Down-Regulating Hexokinase 2 Inhibits Proliferation of Endometrial Stromal Cells Through a Noncanonical Pathway Involving Phosphorylated-STAT1 in Endometriosis

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Research

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Abstract

Background: Endometriosis is a benign gynecologic disease that causes chronic pelvic pain, dysmenorrhea and infertility and shares several characteristics with malignant tumors, afflicting women of reproductive age. Hexokinase 2 (HK2) plays a pivotal role as the first rate-limiting enzyme in the metabolic glycolysis pathway, and its abnormal elevation in tumors is associated with tumor genesis and metastasis. However, the expression and role of HK2 in endometriosis remain unclear.

Methods: We sequenced the primary endometrial stromal cells from patients with endometrioma and adopted immunohistochemistry, quantitative real-time PCR and western blot to determine the expression of HK2. Then wound healing assays, cell invasion assays, cell proliferation assays were performed to explore the functions of HK2 in endometrial stromal cells. Furthermore, mice models of endometriosis were recruited to observe the effects of HK2 inhibitors in vivo. Lastly, glycolysis metabolism detection and transcriptome sequencing were carried out in HK2-knockdown endometrial stromal cells to analyze the mechanism of HK2 affecting cell function.

Results: Endometriotic stromal cells displayed active glycolysis metabolism and elevated expression of HK2. Downregulating HK2 reduced the migration, invasion and proliferation capacity of endometrial stromal cells. Knockdown of HK2 induced upregulation of signal transducer and activator of transcription 1 (STAT1) and their phosphorylation to attenuate the proliferation of endometrial stromal cells.

Conclusions: HK2 was associated with the migration, invasion and proliferation of endometrial stromal cells, which might provide new insights into the pathogenesis and treatment of endometriosis.

Background

Endometriosis is one of the most common gynecologic diseases, characterized by ectopic growth of the endometrium. It afflicts approximately 6%-10% of reproductive-age women (1) with manifestations involving chronic pelvic pain, dysmenorrhea, infertility, and malignant features such as invasion and metastasis (2). However, the pathogenesis has not been elucidated yet (3). Besides the widely accepted retrograde menstruation hypothesis (4), factors from genetics (5, 6), immunology (7), endocrinology (8), hypoxic environment and metabolism (9) might contribute to the pathogenesis of endometriosis. Stromal cells were the major cell type of endometrial tissue, secreting matrix and fiber in the lamina propria of the endometrium (10). They proliferate and differentiate with the transformation of the menstrual cycle and may play roles in the establishment and progression of endometriosis (11), while our understanding is far from sufficient yet.

Hexokinase (HK), the first key rate-limiting enzyme in the glycolysis pathway, catalyzes the formation of glucose 6 phosphates (12). There are five isoforms in mammalian tissues: HK1, HK2, HK3, HK4 and Hexokinase Domain Containing 1 (HKDC1) (13). HK2 is insulin-sensitive, mainly exists in fat, bone, and heart tissue (12). More importantly, it has been found that the high aberrant expression of HK2 in various
tumors is closely related to cell survival (14), and HK2 is regarded as a potential therapeutic target of tumors (15, 16). However, the expression of HK2 and its role in endometriosis remain indistinct.

Signal transducers and activators of transcription (STATs) are a family of proteins, locating at the cytoplasm before being activated. Once activated, STATs form dimers translocate into the nucleus and bind to the promoter of the target genes (17). Signal transducer and activator of transcription 1 (STAT1) is alternately spliced into two protein forms, STAT1a (91 kDa) and STAT1b (84 kDa) (18). Phosphorylation of tyrosine 701 (Y701) loci, situates in the transcriptional activation domain of STAT1, is necessary for the translocation and functions of STAT1 homodimer and heterodimer (19). The classical role of STAT1 is a tumor suppressor depending on the activation of Y701 (20).

