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Follicle-stimulating hormone promoted pyruvate kinase isozyme type M2-induced glycolysis and proliferation of ovarian cancer cells

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Abstract

Purpose Reprogramming of cell metabolism is essential for tumor progression and the best-studied metabolic phenomenon of cancer cells is aerobic glycolysis, in which pyruvate kinase isozyme type M2 (PKM2) plays a critical role. Follicle-stimulating hormone (FSH) contributes to epithelial ovarian cancer progression and has been shown to regulate cell metabolism in ovaries. The aim of this study was to investigate the interaction between FSH and PKM2 and their effect on aerobic glycolysis and cell proliferation in ovarian cancer.

Methods SKOV3 and OVCAR3 ovarian cancer cells were treated with FSH at various doses to investigate its effect on cell proliferation and PKM2 expression. siRNA-PKM2-transfected SKOV3 and OVCAR3 cells were treated with FSH to examine whether the changes induced by FSH could be altered by siRNA-PKM2. Glucose and lactate levels were evaluated to observe the change in glycolysis in these cells.

Results In the current study, FSH upregulated the expression of PKM2 and glycolysis in SKOV3 and OVCAR3 cells. PKM2 knockdown reduced FSH-induced cell growth and glycolysis. Moreover, FSH attenuated apoptosis that was induced by the inhibition of PKM2.

Conclusions Collectively, the findings of this study indicated that FSH promoted glycolysis in epithelial ovarian cancer cells. Knockdown of PKM2 inhibited aerobic glycolysis and cell proliferation induced by FSH.

Keywords Follicle-stimulating hormone · Epithelial ovarian cancer · Glycolysis · Pyruvate kinase isozyme type M2

Introduction

Ovarian cancer is a common malignant tumor of the female reproductive system. Because of its insidious onset, rapid progression, and the lack of effective early detection methods, 70% of patients are diagnosed in the late stage, and the 5-year survival rate in patients with ovarian cancer is only 15–30%. Epithelial ovarian cancer, which arises from simple surface epithelium, constitutes 90% of all ovarian tumors [1–3]. Epidemiological studies have indicated that the majority of epithelial ovarian cancer cases occurs in women with elevated gonadotropins, such as postmenopausal women or those who have received treatment for the induction of ovulation. It has been proposed that repetitive ovulatory trauma and high circulating concentrations of follicle-stimulating hormone (FSH) have growth-promoting effects in normal or immortalized ovarian surface epithelial cells and facilitate the carcinogenesis process [4–6].

In recent years, increasing evidence from research on tumor metabolism has shown that metabolic disorders are a core problem in the process of tumorigenesis and metabolic reprogramming, an indispensable genetic event that mediates malignant transformation. Almost all invasive cancers, regardless of the tissue of origin, exhibit a specific metabolic shift from respiration to fermentation [7, 8]. In the early 1920s, Otto Warburg demonstrated the Warburg effect whereby cancer cells, unlike normal tissues, prefer glycolysis for energy production even when oxygen is abundant. Aerobic glycolysis is characterized by increased glucose uptake and lactate excretion. It ensures a high production rate of glycolytic intermediates for the synthesis of biomass,

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such as ribonucleotides and amino acids, and reduced nicotinamide adenine dinucleotide phosphate production, which can remove reactive oxygen species generated under hypoxic conditions. In addition, upregulation of glycolysis and the consequent reduction in the pH of the tumor microenvironment were proved to promote tumor invasion by favoring cell migration, angiogenesis, and immune escape [9–12].

Pyruvate kinase (PK) regulates the final rate-limiting step of glycolysis and catalyzes the final reaction in glycolysis by transforming the high-energy phosphate from phosphoenolpyruvate into adenosine diphosphate to generate adenosine triphosphate and pyruvate [13]. Pyruvate kinase isozyme type M2 (PKM2) is a subtype of PK, and accumulating evidence has demonstrated the critical participation of PKM2 in tumorigenesis via the promotion of the Warburg effect. In addition to its essential metabolic role, PKM2 also contributes to the process of tumorigenesis through its non-metabolic attributes. It has been reported that the expression of PKM2 was closely associated with the degree of tumor malignancy, angiogenesis, invasion, and metastasis [14–16].

Our previous work has shown that PKM2 contributed to the progression of human ovarian cancer [17]. In this study, we further investigated the interaction between FSH and PKM2, as well as their effect on aerobic glycolysis and the biological behavior of the cancer cells.

