NRF2 is overexpressed in ovarian epithelial carcinoma and is regulated by gonadotrophin and sex-steroid hormones

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Abstract. Aberrant nuclear factor-erythroid 2 (NRF2) expression correlates with tumor development. We investigated NRF2 expression in ovarian epithelial carcinoma (OEC), aiming to identify associations with clinicopathological factors, hormones and induced reactive oxygen species (ROS). Immunohistochemical staining for NRF2 expression was performed on 10 benign cystadenomas, 4 boderline tumors and 64 OECs. Western blotting was used to determine the effects of hormones on NRF2 expression in OEC. NRF2 protein was found to be overexpressed in OEC. Gonadotrophins and estrogen induced ROS production in OEC; these hormones also enhanced NRF2 expression. Therefore, aberrant expression may be induced by hormones through ROS signaling. Increased NRF2 expression may be a molecular event in OEC carcinogenesis.

Introduction

Ovarian cancer is the most complicated malignancy of the female reproductive system, and is the fourth most frequent cause of cancer-related death in women. The mortality rate from ovarian cancer is high as a result of occult metastases within the peritoneal cavity and the typically advanced stage at detection, when curative therapy is ineffective. Over 90% of ovarian cancers are thought to arise in the ovarian surface epithelium. Tumorigenesis has been associated with inflammation, incessant ovulation and persistent stimulation

from high-level gonadotrophins and sex-steroid hormones. Among these etiologies of ovarian epithelial cancer (OEC), hormone-induced tumorigenesis is a popular hypothesis. A growing body of evidence shows a clear trend of decreasing risk with increasing numbers of pregnancies, with the extent of protection being related to the length of the pregnancy and the duration of breast-feeding. There is also a clear decrease in risk with increasing durations of oral contraceptive (OC) use. These protective factors are linked to cyclic hormonal changes. However, the details of the molecular mechanism(s) of these protective effects are not clear.

Recent studies suggest that reactive oxygen species (ROS) play an important role in tumorigenesis. ROS feature in normal cellular function and biological behaviour, including ovulation. However, increased ROS stress is observed in the cancer microenvironment, and excess ROS have been detected in various cancer types. It may be the case that optimal levels of ROS are required for carrying out biological functions, but that excess ROS result in nucleic acid damage, aging, inflammation and cancer. However, whether ROS are involved in OEC development and whether gonadotrophin and sex-steroid hormones could induce ROS remains to be determined.

In response to the harmful effects of excess ROS, cells have developed a system to prevent ROS accumulation in which antioxidant proteins and phase II detoxifying enzymes are activated. The antioxidant genes are coordinately regulated through the antioxidant response element (ARE) in their promoters and the transcription factor nuclear factor-erythroid 2 (NRF2) is the master regulator of ARE-driven genes. Normal levels of NRF2 are kept low by its constant degradation through ubiquitination by Kelch-like ECH-associated protein 1 (KEAP1), a negative regulator of NRF2. Under high ROS levels, NRF2 is liberated from KEAP1 and transactivates ARE-driven gene expression. A recent study indicated that high NRF2 expression levels in gallbladder adenocarcinoma (GC) was associated with shorter overall survival times and concluded that NRF2 expression may be an important factor in identifying a poor prognostic group of GC (1). Jiang *et al* reported that type II endometrial cancer had elevated NRF2 expression, and that high levels of NRF2 resulted in chemoresistance (2). Previous research on the role of NRF2 in the pathogenesis of OEC has focused largely on chemoresistance, and few studies have addressed the relationship

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between NRF2 expression and OEC clinicopathological factors or prognosis, or its association with hormones. The aims of the present study are therefore to investigate NRF2 expression in OEC tissues, to identify its association with clinicopathological factors, and to further determine whether this is related to gonadatrophin exposure and sex-steroid hormone-induced ROS.

