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Original Article

Verteporfin suppressed mitophagy via PINK1/parkin pathway in endometrial cancer

Ming-Ming Zhao[1*], Bo Wang[1*], Wen-Xi Huang[2], Li Zhang[1], Rui Peng[1], Chao Wang[1]

[1]Department of Gynecology, Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, China; [2]School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Shanghai 200032, China. [*]Equal contributors.

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Abstract: Endometrial cancer (EC) is a malignancy that poses a threat to woman's health worldwide. Building upon prior work, we explored the inhibitory effect of verteporfin on EC. We showed that verteporfin can damage the mitochondria of EC cells, leading to a decrease of mitochondrial membrane potential and an increase in ROS (reactive oxygen species). In addition, verteporfin treatment was shown to inhibit the proliferation and migration of EC cells, promote apoptosis, and reduce the expression of mitophagy-related proteins PINK1/parkin and TOM20. The ROS inhibitor N-Acetyl Cysteine was able to rescue the expression of PINK1/parkin proteins. This suggests that verteporfin may inhibit mitophagy by elevating ROS levels, thereby inhibiting EC cell viability. The effect of verteporfin on mitophagy supports further investigation as a potential therapeutic option for EC.

Keywords: Endometrial cancer, verteporfin, mitophagy, PINK1/parkin pathway

Introduction

Endometrial cancer (EC) is one of the most common gynecologic malignancies, being the fourth-most diagnosed cancer and sixth most common cause of cancer-related deaths [1]. With the continuous progress in biotechnology and genetic testing technology, the molecular characteristics of tumors have been increasingly identified and leveraged as therapeutic targets, thus aligning with the core principles of precision medicine. Multiple pathway members are often simultaneously mutated in cancer, including EC. The PI3K/AKT pathway is aberrantly activated in more than 90% of ECs. Thus, EC represents a primarily PI3K-driven disease, which has led to multiple clinical trials targeting different PI3K pathway members. In fact, EC was classified into four subtypes based on the molecular characteristics of tumors in the Cancer Genome Atlas (TCGA): (1) POLE ultra mutated, (2) microsatellite instability hypermutated, (3) copy number low, and (4) copy-number high [2]. Various tumors are characterized by distinct driving mutations that activate corresponding signaling pathways, leading to diverse pathological and physiological effects. Our previous study has also found that Yes-associated Protein1 (YAP1)-mediated upregulation of GRB2-associated binding protein 2 (GAB2) activates the growth factor-promoted PI3K pathway in EC [3]. Furthermore, we also found that verteporfin, a clinical photosensitizer shown to be able to inhibit the YAP-TEAD interaction, reduces proliferation and migration, and induces apoptosis of EC cells through the YAP/TAZ-HIPPO pathway [3]. In addition, verteporfin effectively targets PD-L1 through transcriptional and post-translational mechanisms, and can play a therapeutic role in targeting PD-L1 [4]. In recent years, several studies have also found that verteporfin plays a suppressive role in various tumors by affecting mitochondrial homeostasis. Shin et al. found that the combination of verteporfin and melatonin could affect the proliferation and invasion of head and neck cancer cells by affecting mitochondrial functional homeostasis [5]. Studies in gliomas have shown that verteporfin can inhibit mitochondrial oxidative phosphorylation processes and specifically induce glioma stem cell death [6]. These findings suggest that the photosensitizer verteporfin has great value in tumor therapy, and its mechanisms are varied and complex. In addi-

tion to inhibiting YAP1 protein activity, verteporfin also inhibits tumor progression by affecting mitochondrial function in tumor cells. This effect on mitochondrial function is worthy of further exploration.

Mitophagy is a specific form of autophagy triggered after mitochondrial damage or cellular stress, which selectively degrades mitochondria through the PINK1/parkin and BNIP3/NIX/FUNDC1 pathways. Mitophagy plays an indispensable role in mitochondrial quality control. This type of quality control is necessary for the maintenance of proper mitochondrial functioning and cellular metabolism, and to prevent the accumulation of reactive oxygen species (ROS) that can cause mitochondrial DNA mutations (mtDNA). Sun et al. analyzed data from TCGA and Gene Expression Omnibus databases and found that TOMM40 serves as an oncogene in EC and promotes tumor progression through a mitophagy-related pathway [7]. These energy metabolism-related pathways suggest that mitochondrial energy metabolism plays an important role in tumorigenesis and development, therefore warranting further studies on mitochondria-related pathogenic mechanisms in EC.

PINK1 is a mitochondrial serine-threonine kinase that is stabilized at the outer mitochondrial membrane and senses mitochondrial health status. Upon ΔΨm depolarization, PINK1 recruits parkin, a cytosolic E3 ubiquitin ligase, to the mitochondria and activates mitophagy [8, 9]. The function of mitophagy has been associated with tumor suppression, and PINK1 and parkin have been identified to play a crucial role in various cancer types including colorectal [10], hepatocellular [11], cervical [12] and bladder cancer [13].

Studies on mitophagy in EC have revealed that kinesin family member 4A (KIF4A), which is closely related to mitophagy, promotes the progression of EC through the maintenance of genome stability, and that targeting the KIF4A/TPX2 axis may provide a new strategy for the treatment of EC patients [14]. In another study, pretreatment of EC cells with Mdivi-1, a mitophagy inhibitor, synergistically decreased PINK1/parkin-mediated mitophagy and enhanced chemotherapy-induced oxidative damage, thereby increasing the efficacy of chemotherapy [15]. Another study similarly showed that inhibition of mitophagy by pretreatment of EC cells with Mdivi-1 could enhance the efficacy of chemotherapy [16]. The effect of verteporfin on mitophagy in EC is unknown. Does verteporfin have the potential to inhibit tumors by affecting mitochondrial homeostatic function? To answer this question, we proposed the hypothesis that verteporfin could affect mitophagy through the PINK1/parkin pathway in EC.

Results

Mitophagy-related gene expression is associated with EC patient prognosis

To investigate the effect of differences in mitophagy-related gene expression on the clinical prognosis of EC patients, we searched for PINK1, TOM20, and LC3BI/LC3BII (MAP1LC3B) genes using the TCGA database and analyzed the relationship between the expression of each gene and the survival of patients with EC using the optimal cut-off value (**Figure 1**). Patients with high PINK1 expression showed a trend of having a better prognosis, although the *p* value was 0.06 (>0.05). We speculate that this may be because of the large difference in the number of patients between the two groups. Generally, these results show that patients with high expression of PINK1 (log rank test P=0.06), TOM20 (log rank test P=0.0024), LC3BI (log rank test P=0.027) and LC3BII (log rank test P=0.087) had increased survival. Therefore, patients who retained better mitophagy function may have a better prognosis.

