

Reactive oxygen species regulate FSH-induced expression of vascular endothelial growth factor via Nrf2 and HIF1 α signaling in human epithelial ovarian cancer

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Abstract. Follicle-stimulating hormone (FSH) and the FSH receptor contribute to tumor angiogenesis and are acknowledged risk factors for ovarian epithelial cancer (OEC). Accumulating evidence suggests that FSH can induce vascular endothelial growth factor (VEGF) and hypoxia inducible factor 1 α (HIF1 α) expression. We previously demonstrated that FSH induces reactive oxygen species (ROS) production and activates Nrf2 signaling. This study was performed to investigate whether FSH induces VEGF expression via a ROS-mediated Nrf2 signaling pathway. In the current study, OET cells were treated with FSH; dichlorofluorescein staining was used to determine ROS generation, western blotting was used to quantify Nrf2 expression and VEGF expression was measured using an ELISA. *Nrf2* and *HIF1 α* were knocked down using siRNAs to investigate the role of the Nrf2 and HIF1 α signaling pathways in FSH-induced VEGF expression. The chromatin immunoprecipitation assay (ChIP) was used to determine HIF1 α binding to the *VEGF* promoter. Finally, it was found that FSH induced ROS production and activated Nrf2 signaling; elimination of ROS or knockdown of *Nrf2* blocked FSH-induced VEGF expression. Knockdown of *Nrf2* impaired

HIF1 α signaling activation. Blockage of the FSH-ROS-Nrf2-HIF1 α signaling pathway attenuated FSH-induced binding of HIF1 α to the *VEGF* promoter. Collectively, this study indicates that ROS and aberrant expression of Nrf2 play an important role in FSH-induced angiogenesis in OEC, and provides insight into the mechanisms of FSH-induced VEGF expression. Elimination of ROS or inhibition of Nrf2 may represent potential therapeutic targets for the treatment of ovarian cancer.

Introduction

Ovarian cancer is the most lethal gynecological malignancy. Approximately 80-90% of ovarian cancers originate from the ovarian surface epithelium. The etiology of ovarian epithelial cancer (OEC) is not yet fully clarified; currently, the gonadotropin theory of ovarian cancer proposes that elevated serum gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) contribute significantly to the development of ovarian cancer. Wang *et al* reported that vascular endothelial growth factor (VEGF) plays a role in the development of ovarian cancer, and that elevated gonadotropin levels, as found in menopausal women and most ovarian cancer patients after surgery, could accelerate tumor growth and tumor recurrence by inducing expression of VEGF in OECs (1). However, the detailed molecular mechanisms by which FSH leads to expression of VEGF in OET remain unclear.

Increasing evidence supports the hypothesis that reactive oxygen species (ROS) are involved in the expression and regulation of VEGF and angiogenesis (2-6), and conversely, multiple ROS-mediated cellular functions can be induced by growth factors and hormones (7-10). It has been reported that estrogen-induced ROS-mediated signaling is involved in the development of breast cancer (11). In addition, LH-induced ROS generation contributes to ovulation (12). However, it is not clear whether FSH can induce ROS generation and in turn contribute to FSH-induced VEGF expression.

Cells have developed a variety of protective mechanisms in response to oxidative stress to escape ROS-mediated damage. Activated Nrf2 binds to the antioxidant-response element (ARE) leading to the upregulation of a large number of anti-

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oxidant genes (13-18). In a previous study, we observed that Nrf2 was overexpressed in ovarian epithelial carcinoma, and confirmed that FSH could induce the expression of Nrf2 in OEC cells, suggesting that activation of Nrf2 signaling contributes to the development of OEC (34). Kim *et al.* reported that Nrf2 blockade suppresses angiogenesis in colon cancer by inhibiting hypoxia-induced activation of hypoxia-inducible factor 1 α (HIF1 α) (19), implying that HIF1 α signaling is regulated by Nrf2, and suggesting that both HIF1 α and Nrf2 signaling may regulate the expression of VEGF and cancer angiogenesis.