Materials and methods

Selection of tissues. Seventy-eight tissue samples were studied, with 10 benign cystadenomas used as controls. Sixty-four cases were OEC, including 11 clear cell carcinomas, 9 endometrioid adenocarcinomas, 6 mucinous cystadenocarcinomas and 38 serous cystadenocarcinomas, while the remaining 4 were cases of borderline tumor. The samples were obtained from the Department of Obstetrics and Gynecology of the Shanghai First People's Hospital, affiliated to Shanghai Jiao Tong University. Patients were diagnosed with OEC and had undergone total abdominal hysterectomy and bilateral salpingo-oophorectomy with retroperitoneal lymphadenectomy (radical pelvic and para-aortic lymphadenectomy was performed in all patients), followed by adjuvant chemotherapy with carboplatin/paclitaxel, with residual tumor <2 cm. None of the patients had received any pre-operative therapy, such as radiotherapy or chemotherapy, before the ovarian cancer tissue sample was taken during surgery. Patient age ranged from 22 to 79 years (median, 54 years). The clinical stage distribution of the OEC cases was as follows: 13 cases at stage I, 16 cases at stage II and 35 cases at stage III. Lymph node metastasis was found in 31 OEC cases. All tissues harvested included 13 cases of grade I, 18 cases of grade II and 22 cases of grade III tumors; clear cell carcinoma was excluded. The staging and grading of tumors were determined in accordance with the International Federation of Gynecology and Obstetrics (FIGO). Pathological diagnoses of ovarian lesions were made by two gynecological pathologists on the basis of WHO classification in a double-blinded manner.

Tissue processing. All the collected ovarian lesion samples were fixed in 10% buffered formalin and processed using standard methods for paraffin embedding. The samples were cut as 5-μm sections for immunohistochemistry and placed on positively charged glass slides. Histological diagnoses were confirmed by review of the original H&E-stained tissue sections. Approval for the use of these tissues for research purposes was obtained from the Ethics Committee of the Medical College, Shanghai Jiao Tong University, China and written consent was obtained from all patients.

Immunohistochemical staining. Detection of NRF2 expression was performed using a rabbit polyclonal IgG raised against human NRF2. The paraffin sections were dried at 60°C for 2 h, then deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. Endogenous peroxidase activity was blocked by a 10-min treatment with 3.0% hydrogen peroxide. Slides were then placed in citrate buffer (pH 6.0) and brought to boiling for 10 min in two 5-min sessions in a microwave. They were then incubated for 30 min with 0.01% Trixon, then for 20 min with 5% bovine serum albumin. Subsequently, the sections were incubated for 2 h with the anti-NRF2 antibody

diluted 1:100, followed by a 50-min incubation with a biotinylated secondary antibody (Dako, Carpinteria, CA, USA). Human endometrial cancer tissue sections were used as positive controls for NRF2 IHC, as previously described (2,3). The primary antibody was omitted in endometrial cancer sections as a negative control.

Evaluation of NRF2 expression. NRF2 expression was assessed using a semi-quantitative method, as previously described (4). The total score was AxB, where A represented the proportion of positive cells (≤5% scored 0, 6-25% scored 1, 26-50% scored 2, 51-75% scored 3 and >75% scored 4) and B indicated the intensity of staining (none as 0, weak as 1, moderate as 2 and strong as 3). Therefore, a total score of 0 indicated no NRF2 protein expression (-), 1-4 weak positive (+), 5-8 positive (++) and 9-12 strong positive (+++) expression. All IHC slides were reviewed independently by two investigators.

Cell culture and hormone treatment. Ovarian cancer cells, Hey, SKOV3, HO8910, HO8910PM, ES-2, OVCAR-3 and A2780 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/F12 with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, sodium pyruvate and L-glutamine in humidified 5% CO_2 at 37°C. The Moody cell line was kindly provided by Dr W. Zheng (Arizona University, Tucson, AZ, USA), and is a normal ovarian epithelial cell line that was transfected with *hTERT*. The cells were maintained in MCDB109/M199 medium supplemented with 15% FBS. When these cells reached 80% confluence, the cells were collected for western blot analysis to determine NRF2 expression in different cell lines.

To investigate the effect of hormones on NRF2 expression, Hey cells were plated in 10-cm dishes with $1x10^5$ cells/ml. After 24-h serum-starvation, the cells were treated with 50 mIU/ml follicle-stimulating hormone (FSH), 50 mIU/ml luteinizing hormone (LH), 1 nM β-estradiol (E_2) or 10 μM medroxyprogesterone acetate (MPA) for 48 h, respectively. Proteins were then extracted using standard methods for western blot assays.

