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Paeonol regulates glycolytic metabolism by downregulating BACH1 to ameliorate stemness, angiogenesis, and EMT in SiHa cervical cancer cells

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Summary. As a common reproductive malignancy of the female reproductive system, cervical cancer has increasingly become a public health concern. Paeonol, which is a natural phenolic monomer, has been found to possess substantial anticancer effects in some human cancers. The present study was conceived to explore the role and mechanism of paeonol in cervical cancer. Initially, the cytotoxicity of paeonol on immortalized H8 cervical epithelial cells and the proliferation of SiHa cervical cancer cells with paeonol treatment were detected using the CCK-8 assay. Cell stemness was assessed with the spheroid formation assay while western blot was applied for the measurement of proteins associated with cell stemness. The tube formation assay was used to detect the angiogenesis of human umbilical vein endothelial cells (HUVECs) and western blot was used to estimate the expression of EMT- and angiogenesis-related proteins. The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of cells were appraised via a Seahorse XFe24 Flux Analyzer. Lactate production, glucose consumption, and ATP levels were evaluated with corresponding assay kits. Western blot was applied for the evaluation of GLUT1 and HK2. The mRNA and protein expression of BACH1 before and after transfection were detected using RT-qPCR and western blot. The luciferase reporter assay was used to detect the activities of GLUT1 and HK2 promoters. In this study, we found that paeonol inhibited cell proliferation, cell stemness, EMT progress, angiogenesis, and glycolysis in cervical cancer via downregulating BACH1. In summary, paeonol impeded the progression of cervical cancer by regulating glycolytic metabolism through the inhibition of BACH1.

Key words: Paeonol, Cervical cancer, Glycolytic metabolism, BACH1

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Introduction

Cervical cancer, which takes second place as the world's most prevalent female cancer, is still a primary contributor to cancer-related deaths among women (Cancer Genome Atlas Research Network et al., 2017, Chen et al., 2020). Human papillomavirus (HPV) vaccination is reported to be effective for the prevention of cervical cancer (Singh et al., 2018). However, due to its lower popularity in China, a majority of women missed or postponed the vaccination (Xu et al., 2020). At present, the combination of surgical resection and chemotherapy remains the top priority for controlling the prognosis of patients with cervical cancer; however, patients are still prone to lymph node metastasis as well as distant metastasis, which results in lower survival rates and poorer prognosis (Brucker and Ulrich, 2016; Kontostathi et al., 2016). Therefore, comprehending the mechanism of cervical cancer and exploring more promising therapeutic agents are a great necessity.

Paeonol (2'-hydroxy-4'-methoxyacetophenone), the predominant active component in the extract of peony root, has been well-documented to possess a diverse range of pharmacological actions, such as anti-inflammatory, neuroprotective, antitumor, antidiabetic as well as cardioprotective properties (Li and Gu, 2022). In recent years, the antitumor effect of paeonol has received widespread attention. For instance, paeonol can inhibit cell viability, migration, invasion, and epithelial-mesenchymal transition (EMT) progression in lung cancer (Lv et al., 2022). Also, Li et al. evidenced that paeonol can suppress the proliferation, metastasis, and glycolysis of Apatinib-resistant gastric cancer cells (Li et

al., 2022). Our previous study demonstrated that paeonol can suppress migration and invasion, whereas it facilitates apoptosis in cervical cancer cells (Sheng et al., 2021). However, energy metabolism regulation by paeonol in cervical cancer cells remains elusive.

As a heme-dependent transcription factor, BTB and CNC homology 1 (BACH1) has been acknowledged to be a critical regulator in the physiological modulation of tumor metastasis (Su et al., 2023). According to the HPA database (www.proteinatlas.org), BACH1 expression was upregulated in the tissues of cervical cancer patients. Data in the Kaplan-Meier Plotter database (http://kmplot.com/analysis/index.php?p=background) revealed that BACH1 upregulation was associated with the poor prognosis of cervical cancer patients. To date, the role of BACH1 in cervical cancer has not been reported.

In summary, this study was conceived to explore the efficacy of paeonol on the proliferation, stemness, ETM progress, angiogenesis, and glycolysis of cervical cancer cells as well as to discuss the hidden reaction mechanism.

Materials and methods

Cell culture and treatment

Normal H8 cervical epithelial cells and SiHa cervical cancer cells were provided by BioVector NTCC Inc (Beijing, China). These cells were incubated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin-streptomycin (both from Sigma-Aldrich) at 37°C in the presence of 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were provided by the BeNa Culture Collection and treated with conditioned medium (CM) at 37°C with 5% CO_2. Paeonol (purity >98%) was purchased from Dalian Meilun Biotechnology Co., Ltd. (cat. no. MB1762-S). Paeonol was initially dissolved in dimethyl sulfoxide (DMSO, MERCK, Darmstadt, Germany) and configured as the stock solution, which was further diluted to 0.05, 0.1, and 0.2 mg/ml to pre-treat SiHa cells for 24h (Sheng et al., 2021). Cells were treated with the same volume of DMSO in the drug and control groups.

Cell transfection

SiHa cells harvested in the logarithmic growth phase were inoculated into six-well plates at a density of 1×10^5 cells/mL. For transfection, pc-DNA3.1 vectors containing the complete BACH1 sequence (oe-BACH1) and empty vector (oe-NC) were synthesized by GenePharma (Shanghai, China). Using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.), the above recombinants were transfected into SiHa cells for 48h at 37°C. After 48h of transfection, SiHa cells were collected for follow-up experiments.

Cell counting kit-8 (CCK-8) assay

To explore the cytotoxicity of paeonol on H8 and SiHa cells, a CCK-8 assay was conducted. In brief, cells collected in their logarithmic growth phase were inoculated into 96-well plates at a density of 3×10^4 cells/well and then incubated at 37°C for 24h. After that, 10 μL of CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well, and cells were incubated for an additional 3h. Finally, the optical density (OD) value at 450 nm was determined with a microplate reader.

Spheroid formation assay

SiHa cells were incubated in serum-free DMEM/F12 medium containing B27 supplement, 20 ng/mL EGF, and 20 ng/mL bFGF (ThermoFisher Scientific, USA) in 96-well ultra-low attachment dishes (Ni et al., 2021). After that, cells were inoculated into 200 μL serum-free medium (200 cells/per well) for at least 7 days (Ivanov et al., 2014). The number of spheres larger than 70 μm was counted and a Leica DMI microscope was used for observation.