Aberrant activation of HIF1 α signaling induces expression of VEGF in cancer (20-22). High levels of HIF1 α expression are observed in several types of cancer and correlate with a poor prognosis. We previously confirmed that FSH induced the expression of HIF1 α in ovarian cancer cells (23) and Lee *et al.* revealed that lysophosphatidic acid (LPA) induced the binding of HIF1 α to the VEGF promoter in cancer cells (24). However, it remains to be clarified how FSH-induced HIF1 α activation and VEGF expression occurs in OEC cells. Therefore, in this study, we investigated whether ROS regulate FSH-induced expression of VEGF via Nrf2 and HIF1 α signaling in OEC cells.

Materials and methods

Reagents and antibodies. Human FSH, dichlorofluorescein (DCF) and N-acetyl cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000, DMEM/F12 medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). Anti-Nrf2, GAPDH and HIF1 α primary antibodies were purchased from Abcam (Cambridge, UK).

Cell lines and cell culture. Human ES2 (clear cell adenocarcinoma) and Hey (papillary cystadenocarcinoma) cell lines were obtained from the American Culture Collection (Manassas, VA, USA) and were cultured in 1:1 DMEM/F12 supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum at 37°C in a humidified incubator containing 95% room air and 5% CO₂.

ROS detection. ES2 and Hey cells were seeded in 6-well plates and incubated for 24 h. The culture medium was replaced with OPTI-MEM and the cells were incubated for 24 h. The cells were then treated with 40 mIU/ml FSH for 20 min or 6 h, incubated with 10 μ g/ml DCF for 30 min, and washed three times with PBS, fixed and imaged using a fluorescence microscope.

Western blotting. Western blotting was performed in a routine manner. Briefly, 60 μ g protein samples was loaded on 10% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes, incubated with specific primary antibodies at 4°C overnight, and incubated with the appropriate secondary antibody for 1 h at room temperature. The bands were visualized using the ECL Plus system (Amersham, GE Healthcare; Chalfont St. Giles, UK).

ELISA assay. To investigate the effect of NAC on FSH-induced VEGF expression, Hey cells were pretreated with different concentrations of NAC (10, 50, 100 μ M) for 30 min, and then treated with 40 mIU/ml FSH for 48 h and the cell media were

collected. To determine the effect of blocking ROS signaling and knockdown of Nrf2 or HIF1 α on FSH-induced VEGF expression, Hey cells were treated with NAC in the presence or absence of siNrf2 or siHIF1 α for the indicated times, and then the cell media were harvested. VEGF protein concentration was measured using an ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Chromatin immunoprecipitation assay. A human HIF-1 α chromatin immunoprecipitation (ChIP) assay was performed using a kit purchased from R&D Systems, according to the protocol recommended by the manufacturer. Hey cells were treated as indicated in the figure legends, and then the human VEGF promoter was amplified using the primers: forward, 5'-CCTCAGTTCCTGGCAACATCTG-3' and reverse, 5'-GAAGAATTTGGCACCAAGTTTGT-3'. The amplification products were examined on 2% agarose gels using ethidium bromide staining.

RNA interference. Small interfering RNAs (siRNAs) against HIF-1 α and Nrf2 were designed and synthesized by Dharmacon Thermo Scientific (Waltham, MA, USA). Hey cells were seeded in 6-well plates, cultured to 50% confluence and then serum starved for 24 h. The cells were transiently transfected with siRNA using DharmaFECT transfection reagents according to the manufacturer's instructions, treated with 40 mIU/ml FSH or NAC for 48 h, and the protein expression levels of downstream target genes were determined by western blotting or using an ELISA.

Statistical analysis. Data are presented as the mean \pm standard deviation (SD). Statistical significance was assessed using the Student's t-test or one-way ANOVA with SPSS 11.5 software (SPSS, Chicago, IL, USA); P-values <0.05 were considered significant.

Results

FSH stimulates ROS generation in ovarian epithelial cancer cells. Increasing evidence supports the importance of ROS as secondary messengers in a variety of cellular functions (25-30). For example, ROS, particularly H₂O₂, can mimic LH-induced ovulation (12). To investigate the potential involvement of Nrf2 in FSH-induced VEGF expression, Hey and ES2 ovarian epithelial cancer cells were treated with 40 mIU/ml FSH for 20 min or 6 h. As shown in Fig. 1, FSH potently induced ROS production in both Hey and ES2 cell lines. These observations suggest that ROS play a role in FSH-induced cellular function.

