HSF1 promotes endometriosis development and glycolysis by up-regulating PFKFB3 expression

Yixin Wang  
Weifang Medical University

Jing Xiu  
Weifang Medical University

Tingting Yang  
Weifang Medical University

Chune Ren  
Weifang Medical University

Zhenhai Yu (✉ tomsyu@163.com)  
Weifang Medical University  https://orcid.org/0000-0001-8928-6890

Research

**Keywords:** HSF1, KRIBB11, PFKFB3, Glycolysis, Endometriosis

**DOI:** https://doi.org/10.21203/rs.3.rs-134073/v2

**License:** ☕️ This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

Background Endometriosis is a chronic hormonal inflammatory disease characterized by the presence of endometrial tissue outside the uterus. Endometriosis often causes infertility, which affects the body and mind of patients and their families.

Methods We examined the functions of heat shock factor 1 (HSF1) in endometriosis development through cell count, scratch and clone formation experiments. We used quantitative real-time PCR (qRT-PCR) and Western blot (WB) to detect the functions of HSF1 in endometriosis cells. Glucose and lactate levels were determined using a glucose (GO) assay kit and a lactate assay kit. Furthermore, we established a mouse model of endometriosis by using a HSF1 inhibitor-KRIBB11.

Results Our study demonstrated that HSF1 was highly expressed in endometriosis, and promoted endometriosis development. Interestingly, we found that HSF1 promoted glycolysis in endometriosis cells. Further, HSF1 enhanced glycolysis by up-regulating PFKFB3 in endometriosis cells, which was a key enzyme in glucose metabolism. Moreover, the HSF1 inhibitor KRIBB11 could abrogate endometriosis progression in vivo and in vitro.

Conclusions Findings indicate that HSF1 plays an important role in the development of endometriosis, which might become a new target for the treatment of endometriosis and provide a new idea for the clinical treatment of endometriosis.

Electronic supplementary material Supplementary data are available.

Background

Endometriosis is a disease with features of chronic inflammation, and it is defined as the functional endometrial stroma and glands outside the uterine cavity [1]. 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 with endometriosis develop the disease, and the infertility rate is as high as 50%, seriously affecting the physical health of these women [2]. Endometriosis is mainly affected by estrogen and progesterone, which promotes endometrial tissue proliferation, survival, and inflammation [3]. Further, the development, progression, infertility, and chronic pelvic pain of endometriosis are associated with progesterone resistance [4]. The most common theory leading to endometriosis is the implantation theory, which may also be related to genetic factors and immune inflammatory factors [5, 6]. However, there is still no clear treatment for endometriosis.

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 [7]. The molecular chaperone heat shock factor 1 (HSF1) plays a central role in this process, helping to refold or degrade intracellular proteins [8]. 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 [9]. HSF1 also plays an important role in various fields of tumor biology, promoting the occurrence and development of tumors and affecting the prognosis [10]. For example, HSF1 is highly expressed in prostate cancer PC-3 cells, and plays its functions by increasing levels of its downstream effector HSP27 [11]. Other tumors such as colorectal cancer, breast cancer, oral cancer, and liver cancer have also demonstrated high HSF1 expression [7]. Furthermore, HSF1 can change the survival microenvironment of tumors, promoting their survival under harsh microenvironments and being related to their prognosis [12]. Therefore, HSF1 can be used as a tumor marker and a new therapeutic target. However, the roles of HSF1 in endometriosis are still largely unknown.

In glycolysis process, there is a key enzyme called 6-Phosphofructo-2-kinase/Fructose-2, 6-Biphosphatase 3 (PFKFB3), which belongs to a family of bio-functional proteins and is involved in fructose-2, 6-bisphosphate synthesis and degradation [13]. There are four members of the PFKFB family, but PFKFB3 has the highest kinase/phosphatase ratio in glycolysis [14]. So many studies on glucose metabolism in cancer are based on PFKFB3, which has become a potential target for much drug development [15]. PFKFB3 is widely expressed in tissues, particularly in solid tumors, proliferative tissues, leukemic cells, and transformed cells [13]. As a key enzyme in glycolysis, PFKFB3 regulates glycolysis and plays an important role in the development of many diseases [16]. However, the underlying mechanisms of PFKFB3 functions in endometriosis remain unclear.