Materials and methods

Cell culture

Two human ovarian cell lines, SKOV3 and OVCAR3, which highly express PKM2 as proved in our previous study [17], were obtained from the Shanghai Cell Bank, Chinese Academy of Science (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 100 μg/mL streptomycin, 10% heat-inactivated fetal bovine serum, and 100 U/mL penicillin.

Transient transfection and hormone stimulation

SKOV3 and OVCAR3 cells were collected in the logarithmic growth phase and transfected with 400 nmol/L siRNA-PKM2™ or siRNA (as a negative control) using Lipofectamine 2000 (Invitrogen, Shanghai, China) according to a previous protocol [17]. Cells were seeded in antibiotics-free medium the day before transfection and transfected with 400 nmol/L siRNA-PKM2™ or siRNA negative control. For siRNA-PKM2, RNA oligomers corresponding to human PKM2 were synthesized in sense and antisense directions, containing three sections with the following sequences: at nucleotides 871–893 (5′-GAGGTATTCCGATGCTTATTT-3′), 21 nucleotides; at nucleotides 1335–1357 (5′-TGGTGT TTGCGTCATTCAT-3′), 19 nucleotides; and at nucleotides 1792–1814 (5′-CCGCAAGCTGTTTGAAGAA-3′), 19 nucleotides. Transfected and non-transfected cells were treated with FSH at different concentrations (Sigma-Aldrich, Shanghai, China) and the transfected cells were collected for the following assays.

Cell growth and proliferation assay

Cell Counting Kit-8 (CCK-8, Tongren, Shanghai, China) was used to assess the effects of siRNA-PKM2 and FSH on the viability of SKOV3 and OVCAR3 cells. First, the cells were treated with FSH at various doses (10–80 mIU/mL) for different time periods (0–72 h). CCK-8 reagent was then added to each well containing treated cells at 1:10 (v/v) in 100 μL of medium. After incubation, the optical density (OD) of the supernatant in each well was determined at 450 nm using a microplate reader. As the most significant cell proliferation was observed when the cells were exposed to 40 mIU/mL FSH for 48 h, this treatment condition was selected for further study.

We then added 40 mIU/mL FSH to the siRNA-PKM2-transfected and non-transfected cells, and CCK-8 reagent was added to each well containing cells treated in various ways (control, control + FSH, siRNA negative control + FSH, and siRNA-PKM2 + FSH). After 48 h, the OD was measured at 450 nm. Experiments were performed at least three times, each time in triplicate.

Glucose and lactate assays

To evaluate the concentration of glucose and lactate in culture media, the glucose and lactate assay (F006, A019-2, Jiancheng Bioengineering Institute, Nanjing, China) was conducted. To explore the involvement of PKM2 in cell metabolism, we compared the glucose uptake and lactate production in SKOV3 and OVCAR3 cells transfected with 400 nmol/L siRNA-PKM2™ or siRNA negative control. FSH was then added at 40 mIU/mL and the cells were cultured for an additional 48 h. The medium was harvested and assayed for glucose utilization and lactate production.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The mRNA levels of PKM2 in response to FSH in SKOV3 and OVCAR3 cells were quantified by qRT-PCR. TRIzol reagent(Invitrogen, Japan) was used to isolate total RNA, and the obtained mRNA was detected by agarose gel electrophoresis. cDNA was synthesized from approximately 5 μg of RNA using AMV reverse transcriptase (Fermentas, USA). qRT-PCR reactions were performed in a total volume of 25 μL using SYBR® Green 10 × Supermix (TaKaRa, Japan) on

a Roche Light Cycler® 480II System (Roche Diagnostics Ltd., Switzerland). PrimerExpress Software (Applied Biosystems, Shanghai, China) was used to design the PKM2 primer pairs (Table 1) and glyceraldehyde 3-phosphatedehydrogenase (GAPDH) was used as the internal control. The PCR procedure was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s; one cycle of 95 °C for 15 s, 60 °C for 1 min; one cycle of 95 °C for 15 s and 60 °C for 15 s. The relative expression levels were calculated using the ΔΔCT method by normalizing to the mRNA expression level of GAPDH. All PCR reactions were performed in triplicate.