Verteporfin inhibits proliferation and migration, and induces apoptosis in EC cells

We performed a screen to identify drugs that can regulate mitochondria. We found that verteporfin could inhibit the expression of mitochondria-associated genes, and RNA sequencing showed that the expression of mitochondria-associated genes was downregulated (Figures S1, S2). To determine the suppressive effect of verteporfin on the development of endometrial cells, Ishikawa and Hec1A cells were untreated or treated with multiple concentrations of verteporfin (0, 1, 3, 5 and 10 μM) for 24 h. CCK8 assays were carried out to measure the proliferation of cells at the various concentrations. High concentrations of verteporfin hindered cell proliferation in Ishikawa and Hec1A cell lines (**Figure 2A**, **2B**). We further determined the potential role of verteporfin in the

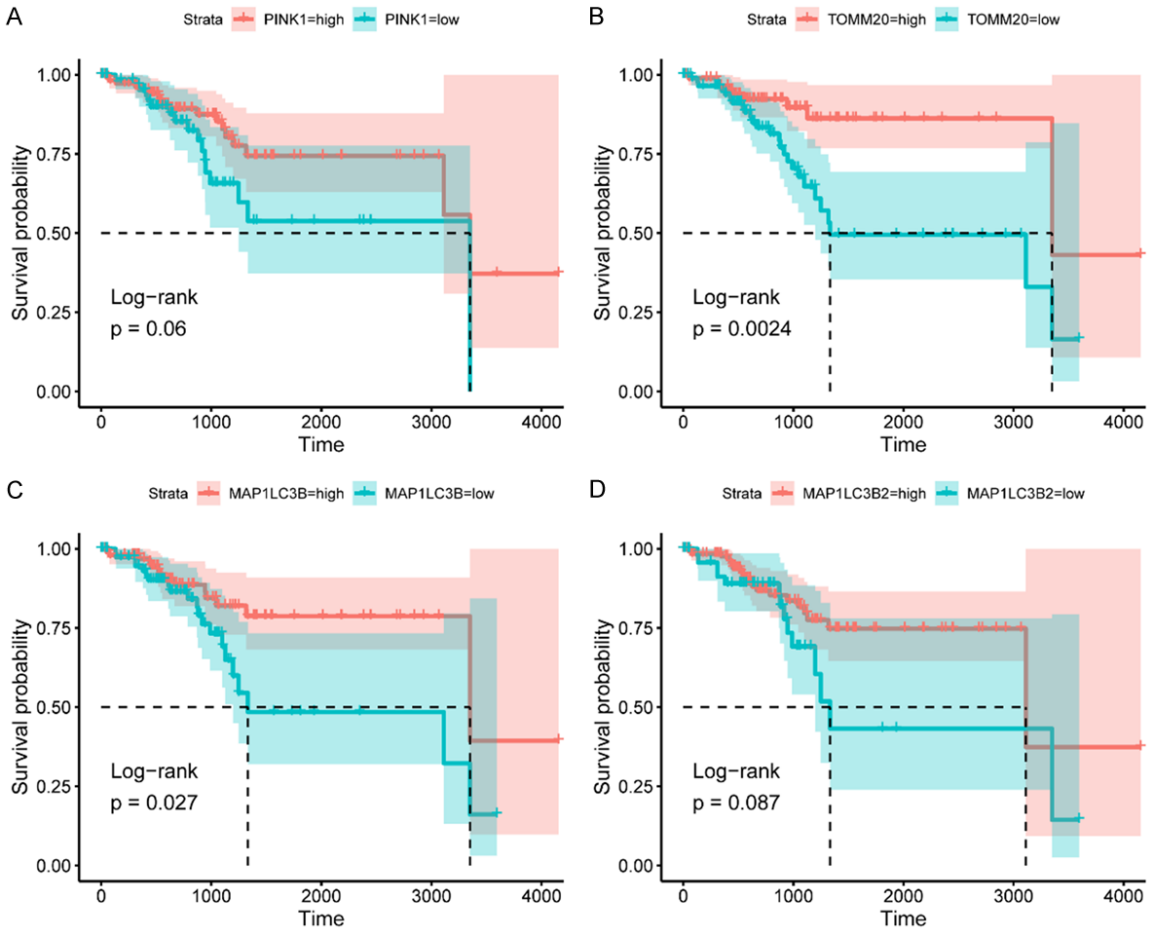

Figure 1. Endometrial cancer patients with high expression of mitophagy-related genes have a superior prognosis. Survival time was longer in EC patients with high PINK1 (A), TOM20 (B), LC3BI (C), and LC3BII (D) gene expression.

migration ability of EC cells. Hec1A and Ishikawa cells were treated with 1 μM or 3 μM of verteporfin for 12 h or 24 h. Verteporfin treatment for 12 h significantly decreased the migration ability compared to the control (**Figure 2C-F**). Flow cytometry was used to assess cell apoptosis. Cells were left untreated or treated with verteporfin 1-4 μM. Verteporfin could induce the apoptosis of EC cells (**Figure 2G**).

Verteporfin disrupts mitochondrial morphology and viability

Transmission electron microscopy was performed to image the mitochondrial structure after 1 μM or 3 μM verteporfin treatment for 24 h. The mitochondrial structure was severely disrupted after verteporfin treatment compared with the control group. Arrows indicate structurally disrupted mitochondria. As shown in **Figure 3A**, **3B**, mitochondria were significantly damaged in the verteporfin-treated group compared with the control group. Mitochondria in cells treated with verteporfin showed prominent structural damages, illustrated by significant mitochondrial deformation, swelling, vacuolization, and lack of intact cristae.

The mitochondrial indicator Mito-Tracker was used to determine whether verteporfin could destroy mitochondria in Hec1A and Ishikawa cells. In normal mitochondria, Mito-Tracker emits red fluorescence and the fluorescence intensity reflects the active mitochondrial levels. Fluorograms showed that the intensity of red fluorescence was significantly reduced after 1 μM or 3 μM of verteporfin treatment for 24 h compared with the control group (**Figure 3C-F**).