Morphologically, endometriosis is a benign disease, but it has some clinical characteristics similar to the tumor process, such as implantable, invasive, and distant metastasis. Moreover, HSF1 was previously reported to be overexpressed in endometriosis [17]. Therefore, we hypothesized that HSF1 also regulated the development of endometriosis. To test this hypothesis, we manipulated HSF1 expression in endometriosis cells, and used a constructed mouse model, which suggested that HSF1 influenced the development of endometriosis. Our study provides a new idea for the clinical treatment of endometriosis by targeting HSF1.

Materials And Methods

Cell culture and antibodies

The endometriotic epithelial cell line (11Z) was established by Professor Anna Strazinski-Powitz [18]. The human endometrial stromal cell line (ESC) was established by Dr. Krikun [19]. 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 100mg/mL penicilin and 100mg/mL streptomycin at 37°C and 5% CO₂.

Mouse anti-β-actin (A1978) was from Sigma-Aldrich, and dilution: 1:5000. Mouse anti-HSF1 (sc-17757) was from Santa Cruz, and dilution: 1:1000. Rabbit anti-PFKFB3 (ab181861) was purchase from ABCAM,
and dilution: 1:2000. KRB11 were obtained from Med Chem Express (MCE), 50mg/kg.

**SiRNA and transfection**

The sequence of small interfering (si) RNAs against HSF1 was 5’- GCAGGUUGUCAUAGUCAGAA-3’. The sequence of siRNA-NC (Negative Control) was 5’-UUCUCCGAACGGUCAGCU-3’ [20]. The method was performed as described previously [21].

**Western blot**

The indicated cells were collected and lysed on ice using lysis buffer, and were centrifuged at 12000rpm at 4°C for 15min. Then, 5’loading buffer was added to the sample, and boiled for 10min. The western blot method was performed as described previously [22].

**Quantitative real-time PCR**

The isolation of total RNA from cells and the synthesis of cDNA were described above [23]. 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**

The indicated cells were transfected with the indicated plasmids, and reseeded in 24-well plates. The cell numbers were counted every 24hr for 4 days [22].

**Colony-formation assay**

The 500 indicated cells were seeded in six-well plates, and cultured at 37°C in 5% CO₂ for 10-14 days. After the clones were formed, the culture medium was removed and fixed at room temperature with 4% paraformaldehyde for 15min. After the fixation, the cells were stained with crystal violet, and then photographed [24].

**Wound healing assay**

The indicated 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. After 24hr, cells were photographed again [25].

**Glucose consumption and lactate production**

The indicated cells were seeded in 6-well plates, and the culture mediums were collected after 24hr to determine the concentration of glucose and lactic acid. The methods were performed as described previously [22, 24].

**Animal experiments**
Animal experiments have been approved by ethics Committee of Weifang Medical University. We used 5-week-old BALB/c female mice, and the donor mice (n=5) were injected with estradiol benzoate to promote endometrial development. Estradiol benzoate was diluted with oil and injected intramuscularly into the thigh of donor mice, 3mg/mouse, 2 times for one week. After one week, the uterus of donor mice was cut into pieces and intraperitoneally injected into experimental (n=7) and control mice (n=7). After one week, the mice in the experimental group were intraperitoneally injected 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. Then, the mice were sacrificed to observe the endometrial lesion.

Tissue Collection and Immunohistochemistry

All tissues were derived from mice model of endometriosis. The sections were embedded in paraffin, dried and dewaxed with xylene. The immunohistochemistry was performed as described previously \[21\]. The immunostaining intensity was quantified using the Image J \[26\].

Statistical analysis

All statistical analyses were used Graphad Prism 5.0 software. The statistical analyses were presented as mean ± SEM, and performed by two-tailed unpaired Student’s t-test. P<0.05 is significant (*p<0.05). n.s. = not significant.

Results

HSF1 promotes the cell proliferation, cell migration and clone formation in endometriosis cells

Endometriosis and tumorigenesis share similar characteristics, and previous studies have shown that HSF1 plays an important role in tumorigenesis \[27\]. To determine whether HSF1 plays a similar role in endometriosis, we performed a series of experiments in endometriosis cells. HSF1 overexpression significantly promoted the cell proliferation, indicating that HSF1 played a significant role in endometriosis (Fig. 1A). Moreover, cell-scratch tests and clone formation experiments revealed that HSF1 overexpression promoted the cell migration and growth in endometriosis cells (Fig. 1B and C). Furthermore, HSF1 knockdown inhibited the growth of endometriosis cells (Fig. 1D and F), and also inhibited the cell migration (Fig. 1E). These findings suggest that HSF1 positively regulates the cell proliferation and migration in endometriosis cells.

