Effects of Forkhead box O1 on lipopolysaccharide-induced mitochondrial dysfunction in human cervical squamous carcinoma SiHa cells

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Abstract. Persistent infection and chronic inflammation play important roles in the development of cervical squamous cell carcinoma. Forkhead box O1 (FOXO1) is a notable regulator of mitochondrial metabolism, which is involved in the occurrence and development of tumors. The present study explored the effects of FOXO1 in human cervical squamous carcinoma SiHa cells. The expression of FOXO1 was examined using reverse transcription-quantitative PCR, western blotting and immunohistochemical staining. SiHa cell migration and proliferation were detected using Transwell and ^{3}H-TdR assays. Mitochondrial functions were assessed based on reactive oxygen species (ROS) generation and changes in the mitochondrial membrane potential ($\Delta\Psi m$). The present study revealed that lipopolysaccharide (LPS) stimulation significantly inhibited the expression of FOXO1 in cervical squamous carcinoma SiHa cells; while silencing FOXO1 resulted in the accumulation of mitochondrial ROS, a decrease in the $\Delta\Psi m$ and abnormal morphology of mitochondria. Accordingly, enhancing FOXO1 expression or treatment with metformin, which protects mitochondrial function, reversed LPS-induced mitochondrial dysfunction, cell pyroptosis, migration and proliferation of cervical squamous carcinoma SiHa cells. Overall, the current study indicated that treatment with FOXO1 could potentially be used as therapeutic strategy to prevent LPS-induced cervical squamous cell carcinoma-related dysfunction in a mitochondria-dependent manner.

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Introduction

Cervical cancer ranks second of all gynecological malignancies with high morbidity and mortality worldwide (1-3). In China, there are >150,000 newly diagnosed cases and >20,000 deaths annually (4). Cervical squamous cell carcinoma accounts for 80-85% of all cervical cancers (5). Molecular and epidemiological studies suggest that multiple genes are involved in the transformation from normal cervical epithelial cells to cervical intraepithelial neoplasia (CIN), and ultimate progression to invasive cervical squamous cell carcinoma (6,7). Therefore, it is very important to clarify the molecular mechanisms to determine prevention and treatment strategies, early diagnostic biomarkers, and therapeutic targets of cervical squamous cell carcinoma.

The relationship between chronic inflammation and cancer has always been a concern. Chronic inflammation is the major cause of prostate cancer (8), cervical cancer (9), and ovarian cancer (10). Some studies have shown that infections trigger a range of inflammatory responses, and inflammation promotes cancer progression (11). The effects of inflammation on tumorigenesis include regulation of the tumor microenvironment through the production of inflammatory cytokines, accumulation of reactive oxygen species (ROS), and activation of transcription factors (12). Lipopolysaccharide (LPS) is a gram-negative bacterial antigen that causes significant systemic inflammation and participates in the progression and metastasis of a variety of tumors (13). It is well known that LPS can promote the progression and metastasis of cervical cancer by stimulating the release of interleukin-1β (IL-1β); thus, promoting downstream cytokines and pyroptosis (14). IL-1β, a generally accepted product of pyroptotic cell death and a crucial cytokine in tumor occurrence and development, has been chosen as a target for cancer immunotherapy (15). However, the underlying mechanism of LPS in cervical carcinoma is not clear.

Forkhead box O1 (FOXO1), a member of the Forkhead box O (FoxO) family, was found to be down-regulated in cervical cancer, and it was considered as a tumor suppressor and negatively modulator of the PI3K/Akt signaling pathway (16). Activation of FOXO1 results in cell proliferation, pyroptosis, autophagy, and DNA repair (17). Therefore this study explored whether FOXO1 expression was altered after LPS treatment in

cervical squamous carcinoma SiHa cells. The role of FOXO1 in mitochondrial function in SiHa cells was checked. Finally, it was tested whether overexpression of FOXO1 could reverse the effects of LPS in SiHa cells. Overall, we confirmed the possibility that FOXO1 could serve as a target for the prevention and treatment of human cervical squamous cell carcinoma.

Materials and methods

Chemicals and reagents. Cervical squamous carcinoma SiHa cells were obtained from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) powder, fetal bovine serum (FBS), and L-glutamine were purchased from Gibco; Thermo Fisher Scientific, Inc. The Lipofectamine® 2000 transfection reagent was obtained from Thermo Fisher Scientific, Inc. Dimethyl sulfoxide (DMSO) and LPS were obtained from Sigma-Aldrich; Merck KGaA. FOXO1 small interfering RNA (siRNA) was designed and synthesized by Wuhan Genesil Biotechnology Co., Ltd. FOXO1 and actin gene primers were designed and synthesized by Shanghai Boya Biotechnology Co., Ltd.

Cell culture and LPS treatment. SiHa cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a 5% CO_2 incubator at 37˚C. The cells were cultured to 80-90% confluence and then treated with 40 μg/ml LPS for 24 h or other reagents at the indicated times and concentrations. The choice of LPS concentrations was based on previous reports (18).