Treatment of EC KLE and ECC1 cell lines with 3 μM verteporfin resulted in decreased mitochondrial oxygen consumption, and cells had no response to oligomycin, carbonyl cyanide

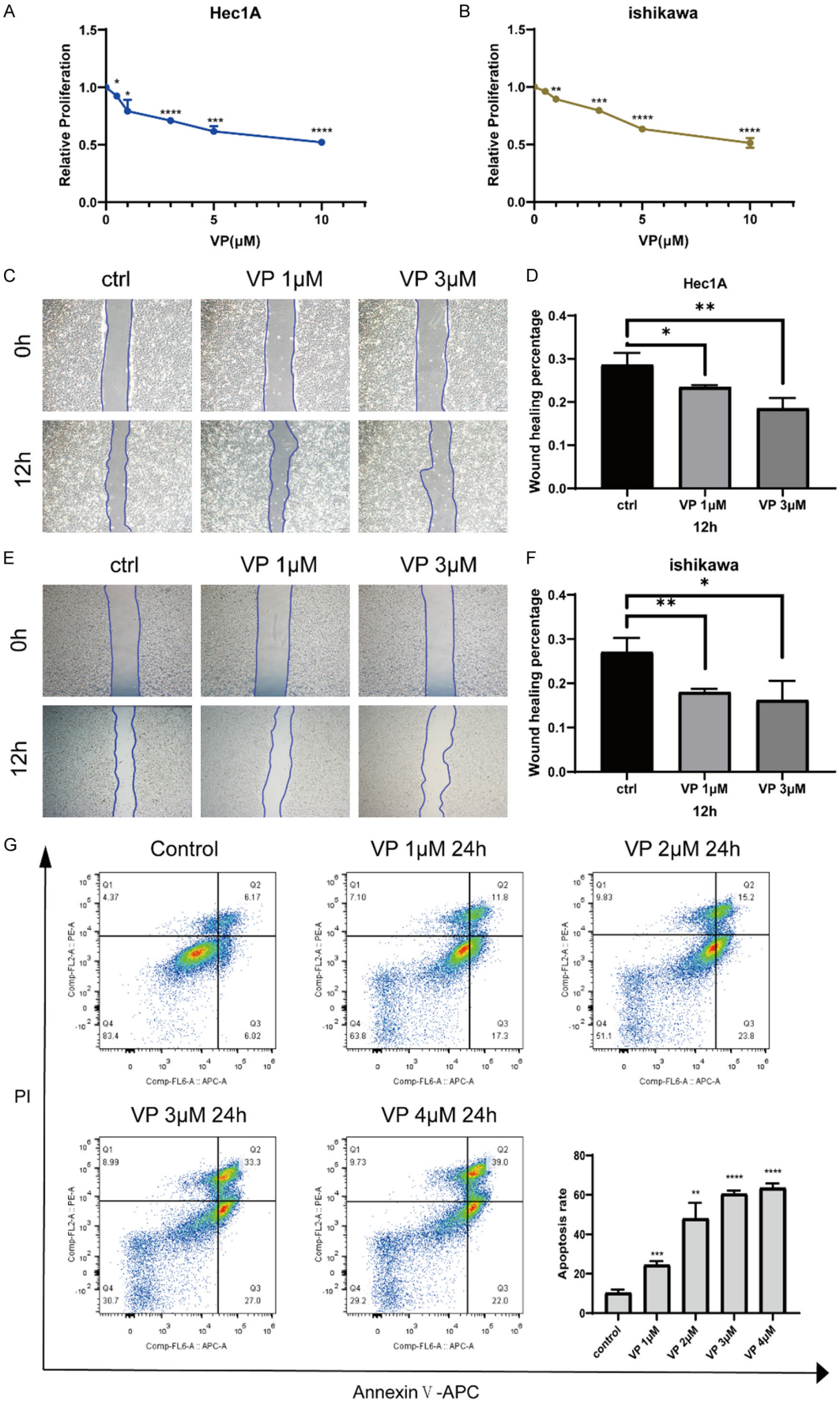
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Figure 2. The effect of verteporfin on the proliferation, migration and apoptosis of EC cells. Concentration-dependent effect of verteporfin on cell viability was analyzed with the CCK-8 assay in Hec1A (A) and Ishikawa (B) cell lines. A scratch assay showed that 1 μM or 3 μM of verteporfin treatment for 12 h significantly inhibited cell migration in Hec1A (C) and Ishikawa (E) cells, and there was a statistically significant difference between the migration rates of the control group and the verteporfin-treated group (D, F). A flow cytometry assay for apoptosis showed that the percentage of apoptosis was elevated in Hec1A (G) cell line after 1-4 μM verteporfin treatment for 24 h. There was a statistically significant difference between the control and verteporfin-treated groups for the Hec1A cell line. Data are presented as means ± SD from three independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with the control group.

p-trifluoro methoxy phenylhydrazone (FCCP), antimycin A, and rotenone treatments (Figure S4A). Basal respiratory values (Basal) and respiratory potential (Spare Respiratory) calculated from OCR (Oxygen Consumption Rate) values were also significantly reduced with verteporfin treatment (Figure S4B).

Verteporfin reduces mitochondrial membrane potential and elevates ROS levels

Mitochondrial membrane potential (MMP) depolarization is the final crucial step of the early stage of cell apoptosis [17]. We employed a JC-1 assay to measure MMP, and carbonyl cyanide 3-chlorophenylhydrazone was used as a positive control. **Figure 4A** and **4C** shows the collapse of the MMP, with the ratio of green to red fluorescence representing the alteration of MMP. There was a statistically significant difference in the fluorescence ratio between the control and the verteporfin-treated groups in Hec1A and Ishikawa cell lines (**Figure 4B**, **4D**).

We next used 2',7'-dichlorofluorescein diacetate (DCFH-DA) to measure the total intracellular ROS production. Green fluorescence intensity represents ROS levels. The level of ROS, a critical factor in oxidative stress, was quantified and depicted in **Figure 4E**. We found that the cellular ROS levels were significantly increased after 1 μM verteporfin treatment for 24 h compared with the control group. In both Hec1A and Ishikawa cell lines, the mean fluorescence intensity was higher in the verteporfin-treated group than in the control group (**Figure 4E-G**).