Western blot

Proteins were extracted from SiHa cells with RIPA lysis buffer (Solarbio) and the protein concentration was quantified with bicinchoninic acid (BCA) protein assay kits (Thermo Fisher Scientific Inc.) following the standard protocol. Following separation with 8% SDS-PAGE, equal amounts of proteins (20 μg per lane) were transferred to PVDF membranes, which were sealed with 5% BSA for 2h at room temperature. After that, membranes were immunoblotted with primary antibodies specific to BACH1 (ab300130; 1:1000; Abcam), Oct4 (ab200834; 1:10000; Abcam), Nanog (ab109250; 1:1000; Abcam), VEGFR1 (ab32152; 1:1000; Abcam), VEGFR2 (ab134191; 1:1000; Abcam), E-Cadherin (ab40772; 1:1000; Abcam), N-Cadherin (ab76011; 1:5000; Abcam), Vimentin (ab92547; 1:1000; Abcam), GLUT1 (ab150299; 1:200; Abcam), HK2 (ab209847; 1:1000; Abcam), or GAPDH (ab9485; 1:2500; Abcam) overnight at 4°C. On the next day, the membranes were exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies (ab6721; 1:2000; Abcam) at room temperature for 1 h. Finally, the protein bands were visualized with enhanced chemiluminescence (ECL) detection reagent (Shanghai Yeasen Biotechnology Co., Ltd.), and ImageJ software (version 1.49, National Institutes of Health) was applied for the analysis of protein density.

Tube formation assay

HUVECs were inoculated into 96-well plates containing Matrigel® gel substrates, which were then exposed to cell supernatants for 8h at 37°C with 5%

CO_2. Subsequently, HUVECs in five randomly selected fields of view were observed under a light microscope and statistical analysis was implemented utilizing ImageJ software.

ECAR and OCR measurement

With the application of the Seahorse XFe24 Flux Analyzer (Seahorse Bioscience, Agilent), the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of SiHa cells were detected with a glycolytic stress test kit (#103020-100; Seahorse Bioscience) and a mitochondrial stress test kit (#103015-100; Seahorse Bioscience). In brief, SiHa cells (1×10^4) inoculated into a 96-well XF Seahorse incubation plate were incubated in XF basal media with sequential addition of glucose (10 mM), glutamine (1 mM), 2-DG (50 mM), and oligomycin (1 µM) at indicated time points according to manufacturer's instructions. The detection of OCR and ECAR was conducted and plotted with Seahorse XF24 software.

Lactate production, glucose consumption, and ATP assays

With the application of the glucose (cat. no. CBA086; Sigma), lactate (cat. no. K607; Biovision), or ATP assay kits (cat. no. MAK190; Sigma), changes in glucose, lactate, or ATP following 24h incubation were detected according to manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA extracted from SiHa cells with TRIzol reagent (Biosharp) was synthesized into complementary DNA by a commercial RevertAid™ cDNA Synthesis kit (Bio-Rad) according to manufacturer's instructions. Following, the templates were amplified on the 7500 Fast Real-time PCR system using the SYBR Green PCR Master Mix (Takara, Toyobo, Japan) according to manufacturer's instructions. The relative gene expression was calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences used were: BACH1 forward (F), 5'-CGCCTCAGCTCTGGT TGATG-3' and reverse (R), 5'-CATCAGCCTGGCCT ACGATT-3', GAPDH F, 5'-TGTGGGCATCAA TGGATTTGG-3' and R, 5'-ACACCATGTATTCC GGGTCAAT-3'.

Luciferase reporter assay

The HumanTFDB and JASPAR databases predicted the binding sites of BACH1 for the GLUT1 and HK2 promoters. Interactions between SOX4 and ADAM17

