

FoxO1, a Potential Therapeutic Target, Regulates Mitochondrial Dysfunction and Pyroptosis in LPS-Induced Human Cervical Cancer Cells

Huizhi Wang

Hongqi Hospital

Zhi Ma

Hongqi hospital

Fanshu Gao

Hongqi hospital

Wei Jiang

Hongqi hospital

Yang Li

Hongqi hospital

Shuping Li (✉ gaolingjuan@njmu.edu.cn)

Hongqi hospital

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Abstract

Background: Chronic inflammation plays an important role in the development of cervical cancer. Studies have demonstrated that transcription factors forkhead box protein O1 (FoxO1) have been reported to play important roles in various cancers.

Aim: The purpose of this study was to investigate the effect of FoxO1 gene on lipopolysaccharide (LPS)-induced inflammation and intracellular pyroptosis in the development and progression of human cervical cancer cells (SiHa).

Methods: In this study, FoxO1 expression was examined using real-time polymerase chain reaction (PCR), western blotting and immunohistochemical staining. SiHa cells migration and proliferation was detected using the transwell assay and ³H-TdR assay. Mitochondrial function was assessed based on reactive oxygen species (ROS) generation and changes in the mitochondrial membrane potential ($\Delta\Psi_m$).

Results: Our study demonstrated that LPS inhibited FoxO1 gene expression, and the silence of FoxO1 gene caused the accumulation of ROS, decreases in the $\Delta\Psi_m$ and mitochondrial morphology change). However, either overexpression of FoxO1 or metformin could reverse the LPS-induced mitochondrial dysfunction, cell pyroptosis, migration and proliferation.

Conclusions: Our study indicated that FoxO1 as a potential therapeutic target to cure against LPS-induced human cervical cancer in a mitochondria-dependent manner.

Background

Cervical cancer is the second most common gynaecological malignant and is responsible for substantial number of morbidities and mortalities worldwide [1]. In China, there were > 150,000 diagnosed newcases and > 20,000 deaths cases annually [2]. Molecular and epidemiological studies have suggested that the normal cervical epithelium cells transforms into preneoplastic cervical intraepithelial neoplasia (CIN), which eventually develops into invasive cervical cancer cells, the development of cervical cancer is multistep process that involving multiple genes[3, 4]. Therefore, it is very important to clarify molecular mechanisms to find the preventive strategies, early clinical diagnostic methods, and effective therapies to treat cervical tumors.

The relationship between chronic inflammation and cancer has always been concerned. Chronic inflammation is the major cause of malignancies, such as prostate cancer [5], cervical cancer [6] and ovarian cancer [7] and so on. Some studies has addressed that infection can trigger a range of inflammatory response, and inflammation can promote the cancer progression [8]. The effects of inflammation on tumorigenesis include the regulation of the tumor microenvironment through producing inflammatory cytokines, reactive oxygen species and transcriptional factors [9]. Lipopolysaccharide (LPS) is a gram-negative bacterial antigen that participates in and promotes the progression and metastasis of a variety of tumors [10]. It is well known that LPS could promote the proliferation and invasion of cervical

cancer cells by stimulating the secretion of interleukin-1 beta (IL-1 β), induction of downstream cytokines and pyroptosis, enhancing the inflammatory microenvironment. IL-1 β , a generally accepted product of pyroptotic cell death and key cytokine in cancer development and progression, has already been chosen as a target for cancer immunotherapy [11]. However, LPS promote the underlying mechanisms of progression of cervical carcinoma is not entirely clear.

FoxO1 is a member of the forkhead box O (FoxO) family, which is recognized as negative regulator of the PI3-k/Akt signaling pathway, and is considered as tumor suppressors [12]. Several tissue culture studies demonstrate that FoxO1 is down-regulated in a wide variety of cancers, such as breast and uterine cervix cancers [13]. Activation of FoxO1 results in regulating cell-cycle arrest, pyroptosis, DNA damage repair, and detoxification of reactive oxygen species [14]. Therefore, this study was to explore whether FoxO1 involved in pyroptosis regulation and mitochondrial dysfunction in SiHa cells. Finally, we confirmed the possibility that FoxO1 served as the therapeutic target to cure human cervical cancer.

Materials And Methods

Chemicals and reagents

The cervical epithelial SiHa cells line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) powder, fetal bovine serum (FBS) and L-glutamine were obtained from the Gibco (Grand Island, NY, USA); The Lipofectamine® 2000 transfection reagent was purchased from Santa Cruz (Santa Cruz, California, USA). Dimethyl sulfoxide (DMSO) and Lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). FoxO1 small-interfering RNA (siRNA) were designed and synthesized by Wuhan Genesil Biotechnology Co. Ltd (Wuhan, China). FoxO1 and actin gene primers were designed and synthesized by Shanghai Boya Biotechnology Co. Ltd (Shanghai, China).

Cell culture

SiHa cells were grown in DMEM medium containing with 1% nonessential amino acids, 2 mM of glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin and 10% FBS. SiHa cells were cultured in a 5 % CO₂ incubator at 37°C to 80-90 % confluence, and then treated with 40 μ g/ml LPS for 24 h or other reagents at the indicated times and concentrations. The choice of LPS concentrations was based on previous reports [21].

Cloning and transfection of FoxO1 vector plasmids

The pcDNA 3.1 expression plasmid (Invitrogen, Carlsbad, CA) expressing pcDNA3.1-FoxO1 vector was created at Hangzhou Hibio Bio-tech Co., Ltd. (Hangzhou, Zhejiang, China). The pcDNA3.1 empty vector was chosen as a negative control. 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 PCR product was digested with BamHI and EcoRI and ligated into pcDNA 3.1 expression plasmid. The resulting pcDNA3.1-FoxO1 vector or pcDNA3.1 empty vector was

then transfected into SiHa cells using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, before transfection, the SiHa cells were serum starved for 24 h, 500 pmol of pcDNA3.1-FoxO1 vector and 10 μ L of Lipofectamine 2000 were diluted in 750 μ L of OptiMEM (Life Technologies, Gaithersburg, MD, USA). The solution was pre-incubated for 45 min at 37 °C, then overlaid onto the SiHa cells for 2 h, finally, 2 ml of growth medium (20% foetal bovine serum) were added for further cultured.

FoxO1 siRNA-expressing plasmid construction

The FoxO1 siRNA expression plasmid was constructed using the primers 5'-CCC AAG GCT TTG GTC CTA TC-3' (forward), 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), 5'-UAC GAA UGA CGU GCG GUA CGU-3' (reverse). The reconstituted FoxO1 siRNA expression plasmid transfected into SiHa cells, along with LPS (40 μ g/ ml). The SiHa cell mitochondrial function and biological function was measured.

Real-time quantitative polymerase chain reaction (real-time qPCR)

Extraction of total RNA from SiHa cells was performed using RNAiso Plus (TakaRa, China). Then, 10 μ L of total RNA was reverse-transcribed into complementary DNA (cDNA) using the PrimeScript cDNA Synthesis Kit (Takara, Japan). The following sequences were used to detect FoxO1 mRNA level: Primer-F: 5'-GCG GGC TGG AAG AAT TCA AT -3' and Primer-R: 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 reactions were performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The relative level of FoxO1 mRNA was calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method [22].

Western blot analysis

The SiHa cells were thawed in lysis buffer containing containing 0.5% Deoxycholate, 50mM Tris-HCl (pH 7.0), 0.1% Triton X-100, 150mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1mM ethylenediaminetetraacetic acid (EDTA). Total proteins were electrophoresed using a 10-15% gradient SDS-polyacrylamide gel and subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were then blocked in 5% non-fat milk in PBST for 1 h, and then incubated with primary antibodies specific to FoxO1 (1: 1000 dilution, Abcam: ab52587), Caspase-1 (1: 500 dilution, Santa Cruz, CA, USA) and actin (1: 2000; ab8227, Abcam). The membrane were washed and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 4000). Visualization of protein band was quantified using the Enhanced Chemiluminescence Western Detection System (Cell Signaling Technology, Beverly, MA, USA).

Immunohistochemistry

The SiHa cells were digested by trypsin and pelleted at 1000 X for 30 min at 4 °C. The mass were cut into 5 μ m slice, then treated with 4% H₂O₂ for 30 min. The antigen retrieval was underwent in 0.01 M citrate

buffer (pH 6.0) for 25 min. The slides were incubated with FoxO1 antibodies (dilution, 1: 100, Santa Cruz Biotechnology) for 30 min at 37°C and 3, 3'-diaminobenzidine Sigma-D8001 staining kit (Sigma Aldrich; Merck KGaA) staining. Positive (brown) staining indicates the presence of the HAX-1 protein, as detected by light microscopy (magnification, x200).

