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ORIGINAL PAPER

17β-estradiol Inhibits Oxidative Stress-Induced Apoptosis in Endometrial Cancer Cells by Promoting FOXM1 Expression

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Abstract

The steroid hormone 17β-estradiol (E2) has a significant impact on the development and progression of tumors. E2 stimulates tumor cell growth and metabolism, leading to an increase in reactive oxygen species (ROS) production. However, the rise in ROS levels is not sufficient to cause severe harm to cancer cells. and the mechanisms that regulate ROS are not well understood. Since FOXM1 plays a crucial role in the production of ROS, we aimed to investigate the impact of E2 on oxidative stress and the involvement of FOXM1 in the Ishikawa endometrial cancer cell line. Our research revealed that E2 controls the levels of ROS inside cells and safeguards them from apoptosis by promoting the expression of FOXM1. We observed a decrease in the expression of FOXM1 alongside an increase in oxidative damage. Moreover, cells demonstrated elevated levels of FOXM1 and ERα upon E2 treatment. Overall, our findings suggest that E2 prevents apoptosis induced by oxidative stress in endometrial cancer cells by encouraging the expression of FOXM1, potentially affecting ERα.

Keywords Endometrial cancer · Estrogen · Estrogen receptor · Oxidative stress · Apoptosis

Introduction

In 2020, 417,000 new cases of endometrial cancer were diagnosed worldwide, making it the sixth most frequent cancer in women and the overall incidence has increased by 132% compared with 30 years ago [1]. Endometrial cancer has high mortality and recurrence rates, especially due to late diagnosis and the development of chemotherapy resistance [2, 3]. It is evident that estrogen plays a crucial role in the female reproductive system, particularly in the development of the endometrium during the menstrual cycle [4]. However, estrogen, specifically E2, has been linked to the development of endometrial cancer. Epidemiological studies have shown that postmenopausal women who take estrogen for extended periods are at a significantly higher risk of developing endometrial cancer [5, 6]. Additionally, previous research has indicated that E2 stimulates the growth of endometrial cancer cells [7]. However, there is no research investigating whether E2 promotes the survival of these cancer cells. It has been suggested that increased metabolic activity in tumor cells leads to higher levels of reactive oxygen species (ROS) [8]. High concentrations of ROS have been shown to induce apoptosis and necrosis [9]. The precise mechanisms by which tumor cells protect themselves from elevated ROS are not well understood. Giovanni Tossetta et al. [3] reported the role of NAD(P)H: quinone oxidoreductase 1 (NQO1) in improving basal antioxidant capacity for ovarian cancer. As an antioxidant, NQO1 prevents the synthesis of extremely reactive semiquinones by reducing quinones to hydroquinones utilizing NADH or NADPH as substrates, which shields cells from oxidative stress. Recent reviews have also highlighted the NRF2/KEAP1 pathway with antioxidant enzymes targeting cervical and endometrial cancer. When ROS activate NRF2, antioxidant enzymes including glutathione peroxidase, heme oxygenase, catalase, and superoxide dismutase are expressed. These enzymes neutralize ROS and shield cells from the damaging effects of oxidative stress [2, 10, 11]. The up-regulated NRF2/KEAP1 signaling pathway in

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prostate cancer has been found [12] Naoko Kanda et al. [13] reported that E2 inhibits oxidative stress-induced apoptosis in keratinocytes, and Burow et al. [14] demonstrated that E2 suppresses apoptosis induced by tumor necrosis factor-α in the MCF-7 breast cancer cell line. However, other researchers have reported contradictory findings, stating that E2 promotes spontaneous apoptosis in MCF-7 cells while inhibiting apoptosis induced by vitamin E succinate [15]. It has been established that the action of E2 is mediated through its binding to two subtypes of estrogen receptor (ER), ERα and ERβ [16]. Mercedes et al. [17] proposed that the effect of E2 on oxidative stress in breast cancer cells is determined by the ratio of ERα to ERβ. Therefore, the different effects of E2 observed may be due to variations in cell types, ERα and ERβ ratios, or experimental conditions. The oncogenic transcription factor Forkhead Box M1 (FOXM1) is known to be overexpressed in proliferating and cancerous cells, while its expression is typically low in normal cells [18]. Additionally, FOXM1 has been associated with tumor invasion, cell proliferation, and angiogenesis [19]. In recent years, FOXM1 has also been implicated in oxidative stress. It has been observed that FOXM1 can protect cells with high levels of ROS from senescence and apoptosis, and the absence of FOXM1 makes cells more susceptible to oxidative stress [20]. Notably, a significant increase in ROS inhibits FOXM1 expression in pancreatic and liver cancer cells [21]. Moreover, previous studies have demonstrated the expression of FOXM1 mRNA and protein was regulated by ER-ligands, including estrogen, with FOXM1 regulating ERα in a positive feedback loop [22, 23]. Given the strong correlation between FOXM1 and ERα, it is plausible that E2 has the potential to inhibit oxidative stress-induced apoptosis by regulating the expression of FOXM1 and ERα. To test this hypothesis, we conducted in vitro experiments to investigate the effects of E2 on H_2O_2-induced apoptosis in human endometrial cancer cells. We chose H_2O_2 as a model to induce apoptosis through reactive oxygen species, and our results showed that E2 inhibits oxidative stress-induced apoptosis in endometrial cancer cells.