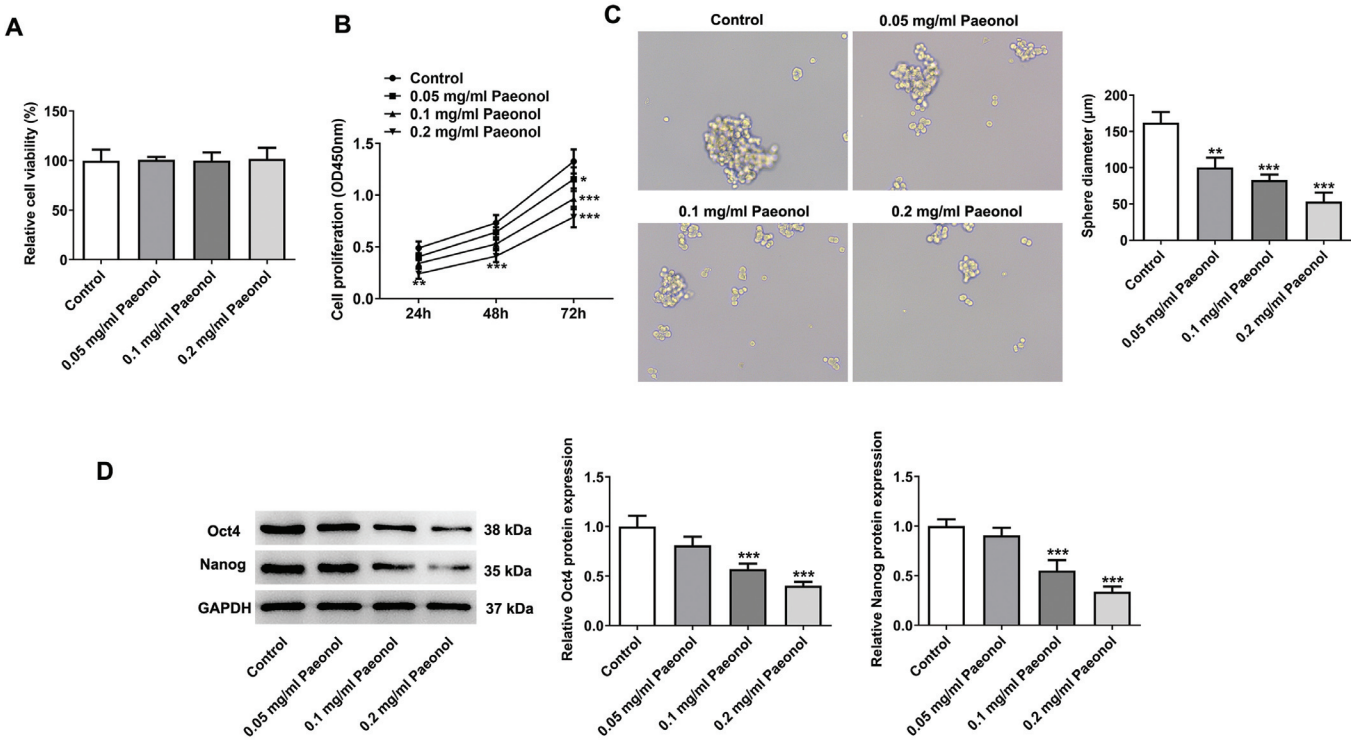

Fig. 1. Paeonol inhibits the proliferation and cell stemness of cervical cancer cells. **A.** The effects of paeonol on H8 viability were detected using the CCK-8 assay. **B.** The proliferation of SiHa cells was detected using the CCK-8 assay. **C.** The cell stemness of SiHa cells was detected using a spheroid formation assay. **D.** The expressions of cell stemness-related proteins were detected using a western blot. *p<0.05, **p<0.01, and ***p<0.001 vs. control.

were verified with the Luciferase Reporter System (Promega). GLUT1 wild-type (WT) and mutant (MUT) reporter plasmids, and HK2WT and HK2-MUT reporter plasmids with oe-BACH1 or oe-NC binding sites were constructed by GenePharma and then transfected into SiHa cells with Lipofectamine 2000 reagent (Thermo Fisher Scientific Inc.) according to manufacturer's instructions. Finally, luciferase activity was normalized to that of Renilla.

Statistical analysis

All experiments were replicated three times. The collected experimental data were analyzed with GraphPad Prism software (version 8.0) and displayed as mean ± standard deviation. For the demonstration of differences among multiple groups, a one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was adopted. p lower than 0.05 was indicative of statistical significance.

Bioinformatics tools

According to the HPA database, BACH1 expression was upregulated in cervical cancer tissues. The Kaplan-Meier Plotter database showed that BACH1 upregulation was associated with the poor prognosis of cervical cancer patients. HumanTFDB and JASPAR databases predicted the binding sites of BACH1 for the GLUT1 and HK2 promoters.

Results

Paeonol inhibits the proliferation and cell stemness of cervical cancer cells

To evaluate the cytotoxicity of paeonol on H8 and SiHa cells, the CCK-8 assay was initially implemented. Compared to the Control group, paeonol treatment had no significant effects on the viability of H8 cells but conspicuously reduced the proliferation of SiHa cells in a concentration-dependent manner (Fig. 1A,B). Results obtained from the spheroid formation assay revealed that paeonol treatment decreased the number of spheres in a dose-dependent manner when compared with the Control group (Fig. 1C). The expression of stemness-related proteins was detected with western blot and the results demonstrated that paeonol treatment concentra-

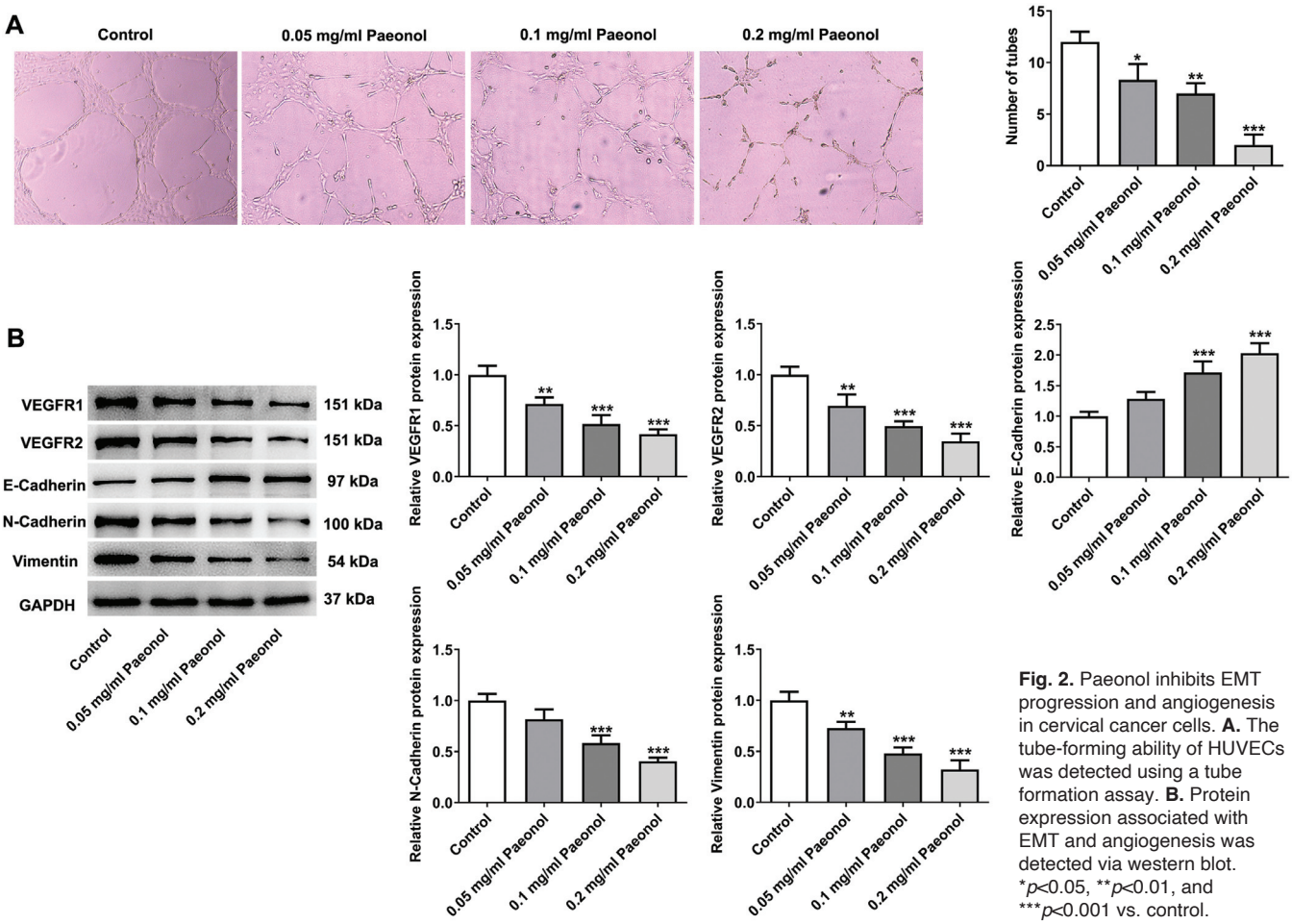

Fig. 2. Paeonol inhibits EMT progression and angiogenesis in cervical cancer cells. **A.** The tube-forming ability of HUVECs was detected using a tube formation assay. **B.** Protein expression associated with EMT and angiogenesis was detected via western blot. *p<0.05, **p<0.01, and ***p<0.001 vs. control.

tion-dependently reduced the protein expressions of Oct4 and Nanog in contrast to the Control group (Fig. 1D).

