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High levels of Nrf2 determine chemoresistance in type II endometrial cancer

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Abstract

Type II endometrial cancer, which mainly presents as serous and clear cell types, has proved to be the most malignant and recurrent carcinoma among various female genital malignancies. The transcription factor, Nrf2, was first described as having chemopreventive activity. Activation of the Nrf2-mediated cellular defense response protects cells against the toxic and carcinogenic effects of environmental insults by upregulating an array of genes that detoxify reactive oxygen species (ROS) and restore cellular redox homeostasis. However, the cancer-promoting role of Nrf2 has recently been revealed. Nrf2 is constitutively upregulated in several types of human cancer tissues and cancer cell lines. Furthermore, inhibition of Nrf2 expression sensitizes cancer cells to chemotherapeutic drugs. In this study, the constitutive level of Nrf2 was compared in different types of human endometrial tumors. It was found that Nrf2 was highly expressed in endometrial serous carcinoma (ESC), whereas complex hyperplasia (CH) and endometrial endometrioid carcinoma (EEC) had no or marginal expression of Nrf2. Likewise, the ESC derived SPEC-2 cell line had a higher level of Nrf2 expression and was more resistant to the toxic effects of cisplatin and paclitaxel than that of the Ishikawa cell line, which was generated from EEC. Silencing of Nrf2 rendered SPEC-2 cells more susceptible to chemotherapeutic drugs while it had a limited effect on Ishikawa cells. Inhibition of Nrf2 expression by overexpressing Keap1 sensitized SPEC-2 cells or SPEC-2-derived xenografts to chemotherapeutic treatments using both cell culture and SCID mouse models. Collectively, we provide a molecular basis for the use of Nrf2 inhibitors to increase the efficacy of chemotherapeutic drugs and to combat chemoresistance, the biggest obstacle in chemotherapy.

Keywords

Nrf2; chemoresistance; and endometrial cancer

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INTRODUCTION

Endometrial cancer is one of the most common gynecologic malignancies in the world. According to the statistics from the American Cancer Society, 42,160 new cases and 7,780 deaths occurred in 2009 (1). Extensive epidemiologic, molecular, and behavioral information suggests that there are two distinct forms of endometrial cancer, referred to as type I and type II. Type I is classified by endometrioid histology and type II consists of serous and clear cell carcinomas. Type I comprises 70% to 80% of newly diagnosed cases of endometrial cancer, while type II consists of only 15% (2–4). Typically, patients diagnosed with type I have a relatively good prognosis, whereas type II has a disproportionally higher death rate. The dismal prognosis for type II endometrial cancer is attributed to its highly aggressive nature, rapid cancer developmental, lack of early diagnostic methods, a high recurrence rate, and a high incidence of chemoresistance (5,6).

Many chemopreventive compounds are able to enhance the expression of Nrf2, a master regulator of an array of genes containing an enhancer sequence known as the antioxidant response element (ARE) (7–13). ARE-bearing genes can be categorized into three major classes: (i) intracellular redox-balancing genes, such as glutamate cysteine ligase (GCLC and GCLM) and heme oxygenase-1 (HO-1). (ii) Phase II detoxifying genes, including glutathione S-transferase, NAD(P)H quinone oxidoreductase-1 (NQO1). (iii) Genes encoding transporters, such as multi-drug resistant proteins (MRPs) and xCT (8,11,14). Based on the functions of these ARE-bearing genes, it is apparent that activation of Nrf2 target genes should enhance the detoxification and removal of xenobiotics, such as pharmacological agents, toxicants, and carcinogens. Indeed, activation of Nrf2 confers protection against many chronic diseases including cancer, cardiovascular diseases, high-fat diet-induced obesity, lung inflammation and fibrosis, and diabetic nephropathy (11,15–21). Furthermore, the beneficial effects of many chemopreventive compounds rely on the activation of the Nrf2-mediated antioxidant response, as demonstrated experimentally in Nrf2-deficient mice, which were unresponsive to chemopreventive compounds (22). Mechanistic studies have demonstrated that Nrf2 is tightly regulated, primarily at the protein level, by a negative regulator named Keap1 (23). Under basal conditions, low levels of Nrf2 are maintained by constant degradation of Nrf2 through the Keap1-dependent ubiquitination and proteasomal machinery. Chemopreventive compounds are able to stabilize Nrf2 through inhibition of Nrf2 degradation, thus enhancing the protein level of Nrf2 and activating the Nrf2-dependant antioxidant response (24,25).