Materials and Methods

Cell Culture

Ishikawa cells were cultured in a medium containing 10% fetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Beyotime, Nanjing, China) and were incubated at a temperature of 37 °C in an environment with 5% CO_2. To assess the effect of E2 treatment, cells were grown in phenol red-free RPMI medium containing 10% FBS for 24 hours prior to treatment.E2 treatments were initiated when the cell cultures reached confluency, then exposed to E2 at concentrations of 50, 100, and 200 nmol/L for a duration of 24 hours. Following this, the cells were treated with H_2O_2(Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for a period of 4 hours for the purpose of mRNA expression and Western blot assays.

CCK8 assay

Ishikawa cells in 96-well plates were washed twice with PBS, and added 110 µl CCK-8 (Dojindo, Japan) intermixture The cells were then incubated for 2 h at 37 °C in 5% CO_2, and detected at 450 by a microplate reader (Clinibio, Australia).

Assay for Apoptosis

Cultured cells were treated with the AnnexinV-FITC/PI Apoptosis Detection kit (KeyGEN BioTECH, Nanjing, China). The cells were collected and then rinsed twice with cold PBS. They were resuspended in 1× binding buffer at a concentration of 5×10^5 cell/mL. Afterwards, 5 µL of Annexin-V was added. The cells were vortexed and then incubated for 10 minutes in the dark at room temperature. The cells were analyzed using flow cytometry (Miltenyi Biotec in Germany), with excitation and emission wavelengths of 488 nm and 578 nm, respectively.

Measurement of ROS

The measurement of changes in intracellular ROS levels was conducted using a kit called Reactive Oxygen Species assay (Beyotime). The cells were first washed with PBS and then treated with 10 umol/L of a cell-permeable dye known as 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for a duration of 20 minutes at a temperature of 37 °C. Subsequently, the collected cells were subjected to examine through flow cytometry, with excitation occurring at a wavelength of 488 nm and emission at a wavelength of 535 nm (Miltenyi Biotec, Germany). Increased values were observed compared to the control group indicating an increase in intracellular ROS levels.

Western Blot Analysis

Cells were extracted for total protein with extraction buffer (Beyotime). Protein concentration was determined using a BCA protein assay kit (Beyotime). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels at a concentration of 8% were used to separate equal amounts of total protein (45 µg). The separated proteins were then transferred to nitrocellulose membranes and blocked with a

western blocking buffer for 2 hours. Rabbit antibodies were diluted to 1:1000 and incubated with the membranes at 4 °C overnight. The sources of related primary antibodies are as follows. The blots were analyzed and scanned using ImageJ software (Wayne Rasband, USA) and normalized to β-actin Tables 1, 2.

Real-time PCR analysis

RNA was extracted from Ishikawa cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc) and subsequently reverse transcribed using a Quant One-Step RT-PCR kit (Applied Biosystems, Waltham, Massachusetts, USA). The resulting complementary (c)DNA was amplified using primers synthesized by Sangon Biotech (Shanghai, China). The total reaction volume was 10 µl, consisting of 0.5 µl cDNA (1:62), 4.1 µl DNase/RNase-free deionized water, 5 µl SYBR Green I Master, and 0.2 µl of 10 µM of each primer. Amplification and analysis of the reactions were performed using the LightCycler 480 software (Roche Applied Science, Switzerland). The primer sequences used were as follows:

Measurement of LDH leakage and SOD activity

The LDH leakage and SOD activity in the obtained supernatant were assessed using commercially accessible kits (Jiancheng Bioengineering, Nanjing, China) according to the guidelines provided by the manufacturer.

SiRNA transfection

Ishikawa cells cultured in 100-mm plates were transfected with FOXM1 siRNA (5′-GGACCACUUUCCCUACUUU-3′) and control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′), which were produced and provided by Sangon Biotech (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen) was used for the transfection process. After 72 hours of transfection, the cells were gathered for protein extract preparation, ROS measurement, and senescence assay.

