HSF1 promotes endometriosis development and glycolysis 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 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% CO_2.

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. KRIBB11 were obtained from Med Chem Express (MCE), 50mg/kg.

SiRNA and transfection

The sequence of small interfering (si) RNAs against HSF1 was 5'- GCAGGUUGUUCAUAGUCAGAA-3'. The sequence of siRNA-NC (Negative Control) was 5'-UUCUCCGAACGGUCACGU-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℃ 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℃ in 5% CO_2 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 KRIBB11, 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 KRIBB11 could suppress glucose metabolism, we cultured endometriosis cells with KRIBB11. As we expected, KRIBB11 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 devistion

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.

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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.

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Figures

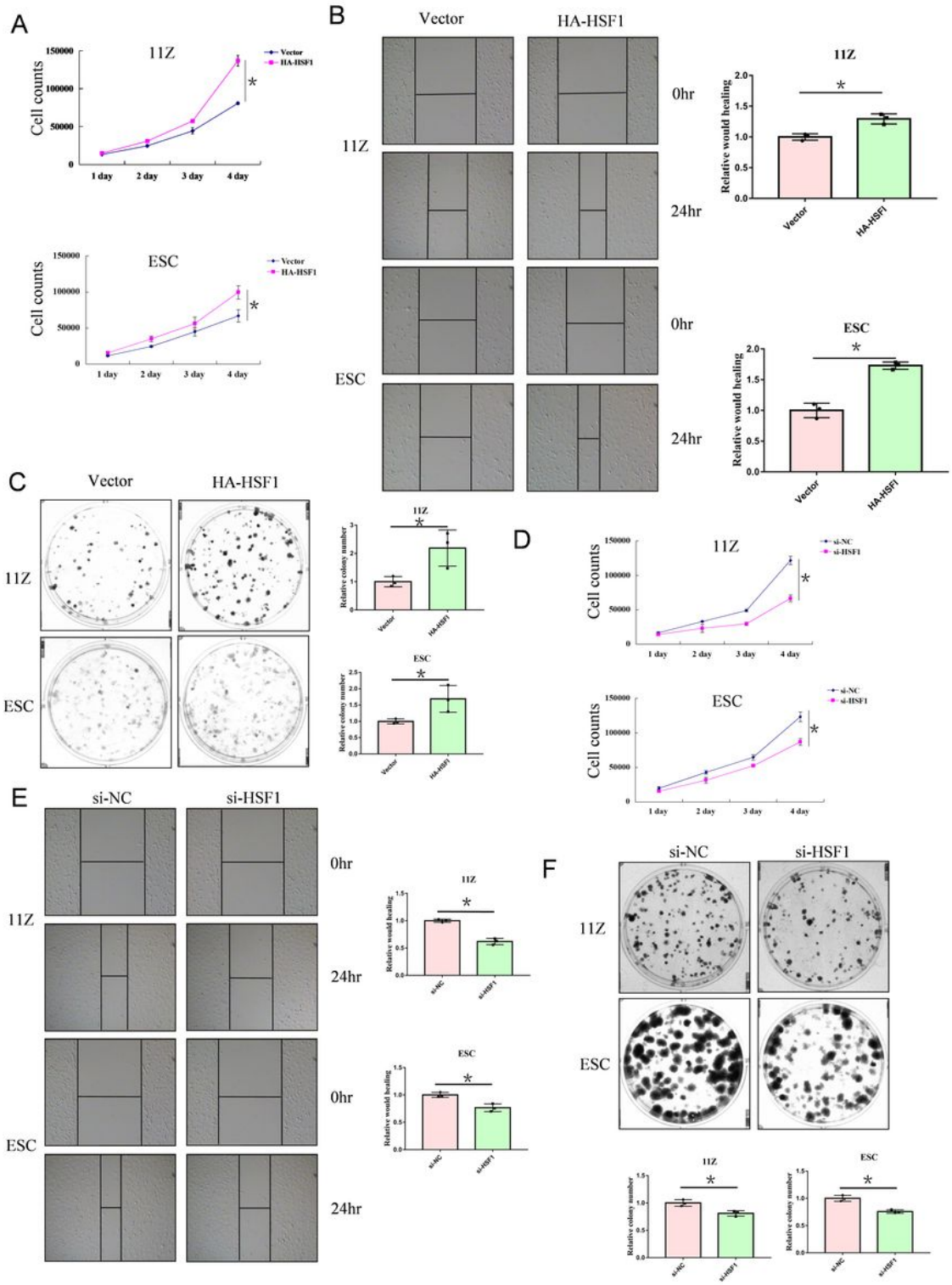

Figure 1

HSF1 promotes the cell proliferation, cell migration and clone formation in endometriosis cells. (A) 11Z and ESC cells were transfected with HA-tagged HSF1 or empoty vector. After one day, cells were re-plated in 24-well plates, and cell counts were performed every 24hr to analyses cell growth. (B) 11Z and ESC cells were transfected with HA-tagged HSF1 or empoty vector. After one day, cells were re-plated in 6-well plates to perform scratch test assay. (C) 11Z and ESC cells were transfected with HA-tagged HSF1 or

empoty vector. After one day, cells were re-plated in 6-well plates, and were cultured for 10-14 days to observe the cell clone formation. (D-F) 11Z and ESC cells were transfected with siRNA-HSF1 or NC. Cell counting, scratching, and cloning were performed. All date are mean ± SD of three independent experiments (*P<0.05).