Recently, the dark side of Nrf2 has been revealed (26). Many mutations in Nrf2 or Keap1 that disrupt the Keap1-Nrf2 interaction have been indentified in several cancer tissues and cancer cell lines (27–30). We have reported that Nrf2 is, at least partially, responsible for chemoresistance, as demonstrated by our findings that overexpression of Nrf2 enhanced chemoresistance whereas knockdown of Nrf2 sensitized cancer cells to chemotherapeutic drugs in neuroblastoma, breast, and lung cancer cell lines (31). Consistent with this notion, Kwak's group provided evidence that inhibition of Nrf2 sensitized ovarian carcinoma cells to cisplatin and doxorubicin (32,33). Most importantly, Biswal's group demonstrated that xenografts, derived from Nrf2-silenced lung cancer cells, had an enhanced response to carboplatin *in vivo* compared to control (34). In this study, the level of Nrf2 expression was compared among the different stages of human endometrial tumors. It was concluded that Nrf2 expression highly correlated with the aggressiveness and chemoresistance of the cancer type, as demonstrated that 90% of ESC tissues had Nrf2 overexpressed. The role of Nrf2 in chemoresistance was further supported by the fact that SPEC-2 cells, expressing a higher level of Nrf2 than Ishikawa cells, were more resistant to cisplatin and paclitaxel than Ishikawa cells. Moreover, inhibiting Nrf2 expression rendered SPEC-2 cells or SPEC-2 derived xenografts more sensitive to chemotherapeutic drugs, providing strong evidence that inhibiting Nrf2 to overcome chemoresistance holds a great promise.

MATERIALS AND METHODS

Case selection, tissue handling and pathological analysis

One hundred and seventeen (117) hysterectomy specimens were studied. These included 46 endometrial serous carcinoma (ESC), 51 endometrial endometrioid carcinoma (EEC), and 20 benign uteri that were resected for a variety of benign indications. The cases that were reviewed in this study included only slides that had adjacent non-cancerous endometrium. No patient with a history of prior radiation or chemotherapy was included in the study. In patients with benign diseases, no personal history of cancer or transplantation therapy was indicated. Any patient with a known history of hormone replacement was excluded. Tissue obtained was formalin fixed and paraffin embedded. The histologic sections containing both benign endometrium (BE) and cancerous areas for all the cancer cases were processed. The diagnosis and histologic classification of the endometrial carcinomas was made using the criteria proposed by the World Health Organization.

Nrf2 immunohistochemical (IHC) analysis

IHC analysis for Nrf2 protein expression was performed as described previously (35). A monoclonal antibody against human Nrf2 (IgG2) was purchased from Abcam, Inc. (Cambridge, MA). Sodium citrate buffer (pH 6) was used as an antigen retrieval solution. A section of lung carcinoma, previously confirmed to have overexpressed Nrf2 protein, was included in every experiment as a positive control. A negative control was carried out by replacing primary antibodies with class-matched mouse IgG proteins on a section. All slides were evaluated in a blinded manner for the percentage (P) of positively stained cells and the intensity (I) of the staining. The intensity was arbitrarily divided into 4 categories (0–3) and was judged based on the positive control. Staining index was calculated according to the following formula: Index of Nrf2 expression = P × I. Only those cases whose indices were more than 25 were regarded as positive.

