usually choose chemotherapy, which has some adverse reactions. However, tumor heterogeneity and drug resistance are major obstacles in the therapy of endometrial cancer. Hence, it is urgent to find a new kind of drug that has more effect and lower adverse reaction to treat endometrial cancer in clinical.

Estrogen is one of crucial hormone in human especially female and it exhibits a vital and broad spectrum of physiological functions including developing and maintaining both the reproductive system and menstrual cycle, modulating the bone density, regulating the brain function and cholesterol mobilization. Nevertheless, estrogen also contributes to pathological complications especially breast cancer and endometrial cancer. After years of investigation, the estrogen through estrogen receptor (ER) has been taken as a classical factor for endometrial cancer. Usually high expressed ER in endometrial cancer is thought to be driven by estrogen. Mostly, the ER of cell surface has been activated after binding to estrogens and initiates many signaling pathways, such as cAMP and MAPK pathway, to regulate the cell proliferation and cell apoptosis associated genes expression[6, 7].

The given of synthetic progestins is main course of treatment for type I endometrial cancer. The progesterone receptor (PR) is activated and block the pro-growth actions of ER in cell autonomous fashion by regulating the related genes expression through binding the similar targeting sites. Progestins is normally combined with tamoxifen in cycling period to enhance the function of PR. Overall, the disorder of hormone could lead to type I endometrial cancer and steroid hormone signaling is taken to support CRC treatment[8-10].

RU486 (mifepristone) is a derivative of synthetic norethindrone and developed as an effective chemopreventive agent against cancer metastasis and exhibit the activity of anti-progesterone and anti-glucocorticoid through block progesterone receptor (PR), androgen receptor (AR) and glucocorticoid receptor (GR)[11, 12]. Mifepristone presents antitumor function that is assessed in many cancer cell lines, mice model and clinical trials[13]. While the exact mechanisms, associated signal pathway and targets towards endometrial cancer are poorly understood. Similarly, metapristone (RU42633) is the primary metabolite of mifepristone (RU486)[14], which is used to terminate pregnancy in the first month in clinic [15]. Some reports had showed that metapristone could inhibit lots of cancer cell proliferation[16, 17]. However, there were few reports that indicated that whether metapristone could treat endometrial cancer. Hence, to find the specific mechanism of metapristone is important for the endometrial cancer treatment.

MicroRNAs (miRNAs) are post-transcriptional regulators that play roles of translational repression and gene silencing, which are involved in many essential biological processes including cell proliferation, apoptosis, and differentiation. Moreover, many studies have reported that miRNAs are associated with many diseases, such as inflammation and tumorigenesis[18]. With the development of studies, it has
become evident that miRNAs are really closed with different kinds of cancers. However, the influence of microRNA to endometrial cancer has been unclear. This study aims to find the specific microRNA to affect endometrial cancer and explore the molecular mechanism.

In this study, we chose the hormone-related inhibitor metapristone to treat endometrial cancer. The results indicated that miR-492 was highly expressed in endometrial cancer cell lines. The study aims to find the role of miR-492 in the development of endometrial cancer in vitro and in vivo, and the molecular mechanism of metapristone effect on the endometrial cancer, which provide the theoretical basis for clinical therapy of endometrial cancer.

Materials And Methods

Cell culture

Endometrial cancer cell lines, including RL95-2 cells (ATCC® CRL-1671™) and Ishikawa cells (ECACC, 99040201) were obtained from American Type Culture Collection (Manassas, VA, USA) and European Collection of Authenticated Cell Cultures (Porton Down, UK). These cells were cultured with Dulbecco's modified Eagle's medium (DMEM) which contained 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific Inc.) and 10% FBS. The cultured condition was at 37°C in 5% CO2 humidified atmosphere.