ELISA analysis

The production of IL-1 β was detected by ELISA kit (R&D System, Minneapolis, MN). 200 μ L of supernatant was added to each well and incubated for 45 min at 37 °C. After incubation, the plate was washed four times, and was then added with 200 μ l of conjugate for 45 min at 37 °C. After washing four times, 200 μ L substrate solution was added for 20 min in the dark. The absorbance was read using an ELISA Reader at 450 nm after the addition of 2 M sulfuric acid. The level of IL-1 β in culture media was calculated according to a standard curve.

Electron microscope

The cultured SiHa cells were digested by trypsin and pelleted at 12000 \times g for 10 min at 4 °C. The cells mass were fixed with 2 % paraformaldehyde and placed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Then the pellet was washed twice and dehydrated in a gradient series of ethanol solutions (25%, 50%, 70%, 80%, 90% and twice 100% for 15 min). The sample was treated with acetone and flat-embedded in Durcupan (Fluka Chemic AG, Buchs, Switzerland), and then sectioned to 60-70 nm thickness on 300 mesh copper slot grids. The ultrastructure of cells was examined at 5200 X magnification (Observation of mitochondrial morphology) and 2500 X magnification (Observation of cell apoptosis morphology), and the images were viewed under a Zeiss 10⁹ electron microscope (Carl Zeiss, Oberkochen, Germany).

Assay of intracellular ROS

The production of intracellular ROS was measured by a H₂DCFDA based ROS assay kit (Beyotime, Shanghai, China). Briefly, the SiHa cells were applied with different treatments, cells were harvested, and then incubated with 10 μ M final concentration of H₂DCFDA for 30 min in the dark at 37°C. Flow cytometry (BD FACSCalibur, San Jose, CA, USA) was used to detect the intracellular ROS generation with excitation wavelength and the emission wavelength at 488 nm and 530 nm respectively.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was examined in SiHa cells using the fluorescent cationic dye JC-1 (Molecular Probes; Thermo Fisher Scientific, Inc.). The SiHa cells were applied with different treatments and then stained with 10 μ M JC-1 for 20 min at RT. The fluorescence intensities with the excitation wavelength and the emission wavelength at 485 nm and 530 nm respectively using fluorescence microscopy (magnification, x200).

SiHa cells migration analysis

The siHa cells migration were detected using the transwell (Corning Incorporated, Corning, NY, USA) assay. The SiHa cells were starved for 24 h and were then harvested. SiHa cells were resuspended in culture medium at least 2×10^6 cells/ml. The upper chamber was filled with 100 μ l cell suspension and the lower chamber was added with 600 μ l culture medium containing 20% FBS. The chambers were incubated for 24 h at 37°C. The migration ability of SiHa cells were fixed with 95% ethanol and then stained with 0.1% crystal violet staining. In five different fields per filter, the migrated cells were counted under a microscopic ($\times 400$).

SiHa cell proliferation assay

The SiHa cells were starved for 24 h and were then harvested. SiHa cells were resuspended in culture medium at least 2×10^6 cells/ml. ^3H -thymidine were incorporated into SiHa cells to indicate DNA synthesis. The cultured SiHa cells were trypsinized and were then harvested onto a glass fiber filter paper. Scintillation solution was added to detect the radioactivity, which was counted by a TopCount NXT scintillation counter (LKB Instruments, Mount Waverly, Victoria, Australia).

Statistical analysis

All data are presented as mean \pm standard deviation (SD). Student's t test was used to compare the means of two groups. p -values less than 0.05 were considered significant (* $p < 0.05$; ** $p < 0.01$; # $p > 0.05$). Statistical significance was calculated using SPSS18.0. All experiments were performed at least three independent experiments.

Results

The effects of LPS on the FoxO1 expression in SiHa cells

Firstly, the effect of LPS on the expression of FoxO1 in SiHa cells was evaluated. qRT-PCR showed that LPS stimulation apparently inhibited the mRNA level of FoxO1 gene (Fig. 1a). Western blot results showed that the expression of FoxO1 protein in LPS group were obviously decreased when compared with the DMSO group (Fig. 1b). In addition, immunohistochemical staining indicated that FoxO1 expression was significantly decreased in the cytoplasm of SiHa cells in LPS group (Fig. 1c).

Silencing of FoxO1 induced mitochondrial dysfunction in SiHa cell

The effect of the FoxO1 gene on the production of ROS and changes in the mitochondrial membrane potential in SiHa cells was explored in this study. Data indicated that ROS accumulation in the FoxO1 siRNA vector group were enhanced by approximately 2.85-fold compared with mock treatment (Fig. 2a). In addition, the silence of the FoxO1 decreased mitochondrial membrane potential approximately 63.4% compared to those in mock group ($p < 0.01$). However, the negative siRNA group, the mitochondrial membrane potential showed no obvious changes when compared with mock group (Fig. 2b).

Transmission electron microscopy images indicated the the silence of the FoxO1 gene induced mitochondrial morphology change containing mitochondrial swelling and vesicular formation (Fig. 2c).

Effect of FoxO1 overexpression on mitochondrial function in LPS-stimulated SiHa cells

Pretransfection with FoxO1 vector prevented LPS-induced ROS production, the ROS levels in the LPS (+), FoxO1 vector (+) group were decreased compared with those in the LPS alone-treated group ($p < 0.01$). When compared with LPS (+), FoxO1 vector (+) treatment, treatment with LPS (+) and empty vector (+) caused a significant increase in ROS accumulation. In addition, treatment with metformin may reverse LPS-induced ROS production ($p < 0.01$, Fig. 3a, metformin can protect mitochondrial function and promote mitochondrial metabolism). Mitochondrial dysfunction was manifested as decreases in the mitochondrial membrane potential. We observed that overexpression of FoxO1 or protect mitochondrial function maintained the mitochondrial membrane potential at normal levels in cells exposed to LPS. The mitochondrial membrane potential in the LPS (+) and empty vector (+) group were significantly decreased compared to those in the LPS (+) and FoxO1 vector (+) group ($p < 0.01$). In addition, the mitochondrial membrane potential in the LPS (+) and metformin (+) group were significantly increased compared to those in the LPS (+) and empty vector (+) group ($p < 0.01$, Fig. 3b). As shown in Fig. 3c, transmission electron microscopy images indicated the mitochondrial morphology change including mitochondrial swelling and vesicular formation in LPS alone-treated group or LPS (+) and empty vector (+) group. However, enhanced FoxO1 gene or protect mitochondrial function could maintain the integrity of mitochondrial morphology.

Effect of FoxO1 overexpression on biological function in LPS-stimulated SiHa cells

In this study, ELISA analysis showed that LPS stimulation significantly increased the extracellular levels of IL-1 β in culture media. Pretransfection with FoxO1 vector or metformin attenuated LPS-induced the IL-1 β generation, the levels of IL-1 β in the LPS (+), FoxO1 vector (+) group were reduced compared with those in the LPS alone-treated group ($p < 0.001$). When compared with LPS (+), FoxO1 vector (+) treatment, treatment with LPS (+) and empty (+) caused a significant increase in IL-1 β levels ($p < 0.01$). Treatment with metformin may reduce LPS-induced IL-1 β production ($p < 0.01$, Fig. 4a). Activation of caspase-1 is required during IL-1 β production, as shown in Fig. 4b, LPS increased the cleavage of caspase-1, while treatment with FoxO1 vector or metformin decreased LPS-induced the expression of caspase-1, however, compared with LPS (+) and FoxO1 vector (+) treatment, treatment with LPS (+) and empty (+) caused a significant increase in caspase-1 levels ($p < 0.01$). For further evaluation the role of FoxO1 in LPS-induced SiHa cell migration and proliferation ability, transwell experiments and $^3\text{HTdR}$ incorporation assay showed that the migration and proliferation cells in the LPS (+), FoxO1 vector (+)-treated group or LPS (+), metformin (+) group reduced compared with the LPS alone group ($p < 0.01$). However, compared with LPS (+), FoxO1 vector (+) treatment, treatment with LPS (+) and empty vector (+) caused a significantly enhanced in the migration and proliferation cells ($p < 0.01$, Fig. 4c-d).