Paeonol inhibits EMT progression and angiogenesis in cervical cancer cells

As Figure 2A demonstrates, paeonol treatment markedly inhibited the tube-forming ability of HUVECs in a concentration-dependent manner in comparison with the Control group. The expression of proteins associated with EMT and angiogenesis was assessed with western blot, finding that paeonol treatment increased E-Cadherin expression but decreased VEGFR1, VEGFR2, N-Cadherin, and Vimentin expression in SiHa cells in a dose-dependent manner (Fig. 2B).

Paeonol inhibits glycolysis in cervical cancer cells

Results obtained from the Seahorse XFe24 Flux Analyzer revealed that paeonol treatment decreased the ECAR while increasing the OCR in SiHa cells (Fig. 3A,B). Furthermore, lactate production, glucose consumption, and ATP levels in SiHa cells were also concentration-dependently reduced by paeonol treatment in contrast to the Control group (Fig. 3C-E). Moreover, results obtained from the western blot demonstrated that paeonol treatment decreased the protein expression of GLUT1 and HK2 in a concentration-dependent manner in comparison with the Control group (Fig. 3F).

Paeonol regulates the expression levels of glycolysis-related proteins by downregulating BACH1

According to the HPA database, BACH1 expression was upregulated in cervical cancer tissues (Fig. 4A). Data in the Kaplan-Meier Plotter database revealed that BACH1 upregulation was associated with the poor prognosis of cervical cancer patients (Fig. 4B). To explore the expression of BACH1 in cervical cancer cells, RT-qPCR and western blot were conducted and the results illustrated that paeonol treatment greatly increased the mRNA and protein expression of BACH1 in SiHa cells (Fig. 4C). It was noted that BACH1 had the highest expression in the 0.2 mg/ml Paeonol group; therefore, this concentration was selected for subsequent experiments. To overexpress BACH1, oe-BACH1 was transfected into SiHa cells and the transfection efficiency was examined with RT-qPCR and western blot. As Figure 4D depicts, the expression of BACH1 was greatly upregulated in SiHa cells following transfection with

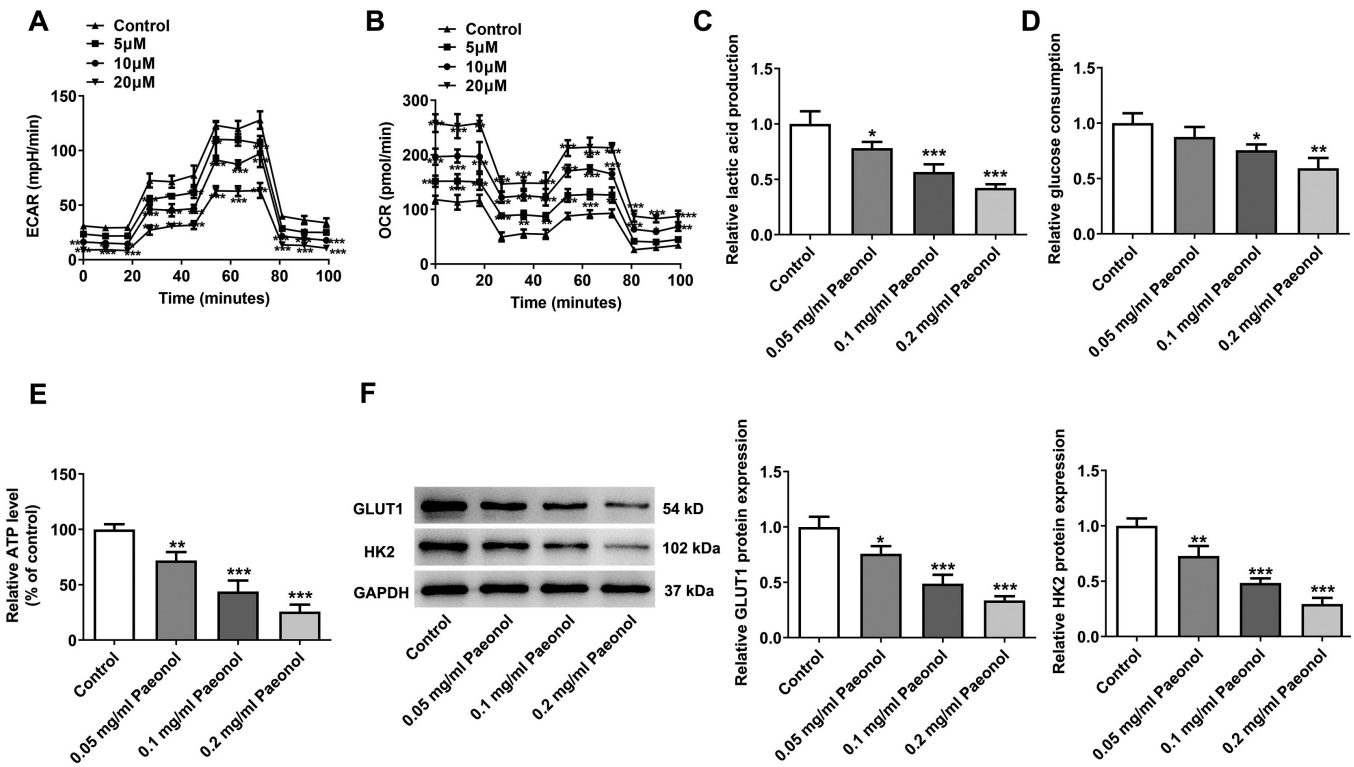

Fig. 3. Paeonol inhibits glycolysis in cervical cancer cells. ECAR **(A)** and OCR **(B)** were detected using Seahorse XFe24 Flux Analyzer. **C.** Lactate production was detected using lactate assay kits. **D.** Glucose consumption was detected using glucose assay kits. **E.** ATP levels were detected using ATP assay kits. **F.** The expression of GLUT1 and HK2 were detected using a western blot. *p<0.05, **p<0.01, and ***p<0.001 vs. control.

oe-BACH1. The binding sites of BACH1 for GLUT1 and HK2 were predicted by the HumanTFDB and JASPAR databases (Fig. 4E). The activity of GLUT1 and HK2 promoters was detected with the luciferase reporter assay, finding that the activity of the GLUT1 or HK2 promoter was dramatically increased after BACH1 overexpression when compared with the GLUT1-WT+oe-NC or HK2-WT+oe-NC group (Fig. 4F).