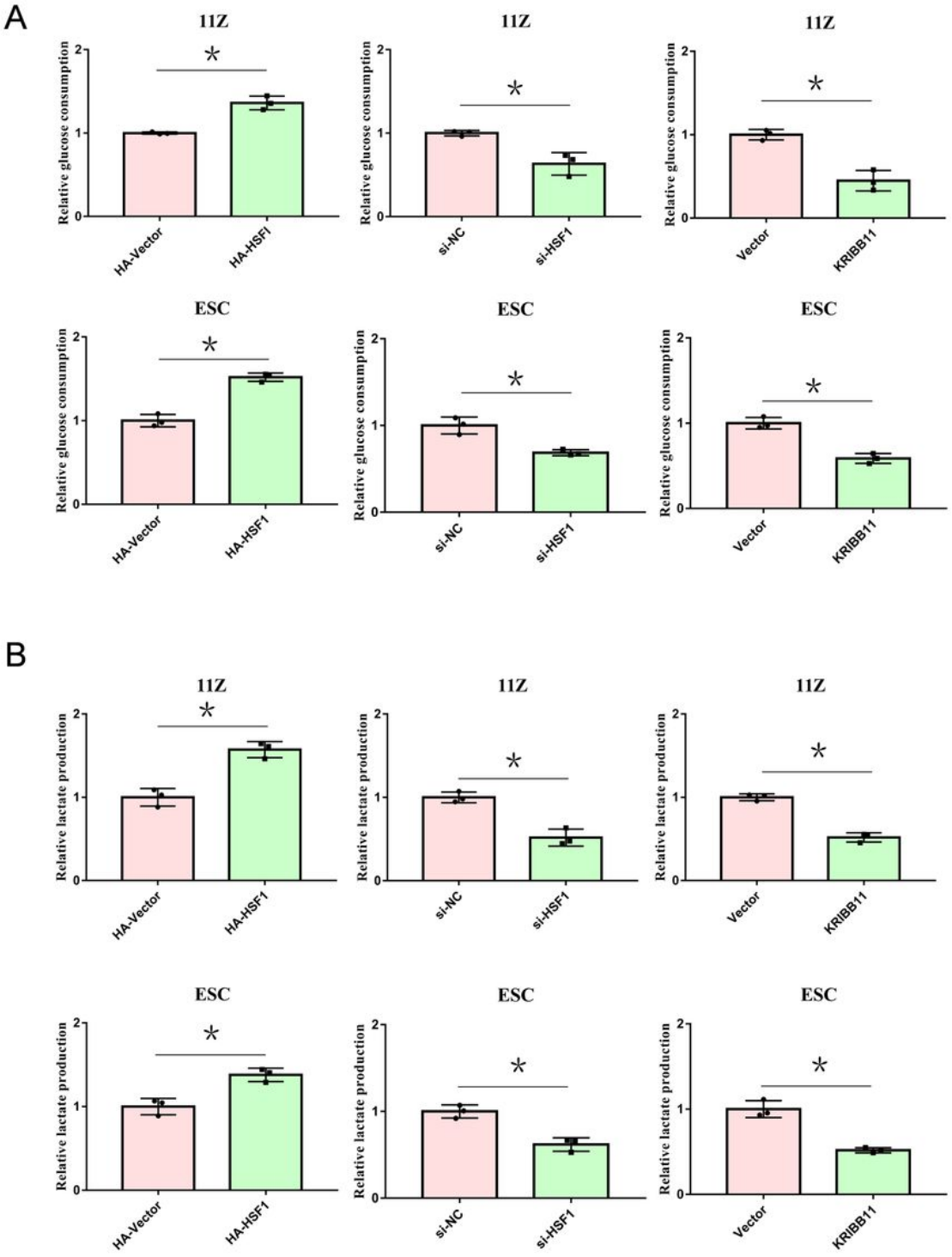

Figure 2

HSF1 enhances glycolysis in endometriosis cells. (A, B) 11Z and ESC cells were transfected with HA-tagged HSF1 or empoty vector, siRNA or NC. Cells were re-plated in 6-well plates. After 24hr, glucose and lactic acid concentrations in culture medium were determine using glucose and lactic acid kits. 11Z and ESC cells were cultured with KRIBB11 for 24hr, and the concentration of glucose and lactic acid in the super-medium was determined. All date are mean ± SD of three independent experiments (*P<0.05).