Western blot

The relationship between PKM2 and FSH was explored by western blot. Transfected cells were harvested, washed twice with phosphate-buffered saline, lysed in ice-cold radio immunoprecipitation assay buffer (Beyotime, Shanghai, China) containing 0.01% protease and phosphatase inhibitor (Sigma-Aldrich, Shanghai, China), and incubated on ice for 30 min. The cell lysate was obtained and centrifuged at 12,000×*g* at 4 °C for 10 min. Proteins in the supernatant were quantified by a bicinchoninic acid protein quantitation kit (PICPI23223; Thermo Fisher Scientific). Approximately 20–30 μg of protein sample in each load was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). The membrane was blocked with 5% bovine serum albumin in PBS/Tween 20 and incubated with primary antibodies against PKM2 (Ab133490; 1:1000; Abcam) and GAPDH (5174; 1:1500; Cell Signaling Technology). The blots were incubated for 1 h at 37 °C with goat anti-mouse or anti-rabbit secondary antibodies (Beyotime), and the band intensities were measured by enhanced chemiluminescence (Thermo Scientific, Shanghai, China).

Flow cytometry

Cell cycle progression was measured using propidium iodide (PI) flow cytometry. Cells were incubated in culture solution for 60 min and seeded in 6-well plates at a density of 3×10^5 cells/well. After 48 h of incubation, the cells were washed with PBS, trypsinized, and centrifuged at 1000×*g* at 4 °C for 5 min. The obtained pellets were suspended with 300 μL of PBS containing 10% fetal bovine serum and fixed in 700 μL of cold ethanol at − 20 °C for 24 h. Then the cells were washed twice with PBS and incubated in PBS containing RNase A (1 mg/mL) for 10 min at 37 °C. The samples were stained with PI (1 mg/mL) for 10 min, shielded from light, at room temperature. The cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed using CellQuest software (BD Biosciences). Apoptotic cells were identified by flow cytometry using the Annexin-V/fluorescein isothiocyanate (FITC) kit (BD Biosciences). SKOV3 and OVCAR3 cells were collected, resuspended in 200 μL of binding buffer containing 5 μL of Annexin-V/FITC, and stained at 20–25 °C in the dark for 10 min. The cells were then centrifuged at room temperature at 1000×*g* for 5 min and resuspended in 200 μL of binding buffer. Then, 10 μL of PI was added and mixed gently, and the cells were stained in an ice bath in the absence of light, after which flow cytometry was performed.

Statistical analysis

All statistical analyses were conducted using the GraphPad Prism 6.0 software. Data were analyzed by *t* tests. All experiments were performed with values expressed as mean ± standard deviation. A value of $p<0.05$ was considered statistically significant in all statistical comparisons.

Results

PKM2-mediated FSH-induced ovarian cell growth

Cell growth (OD at 450 nm) and proliferation rates were measured by CCK-8. After FSH was added for 24, 48, and 72 h, the viability of both SKOV3 and OVCAR3 cells was markedly increased in a dose-dependent and time-dependent manner (Fig. 1). The most significant cell proliferation was observed when the cells were exposed to 40 mIU/mL FSH for 48 h.

Table 1 Primers used in qRT-PCR analysis

Gene	Gene primer sequence	Species	Amplicon size (bp)
PKM2	Forward: 5′-TCTCCAGGGCACACCGTATTC-3′	Human	108
	Reverse: 5′-GCTGCTGAGGTCCTTTGGTTC-3′		
GAPDH	Forward: 5′-CACCCACTCCTCCACCTTTG-3′	Human	110
	Reverse: 5′-CCACCACCCTGTTGCTGTAG-3′		