Discussion

LPS is a common inflammatory inducer, and its exposure activates many components of chronic inflammation, such as the production and release of cytokines [15]. LPS exposure is closely related to the survival, proliferation, invasion and metastasis of tumor cells by stimulating the production of TNF- α and IL-6, and the serum levels of these cytokines are associated with prostate cancer stage [16]. The immunological stress model provided by LPS is used to release of excessive inflammatory mediators, such as IL-1 β , which can also cause the occurrence of cervical cancer [17]. Previous studies have indicated that LPS stimulation could activate the TLR4-NF- κ B signalling pathway in SiHa cells, which activates the inflammatory mediators such as IL-1 β [18]. The present study firstly showed that LPS stimulation inhibited the mRNA and protein expression of FoxO1 in SiHa cells, and consequently promoting the release of IL-1 β , which illustrated that FoxO1 gene might also play a role in the promoting the progression of cervical cancer.

Transcription factor FoxO1 participates in many important functions such as the regulating cell cycle progression, apoptosis, tumor suppression, cell differentiation and metabolism, and oxidative stress resistance [19–21]. Previous studies have shown that FoxO1 is significantly upregulated in epithelial ovarian cancer, whereas FoxO1 is significantly down-regulated in tumors of other organs, such as cervix, prostate, breast, and endometrium [22–24]. The function of FoxO1 in the carcinogenesis is also controversial, it was reported that siRNA-mediated FoxO1 silence in OVCA429 and OVCA433 cells resulted in decreased cell proliferation. In addition, migration experiment showed that FoxO1 knockdown had a significant negative effect on the epithelial ovarian cancer cells migration [25]. However recent studies have shown that FoxO1 plays an anti-tumor role by regulating pro-apoptotic genes such as BIM, FasL and TRAIL, which in turn inhibits the growth of various tumors such as glioblastoma and cervical cancer [26–27]. In our study, we examined the role of FoxO1 in human cervical cancer cells growth (proliferation) and metastasis (migration) in functional studies. Our results indicated that overexpression of FoxO1 gene attenuated LPS-induced migration and proliferation of SiHa cell.

The form of FoxO1 is associated with protection of mitochondrial function [28]. Studies have shown that mitochondrial dysfunction is associated with loss of cell function, apoptosis and pyroptosis induced by oxidative stress, characterized by excessive ROS production in defective mitochondria [29]. Mitochondrial dysfunction is a kind of dysfunction in biochemical process, which is characterized by the accumulation of ROS can lead to the loss of ATP, which disrupts the integrity of mitochondrial membrane, leading to mitochondrial dysfunction and pyroptosis [30]. Previous studies have shown that cobalt protoporphyrin upregulates FoxO1 expression, further increases the expression of HO-1 gene and other oxidative stress-responsive genes and reduces the production of mitochondria-derived ROS, which are partially mediated by FoxO1 [31]. Our study indicates that FoxO1 gene silencing increases ROS production and loss of the $\Delta\Psi_m$, and changes the mitochondrial morphology. However, either overexpression of FoxO1 or metformin could reverse the LPS-induced mitochondrial dysfunction, cell pyroptosis, migration and proliferation. These data indicated the role of FoxO1-mediated mitochondrial function in LPS-induced pyroptosis of SiHa cells.

Conclusion

The current study indicates that LPS induces the release of IL-1 β and other inflammatory cytokines modulated partially by FoxO1, and that FoxO1 plays an important role in protecting mitochondrial function, including reducing of ROS generation and maintaining the mitochondrial membrane integrity. Either overexpression of FoxO1 or metformin could reverse the LPS-induced mitochondrial dysfunction, cell pyroptosis, migration and proliferation. Taken together, our study identified FoxO1 as a potential therapeutic target for human cervical cancer in a mitochondrial-dependent manner.

Abbreviations

CIN: Cervical intraepithelial neoplasia; FoxO1: Forkhead box protein O1; LPS: Lipopolysaccharide; Real-time PCR: Real-time polymerase chain reaction; ROS: Reactive oxygen species; IL-1 β : Interleukin-1 beta; ATCC: American Type Culture Collection; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; siRNA: small-interfering RNA; SDS: Sodium dodecyl sulfate; EDTA: Ethylenediaminetetraacetic acid; PVDF: Polyvinylidene fluoride; HRP: Horseradish peroxidase; ³H-TdR: ³H-thymidine; SD: standard deviation.

Declarations

Acknowledgements

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Authors' Contributions

SPL made substantial contributions to the conception and design of the study. HZW drafted the manuscript and revised it critically for important intellectual content. ZM, FSG, WJ and YL have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Figures

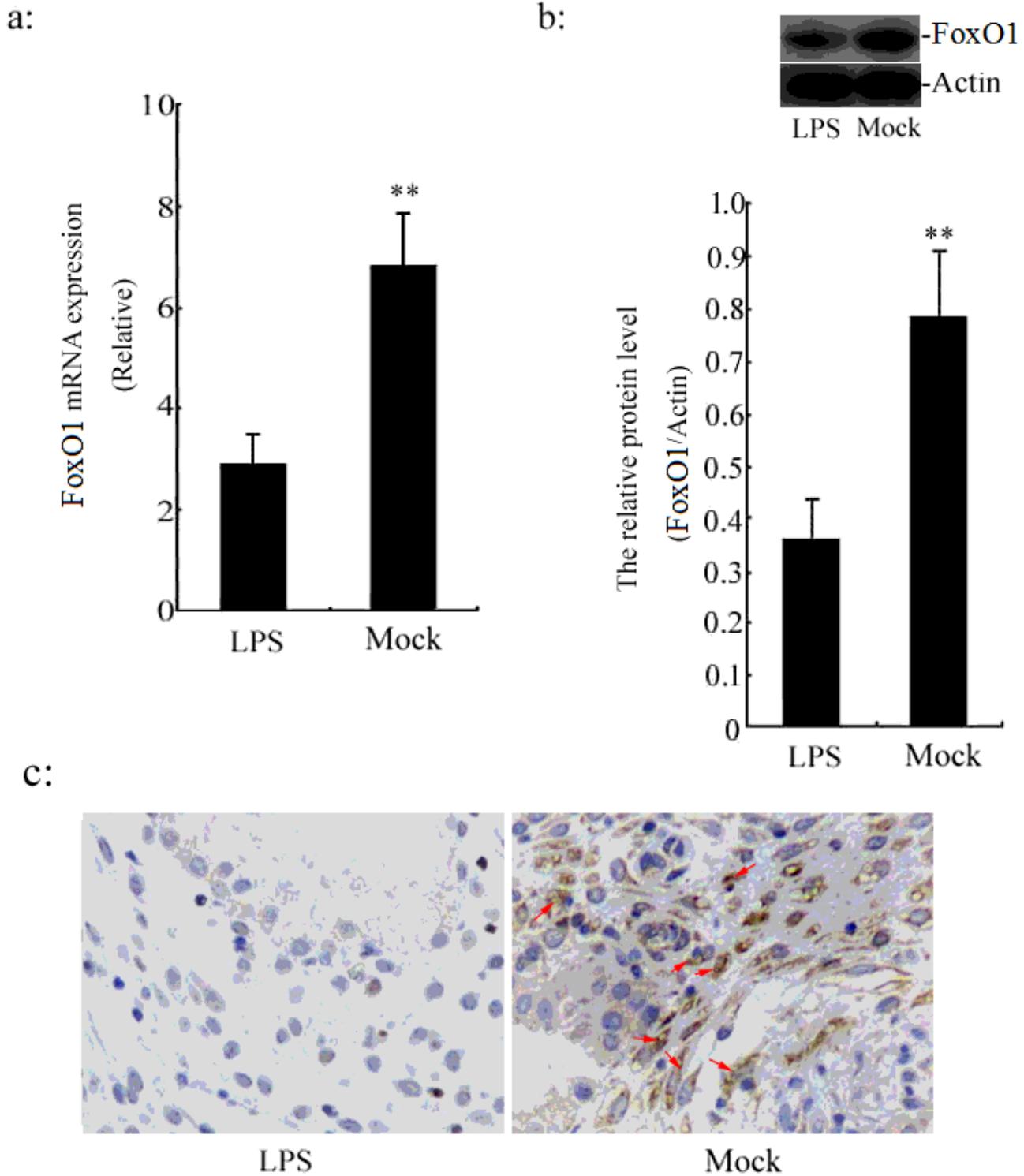
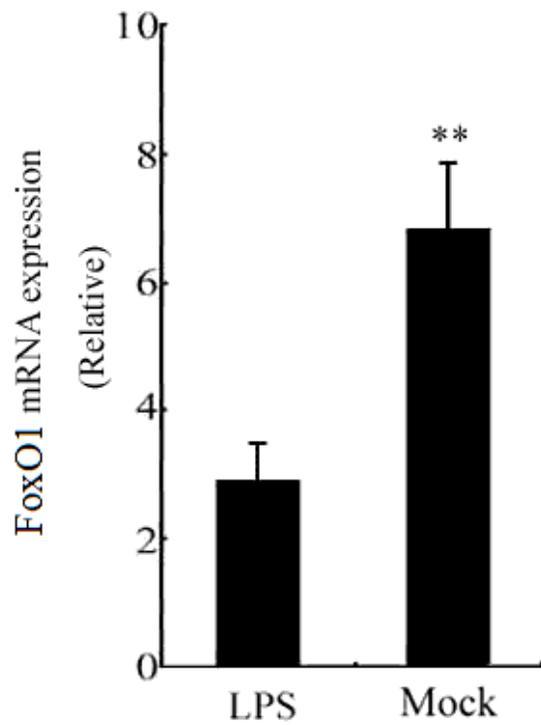