ROS generation is required for FSH-induced Nrf2 signaling. Nrf2 is one of the most important cellular defense mechanisms against oxidative stress. Our observation of increased ROS production in FSH-treated cells prompted us to investigate whether Nrf2 is also involved in FSH-induced ovarian cellular function. As expected, FSH treatment induced Nrf2 expression in a dose-dependent manner (Fig. 2A). The peak Nrf2 expression level was observed in Hey and ES2 cells exposed to 40 mIU/ml FSH for 48 h (Fig. 2B).

To confirm that ROS production is required for FSH-induced Nrf2 signaling, we pretreated ovarian cancer cells with the

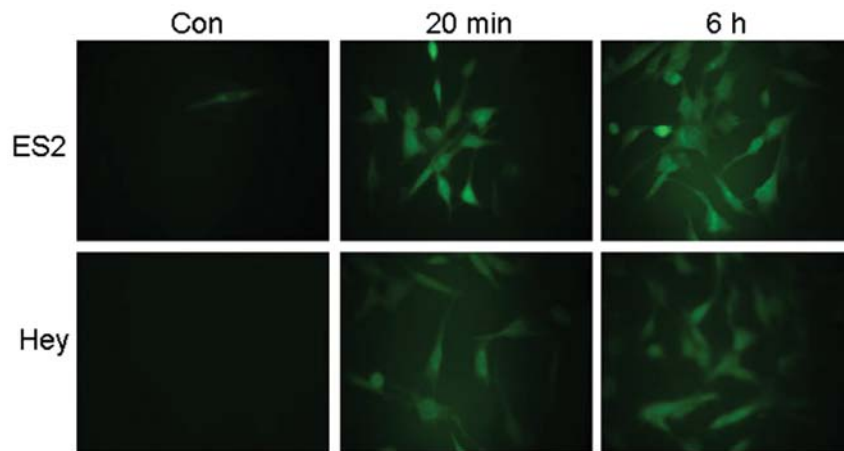


Figure 1. FSH treatment induces ROS production in OET cells. ES2 and Hey cells were seeded in 6-well plates, serum starved for 24 h, treated with 40 mIU/ml FSH for 20 min or 6 h, incubated with dichlorofluorescein. Fluorescent images are shown.

