HSF1 Promotes the Development of Endometriosis by Up-regulating PFKFB3 Expression

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Research

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Abstract

Background

Endometriosis is a chronic hormonal inflammatory disease characterized by the presence of endometrial tissue (glands and stroma) outside the uterus. Endometriosis seriously affects the physical health of women of childbearing age, often causes infertility, and affects the body and mind of patients and their families.

Methods

We examined the effect of HSF1 on endometriosis through cell count, scratch and clone formation experiments. We used real-time quantitative PCR and western blotting to detect the effect of HSF1 on mRNA and protein of endometriosis cells. Collect the cell culture medium and Glucose levels and lactate levels were determined using a glucose (G0) assay kit and a lactate assay kit. Furthermore, we established a mouse model of endometriosis, and the effect of HSF1 on endometriosis was observed by inhibiting HSF1 with K Ribbin in the mice.

Results

HSF1 is highly expressed in endometriosis and plays an indispensable role in endometriosis development in both cell and animal studies. We found that HSF1 promotes endometriosis development and glucose consumption and lactic acid production. Further research showed that HSF1 functions in endometriosis by up-regulating PFKFB3, a key enzyme in glycolysis. And the HSF1 inhibitor K Ribbin can abrogate all of the above experimental effects both in vivo and in vitro.

Conclusions

Our study shows that HSF1 plays a significant role in the occurrence and development of endometriosis, which may become a new target for the treatment of endometriosis and provide a new idea for the clinical treatment of endometriosis.

Background

Endometriosis is a chronic hormonal inflammatory disease characterized by the presence of endometrial tissue (glands and stroma) outside the uterus [1]. Most of the ectopic tissues are located in the pelvic viscera and wall peritoneum, with the ovary and uterine sacral ligament being the most common, followed by the uterus and other visceral peritoneum, and the vaginal and rectal septum [2]. The main clinical manifestations of endometriosis are lower abdominal pain, dysmenorrhea, infertility, sexual discomfort, abnormal menstruation and local periodic pain, bleeding and a mass. Approximately 6%-10% of women of childbearing age develop the disease, 50%-60% of whom have pelvic pain, and the infertility rate is as high as 50%, seriously affecting the physical health of these women [3]. The pathogenesis of endometriosis remains unclear, the most common theory leading to endometriosis is the implantation
theory, followed by the theory of intrathecal metaplasia and induction theory, which may also be related
to genetic factors and immune inflammatory factors [4].

In eukaryotes, various in vivo and in vitro stressors cause protein damage which induces an evolutionally
conserved cellular protective mechanism, the heat shock response (HSR), to maintain protein stability.
The molecular chaperone heat shock factor 1 (HSF1) plays a central role in this process, helping to refold
or degrade intracellular proteins [5]. HSF1 also plays an important role in various fields of tumor biology,
promoting the occurrence and development of tumors and affecting the prognosis. HSF1 drives the
migration, invasion, and growth of cancer cells independent of anchor points, and HSF1 was reported to
be highly expressed in PC-3 cells for the first time in prostate cancer, other tumors such as colorectal
cancer, breast cancer, oral cancer, and liver cancer have also demonstrated high HSF1 expression [6].

6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) is a key enzyme of glycolysis that
belongs to a family of bio-functional proteins and is involved in fructose-2,6-bisphosphate synthesis and
degradation. The PFKFB3 gene is located on chromosome 10p15.1 and the 3′UTR with multiple AUUUA
copies, and PFKFB3 is widely expressed in tissues, particularly in solid tumors, proliferative tissues,
leukemic cells, and transformed cells [7]. As a key enzyme in glycolysis, PFKFB3 regulates glycolysis and
plays an important role in the development of most diseases [8]. However, the underlying mechanisms
regarding how PFKFB3 functions in endometriosis remain unclear.