Paeonol inhibits the proliferation and cell stemness of cervical cancer cells by downregulating BACH1

To explore the mechanism of paeonol associated with BACH1 in cervical cancer cells, oe-BACH1 was transfected into SiHa cells treated with paeonol, and the above functional experiments were repeated. Compared with the Paeonol+oe-NC group, BACH1 overexpression revived the proliferation of SiHa cells (Fig. 5A).

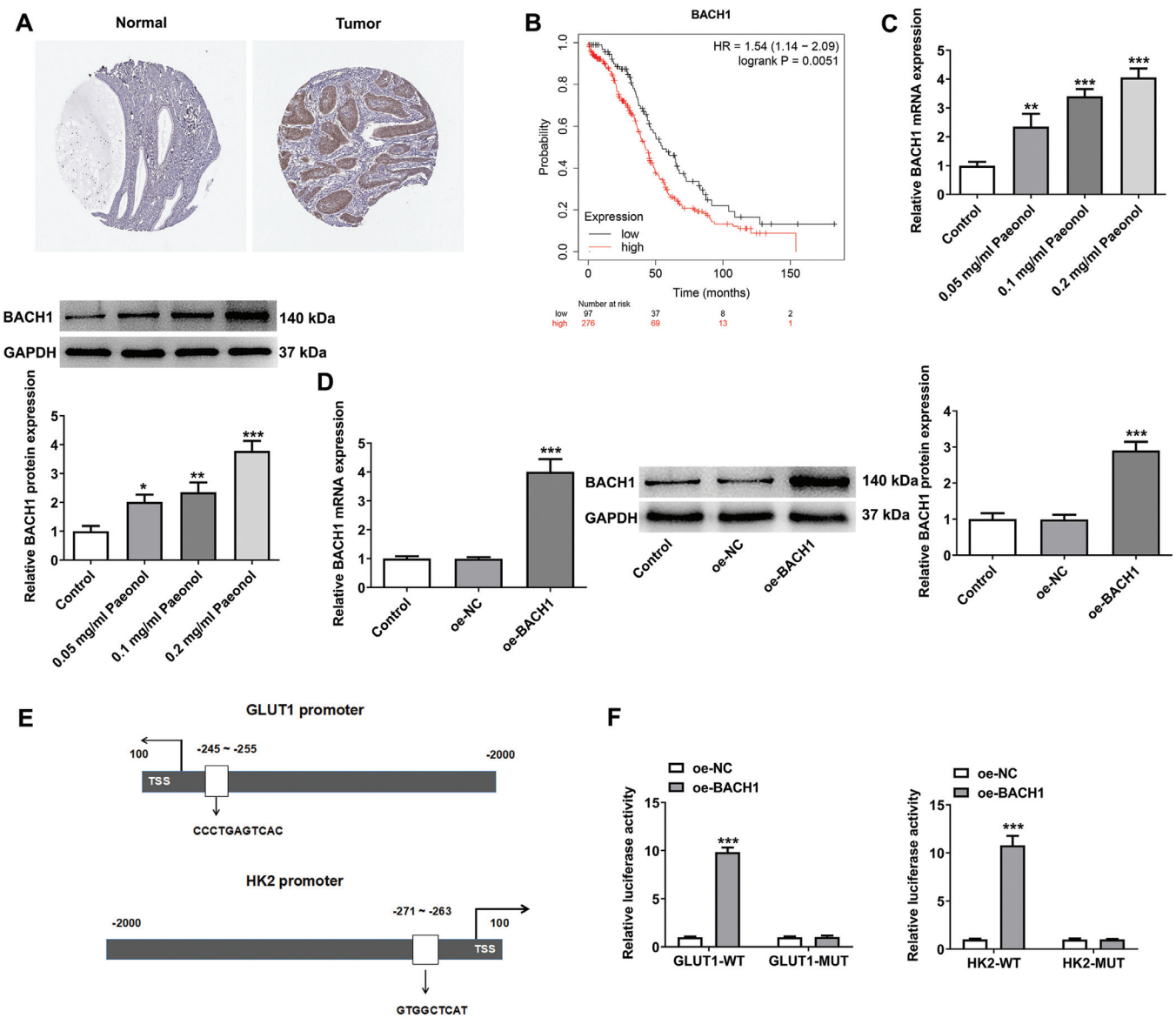

Fig. 4. Paeonol regulates the expression levels of glycolysis-related proteins by downregulating BACH1. **A.** The HPA database showed that BACH1 expression was upregulated in cervical cancer tissues. **B.** The Kaplan-Meier Plotter database showed that BACH1 upregulation was associated with the poor prognosis of cervical cancer patients. **C.** mRNA and protein expression of BACH1 was detected using RT-qPCR and western blot. *p<0.05, **p<0.01, and ***p<0.001 vs. control. **D.** The transfection efficiency of oe-BACH1 was examined with RT-qPCR and western blot. ***p<0.001 vs. oe-NC. **E.** The binding sites of BACH1 for GLUT1 and HK2 promoters were predicted by the HumanTFDB and JASPAR databases. **F.** The activities of the GLUT1 and HK2 promoters were assessed with the luciferase reporter assay. ***p<0.001 vs. oe-NC.

Meanwhile, the reduced number of spheres in SiHa cells with paeonol treatment was increased following transfection with oe-BACH1 (Fig. 5B). Additionally, the reduced expression of Oct4 and Nanog in paeonol-treated SiHa cells was increased after BACH1 overexpression (Fig. 5C).