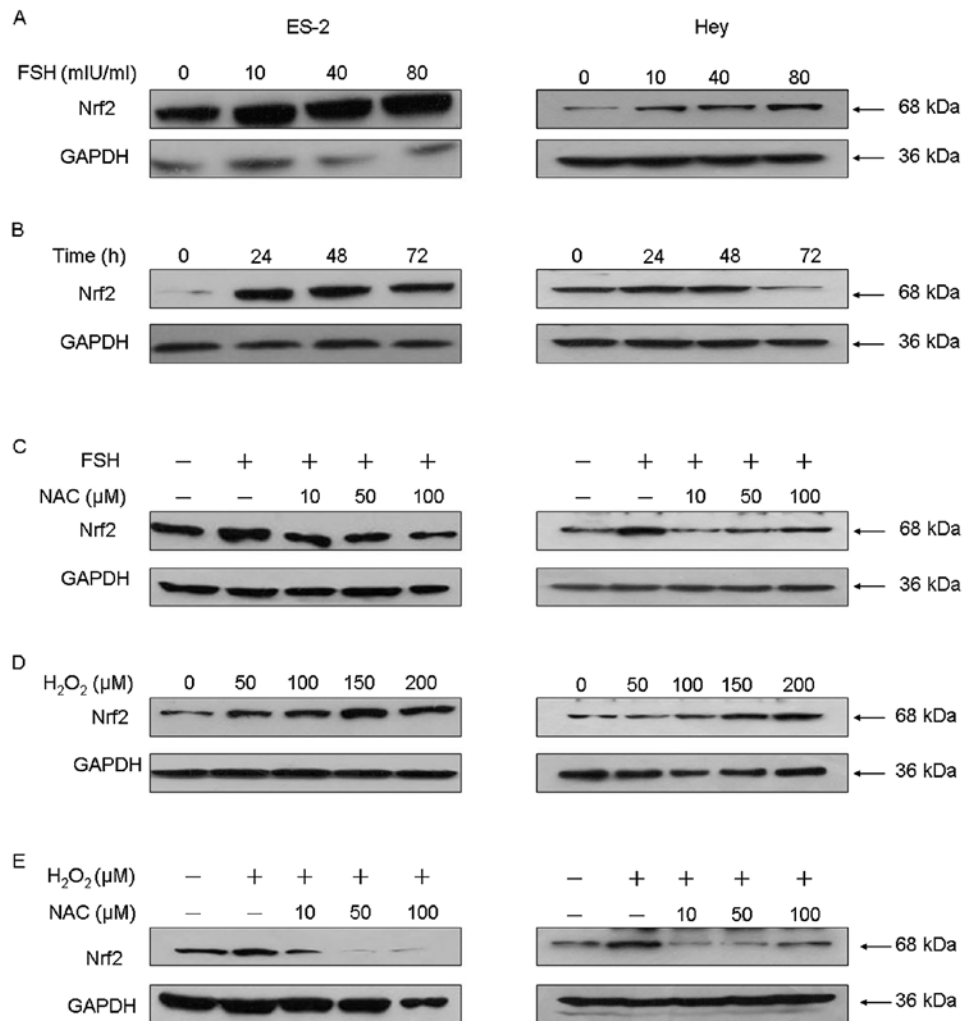


Figure 2. ROS production is necessary for FSH-induced Nrf2 expression in OET cells. Western blot assay of Nrf2 expression in ES2 and Hey cells treated with (A) various doses of FSH for 48 h or (B) 40 mIU/ml FSH for different periods of time. (C) NAC attenuates FSH-induced expression of Nrf2. Western blot analysis of Nrf2 protein expression in ES2 and Hey cells pretreated with the indicated concentrations of NAC for 30 min, and then treated with 40 mIU/ml FSH for 48 h. (D) H₂O₂ induces expression of Nrf2. Western blot analysis of Nrf2 protein expression in serum-starved ES2 and Hey cells treated with H₂O₂ (0, 50, 100, 150, 200 μ M) for 48 h. (E) NAC attenuates H₂O₂-induced expression of Nrf2. Western blot analysis of Nrf2 protein expression in ES2 and Hey cells pretreated with the indicated concentrations of NAC for 30 min, and then treated with 150 μ M H₂O₂ for 48 h.

broad-range ROS scavenger N-acetyl cysteine (NAC) at various concentrations for 30 min, and then treated the cells with

40 mIU/ml FSH for 48 h. As shown in Fig. 2C, NAC significantly and dose-dependently attenuated the ability of FSH to

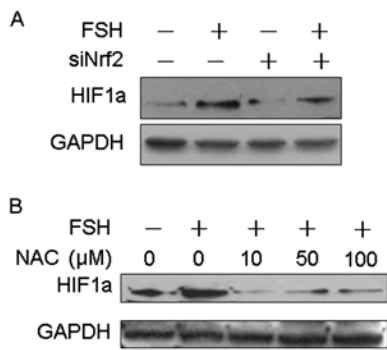


Figure 3. FSH-induced HIF1α expression is dependent on ROS generation and Nrf2 signaling activation. (A) Knockdown of Nrf2 blocks FSH-induced HIF1α expression. Western blot analysis of HIF1α protein expression in serum-starved Hey cells transfected with Nrf2 siRNA (siNrf2) and then treated with 40 mIU/ml FSH for 48 h. (B) NAC attenuates FSH-induced HIF1α expression. Western blot analysis of HIF1α protein expression in Hey cells pretreated with NAC for 30 min, and then treated with 40 mIU/ml FSH for 48 h.

induce Nrf2 protein expression. As expected, H₂O₂ treatment also effectively induced Nrf2 expression in a dose-dependent manner (Fig. 2D), mimicking the effect of FSH; this effect was also inhibited by pretreatment with NAC (Fig. 2E). Collectively, these results suggest that ROS, especially H₂O₂, are required for FSH-induced activation of Nrf2 signaling.