In this study, we aimed to investigate the metabolic disorder and the role of HK2 in endometrial stromal cells endometriosis. Furthermore, we explored the non-catalytic effect of HK2, upregulating phosphorylation of STAT1. These findings unveil the noncanonical function of HK2 and provide a promising therapeutic target for endometriosis.

**Methods**

**Patients and samples**

Forty-six participants were enrolled in Shanghai First Maternity and Infant Hospital from April 2019 to September 2020. The collection of the samples was approved by the Ethics Committee and obtained informed consent from all patients. The experimental group included 23 cases of ectopic cyst wall of the ovary endometrioma confirmed by pathology. The endometrium was obtained from 23 donors who received hysteroscopy for benign gynecological diseases, excluding endometriosis in the control group. None of the patients had other immune diseases, acute inflammation, or estrogen-dependent diseases, and no hormone medications were used within three months before surgery. Specimens from both the experimental and control groups were collected during surgery. The specimen was achieved and immediately placed into the specimen preservation solution and then transferred to the laboratory.

**Primary cells isolation and culture**

Endometrial stromal cells were isolated and cultured according to the methods described previously (44, 45) and with slight modification. Specimen tissues were washed with phosphate buffered saline (PBS) and cut into fragments. 1 mg/mL collagenase type IV (Sigma, USA) was added and digested for 20-40 min in a shaker at 37°C. Tissue homogenate was filtered through a 40 μm cell strainer (Falcon, USA), and the cell suspension was collected and centrifuged for 10 min to collect cells. The isolated primary cells were resuspended with complete medium DMEM/F12 (Hyclone, USA) containing 10% fetal bovine serum (FBS, Sciencell, USA) and cultured in an incubator at 37°C in a humidified atmosphere with 5% CO₂.

**Cell transfection**
HESCs were purchased from American Type Culture Collection and cultured under the same conditions as primary cells. Three designed HK2-short hairpin RNAs (shRNAs) cloned into pGMLV-SC5-puro vector plasmids and scrambled control vector plasmids were transfected respectively with the lentiviral packaging mix into 293T cells to produce the virus for 48 h. The supernatant containing the virus was filtered through a 0.45 μm filter and used to infect HESCs. Seventy-two hours later, the infected HESCs were selected with 30 μg/mL puromycin (Invitrogen, USA), and the knockdown efficiency was detected. The sequences of shRNAs were listed in Supplementary Table 1.

**RNA extraction and quantitative real-time PCR (qRT-PCR) analysis**

According to the reagent instructions, total RNA was extracted from tissues and cells using RNAiso Plus and transcribed into cDNA (Takara, Japan). Then qRT-PCR was carried out in triplicate on a 384-well plate using a QuantStudio 5 Flex Real-time PCR system (Applied Biosystems, USA). The expression of the target gene relative to ACTB was determined using the $2^{-\Delta\Delta CT}$ method. The sequences of primers were listed in Supplementary Table 1.

**Western Blotting**

Tissue and cellular proteins were extracted with RIPA lysate mixed with phosphatase inhibitors and quantified by the BCA method. Following the manufacturer's instructions (Beyotime Biotechnology, China), cytoplasm and nucleus proteins were extracted. The exact amount of protein was electrophoresis with SDS-polyacrylamide gel and then transferred onto a piece of PVDF membrane (Millipore, USA). After blocking, the membrane was incubated with the primary antibody against the target protein on a shaking table at 4°C overnight. Then the membrane was incubated with a secondary antibody, and the protein bands were quantitated by electrochemiluminescence assay. The antibody details were listed in Supplementary Table 2.

**Immunohistochemistry, immunocytochemistry and hematoxylin-eosin (HE) staining**

Tissue sections were deparaffinized, rehydrated, retrieved antigen and removed endogenous peroxidase (Biotech Well, China). After being blocked and washed, these sections were incubated with the primary antibody at 4°C overnight. The next day, secondary antibody incubation and chromogenic detection were performed. The sections were then counterstained with hematoxylin, dehydrated, mounted, and examined.