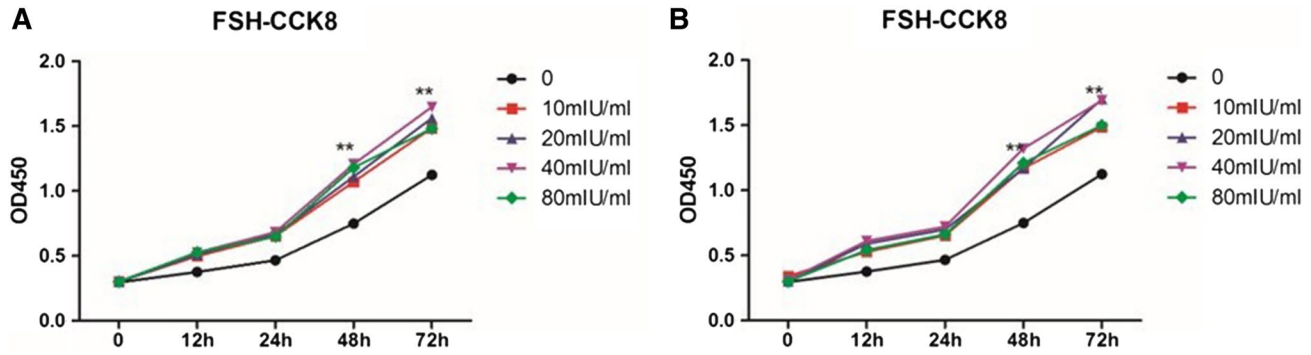

Fig. 1 FSH simulated the growth of cancer cells. FSH simulated the growth of **a** SKOV3 and **b** OVCAR3 ovarian cancer cells in a dose-dependent manner. Significant stimulatory effects were apparent 48 h after treatment with FSH at a concentration of 40 mIU/mL in both cells. $^{**}p < 0.01$

We then determined whether the pro-proliferative effect of FSH could be attenuated by siRNA-PKM2. Among the four tested groups (control, FSH treatment at 40 mIU/mL for 48 h, siRNA negative control + FSH, and siRNA-PKM2 + FSH), the viability of both SKOV3 and OVCAR3 cells was evidently weakened by siRNA-PKM2 (Fig. 2). This result indicated that PKM2 may at least partially mediate FSH-induced ovarian cell growth.

PKM2 expression was upregulated by FSH

To evaluate the relationship between FSH and PKM2 overexpression in ovarian cancer cells, the effect of FSH administration on PKM2 expression was studied. As shown in Fig. 3, FSH stimulation resulted in a significant increase in the mRNA and protein expression of PKM2 in a dose-dependent and time-dependent manner in both SKOV3 and OVCAR3 cells. The most significant upregulation of PKM2 was observed when the cells were exposed to 80 mIU/mL FSH for 48 h.

PKM2 knockdown reduced FSH-induced cell glycolysis

To explore the role of PKM2 in ovarian cancer cell glycolysis, we examined the glucose uptake and lactate production in cells transfected with siRNA-PKM2 and control cells (Fig. 4). We observed that glucose uptake and lactate secretion were significantly reduced in PKM2-knockdown cells compared with those in the control cells. We then added 40 mIU/mL FSH to the cells and examined the glucose uptake and lactate secretion after FSH treatment. Much higher glucose uptake and lactate secretion were observed in cells stimulated with FSH, but the increase was markedly reduced by PKM2 knockdown (Fig. 5). In addition, FSH-induced promotion of cell proliferation was inhibited by PKM2 knockdown (Fig. 2). Taken together, these results suggested that FSH is involved in Warburg metabolism through the regulation of PKM2 expression.

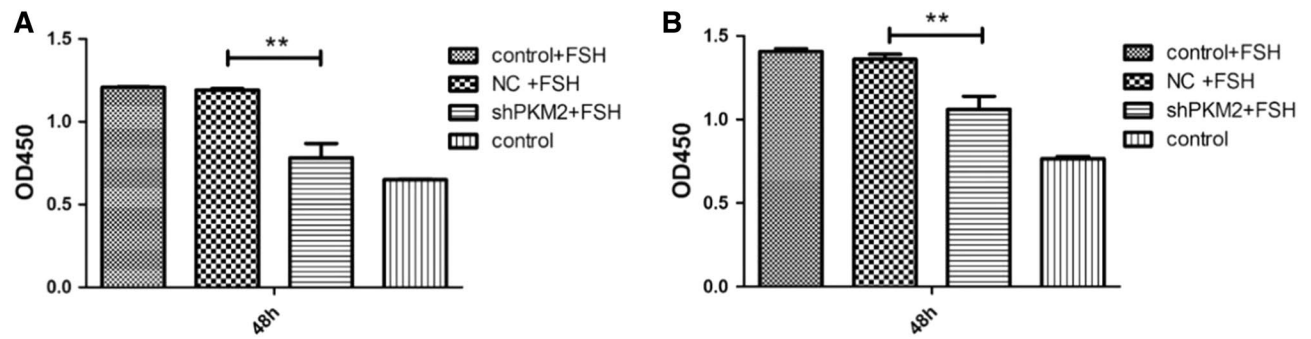

Fig. 2 PKM2 knockdown reduced FSH-induced cell proliferation. siRNA-PKM2 markedly inhibited the FSH-induced proliferation of **a** SKOV3 and **b** OVCAR3 cells in a time-dependent manner. $^{**}p < 0.01$

