

PFKFB4 promotes endometrial cancer by regulating glycolysis through SRC-3 phosphorylation

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Abstract. The present study aimed to investigate the role of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4) in endometrial cancer cells and to explore its potential molecular mechanisms. PFKFB4 expression in endometrial cancer tissues was detected by immunohistochemistry. Cell Counting Kit-8, Transwell assays and flow cytometry were used to detect cell proliferation, invasion and apoptosis in endometrial cancer cells after PFKFB4 knockdown. An enzyme-linked immunosorbent assay was used to detect the glucose and lactic acid contents. Western blotting was performed to detect the levels of glycolysis-related enzymes, steroid receptor coactivator-3 (SRC-3), and phosphorylated SRC-3. *In vivo* experiments were performed to investigate the tumorigenic potential of PFKFB4. PFKFB4 expression was upregulated in endometrial cancer tissues compared with that in normal controls, and its upregulation was positively correlated with the depth of myometrial invasion, lymph node metastasis, surgical pathological stage and vascular invasion. PFKFB4 knockdown significantly inhibited proliferation and

invasion, increased apoptosis, and decreased oxygen consumption and lactic acid production in endometrial cancer cells. PFKFB4 knockdown decreased SRC-3 phosphorylation. After simultaneous PFKFB4 knockdown and SRC-3 overexpression in cancer cells, oxygen consumption, lactic acid production, and glycolysis-related protein expression were increased compared with those in control cells. PFKFB4 knockdown inhibited tumor proliferation, apoptosis and the expression of Ki-67. PFKFB4 may regulate glycolysis in endometrial cancer cells by targeting SRC-3, thus promoting endometrial cancer progression.

Introduction

Endometrial cancer is a common gynecological malignancy of the inner epithelial lining of the uterus (1,2). It is the sixth most common cancer in females worldwide, with an increasing incidence and mortality (3,4). Although most patients with early stage and low-grade endometrial cancer can achieve favorable survival results through surgery alone, patients with advanced and metastatic disease progress rapidly within one year (5,6) and the five-year overall survival rate for patients with stage IV endometrial cancer is ~15% (7). Endometrial cancer cell invasion through the myometrium and metastasis to nearby lymph nodes are key factors affecting the prognosis (8). Therefore, elucidating the mechanisms underlying endometrial cancer progression is of great significance for disease prevention and treatment.

Normal cells rely primarily on the tricarboxylic acid cycle and oxidative phosphorylation as their main sources of adenosine triphosphate when oxygen is sufficient. By contrast, cancer cells rely on glycolysis as their primary energy source, even under oxygen-rich conditions when it is called aerobic glycolysis (9-11). This continuous use of aerobic glycolysis, known as the Warburg effect, is considered a hallmark of numerous cancers (12,13). 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4) belongs to a family of bifocal enzymes that regulate the balance between fructose-6-phosphate and fructose-2, 6-bisphosphatase, and is a key enzyme in glycolysis. PFKFB4 has both kinase and phosphatase activities and can maintain the intracellular level of fructose-2,

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Abbreviations: EdU, -ethynyl-2'-deoxyuridine; GLUT1, glucose transporter 1; HK2, hexokinase 2; IHC, immunohistochemistry; LDHA, lactate dehydrogenase A; NC, negative control; PBS, phosphate-buffered saline; PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4; PKM2, pyruvate kinase M2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA; SRC, steroid receptor coactivator-3; SRC-3-OE, SRC-3 overexpression plasmid; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

Key words: PFKFB4, endometrial cancer, SRC-3, aerobic glycolysis

6-diphosphate, the main allosteric activator of the glycolytic rate-rater 3-phosphoinositide dependent protein kinase-1, through the dual action of phosphorylation and dephosphorylation. Therefore, PFKFB4 controls glycolysis by regulating the level of fructose-2,6-diphosphate (14). Importantly, PFKFB4 has been demonstrated to be involved in numerous cancer-related processes such as proliferation, metastasis and invasion, and PFKFB4 overexpression has been identified in gastric, bladder, lung and breast cancers (15-18). PFKFB4 affects carcinogenesis and cancer metabolism by participating in glucose metabolism regulation by enhancing glycolysis and the pentose phosphate pathway (19). Enhanced glycolysis increases the cancer cell survival rate in a microenvironment with limited oxygen supply, thus promoting metastatic development (20). It has been reported that PFKFB4 mediated CD44-driven proliferation in prostate cancer (21). Lu *et al* (22) reported that PFKFB4 is abnormally highly expressed in endometriosis, and that phosphorylation of PFKFB4 results in endometriosis, glycolysis, and development via proviral insertion in murine lymphomas 2. However, the molecular mechanisms of PFKFB4 in endometrial cancer have not yet been fully elucidated.

Steroid receptor coactivator-3 (SRC-3), is a member of the SRC family that regulates the transcriptional activity of steroid hormone receptors and other transcription factors (23). It also acts as a key modulator of regulatory T cell-mediated tumor evasion to promote cancer progression (24) and inhibition of SRC-3 has been shown to be a potential therapeutic strategy for cancers such as aggressive mantle cell lymphoma and breast cancer (25,26). PFKFB4 was shown to phosphorylate oncogenic SRC-3 at serine 857 and enhance its transcriptional activity in breast cancer cells, resulting in a pro-proliferative effect (15). However, the role of PFKFB4 on SRC-3 phosphorylation and function in endometrial cancer remains unclear. Thus, exploring the mechanism of action of PFKFB4 in endometrial cancer will facilitate the development of new drugs for endometrial cancer treatment.

The objective of the present study was to investigate the role and molecular mechanism of PFKFB4 in endometrial cancer from the perspective of glucose metabolism. It was hypothesized that PFKFB4 promotes the endometrial cancer progression by regulating SRC-3. The results of the present study may provide new methods for studying endometrial cancer pathogenesis and treatment.

Materials and methods

Patients. The present study included two parts of tissue specimens: one part of specimens were 180 paraffin-embedded endometrial cancer tissues (median age: 55 years old, age range: 35-81) and 60 paraffin-embedded normal endometrial tissues. The cancer tissues were collected from patients with endometrial cancer who underwent surgical treatment in strict accordance with surgical indications between January 1, 2017 and March 31, 2021 in our hospital. The normal endometrial tissues were collected from participants who underwent hysterectomy for uterine fibroids in the same period. The other part of tissue specimens were frozen cancer and para-carcinoma tissues collected from ten patients with endometrial cancer (median age: 56 years old; age range: 37-80) who underwent

surgical treatment in our hospital from March 1, 2022 to July 31, 2022. All the patients included in the study were first diagnosed, without obvious distant metastasis lesions. They were treated with surgery and had no radiotherapy or preoperative chemotherapy, and the malignancy was evaluated according to the international TNM clinical staging standard.