Table 1 Primary antibodies of Western blot analysis

Primary antibody (Rabbit)	Source
FOXM1	Cell Signaling Technology, 5436 T
ERα	Abcam, ab32063
ERβ	Abcam, ab133467
β-actin	Bioworld, No. AP0060

Statistics

The statistical analysis was performed using SPSS statistical software (SPSS 16.0). The data were presented as the average ± standard deviation. Statistical disparities between the groups were evaluated through one-way analysis of variance (ANOVA). All measurements were carried out in triplicate. The significance level for statistical analysis was established at $P \le 0.05$.

Results

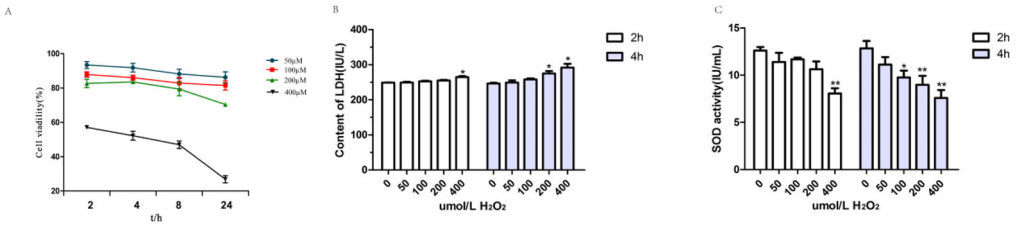
Establishment of an Oxidative Stress Model of Ishikawa cells

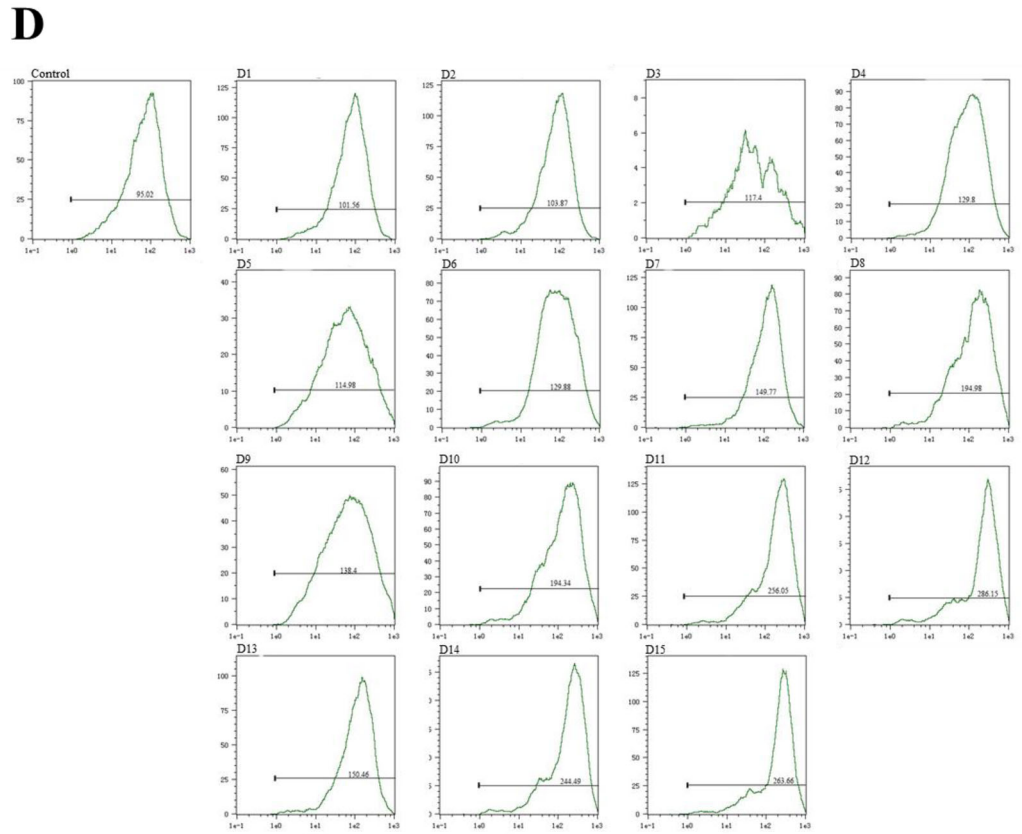
ROS levels are elevated in the majority of cancer cells. As E2 has been documented to counteract the effects of oxidative stress, it is preferable to establish a cellular model of oxidative stress to gain a deeper understanding of this process. The Ishikawa cells were subjected to gradient concentrations of H_2O_2 at different durations. It was discovered that the treatment with 400 µmol/L H_2O_2 resulted in maximum cellular damage among these treatments, all of which exhibited a cell survival rate of at least 70% (Fig. 1A). Subsequently, the levels of intracellular ROS and apoptosis were assessed using oxidative markers and flow cytometry. Intracellular ROS production and the incidence of apoptosis increased with increasing H2O2 concentration and incubation time. Absent from the data is the 400 µmol/L H_2O_2 treatment for 24 hours due to complete cell death (Fig. 1D, E).