Cloning and transfection of the FOXO1 vector plasmids. The pcDNA3.1-FOXO1 plasmid was synthesized by Hangzhou Hibio Biotech Co., Ltd. (Hangzhou, Zhejiang, China). FOXO1: Primer-F: 5'-GCG GGC TGG AAG AAT TCA AT-3' and Primer-R: 5'-TCC AGT TCC TTC ATT CTG CA-3'. The resulting pcDNA3.1-FOXO1 plasmids or pcDNA3.1 plasmids were then transfected into SiHa cells using Lipofectamine 2000. Briefly, the SiHa cells were added with serum-free DMEM for 24 h; then 2.5 μg pcDNA3.1-FOXO1 plasmid and 8 μl of Lipofectamine 2000 were diluted in 750 μl of OptiMEM (Life Technologies), and incubated for 45 min and then overlaid onto SiHa cells for 6 h at 37˚C. Finally, cell medium containing 20% fetal bovine serum was added for further culture for 48 h.

FoxO1 siRNA-expressing plasmid construction. The FOXO1 siRNA plasmid was constructed using the primers 5'-CCC AAG GCT TTG GTC CTA TC-3' (forward) and 5'-GCC GGA TTC ACT GTA TTC TTG-3' (reverse). Negative siRNAs were sequenced (an unrelated gene) as follows: 5'-GUA CCG CAC GUC AUU CGU AUC-3' (forward) and 5'-UAC GAA UGA CGU GCG GUA CGU-3' (reverse). The reconstituted FOXO1 siRNA or negative siRNA expression plasmid was transfected into SiHa cells, along with or without LPS (40 μg/ml) treatment. Mitochondrial function and biological function of SiHa cells were measured.

Reverse transcription-quantitative PCR (qPCR). Extraction of total RNA from SiHa cells was performed using RNAiso Plus (Takara). Then, 1 μg of RNA was reverse transcribed into complementary DNA (cDNA) by a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). Specific primers used in this experiment were as follows: FOXO1 sense, 5'-GCG GGC TGG AAG AAT TCA AT-3' and antisense, 5'-TCC AGT TCC TTC ATT CTG CA-3'; β-actin sense, 5'-CGA GCG GGA AAT CGT GCG TGA CAT-3'; and antisense, 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT-3'. Real-time qPCR was performed on a StepOne real-time PCR System (Applied Biosystems). The relative level of the FOXO1 mRNA was calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method (19).

Western blot analysis. SiHa cells were thawed using lysis buffer containing 0.5% deoxycholate, 50 mM Tris-HCl (pH 7.0), 0.1% Triton X-100, 150 mM sodium chloride (NaCl), 0.1% sodium dodecyl sulfate (SDS), and 2 mM ethylene diamine tetraacetic acid (EDTA). Total proteins were electrophoresed using a 10-15% gradient SDS-polyacrylamide gel and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking in 5% nonfat milk in Tris-buffered saline for 2 h, the PVDF membranes were probed with primary antibodies specific to FOXO1 (1:1,000 dilution; Abcam: ab52857), Caspase-1 (1:500 dilution; Santa Cruz Biotechnology, Inc.), and β-actin (1:2,000; ab8227; Abcam). The membranes were washed and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:4,000) for 1 h at room temperature. The proteins were visualized by the ECL Chemiluminescence Western Detection System (Cell Signaling Technology).

Immunohistochemistry. SiHa cells were digested by trypsin and centrifuged at 1,000 x g for 10 min at 4˚C. The cell masses were cut into 5 μm slices and then treated with 4% H_2O_2 for 30 min. Antigen retrieval was performed in 0.01 M citrate buffer for 25 min. The slides were incubated with FOXO1 antibodies (dilution, 1:100; Santa Cruz Biotechnology, Inc.) for 30 min at 37˚C and a 3,3'-diaminobenzidine Sigma-D8001 staining kit (Sigma-Aldrich; Merck KGaA). Positive (brown) staining indicated the presence of the FOXO1 protein, as detected by light microscopy (magnification, x200).

Enzyme-linked immunosorbent assay (ELISA) analysis. The production of IL-1β was detected by an ELISA kit (R&D System). Two hundred microliters of the supernatant were added and incubated for 45 min at 37˚C. After incubation, the plates were washed 4 times and 200 μl of conjugates were added for 35 min at 37˚C. After washing 4 times, 200 μl of the substrate solution was added. The absorbance was read using an ELISA reader at 450 nm.

Electron microscope. Cell masses were fixed with 2% paraformaldehyde and placed in 2.5% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4). Then, the pellets were washed twice and dehydrated in a gradient series of ethanol solutions (25, 50, 70, 80, 90, and twice at 100% for 15 min). The samples were treated with acetone and flat-embedded in Durcupan (Fluka Chemic AG) and then sectioned to 60-70 nM thickness on 300 mesh copper slot grids. The ultrastructures of cells, including mitochondrial morphology, were examined (magnification, x3,700, x12,500), and the images were viewed under a Zeiss 109 electron microscope (Carl Zeiss).

Assay of intracellular ROS. The production of intracellular ROS was measured by a H_2DCFDA-based ROS assay kit (Beyotime).

Briefly, SiHa cells were subjected to different treatments, harvested, and then incubated with a final concentration of H_2DCFDA (10 μM) for 30 min in the dark at 37˚C. The fluorescence intensity of dichlorofluorescein (DCF) was observed by a fluorescence microscope. Flow cytometry (BD FACSCalibur) was used to detect the intracellular ROS generation.