The present study was approved by the Ethics Committee of the People's Hospital of Shanxi (approval no. 13; Taiyuan, China), and all participants provided written informed consent in accordance with the Declaration of Helsinki.

Cell culture. Endometrial cancer cell lines HEC-1A, Ishikawa, and AN3CA, as well as the human normal endometrial epithelial cell line HEEC, were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in RPMI-1640 (Corning, Inc.) containing 10% fetal bovine serum (Ausbian, <http://aipunuobio.com/mxproduct/1440.html>) and 100 U/ml penicillin/100 µg/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. When the cells reached 90-100% confluence, pancreatin was added for subculture.

Cell transfection. Three small interfering RNAs (siRNAs) targeting PFKFB4, including si-PFKFB4-1 (GACGTGGTC AAGACCTACAAA), si-PFKFB4-2 (CCGCATCGTATATTA CCTCAT), and si-PFKFB4-3 (GCTGGCCTACTTCCTCGA CAA) and the corresponding negative control (NC) (si-NC, TCTCCGAACGTGTACAGT) were designed by Guangzhou RiboBio Co., Ltd. The siRNAs (50 nM), SRC-3 overexpression plasmid (SRC-3-OE, 1 µg/µl), and corresponding NC were transfected into HEC-1A cells using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) at a temperature of 37°C for a duration of 6 h. Cells were collected for subsequent experimentation after 48 h. Transfection efficiency was determined using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR. Total RNA was extracted using TRIzol (MilliporeSigma) and reverse transcription was conducted using HiScript QRT Super Mix (Vazyme Biotech Co., Ltd.). The quantitative PCR was conducted using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.). The qPCR thermal cycling conditions were as follows: Initial denaturation at 95°C for 60 sec; followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. Primers used are listed in Table SI. GAPDH was used as a reference gene. Relative changes in the transcriptional levels of all genes were analyzed using the 2^{-ΔΔCq} method (27).

Western blotting. Tissues or cells were homogenized in RIPA solution containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and sodium orthovanadate (Beyotime Institute of Biotechnology) and centrifuged at 15,000 x g and 4°C for 10 min, and the total protein content was determined using the BCA method. Proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride membrane. After blocking in 5% skim milk 1 h at room temperature (around 25°C), the membrane was incubated with primary antibodies,

including anti-PFKFB4 (1:1,000; cat. no. A17915; Abclonal Biotech Co., Ltd.), anti-hexokinase 2 (HK2; 1:3,000; cat. no. 66974-1-Ig; Proteintech Group, Inc.), anti-SRC3 (1:1,000; cat. no. ab133611; Abcam), anti-phosphorylated (p)-SRC3 (1:1,000; cat. no. PA5-64836; Thermo Fisher Scientific, Inc.), anti-pyruvate kinase M2 (PKM2; 1:5,000; cat. no. 15822-1-AP; Proteintech Group, Inc.), anti-lactate dehydrogenase A (LDHA; 1:1,000; cat. no. 66287-1-Ig; Proteintech Group, Inc.), anti-glucose transporter 1 (GLUT1; 1:750; cat. no. bs-4855R; BIOSS), anti-hypoxia-inducible factor 1 α (1:2,000; cat. no. 20960-1-AP; Proteintech Group, Inc.), anti-cleaved caspase 3 (1:500; cat. no. AF7022; Affinity Biosciences), anti-caspase 3 (1:500; cat. no. AF6311; Affinity Biosciences), anti-Bax (1:500; cat. no. AF0120; Affinity Biosciences), anti- β -actin (1:5,000; cat. no. 200068-8F10; Chengdu Zhongneng Biotechnology Co., Ltd.) and anti-GAPDH (1:30,000; cat. no. 60004-1-Ig; Proteintech Group, Inc.) overnight at 4°C and then with the HRP-conjugated secondary antibody (1:3,000; cat. no. A0208/A0216; Beyotime Institute of Biotechnology) at 37°C for 1 h. Finally, specific binding sites were analyzed using enhanced chemiluminescence detection solutions (MilliporeSigma). ImageJ software (version 1.53t, National Institutes of Health) was used for densitometric analysis.

Cell Counting Kit-8 (CCK-8) assay. HEC-1A cells were seeded into a 96-well plate at a density of 5×10^3 cells/well and treated with 10 μ l of CCK-8 reagent (cat. no. 96992; MilliporeSigma) for 1-4 h. Absorption was measured at 450 nm on a multi-mode microplate reader (M2009PR; Tecan Group, Ltd.).

5-ethynyl-2'-deoxyuridine (EdU) assay. Cells seeded in a 96-well plate at a density of 6×10^3 cells/well were labeled with EdU (Guangzhou RiboBio Co., Ltd.), fixed with 4% paraformaldehyde at room temperature (~25°C) for 20 min, and images were captured using a fluorescence microscope (Olympus Corporation).

Transwell migration assay. Transwell chambers (8.0 μ m, Corning, Inc.) were placed in 24-well plates. Serum-free medium (100 μ l) was added to the chamber for 1-2 h and then removed. Next, 600 μ l of medium with 30% fetal bovine serum was added to the lower chamber, and 1×10^5 cells/well were added to the upper chamber. A total of 24 h later, the cells that remained on the upper surface were removed, and the migratory cells on the lower surface were stained with 0.1% crystal violet at room temperature (~25°C) for 15 min. After staining, five images of the migratory cells were randomly captured using an inverted microscope (CKX31; Olympus Corporation).

Cell apoptosis assay. Cell supernatants from different groups were collected in centrifuge tubes. Then, the cells in the culture plates were washed and digested with trypsin. Complete culture medium was then added to terminate the digestion. The cells were collected in the same centrifuge tube as their supernatant. After centrifugation at 400 x g at 4°C for 10 min, the cells were collected and resuspended in 200 μ l of 1X apoptosis buffer, followed by staining with 5 μ l annexin V-APC and 5 μ l propidium iodide for 15 min. Then, 800 μ l

of 1X apoptosis buffer was added to resuspend the cells in 1 ml. The cell suspension was added to a 96-well plate and analyzed by FlowJo (version 10.8.1; BD Biosciences) using a flow cytometer (cat. no. ABC250-22INT; MilliporeSigma).

Glucose and lactic acid content assays. Glucose or lactic acid contents of fresh tissues or HEC-1A cells were detected using a glucose colorimetric assay kit (cat. no. E-BC-K234-M) or a D-lactic acid colorimetric assay kit (cat. no. E-BC-K002; both from Elabscience Biotechnology, Inc.), respectively.