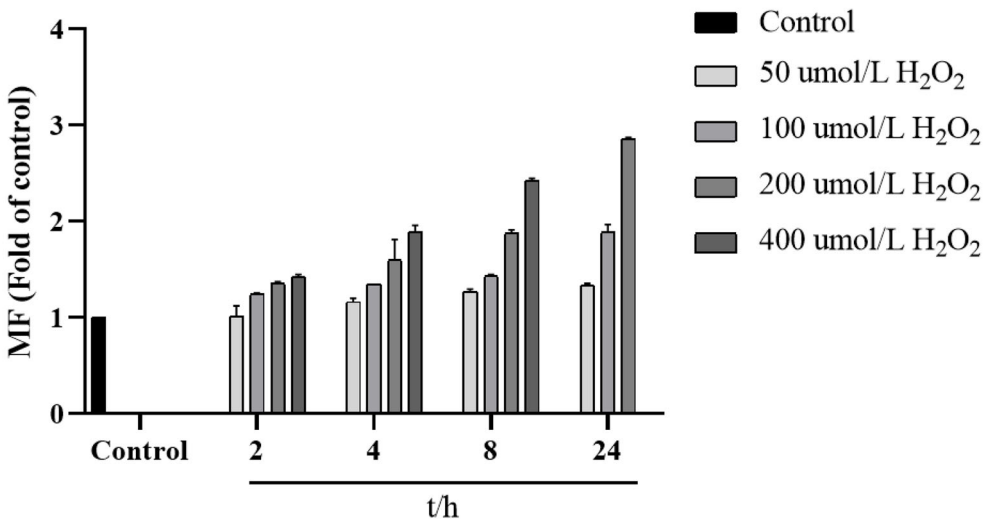
To evaluate the detrimental effects of oxidative stress on cells, LDH leakage, and SOD activity were measured in the collected supernatant. These assays provide an indication of the extent of oxidative damage to the cell membrane and the

Table 2 Primers used of Real-time PCR analysis

The related gene	Forward	Reverse
ERα	5′-TAAATGCTGCCATGTTCCAA-3′	5′-GTGAATGCAAAGGGGTCTGT-3′
ERβ	5′-GAGCTGTTGGATGGAGGTGT-3′	5′-CTTCTACGCATTTCCCCTCA-3′
FOXM1	5′-TGCAGCTAGGGATGTGAATCTTC-3′	5′-GGAGCCCAGTCCATCAGAACT-3′ (reverse)
GAPDH	5′-CCTCCTGCACCACCAACTGCTTAG-3′ (forward)	5′-TTGACGGTTTGGTTTGTGTG-3′







cell's ability to withstand oxidative stress. The results revealed a gradual increase in LDH leakage and a slow decrease in SOD activity (Fig. 1B, C).

These findings suggest that as the concentration of H_2O_2 and the duration of treatment increase, cell viability decreases, whereas intracellular ROS levels and the ratio of