Verteporfin inhibits mitophagy by decreasing PINK1/parkin protein expression from ROS

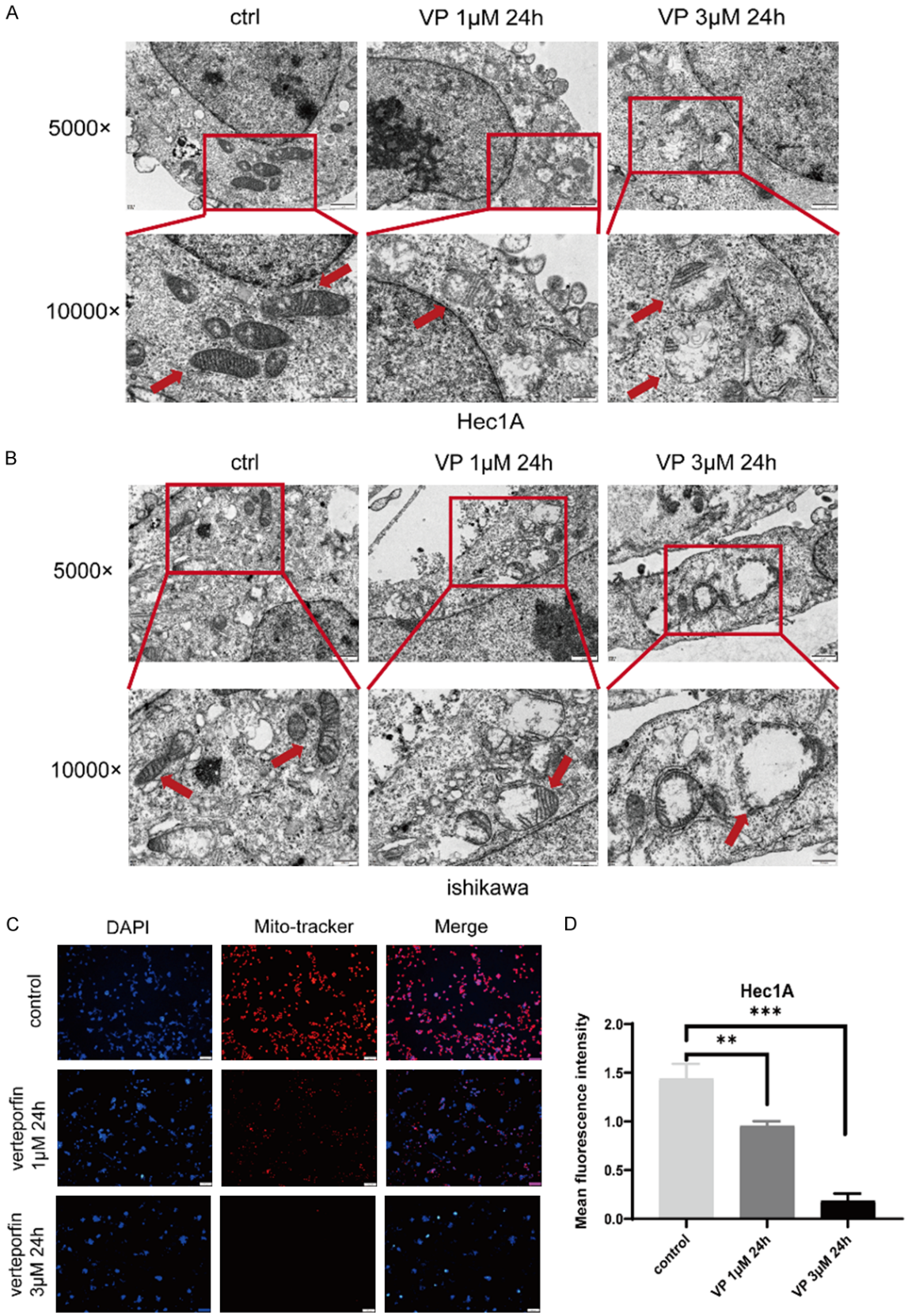
To investigate the mechanism of verteporfin in mitophagy, we treated cancer cells with different concentrations (0.5, 1, 3 μM) of verteporfin for 24 h. Quantified western blot results demonstrated that the expression of YAP as well as PINK1/parkin proteins significantly decreased with the increase of verteporfin concentration (**Figure 5A-D**). These data suggest that verteporfin inhibits mitophagy by downregulating PINK1/parkin protein levels, which subsequently affects mitochondrial function.

To explore the role of the PINK1/parkin signaling pathway in oxidative stress-mediated mitophagy associated with cell proliferation or apoptosis, EC cells were stimulated with verteporfin and treated with the ROS inhibitor N-Acetyl Cysteine (NAC). We found that oxidative stress inhibited the expression of PINK1 and parkin, which was reversed by NAC treatment (**Figure 5E**, **5F**). Degradation of TOM20 is a marker of mitophagy [18], and the expression of TOM20 was used for measuring mitochondrial mass. These results indicate that verteporfin inhibits the PINK1/parkin signaling pathway in EC mitochondria through oxidative stress.

Discussion

Mitochondria are at the core of cellular metabolism and play a crucial role in key metabolic activities from energy production to cell signaling [19]. Mitochondria continuously fuse and divide, which supports cell viability [20]. They also play an important role in the limitless proliferation of tumor cells, thereby affecting the prognosis of tumors [21-23]. Our analysis of data from EC patients in the TCGA database also revealed that patients with high expression of mitophagy-related genes such as TOM20/PINK1 had a better prognosis (**Figure 1**). Studies on mitophagy-related gene expression in multiple myeloma and papillary renal cell carcinoma have shown that patients with low expression of the PINK1 gene have a worse prognosis. In addition, PINK1 plays a protective role in liver hepatocellular carcinoma patients, which is consistent with our findings [24-26].