As for immunocytochemistry, cells were seeded in each compartment of slides (Millipore, USA). After fixing with 4% paraformaldehyde and incubating with PBS containing 0.5% Triton X-100, endogenous peroxidase removal and subsequent steps were the same as immunohistochemistry. The antibody details were listed in Supplementary Table 2.

Tissue sections for HE staining were deparaffinized and rehydrated. They were treated with hematoxylin for 5 min, ethanol containing 1% hydrochloric for 45 s, and then immersed in eosin for 5 min. Dehydration
and subsequent steps were similar to immunohistochemistry.

**Wound healing assay**

Cells were seeded in a 6-well plate cultured to 80-90% confluence and then scratched with a pipette tip vertical to the six-well plate. Cell debris was gently washed off with PBS and added FBS-free medium. Images of cells were recorded at 0 h, 24 h, and 48 h after scraping, and scratch areas were measured.

**Cell invasion and migration assay**

For the invasion assay, 50 μL diluted Matrigel (BD Bioscience, USA) was added to each transwell chamber (Millipore, USA) and solidified at 37℃ before cells were seeded. For both invasion and migration assay, 5×10^4 cells resuspended in 200 μL of FBS-free medium were seeded in the upper compartment of the chamber, and 600 μL complete medium was added to the lower compartment. After 48 h-incubation, cells on the upper compartment were wiped with a cotton swab. The invaded cells at the lower compartment were fixed in 4% paraformaldehyde and stained with crystal violet. Then invaded cells were photographed under a microscope.

**Cell proliferation assay**

A total of 100 μL suspension containing 3 000 cells were seeded in each well of a 96-well plate. After different treatments for an appropriate time, 10μL cell counting kit-8 reagent (CCK8, Dojindo, Japan) was added to the culture medium at specific time points of 0 h, 24 h, 48 h, 72 h. After incubating for 4 h at 37℃ in 5% CO2, the optical density was measured at 450 nm using a microplate reader.

**Detection of glucose consumption, lactic acid production and the activity of hexokinase**

Cultured primary endometrial stromal cells were trypsinized and counted and then seeded into a 96-well plate. After 24 h, the experiments of glucose consumption, lactic acid production, the activity of hexokinase detection were performed following the manufacturer's instructions (Solarbio, China).

**Extracellular acidification rate (ECAR) detection**

1×10^5 cells were seeded in each well of a 96-well plate. The XF96 extracellular flux kits were pretreated, and the essential medium was prepared. The next day, 175 μL essential medium was added to each well of the 96-well plate which would be incubated in a CO2-free incubator at 37 ℃ for 1 h. In addition, 25 μL D-glucose (10 mM), Oligomycin (1 μM) and 2-DG (50 mM) per well were added into the upper part of XF96 extracellular flux kits, which were put into a seahorse XF96 cellular flux analyzer. After 30 min, the lower layer of the XF96 extracellular flux kits was replaced with the cell plate, and detection was proceeded according to the standard procedures.

**Mice model of endometriosis**
The intraperitoneal endometriosis model was constructed according to a previously described method (46) with minor alterations. The uteruses of donor mice were removed and minced, and then injected into recipient mice intraperitoneally. The recipient mice were divided into experimental group and control group randomly. Drug solutions and solvents were intraperitoneally injected into the mice of the experimental group and control group, respectively. All recipient mice were sacrificed 14 days after implantation, and the endometriosis lesions were collected.

**Statistical analysis**

The statistical analysis was carried out using GraphPad Prism version 6.02. Results are reported as the mean ± SEM from triplicate experiments. Student’s t-test and one-way ANOVA were used to analyzed statistics following correction. There was a statistical difference when P < 0.05.