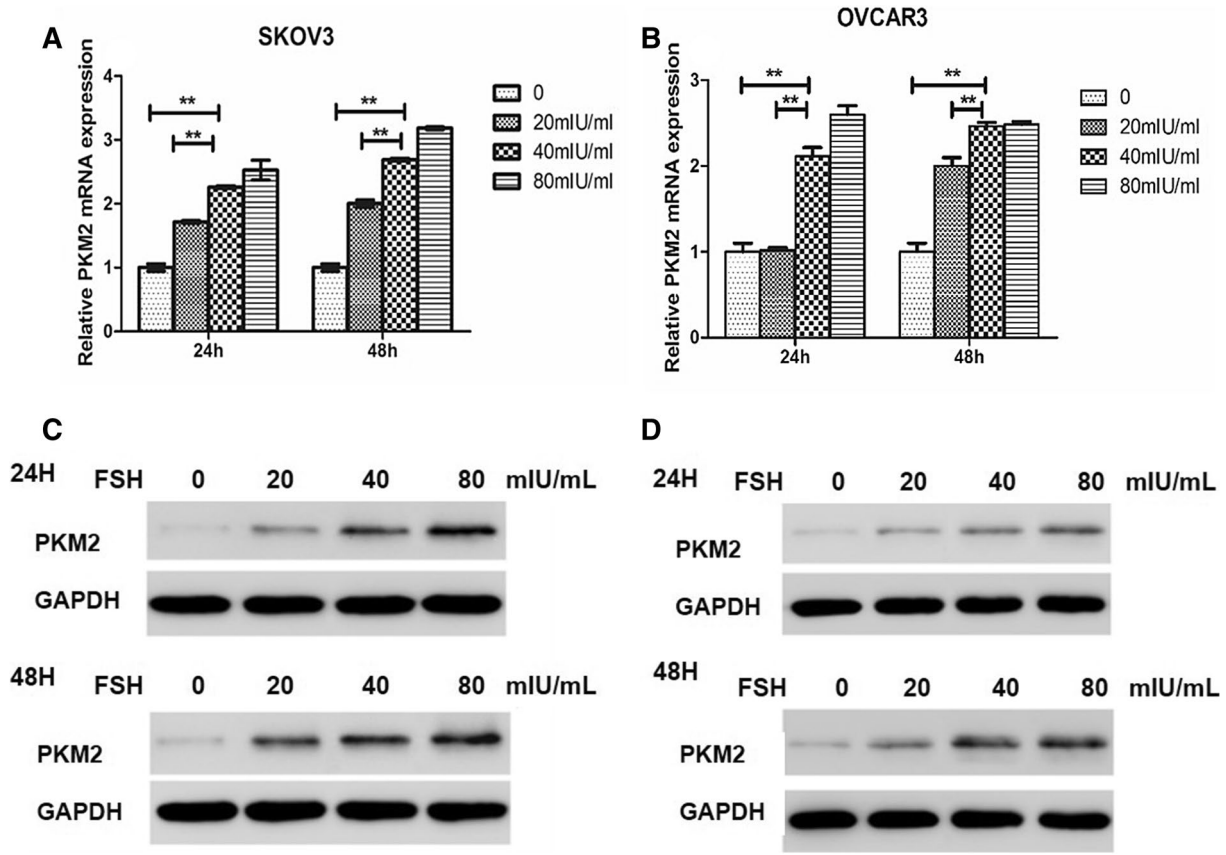

Fig. 3 PKM2 overexpression in ovarian cancer cells with FSH stimulation. Relative PKM2 mRNA expression in **a** OVCAR3 and **b** SKOV3 cells treated with various doses of FSH for 24 or 48 h. PKM2 protein expression in **c** OVCAR3 and **d** SKOV3 treated with various doses of FSH for 24 or 48 h. $^{**}p < 0.01$