HSF1 enhances glycolysis in endometriosis cells

The endometriosis cells require high glycolysis during its rapid metastasis and growth \[28\]. To validate the effects of HSF1 on glycolysis, we overexpressed or knocked down HSF1 in endometriosis cells. Interestingly, we found that HSF1 could increase both glucose consumption and lactate production (Fig. 2A and B). Subsequently, to determine whether the HSF1 inhibitor KRIIB11 could suppress glucose metabolism, we cultured endometriosis cells with KRIIB11. As we expected, KRIIB11 reduced the
glucose consumption and lactic acid generation in endometriosis cells (Fig. 2A and B). These data show that HSF1 enhances glycolysis in endometriosis cells.

**HSF1 promotes PFKFB3 expression in endometriosis cells**

In previous studies, we confirmed that HSF1 promoted glycolysis in endometriosis cells. Therefore, we hypothesized that HSF1 regulation glycolysis may depend on key glycolytic enzymes. By treating cells with heat shock in a time-dependent manner, the expression levels of PFKFB3 were increased (Fig. 3A and B). But HSF1 activation had little effect on the *PKM2* and *HK2* expressions (Supplementary Fig. 1A and B). In addition, overexpression HSF1 increased the expression of PFKFB3 (Fig. 3C and D). Furthermore, HSF1 knockdown resulted in a decrease in the expression of PFKFB3 (Fig. 3E and F). Taken together, our results indicate that HSF1 promotes PFKFB3 expression in endometriosis cells.

**KRIBB11 inhibits endometriosis cell growth by targeting 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 [29]. The Cells were seeded onto 24-well plates were treated with increasing concentrations of KRIBB11, and the IC$_{50}$ values of the two cell lines were measured (Fig. 4A). As we expected, KRIBB11 inhibited the growth of endometriosis cells (Fig. 4B and C). Cell-scratch tests indicated that KRIBB11 inhibited the migration of endometrial cells (Fig. 4D). Moreover, western blot showed that the PFKFB3 protein 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 endometriosis cells.

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

To determine whether KRIBB11 regulates endometriosis *in vivo*, the endometria of donor mice were cut up and intraperitoneally injected into recipient mice, and a mouse model of endometriosis was established after one week (Fig. 5A). Two days after the last injection, the mice were sacrificed, and the abdominal cavity was opened to observe the ectopic lesion. Interestingly, the endometriosis tissues were observed in all control mice, but only two in seven experimental mice (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 mice, and HSF1 expression was significantly lower in the mice with KRIBB11 (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 [6]. The diagnosis of endometriosis is difficult, because experienced
obstetricians and gynecologists are required to assess the clinical symptoms of the disease and assess the existence of ectopic endometrium in the abdominal cavity and pelvis [30]. In recent years, more studies have been published on how to treat endometriosis. However, the treatment of endometriosis is still a challenge in clinical, which causes increased burdens to women of childbearing age. Moreover, endometriosis has the characteristics of invasion and metastasis, which is similar to tumor behavior. HSF1 is an oncogene to promote tumor progress, so we speculate that HSF1 plays a similar role in the development of endometriosis. Our hypothesis is supported by the finding that HSF1 promotes endometriosis development through a series of experiments, including glycolysis, cell counting, cloning, and cell scratching.

Because endometriosis cells must get more energy to support rapid cell proliferation, glycolysis must be enhanced. As we expected, we find that HSF1 up-regulates PFKFB3 expression to promote glycolysis, thus accelerating the development 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. Interestingly, these effects are abrogated using the HSF1-specific inhibitor KRIBB11, which bind to HSF1 to prevent HSF1-dependent recruitment of p-TEFb to HSP70 promoters [31]. The effects of KRIBB11 are demonstrated in vitro and in vivo. Therefore, HSF1 is a potential target for the treatment of endometriosis. However, the regulatory role of HSF1 in endometriosis is still elusive. Other mechanisms may be studied in the future. Our findings provide some new insights into the functions of HSF1 in endometriosis, which identifies a new pathway to treat endometriosis (Fig. 5E).

Conclusions

We have verified the important roles of HSF1 in endometriosis, which will provide new ideas for the treatment of endometriosis in the future.