Cell culture, transient transfection of small interfering RNA (siRNA) and establishment of stable cell lines

All the chemicals used in this study, including cisplatin and paclitaxel, were purchased from Sigma (Saint Louis, MO). Ishikawa cells, which were derived from well-differentiated endometrioid carcinoma, were bought from American Type Culture Collection (ATCC) (Manassas, VA). SPEC-2 cells, which were derived from endometrial serous carcinoma, were kindly provided by Dr. Janet Price at MD Anderson Cancer Center (36). Nrf2- siRNA and Hiperfect transfection reagent were purchased from Qiagen (Valencia, CA). Transfection of Nrf2-siRNA was performed according to the manufacturer's instructions. SPEC-2 derived stable cell lines, with stable incorporation of Keap1-CBD or an empty vector, were established using a retrovirus system as described previously (31). Stable SPEC-2 cell lines were continuously cultured in medium containing 1.5 μg/ml puromycin (Sigma).

Immunoblot analysis

The antibodies for Keap1, NQO1, HO-1, γ-GCS, Keap1, GAPDH, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-CBD antibody (New England Biolabs, Ipswich, MA) and Ki67 antibody (Vector Lab, Burlingame, CA) were purchased from commercial sources. Immunoblot analysis for cultured cells and tissues were perfomed as previously reported (37).

Cell viability assay, apoptotic cell death, NQO1 activity, and glutathione level

Cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as reported previously (31). An *in situ* cell death detection kit (Roche)

was used for detecting apoptotic cell death in tumor tissue according to the manufacturer's instructions and analyzed under a fluorescence microscope (Zeiss Observer Z1, Marianas™ Digital Microscopy workstation). NQO1 activity was measured as the dicoumarol-inhibitable fraction of DCPIP reduction (31). The intracellular glutathione concentration was measured using QuantiChrom glutathione assay kit from BioAssay Systems (Hayward, CA) (31). All the experiments were performed in triplicate to obtain means and standard deviations, and data are presented as mean ± SD.

Real-time reverse transcription-polymerase chain reaction

Total mRNA extraction, reverse-transcription, and qRT-PCR conditions have been previously reported (37). Taqman probes were from the universal probe library (Roche): hNrf2 (#70), hKeap1 (#10), hNQO1 (#87), hHO-1 (#25), hMRP2 (#25), hGCLC (#25), hGCLM (#18), and hGAPDH (#25). Primers were synthesized by Integrated DNA Technologies and their sequences are listed in Table 1. Duplicated reactions were performed for each sample and the same experiment was repeated twice. GAPDH was included as a reference gene.

Endometrial serous carcinoma models in SCID mice

Two SPEC-2-derived cell lines (SPEC-2-control, SPEC-2-Keap1$^+$) were harvested and resuspended at the concentration of 1×10^7 cells/200 μl sterile saline. Mice (16/group) were injected subcutaneously in the subdermal space on the medial side of the thigh just above the level of the stifle with 1×10^7 SPEC-2-control or SPEC-2-Keap1$^+$ cells at the age of 6 weeks. Mice injected with control cells or Keap1$^+$ cells were further divided into two groups: one treated with saline, and the other with cisplatin. Mice were given saline or cisplatin (2 mg/kg mice) intraperitoneally every other day for a total of 10 days (5 injections total). Tumor volume (mm^3) was measured every other day and calculated using the formula ($a^2\times b$)/2 (a: the smallest diameter; b: the largest diameter). Mice were sacrificed 24 days after cell injection. Tumors were dissected and weighed. Formalin-fixed, paraffin-embedded tumor tissue sections were used for immunohistochemistry and TUNEL analysis, whereas snap-frozen tissues were subjected to immunoblot analysis.

Statistical test

Results are expressed as mean ± SD. Statistical tests were performed with SPSS 10.0. Unpaired student t-tests were used to compare the means of two groups. One-way ANOVA was applied to compare the means of three or more groups. $P<0.05$ was considered to be significant.