Figure 1

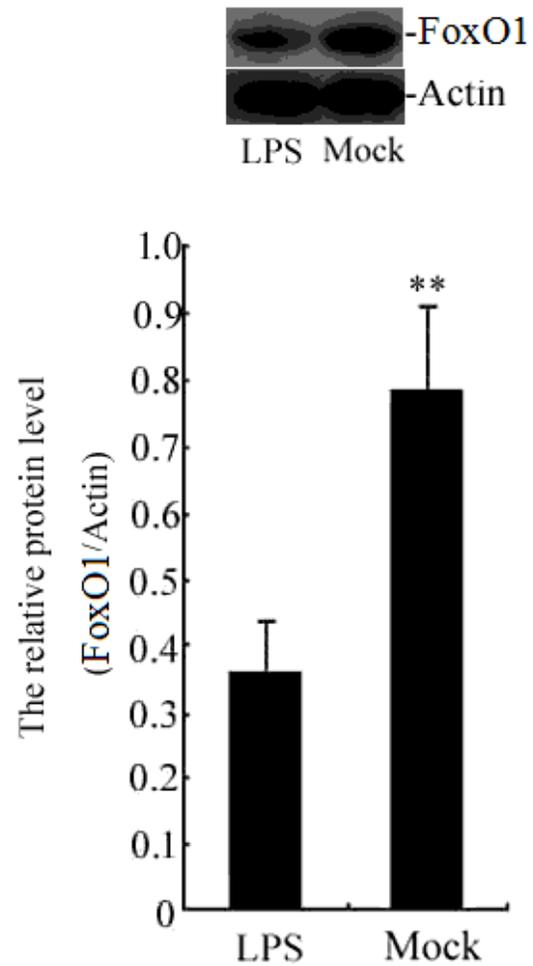
Effects of LPS on FoxO1 expression in SiHa cells. SiHa cells were stimulated with 40 $\mu\text{g/ml}$ LPS for 24 h. (a) The mRNA level of FoxO1 was examined by qRT-PCR analysis. (b) The protein levels of FoxO1 in lysates of SiHa cells were measured by western blot assay. The bar chart showed the ratio of FoxO1 to β -actin at each groups. (c) The expression level and localization of FoxO1 protein was detected using

immunohistochemical staining analysis. SiHa cells with brown cytoplasm were positive (Red arrows). Data are the mean \pm S.D. of three independent experiments. ** $p < 0.01$ versus mock group.

a:



b:



c:

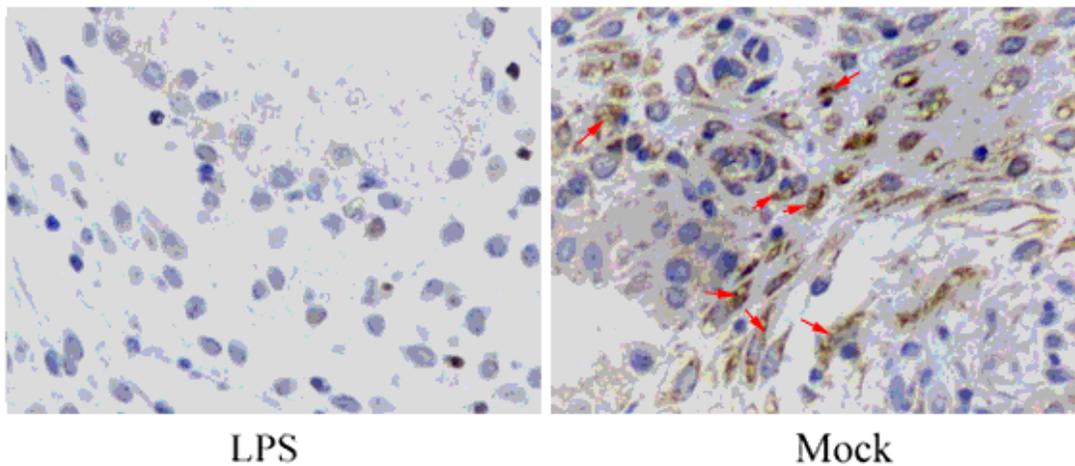


Figure 1

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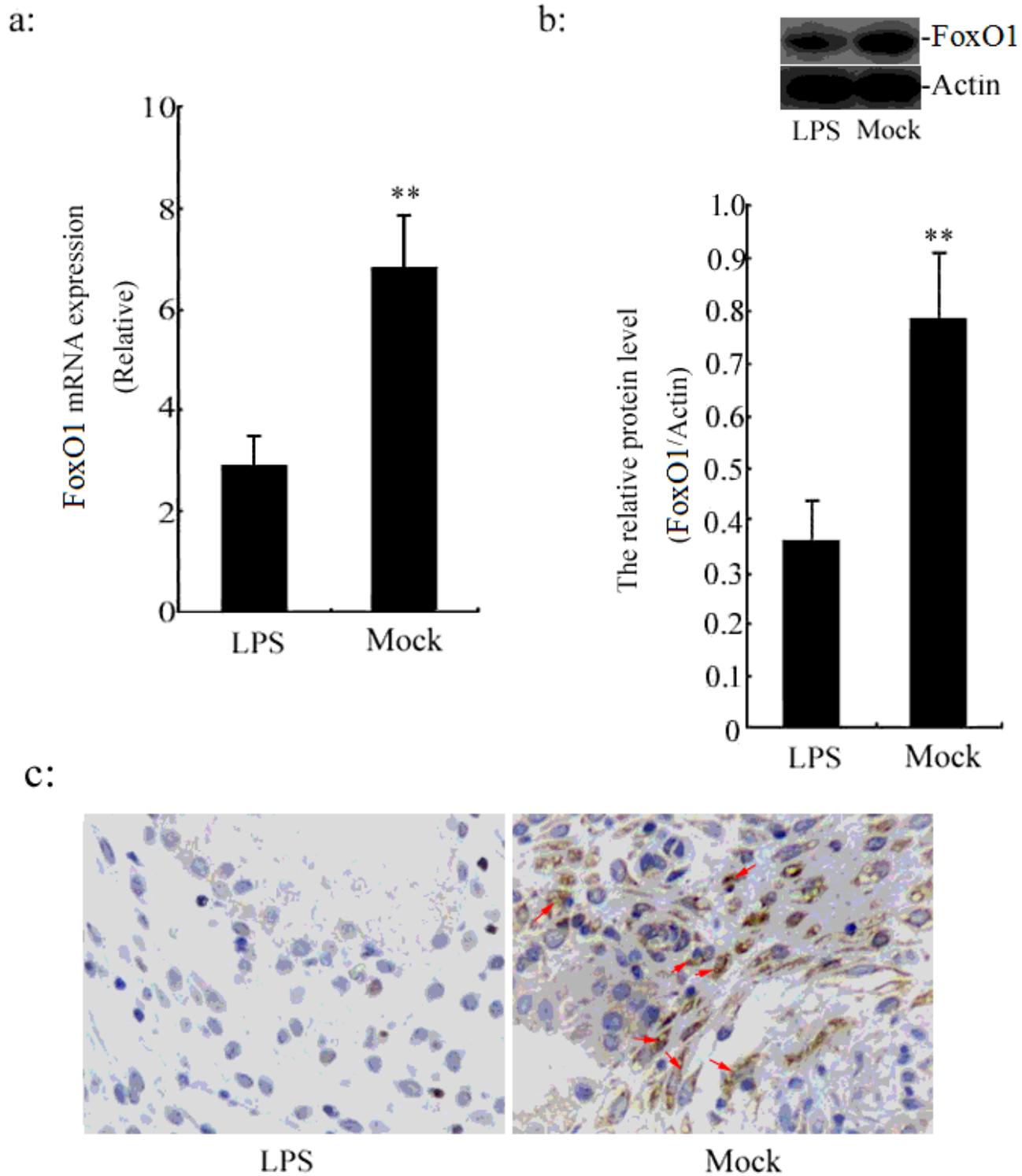