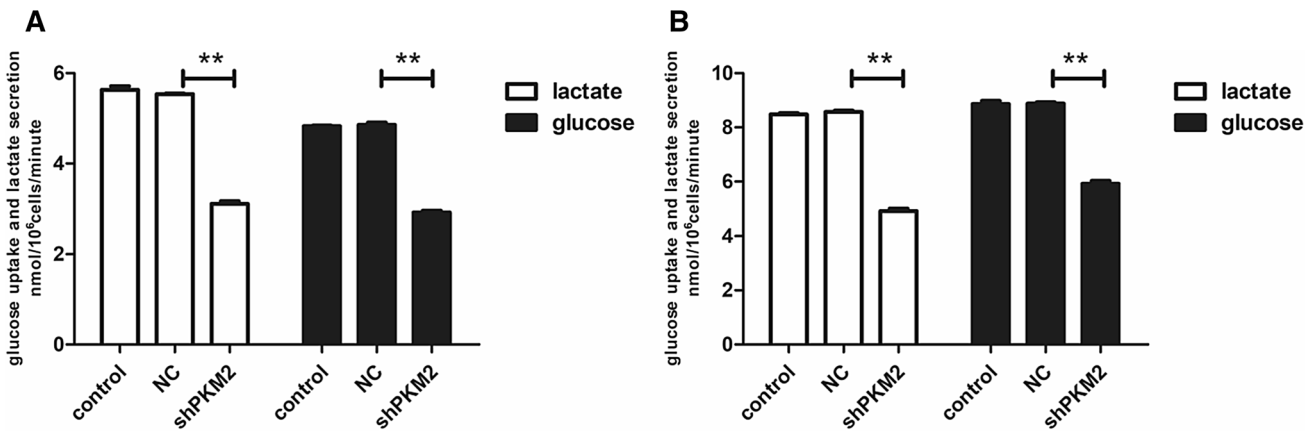

Fig. 4 PKM2 knockdown reduced cell glycolysis. Glucose uptake and lactate secretion were significantly reduced by PKM2 knockdown in **a** SKOV3 and **b** OVCAR3 cells. $^{**}p < 0.01$

Apoptosis induced by PKM2 knockdown was attenuated by FSH

Our previous study showed that knocking down PKM2 resulted in a significant increase in early apoptosis rate in SKOV3 and OVCAR3 cells [17]. To verify whether PKM2 participates in FSH-induced inhibition of apoptosis, FSH (40 mIU/mL) was added to these cells and PKM2 was knocked

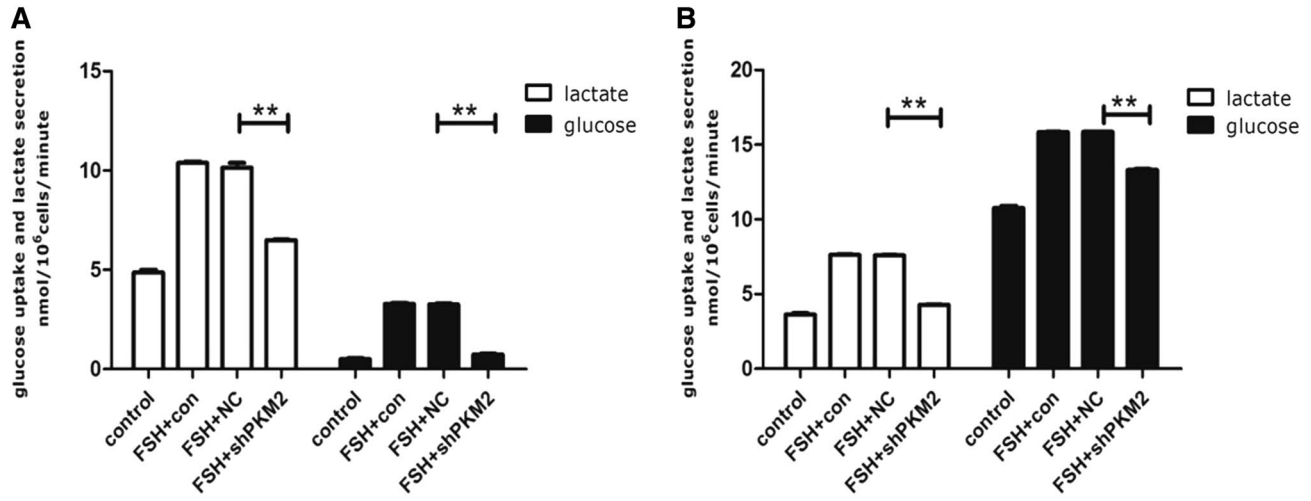

Fig. 5 PKM2 knockdown reduced FSH-induced cell glycolysis. Glucose uptake and lactate secretion were significantly increased with FSH treatment (40 mIU/mL). The increase in glucose uptake and lactate secretion were reduced by PKM2 knockdown in **a** SKOV3 and **b** OVCAR3 cells. $^{**}p < 0.01$

down by siRNA. We found that FSH inhibited apoptosis and decreased the early apoptosis rate induced by PKM2 knockdown in both cell lines (Fig. 6).