Paeonol inhibits EMT progression and angiogenesis in cervical cancer cells by downregulating BACH1

Compared with the Paeonol+oe-NC group, the inhibited tube-forming ability of HUVECs was evidently increased by BACH1 overexpression (Fig. 6A). In addition, paeonol treatment increased E-Cadherin content whereas it decreased the contents of VEGFR1, VEGFR2, N-Cadherin, and Vimentin expression in SiHa cells when compared with the Control group. BACH1 over-expression exhibited opposing effects on these proteins, evidenced by decreased content of E-Cadherin and increased VEGFR1, VEGFR2, N-Cadherin, and Vimentin content in the Paeonol+oe-BACH1 group (Fig. 6B).

Paeonol inhibits glycolysis in cervical cancer cells by downregulating BACH1

Compared with the Paeonol+oe-NC group, BACH1 overexpression increased the ECAR whereas it reduced OCR in SiHa cells (Fig. 7A,B). In comparison with the Control group, paeonol treatment decreased lactate production, glucose consumption, and ATP levels in SiHa cells, which were all reversed by BACH1 over-expression (Fig. 7C-E). Moreover, the reduced protein expression of GLUT1 and HK2 in paeonol treated-SiHa cells was partially increased following transfection with oe-BACH1 (Fig. 7F).

Discussion

Cervical cancer, a gynecological malignancy (Liu et al., 2023; Xie et al., 2023). Hence, the investigation into prospective therapeutic agents to manage cervical cancer is of great significance. In the present study, paeonol was evidenced to inhibit cell proliferation, cell stemness, ETM progression, and glycolysis through the downregulation of BACH1. This preliminary study showed that paeonol might be a promising therapeutic candidate for the treatment of cervical cancer.

Paeonol has been extensively applied to cancer treatment due to its low toxicity and anti-tumor properties (Gao et al., 2019; Zhou et al., 2020). In this study, we found that paeonol treatment had no significant effects on the viability of normal H8 cervical epithelial cells. Uncontrolled and abnormal proliferation is a typical characteristic of cancer and the suppression of cell proliferation is believed to be effective for the control of tumor advancement (Loftus et al., 2022). A previous study illustrated that paeonol can suppress the proliferation of T24 and 5637 cells in bladder cancer (Zhang et al., 2021). Similarly, the present study also attested that the proliferation of SiHa cells in cervical cancer was inhibited by paeonol treatment, which was consistent with the results of the previous study (Du et al., 2022). As we all know, tumor cell stemness is deemed a pivotal driver of tumor recurrence and metastasis (Ni et al., 2021). The stemness-associated markers Oct4 and Nanog are expressed in many cancers, indicating the existence of cancer stem cells (Paterson et

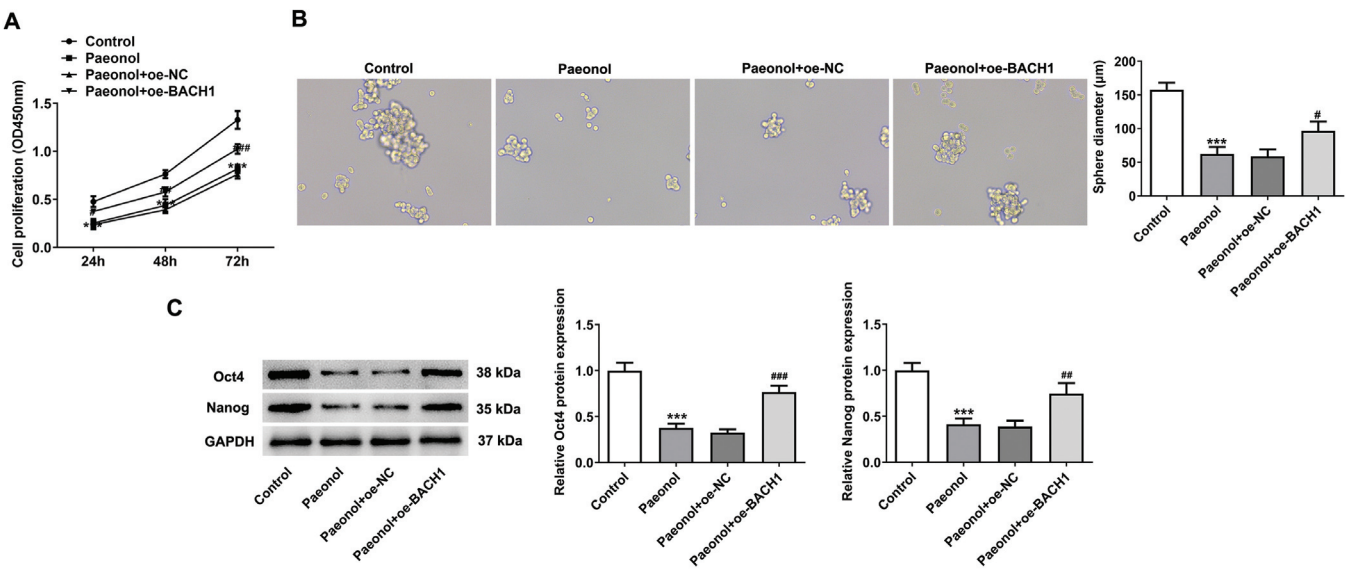

Fig. 5. Paeonol inhibits the proliferation and cell stemness of cervical cancer cells by downregulating BACH1. **A.** Cell proliferation was detected using the CCK-8 assay. **B.** The cell stemness of SiHa cells was detected using a spheroid formation assay. **C.** The expression of cell stemness-related proteins was detected by western blot. ***p<0.001 vs. control, $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 vs. Paeonol+oe-NC.

al., 2021). Paeonol treatment was hereby found to inhibit cell stemness in cervical cancer, accompanied by reduced contents of Oct4 and Nanog.

EMT has been extensively supposed to be a critical part of metastasis, which facilitates the acquisition of malignant traits by cancer cells (Tian et al., 2022). The abnormal promotion of angiogenesis supplies the necessary oxygen and nutrients for tumor growth and metastasis (Rajabi and Mousa, 2017). Paeonol has been found to suppress EMT progression in non-small-cell lung cancer (Zhang et al., 2020). Moreover, Kim et al. elucidated that paeonol can be viewed as a potent suppressor of angiogenesis and metastasis (Kim et al., 2009). The present study showed that paeonol could suppress the tube-forming capability of HUVECs. VEGF is believed to be a pivotal angiogenesis activator, and targeting VEGF might be a favorable method to impede tumor progression (Chang et al., 2023). A previous study disclosed that paeonol can suppress VEGF expression in human glioblastoma cells (Tang et al., 2011). Additionally, Vimentin and E-cadherin molecular markers play critical roles in EMT, which has been shown to correlate with tumor progression (Luo et al., 2017). Lv et al. clarified that paeonol reduced the expression of Vimentin and N-cadherin, whereas it increased E-cadherin expression (Lv et al., 2022). In this regard, the expression of ETM- and angiogenesis-related proteins were also assessed with western blot and the results demonstrated that paeonol reduced the contents of VEGFR1, VEGFR2, N-Cadherin, and Vimentin whereas it increased E-Cadherin expression in SiHa cells in a concentration-dependent manner.