Mitophagy is a form of autophagy that selectively degrades damaged mitochondria as well as maintains healthy mitochondria [27]. Mitophagy requires recognition and clearance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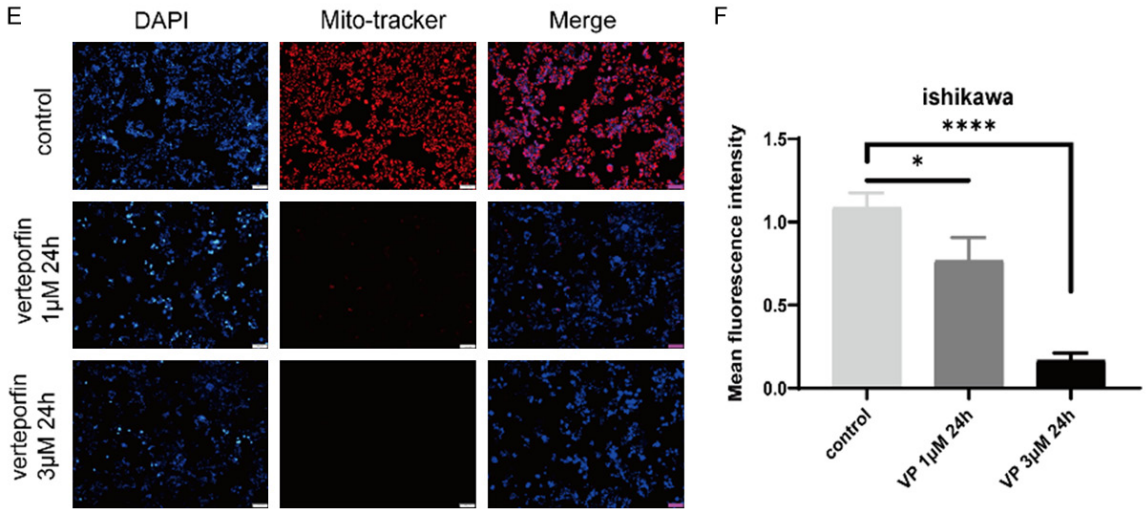

Figure 3. Verteporfin disrupts mitochondrial morphology and inhibits mitochondrial viability. Mitochondrial ultrastructure (A, B) and immunofluorescence (C, E) of DAPI and MitoTracker were evaluated after treatment with 1 μM or 3 μM verteporfin in Hec1A and Ishikawa cell lines. The difference in mean fluorescence intensity (D, F) between the control and verteporfin-treated groups was statistically significant. Data are presented as means ± SD from three independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with control group.

of mitochondria in response to accumulation of misfolded mitochondrial proteins or depolarization of mitochondria, which is required to maintain mitochondrial quality control [28]. Under physiological conditions, mitophagy plays a vital role in keeping proper mitochondrial function and homeostasis by eliminating damaged or superfluous mitochondria through the PINK1/parkin pathway [29], a well-known mitochondrial stress signaling pathway that mediates mitophagy [30]. PINK1 is a sensor of mitochondrial damage and acts to maintain mitochondrial health. Furthermore, PINK1 recruits parkin to damaged mitochondria and promotes autophagic clearance in a parkin-dependent ubiquitination process [31]. Persistent and/or excessive mitophagy is an important cause for mitochondrial dysfunction and cell death. Parkin regulates IGF2BP3 (insulin-like growth factor 2 mRNA-binding protein 3) through ubiquitination in cervical cancer, leading to inactivation of the PI3K and MAPK signaling pathways, which results in the inhibition of cervical cancer cell growth and tumorigenesis. IGF2BP3 mutation also leads to the attenuation of parkin-mediated mitophagy and inhibits cervical tumor development [32]. Valine activates mitochondrial apoptosis via the PINK1/parkin pathway leading to loss of mitochondrial membrane potential in colorectal cancer [33]. Jiang et al. discovered that Caveolin-1 suppresses the PINK1/MAPK signaling pathway, thereby inhibiting the initiation of mitophagy, this inhibition leads to the accumulation of damaged mitochondria and the production of ROS, which triggers cell apoptosis [34]. Yin et al. found that doxorubicin induces mitophagy and mitochondrial damage by dysregulating the PINK1/parkin pathway [35]. Similarly, we found that verteporfin inhibits mitophagy by downregulating the levels of PINK1/parkin proteins in EC.

ROS may act as upstream factors in the PINK1/parkin signaling pathway and can activate this signaling axis [36]. Mitochondria are considered the main source of ROS in the cell. In addition, ΔΨm depolarization results in the accumulation of damaged mitochondria and ROS release, which plays a central role in cell apoptosis [37].

In our study, we found that verteporfin elevated ROS levels, decreased mitochondrial membrane potential (**Figure 4**) and inhibited mitochondrial function (Figures S3, S4), ultimately exerting an inhibitory effect on EC cells.

A previous study found that mitochondria are key players in drug-induced toxicity of central organs [38]. Verteporfin has been defined as a clinical photosensitizer and is an inhibitor of the YAP/TEAD interaction. Our previous study also found that verteporfin reduced proliferation, migration and induced apoptosis of EC cells