Abbreviations

**HSF1:** Heat shock factor 1  
**HSR:** Heat shock response  
**HSP:** Heat shock protein  
**PCR:** Polymerase chain reaction  
**qRT-PCR:** Quantitative real-time PCR  
**WB:** Western blot  
**PFKFB3:** 6-Phosphofructo-2-kinase/Fructose-2, 6-Biphosphatase 3
DMEM/F-12: Dulbecco's Modified Eagle Medium/Ham's F-12 50/50 Mix

FBS: Fetal bovine serum

RNA: Ribonucleic acid

siRNA: Small interfering RNA

DNA: Deoxyribonucleic acid

cDNA: Complementary DNA

PBS: Phosphate buffer saline

PKM2: pyruvate kinase 2

HK2: Hexokinase 2

IC$_{50}$: 50% inhibiting concentration

p-TEFb: Positive transcription elongation factor b

SEM: Standard error of the means

SD: Standard deviation

n.s.: Not significant

Declarations

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.

Availability of supporting data

The data used in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.
Funding

The study was supported by research grants from National Natural Science Foundation of China (Grant no. 81972489) and National Natural Science Foundation of Shandong Province (Grant no. ZR2020YQ58).

Authors’ contributions

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

Acknowledgments

Not applicable.

Authors’ information

Affiliations

Department of Reproductive Medicine, Affiliated Hospital of Weifang Medical University, Weifang, 261000, Shandong Province, P.R. China.

Yixin Wang, Jing Xiu, Tingting Yang, Chune Ren and Zhenhai Yu

Corresponding author

Correspondence to Chune Ren and Zhenhai Yu.

References

1. Mehedintu C, Plotogea MN, Ionescu S, Antonovici M: Endometriosis still a challenge. J Med Life 2014, 7:349-357.

2. Giudice LC: Clinical practice. Endometriosis. N Engl J Med 2010, 362:2389-2398.

3. Olsarova K, Mishra GD: Early life factors for endometriosis: a systematic review. Hum Reprod Update 2020, 26:412-422.

4. Chen H, Malentacchi F, Fambrini M, Harrath AH, Huang H, Petraglia F: Epigenetics of Estrogen and Progesterone Receptors in Endometriosis. 2020, 27:1967-1974.

5. Vercellini P, Viganò P, Somigliana E, Fedele L: Endometriosis: pathogenesis and treatment. Nat Rev Endocrinol 2014, 10:261-275.

6. Wang Y, Nicholes K, Shih IM: The Origin and Pathogenesis of Endometriosis. Annu Rev Pathol 2020, 15:71-95.

7. Dong B, Jaeger AM, Thiele DJ: Inhibiting Heat Shock Factor 1 in Cancer: A Unique Therapeutic Opportunity. Trends Pharmacol Sci 2019, 40:986-1005.
8. Kovács D, Sigmond T, Hotzi B, Bohár B, Fazekas D, Deák V, Vellai T, Barna J: HSF1Base: A Comprehensive Database of HSF1 (Heat Shock Factor 1) Target Genes. 2019, 20.

9. Wang G, Cao P, Fan Y, Tan K: Emerging roles of HSF1 in cancer: Cellular and molecular episodes. Biochim Biophys Acta Rev Cancer 2020, 1874:188390.

10. Dai C, Sampson SB: HSF1: Guardian of Proteostasis in Cancer. Trends Cell Biol 2016, 26:17-28.

11. Hoang AT, Huang J, Rudra-Ganguly N, Zheng J, Powell WC, Rabindran SK, Wu C, Roy-Burman P: A novel association between the human heat shock transcription factor 1 (HSF1) and prostate adenocarcinoma. Am J Pathol 2000, 156:857-864.

12. Grunberg N, Levi-Galibov O, Scherz-Shouval R: The Role of HSF1 and the Chaperone Network in the Tumor Microenvironment. Adv Exp Med Biol 2020, 1243:101-111.

13. Shi L, Pan H, Liu Z, Xie J, Han W: Roles of PFKFB3 in cancer. Signal Transduct Target Ther 2017, 2:17044.

14. Yi M, Ban Y, Tan Y, Xiong W, Li G, Xiang B: 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 and 4: A pair of valves for fine-tuning of glucose metabolism in human cancer. Mol Metab 2019, 20:1-13.

15. Wang Y, Qu C, Liu T, Wang C: PFKFB3 inhibitors as potential anticancer agents: Mechanisms of action, current developments, and structure-activity relationships. Eur J Med Chem 2020, 203:112612.

16. De Bock K, Georgiadou M, Schoors S, Kuchnio A, Wong BW, Cantelmo AR, Quaegebeur A, Ghesquière B, Cauwenberghs S, Eelen G, et al: Role of PFKFB3-driven glycolysis in vessel sprouting. Cell 2013, 154:651-663.