Aerobic glycolysis is a critical glycolytic fermentation for transforming glucose to lactic acid to provide energy for tumor cells (Orang et al., 2019), thus acting as a pivotal regulator in tumor progression (Nie et al., 2015). The promotion of glycolysis drives cancer cells to surpass a deficient nutrient and energy supply and thus has a close affinity with cancer advancement and metastasis (Yang et al., 2020). Of note, Wang et al.

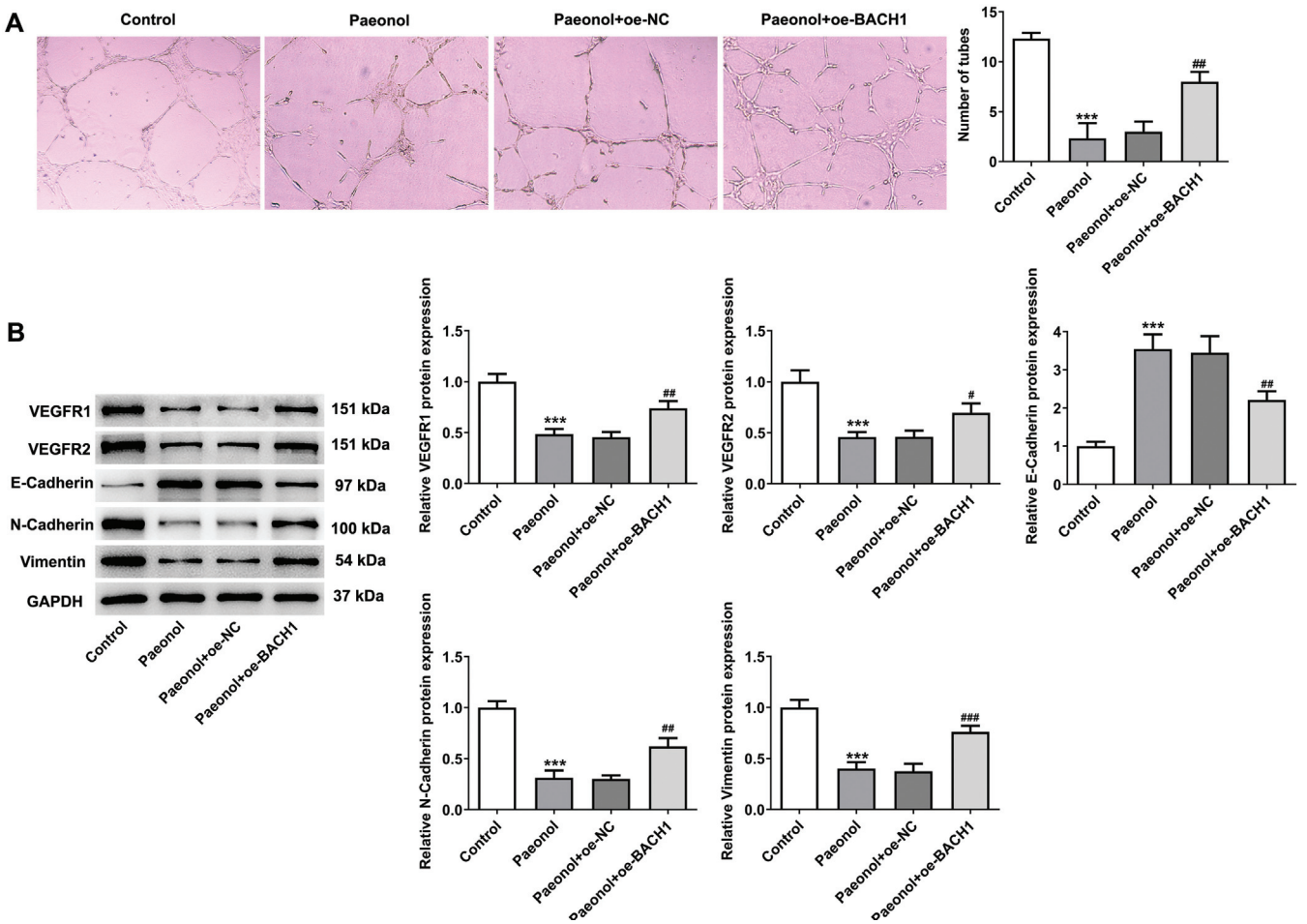

Fig. 6. Paeonol inhibits EMT progression and angiogenesis in cervical cancer cells by downregulating BACH1. **A.** The tube-forming ability of HUVECs was detected using a tube formation assay. **B.** The expression of proteins associated with EMT and angiogenesis was detected using a western blot. ***p<0.001 vs. control, #p<0.05, ##p<0.01, and ###p<0.001 vs. Paeonol+oe-NC.

proved that the inhibition of glycolysis by circMYC knockdown can alleviate the malignant behavior of cervical cancer cells, demonstrated by decreased ATP generation, lactate production, and glucose uptake in SiHa cells (Wang et al., 2021). Moreover, previous studies showed that paeonol has potent inhibitory effects on glycolysis in cancers (Li et al., 2022; Zhang et al., 2023). In the present study, the increased lactate production, glucose uptake, and ATP generation in SiHa cells were concentration-dependently decreased by paeonol treatment. Additionally, ECAR reflects aerobic glycolysis flux while OCR is an indicator of the state of mitochondrial oxidative respiration (Hu et al., 2022). Furthermore, the downregulation of GLUT1 and HK2 by curcumin was illustrated to suppress glycolysis in pancreatic cancer (Guo et al., 2022). Notably, our data showed that paeonol treatment concentration-dependently decreased ECAR, increased OCR, reduced lactate production, glucose consumption, and ATP levels, and decreased GLUT1 and HK2 expression, which further implied the suppressive effects of paeonol on glycolysis in cervical cancer.