**Results**

**Glycolysis metabolism was enhanced in ESCs**

To investigate the differences between ectopic stromal cells (ESCs) with normal stromal cells (NSCs), we performed transcriptome sequencing (Figure 1A). Gene ontology (GO) enrichment of upregulated differently expressed genes showed that the metabolic processes and glucose metabolic processes vary (Figure 1B). Furthermore, we found that most glycolysis-related genes were upregulated in ESCs (Figure 1C), suggesting that the glycolysis pathway might be vibrant in ectopic stromal cells. Interestingly, we noted that HK2 was differently expressed merely among the four hexokinases in endometrial stromal cells (Figure 1D). To further investigate glycolysis metabolism in ESCs and NSCs, we detected the glucose consumption, lactic acid production and the activity of hexokinase. The results indicated that glucose consumption (Figure 1E) and lactic acid production (Figure 1F) are increased in ESCs compared to NSCs. Besides, the activity of hexokinase was also significantly enhanced in ESCs (Figure 1G). Next, the extracellular acidication rate (ECAR) was detected to evaluate the glycolysis metabolism (Figure 1H). Statistical results revealed that the basal glycolysis level of ESCs was elevated compared with NSCs (Figure 1I), and glycolysis capacity was enhanced (Figure 1J).

**HK2 is upregulated in ectopic tissue and ESCs**

To explore the role of HK2 in endometriosis, we detected the expression of HK2 in tissues and primary stromal cells, respectively. Immunohistochemistry results indicated that HK2 mostly expressed in stromal tissues and distributed more in ectopic tissues (Figure 2A) than in the control group. The expression of mRNA (Figure 2B) and protein of HK2 (Figure 2C and 2D) was upregulated in ectopic lesions compared to the control endometrium. Subsequently, primary stromal cells were isolated and stained for cytokeratin-7 and vimentin for identification (Figure 2E). The results showed HK2 was overexpressed in ESCs (Figure 2G and 2H).

**Inhibiting HK2 suppressed proliferation, invasion and migration of HESCs**
Next, we studied the effect of HK2 through treating on Human endometrial stromal cells (HESCs) with its inhibitor. 2-Deoxy-D-glucose (2-DG) is a glucose analog that serves as a competitive inhibitor of glucose metabolism, inhibiting glycolysis via acting on hexokinase (21). As shown in the wound healing assays, we found that the migration of HESCs decreased when the 2-DG supplement (Figure 3A), and the transwell migration experiment also proved this conclusion (Figure 3B). Notably, the invasion capacity of HESCs was inhibited (Figure 3C). Furthermore, the effect of the inhibitor on the proliferation of stromal cells was detected. The higher the inhibitor concentration was, the lower the cell proliferation ability was (Figure 3D).

**Inhibiting HK2 alleviated endometriosis in vivo**

3-Bromopyruvate (3-BP), another inhibitor of HK2 (22), was utilized intraperitoneally into constructed mice models of endometriosis (Figure 4A). We found that the lesions of mice injected with 3-BP were significantly smaller than those in the solvent control group. Next, we measured the gross weight of each lesion, and the results showed a remarkable difference between the treatment and control group (Figure 4B). HE staining of the lesions confirmed the distribution of stroma and glands, similar to the uterus endometrial tissue, indicating that the mice model was successfully established (Figure 4C). Ki67 staining demonstrated reduced proliferation in the 3-BP treatment group (Figure 4F).

**HK2 knockdown attenuated the migration, invasion and proliferation of HESCs**

To further determine the effect of HK2 on cell function, we transfected HESCs with lentivirus to downregulate HK2 (Figure 5A-5D), and we chose cell line transfected with shRNA2 for the following experiment. Significantly, the sh-HK2 cells appeared reduced ability of migration (Figure 5E and 5F), invasion (Figure 5G and 5H) and proliferation (Figure 5I).

**HIF-1α upregulated HK2**

Studies have shown that endometriosis lesions are in anoxic environments, and the expression of hypoxia-inducible factor-1α (HIF-1α) was increased (23, 24). We validated it through an immunohistochemistry assay (Figure 6A). According to the literature, we hypothesize that the overexpression of HK2 in ectopic lesions may attribute to the hypoxic environment. We treated HESCs with dimethylallyl glycine (DMOG), inducing the accumulation and stabilization of HIF-1α. The results indicated that with the increase of HIF-1α, the expression of HK2 elevated (Figure 6B).