Endometriosis is morphologically benign but clinically ethologically similar to tumors, such as
implantation, invasion, and distant metastasis. Therefore, we hypothesized that HSF1 also influenced the
development and progression of endometriosis because HSF1 was previously reported to be
overexpressed in endometriosis [9]. To test our hypothesis, we knocked out and overexpressed this gene
in cell experiments and constructed animal models, revealing that HSF1 influences the development and
progression of endometriosis. Our study provides a new idea for the future clinical treatment of
endometriosis through HSF1.

Materials And Methods

Cell culture

All cell lines were cultured in Dulbecco's Modified Eagle Medium / Ham's F-12 50/50 Mix (DMEM/F-12)
supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) with 100 µg.mL⁻¹ penicilin and 100 µg.mL⁻¹
streptomycin at 37°C and 5% CO₂.

SiRNA and transfection

The sequence of small interfering (si) RNAs against HSF1 was 5'- GCAGGUGUUCAGUCAGAA-3' [10].
The method of cell transfection is just as described previously [11].

Western blot
The cells are collected and lysed on ice with cell lysates for 30 minutes, shaking every 10 minutes, lysis buffer was centrifuged at 12000 rpm at 4 °C for 15 min, 5-loading buffer was added to the sample, and the protein was quantified and boiled for 10 min, the western blotting method is the same as in our previous paper [12].

**Quantitative real-time PCR**

The isolation of total RNA from cells and the synthesis of cDNA are described above [11]. Performing quantitative real-time PCR using SYBR Green PCR Master Mix (Takara) with CFX96 Real-Time PCR detection system (Bio-Rad, Shanghai, China).

**Cell proliferation assay**

Cells are pre-transfected with the correct plasmids, in a 24-well plate, 20,000 cells were planted in each well, repeated three times, and cell count was performed every 24 hr.

**Colony-formation assay**

Cells were planted on six-well plates, 500 cells per well, 2 ml cell culture medium was added and cultured at 37°C in 5% CO₂ for 10–14 days. After the clone was formed, the culture medium was removed and fixed at room temperature with 1 mL 4% paraformaldehyde for 15 min, after the fixation, the cells were stained with crystal violet for 20 min, PBS washed, dried and photographed.

**Wound healing assay**

The cells were inoculated in 6-well plates and cultured in medium until overgrown, the pipette tip was used to draw a fine line and washed with PBS, 100 times magnification under an optical microscope, original photographic record, after 24 hr, cells were photographed again.

**Glucose consumption and lactate production**

The transfected cells were replanted in a 6-well plate, and the culture medium was collected after 24 h to determine the concentration of glucose and lactic acid in the medium. Please refer to our previous article for the kit we used and the specific method [12].

**Animal experiments**

Animal experiments have been approved by ethics Committee of Weifang Medical University. Purchase 3–4 weeks age mice, One week after feeding, donor mice were injected with estradiol to promote endometrial development, one week later, the uterus of donor mice was cut into pieces and intraperitoneally injected into experimental and control mice (n = 7), the mouse model of endometriosis was established one week later, then the mice in the experimental group were injected intraperitoneal injection of HSF1 inhibitor KRIIB11, and the mice in the control group were injected with normal saline in the same amount, 2 times a week for one month. After injection, the mice were opened to observe the endometrial lesion.

**Tissue Collection and Immunohistochemistry**
All tissues were derived from our mouse model of endometriosis. The tissue sections were embedded in paraffin, dried and dewaxed with xylene. Please refer to our previous article for further detailed steps in immunohistochemistry [11].

**Statistical analysis**

All statistical analyses were used Graphpad Prism 5.0 software, and statistical analyses were presented as mean ± SEM, and performed by two-tailed unpaired Student's t-test. When p < 0.05, the difference is statistically significant. ∗p < 0.05, n.s.= not significant.