ROS detection. Hey cells were plated onto glass slips in 6-well plates at $1x10^4$ cells/well. After 24-h serum-starvation, cells were treated with 50 mIU/ml FSH, 50 mIU/ml LH, 1 nM E_2 or 10 μM MPA for 24 h. For ROS determination, cells were incubated with dichlorofluorescein (10 μg/ml) for 30 min and were then fixed with 10% buffered formalin. Images were obtained with a fluorescence microscope.

Immunoblotting. All cells were harvested and lysed. Total protein extracts (60 μg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk for 1 h and incubated overnight with anti-NRF2 primary antibody, followed by 1 h of incubation with the appropriate secondary antibody. Proteins were detected using an Enhanced Chemiluminescence reagent.

Statistical analyses. The statistical significance of intergroup differences was evaluated by the Fisher's exact test and the χ^2 test. All statistical tests were two-sided and the signifi-
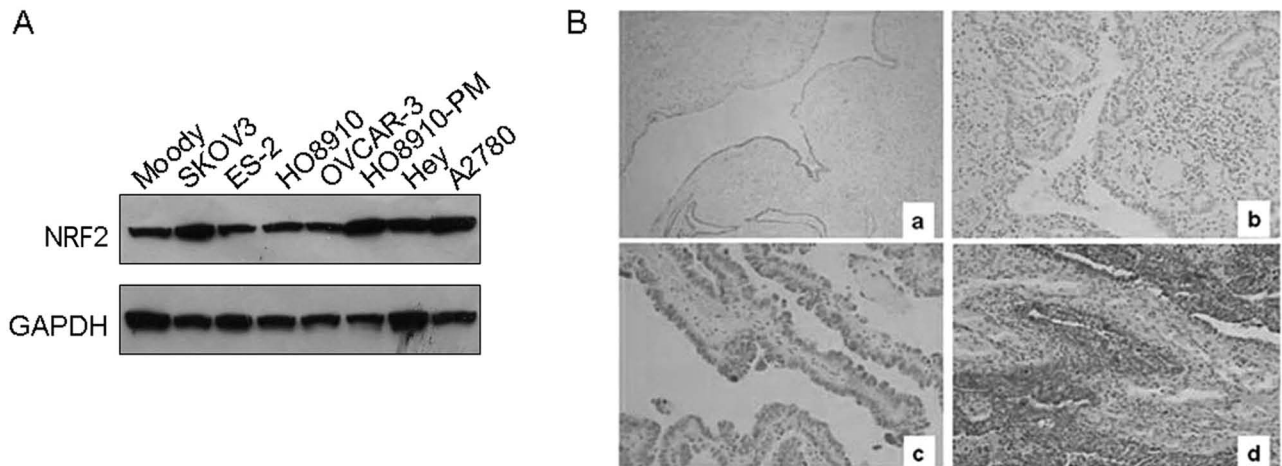
Figure 1. NRF2 protein expression in the ovarian surface epithelial cell line, ovarian cancer cell lines and ovarian tissues (cystadenoma, borderline tumor, high-grade serous carcinoma and low-grade serous carcinoma). (A) The expression pattern of NRF2 in ovarian surface epithelial cell line and ovarian cancer cell lines. (B) Immunohistochemical staining of benign ovarian cystadenomas tissues (a), borderline tumors (b), high-grade serous carcinoma (c) and low-grade serous carcinoma (d) tissues for NRF2 protein expression. Magnification, x100.

Table I. The expression of NRF2 in distinct tissue types.

Tissue type	Total	Positive			Negative	% positive	P-value
		+	++	+++			
Cystadenomas	10	3	-	-	7	30	0.006
Borderline tumor	4	-	-	-	4	100	
Carcinomas	64	13	10	19	22	65.6	

Fisher's exact test, P=0.006.

Table II. The expression of NRF2 in different OEC pathological types.