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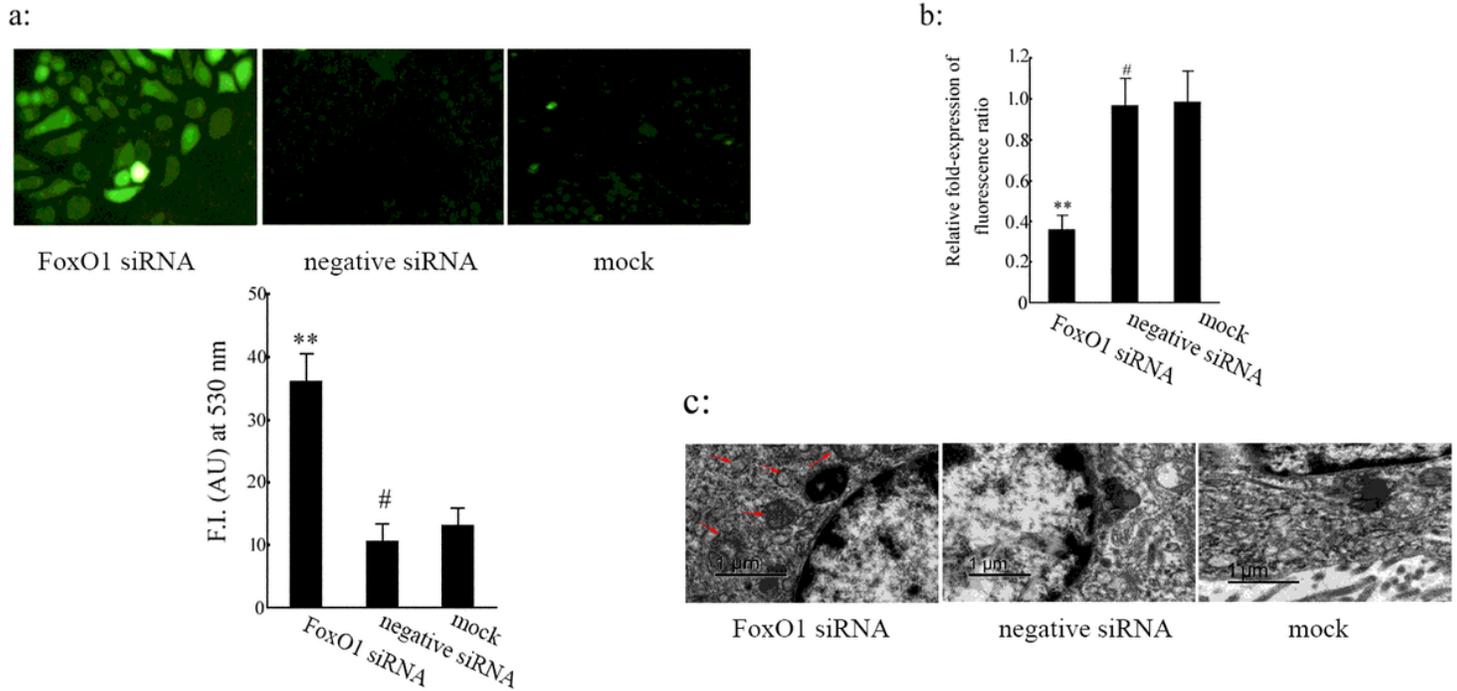


Figure 2

Effect of silencing of FoxO1 gene on mitochondrial function of SiHa cells. The SiHa cells were transfected with FoxO1 siRNA or negative siRNA for 48 h. (a) Intracellular ROS production was detected by H2DCFDA fluorescence (Green). The data are means \pm S.D. of three separate experiments. $**p < 0.01$, $\#p > 0.05$ versus mock group. (b) The change of mitochondrial membrane potential was analyzed by staining with fluorescence of JC-1 (590: 527 nm fluorescence ratio). The data are presented as mean \pm S.D. (n = 3). $**p < 0.01$, $\#p > 0.05$ versus mock group. (c) Representative images of mitochondrial morphology visualized by transmission electron microscopy. Mitochondrial swelling and vesicular formation were observed (Red arrows). Scale bar: 1 μ m.