Transfection

The sequence of miR-492 was obtained from National Center for Biotechnology Information (NCBI). The small interfering RNA (siRNA) targeting miR-492 (si-miR492) and negative control (si-NC), miR-492 mimic and negative control (miR-NC) were purchased from Genepharma (Shanghai, China). Thereafter, both of these plasmids were transfected into RL95-2 cells and Ishikawa cells in 100nM for 48h by use of lipofectamine 2000 (Invitrogen, Carlsbad, CA). The sequences were shown in Table 1

MTT assay

Thiazolyl blue tetrazolium bromide (MTT) (KeyGentech, China) was normally used to determine the cell survival. The living cell could convert the MTT to insoluble formazan by the cell’s redox potential. In brief, the total of 2000 RL95-2 cells and Ishikawa cells were plated into 96-well plates each well. The cells were treated without or with metapristone (50μM) or transfected with miR-492 mimic or miR-NC for 5 days. The blank were the non-cell wells with medium. The culture medium was removed and 20μl MTT (5 mg/ml) were added to each well for four hours at 37°C. Therefore, 150μl of DMSO were added and incubate for 15 min. Cell numbers were counted at a wavelength of 570 nm by the Model 680 Microplate Reader (Bio-Rad Laboratories). The six replicates were in each treatment and three times were repeated for MTT assay.

Cell colony formation assay
A total of 800 RL95-2 cells and Ishikawa cells were plated to 6-well plates with soft agar each well with or without the treatment of metapristone (50μM) or transfected with miR-492 mimic or miR-NC. Two weeks later, cells were fixed with formaldehyde after being washed with phosphate-buffered saline (PBS) three times, and stained with Giemsa staining solution for 30 min under room temperature. Visible clones were counted by an Olympus inverted microscope (Japan).

**EdU Assay**

A total of 5000 RL95-2 cells and Ishikawa cells were plated to 6-well plates each well and treated with metapristone (50μM, 48h). EdU staining proliferation kit was purchased from Abcam (ab219801). The plate was added with EdU solution and incubate for 3 hours and then treated with 4% formaldehyde. After the process, the cells were stained with DAPI and performed as the instruction described by Olympus inverted microscope (Japan).

**Luciferase reporter assay**

Luciferase reporter assay was performed as paper described[19]. In brief, the wild type or mutated 3' UTR sequence of Klf5 and Nrf1 was cloned into pGL3-luc vector (Promega, USA). The reporter constructs and the miR-492 were co-transfected into RL95-2 or ISK cells by using Lipofectamine 2000. 48 h after transfection, the cells were lysed and the relative luciferase activity was measured by using dual-luciferase reporter assay system (Promega, USA).

**Western Blot**

The RIPA buffer (Beyotime, China) were used to lyse all the cells sample and tumors tissue and total protein amounts was determined by BCA protein assay (Pierce Biotechnology) and then denatured with Laemmli buffer at 95°C for 10 min. Total protein (30mg) was separated by 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore). Membranes were blocked at room temperature for 1 h with 5% non-fat milk in Tris-buffered saline with Tween 20(TBS-T). Then, the membranes with antibodies were incubated at 4°C overnight. The primary antibodies were as followed: Cleaved-Caspase3 (dilution,1:1000; Cell Signaling Technology, 9661), Cleaved-Caspase-9 (dilution, 1:1000, Cell Signaling Technology, 20750), Bax (dilution, 1:1000, Cell Signaling Technology, 2774), Bcl-2 (dilution, 1:1000, Cell Signaling Technology, 15071), Klf5 (dilution, 1:1000, Cell Signaling Technology, 51586), Nrf1 (dilution, 1:1000; Santa Cruz, sc-28379) and GAPDH (dilution, 1:1000; easybio, BE0023). Horseradish peroxidase-conjugated secondary antibodies were as followed: rabbit-HRP (easybio, 1:20000; BE0101-100). The results were detected by ECL Western Boltting Substrate (Pierce, Thermo Fisher) and captured with the ImageQuant LAS 400 imaging system (GE Healthcare Life Sciences).