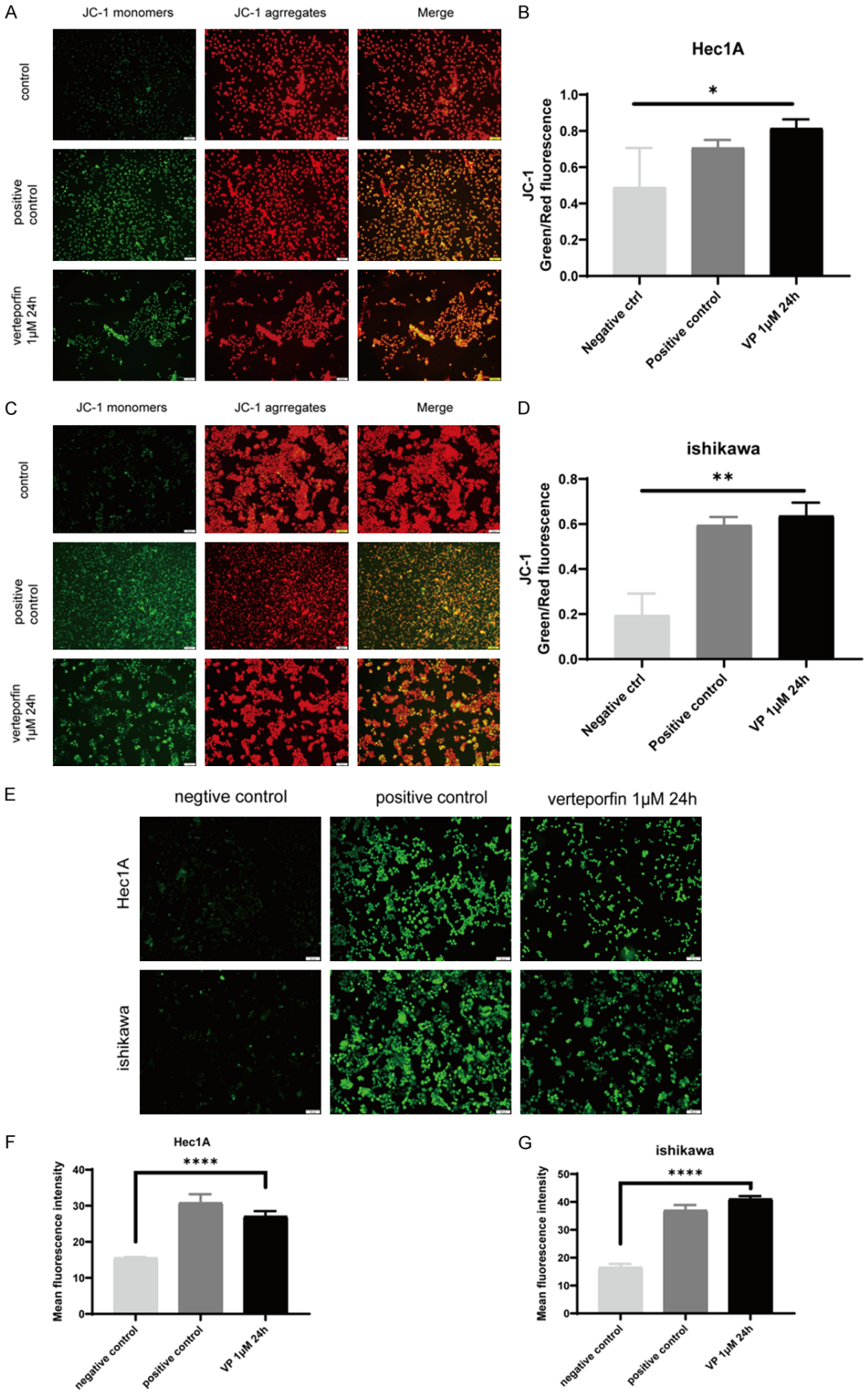
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Figure 4. Verteporfin decreases mitochondrial membrane potential and increases ROS levels in EC cells. A-D. JC-1 staining is depicted. The ratio of red/green fluorescence reflects changes in the mitochondrial membrane potential. Scale bar: 100 μm. E-G. Measurement of ROS by DCFH-DA after treatment with verteporfin. Scale bar: 100 μm. Data are presented as means ± SD from three independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with the control group.

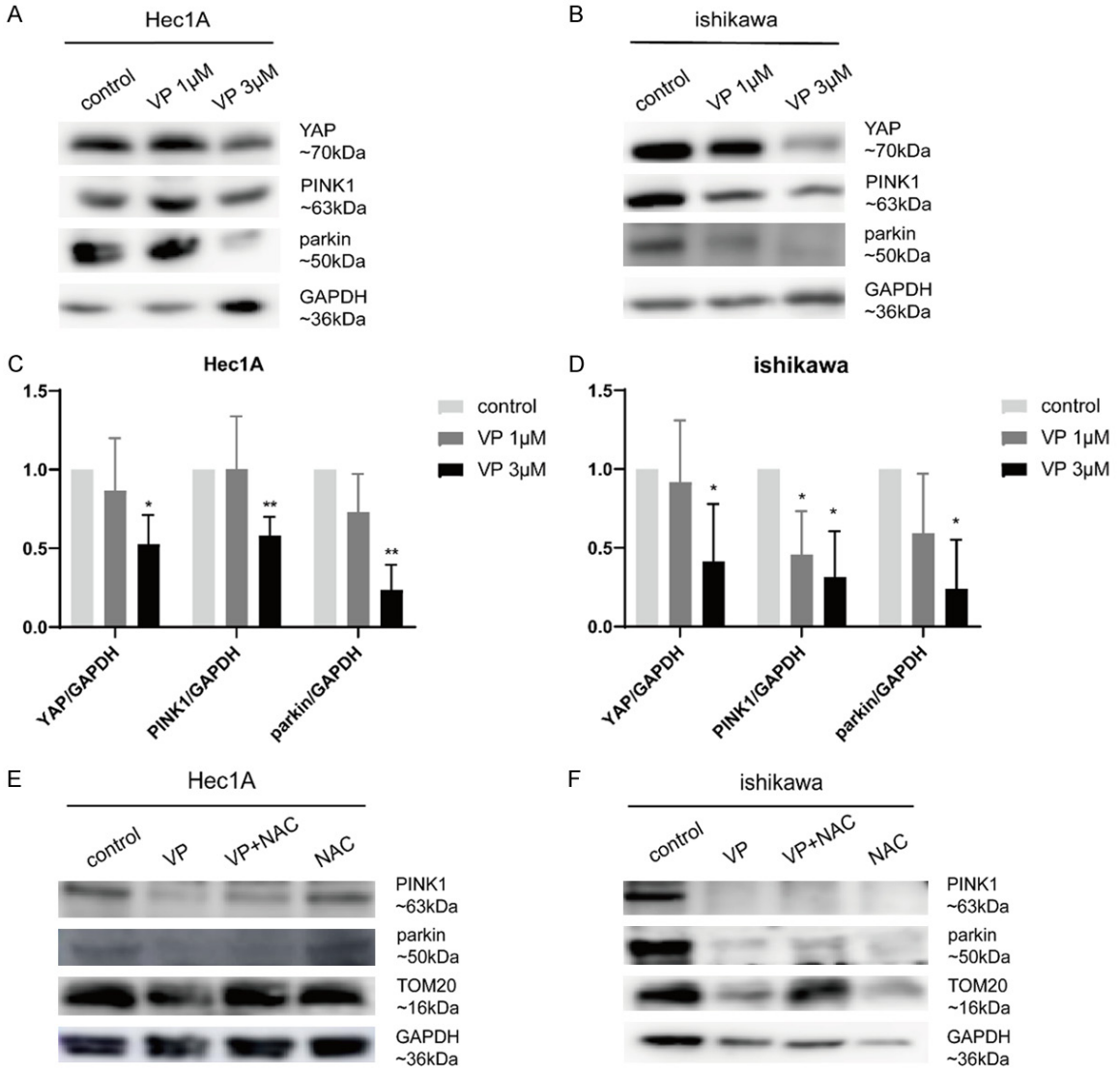

Figure 5. Verteporfin reduces the expression level of the mitophagy-related protein PINK1/parkin in endometrial cells. Cells were treated with verteporfin (1 μM or 3 μM) alone or verteporfin plus NAC (2 mM) for 24 h. Representative western blot images of PINK/parkin in Hec1A (A) and Ishikawa (B) cell lines are shown. Quantitative analysis of YAP/GAPDH, PINK1/GAPDH and parkin/GAPDH in the Hec1A cell line (C), YAP/GAPDH, PINK1/GAPDH and parkin/GAPDH in the Ishikawa cell line (D) are presented. Data are shown as means ± SD from three independent experiments. *P<0.05, **P<0.01, compared with the control group. Western blot image of PINK1/parkin and TOM20 treated by verteporfin and the ROS inhibitor NAC in Hec1A (E) and Ishikawa (F) cell lines.