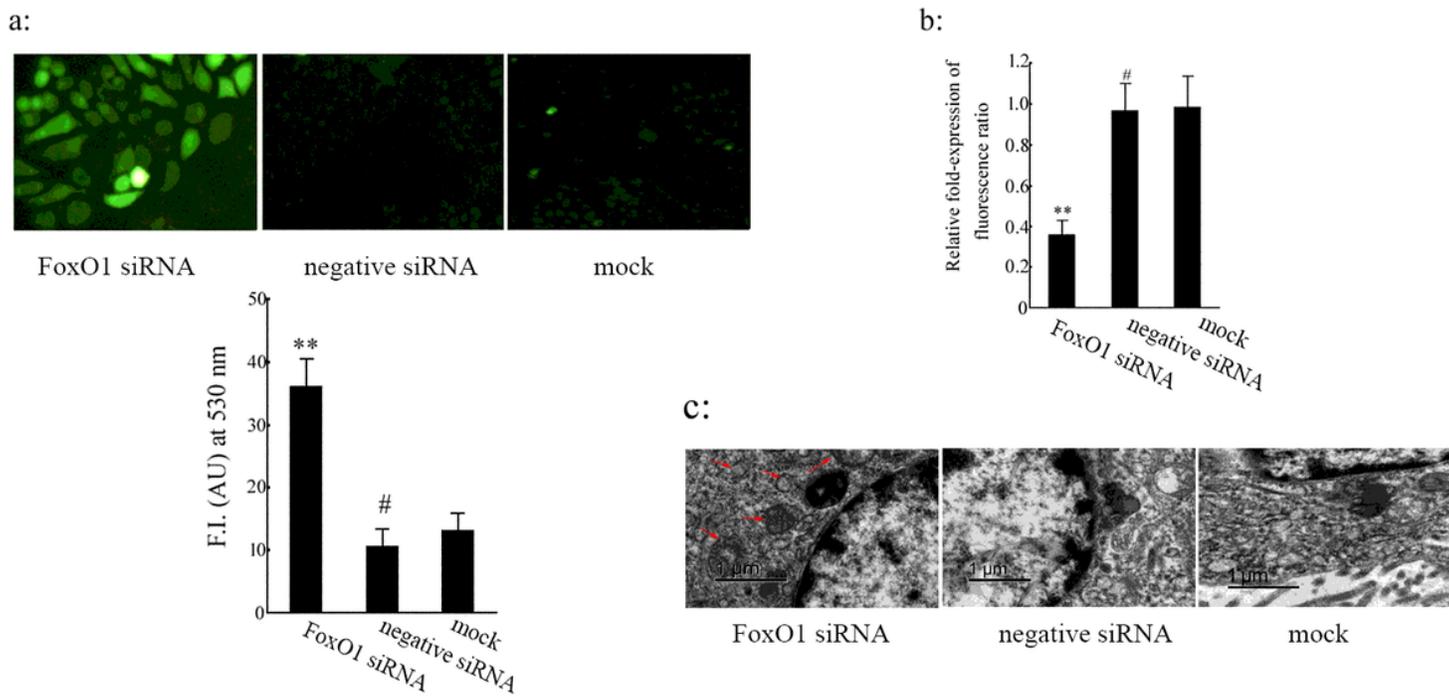


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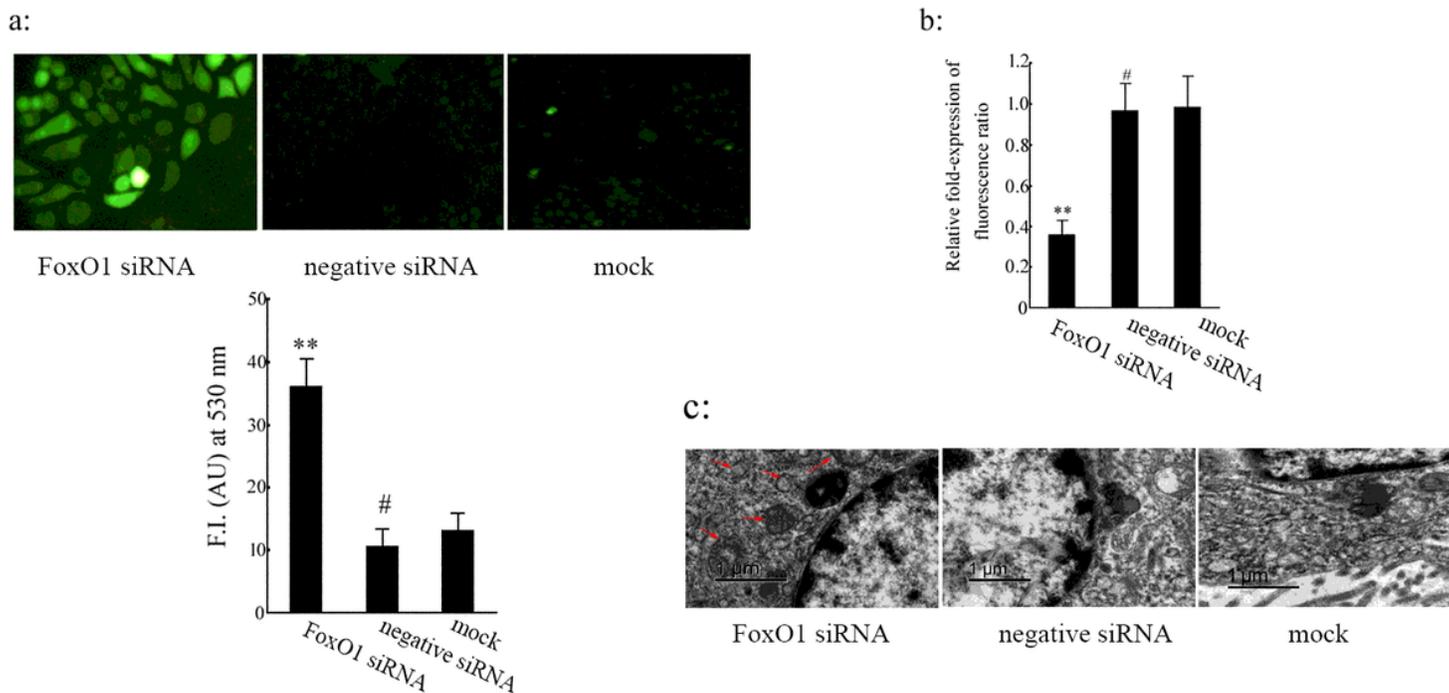


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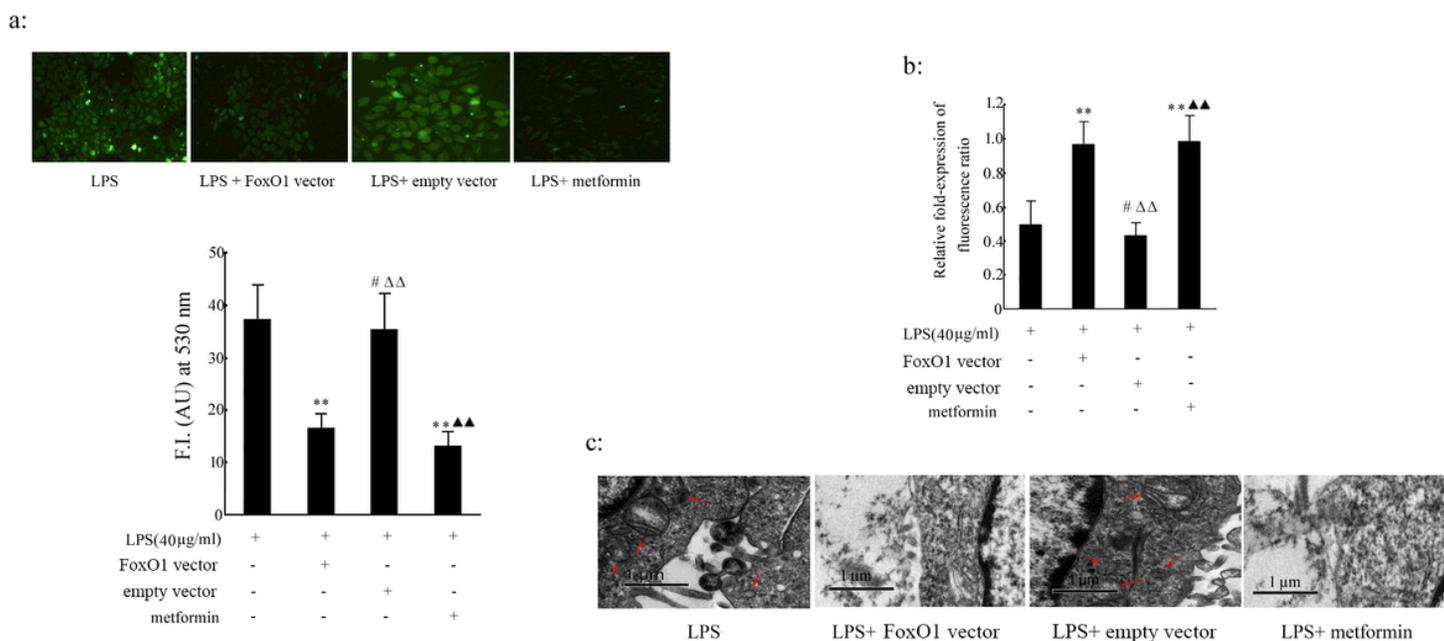
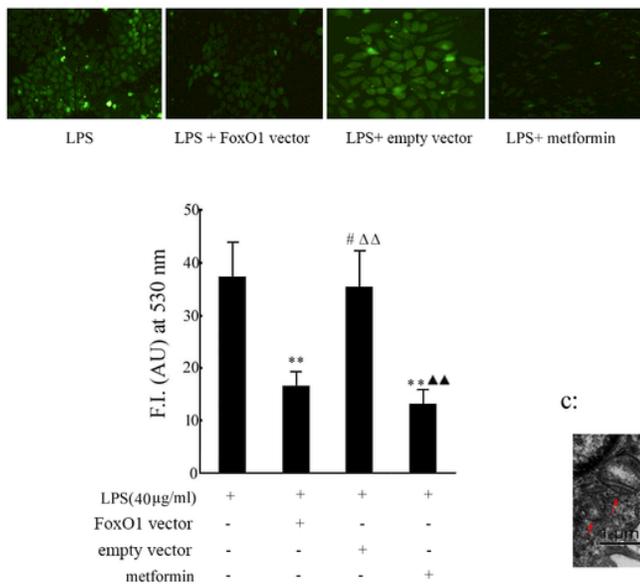


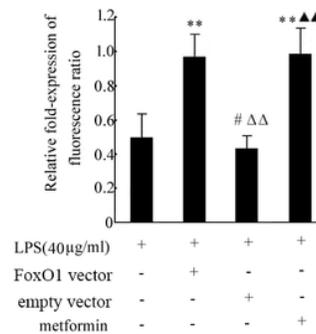
Figure 3

Effect of overexpression of FoxO1 on mitochondrial function in LPS-stimulated SiHa cells. The SiHa cells were stimulated with 40 $\mu\text{g/ml}$ LPS for 24 h under transfection with pcDNA3.1-FoxO1 vector (2 $\mu\text{g/ml}$) or pcDNA3.1-empty vector (2 $\mu\text{g/ml}$) for 48 h respectively. (a) Intracellular ROS levels were measured by H2DCFDA fluorescence (Green). Data represent the means \pm S.D. of three independent experiments (n = 3). **p < 0.01, #p > 0.05 versus LPS alone-treated group; $\Delta\Delta$ p < 0.01 versus LPS (+) and FoxO1 vector (+) group; $\blacktriangle\blacktriangle$ p < 0.01 versus LPS (+) and empty vector (+) group. (b) Mitochondrial membrane potential was detected by the change of JC-1 fluorescence. **p < 0.01, #p > 0.05 versus LPS alone-treated group; $\Delta\Delta$ p < 0.01 versus LPS (+) and FoxO1 vector (+) group; $\blacktriangle\blacktriangle$ p < 0.01 versus LPS (+) and empty vector (+) group. (c) Representative transmission electron microscopy images. Mitochondria became smaller, punctate, and the length was significantly shortened (Red arrows). Scale bar: 1 μm .

a:



b:



c:

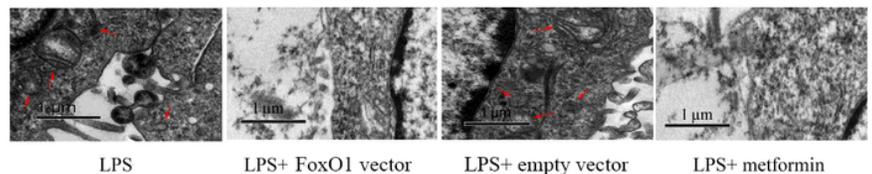


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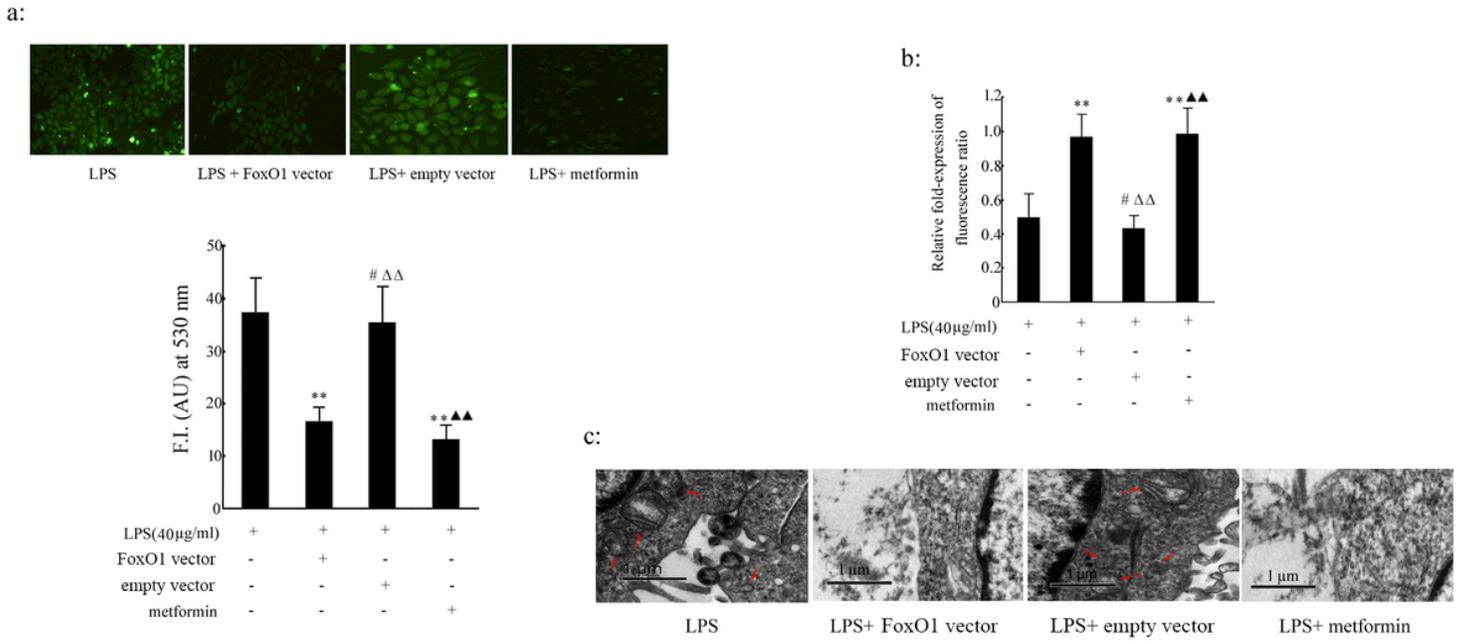


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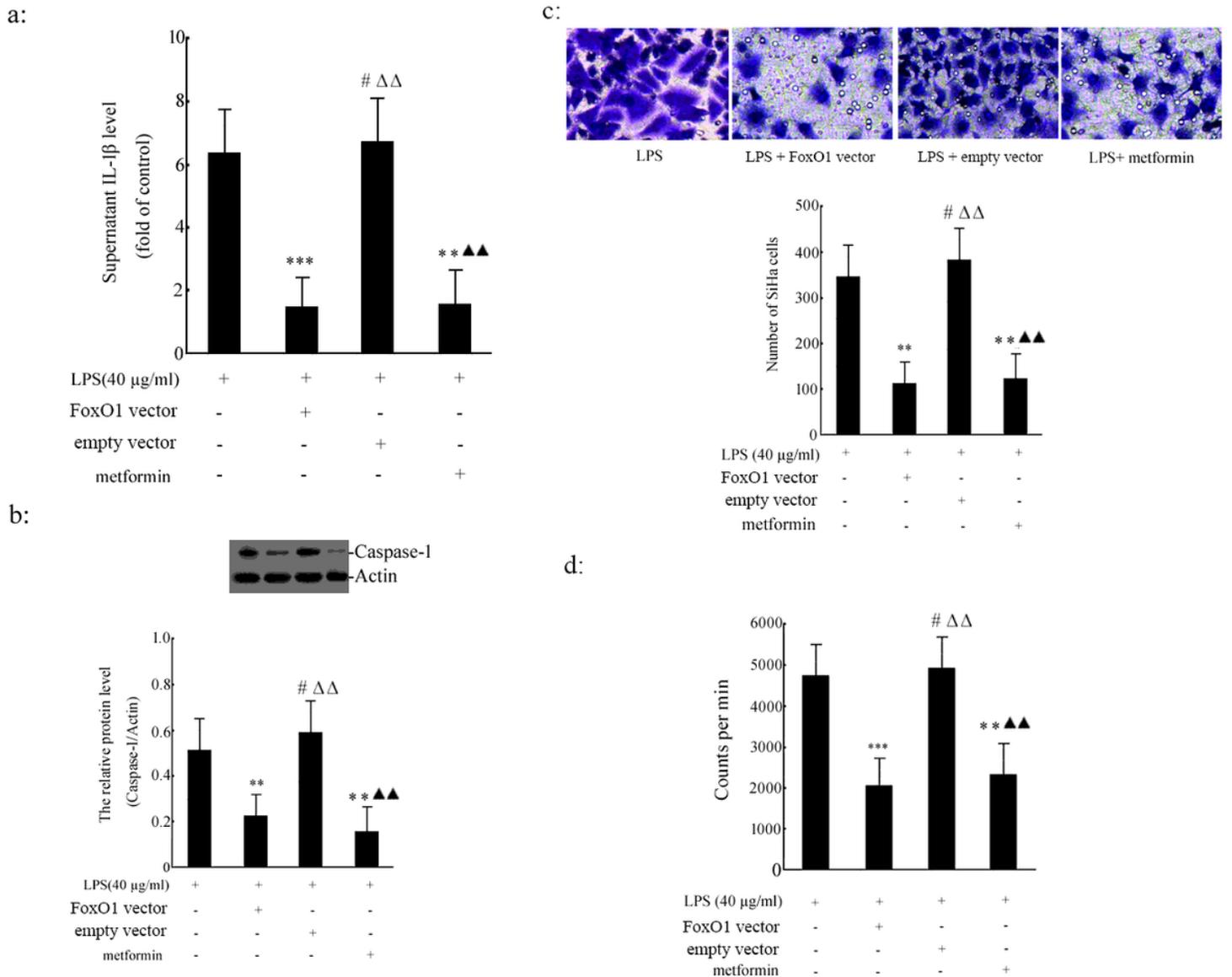


Figure 4

Effect of overexpression of FoxO1 on biological function in LPS-stimulated SiHa cells. The SiHa cells were stimulated with 40 μg/ml LPS for 24 h under transfection with pcDNA3.1-FoxO1 vector (2 μg/ml) or pcDNA3.1-empty vector (2 μg/ml) for 48 h respectively. (a) The extracellular levels of IL-1β were examined by ELISA assay. Data are the mean ± S.D. of three independent experiments. *** $p < 0.001$, ** $p < 0.01$, # $p > 0.05$ versus LPS alone-treated group; ΔΔ $p < 0.01$ versus LPS (+) and FoxO1 vector (+) group; ▲▲ $p < 0.01$ versus LPS (+) and empty vector (+) group. (b) The protein levels of caspase-1 in lysates of SiHa cells were measured by western blot assay. The bar chart showed the ratio of caspase-1 to β-actin at each groups (n = 3). ** $p < 0.01$, # $p > 0.05$ versus LPS alone-treated group; ΔΔ $p < 0.01$ versus LPS (+) and FoxO1 vector (+) group; ▲▲ $p < 0.01$ versus LPS (+) and empty vector (+) group. (c) The SiHa cells migration were measured by Transwell migration assay (n = 3). ** $p < 0.01$, # $p > 0.05$ versus LPS alone-treated group; ΔΔ $p < 0.01$ versus LPS (+) and FoxO1 vector (+) group; ▲▲ $p < 0.01$ versus LPS (+) and empty vector (+) group. (d) 3H TdR incorporation was executed to assess the proliferation of SiHa cells.