Animals. Animal experiments were conducted according to the ARRIVE guidelines (28) and approved by the Ethics Committee of the People's Hospital of Shanxi (approval no. 9; Taiyuan, China). Eighteen Female, 4-week-old specific pathogen free BALB/c nude mice, weighted 16-18 g (HFK Bioscience Co. Ltd.) were housed in an 12/12-h photoperiods at 21-23°C and 40-60% humidity environment with free access to food and water. They were divided into three groups: blank, si-NC, and si-PFKFB4 (n=6) after 1-week of acclimation, and were implanted subcutaneously with HEC-1A cells, HEC-1A transfected with si-NC, and HEC-1A transfected with si-PFKFB4 (5×10^6 cells for each mouse), respectively. The maximum length and width of the tumors were measured every 2-3 days after one-week of cell implantation. Tumor volume was calculated by length x width²/2. All mice were anesthetized with 2.5% isoflurane inhalation and euthanized by cervical dislocation at the end of the experiment (day 15). The tumors were excised, weighed, and fixed in 4% paraformaldehyde at 4°C for 12 h for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and immunohistochemistry (IHC) or stored at -80°C for western blotting.

TUNEL staining. The tumor tissues fixed in 4% paraformaldehyde were embedded in paraffin and cut into 4- μ m slides. The slides were then deparaffinized in xylene, rehydrated with gradient ethanol, and stained with TUNEL (Beyotime Institute of Biotechnology). After washing with phosphate-buffered saline (PBS) and stained with 5 μ g/ml DAPI at room temperature (~25°C) for 10 min to visualize nuclei, the slides were sealed with anti-fluorescence quenching mounting medium and observed under a fluorescence microscope (Olympus Corporation).

IHC staining. Formalin-fixed and paraffin-embedded tissue sections (4 μ m) were subjected to IHC (29). After dewaxing and rehydration, the endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol. The sections were then incubated with rabbit polyclonal antibodies against PFKFB4 (1:100; cat. no. AB137785; Abcam) and Ki-67 (1:100; cat. no. AF0198; Affinity Biosciences) at 4°C overnight. Then, enzyme-labeled anti-rabbit IgG was added and incubated for 30 min. A NC was obtained by replacing the antibodies with normal PBS. Staining was developed by 3,3'-diaminobenzidine, followed by counterstaining with hematoxylin, dehydration in gradients of ethanol, and clearing in xylene.

The expression level of PFKFB4 or Ki-67 was calculated by multiplying the staining intensity by the percentage of stained cells in ten random fields of view. Staining intensity was scored as 0 for negative staining, 1 for weakly positive

staining, 2 for moderately positive staining, and 3 for strongly positive staining (30). The percentage of positively stained cells was scored as 1 for 1-25% positive staining, 2 for 26-50% positive staining, 3 for 51-75% positive staining, or 4 for 76-100% staining. A final score of 0-1 indicated negative expression, 2-5 low expression, and 6-12 high expression.

Statistical analysis. SPSS 25.0 (IBM Corp.) was used for data analyses. For measurement data, the Shapiro-Wilk test was first performed to determine whether the data followed a normal distribution. Data that conformed to a normal distribution were expressed as the mean \pm standard deviation. The differences between groups were calculated using an unpaired Student's t-test or one-way analysis of variance followed by a Bonferroni post-hoc test. Data that did not conform to a normal distribution were expressed as median (interquartile range), and the differences between groups were calculated using the Mann-Whitney U-test or Kruskal-Wallis test. Categorical data were expressed as number (percentage), and the differences between two groups were calculated using the Chi-square test or Fisher's exact test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PFKFB4 expression and glycolysis increase in endometrial cancer tissues. Data from 180 patients with human endometrial cancer and 60 patients with a normal endometrium were analyzed. The average age of patients with cancer and normal endometrium was 55.1 and 50.8 years, respectively. Tumor samples underwent IHC staining for PFKFB4. PFKFB4-positive staining was primarily observed in the cytoplasm of the endometrial stroma and glandular epithelium, and most intra- or extra-tumoral stromal cells were PFKFB4 negative (Fig. 1A). In the normal endometrium, most endometrial glands showed negative expression and a few exhibited low expression of PFKFB4. The expression of PFKFB4 increased with increasing stage ($P < 0.05$; Fig. 1B). In addition, glucose and lactic acid levels were significantly increased in cancer tissues compared with those in normal endometrium ($P < 0.05$; Fig. 1C and D).

Among the 180 cases of endometrial cancer, 29 had high PFKFB4 expression and 151 had low expression, with a high expression rate of 16.11% (29/180). In the control group of 60 cases, 3 cases had high PFKFB4 expression and 57 cases had low expression, with a high expression rate of 5% (3/60), which was significantly lower than that in the endometrial cancer group ($P = 0.028$; Table I).

In addition, both glucose consumption and lactic acid production increased significantly in endometrial cancer tissues compared with those in normal endometrium ($P < 0.05$).

PFKFB4 expression is associated with clinicopathological parameters of endometrial cancer. Subsequently, the relationship between PFKFB4 expression and clinicopathological parameters in endometrial cancer tissues was analyzed. PFKFB4 upregulation was positively correlated with the depth of myometrial invasion, vascular invasion, surgical pathological stage, and lymph node metastasis (Table II).

Among the 180 endometrial cancer patients, 130 had a muscle layer invasion depth of $< 1/2$, among which 11 had high

Table I. Comparison of the PFKFB4 expression in endometrial cancer and control groups.

Group	Expression level of PFKFB4		χ^2	P-value
	Low, n (%)	High, n (%)		
Endometrial cancer	151 (83.9)	29 (16.1)	4.808	0.028
Control	57 (95)	3 (5)		
Total	208	32		

PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4.

PFKFB4 expression (11/130; 8.46%), 119 had low PFKFB4 expression. Fifty of the endometrial cancer patients had a muscle layer invasion depth of $\geq 1/2$, among which 18 had high PFKFB4 expression (18/50; 36%), and 32 cases had low expression. The high PFKFB4 expression rate at a muscle layer invasion depth of $\geq 1/2$ was significantly lower than that at a muscle layer invasion depth of $< 1/2$ ($P < 0.001$). There were 154 patients without vascular invasion, including 19 with high PFKFB4 expression (19/154, 12.34%). Among the 26 patients with vascular invasion, 10 had high PFKFB4 expression, with a high expression rate of 38.46% (10/26), which was significantly higher than in patients without vascular invasion ($P = 0.002$). According to the surgical pathological stages of endometrial cancer (FIGO, 2009), 158 patients were in stages I-II, and 22 were in stages III-IV. The high PFKFB4 expression rates were 5.70% (9/158) for stages I-II and 90.90% (20/22) for stages III-IV, with a significant difference ($P < 0.001$). Additionally, 165 patients did not have lymph node metastases, including 19 with high PFKFB4 expression (19/165, 11.52%). Among the 15 patients with lymph node metastases, 10 showed high PFKFB4 expression (10/15, 66.67%). The difference between the two groups was statistically significant ($P < 0.001$).