Measurement of the mitochondrial membrane potential (ΔΨm). Loss of the mitochondrial membrane potential (ΔΨm) was examined in SiHa cells using the fluorescent cationic dye JC-1 (Molecular Probes; Thermo Fisher Scientific, Inc.). SiHa cells were subjected to different treatments and then stained with 10 μM of JC-1 for 20 min at room temperature. The fluorescence levels were measured at two different wavelengths, 488 and 530 nm, using flow cytometry (BD FACSCalibur).

SiHa cell migration analysis. SiHa cell migration was detected using the Transwell (Corning Incorporated) assay. The upper chamber was filled with 100 μl cell suspension, and 600 μl culture medium containing 20% of FBS was added to the lower chamber. Migrated SiHa cells were fixed with 95% ethanol and then stained with 0.1% crystal violet staining. Migrated cells were counted under a microscope (x400).

SiHa cell proliferation assay. ^{3}H-thymidine was incorporated into SiHa cells to indicate DNA synthesis. The cultured SiHa cells were trypsinized and then harvested onto a glass fiber filter paper. A scintillation solution was added to detect the radioactivity, which was counted by a TopCount NxT scintillation counter (LKB Instruments).

Statistical analysis. Data are shown as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp.). For the comparison of two groups, unpaired two-tailed Student's t-test was used. For the comparison of multiple groups, one-way ANOVA with Tukey's post hoc test was used. $P<0.05$ was considered statistically significant. All experiments were repeated 3 times and performed in triplicate.

Results

Localization and expression of FOXO1 in LPS-stimulated cervical squamous carcinoma SiHa cells. To detect the effects of LPS on FOXO1 expression, we carried out RT-qPCR, western blot, and immunohistochemical staining in cervical squamous carcinoma SiHa cells. According to this paper (18), LPS at 40 μg/ml leads to the most expression of TLR4. Therefore, we use this concentration of LPS to detect the expression changes of FOXO1 in our study. The RT-qPCR results indicated that LPS stimulation significantly inhibited the mRNA level of FOXO1 (Fig. 1A). Western blot data showed that the protein expression of FOXO1 was decreased in the LPS group compared with the mock group (Fig. 1B). In addition, immunohistochemical staining results showed that FOXO1 expression in the cytoplasm of SiHa cells was significantly decreased in the LPS-treated group (Fig. 1C).

Silencing FOXO1 leads to mitochondrial dysfunction in cervical squamous carcinoma SiHa cells. To study the effects

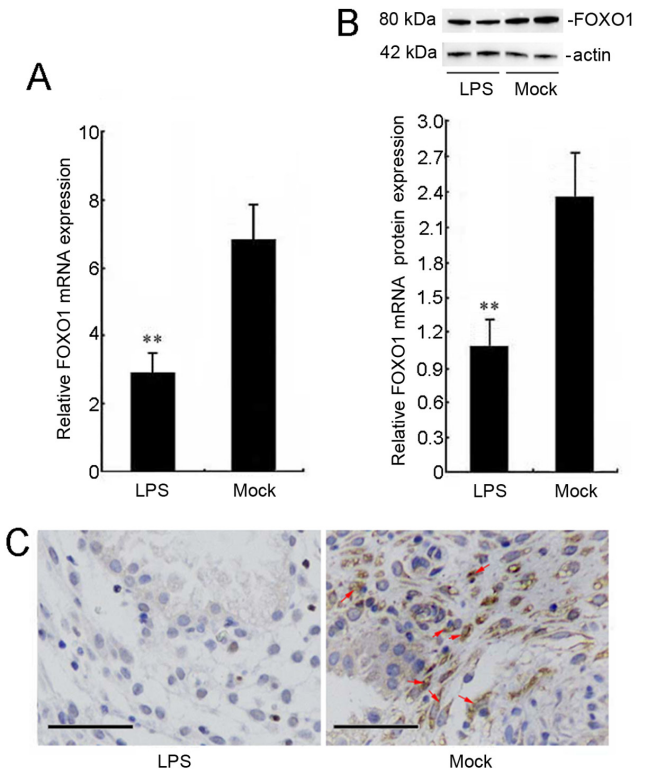

Figure. 1. Localization and expression of FOXO1 in LPS-stimulated cervical squamous carcinoma SiHa cells. SiHa cells were stimulated with LPS (40 μg/ml) for 24 h. (A) mRNA level of FOXO1 was examined using reverse transcription-quantitative PCR analysis. (B) Protein level of FOXO1 in lysates of SiHa cells was detected using western blot assay. The bar chart shows the ratio of FOXO1 to β-actin in each group. (C) Expression levels and localization of FOXO1 were detected using immunohistochemical staining analysis. SiHa cells that stained with brown cytoplasm were positive for FOXO1 (red arrows). Scale bars, 50 μm. $^{**}P<0.01$ versus the mock group. FOXO1, Forkhead box O1; LPS, lipopolysaccharide.