FSH-induced HIF1α expression requires ROS generation and Nrf2 signaling activation. HIF1α accumulation is necessary for the induction of VEGF expression, and our previous research demonstrated that HIF1α is involved in FSH-induced VEGF expression. Therefore, we hypothesized that ROS production and activation of Nrf2 are necessary for FSH-induced HIF1α expression. To address this, *Nrf2* was knocked down using *Nrf2*-specific siRNA. Knockdown of *Nrf2* reduced the expression of HIF1α and abolished FSH-induced HIF1α expression, compared to control siRNA-transfected cells (Fig. 3A). To further confirm the role of ROS as a mediator in FSH-induced HIF1α expression, the cells were pretreated with various doses of NAC prior to treatment with 40 mIU/ml FSH for 48 h. FSH-induced HIF1α expression was considerably attenuated by NAC, suggesting that ROS play a role in FSH-induced HIF1α expression (Fig. 3B).

FSH induces VEGF expression through ROS production and activation of Nrf2-HIF1α signaling. As demonstrated in Fig. 4A, treatment of Hey cells with FSH dramatically enhanced the expression of VEGF; this effect was attenuated in a dose-dependent manner by pretreatment of the cells with NAC. Furthermore, siRNA-mediated knockdown of *Nrf2* decreased FSH-induced VEGF expression in a dose-dependent manner (Fig. 4B). ELISA assays were performed to investigate the effect of combined NAC pretreatment and knockdown of