**Quantitative real-time polymerase chain reaction (q-PCR)**

The total RNA of cells was extracted with Trizol (Invitrogen, USA), and then had the process of reverse transcription to cDNA Reverse Transcriptase M-MLV (TakaRa). Q-PCR was performed with SYBR Green
PCR reagents (CoWin Biosciences) on a Real-Time PCR System (Carlsbad, USA), and analyzed with StepOne Software (Thermo Fisher, USA). The primers were performed in supplementary table.

Drug administration

Mifepristone was purchased from Shanghai New Hualian pharmaceutical Co., with purity >98%. Metapristone was synthesized, using mifepristone as the starting material as described previously [20].

Immunohistochemistry

The tissue sections were divided into 5μm thickness and the dehydration was reached with the different concentration ethanol after dewaxed in xylene. The sections were oven heating in 0.01 M sodium citrate buffer (pH 6.0, Beyotime China) for 10 min to antigen retrieval. 3% hydrogen peroxide and 5% BSA (Amresco, USA) were used to block endogenous peroxidase activity and nonspecific staining for 20 min and 1 h. The primary antibody of anti-Klf5 (1:100, Cell Signaling Technology, 51586) and anti-Nrf1 (1:1000, Santa Cruz, sc-28379) were incubated with tissue sections for overnight in 4℃ and incubated with secondary for 1 h in room temperature. And the cultured section with streptavidin peroxidase for 10 min and 3,3- Diaminobenzidine tetrachloride (DAB, TIANGEN China) were used to detect peroxidase activity. All the experimental processes were performed at room temperature and the section were washed with PBS for three times. At last, sections were dehydrated in alcohol and cleared in xylene.

Tumor xenograft model

Female nude mice (20g, 7-8 weeks) were obtained from Vital River Laboratory Animal Technology (Beijing, China) and were kept in standard environment with the unified breeding. Mice were sacrificed by carbon dioxide asphyxiation. All the experiment was approved by the Animal Research Committee of the Beijing Friendship Hospital Affiliated to Capital Medical University and conform to the ethical standards with the guidelines of the National Animal Care and Ethics Institution. Two million of RL95-2 cells and Ishikawa cells were diluted in 0.1ml of saline and injected subcutaneously into 7-week old female nude mice. After the cell injection, the experimental group mice (more than 6 mice per trial) were intraperitoneally injected with metapristone (45mg/kg, three times for one week) during two weeks and the control were intraperitoneally injected with vehicle at the same time points. Tumor xenografts were measured using a caliper during the experiment. The tumor volume was calculated by this formula: volume = (length × width²)/2. After sacrificed, the tumors were separated for future analysis. Each group contained more than 6 mice.

Statistical analysis

All the results were analyzed with Mean ± S.D. or ±S.E.M. The significance analysis of all the experiments by student’s two-tailed non-paired t-test. P < 0.05 was considered to have statistical significance.

Results
1. Metapristone inhibited endometrial cancer cell growth and promoted endometrial cancer cell apoptosis

Firstly, IC50 values of metapristone were observed in different tumor cells and the results demonstrated that RL95-2 cells and Ishikawa cells were more susceptible to metapristone (Supplementary Fig. 1). RL95-2 cells and Ishikawa cells were used to future investigation. The cell growth curve results showed that metapristone could inhibit the cell growth of endometrial cancer cell lines RL95-2 cells and Ishikawa cells (Fig. 1A). The cell colony formation assay results also showed that the cell colony number was highly decreased with the treatment of metapristone than the cells without metapristone treatment (Fig. 1B). Moreover, EdU assay results showed that the positive staining cell number was significantly decreased when RL95-2 cells and Ishikawa cells were treated with metapristone, which indicated that metapristone could inhibit the cell proliferation (Fig. 1C). To further explore the signaling pathway that metapristone affected, the western blot was detected. The results demonstrated that metapristone could increase the expression of cleaved-caspase3, cleaved-caspase9, Bax and decrease the expression of Bcl-2, which indicated that metapristone affected cell apoptosis related signaling pathways. All these data indicated that metapristone inhibited endometrial cancer cell growth and promoted endometrial cancer cell apoptosis.