through YAP [39]. Verteporfin also has been shown to induce cell apoptosis through the regulation of ROS levels and mitophagy in cancers [40-43]. Researchers also found that excessive oxidative stress leads to mitophagy in myocardial apoptosis [44].

In our study, we found that levels of mitophagy markers were significantly reduced by verteporfin treatment in Ishikawa and Hec1A cells. Notably, verteporfin significantly decreased mitophagy-related proteins PINK1 and parkin (**Figure 5A-D**) in EC cells, suggesting that verte-

porfin effectively reduces endometrial cell viability by targeting mitophagy. We also found that verteporfin induces mitochondrial dysfunction by decreasing the incidence of mitophagy in endometrial cells. PINK1/parkin was found to be responsible for this effect, which could be reversed by the ROS inhibitor NAC (**Figure 5E**, **5F**). Fan et al. found that PINK1-dependent mitophagy regulates migration and homing of multiple myeloma cells through the Hippo-YAP/TAZ pathway [45]. Researchers showed that PINK1 interacts with YAP1 during viral infection and impairs YAP1/IRF3 complex formation, positively regulating retinoic-acid-inducible-gene-I-like receptors (RLR)-triggered innate immune responses [46]. Based on the above findings, we speculate that verteporfin may also downregulate the PINK1 protein by inhibiting the YAP protein. Therefore, the relationship between YAP and PINK1 protein deserves further investigation.

In summary, our study found that survival time was longer in EC patients with higher expression of mitophagy-related genes, and we identified the tumor-suppressive effects of verteporfin through mitochondrial dysfunction and inhibiting mitophagy through the PINK1/parkin pathway. We have demonstrated that the regulatory mechanism of the effect of verteporfin in mitophagy has been explored in EC for the first time. However, one limitation of our study is the lack of in vivo investigation. This work may inform clinical prediction of patient survival. In addition, we believe this work supports the idea that verteporfin may be suitable as a novel application for the treatment of EC.

Disclosure of conflict of interest

None.

Address correspondence to: Chao Wang, Department of Gynecology, Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, China. E-mail: wang1980-55@163.com

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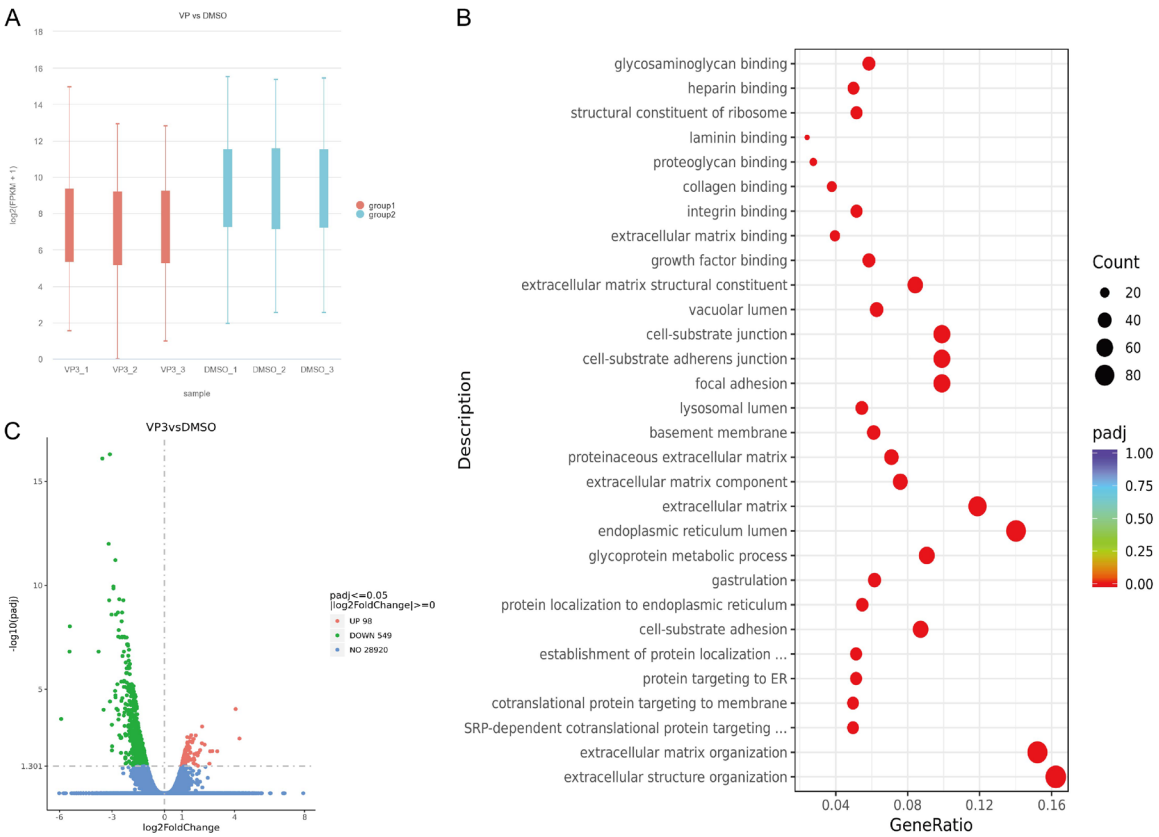

Figure S1. Transcriptomic difference and GO enrichment analyses of samples from the verteporfin-treated group and normal control samples.