Pathological type	Total	Expression		% positive	P-value
		Positive	Negative		
Clear cell carcinoma	11	3	8	27.3	0.028
Endometrioid adenocarcinoma	9	7	2	77.8	
Mucinous cystadenocarcinoma	6	5	1	83.3	
Serous cystadenocarcinoma	38	27	11	71.1	

χ^2 test, P=0.028.

cance level was established at 0.05. All statistical analysis was performed using SPSS11.0 (SPSS Inc., Chicago, IL) or Prism 5.0 (GraphPad Software).

Results

Overexpression of NRF2 in ovarian epithelial carcinoma. As shown in Fig. 1A, NRF2 overexpressed in several ovarian cancer cell lines, including SKOV3, HO8910PM, Hey and A2780, when compared with normal ovarian epithelial Moody cell line. The expression of NRF2 in cystadenoma, borderline tumors and OEC cases was also evaluated. NRF2 was expressed in epithelial cells in 3/10 (30%) benign ovarian cystadenoma samples (Table I); in these cases, very few positive cells were observed (Fig. 1Ba). Positive staining of NRF2 in borderline tumors was not observed (Fig. 1Bb). However, in ovarian carcinomas, NRF2 expression was enhanced in 44/64 (65.6%) samples (Table I). Of these, 3/11 (27.3%) clear cell carcinomas showed weak positive staining in the nucleus, 7/9 (77.8%) endometrioid adenocarcinomas showed positive staining, 5/6
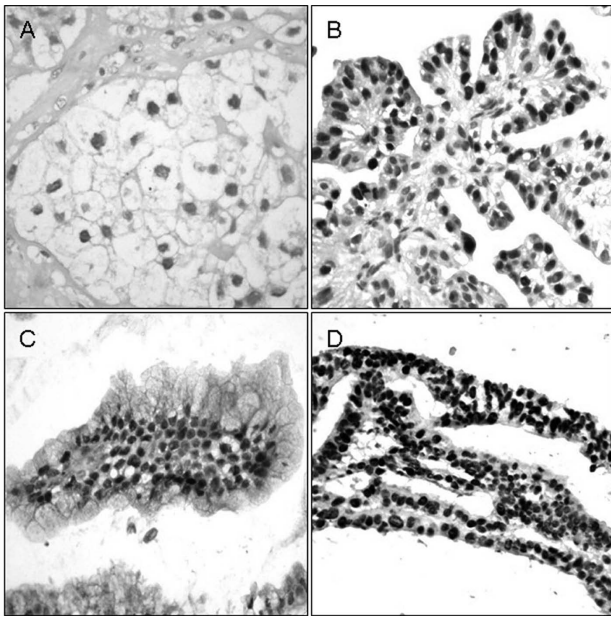
Figure 2. NRF2 protein expression in ovarian carcinoma of different pathological types. Immunohistochemical staining of clear cell carcinoma (A), endometrioid adenocarcinoma (B), mucinous cystadenocarcinoma (C) and serous cystadenocarcinoma (D) tissues for NRF2 protein expression. Magnification, x400.
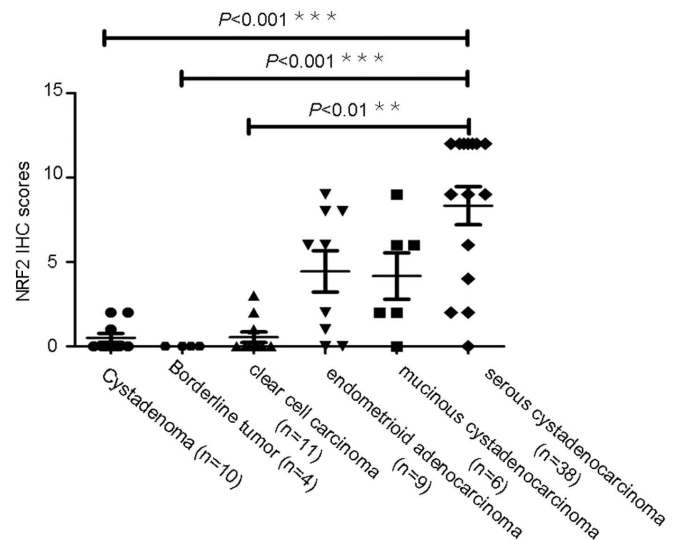

Figure 3. Comparison NRF2 protein expression among benign cystadenomas and different pathological types of ovarian carcinoma. *Statistically significant compared with serous cystadenocarcinoma (P<0.05).