2. MiR-492 promoted the cell proliferation of endometrial cancer cell and had resistance to cell apoptosis.

The previous studies has reported that miR-492 was involved in the development of some cancers, such as cervical squamous cell carcinomas[21], hepatoblastoma[22] and colon cancer cells[23]. We chose different types of cancer cell lines to detect the expression of miR-492. Interestingly, the result showed that miR-492 was relatively peculiarly highly-expressed in endometrial cancer cell lines RL95-2 cells and Ishikawa cells than other cancer cell lines, including breast cancer cell lines MCF7 cells and BT474 cells, lung cancer cell line A549 cell and stomach cancer cell line MGC803 cell (Fig. 2A). To better investigate the role of miR-492 in the development in endometrial cancer, the overexpression or knockdown of miR-492 experiment was designed. In the cell growth curve results, overexpression of miR-492 could significantly promote the endometrial cancer cell growth, on the contrary, knockdown of miR-492 inhibited the endometrial cancer cell growth (Fig. 2B). Analogously, the cell colony formation results showed that overexpression of miR-492 could form more cell clones and the opposite results were shown in the si-miR-492 group (Fig. 2C). Moreover, the EdU assay results also indicated that miR-492 promoted the cell proliferation of endometrial cancer cell lines RL95-2 cells and Ishikawa cells. Expectedly, the cell proliferation was inhibited with the treatment of si-miR-492 (Fig. 2D). Moreover, the western blot results showed that miR-492 decreased the expression of cleaved-caspase3 and changed the pro-apoptotic protein Bax, which suggested that overexpression of miR-492 could inhibit the cell apoptosis (Fig. 2E). The absence of miR-492 also exerted the completely different cell apoptosis related protein expression, which further proved the role of miR-492 in endometrial cancer cells.

3. Klf5 and Nrf1 were downstream target genes of miR-492.
To further study the specific molecular mechanism of miR-492 effect on the endometrial cancer cells, the potential downstream target genes of miR-492 were predicted online. The results suggested us two target genes, \textit{Klf5} and \textit{Nrf1}, which were both transcription factors. The results of real-time PCR showed that the mRNA expression of \textit{Klf5} and \textit{Nrf1} were obviously increased when miR-492 was overexpressed and were decreased with the treatment of si-miR-492 in the RL95-2 cells and Ishikawa cells (Fig. 3A). On the other hand, the protein expression level was also detected with the overexpression of miR-492 or knockdown of miR-492. As Fig3 B shown, miR-492 also promoted the protein expression of Klf5 and Nrf1, while inhibited the protein expression in knockdown experiments. All these data indicated that \textit{Klf5} and \textit{Nrf1} were potential target genes of miR-492. Furthermore, the luciferase reporter assay results also directly proved that \textit{Klf5} and \textit{Nrf1} were downstream target genes of miR-492. As Fig. 3C shown, mutant \textit{Klf5} or \textit{Nrf1} expression was not changed with the overexpression of miR-492, but wildtype \textit{Klf5} or \textit{Nrf1} expression was up-regulated obviously in the existence of miR-492.