**HK2 knockdown reduced cell proliferation by affecting phosphorylation of STAT1 rather than glycolysis pathway**

To uncover how HK2 affected cell function, ECARs of sh-HK2 and sh-NC were determined (Figure 7A). Surprisingly, the results confirmed no significant difference in basal glycolysis level and glycolysis ability of sh-HK2 cells compared with sh-NC (Figure 7B and 7C), suggesting HK2 regulated cell migration, invasion, and proliferation without influencing the glycolysis process. Furthermore, results revealed no differentially expressed genes of glycolysis pathways except HK2 (Supplementary Figure A) or
enrichment information related to glycolysis in differential expressed genes of sh-HK2 and sh-NC cells (Supplementary Figure B and C).

Nevertheless, we found that knockdown of HK2 induced an increase of STAT1 expression and phosphorylation of its Y701 loci (Figure 7D). As reported, STAT1 enters the nucleus after being activated to inhibit cell proliferation and promote apoptosis as a tumor suppressor (25, 26). To unveil the distribution of STAT1 under the impact of HK2, we extracted protein from the cell cytoplasm and nucleus, respectively. HK2 was accidentally detected in the nucleus protein. Crucially, the phosphorylated-STAT1 (p-STAT1) ratio to total STAT1 in the nucleus increased remarkably in sh-HK2 compared to sh-NC cells. Moreover, FBS treatment stimulated an increase of the ratio in the nucleus, while the cytoplasmic changed inconspicuously (Figure 6E). These results suggest that HK2 may also play a non-canonical role as a signaling molecule other than participating in glycolysis as an enzyme.

Next, we treated shHK2 cells and HESCs with udarabine, a phosphorylation inhibitor of STAT1, and observed rescue changes in cell proliferation. The addition of STAT1 inhibitor recovered the reduced proliferation ability caused by knockdown of HK2 to a certain extent (Figure 6F). We concluded that the effect of HK2 on the proliferation of HESCs is partly dependent on the phosphorylation of STAT1.

**Discussion**

Currently, endometriosis therapy is limited to hormonal treatment and surgery, which cannot essentially cure the disorder (27). Kasvandik et al. reported metabolic reprogramming of ESCs by deep quantitative proteomics (28), and Young et al. reported the Warburg-like effects in ectopic lesions (29). Recently, Lee et al. confirmed the altered metabolism of ESCs and over-expressed pyruvate dehydrogenase kinase 1, a glycolysis enzyme (30). In this study, we further identified the abnormal glucose metabolism and increased expression of HK2 in ESCs, the first key enzyme of the glycolysis pathway. Moreover, the proliferation, migration, and invasion cell ability were reduced when HK2 was inhibited and knocked down, and an inhibitor of HK2 could alleviate endometriosis in vivo. These results suggested that HK2 may be related to endometriosis progression and is a potential therapeutic target that needs further study.

HIF-1α is closely associated with endometriosis, according to various studies (31). Retrograde endometrial fragments suffer from more hypoxic stress, and HIF-1α accumulates in ectopic areas (24). Further, HIF-1α could affect epithelial to mesenchymal transition (32), growth of vascular endothelial cells (33), and adhesion (34), invasion (35), migration (36) of ESCs. Herein, we unveiled another working pathway of HIF-1α via up-regulating HK2, a realistic and feasible target to treat endometriosis since anoxic state and HIF-1α accumulation are inevitable.

Conventionally, STATs were activated by cell surface receptor JAKs binding to the ligand and regulated the immune system, inhibited cell proliferation, and promoted apoptosis by regulating genes expression associated with the cell cycle (37, 38). In recent years, studies have found that STAT1 may act as a tumor promoter (39), suggesting that the function of STAT1 might change with the cellular and external environment (40). Elevation of p-STAT1 has previously been reported to promote apoptosis of ectopic
endometrial stromal cells (41). We found that the effect of HK2 on cell proliferation was partially dependent on p-STAT1.