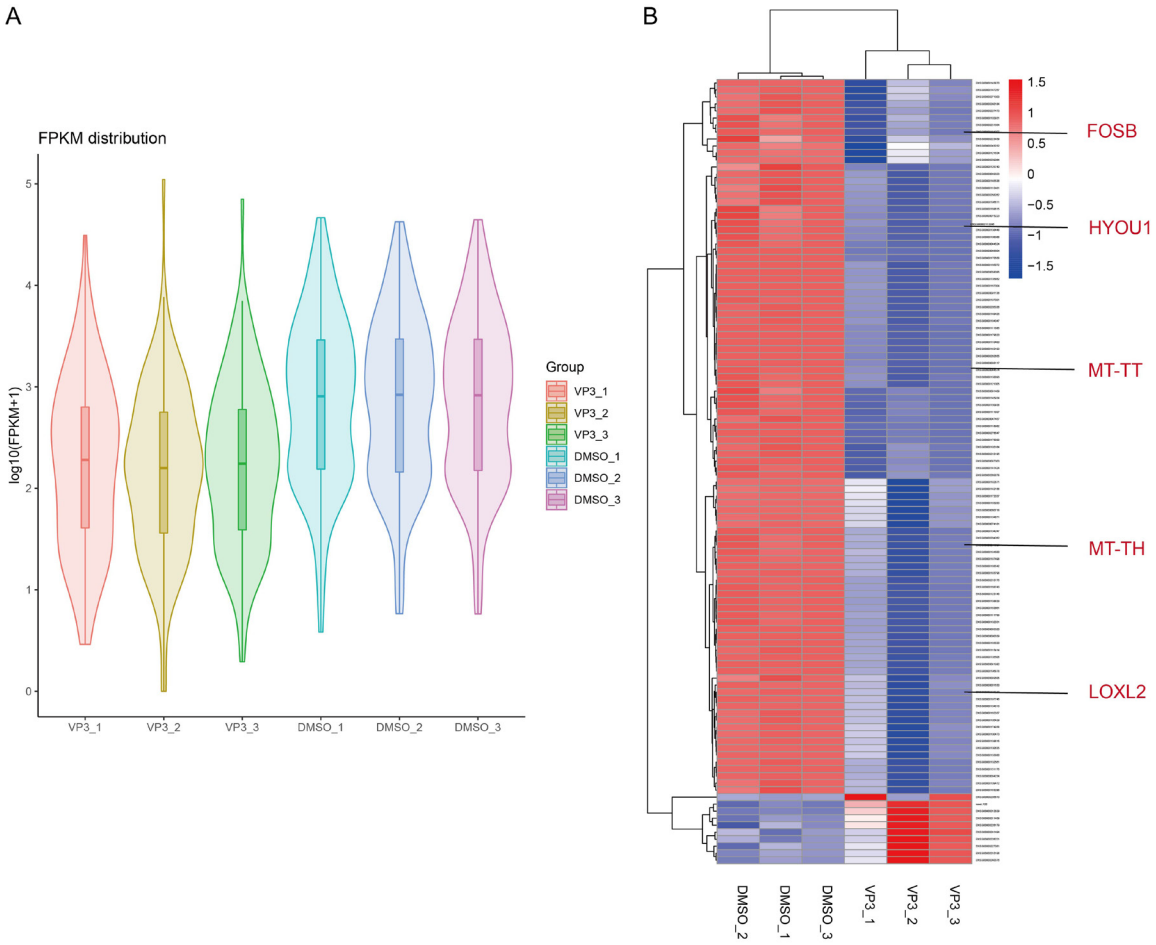

Figure S2. Altered gene expression profiles in verteporfin-treated EC cells.

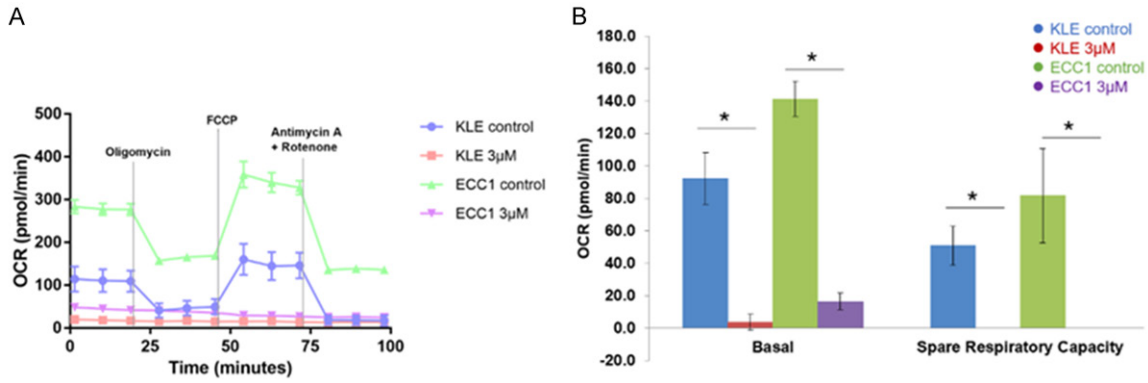

Figure S3. Verteporfin inhibits mitochondrial oxygen consumption in EC cells.

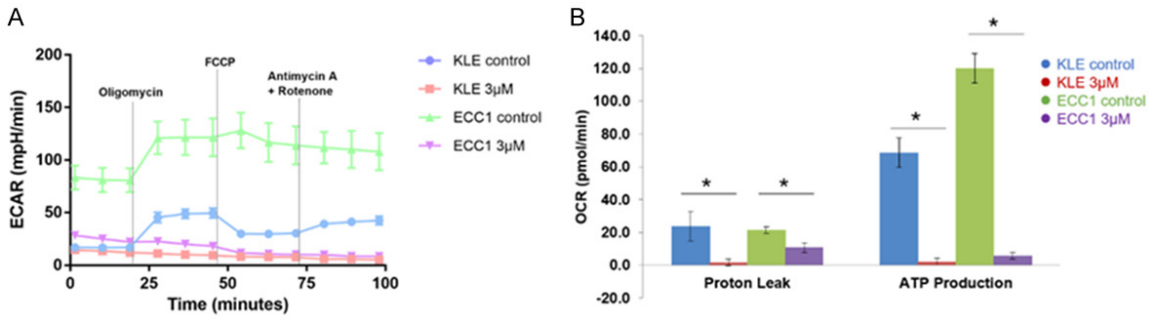

Figure S4. Verteporfin inhibits extracellular acidification rate (ECAR) and ATP production in EC cells.