4. MiR-492 rescued the antitumor function of metapristone that inhibited the expression of miR-492.

To further clear the mechanism of metapristone effect on endometrial cancer cell that whether through miR-492, the miR-492 expression was detected when RL95-2 cells and Ishikawa cells were treated with metapristone. As Fig. 4A shown, metapristone could decrease the expression of miR-492. Moreover, the downstream target genes were also detected at the mRNA level. The results of Fig. 4B showed that the mRNA expression of \textit{Klf5} and \textit{Nrf1} were down-regulated with the treatment of metapristone. Interestingly, metapristone did not completely inhibit the expression of miR-492 when the miR-492 was exogenous overexpressed. Subsequently, the results of Fig. 4C showed that administration of both miR-492 and metapristone promoted the endometrial cancer cell growth, which suggested that miR-492 could rescue the anti-tumor function of metapristone. Similarly, the EdU assay showed that the combination of miR-492 and metapristone exhibited the strongest cell proliferation, which was totally different from the administration of metapristone (Fig. 4D). Moreover, the western blot results indicated that the combination of miR-492 and metapristone affected the cell apoptosis related signaling pathway, including decreasing the expression of cleaved caspase 3 and Bax (Fig. 4E). All these results demonstrated that metapristone inhibited the expression of miR-492 and its downstream target genes \textit{Klf5} and \textit{Nrf1}. However, overexpression of miR-492 could destroy the antitumor effect of metapristone.

5. Metapristone inhibited the tumor growth through miR-492 and its downstream target genes Klf5 and Nrf1 \textit{in vivo}.

Previous data suggested us that metapristone had a prefect inhibitory effect on the endometrial cancer cell lines RL95-2 cells and Ishikawa cells \textit{in vitro}. We established the tumor xenograft model. As Fig. 5A shown, the growth of tumors was obviously inhibited when the mice were treated with metapristone both in the model of RL95-2 cells and Ishikawa cells. The IHC results of tumor tissues demonstrated that Klf5 and Nrf1 expression were down-regulated with metapristone treatment (Fig. 5B). Moreover, we extracted the mRNA and protein of both RL95-2 cells and Ishikawa cells tumor tissues. The results of Fig. 5C demonstrated that both the expression of miR-492 and its downstream target genes were decreased
when the mice were treated with metapristone. Furthermore, the protein expression level of Klf5 and Nrf1 were also deceased with the metapristone treatment (Fig. 5D). Taken together, all the results showed that metapristone could inhibit the endometrial cancer cell growth in vitro and in vivo through regulating miR-492/Klf5/Nrf1 axis and the cell apoptosis related pathways.

**Discussion**

Endometrial cancer is a kind of cancer that is closed related to hormone secretion. Abnormal hormone secretion can lead to the tumorigenesis and development of endometrial cancer[1]. Hence, we chose a hormone-related drug, metapristone (RU42633), to treat endometrial cancer. In addition, the specific mechanism of metapristone on endometrial cancer is helpful for the clinical treatment. Upon the present research, chemotherapy is still the mainly and first-line therapeutic options for endometrial cancer. However, chemotherapy has inevitable side effects which limit the application in clinic. In our study, we found that metapristone could obviously inhibit the endometrial cancer cell growth through promoting cell proliferation and inhibiting cell apoptosis. These may provide some theoretical basis for the clinical therapy of endometrial cancer.

Many studies provided strong evidence that metapristone is one of mifepristone metabolites with oral administration in mammals and its metabolic pathway is through blood circulation[24, 25]. The recent report indicated that metapristone had lower cytotoxicity in the systematic monitoring than mifepristone, and was more suitable for the treatment of metastatic tumors therapy[26]. Our results also showed that metapristone maintained more biosecurity in vitro and in vivo (data not shown), which was instructive for its application. Since the occurrence and development of most gender-related tumors have been associated with sex hormones, mifepristone or metapristone has also been clinically applicable to most hormone-related diseases, including contraception and breast cancer[27]. Most of known mechanisms are about estrogen, androgen or their receptors. While our research aimed to find out the new molecular mechanism that are related with miRNAs.