(83.3%) mucinous cystadenocarcinomas had positive nucleus staining, 27/38 (71.1%) serous cystadenocarcinomas showed strong positive nucleus staining, and the remaining cases exhibited negative NRF2 expression (Table II). In these serous carcinomas cases, low-grade serous carcinomas shows an intensive positive NRF2 staining (Fig. 1Bd), whereas high-grade serous carcinomas shows relative weak positive staining (Fig. 1Bc). Compared with the cystadenoma samples, OEC samples showed an increased NRF2 expression (P=0.006). Representative pictures indicate low expression of NRF2 in cystadenoma samples (Fig. 1Ba) and strong positive staining in carcinomas (Fig. 1Bd). Although increased NRF2 expression in OEC was observed, the expression of NRF2 among different pathological types of tumor was variable. The majority of cases with high expression levels of NRF2 were serous cystadenocarcinomas (Fig. 3). Both endometrioid adenocarcinoma and mucinous cystadenocarcinoma also exhibited positive NRF2 protein expression; however, clear cell carcinoma showed a relatively low level of NRF2 expression (P=0.028) (Table II), compared with the other three pathological types. Representative pictures are shown in Fig. 2.

The relationship between prognosis and NRF2 expression. Table III shows the distribution of nuclear NRF2 positivity according to the clinicopathological characteristics of the malignant sample group. Within the age range of 22-79 years, 19/30 (63.3%) cases in patients >55 years old and 23/34 (67.6%) cases <55 years old showed positive NRF2 staining. No significant correlation was observed between age and NRF2 expression.

The correlation between NRF2 expression and histological grade was also investigated. The malignant cases were classified into three groups according to the degree of differentiation. Expression of NRF2 in ovarian carcinoma did not show statistically significant differences according to tumor grade (Table III). Similarly, no correlation between NRF2 expression and stage was observed.

Lymph node metastasis is an important prognostic factor for OEC, thus the relationship between NRF2 expression and lymph node metastasis was determined. NRF2 positive staining was detected in 21/33 (63.6%) cases without lymph node metastasis, while 20/31 (64.5%) cases with metastasis exhibited NRF2 expression (Table III). There was no correlation between NRF2 expression and lymph node metastasis.

The effects of hormones on ROS level in OEC. Gonadotrophins and sex-steroid hormones are high risk factors for OEC development. Increased NRF2 expression is generally accompanied by elevated ROS levels, so the ROS levels after hormone treatment were examined in Hey cells. Stimulation with 50 mIU/ml FSH, 50 mIU/ml LH or 1 nM E_2 resulted in increased ROS levels, as shown by increased green fluorescence (Fig. 4). Of these hormones, FSH had the greatest effect on ROS production. However, 10 μM MPA treatment had no obvious effect on ROS production (Fig. 4).

The effects of hormones on NRF2 expression. We investigated the effects of hormones on NRF2 protein expression in ovarian cancer cells. FSH, LH, E_2 and MPA significantly upregulated NRF2 expression, but by differing amounts, which suggests that NRF2 expression responds unequally to different hormonal stimuli (Fig. 5).

Discussion

NRF2 is a key transcriptional regulator of an intracellular antioxidant response through transcriptional activation of a battery of genes, including antioxidants and phase II detoxifying enzymes. NRF2 participates in multiple physiological processes, such as phase II drug metabolism and oxidative

Table III. Correlations of NRF2 and major clinical pathological factors (n=64).