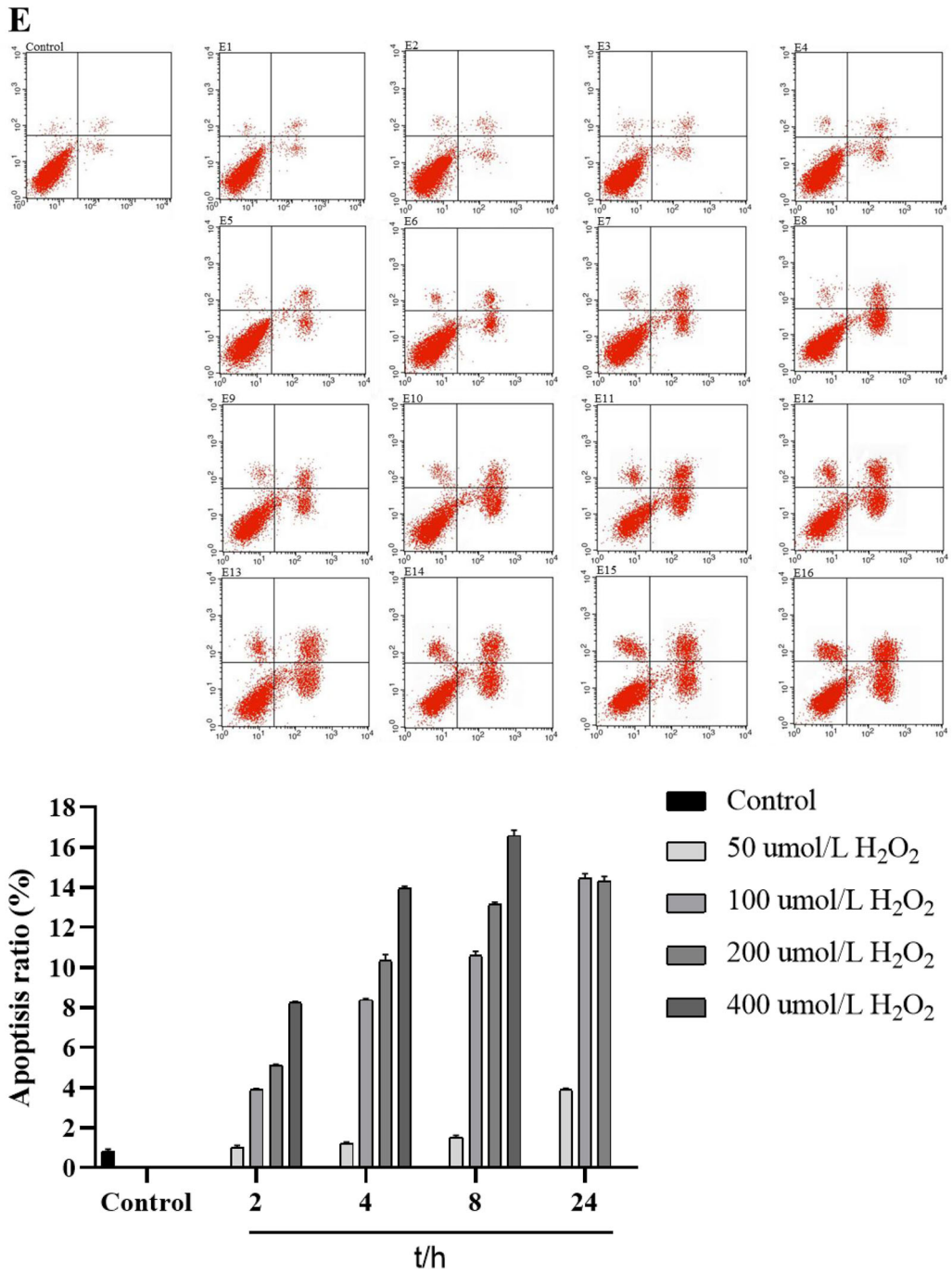


Fig. 1 Establishment of an oxidative stress model of Ishikawa cells. Ishikawa cells were treated with different concentrations (50, 100, 200, 400 μmol/L) of H_2O_2 for different times (2, 4, 8, 24 h). **A** Cell viability was determined by CCK-8 assay. The cell survival rate reached 82.83% in 200 μmol/L of H_2O_2 for 4 h. **B**, **C** LDH leakage and SOD activity were measured by a microplate reader. The data were presented as the mean ± S.D.; $n = 3$; *, **$P < 0.05$, 0.01 respectively, compared with the same time control group. The cells are severely damaged in the 8 and 24 h time point; therefore, we did not report it in the figure. **D** ROS levels were determined by FACS after DCFH-DA treatment. D-1 to D-4: 50 μmol/L H_2O_2 for 2, 4, 8, 24 h; D-5 to D-8: 100 μmol/L H_2O_2 for 2, 4, 8, 24 h; D-9 to D-12: 200 μmol/L H_2O_2 for 2, 4, 8, 24 h; D-13 to D-15: 400 μmol/L H_2O_2 for 2, 4, 8, 24 h. ROS levels were described by mean fluorescence intensity (MFI) and presented as the mean ± S.D.; *, **$P < 0.05$, 0.01, respectively, *vs.* control. **E** Apoptosis was tested by flow cytometry. The group and statistics description are the same as the ROS. ($n = 3$, cell density = $1*10^{6}$/ml)

apoptosis continue to rise. Simultaneously, there is an increase in LDH leakage and a decrease in SOD activity. Following treatment with 200 μmol/L H_2O_2 for 4 hours, there was a significant elevation in intracellular ROS production and the proportion of apoptosis, along with a significant decrease in SOD activity and a notable LDH

leakage. The cell survival rate reached 82.83. In conclusion, the optimal conditions for establishing the Ishikawa oxidative stress model are treatment with 200 μmol/L H_2O_2 for 4 hours.