of FOXO1 on mitochondrial function, we transfected FOXO1 siRNA into SiHa cells. The transfection efficiency of FOXO1 siRNA was good as shown in Fig. 2A and B. The mRNA expression of FOXO1 was reduced to 25% compared to the mock group (Fig. 2A). And the protein expression of FOXO1 was reduced to 35% compared to the mock group (Fig. 2B). Then the ROS production and mitochondrial membrane potential in SiHa cells were checked. These data indicated that ROS accumulation was enhanced by ~2.85-fold in the FOXO1 siRNA group compared with the mock treatment group (Fig. 2C). In addition, silencing FOXO1 decreased the mitochondrial membrane potential by ~63.4% compared with that in the mock group (Fig. 2D). However, in the negative siRNA group, the ROS accumulation and mitochondrial membrane potential showed no obvious changes compared with the mock group. Transmission electron microscopy images indicated that silencing FOXO1 caused changes in the mitochondrial morphology, including damage or disappearance of the mitochondrial cristae, and mitochondrial swelling (Fig. 2E).

Effects of FOXO1 on mitochondrial function in LPS-stimulated SiHa cells. Since LPS treatment caused downregulation of FOXO1, and silencing FOXO1 effectively induced mitochondrial dysfunction, including ROS accumulation and mitochondrial membrane disruption, we hypothesized that overexpression