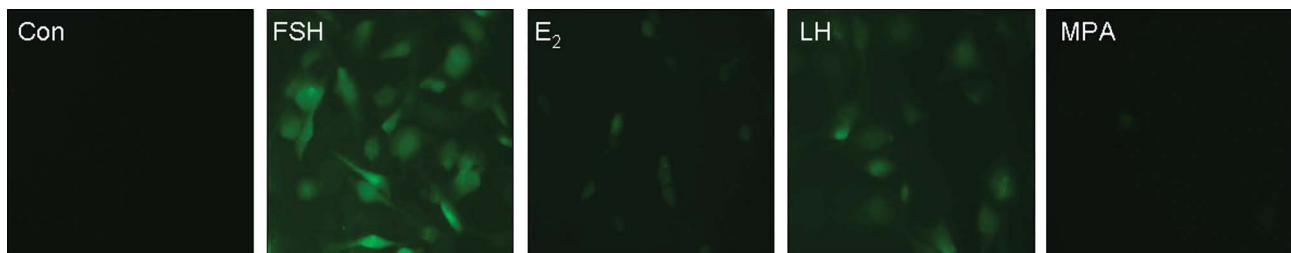| Factors | No. of patients | NRF2 expression | | P-value |
		Positive	Negative	
Age (years)				
<55	34	23	11	0.795
≥55	30	19	11	
Histological grade				
Low (I)	22	16	6	0.531
Intermediate (II)	18	12	6	
High (III)	13	11	2	
Clinical stage				
I	13	7	6	0.499
II	16	10	6	
III	35	25	10	
Lymph node metastasis				
No	33	21	12	1.000
Yes	31	20	11	

Figure 4. The effects of gonadotrophins and sex-steroid hormones on ROS production. Hey cells were plated onto a glass slip in the 6-well plate at 1x10^4 cells/well, after 24-h serum-starvation, the cells were treated with 50 mIU/ml FSH, 50 mIU/ml LH, 1 nM β-estradiol (E$_2$) and 10 μM MPA for 24 h, receptively. After incubation with dichlorofluorescein (10 μg/ml) for another 30 min, the images were captured with a fluorescence microscope. Magnification, x400.
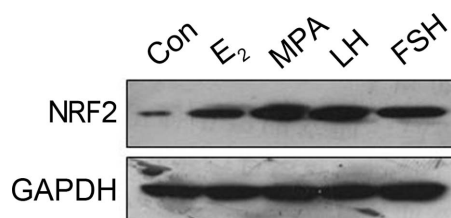

Figure 5. Gonadotrophins and sex-steroid hormones enhance NRF2 protein expression. Western blot analysis was used to determine NRF2 protein levels after treatment with 50 mIU/ml FSH, 50 mIU/ml LH, 1 nM β-estradiol (E$_2$) and 10 μM MPA for 48 h. GAPDH served as a loading control.

stress, and is negatively regulated by KEAP1. Aberrant NRF2 expression is found in various cancer types (1,5-9) and the dysregulation of NRF2 affects cellular growth and response to therapy. Wang et al reported that overexpression of NRF2 in gallbladder adenocarcinoma was correlated with tumor differentiation, staging and metastasis (1). They also concluded that NRF2 was an independent prognostic factor, due to shorter overall survival times in patients with high levels of NRF2 expression (1). In addition, Chen et al reported that NRF2 was significantly highly expressed in endometrial serous carcinoma (ESC) and some endometrial precursor lesions, compared with atypical endometrial hyperplasia or endometrial intraepithelial neoplasia (3). Their study demonstrated that alteration of NRF2 expression may represent one of several early molecular events in ESC carcinogenesis. Similarly, Jiang et al showed that NRF2 is overexpressed in Type II endometrial cancer, and that this was correlated with resistance to chemotherapeutic drugs (2). Inhibition of NRF2 signaling may therefore increase the efficacy of chemotherapy, making NRF2 a potential target molecule for endometrial cancer therapy (2). However, information concerning NRF2 expression in OEC is scarce. In the current study, we observed that NRF2 overexpressed in several ovarian cancer cell lines. It was found that NRF2 expression was enhanced in 65.6% OEC cases, and was mainly localized to cell nuclei, by immunohistochemistry assay. Only a few weakly positive NRF2 staining cells could be observed in benign cystadenoma cases; however, a significant increase in NRF2 expression was exhibited in OEC cases. Further studies

demonstrated that overexpression of NRF2 was more common in serous cystadenocarcinoma, endometrioid adenocarcinoma and mucinous cystadenocarcinoma than in clear-cell OEC. The lattermost pathological subtype showed increased NRF2 expression in only 27.3% of cases, which suggested that NRF2 expression was associated with pathological type in OEC. Although NRF2 is overexpressed in OEC, whether it could be used as an independent prognostic factor is not clear. In the present investigation, no relationships between NRF2 expression and patient age, tumor differentiation, staging or lymph node metastasis were found. In spite of being of limited use for prognostic assay, NRF2 expression may allow benign cystadenomas to be distinguished from malignant carcinomas. In addition, elevated NRF2 expression may be a molecular event in the process of OEC development; the reasons for increased NRF2 expression in OEC warrant further study.