**Results**

**HSF1 promotes the proliferation, migration and clone formation of endometriosis**

Heat shock transcription factors (HSFs) are a family of proteins with broad homology in structure and function in eukaryotic cells and include HSF1, HSF2, HSF3, and HSF4. HSF1 is the main regulator of heat shock protein expression and a very highly conserved protective protein. Previous studies have shown that HSF1 plays an important role in tumorigenesis. Endometriosis and tumorigenesis share similar characteristics. To determine whether HSF1 plays a similar role in endometriosis, we conducted a series of experiments in endometrial epithelial cells Z11 and stromal cells ESCs. HSF1 overexpression significantly promoted the proliferation of both ESCs and Z11, indicating that HSF1 plays a significant role in endometriosis (Fig. 1A). Moreover, cell-scratch tests and clone formation experiments revealed that HSF1 overexpression promotes the growth of endometriosis cells (Fig. 1B and C). Furthermore, HSF1 knockdown in endometrial cells by transfecting HSF1 small interfering RNAs (si-RNAs) inhibited the growth of endometrial cells (Fig. 1D). HSF1 gene deletion also inhibited the proliferation and clone formation of endometriosis (Fig. 1E and F). These findings suggest that HSF1 positively regulates the proliferation, migration, and clone formation of endometriosis.

**HSF1 regulates glucose metabolism and lactic acid production in endometriosis**

Glucose is an essential energy source for the body's vital activities—that is, endometriosis requires high amounts of energy during its rapid metastasis and growth. Because this process consumes glucose, more lactic acid must be produced. To validate our hypothesis, we overexpressed HSF1 in both Z11 and ESCs. Interestingly, we found that HSF1 overexpression increased both glucose consumption and lactate production (Fig. 2A and B). Similarly, we knocked out HSF1 in both cell lines, revealing that the knockdown of HSF1 decreased glucose consumption and lactate production (Fig. 2A and B).

Subsequently, to verify whether the HSF1 inhibitor KRRBB11 could suppress glucose metabolism by inhibiting HSF1, we cultured both cell lines with KRRBB11 and then measured the concentration of glucose and lactic acid in the cell culture medium. KRRBB11 reduced the glucose consumption and lactic acid generation by inhibiting HSF1 in Z11 and ESCs (Fig. 2A and B). The above results show that, in Z11
and ESCs, HSF1 regulates the growth of endometriosis by increasing glucose consumption and lactic acid production.

**HSF1 promotes endometriosis by regulating PFKFB3**

Glycolysis is a common process that all organisms use to metabolize glucose. Moreover, the rapid growth of tumors and endometrial cells requires more energy than normal cells. In previous experiments, we confirmed that HSF1 promotes glucose consumption and lactic acid production in endometriosis. Therefore, we hypothesized that HSF1 regulation of endometriosis depended on key glycolytic enzymes and then analyzed three key enzymes involved in glycolysis, HK2, PKM2, and PFKFB3 by quantitative real-time PCR. The application of 30 minutes of heat shock in both Z11 and ESC cells to maximize HSF1 activation had little effect on the PKM2 and HK2 mRNA levels (Supplementary Fig. 1A and B), suggesting that HSF1 does not function through PKM2 and HK2. By treating cells with heat shock in a time-dependent manner, the expression levels of PFKFB3 increased with the increasing expression of HSF1 protein, and the mRNA levels of PFKFB3 were also increased in a concentration-dependent manner (Fig. 3A and B). In addition, the overexpression of HA-tagged HSF1 increased the levels of PFKFB3 protein and mRNA (Fig. 3C and D). Furthermore, HSF1 knockdown by si-RNA resulted in a decrease in the PFKFB3 protein and mRNA levels (Fig. 3E and F). Taken together, our results indicated that HSF1 promotes endometriosis by increasing the key glycolytic enzyme PFKFB3.