Discussion

Metabolic alterations are essential for cancer cells for continued growth, proliferation, and survival [18, 19]. Increased aerobic glycolysis not only enables rapid adenosine triphosphate generation, but also has biosynthetic advantages and contributes to the proper control of redox balance in tumor cells. The tumorigenicity of cancer cells has been greatly compromised by reversing the Warburg effect in these cells, which indicates that targeting metabolic changes could be a feasible strategy for cancer treatment [20–22].

The tumor metabolic network is complex and aerobic glycolysis is a hallmark of cancer that can be regulated by diverse factors. The activation of oncogenes, such as Myc and hypoxia-inducible factor-1 (HIF-1), and inactivation of tumor suppressors, such as p53, phosphatase and tensin homolog, tuberous sclerosis complex 2, and liver kinase B1, in cancer cells have been shown to contribute to metabolic alterations [23–26]. In addition, activation of phosphatidylinositol-4, 5-bisphosphate 3-kinase/protein kinase B signaling is vital for the activation of glycolysis, and the mammalian target of rapamycin (mTOR) signaling pathway also plays a critical role in tumorigenesis and metabolism. Further research has reported that the activation of mTOR induced HIF-1α, which in turn induced PKM2 expression to promote glycolysis [27].

The expression of the PKM2 isoform has been closely linked to embryogenesis, tissue repair, and cancer. PKM2 is located at a critical position in the glycolytic flux to respond to various stimuli that are crucial for the Warburg effect. It catalyzes the final step of glycolysis and plays an important role in reprogramming the glycolytic metabolism to feed the demands of proliferating cells [15, 28, 29]. PKM2 is overexpressed in essentially all human cancer cells and commonly acts as a new cancer biomarker. It facilitates tumor proliferation by promoting anabolic metabolism and, in addition, interacts with oncogenic signaling in response to extracellular stimulation [30–35]. Sun et al. suggested that mTOR signaling during multistep oncogenic processes contributed to the development of the Warburg effect through regulating PKM2 [27]. In our previous reports, we found that PKM2 depletion markedly inhibited cell proliferation, induced apoptosis, and caused cell cycle arrest at the G0/G1 phase [17]. In the current study, we observed a decrease in glucose consumption and lactate production in ovarian cancer cells by knocking down PKM2, and the downregulation of glycolysis was related to the proliferation of cancer cells. The results implied that PKM2 may be involved in inhibiting apoptosis and promoting proliferation in ovarian cancer cells.

FSH has previously been shown to stimulate the growth of human epithelial ovarian cancer tissues and cells [36, 37]. Although the expression of FSH receptors was much lower in SKOV3 cells compared with that in OVCAR3 cells [36], FSH increased the growth and invasion in SKOV3 cells [37–39], indicating that the signaling pathways activated by FSH stimulation in ovarian cancer are not fully understood. Moreover, little is known about the alterations in metabolism induced by FSH. The modulation in the glucose metabolic capacity of human preantral follicles in vitro by gonadotropins was evaluated, and FSH and luteinizing hormone were