Reactive oxygen species (ROS) play an important role in multiple normal physiological functions, such as ovulation (10). However, ROS are also involved in the development of cancer, due to induction of cellular DNA damage. In order to avoid the deleterious effects of excess ROS, NRF2 signaling is commonly activated by an antioxidant response that decreases ROS levels. However, if excess ROS cause constitutive activation of NRF2 signaling, the elevated NRF2 levels will result in chemoresistance, increased DNA repair, inflammatory responses and angiogenesis (11). ROS are commonly induced by a variety of external stimuli, including chemotherapeutics, growth factors and hormones (12-16). Generally, elevated ROS will induce a protective antioxidant response, enhancing the expression of NRF2. NRF2 in turn regulates an array of genes containing an enhancer sequence known as the ARE, which include glutamate cysteine ligase, heme oxygenase-1 (HO-1) and NAD(P)H quinine oxidoreductase-1 (NQ-1). However, the role of ROS production in response to gonadatrophins and sex-steroid hormones as high risk factors for OEC development has not been studied to date. In this study, we found that FSH, LH and estrogen (but not MPA) could induce ROS production at different levels. These hormones may therefore be involved in the cancer development process by inducing ROS.

Since some hormones are able to induce ROS production, they may also indirectly regulate NRF2 expression. To further determine the possible relationship between gonadotrophins, sex-steroid hormones and NRF2 expression, we treated Hey cells with 50 mIU/ml FSH and LH, 1 nM E_2 and 10 μM MPA. Our results indicate that FSH, LH, MPA and E_2 cause the upregulation of NRF2 expression. FSH enhances OEC cell proliferation (17,18), which may be mediated by elevated NRF2 levels, since NRF2 is implicated in the cellular growth of various cancers (19,20). Ovulation is stimulated by the preovulatory surge of the pituitary LH, and a very recent report showed that LH-induced ROS is able to mimic the effect of LH and trigger ovulation (10). Under pathological conditions, LH may be involved in OEC development. It can therefore be speculated that LH-induced NRF2 may be associated with LH-mediated invasive and anti-apoptotic activity in OEC, but further studies to investigate this mechanism are required. In addition, excess estrogen exposure is a risk factor not only for OEC but also for breast cancer. Certain findings suggest that E_2 promotes proliferation of ovarian epithelial cells. Studies done in the 1980s and 1990s of 'traditional' Asian women showed

that their average serum E_2 level in the premenopausal period was 75-80% of that of US women (21). These lower E_2 levels may account for the lower ovarian cancer rates in Asian women. Although estrogen receptor (ER) positivity is found in 50-70% of ovarian cancers (22), whether E_2 enhances OEC development through ER signaling is still not clear. In breast carcinogenesis, some studies indicate that induction of ER-mediated signaling is not sufficient for the development of breast cancer and the underlying mechanisms of breast susceptibility to estrogen's carcinogenic effect may correlate with ROS induced by E_2 (23). Here we showed elevated levels of NRF2 induced by E_2 which are associated with E_2-induced ROS. Based on this finding, we suggest that E_2-induced ROS and NRF2 are the molecular basis for OEC development. However, we did not observe this relationship between increased NRF2 expression induced by MPA and ROS levels induced by MPA. Therefore, we speculate that MPA upregulates NRF2 expression through another signaling mechanism, rather than ROS signaling.

This study demonstrated that NRF2 is overexpressed in human OEC tissues compared with benign cystadenomas, indicating that NRF2 may play a role in OEC development. This elevated NRF2 expression may be induced by gonadotrophins and sex-steroid hormones, which suggest that these hormones are involved in OEC development via modulation of NRF2 signaling. Therefore, the inhibition of NRF2 may represent an effective therapeutic strategy for the treatment of OEC.

Acknowledgements

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