**KRIBB11 regulates endometriosis by inhibiting HSF1**

KRIBB11, a specific inhibitor of HSF1, effectively inhibits HSF1 activity, leading to cell cycle arrest in the G2/M phase, cell apoptosis, and inhibition of tumor cell proliferation [13]. Cells seeded onto twenty-four well plates were treated with increasing concentrations of KRIBB11, and the IC50 values of the two cell lines were measured (Fig. 4A). Regarding cell proliferation analysis, KRIBB11 inhibited the growth of endometrial epithelial and stromal cells (Fig. 4B). Clone formation experiments and cell-scratch tests indicated that KRIBB11 inhibited the clone formation and migration of endometrial cells (Fig. 4C and D). Western blotting showed that the PFKFB3 level was reduced after HSF1 inhibition by KRIBB11 (Fig. 4E). Thus, these data reveal that the HSF1-specific inhibitor KRIBB11 reduces the expression of the key glycolytic enzyme PFKFB3 by inhibiting HSF1 expression, and ultimately inhibits the proliferation of endometrial cells.

**KRIBB11 plays a therapeutic role in a mouse model of endometriosis**

To determine whether KRIBB11 regulates endometriosis in vivo, the endometrium of donor mice was cut up and intraperitoneally injected into recipient mice, and a model of endometriosis was established one week later. The control group was injected with normal saline, 2 times a week for 1 month, and the experimental group was injected with KRIBB11, 2 times a week for 1 month (Fig. 5A). Two days after the last injection, the mice were sacrificed, and the abdominal cavity was opened to observe the ectopic lesion. The experimental group showed endometriosis lesions, while only two cases in the control group showed lesions. Additionally, mice in the experimental group had lower body weights than those in the
control group (Fig. 5B). Ectopic lesions without KRIBB11 grew significantly faster than those in the experimental group, and the weight of ectopic lesions with KRIBB11 was substantially lower than that of ectopic lesions in the control group (Fig. 5C). We performed immunohistochemical staining of ectopic tissue collected from our mice, and HSF1 expression was significantly lower in the experimental mice (Fig. 5D). The above results indicate that the HSF1-specific inhibitor KRIBB11 plays a therapeutic role in the mouse model of endometriosis.

Discussion

Endometriosis is an age-related disease of the reproductive system, and its prevalence is up to 10% in premenopausal women worldwide. Its diagnosis is difficult because experienced obstetricians and gynecologists are required to correctly assess the clinical symptoms of the disease and assess the existence of ectopic endometrium in the abdominal cavity and pelvis [14]. Known hypotheses for endometriosis include embryonic origin, retrograde menstruation, and supraventricular metaplasia. However, many factors contribute to this complex pathogenesis, such as abnormal angiogenesis, an abnormal inflammatory response, estrogen dependence, genetic changes, and epigenetic changes, studies have shown that endometriosis is mainly affected by estrogen and progesterone. Estrogen promotes endometrial tissue proliferation, survival, and inflammation. However, the development, progression, infertility, and chronic pelvic pain of endometriosis are associated with progesterone resistance [15]. In recent years, more studies have been published on how to treat endometriosis. However, a radical cure is elusive, causing increased burdens to women of childbearing age and challenges to clinical work. Moreover, endometriosis has the characteristics of invasion and metastasis, very similar to tumor behavior, making the treatment of endometriosis a challenge clinically.

HSF1 is an evolutionarily conserved transcription factor that can respond to endogenous and exogenous cellular stresses by inducing HSP expression and ultimately maintaining intracellular protein stability. HSF1 responds to stress by up-regulating HSP27 and HSP40, however, HSP70 and HSP90 facilitate the refolding of misfolded proteins [16]. HSF1 can change the survival microenvironment of tumors, promoting their survival under harsh microenvironments and being related to their prognosis [17]. HSF1 plays an indispensable role in tumor migration, invasion, metastasis, proliferation, and cell metabolism. Therefore, HSF1 can be used as a tumor marker and a new therapeutic target [18]. Based on endometriosis showing invasion and metastasis characteristics similar to those of tumors, we suspect that HSF1 plays a similar role in the development of endometriosis. Our hypothesis was supported by the finding that HSF1 promoted endometriosis growth through a series of experiments, including cell counting, cloning, and cell scratching.