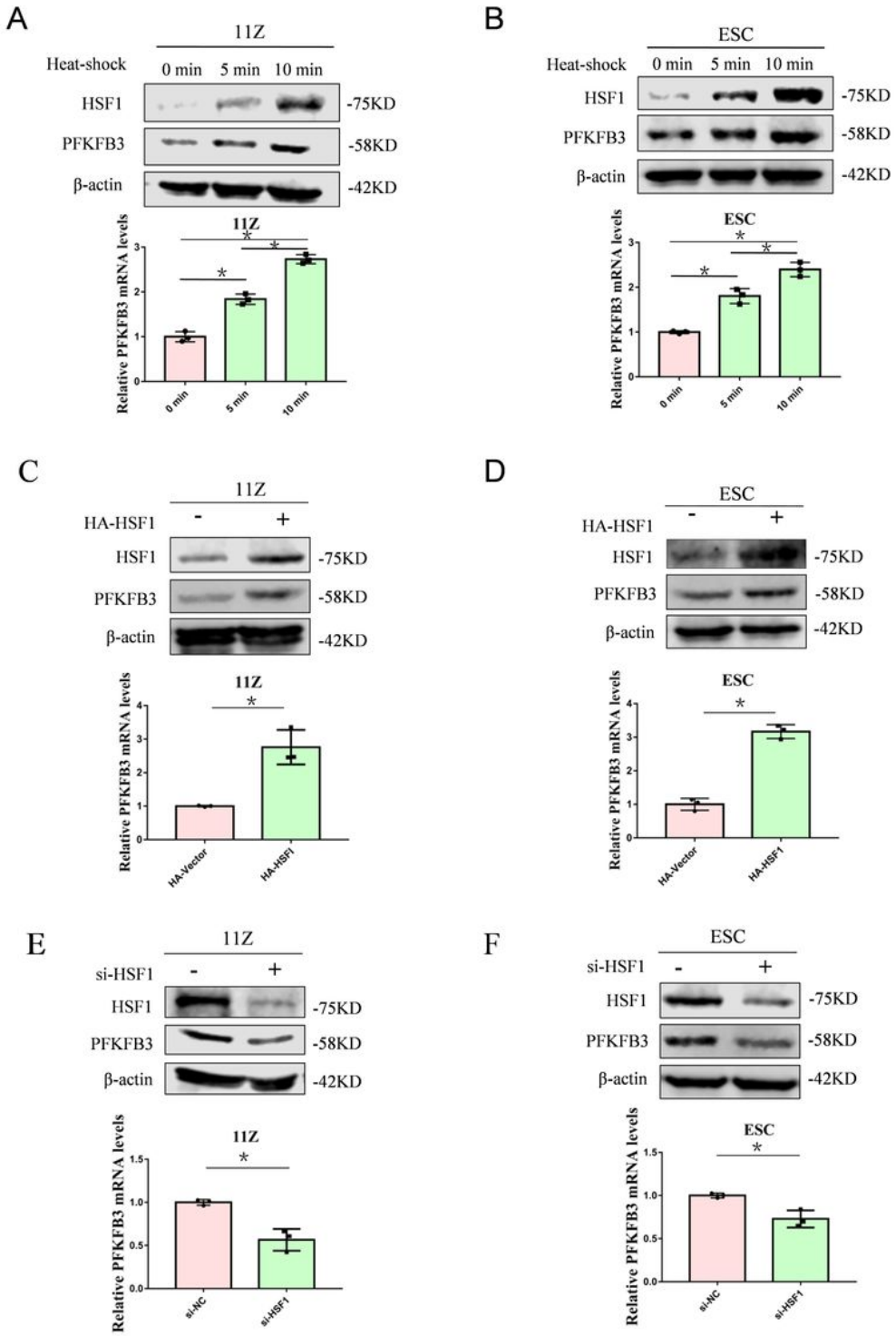

Figure 3

HSF1 promotes PFKFB3 expression in endometriosis cells. (A, B) 11Z and ESC cells were heat shocked in a time-dependent manner. The expression of PFKFB3 was determined by Western blot and qRT-PCR. (C, D) 11Z and ESC cells were transfected with HA-tagged HSF1 or empoty vector, and the expressions of PFKFB3 were detected. (E, F) 11Z and ESC cells were transfected with siRNA-HSF1 or NC. The expressions of PFKFB3 were detected after 2 days. All date are mean ± SD of three independent experiments (*P<0.05).