There is plenty of evidence to suggest that miRNAs regulate various biological processes in tumorigenesis, development and metastasis of different kinds of cancers[28]. Although miRNAs expression is abnormal in the tumor development and metastasis, the specific function of individual miRNA has been still under investigation. The functional role of miR-492 has been reported in few types of cancers[29-31]. In this project, we investigated the functional impact of miR-492 on endometrial cancer in vitro and in vivo. The results add to the understanding of its role in endometrial cancer progression and indicate its diagnostic and prognostic relevance. Our data firstly indicated that miR-492 was specific highly-expressed in endometrial cancer cell lines, which indicated that miR-492 was essential for the development of endometrial cancer. The further study also showed that *Klf5* and *Nrf1* as the transcription factors were the new targets of miR-492. Klf5 (also known as BTEB2 and IKLF), a member of the Kruppel-like family of transcription factors, has been implicated as an oncogene and therapeutic target in a number of cancers including breast, colon, bladder, lung, stomach, and ovarian cancer[32]. The specific biological function of Klf5 is considered as transcription factor to regulate cell proliferation, apoptosis,
migration, and differentiation[33, 34]. The other study also indicated that Nrf1 was involved in the melanoma carcinogenesis [35]. Hence, our results provided a new research direction for the research of Klf5 and Nrf1. Both of them could regulate endometrial cancer cell growth through miR-492. But further functional research needs to be confirmed by more experiments.

Conclusion

Taken together, our research proved that metapristone inhibited endometrial cancer cell growth through regulating miR-492 and its new target genes Klf5 and Nrf1 \textit{in vitro} and \textit{in vivo}. Meanwhile, metapristone also regulated cell apoptosis related signaling pathway to treat endometrial cancer. All these results provided a new horizon for treatment of endometrial cancer.

Declarations

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Consent for publication

Not applicable.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Beijing Friendship Hospital Affiliated to Capital Medical University.

Competing Interests

The authors declare that there were no conflict of interests.

Author Contributions

Yun Liu conceptualized the project, designed research, supervised experiments, and wrote the manuscript. Yue Chang designed research, performed experiments, analyzed data, and wrote the manuscript. Min Hao contribute to collate and analyze the new data and revised the manuscript. Ru Jia performed the si-RNA
and related experiments. Yihui Zhao performed the western blot experiments. Yixuan Cai contributed to modify the manuscript. All authors have read and approved the final submitted manuscript.

**Availability of data and materials**

The data supporting the conclusions of this study are include within the article.

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**Figures**
Figure 1
Metapristone inhibited endometrial cancer cell growth and promoted endometrial cancer cell apoptosis (A) Cell growth curves of RL95-2 cells and Ishikawa cells with metapristone (50μM) treatment. Non-treated cells were as control. Results represent mean ± standard error of the mean (SEM) (n = 6). ****p < 0.0001. (B) Cell colony formation of RL95-2 cells and Ishikawa cells with metapristone (50μM) treatment. Non-treated cells were as control. Values are mean ± S.D. (n = 3 per group). (C) EdU assay was used to detect the cell proliferation phenotype with or without the treatment of metapristone (50μM 48 h). (D) The protein levels of Cleaved-Caspase3, Cleaved-Caspase9, Bax and Bcl-2 in RL95-2 cells and Ishikawa cells with or without metapristone treatment were test by western blot. GAPDH was used as the control.
Figure 2
MiR-492 promoted the cell proliferation of endometrial cancer cell and had resistance to cell apoptosis. (A) The mRNA expression of miR-492 of RL95-2, Ishikawa, MCF7, BT474, A549 and MGC0803 cells were measured with real-time PCR. Values are means ± S.D. (n=3). ****P<0.0001. (B) Cell growth curves of RL95-2 cells and Ishikawa cells with miR-492 (100nM) and si-miR-492 (100nM) treatment. Non-treated and mir expression system cells were as control. Results represent mean ± standard deviation (S.D.) (n = 3 per group). (C) Cell colony formation of RL95-2 cells and Ishikawa cells with miR-492 (100nM) and si-miR-492 (100nM) treatment. Non-treated and mir expression system cells were as control. Values are mean ± S.D. (n = 3). (D) EdU assay was used to detect the cell proliferation phenotype with or without the treatment of miR-492 (100nM, 48h) and si-miR-492 (100nM). (E) The protein levels of Cleaved-Caspase3, Cleaved-Caspase9, Bax and Bcl-2 in RL95-2 cells and Ishikawa cells with or without miR-492 and si-miR-492 treatment (100nM, 48h) were test by western blot. GAPDH was used as the internal control.
Figure 3