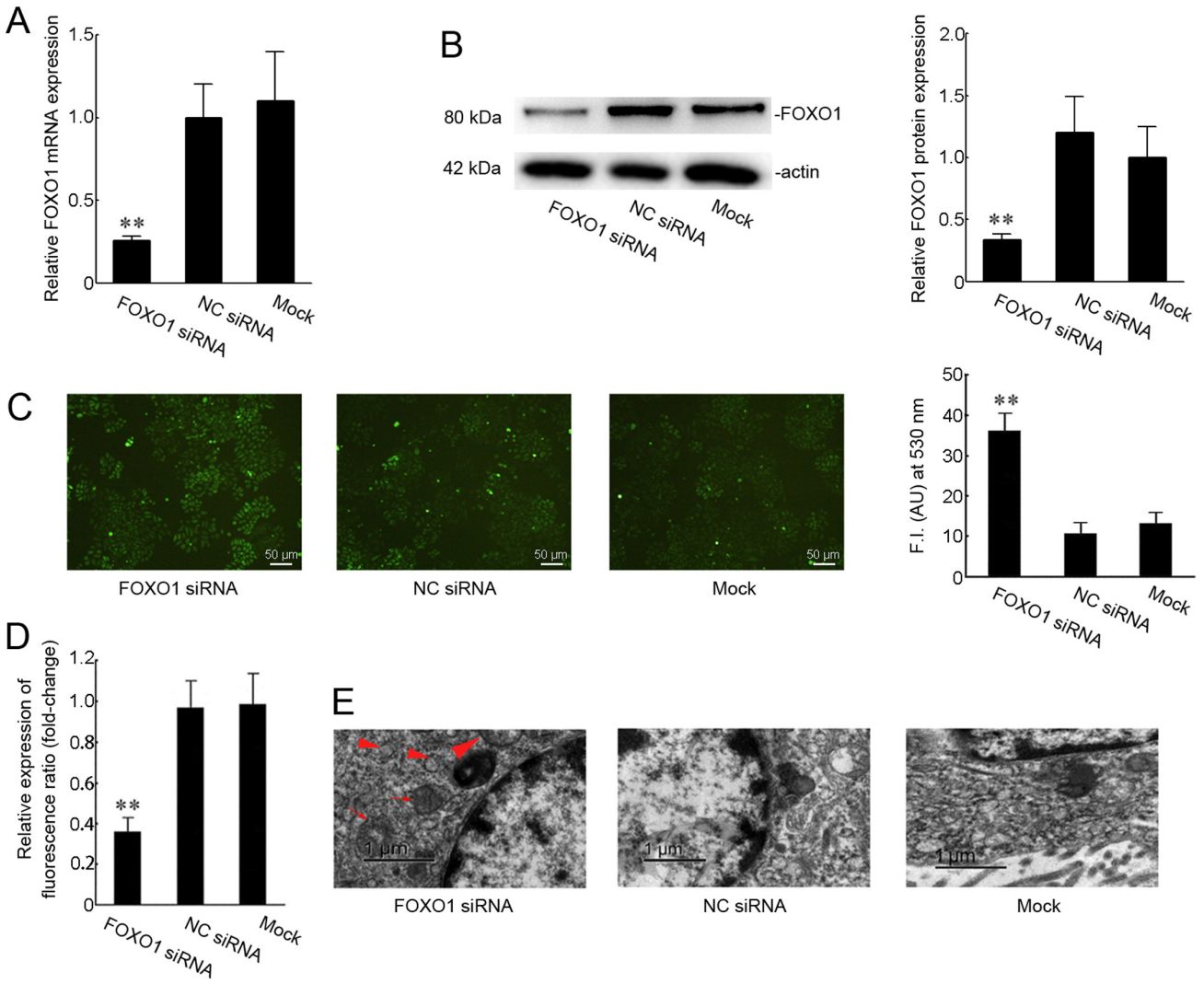

Figure 2. Effect of FOXO1 on the mitochondrial function in SiHa cells. (A) Transfection success was confirmed using reverse transcription-quantitative PCR. Expression of FOXO1 was measured when SiHa cells were transfected with FOXO1 siRNA or NC siRNA for 48 h. (B) Protein expression levels of FOXO1 were checked in SiHa cells transfected with FOXO1 siRNA or NC siRNA for 48 h. (C) Production of intracellular reactive oxygen species was measured using H_2DCFDA fluorescence (green). Scale bars, 50 μm. (D) Change in the mitochondrial membrane potential was analyzed by staining for JC-1 fluorescence. (E) Representative images of mitochondrial morphology visualized by transmission electron microscopy. The mitochondrial cristae were broken or disappeared (red triangles) and mitochondrial swelling was noted (red arrows). Scale bar, 1 μm. **P<0.01 versus the mock group. NC, negative control; si, short interfering; FOXO1, Forkhead box O1.

of FOXO1 could reverse the effects of LPS on mitochondrial function in SiHa cells. To test this hypothesis, we transfected FOXO1 overexpression plasmids into SiHa cells and checked the transfection efficiency. The mRNA expression of FOXO1 was increased to 35-fold compared to the mock group (Fig. 3A). And the protein expression of FOXO1 was increased to 7 fold compared to the mock group (Fig. 3B). Then we performed H_2DCFDA-based ROS assay and JC-1 staining, as well as transmission electron microscopy assay. The results revealed that mitochondrial ROS were maintained at a high level under 40 μg/ml LPS stimulation, and the ROS levels in the LPS (+) and FOXO1 vector (+) groups were decreased compared with those in the LPS alone-treated group (P<0.01; Fig. 3C). Treatment with LPS (+) and empty vector (+) caused no changes in ROS accumulation when compared with that in the LPS alone-treated group (Fig. 3C). Metformin was previously reported to protect the mitochondrial function and promote mitochondrial metabolism (20,21). Our results revealed that treatment with metformin attenuated LPS-induced ROS production (P<0.01; Fig. 3C). The mitochondrial membrane potentials were significantly increased in the LPS (+) and FOXO1 vector (+) group or the metformin treatment group compared with the LPS (+) and empty vector (+) group or the LPS alone-treated group (Fig. 3D). As shown in Fig. 3E, transmission electron microscopy images indicated changes in the mitochondrial morphology, including mitochondrial swelling and vesicular formation in the LPS alone-treated group or the LPS (+) and empty vector (+) group. However, enhancing FOXO1 expression or administration of metformin maintained the integrity of mitochondrial morphology (Fig. 3E).

Effects of FOXO1 on biological function in LPS-stimulated SiHa cells. A previous study has shown that LPS promoted the progression and metastasis of cervical cancer by stimulating the release of IL-1β; thus, promoting downstream cytokines and pyroptosis (14). LPS markedly induced pyroptosis by