Studies have shown that various metabolic enzymes in the cytoplasm and mitochondria could translocate into the nucleus to modify histones and DNA, regulating gene expression (42). A report stated HK2 could translocate into the nucleus as a coactivator participating in redox homeostasis (43). In our findings, HK2 affected the expression and distribution of phosphorylation of STAT1. So far, we have associated HK2 with STAT1 and revealed the noncanonical function of HK2.

Conclusions

In summary, we demonstrate the vibrant glycolysis metabolism and overexpression of HK2 in ESCs. Inhibiting HK2 reduces cell migration, invasion, and proliferation in vitro and alleviates endometriosis in vivo. HIF-1α upregulates the expression of HK2, and knockdown of HK2 attenuates cell proliferation by upregulating p-STAT1. The noncanonical role of HK2 is revealed, expecting to shed light on the pathogenesis and potential treatment of endometriosis.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| HK2          | Hexokinase 2 |
| STAT1        | Signal transducer and activator of transcription 1 |
| HK           | Hexokinase |
| HKDC1        | Hexokinase Domain Containing 1 |
| Y701         | Tyrosine 701 |
| ESC          | Ectopic stromal cell |
| NSC          | Normal stromal cell |
| GO           | Gene Ontology |
| ECAR         | Extracellular acidification rate |
| HESC         | Human endometrial stromal cell |
| 2-DG         | 2-Deoxy-D-glucose |
| 3-BP         | 3-Bromopyruvate |
| HIF-1α       | Hypoxia-inducible factor-1α |
DMOG  Dimethylallyl glycine
p-STAT1  phosphorylated STAT1
PBS  Phosphate buffered saline
FBS  Fetal bovine serum
shRNA  short hairpin RNA
qRT-PCR  Quantitative real-time polymerase chain reaction
HE  Hematoxylin and eosin
CCK8  Cell counting kit-8

**Declarations**

**Ethics approval and consent to participate**

The study has been approved by the ethics committee of Shanghai First Maternity and informed consent was obtained from all patients. The animal experiment was approved and monitored by the institutional animal care.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its additional files.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

SH designed the study, mainly performed the experiments, and wrote the manuscript under the direction of DZ; SL, HP, LW and SL participated in the experiments and analysis; ML participated in study design
and advised the paper. DZ contributed to experimental design, interpretation of results, and article revision.

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Figures
Glycolysis metabolism was enhanced in ESCs. (A) Heatmap of the differentially expressed genes between ectopic stromal cells (ESC) vs. normal stromal cells (NSC) groups. (B) Top 20 GO items enriched from upregulated differentially expressed genes in ESC vs. NSC groups. (C) Expression of genes related to glycolysis process in NSCs and ESCs. (D) Expression of four hexokinases in NSCs and ESCs. (E-G) Determination of glucose uptake, lactic acid production and hexokinase activity in NSCs and ESCs using kits, respectively. (H-J) Extracellular acidification rates of NSCs and ESCs were shown in Figure 1H. The response inducing by the addition of glucose (GLU) reflects cellular basal glycolysis level, and statistical results were shown in Figure 1I. The response inducing by the addition of oligomycin (OLI) reflects cellular maximal glycolysis capacity, and statistical results were shown in Figure 1J. The data are expressed as the mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001; ns, no statistical difference.

**Figure 2**

HK2 is upregulated in ectopic tissue and ESCs. (A) Immunohistochemistry revealed the expression of HK2 in tissues from negative control (NC) and endometriosis (EMS) groups. (B) The mRNA level of HK2 in NC and EMS groups was determined by qRT-PCR. (C, D) The protein level of HK2 in NC and EMS groups was analyzed by western blotting and quantified normalized to α-tubulin. (E) Primary endometrial stromal
cells were identified with vimentin and cytokeratin 7 (CK7) staining. (F) The mRNA level of HK2 in NSCs and ESCs determined by qRT-PCR. (G, H) The protein level of HK2 in NSCs and ESCs groups was analyzed by western blotting and quantified normalized to α-tubulin. The data are expressed as the mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001; ns, no statistical difference.