Klf5 and Nrf1 were downstream target genes of miR-492. (A) The mRNA level of Klf5 and Nrf1 in RL95-2 cells and Ishikawa cells with or without miR-492 and si-miR-492 treatment (100nM, 48h) were measured with by real-time PCR. Values are means ±S.D. (n=3). ***P<0.001, ****P<0.0001. (B) The protein level of Klf5 and Nrf1 in RL95-2 cells and Ishikawa cells with or without miR-492 and si-miR-492 treatment (100nM, 48h) were detected by western blot (n=3 per group). Values are means ±S.D. ***P<0.001, ****P<0.0001. (C) Prediction of the binding sites for miR-492 in wild-type (wt) of Klf5 and Nrf1. The mutated (mut) LIFR-Klf5 and mutated (mut) LIFR-Nrf1 showed the disruption of miR-492 binding sites in RL95-2 and ISK cells. Values are means ±S.D. ***P<0.001, ****P<0.0001.
Figure 4
MiR-492 rescued the antitumor function of metapristone that inhibited the expression of miR-492. (A) The mRNA expression of miR-492 of RL95-2 cells and Ishikawa cells with or without metapristone treatment (50μM 48 h) were measured with real-time PCR. Values are means ±S.D. (n=3 per group). **P<0.01, ***P<0.001. (B) The mRNA expression of Klf5 and Nrf1 in RL95-2 cells and Ishikawa cells with or without metapristone and miR-492 +metapristone treatment (50μM 48 h) were detected by real-time PCR. Non-treated and mir expression system cells were as control. Values are means ±S.D. (n=3 per group). **P<0.01, ***P<0.001. (C) Cell growth curves by MTT assay of RL95-2 cells and Ishikawa cells with or without metapristone and miR-492 +metapristone treatment (50μM 48 h) treatment. Non-treated and mir expression system cells were as control. Results represent mean ± standard deviation (S.D.) (n = 6 per group). (D) Cell growth curves by EDU assay of RL95-2 cells and Ishikawa cells with or without metapristone and miR-492 +metapristone treatment (50μM 48 h) treatment. Non-treated and mir expression system cells were as control. Results represent mean ± standard deviation (S.D.) (n = 6 per group). (E) The protein expression of Klf5 and Nrf1 in RL95-2 cells and Ishikawa cells with or without metapristone and miR-492 +metapristone treatment (50μM 48 h) were detected by western blot. Values are means ±S.D. Non-treated and mir expression system cells were as control. (n=6 per group).
Figure 5
Metapristone inhibited the tumor growth through miR-492 and its downstream target genes Klf5 and Nrf1 in vivo. (A) The tumor xenograft model of RL95-2 cells and Ishikawa cells (n=6 per group). (B) The expression of Klf5 and Nrf1 in the tumor xenograft of RL95-2 cells and Ishikawa cells with or without metaprostione treatment were detected by immunohistochemistry. (C) The mRNA expression of miR-492, Klf5 and Nrf1 in the tumor xenograft of RL95-2 cells and Ishikawa cells with or without metapristone treatment were measured with real-time PCR (n=6 per group). Values are means ±S.D. *P<0.05, **P<0.01, ***P<0.001. (D) The protein of Klf5 and Nrf1 in the tumor xenograft of RL95-2 cells and Ishikawa cells with or without metapristone treatment were measured with western blot (n=6 per group). Values are means ±S.D. *P<0.05, **P<0.01, ***P<0.001.

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