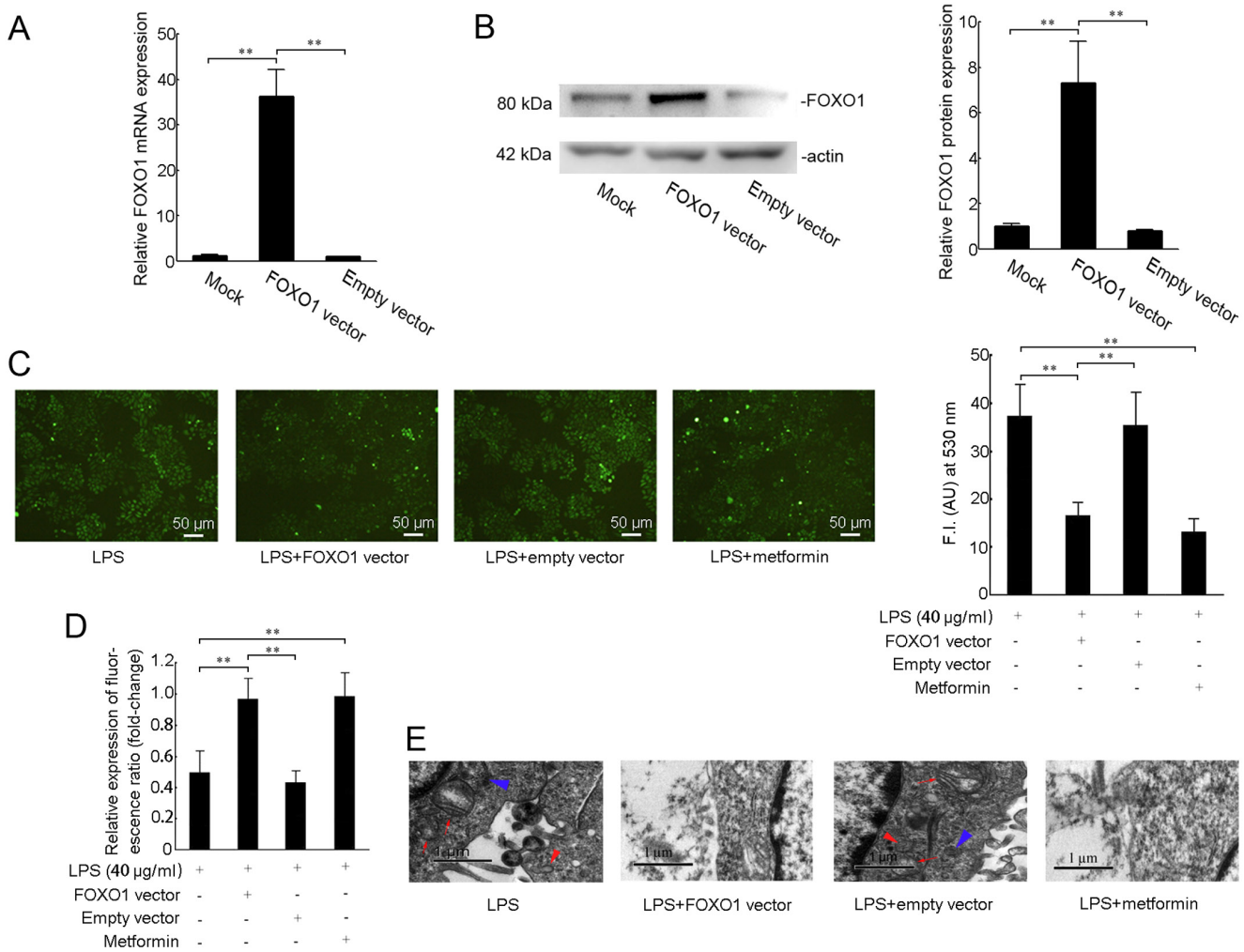

Figure 3. Effect of FOXO1 on mitochondrial function in LPS-stimulated cervical squamous carcinoma SiHa cells. (A) mRNA and (B) protein expression levels of FOXO1 were measured after SiHa cells were transfected with pcDNA3.1-FOXO1 vector (2 μg/ml) or pcDNA3.1 empty vector for 48 h. (C) Cells were stimulated with LPS (40 μg/ml) for 24 h after transfection with the pcDNA3.1-FOXO1 vector (2 μg/ml), pcDNA3.1 empty vector or treatment with metformin for 48 h. Intracellular reactive oxygen species levels were measured using H_2DCFDA fluorescence (green). Scale bars, 50 μm. (D) Mitochondrial membrane potential was detected by the change in JC-1 fluorescence. (E) Representative transmission electron microscopy images. The image shows that the mitochondria were swollen (red arrows), vesicles were formed (red triangle) or the length was significantly shortened (blue triangle). Scale bar, 1 μm. **P<0.01. FOXO1, Forkhead box O1; LPS, lipopolysaccharide.

increasing the protein levels of caspase-1 (22). Metformin inhibits cancer cell proliferation, migration and invasion (23). In this study, ELISA analysis showed that 40 μg/ml LPS stimulation significantly increased the extracellular levels of IL-1β in culture media. Pre-transfection with the FOXO1 vector or pre-treatment with metformin attenuated LPS-induced IL-1β generation (Fig. 4A). Activation of caspase-1 is required during IL-1β production. As shown in Fig. 4B, 40 μg/ml LPS stimulation increased the expression of caspase-1, while overexpression of FOXO1 or treatment with metformin decreased the LPS-induced expression of caspase-1 (Fig. 4B). To further evaluate the role of FOXO1 in LPS-induced SiHa cell migration and proliferation ability, Transwell experiments and ^{3}H-TdR incorporation assays showed that the migration and proliferation of cells were reduced in the LPS (+) and FOXO1 vector (+)-treated group or LPS (+) and metformin (+) group compared with the LPS alone group (P<0.01) (Figs. 4C and D). However, treatment with LPS (+) and empty vector (+) caused no difference compared with LPS alone-treated group (Fig. 4A-D).

Discussion

The development of cervical squamous cell carcinoma is strongly associated with persistent infection and chronic inflammation. LPS is a common inducer of inflammation, and various signaling pathways, including Janus kinase/signal transducers and activators of transcription (JAK/STAT), mitogen-activated protein kinases (MAPKs), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are activated under stimulation with LPS (24). LPS exposure is positively related to the survival, invasion, proliferation, and metastasis of tumor cells, by stimulating the release of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β (25-27). In this work, we first tested the influence of LPS on FOXO1 expression. The results showed that LPS could downregulate FOXO1 expression in cervical squamous carcinoma SiHa cells. The mechanisms may be strongly associated with signaling pathways, including JAK/STAT, MAPKs, and NF-κB. Largely consistent with our results, exposure to LPS was found to cause downregulation of FOXO1 in renal tubular epithelial cells (28).