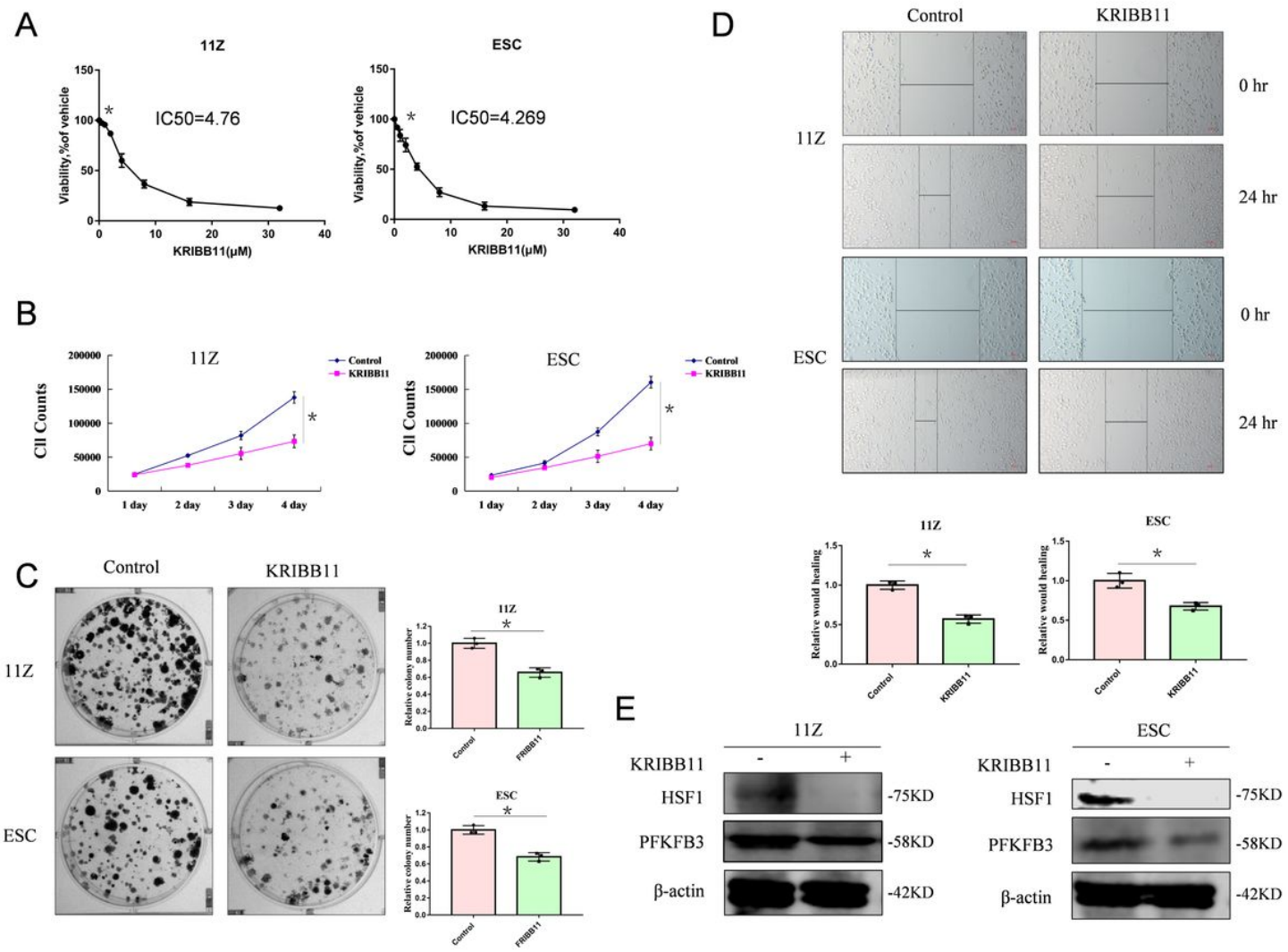

Figure 4

KRIBB11 inhibits endometriosis cell growth by targeting HSF1 A) 11Z and ESC cells were plated in 24-well plates. KRIBB11 was given in a concentration-dependent manner, and the IC50 value of the drug was measured by cell counting. (B) 11Z and ESC cells were plated in 24-well plates, and the experimental group treated with HSF1 inhibitor KRIBB11. The cell counts were performed every 24hr for 4 days. (C) 11Z and ESC cells were plated in 6-well plates, and the experimental group treated with HSF1 inhibitor KRIBB11. After 10-14 days, the cell clones were analyzed. (D) 11Z and ESC cells were plated in 6-well plates, and the scratches were made by pipette tip. Experimental group was treated with HSF1 inhibitor KRIBB11. After 24hr, the wound healing was analyzed. (E) 11Z and ESC cells were plated in 6-well plates.

The experimental group was treated with HSF1 inhibitor KRIBB11. After 24hr, the cells are lysed for westen blot. All date are mean±SD of three independent experiments (*P<0.05).