E2 modulates the expression of FOXM1 protein and mRNA levels

Previous research has demonstrated that E2 modifies oxidative stress and promotes cancer growth [13]. Given the crucial role of FOXM1 in both proliferation and oxidative stress [24], we propose that there may be a correlation between E2 and FOXM1. To investigate whether E2 actively regulates FOXM1, we exposed cell models with oxidative stress to different concentrations of E2 for a duration of 24 hours. Subsequently, the cells were collected for western blotting and RT-PCR analysis. Our findings indicate that E2 increases the expression level of both the mRNA and protein of FOXM1 (Fig. 2A, B). Interestingly, the expression of FOXM1 in the model decreased in comparison to the control. Therefore, we hypothesize that the consumption of FOXM1 by ROS may occur before any changes in gene expression can be rescued. Furthermore, the results indicate a significant increase in ERα levels following E2 activity, while no significant change is observed in ERβ levels (Fig. 2A, B). Consequently, we conclude that E2 regulates the expression of FOXM1 and ERα, and hypothesize that it is plausible that E2 has effects on FOXM1 levels through ERα.

FOXM1 protects cells from oxidative stress-induced apoptosis

Based on the data presented in Fig. 3A, B, it can be observed that the E2-treated oxidative stress model of Ishikawa cells exhibited a significant reduction in the production of ROS and apoptosis ratio as determined through flow cytometry. Hence, it is presumed that FOXM1 plays a significant role in the observed alterations. In order to examine whether FOXM1 controls the intracellular levels of ROS, we conducted siRNA transfection to silence FOXM1 (refer to Fig. 4A). Our findings indicate that the depletion of FOXM1 through siRNA results in heightened levels of ROS in comparison to the control cells (see Fig. 4B). Furthermore, it was observed that the cells without FOXM1 were more prone to cell death induced by 200umol/L H_2O_2 (as indicated in Fig. 4C), suggesting that FOXM1 plays a crucial role in safeguarding cells against oxidative stress, which aligns with a previous study [25]. Collectively, our outcomes demonstrate that FOXM1 regulates the intracellular levels of ROS and contributes to the pathway of cancer transformation that hampers the senescence program due to oxidative stress.

Discussion

The destiny of cancer cells can potentially rely on the levels of reactive oxygen species (ROS). Lower concentrations of ROS could function as signaling messengers to enhance cell growth and replication; nonetheless, excessive levels of ROS can harm proteins, lipids, and DNA, and even trigger apoptosis or necrosis [26]. The dual characteristics of ROS necessitate precise regulation of intracellular ROS levels by the cell. Considerable energy levels are required for cellular replication, and it is vital to maintain stable ROS production and elimination. This also suggests the need for equilibrium in the interplay between cellular metabolism and replication. In this study, we demonstrate that the expression of FOXM1, a suppressor of apoptosis induced by oxidative stress, is amplified by E2.

E2 has a protective function in the brain, skeletal system, cardiovascular system, and reproductive tract [27–29]. However, prolonged exposure to high levels of endogenous estrogen is closely linked to the progression of type I endometrial cancer [30]. Additionally, E2 binds with two estrogen receptor (ER) subtypes, ERα and ERβ, to carry out its actions. It is tempting to speculate that E2 increases the risk of endometrial cancer development and supports the survival of carcinoma cells through the ER. Our findings demonstrate that E2 can enhance the expression of FOXM1 and ERα at both the mRNA and protein levels, but not ERβ. Previous studies by Millour [22] and Madureira [23] have shown a reciprocal regulation between FOXM1 and ERα in breast cancer MCF-7 cells. Additionally, DA Sanders et al. [31] discovered a significant overlap between FOXM1 and ERα binding in MCF-7 cells through ChIP assays. Taking our experiments into account, these findings suggest that FOXM1 expression is crucial for ERα-induced proliferation. However, Yoshiya et al. [32] observed that when ERβ is ectopically expressed in MCF-7 cells, it displaces ERα from the ERE region of the FOXM1 promoter, indicating that ERβ competes with ERα for ERE binding. This may provide a mechanistic explanation for how ERα counteracts ERβ's transcriptional output in the regulation of FOXM1 expression during E2 treatment. To gain a deeper understanding of the interaction between ERα, ERβ, and FOXM1, further investigation is required.