PFKFB4 expression increases in endometrial cancer cells. The protein expression of PFKFB4 in ten pairs of endometrial cancer tissues and normal endometrium was measured by western blotting. The protein expression of PFKFB4 was significantly increased compared with that in the normal endometrium ($P < 0.001$; Fig. 2A). The mRNA expression levels of *PFKFB4* in the three endometrial cancer cell lines and the human normal endometrial epithelial cell line, HEEC, are shown in Fig. 2B. The mRNA levels of *PFKFB4* in Ishikawa and HEC-1A cells were higher than those in HEECs ($P < 0.01$). The protein levels of PFKFB4 in the four cell lines were measured. The protein levels of PFKFB4 in cancer cells were significantly higher than in normal cells ($P < 0.01$; Fig. 2C). Among the three cancer cell lines, HEC-1A revealed the most significant upregulation; therefore, HEC-1A cells were selected for subsequent experiments.

PFKFB4 promotes proliferation and migration and inhibits apoptosis of endometrial cancer cells in vitro and in vivo. To further investigate the function of PFKFB4 in endometrial cancer, a PFKFB4 knockdown plasmid was constructed and

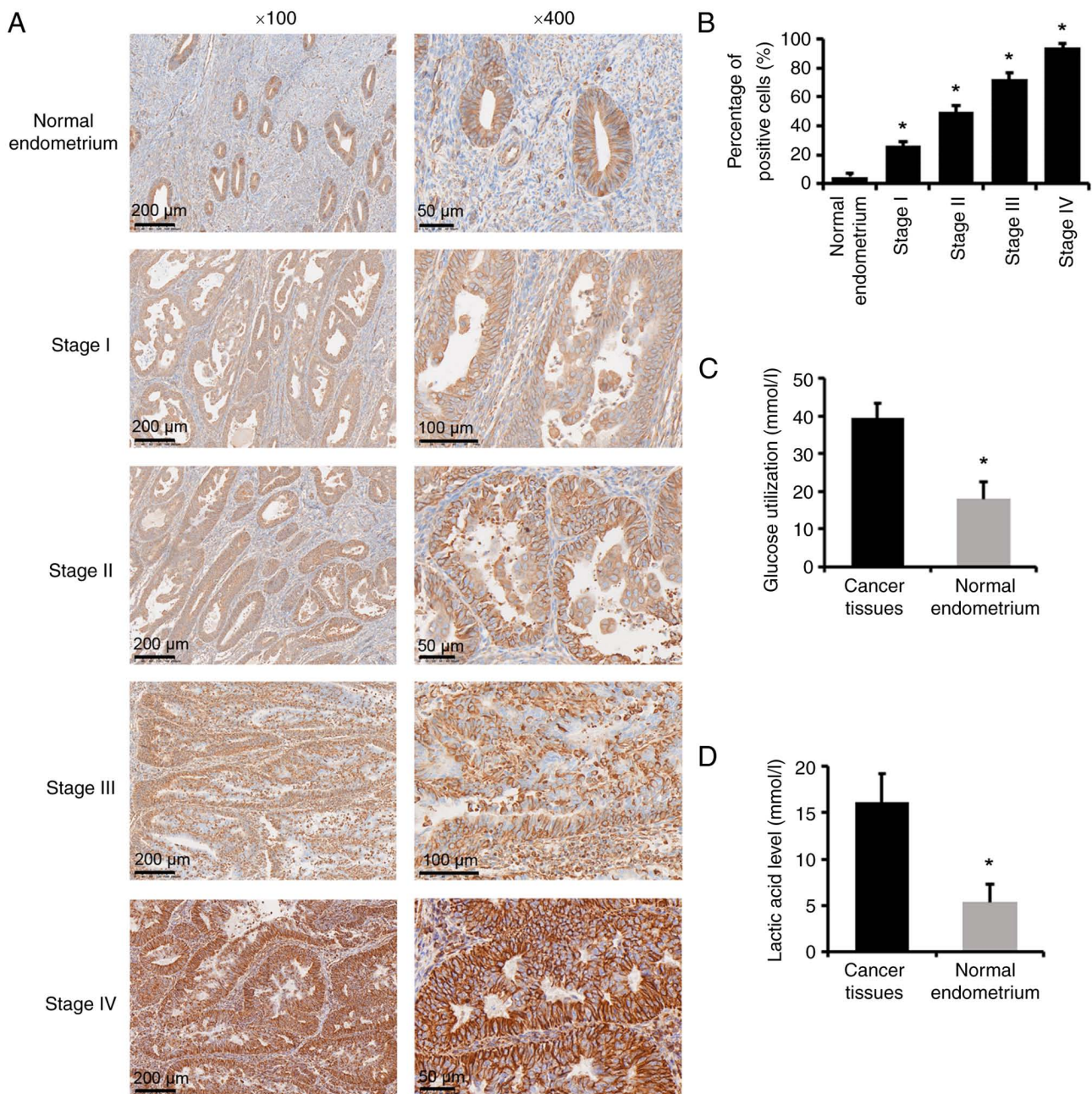


Figure 1. PFKFB4 expression and glycolysis increase in endometrial cancer tissues. (A) Representative images of PFKFB4 IHC staining in normal endometrium and endometrial cancer specimens at low magnification ($\times 100$) and high magnification ($\times 400$). (B) Quantitative data of IHC staining. (C) Glucose consumption in endometrial cancer and normal endometrium. (D) Lactic acid production in endometrial cancer and normal endometrium. * $P < 0.05$ compared with control. PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; IHC, immunohistochemistry.

the knockdown efficiency was determined by RT-qPCR. Compared with the si-NC group, the mRNA levels of *PFKFB4* in the si-PFKFB4-1, si-PFKFB4-2, and si-PFKFB4-3 groups decreased significantly ($P < 0.01$), suggesting that the knock-down plasmids were successfully constructed (Fig. 3A).

CCK-8 and EdU assays were performed to detect the endometrial cancer cell proliferation following PFKFB4 knockdown. PFKFB4 knockdown significantly decreased the proliferation of endometrial cancer cells compared with that in the control ($P < 0.001$; Fig. 3B and C). Transwell assays revealed that, compared with the control, PFKFB4 knockdown inhibited the migration of endometrial cancer cells ($P < 0.001$; Fig. 3D). Furthermore, PFKFB4 knockdown significantly

increased the apoptosis of cancer cells ($P < 0.001$; Fig. 3E) and upregulated the protein expression of cleaved Caspase 3 and Bax (Fig. 3F).

The effects of the PFKFB4 knockdown were further investigated *in vivo*. The maximum tumor diameter measured was 15.7 mm and maximum volume was 1478.9 mm³ in a mouse in the blank group. The tumor volume and weight were significantly lower in si-PFKFB4 group, compared with those in the blank and si-NC groups ($P < 0.05$; Fig. 3G and H). TUNEL staining demonstrated that the percentage of TUNEL positive cells was significantly higher in the si-PFKFB4 group, compared with those in the blank and si-NC groups ($P < 0.05$; Fig. 3I). IHC staining demonstrated

Table II. Correlation of PFKFB4 expression with clinicopathological parameters.