Figure 3

Inhibiting HK2 suppressed proliferation, invasion and migration of HESCs. (A, B) Human endometrial stromal cells (HESCs) were treated with HK2 inhibitors, 2-Deoxy-D-glucose (2-DG, 0.25mM), and DMSO, a negative control to observe the effect on cell migration by wound healing assay. The wound area at 12 h and 24 h relative to 0 h of the two groups were calculated and compared. (C, D) HESCs were treated with 2-DG (0.1 mM, 0.25mM) and DMSO to observe the effect on cell migration and invasion by transwell assay. The invaded cells of the three groups were counted and compared. (E) HESCs were treated with 2-DG (0.1 mM, 0.25mM) and DMSO as a negative control to observe the effect on cell proliferation. The data are expressed as the mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001; ns, no statistical difference.
Figure 4

Inhibiting HK2 alleviated endometriosis in vivo. (A) The diagram showed the procedures of establishing the endometriosis mice model and the experimental scheme. (B) The ectopic lesions in the mouse peritoneal cavity of the solvent group and two treatment groups (1 mg/kg and 2.5 mg/kg) were collected and weighed. (C) HE staining of the ectopic lesions showed that stromal cells and glands were distributed in the lesion tissue similar to uterus endometrium. (D) Ki67 staining of the ectopic lesions from the control and treatment group. The data are expressed as the mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001; ns, no statistical difference.
Figure 5

HK2 knockdown attenuated the migration, invasion and proliferation of HESCs (A) The transfection efficiency of sh-NC and sh-HK2 lentivirus was showed by green fluorescence. (B-D) The HK2 knockdown efficiency of negative control (NC), sh-NC, and three designed sh-RNA targeted HK2 were confirmed by qRT-PCR, Western Blotting and immunocytochemistry. (E, F) The wound healing assay was performed to observe the migration of sh-NC and sh-HK2 cells. The wound area at 12 h and 24 h relative to 0 h of the two groups were calculated and compared. (G, H) Transwell assay was performed to observe the migration and invasion of sh-NC and sh-HK2 cells. The invaded cells of the two groups were counted and compared. (I) Comparison of cell proliferation between sh-NC and sh-HK2 cells. The data are expressed as the mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001; ns, no statistical difference.
Figure 6

HK2 was upregulated by HIF-1α. (A) Immunohistochemistry exhibited the expression of HIF-1α in tissues from NC and EMS groups. (B) HESCs were treated with the indicated concentrations of Dimethyl sulfoxide (DMOG), and the protein level of HIF-1α and HK2 was detected. The data are expressed as the mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001; ns, no statistical difference.

Figure 7

HK2 knockdown reduced cell proliferation by affecting phosphorylation of STAT1 rather than glycolysis pathway. (A-C) Extracellular acidification rates of sh-NC and sh-HK2 cells were shown in figure 7A. The response inducing by the addition of glucose (GLU) reflects cellular basal glycolysis level, and statistical
results were shown in figure 7B. The response inducing by the addition of oligomycin (OLI) reflects cellular maximal glycolysis capacity, and statistical results were shown in figure 7C. (D) The protein level of signal transducer and activator of transcription 1 (STAT1) and phosphorylation of Tyrosine701 (Tyr701) loci in blank control, sh-NC and sh-HK2 cells detected by Western Blotting, and VINCULIN acted as an internal reference. (E) The protein level of HK2, STAT1, p-STAT1 in nucleus and cytoplasm of sh-NC and sh-HK2 cells with FBS or not detected by Western Blotting. Histone H3 and α-tubulin acted as the internal reference of the nucleus and cytoplasm, respectively. (F) sh-HK2 cells were treated with fludarabine (5μM), inhibiting phosphorylation of STAT1 and PBS as a negative control to observe the effect on cell proliferation. The data are expressed as the mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001; ns, no statistical difference.

**Supplementary Files**

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