The role of E2 in promoting endometrial cancer has been acknowledged for many years [33]. However, the precise mechanisms through which E2 increases carcinogenesis have remained unclear. Studies conducted by Lisi Yuan et al. [34] suggest that the downregulation of Cu/Zn superoxide dismutase (SOD1) and apurinic endonuclease (Ape1) caused by E2 treatment could lead to the disruption of ROS regulation, ultimately contributing to endometrial carcinogenesis. In our study, we have also found that E2 is involved in the regulation of ROS levels in endometrial

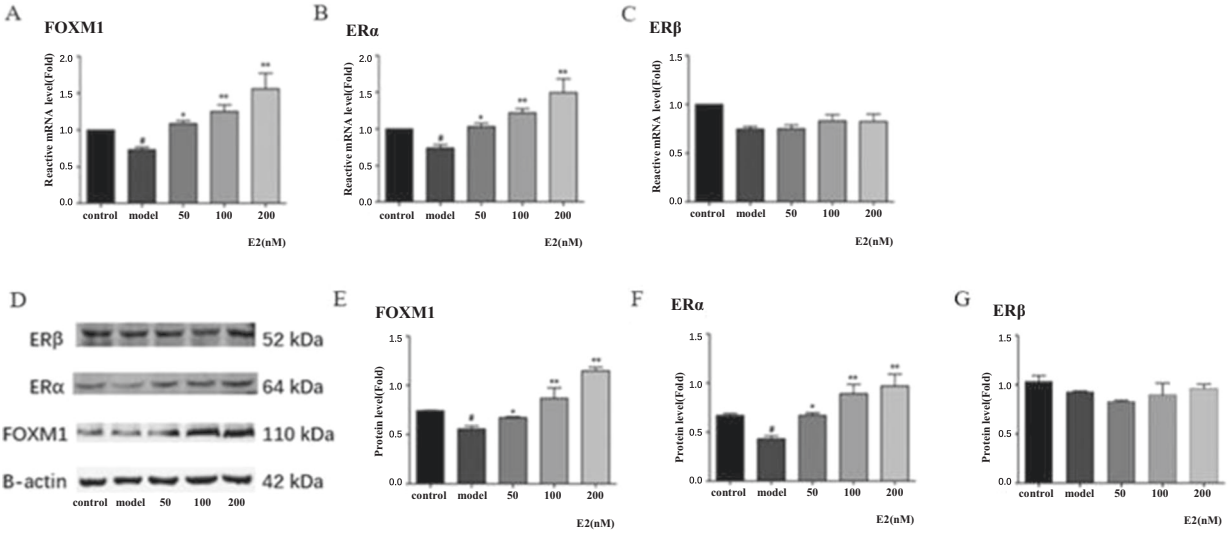


Fig. 2 **A** E_2 modulates the expression of FOXM1 protein and mRNA levels. (A) RNA was isolated from the model cells after being treated with E_2 in different concentrations. cDNA was synthesized, and ERα, ERβ, and FOXM1 mRNA levels were determined using RT-PCR. Each sample was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA present. Compared with the control group, the ERα, and FOXM1 in the model group decreased significantly ($^{\#}P < 0.05$). E_2 induced a significant increase in ERα ($^{*}P < 0.05$, $^{**}P < 0.01$) vs. the model group, FOXM1 ($^{*}P < 0.05$, $^{**}P < 0.01$) vs. model group, but not ERβ ($P > 0.05$ vs. model group). **B** Whole cell extracts were prepared from the model cells treated with E_2 and subjected to quantitative western blot analysis. The ERα and FOXM1 in the model group significantly decreased ($^{\#}P < 0.05$ vs. control group). The levels of ERα ($^{*}P < 0.05$, $^{**}P < 0.01$) *vs* the model group and FOXM1 ($^{*}P < 0.05$, $^{**}P < 0.01$) *vs* the model group protein were significantly different in E_2 treated cells, but not ERβ ($P > 0.05$) vs. the model group. The expression of each protein was normalized to the amount of β-actin present. ($n = 3$, cell density= $1*10^6$/ml)

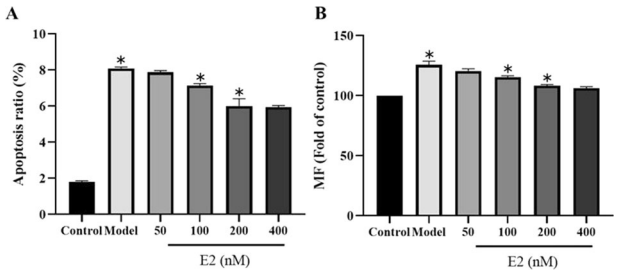


Fig. 3 E_2 reduces ROS production and the apoptosis proportion in Ishikawa cells. **A** There is a significant increase in the model group compared with the control group ($^{*}P < 0.05$). The oxidative stress model of Ishikawa cells was treated with different concentrations E_2, and the apoptosis ratio was significantly decreased in the cells treated with 100, 200 nmol/L E_2. **B** ROS levels were detected by FACS and described by mean fluorescence intensity (MFI). Compared with the control group, the model group showed a significant increase ($^{*}P < 0.05$). The E_2 treated groups treated with 100, 200 nmol/L E_2 were significantly decreased ($^{*}P < 0.05$) *vs* the model group. ($n = 3$, cell density= $1*10^6$/ml)