Parameters	Expression level of PFKFB4		χ^2	P-value
	Low, n (%)	High, n (%)		
Age, years			1.833	0.176
<55	78 (87.6)	11 (12.4)		
≥55	73 (80.2)	18 (19.8)		
Menopausal or not			1.608	0.205
No	66 (88)	9 (12.1)		
Yes	85 (81)	20 (19)		
Histological subtype				0.23
Endometrioid adenocarcinoma	142 (85)	25 (15)		
Non-endometrioid adenocarcinoma	9 (69.2)	4 (30.8)		
Histopathological grade				0.583
High	28 (90.3)	3 (9.7)		
Median	95 (81.9)	21 (18.1)		
Low	28 (84.8)	5 (15.2)		
Depth of myometrium invasion			20.262	<0.001
<1/2	119 (91.5)	11 (8.5)		
≥1/2	32 (64)	18 (36)		
Lymph node metastasis				<0.001
No	146 (88.5)	19 (11.5)		
Yes	5 (33.3)	10 (66.7)		
Surgical pathological stage				<0.001
I + II	149 (94.3)	9 (5.7)		
III + IV	2 (9.1)	20 (90.9)		
Vascular invasion				0.002
No	135 (87.7)	19 (12.3)		
Yes	16 (61.5)	10 (38.5)		

PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4.

that the protein expression of Ki-67 and PFKFB4 decreased in tumors from the si-PFKFB4 group compared with those in the blank and si-NC groups (Fig. 3J). Taken together, these results suggest that PFKFB4 downregulation is beneficial for the proliferation and tumorigenesis of endometrial cancer cells.

PFKFB4 increases glycolysis in endometrial cancer cells. To analyze the effect of PFKFB4 on glycolysis in endometrial cancer cells, an enzyme-linked immunosorbent assay was used to measure glucose and lactic acid content after PFKFB4 knockdown. Compared with the control group, both glucose and lactic acid levels decreased significantly in PFKFB4 knockdown group (Fig. 4A and B). The levels of glycolysis-related enzymes, including GLUT1, HK2, LDHA and PKM2, were downregulated after PFKFB4 knockdown compared with those in the control (Fig. 4C).

PFKFB4-phosphorylated SRC-3 regulates glycolysis in endometrial cancer cells. SRC-3 may be a downstream target of PFKFB4 (15). Thus, the expression level of

p-SRC-3 was measured after PFKFB4 knockdown using western blotting. The results revealed that p-SRC-3 was significantly downregulated after PFKFB4 knockdown compared with that in the control, indicating that PFKFB4 could phosphorylate SRC-3 (Fig. 5A). SRC-3 overexpression induced changes in glycolysis in endometrial cancer cells. The efficiency of SRC-3 overexpression is shown in Fig. 5B. Compared with the NC group, PFKFB4 expression in the NC + SRC-3-OE group revealed no significant change, whereas SRC-3 expression was significantly higher ($P < 0.001$, Fig. 5C). In the PFKFB4-KD + NC group, PFKFB4 and SRC-3 expression levels were significantly lower than in those in the NC group ($P < 0.05$, Fig. 5C). Additionally, compared with the PFKFB4-KD + NC group, PFKFB4 expression in the PFKFB4-KD + SRC-3-OE group did not change significantly, whereas SRC-3 expression increased significantly ($P < 0.001$, Fig. 5C). Subsequently, glucose and lactic acid contents were detected after SRC-3 overexpression. Compared with the NC group, glucose consumption and lactic acid production after SRC-3 overexpression increased significantly ($P < 0.001$), whereas after PFKFB4 knockdown,