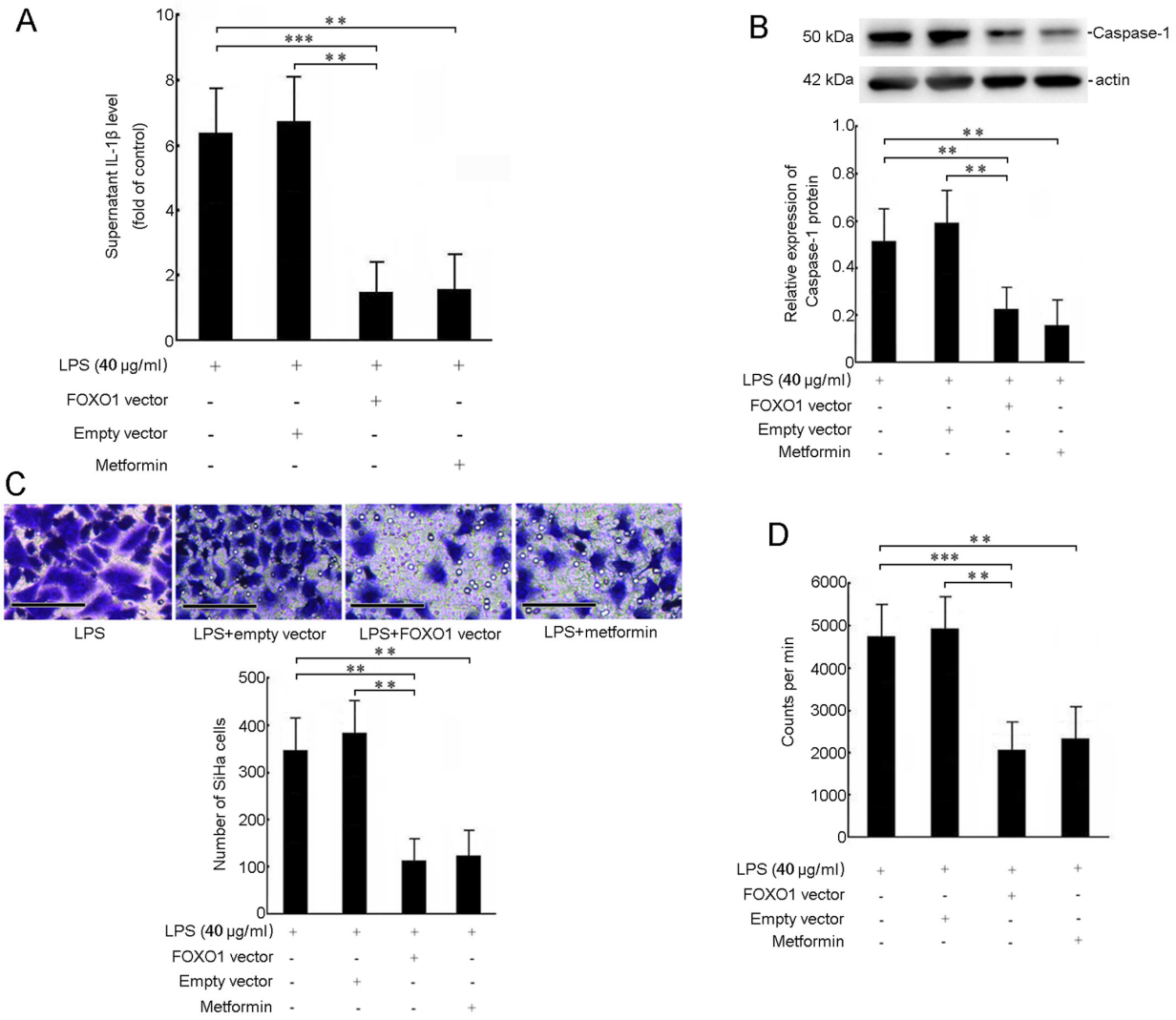

Figure 4. Effect of FOXO1 on biological function in LPS-stimulated SiHa cells. Cells were stimulated with LPS (40 μg/ml) for 24 h after transfection with the pcDNA3.1-FOXO1 vector (2 μg/ml), pcDNA3.1 empty vector (2 μg/ml) or treatment with metformin for 48 h. (A) Extracellular levels of IL-1β were determined using ELISA. (B) Protein levels of caspase-1 in lysates of SiHa cells were measured using western blotting. (C) SiHa cell migration was measured using a Transwell migration assay (n=3). Scale bar, 50 μm. (D) ^{3}H-TdR incorporation was executed to assess the proliferation of SiHa cells. The absolute value of CPM indicated the proliferation of cells (cpm/10^{6} cells). **P<0.01, ***P<0.001. FOXO1, Forkhead box O1; LPS, lipopolysaccharide; CPM, counts per min.

Mitochondria are the major physiological source of ROS, and they cause an increase in oxidative stress when they become dysfunctional (29,30). Mitochondrial dysfunction is characterized by excessive ROS production in defective mitochondria, loss of ATP, and disruption of the integrity of the mitochondrial membrane. Previously published studies have shown that the transcription factor FOXO1 regulates cell cycle progression, oxidative stress, apoptosis, autophagy, tumor suppression, cell differentiation, and energy metabolism (31,32). Particularly, FOXO1 is a critical regulator of the mitochondrial metabolism, which is involved in the occurrence and development of tumors. In this study, we found that silencing FOXO1 increases ROS production, and leads to $\Delta\Psi m$ loss and disruptions of mitochondrial morphology in cervical squamous carcinoma SiHa cells. These observations imply the roles of FOXO1 in mitochondrial dysfunction in SiHa cells.