Previous studies reported that BACH1 expression is upregulated in breast and lung cancer (Jiang et al., 2021; Huang et al., 2022). Also, BACH1 can facilitate the invasion and metastasis of cancer cells and BACH1 upregulation indicates poor outcomes in breast and lung cancer patients (Padilla and Lee, 2021). BACH1 is involved in the progression of many human cancers. According to our research in the HPA database, we found that BACH1 expression was greatly increased in the tissues of cervical cancer patients. The Kaplan-Meier Plotter database revealed that BACH1 upregulation was associated with the poor prognosis of cervical cancer patients. Following treatment with paeonol, BACH1 expression was significantly increased in SiHa cells, which was consistent with the findings of a previous study (Saahene et al., 2018). It has been validated that BACH1 enhances the glycolysis pathway by increasing the expression of HK2 (Wiel et al., 2019). In addition, the HumanTFDB and JASPAR databases predicted the binding sites of BACH1 for the GLUT1 and HK2 promoters. In this study, results obtained from the luciferase reporter assay disclosed that BACH1 overexpression increased the activities of the GLUT1 and HK2 promoters in cervical cancer cells. Collectively, paeonol treatment reduced the expression of the glycolysis-related proteins GLUT1 and HK2 by decreasing BACH1 expression.

To further explore the mechanism of paeonol associated with BACH1 in SiHa cells, rescue experiments were conducted and the results indicated

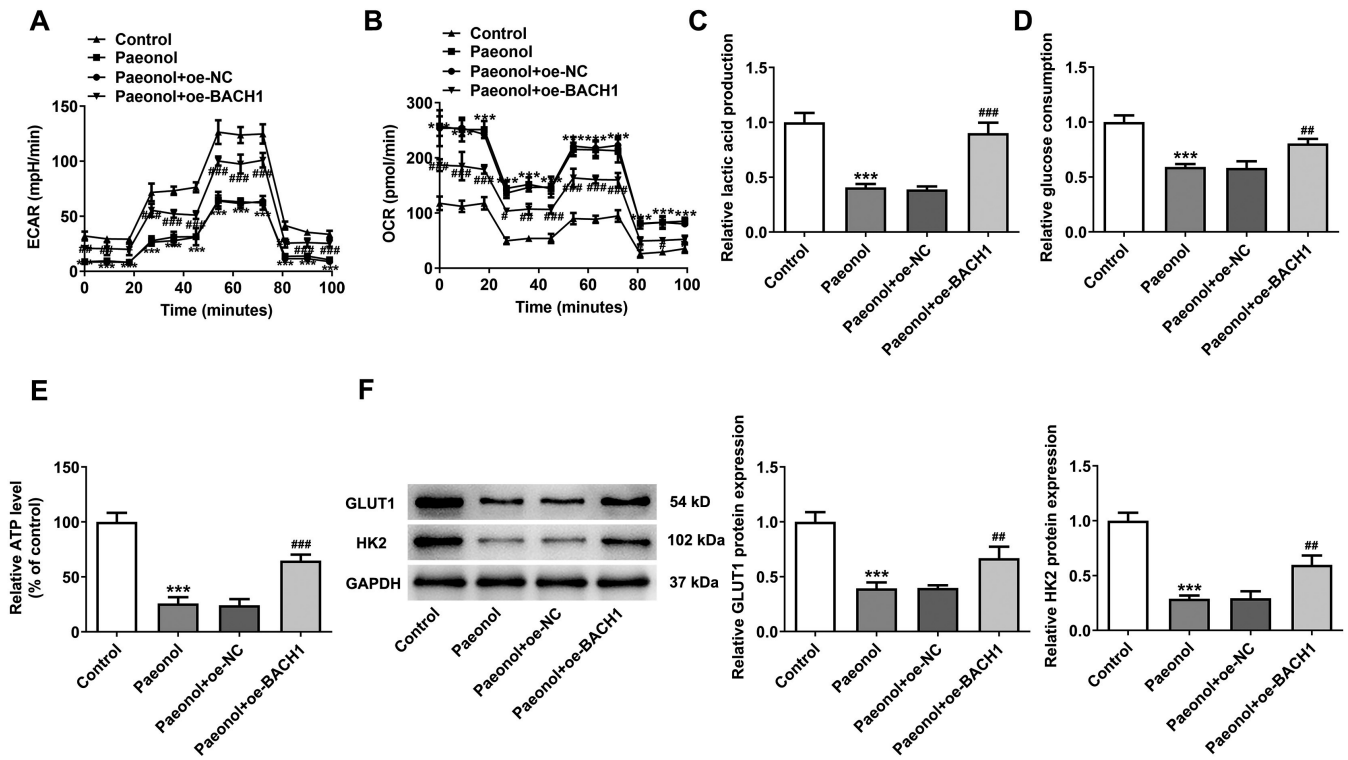

Fig. 7. Paeonol inhibits glycolysis in cervical cancer cells by downregulating BACH1. ECAR **(A)** and OCR **(B)** were detected using Seahorse XFe24 Flux Analyzer. **C.** Lactate production was detected using lactate assay kits. **D.** Glucose consumption was detected using glucose assay kits. **E.** ATP levels were detected using ATP assay kits. **F.** The expression of GLUT1 and HK2 was detected using a western blot. $^{***}p<0.001$ vs. control, $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$ vs. Paeonol+oe-NC.

that BACH1 overexpression partially counteracted the suppressive effects of paeonol treatment on the proliferation, cell stemness, EMT progression, angiogenesis, and glycolysis in SiHa cells.

Conclusion

This study disclosed that paeonol inhibited proliferation, cell stemness, EMT progression, angiogenesis, and glycolysis in SiHa cells through the downregulation of BACH1, revealing, for the first time, the mechanism by which paeonol protects against cervical cancer. This study provides a theoretical basis for paeonol as a therapeutic agent for the treatment of cervical cancer.

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Availability of data and materials. The analyzed data sets generated during the present study are available from the corresponding author upon reasonable request.

Authors' contributions. Shaoqin Sheng and Jing He conceived the experiments. Shaoqin Sheng performed the experiments. Shaoqin Sheng, Jing Xu, and Danhong Hu analyzed the data. Shaoqin Sheng, Weiwei Qian, and Xiangqian Xu confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate. Not applicable.

Patient consent for publication. Not applicable.

Competing interests. The authors declare no competing financial interests.

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