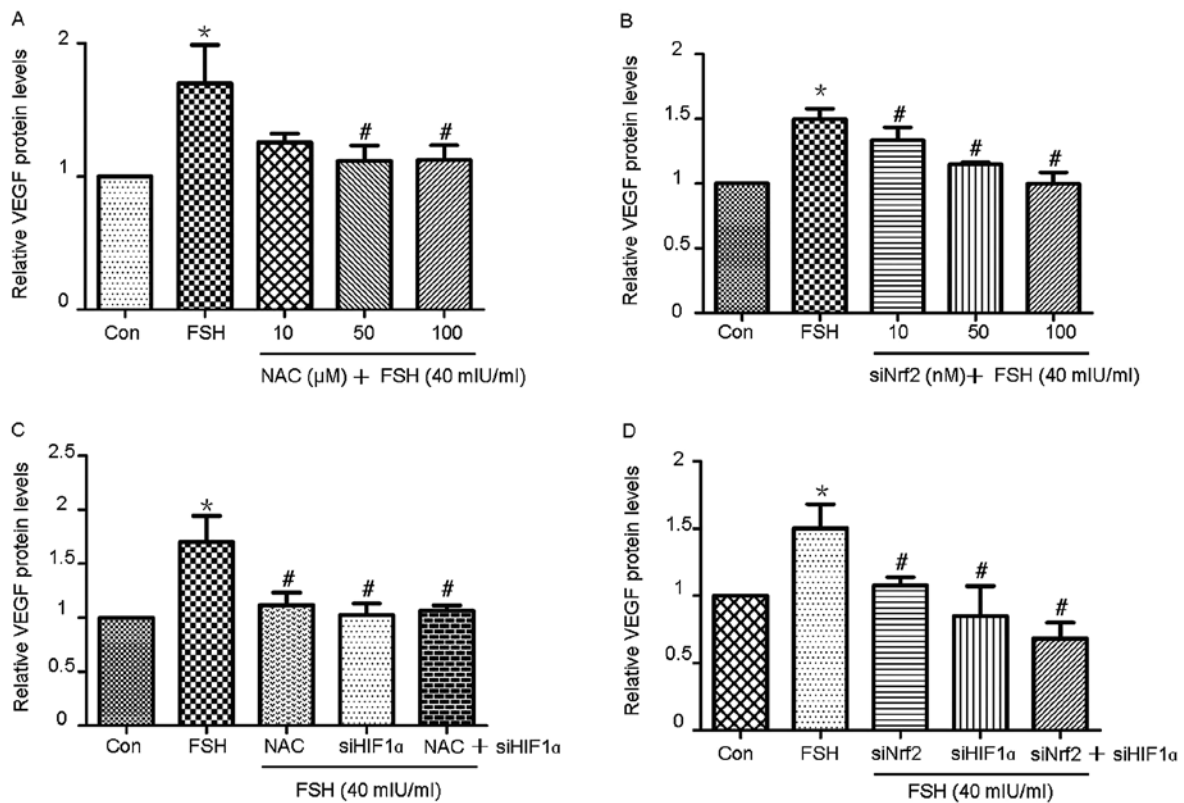


Figure 4. FSH-induced expression of VEGF requires ROS-mediated activation of the Nrf2 and HIF1α signaling pathways. (A) NAC attenuates FSH-induced VEGF expression. ELISA of VEGF protein expression in Hey cells pretreated with NAC for 30 min, and then treated with 40 mIU/ml FSH for 48 h. (B) Knockdown of Nrf2 attenuates FSH-induced VEGF expression. ELISA of VEGF protein expression in serum-starved Hey cells transfected with Nrf2 siRNA (siNrf2) and then treated with 40 mIU/ml FSH for 48 h. (C) Combined NAC pretreatment and knockdown of HIF1α attenuate FSH-induced VEGF expression. ELISA of VEGF protein expression in Hey cells pretreated with NAC and/or transfected with HIF1α siRNA (siHIF1α), and then treated with 40 mIU/ml FSH for 48 h. (D) Double knockdown of HIF1α and Nrf2 attenuate FSH-induced VEGF expression. ELISA of VEGF protein expression in Hey cells transfected with siNrf2 and/or siHIF1α and then treated with 40 mIU/ml FSH for 48 h. Each experiment was repeated three times, *P<0.05 vs. control group; #P<0.05 vs. FSH-treated groups.

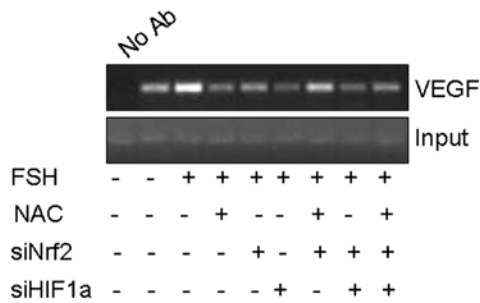


Figure 5. FSH increases the ability of HIF1 α to bind the VEGF promoter. ChIP assay for HIF1 α . Serum-starved Hey cells were treated as indicated, then the immunoprecipitated DNA was purified and the VEGF promoter was amplified by PCR.

HIF1 α and/or Nrf2 on FSH-induced VEGF expression. NAC pretreatment, knockdown of HIF1 α or knockdown of Nrf2 alone potentially attenuated FSH-induced VEGF expression. Combined NAC pretreatment and knockdown of HIF1 α did not lead to a significant reduction in FSH-induced VEGF expression, compared to pretreatment with NAC or knockdown of HIF1 α alone (Fig. 4C). However, double knockdown of HIF1 α and Nrf2 significantly inhibited FSH-induced VEGF expression, compared to pretreatment with FSH, knockdown of HIF1 α or knockdown of Nrf2 alone (Fig. 4D).

FSH enhances binding of HIF1 α to the VEGF promoter. To further investigate the role of HIF1 α in FSH-induced VEGF expression, we explored the ability of FSH to affect the interaction of HIF1 α with the native hypoxia response element of the VEGF promoter using the chromatin immunoprecipitation assay (ChIP). As shown in Fig. 5, HIF1 α bound to the VEGF promoter could be precipitated using an anti-HIF1 α antibody. Treatment of Hey cells with 40 mIU/ml FSH significantly enhanced the ability of HIF1 α to bind the VEGF promoter; however, treatment with NAC, siNrf2 or siHIF1 α alone or in combination blocked FSH-induced binding of HIF1 α to the VEGF promoter.

Discussion

In the present study, our *in vitro* studies and molecular analyses provided evidence that ROS are necessary for FSH-induced VEGF expression in ovarian cancer, as ablation of ROS or knockdown of Nrf2 attenuated FSH-induced VEGF expression. Our data also demonstrated that the Nrf2 signaling pathway is involved in FSH-induced cellular function, and that FSH enhances the ability of HIF1 α to bind the VEGF promoter. Increased FSH levels are a significant risk factor for the development of ovarian cancer. Our previous study demonstrated that activation of the PI3K/AKT pathway mediated FSH-stimulated VEGF expression in ovarian serous cystadenocarcinoma (23). That study also indicates that HIF1 α is involved in FSH-induced VEGF expression.