Recent studies have proved that LPS stimulation induced mitochondrial damage-mediated ROS production in HepG2 cells (24), increased the levels of inflammatory cytokines in the brain, and deregulated the mitochondrial function in mice (33). ROS accumulation induced by LPS leads to the state of oxidative stress, which also promotes inflammatory progression (34). In this work, we found that LPS induced ROS production, inhibited the mitochondrial membrane potential, and disrupted the mitochondrial morphology in cervical squamous carcinoma SiHa cells. These data uncovered that LPS-induced mitochondrial dysfunction may promote inflammatory progression in cervical squamous cell carcinoma.

Previous studies have revealed that overexpression of FOXO1 improved LPS-induced renal injury and mitochondrial dysfunction partly through PGC1-α signaling (28). Other studies have reported that cobalt protoporphyrin upregulated FOXO1 expression, further increased the expression of the heme oxygenase-1 (HO-1) gene and other oxidative stress-responsive genes, and reduced the production of mitochondria-derived ROS (35). These findings proposed the roles of FOXO1 in improving mitochondrial function during LPS exposure. Also, decreased expression of FOXO1 mediated the

role of LPS in mitochondrial dysfunction in SiHa cells. The mechanism may occur partly through PGC1-α signaling or upregulation of the HO-1 gene. However, further studies are needed to explore the detailed mechanisms.

As previously described, mitochondrial dysfunction contributes to the loss of cell function, apoptosis, and pyroptosis (36,37). Pyroptosis is a form of programmed cell death dependent on the activation of caspase-1. Caspase-1 was a marker of cell pyroptosis (38). NLRP3 inflammasome (a complex of NLRP3, apoptosis-associated speck-like (ASC), and procaspase-1) cleaves procaspase-1 as an activated form of caspase-1, leading to gasdermin D (GSDMD) cleavage. Then the cleaved GSDMD forms a pore in the cellular membrane and induces pyroptosis. Additionally, cleaved caspase-1, enhances the maturation of proinflammatory cytokines, such as IL-1β (38). LPS stimulation activated the TLR4-NF-κB signaling pathway and inflammatory mediators, such as IL-1β, in cervical squamous carcinoma cells (18). LPS markedly induced pyroptosis by increasing the protein levels of caspase-1 (22). Cell migration and proliferation were also promoted by LPS in cervical cancer SiHa cells (39). FOXO1 is an important transcription factor with a regulatory role in cell proliferation and differentiation (40). The present study revealed that overexpression of FOXO1 could reverse LPS-induced IL-1β secretion, caspase-1 expression, cell migration, and proliferation of SiHa cells. These results suggested that decreased expression of FOXO1 mediated the role of LPS in SiHa cell pyroptosis, migration, and proliferation. However, further studies are needed to explore the detailed mechanisms. For the pyroptosis, although we determined caspase-1 and IL-1β expression in this study, the mRNA and protein levels of NLRP3, ASC, GSDMD should be checked in the future.

In Figs. 3 and 4, 'no LPS' was not used. Our study focus on the roles of rescue effects of FOXO1 in LPS-induced situation. And the effects of LPS on mitochondrial function in Fig. 3 could be compared to the mock group in Fig. 2. In Fig. 4, various study demonstrated that LPS could increase IL-1β expression, caspase-1 expression, cell migration and proliferation compared to the no LPS control (18,22,39,40). Thus, 'no LPS' was not used.

Prior studies have implicated that the functions of FOXO1 in cancer progression were controversial. It has been reported that FOXO1 silencing in OVCA429 and OVCA433 cells resulted in decreased cell proliferation. In addition, knockout of FOXO1 had a significant negative effect on the epithelial ovarian cancer cell migration (41). However, recent studies have indicated that FOXO1 played an antitumor role by regulating proapoptotic genes, such as Bcl-2 interacting mediator of cell death (BIM), fas ligand protein (FasL), and TNF related apoptosis inducing ligand (TRAIL), which in turn inhibited the growth of various tumors, such as glioblastoma and cervical squamous cell carcinoma (42). In this study, we examined the roles of FOXO1 in human cervical squamous carcinoma SiHa cell growth (proliferation) and metastasis (migration) in LPS-induced functional studies. Our results showed that overexpression of FOXO1 attenuated LPS-induced migration and proliferation of cervical squamous carcinoma SiHa cells. Another study has implicated that metformin inhibited estrogen-dependent endometrial cancer cell growth by activating the AMPK-FOXO1 signal pathway (43). The present study demonstrated that metformin reversed the roles of LPS in mitochondrial function, cell pyroptosis, proliferation, and migration. The mechanism may be that metformin upregulated the FOXO1 activity by AMPK activation, and then reversed the roles of LPS. However, further mechanism studies are required.

Taken together, our study uncovered that decreased expression of FOXO1 may mediate the functions of LPS in cervical squamous carcinoma SiHa cell mitochondrial function, pyroptosis, proliferation, and migration. Also, FOXO1 may be a potential therapeutic target for prevention and treatment of human cervical squamous cell carcinoma from the mitochondrial perspective. However, further studies are required to investigate the therapeutic potential of FOXO1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL made substantial contributions to the conception and design of the study. HW and SL confirm the authenticity of all the raw data. HW and SL designed the experiments and wrote and edited the manuscript. ZM, FG, WJ and YL performed the experiments, analyzed and interpreted the data and performed the statistical analysis. 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 that they have no competing interests.

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