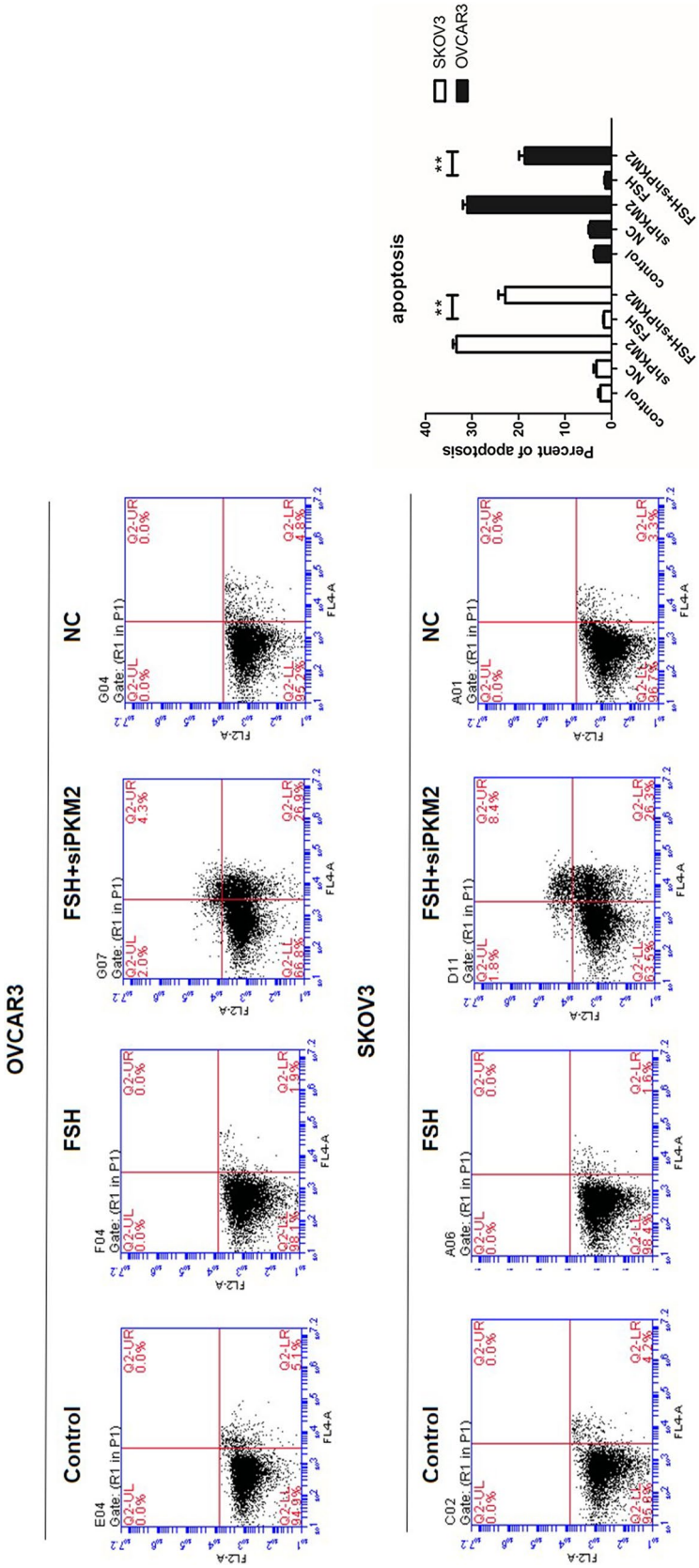

Fig. 6 Apoptosis induced by PKM2 knockdown was attenuated by FSH. The early apoptosis rate was increased in the presence of FSH (40 mIU/mL) and decreased by PKM2 knockdown in **a** SKOV3 and **b** OVCAR3 cells. $^{**}p < 0.01$

found to significantly stimulate the activities of phosphofructokinase and PK in class 1 and 2 follicles, inferring that FSH may be associated with glucose metabolism in ovarian cells [40]. It was observed in our study that FSH treatment significantly increased ovarian cancer cell proliferation, glucose consumption, and lactate production. These effects, however, were suppressed by specific siRNA-PKM2. Our findings indicated that FSH may promote abnormal anabolic metabolism and cell growth in epithelial ovarian cancer cells due to the upregulation of PKM2.

Conclusion

In conclusion, we showed that glycolysis induced by PKM2 may play a role in epithelial ovarian cancer development. We further found that FSH elevated PKM2 expression and facilitated tumor proliferation by promoting anabolic metabolism. Our findings could be an important supplement to the mechanism of FSH in promoting epithelial ovarian cancer cell proliferation and provide novel insights into FSH-induced apoptosis inhibition and abnormal cell metabolism in epithelial ovarian cancer.

Author contributions SL: data collection, data analysis and interpretation, drafting the article, final approval of the version to be published. XJ: cell experiment and data collection, final approval of the version to be published. RW: cell experiment, final approval of the version to be published. YM: conception or design of the work, critical revision of the article, drafting the article, final approval of the version to be published.

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Compliance with ethical standards

Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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