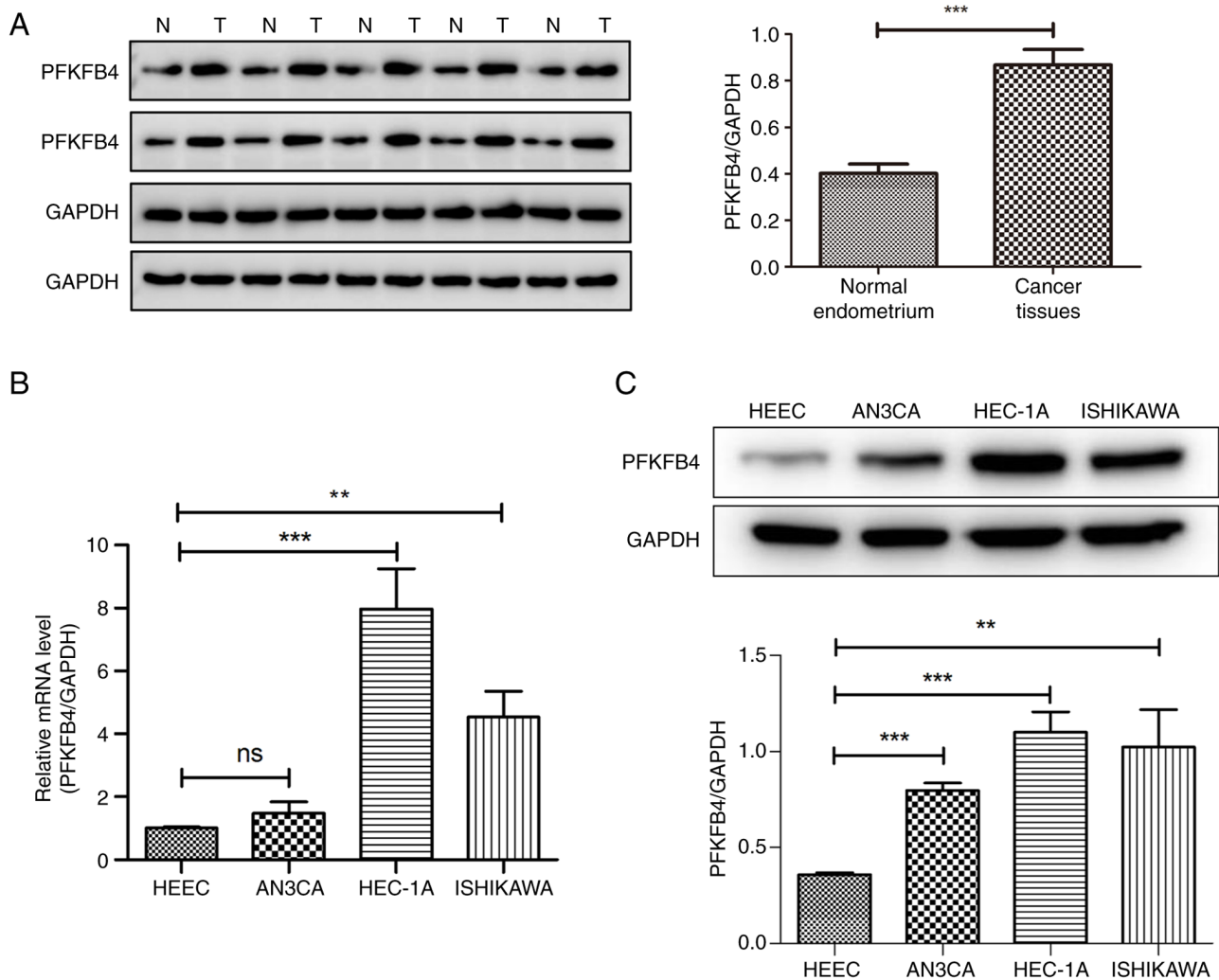


Figure 2. PFKFB4 expression is increased in endometrial cancer cells. (A) The protein expression of PFKFB4 in endometrial cancer tissues and normal endometrium. T, tumor; N, normal control. (B) The mRNA level of *PFKFB4* in endometrial cancer cell lines Ishikawa, HEC-1A and AN3CA, and the human normal endometrial epithelial cell line HEEC was detected by reverse transcription-quantitative PCR. (C) The protein level of PFKFB4 in endometrial cancer cell lines was quantified by western blotting. ** $P < 0.01$ and *** $P < 0.001$, compared with normal endometrium or normal endometrial epithelial cell line. PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; ns, not significant.

they decreased significantly ($P < 0.001$). Compared with the PFKFB4-KD + NC group, glucose consumption and lactic acid production in the PFKFB4-KD + SRC-3-OE group increased ($P < 0.001$), suggesting that SRC-3 overexpression reversed the effect of PFKFB4 knockdown on glycolysis in cancer cells (Fig. 5D and E).

In addition, the protein levels of glycolysis-related enzymes (PKM2, GLUT1, HK2, LDHA and PFKFB4) and p-SRC-3 were determined (Fig. 5F). Compared with the NC group, PFKFB4 expression was downregulated whereas PKM2, GLUT1, HK-II, LDHA and p-SRC-3 expression were upregulated in the NC + SRC-3-OE group. The expression of PKM2, GLUT1, HK2, LDHA, and p-SRC-3 was downregulated in the PFKFB4-KD + NC group. Compared with the PFKFB4-KD+NC group, PFKFB4 expression was downregulated, and PKM2, GLUT1, HK-II, LDHA and p-SRC-3 expression were upregulated in the PFKFB4-KD + SRC-3-OE group, suggesting that SRC-3 overexpression reversed the effect of PFKFB4 knockdown on the expression of glycolysis-related enzymes.

Discussion

Metabolism is critical for the development of cancer (31,32). The key enzymes involved in the core metabolic processes of tumor cells play indispensable roles in tumor cell survival and proliferation (33-35). PFKFB4 is an important enzyme that regulates the energy pathways in tumors and is involved in the movement of tumor and stem cells, thereby acting as an energy regulator during tumor cell metastasis (36). In the present study, the protein level of PFKFB4 in endometrial cancer tissues and cells was upregulated compared with that in normal endometrial tissues and cells.

Dasgupta *et al* (15) demonstrated that PFKFB4 is a major regulator of the SRC3-dependent cancer cell proliferation. PFKFB4 phosphorylates SRC-3, thereby promoting estrogen receptor coactivation and cell proliferation. High expression of SRC-3 and PFKFB4 indicates poor prognosis in patients with breast cancer. In the present study, PFKFB4 was overexpressed in endometrial cancer tissues compared with normal endometrial tissues, and its high expression correlated with the