The absolute value of CPM showed the proliferation of SiHa cells (cpm/106 cells). Data represent the means \pm S.D. of three independent experiments, *** $p < 0.001$, ** $p < 0.01$, # $p > 0.05$ versus LPS alone-treated group; $\Delta\Delta$ $p < 0.01$ versus LPS (+) and FoxO1 vector (+) group; $\blacktriangle\blacktriangle$ $p < 0.01$ versus LPS (+) and empty vector (+) group.

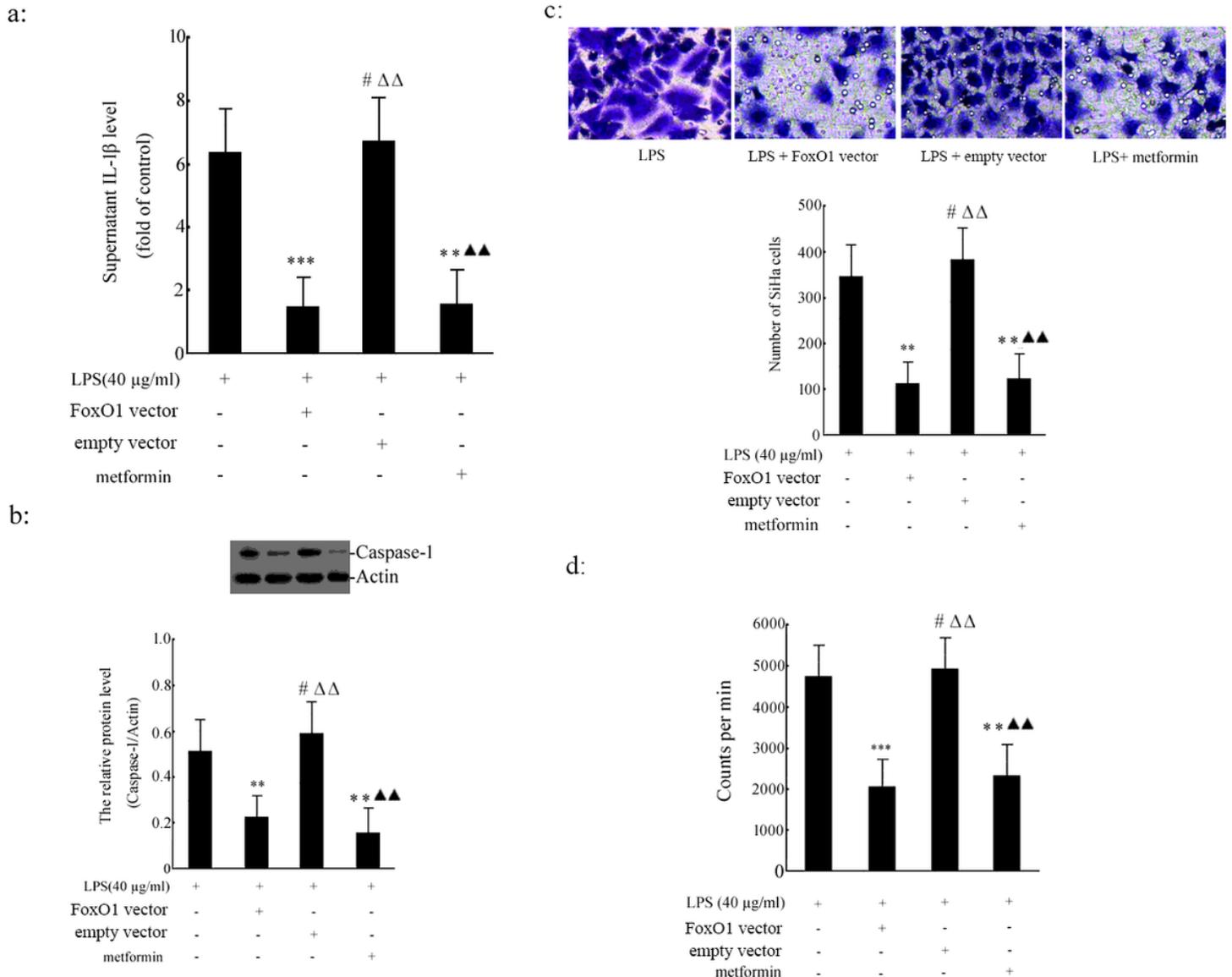


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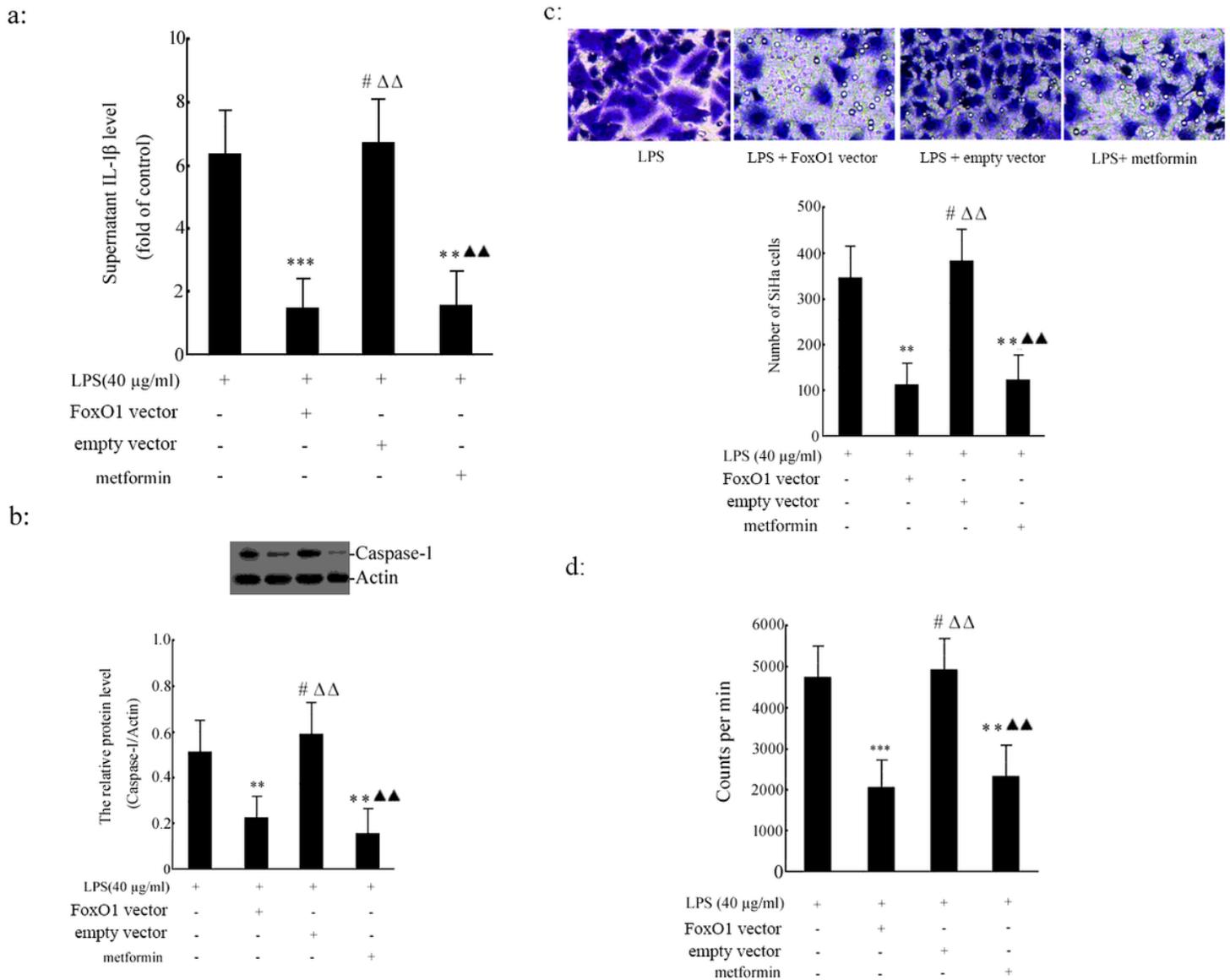


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