17. Stephens AN, Hannan NJ, Rainczuk A, Meehan KL, Chen J, Nicholls PK, Rombauts LJ, Stanton PG, Robertson DM, Salamonsen LA: Post-translational modifications and protein-specific isoforms in endometriosis revealed by 2D DIGE. J Proteome Res 2010, 9:2438-2449.

18. Gaetje R, Kotzian S, Herrmann G, Baumann R, Starzinski-Powitz A: Nonmalignant epithelial cells, potentially invasive in human endometriosis, lack the tumor suppressor molecule E-cadherin. Am J Pathol 1997, 150:461-467.

19. Krikun G, Mor G, Alvero A, Guller S, Schatz F, Sapi E, Rahman M, Caze R, Qumsiyeh M, Lockwood CJ: A novel immortalized human endometrial stromal cell line with normal progestational response. Endocrinology 2004, 145:2291-2296.

20. Yang T, Ren C, Lu C, Qiao P, Han X, Wang L, Wang D, Lv S, Sun Y, Yu Z: Phosphorylation of HSF1 by PIM2 Induces PD-L1 Expression and Promotes Tumor Growth in Breast Cancer. Cancer Res 2019, 79:5233-5244.

21. Ren C, Yang T, Qiao P, Wang L, Han X, Lv S, Sun Y, Liu Z, Du Y, Yu Z: PIM2 interacts with tristetraprolin and promotes breast cancer tumorigenesis. Mol Oncol 2018, 12:690-704.

22. Yang T, Ren C, Qiao P, Han X, Wang L, Lv S, Sun Y, Liu Z, Du Y, Yu Z: PIM2-mediated phosphorylation of hexokinase 2 is critical for tumor growth and paclitaxel resistance in breast cancer. Oncogene 2018, 37:5997-6009.
23. Lu C, Ren C, Yang T, Sun Y, Qiao P, Wang D, Lv S, Yu Z: A Noncanonical Role of Fructose-1, 6-Bisphosphatase 1 Is Essential for Inhibition of Notch1 in Breast Cancer. *Mol Cancer Res* 2020, **18**:787-796.

24. Han X, Ren C, Yang T, Qiao P, Wang L, Jiang A, Meng Y, Liu Z, Du Y, Yu Z: Negative regulation of AMPKalpha1 by PIM2 promotes aerobic glycolysis and tumorigenesis in endometrial cancer. *Oncogene* 2019, **38**:6537-6549.

25. Lu C, Ren C, Yang T, Sun Y, Qiao P, Han X, Yu Z: Fructose-1, 6-bisphosphatase 1 interacts with NF-kappaB p65 to regulate breast tumorigenesis via PIM2 induced phosphorylation. *Theranostics* 2020, **10**:8606-8618.

26. Han SJ, Jung SY, Wu SP, Hawkins SM, Park MJ, Kyo S, Qin J, Lydon JP, Tsai SY, Tsai MJ, et al: Estrogen Receptor beta Modulates Apoptosis Complexes and the Inflammasome to Drive the Pathogenesis of Endometriosis. *Cell* 2015, **163**:960-974.

27. Gomez-Pastor R, Burchfield ET, Thiele DJ: Regulation of heat shock transcription factors and their roles in physiology and disease. *Nat Rev Mol Cell Biol* 2018, **19**:4-19.

28. Qi X, Zhang Y, Ji H, Wu X, Wang F, Xie M, Shu L, Jiang S, Mao Y, Cui Y, Liu J: Knockdown of prohibitin expression promotes glucose metabolism in eutopic endometrial stromal cells from women with endometriosis. *Reprod Biomed Online* 2014, **29**:761-770.

29. Yoon YJ, Kim JA, Shin KD, Shin DS, Han YM, Lee YJ, Lee JS, Kwon BM, Han DC: KIRRIB11 inhibits HSP70 synthesis through inhibition of heat shock factor 1 function by impairing the recruitment of positive transcription elongation factor b to the hsp70 promoter. *J Biol Chem* 2011, **286**:1737-1747.

30. Malvezzi H, Marengo EB, Podgaec S, Piccinato CA: Endometriosis: current challenges in modeling a multifactorial disease of unknown etiology. *J Transl Med* 2020, **18**:311.

31. Kijima T, Prince T, Neckers L, Koga F, Fujii Y: Heat shock factor 1 (HSF1)-targeted anticancer therapeutics: overview of current preclinical progress. *Expert Opin Ther Targets* 2019, **23**:369-377.