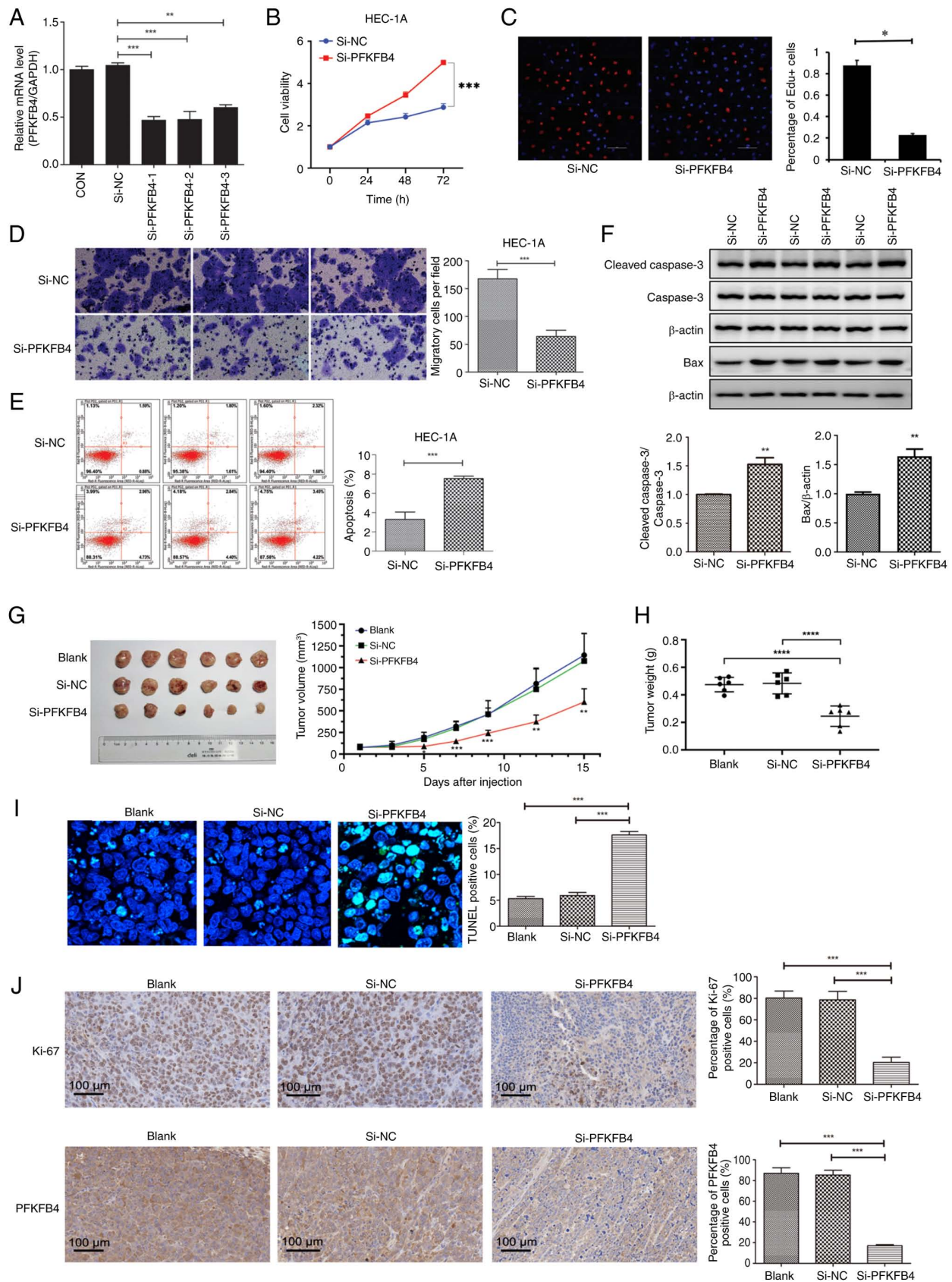


Figure 3. PFKFB4 promotes proliferation and migration and inhibits apoptosis of endometrial cancer cells *in vitro* and *in vivo*. (A) PFKFB4 knockdown efficiency was detected by reverse transcription-quantitative PCR. (B) The viability of endometrial cancer cells after PFKFB4 knockdown was detected by Cell Counting Kit-8. (C) The proliferation of endometrial cancer cells was detected by EdU assay. (D) The migration ability of endometrial cancer cells was tested in a Transwell assay. (E) The apoptosis of endometrial cancer cells was detected by flow cytometry. (F) The protein expression of apoptosis indicators was analyzed by western blotting. (G) Tumor growth curves of PFKFB4 knockdown cells or NC cells in the xenograft mouse model. (H) Tumor weight. (I) TUNEL staining of tumor tissues (magnification, x400). (J) IHC staining of Ki-67, PFKFB4 and SRC-3 (magnification, x400). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared with control or si-NC. PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; IHC, immunohistochemistry; si-, small interfering; NC, negative control.

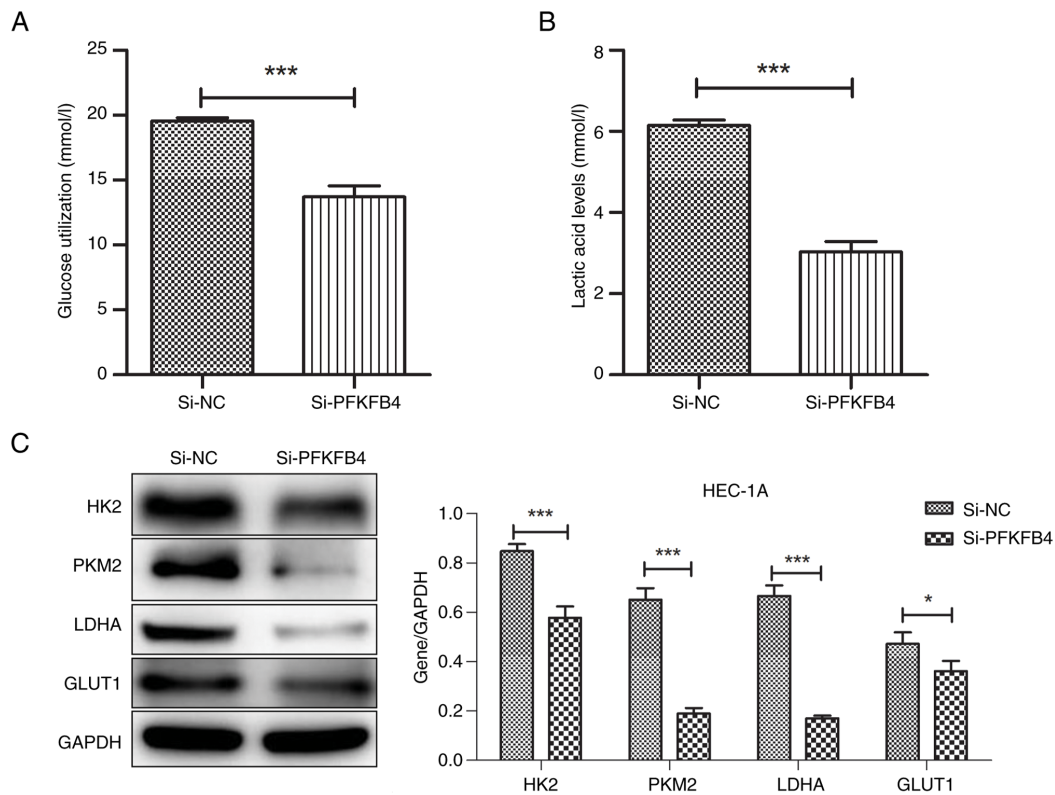


Figure 4. PFKFB4 increases glycolysis of endometrial cancer cells. (A and B) Glucose and lactic acid production after PFKFB4 knockdown, as measured by ELISA. (C) The expression levels of glycolysis-related enzymes were detected by western blotting. * $P < 0.05$ and *** $P < 0.001$ compared with si-NC. PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; LDHA, lactate dehydrogenase A; HK2, hexokinase 2; PKM2, pyruvate kinase M2; GLUT1, glucose transporter 1; si-, small interfering; NC, negative control.