ROS production was observed in OEC cells treated with FSH. ROS, such as hydrogen peroxide, the hydroxyl radical and superoxide anion radical are produced in measurable quantities by every aerobic system, and are considered to be toxic to living cells in high concentrations. Oxyradicals can

act as important secondary messengers to regulate a variety of cellular functions. For example, estrogen-induced ROS production contributes to the development of breast cancer (11) and ROS can mimic LH-induced ovulation (12). In agreement with these reports which indicate that ROS mediated-signaling contributes to hormone-induced cellular function, our results clearly demonstrate that ROS are involved in FSH-induced VEGF expression. FSH-induced binding of HIF1 α to the VEGF promoter and FSH-induced VEGF expression were attenuated by the antioxidant supplement NAC (Figs. 4A and 5), confirming that ROS are necessary for FSH-induced VEGF expression.

The transcription factor Nrf2 regulates the cellular antioxidant response which protects cells from various insults (17) and facilitates cell survival by inducing intracellular antioxidants, phase II detoxifying enzymes and other molecules that detoxify xenobiotics and neutralize ROS (31-33). In a previous study, we confirmed that Nrf2 was overexpressed in ovarian cancer tissues (34). In agreement with our previous study (34), the present study confirmed that FSH upregulated the expression of Nrf2 in ovarian cancer cells in a dose- and time-dependent manner (Fig. 2A and B). Moreover, H₂O₂ treatment mimicked FSH-induced Nrf2 expression; this effect was abolished by NAC (Fig. 2C-E). Moreover, knockdown of Nrf2 impaired FSH-induced VEGF expression and reduced the ability of HIF1 α to bind the VEGF promoter (Figs. 4B and 5). These data imply that Nrf2 plays a critical role in ROS-mediated FSH-induced VEGF expression. Nrf2 normally exerts a protective role when oxyradicals are present, which raises the question of how the Nrf2-mediated antioxidant response fails to protect ovarian cells in patients with ovarian cancer. We suggest that Nrf2 signaling can easily eliminate FSH-induced ROS generation and prevent ROS-induced damage in the early stages of ovarian epithelial cancer.

According to the gonadotropin theory, persistent stimulation with high concentrations of FSH contributes to the progression of ovarian epithelial cancer. This stimulation may induce persistent ROS generation; therefore, the Nrf2-mediated protective mechanism may become saturated by excessive ROS, resulting in the development of ovarian epithelial cancer. Our data indicate that aberrant activation of Nrf2 in a highly oxidizing environment may facilitate angiogenesis and tumor cell survival in ovarian epithelial cancer.

In a previous study, we demonstrated that FSH regulated the expression of HIF1 α in a dose-dependent manner (23). In the present study, we showed that knockdown of Nrf2 impaired FSH-induced HIF1 α expression (Fig. 3A). In addition, elimination of ROS using the antioxidant NAC also abolished FSH-induced HIF1 α expression (Fig. 3B). These data suggest that ROS and Nrf2 signaling are involved in the regulation of HIF1 α expression. Zhou *et al* reported that HIF1 α is indispensable during insulin-induced VEGF transcriptional activation (35). Another study demonstrated that Nrf2-deficient colon cancer cells failed to accumulate HIF1 α protein, which limited the expression of VEGF and other HIF1 α target genes (19). Our research highlights Nrf2 as a potential candidate molecular target for the control of tumor angiogenesis, as inhibition of Nrf2 may block HIF1 α signaling (19). Blockage of ROS using NAC, knockdown of HIF1 α or knockdown of Nrf2 attenuated FSH-induced binding of HIF1 α to the VEGF

promoter. However, knockdown of *HIF1 α* combined with NAC and/or knockdown of *Nrf2* to a more significant reduction in VEGF expression (Fig. 4C and D), indicating that Nrf2/HIF1 α signaling is involved in FSH-induced VEGF expression. Most importantly, FSH induced the binding of HIF1 α to the *VEGF* promoter, which explains why depletion of *HIF1 α* abolished the expression of VEGF in our previous study (23).

In summary, this study suggests that FSH induces ROS generation, which activates Nrf2 signaling, which in turn triggers HIF1 α signaling and promotes the binding of HIF1 α to the VEGF promoter, which facilitates ovarian epithelial cancer progression. Prevention of ROS accumulation and targeting of the Nrf2/HIF1 α signaling pathway may represent potential strategies to prevent the development of ovarian epithelial cancer.

Acknowledgements

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