cancer Ishikawa cells. In the research conducted by Sugioka et al. [35], it was discovered that E2 inhibits the peroxidation of membrane phospholipids and acts as an antioxidant. FOXM1, which acts as a link between proliferation and metabolism, appears to inhibit ROS-induced apoptosis based on our findings. Li et al. [25] have proposed that FOXM1 prevents premature senescence by stimulating the expression of Bmi1. Furthermore, the results of Park et al. [20] have demonstrated that FOXM1 stimulates the expression of detoxifying enzymes, such as catalase, manganese superoxide dismutase (MnSOD), and peroxiredoxin 3 (PRDX3), to regulate intracellular ROS levels in a negative manner. However, the levels of intracellular ROS and antioxidant enzyme expression, which are regulated by E2 or FOXM1, may be influenced by the cellular type and the ratio of ERα/Erβ [17, 36–38]. Nevertheless, further investigation is necessary using different endometrial cancer cell models.

Our research findings indicate that E2 plays a crucial role in safeguarding cells against apoptosis caused by oxidative stress, through the regulation of FOXM1. This suggests that E2 can be beneficial for the progression of endometrial cancer. Conversely, increased levels of ROS suggest that cancer cells become more susceptible to oxidative stress, and the manipulation of such vulnerabilities can be utilized to selectively eliminate cancer cells by intensifying oxidative stress [39]. Carr JR et al. [40] conducted a study revealing that FOXM1 contributes to the resistance against Herceptin and paclitaxel in the treatment of breast cancer. Previous studies have demonstrated that the combined impact of oxidative stress and FOXM1 inhibitors induces apoptosis in cancer cells [41]. In addition, proteasome inhibitors that inhibit FOXM1 have emerged as promising approaches in cancer treatment [42]. Consequently, FOXM1 may potentially be identified as a novel target in the clinical management of tumors in the future.

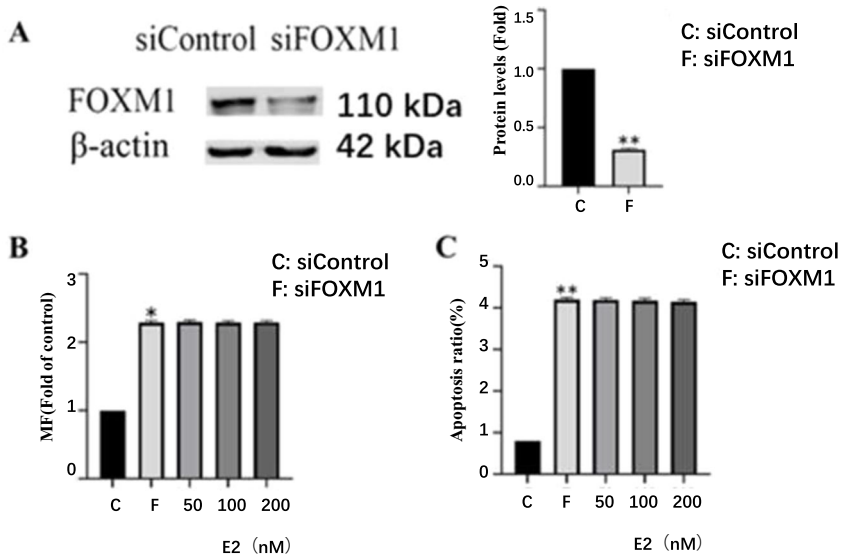


Fig. 4 FOXM1 protects cells from oxidative stress. Ishikawa cells were transfected with control siRNA or FOXM1 siRNA (50 nM) for 72 h and treated by 200 umol/L H_2O_2. **A** Cell extracts (60 μg) were prepared for western blot to analyze FOXM1 knockdown efficiency. The expression levels of FOXM1 were significantly decreased *vs* control (**$P < 0.01$). **B** Intracellular ROS levels were measured using DCFH-DA fluorescence probes. The data are displayed as the mean ± S.D. of triplicates (*$P < 0.01$) compared with control siRNA-transfected cells. **C** Apoptosis ratios were tested by AnnexinV-FITC/PI. The data are displayed as the mean±S.D. (**$P < 0.01$). ($n = 3$, cell density = 1*10 ^{6}/ml)

Author contributions G.X. and J.A.W. wrote the main manuscript text and X.J.M. and M.H.X. prepared figures 1-4. All authors reviewed the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

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