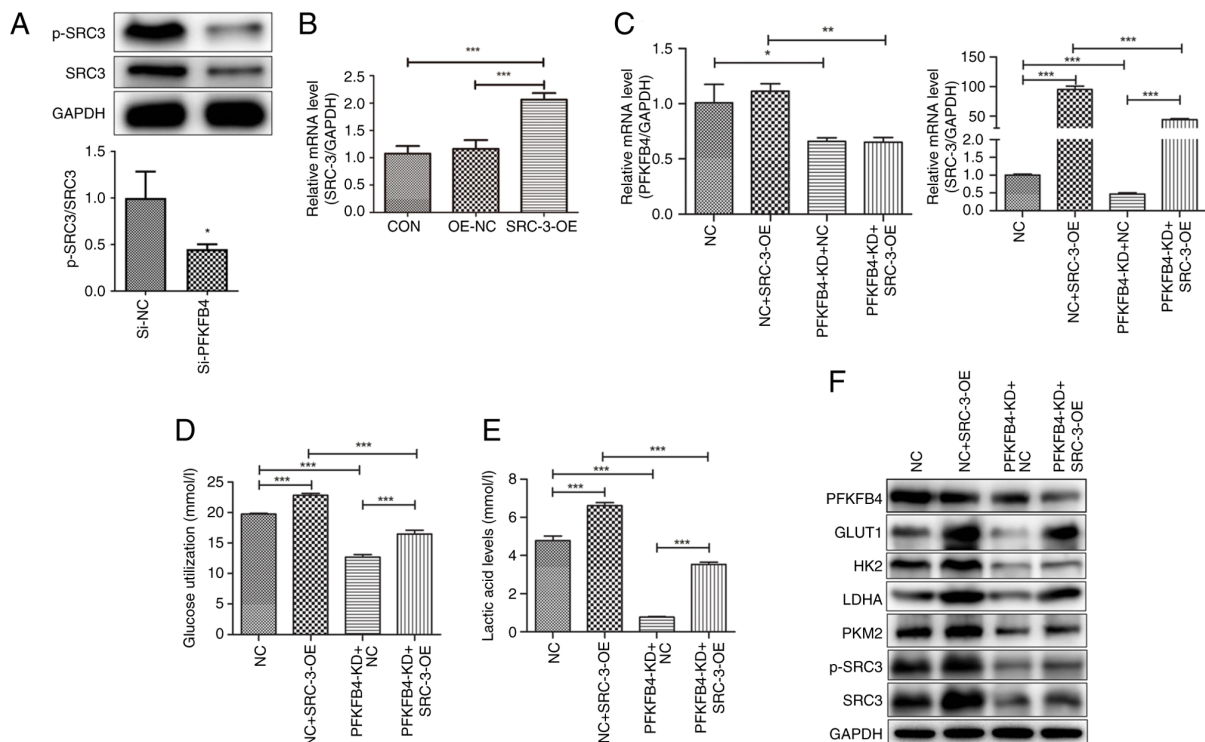


Figure 5. PFKFB4-phosphorylated SRC-3 regulates glycolysis in endometrial cancer cells. (A) The protein level of p-SRC-3 was detected by western blotting. (B) The efficiency of SRC-3 overexpression detected by RT-qPCR. (C) The effects of PFKFB4 KD and/or SRC-3 overexpression on the mRNA expression of PFKFB4 and SRC-3 detected by RT-qPCR. (D and E) Glucose and lactic acid production after PFKFB4 knockdown or SRC-3 overexpression measured by ELISA. (F) The expression levels of glycolysis-related enzymes detected by western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; p-, phosphorylated; SRC, steroid receptor coactivator-3; SRC-3-OE, SRC-3 overexpression plasmid; RT-qPCR, reverse transcription-quantitative PCR; KD, knockdown; p-, phosphorylated; NC, negative control.

depth of myometrial invasion, lymph node metastasis, surgical pathological stage, vascular invasion, and other adverse prognostic factors. Moreover, the present study revealed that the higher the tumor malignancy, the higher the PFKFB4 expression level, indicating that PFKFB4 may serve as a potential target for treating patients with malignant endometrial cancer. Yun *et al* (18) reported that PFKFB4 is a prognostic biomarker of non-muscle-invasive bladder cancer. Ros *et al* (37) also found that PFKFB4 upregulation predicts shorter survival in patients with non-small cell lung or breast cancers. Taken together, these results suggest that PFKFB4 is a potential therapeutic target for tumors.

PFKFB4 is also a key glycolytic enzyme that is highly expressed in numerous tumors (38–41). For instance, PFKFB4 is necessary in prostate cancer to maintain a balance between energy generation and glycolytic activity (21). PFKFB4 is also a key metabolic enzyme in pancreatic and breast cancers and acts as a regulator of the glycolytic pathway in these tumors (15,42). In the present study, the function of PFKFB4 was explored by knocking down PFKFB4 in HEC-1A cells and found that PFKFB4 knockdown suppressed the proliferation and migration of endometrial cancer cells and promoted their apoptosis, suggesting a carcinogenic effect of PFKFB4 in endometrial cancer. The current findings are consistent with those of PFKFB4 in other cancers (35,43).

Even when oxygen is abundant in the microenvironment, tumor cells metabolize glucose at a high rate, a unique metabolic pattern known as aerobic glycolysis (Warburg effect) (9). Metabolic reprogramming induces changes in metabolic enzymes and patterns in tumor cells. Glycolysis in tumor cells is regulated by different enzymatic reactions that allow glucose-derived carbon units to enter other anabolic pathways such as protein and nucleotide biosynthesis. PFKFB4 balances anabolism and redox homeostasis through allosteric regulation to control metabolic fluxes in the glycolysis and pentose phosphate pathways. PFKFB4 plays a critical role in regulating glucose metabolism and guiding metabolic pathways required for macromolecular biosynthesis to maintain cancer cell proliferation (44). In addition, PFKFB4 has been reported to be necessary for hypoxia-induced glycolysis (45). The present study explored the effects of PFKFB4 knockdown on glycolysis in endometrial cancer cells and revealed that PFKFB4 promotes glycolysis in cancer cells, which is consistent with previous studies. Thus, PFKFB4 may increase the proliferation and migration of endometrial cancer cells and inhibit apoptosis by enhancing glycolysis.

Furthermore, PFKFB4 may be involved in cancer cell metastasis via signal transduction. A previous study reported that the interaction between PFKFB4 and SRC-3 is a key regulatory mechanism in breast cancer (15). The interaction between PFKFB4 and SRC-3 was further investigated and it was found that PFKFB4 phosphorylated SRC-3, affected glucose metabolism, and promoted glycolysis in endometrial cancer cells. Improvement of glycolysis in cancer cells helps them cope with hypoxia and nutritional deficiency, thus promoting their proliferation and migration (46). Overall, the current results indicated that PFKFB4 may serve as a potential therapeutic target for cancer.

However, the present study had several limitations. The role of PFKFB4 in regulating endometrial cancer glycolysis

and cell malignant behaviors was only investigated using the loss-of-function method in one cell line owing to funding limitations. Next, the effects of PFKFB4 on SRC-3 were investigated; however, PFKFB4 can signal through additional the downstream molecules of PFKFB4 besides SRC3. Thus, RNA sequencing could be used in the future to more comprehensively identify PFKFB4-regulated signaling pathways. Further in-depth experiments in other endometrial cancer cells should be performed to comprehensively confirm the function and molecular mechanism of PFKFB4 in the regulation of glycolysis in endometrial carcinoma.

In conclusion, the present study suggests that PFKFB4 promotes proliferation and migration and inhibits apoptosis in endometrial cancer cells. Furthermore, it was revealed that PFKFB4 promotes glycolysis in endometrial cancer cells by phosphorylating SRC-3, suggesting a pro-tumor role of the PFKFB4-SRC-3-glycolysis axis in endometrial cancer. The present study provides *in vitro* and *in vivo* evidence of the potential application of PFKFB4 in endometrial cancer.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

YW and YW were involved in the conception and design, obtained the fundings. YW, JZ, SZ and JL performed the experiments, analysis and interpretation of the data. JL performed the statistical analysis. YW drafted the paper. YW and YW confirm the authenticity of all the raw data. All authors revised the manuscript critically for intellectual content, agree to be accountable for all aspects of the work, and read and approved the final version of the manuscript.

Ethics approval and consent to participate

Human studies were approved by the Ethics Committee of the People's Hospital of Shanxi (approval no. 13; Taiyuan, China), and all participants provided written informed consent in accordance with the Declaration of Helsinki. Animal experiments were conducted according to the ARRIVE guidelines and approved by the Ethics Committee of the People's Hospital of Shanxi (approval no. 9; Taiyuan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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