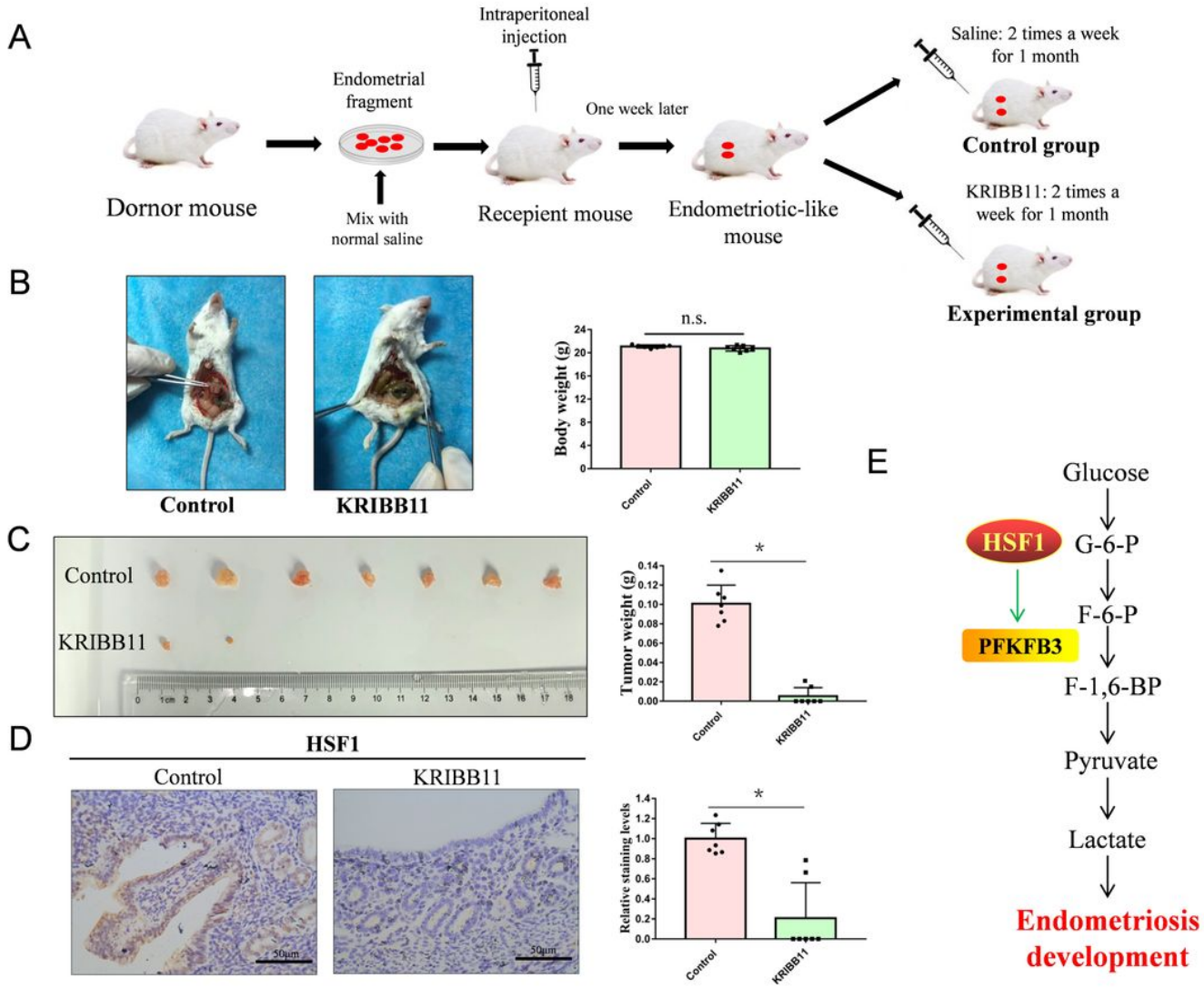

Figure 5

KRIBB11 plays a therapeutic role in a mouse model of endometriosis. (A) Endometriosis model was established using 5-week-old BALB/c female mice. (B) Mice were sacrificed, the endometriosis tissues and the weight of mice were analyzed (*P<0.05). (C) The size of the ectopic tissues was observed and heterotopic tissues were weighed (*P<0.05). (D) Immunohistochemical staining was performed to determine the HSF1 expression in the control group and the experimental group (Scale bars, 50μm). Quantitative analyses of HSF1 expression was performed. (E) HSF1 promoted glycolysis by up-regulating the expression of PFKFB3, which induced the development of endometriosis.

Supplementary Files

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- FigureS1.pdf