The catalytic conversion of F-6-P to F-1,6-BP by PFK-1 is the main rate-limiting step in glycolysis, and the homeostatic state of F-1,6-BP is regulated by the bifunctional enzyme PFKFB. PFKFB has two regulatory domains: the 2-Kase domain, which has kinase activity and phosphorylates F-6-P to F-2,6-BP, and the 2-Pase domain, which has phosphatase activity and dephosphorylates F-2,6-BP back to F-6-P (Fig. 5E). There are four members of the PFKFB family, among which PFKFB3 has the highest kinase/phosphatase
ratio and is a key enzyme in glycolysis. Therefore, many studies on glucose metabolism in cancer are based on PFKFB3, which has become a potential target for much drug development [19]. Because endometriosis cells must consume high amounts of energy in the process of migration and metastasis, glucose must be consumed. Additionally, when the effects of other key enzymes such as HK2 and PKM2 were excluded by real-time quantitative PCR, we found that HSF1 upregulated PFKFB3 expression to improve the glycolysis process, thus accelerating the development of endometriosis. Interestingly, these effects were reversed using the HSF1-specific inhibitor KRIBB11. KRIBB11 binds to HSF1 to prevent HSF1-dependent recruitment of p-TEFb to HSP70 promoters [20]. The effect of KRIBB11 was demonstrated both at the cellular level and in animal experiments.

Therefore, HSF1 is a potential target for the treatment of endometriosis. By increasing the expression of PFKFB3, the efficiency of glycolysis can be rapidly improved. Importantly, HSF1 regulates glucose metabolism through PFKFB3, ultimately influencing the development of endometriosis. However, the regulatory role of HSF1 in endometriosis is complex, other compensatory mechanisms may be present. Many factors must be considered in future drug development and clinical applications, and we have provided some insight into the role of HSF1 in endometriosis. Further studies are warranted, but we believe our study has uncovered a new pathway to treat endometriosis.

Conclusions

We have verified the important role of HSF1 in endometriosis through cell experiments and animal experiments, which will provide new ideas for the treatment of endometriosis in the future.

Declarations

Acknowledgments:

Not applicable.

Authors' contributions:

Z.Y. and C.R. designed research; Y.W. wrote and revised the paper. All authors read and approved the final manuscript.

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Ethics approval and consent to participate:

All procedures performed in this study involving were in accordance with the ethical standards of the institutional research committee of Weifang medical university.
Consent for publication:

Not applicable.

Competing interests:

The authors declare that they have no competing interests.

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**Figures**
KRBEB11 inhibits the progression of endometriosis in mice (A) Mice aged 3-4 weeks were purchased, estradiol benzoate was diluted with oil and injected intramuscularly into the thigh of mice, 3μg/mouse, 2 times for 1 week, one week later, the donor endometrium was cut into pieces and the recipient mice were intraperitoneally injected with the fragments, endometriosis model was established one week after growth. Mice in the control group were intraperitoneally injected with normal saline, 2 times a week for 1 month (n=7). The mice in the experimental group were injected with the HSF1 inhibitor KRBEB11, 2 times a week for 1 month (n=7). (B) After the mice were sacrificed, the abdominal cavity was opened to observe the endometriosis tissue, the weight of mice was statistically analyzed (*p<0.05). (C) The size of the ectopic tissue was observed and heterotopic tissues were weighed and analyzed statistically (*p<0.05). (D) Immunohistochemical staining was performed on the heterotopic tissues of mice in the control group and the experimental group, the expression level of HSF1 in the control group and the experimental group was observed by microscope (Scale bars, 50 μm). (E) HSF1 promote the efficiency of glycolysis by up-regulating the expression of key glycolysis enzyme PFKFB3, thus promoting the proliferation of endometriosis.