RESULTS

Selected Clinical and Pathological Features

The age of patients undergoing hysterectomies for cancer ranged from 45 to 91 years with a mean of 58 years. The mean age of patients with ESC was 65 years (ranged from 58 to 91), while the mean age of patients with EEC was 57 years (ranged from 45 to 77). The average age of patients with positive Nrf2 expression ranged from 50 to 79 years with a mean of 59 years. All but 23 cancer patients presented had postmenopausal bleeding. Among the 23 patients who did not have postmenopausal bleeding, 15 had an abnormal pap smear, 6 had an increased endometrial stripe thickness found by ultrasound, and 2 were found to have a pelvic mass by a routine pelvic examination. Among the benign control group, average patients' age ranged from 46 to 82 with an average age of 58.

Endometrial Serous Carcinoma had elevated Nrf2 expression

A total of 117 cases including 46 ESC, 51 EEC, and 20 benign endometria were studied. H&E staining was examined to differentiate the type of endometrial tumors. Representative images

are shown (Fig. 1A, panel a, c, and e). A consecutive tissue section from the same patient was also analyzed for Nrf2 expression by IHC (Fig 1A, panel b, d, and f). Nrf2 expression was scored as positive or negative for each case, according to the criteria described in the materials and methods section. The data are summarized in Table 2. Among the 46 ESC cases, 41 (89%) were positive for Nrf2 expression. In contrast, Nrf2 was only expressed in 14 of 51 (28%) EEC cases. The difference in Nrf2 expression was statistically significant between the two types of endometrial cancer ($P<0.0001$). Furthermore, there was no Nrf2 expression in all 20 benign endometria consisting of 5 atrophic, 5 weakly proliferative, 6 proliferative, and 4 hyperplastic endometria. These results implicate a strong correlation between elevated Nrf2 expression and the aggressiveness of cancer, indicated by higher Nrf2 expression in type-II endometrial cancer tissues. Furthermore, overexpression of Nrf2 correlated with high expression of HO-1, a Nrf2 target gene. HO-1 was highly expressed in ESC tissue sections while there was no expression detected in benign tissue sections (Fig 1B). These data demonstrate that Nrf2 was functional in ESC and was able to activate its downstream genes.

SPEC-2 cells express a higher level of Nrf2 than Ishikawa cells and are more resistant to chemotherapeutic drugs

To investigate if there is a correlation between basal Nrf2 protein level and resistance to chemotherapeutic drugs two cell lines, Ishikawa and SPEC-2, were used. Ishikawa and SPEC-2 were isolated from tumors of patients with type I (EEC) or type II (ESC) endometrial carcinomas, respectively. Although no difference in Nrf2 mRNA transcription was observed (Fig 2A, Nrf2 panel), the mRNA expression of Nrf2-downstream genes, including NQO1, HO-1, MRP2, GCLC, and GCLM, was increased in SPEC-2 cells (Fig 2A), indicating the activation of the Nrf2-mediated response. Transcription of MRP2 was increased almost 9-fold in SPEC-2 cells compared to that in Ishikawa cells. Interestingly, Keap1 mRNA was decreased about 30% in SPEC-2 cells compared to Ishikawa cells (Fig 2A). Next, the protein level of Nrf2 and two of its downstream genes was measured under both basal and tert-Butylhydroquinone (tBHQ)-induced conditions. SPEC-2 had higher levels of Nrf2, NQO1, and γ-GCS compared to Ishikawa under both basal and induced conditions (Fig 2B, compare lane 1 to 3 and lane 2 to 4). Consistent with a decrease in Keap1 mRNA, there was a decrease in Keap1 protein level in SPEC-2, compared to Ishikawa (Fig. 2B, Keap1 panel and the bar graph). Next, the sensitivity of these two cell lines to cisplatin and paclitaxel, two commonly used chemotherapeutic drugs for treating endometrial carcinoma, was measured. Ishikawa cells were more sensitive to cisplatin and paclitaxel treatment than SPEC-2 (Fig 2C). Collectively, these data establish a correlation between the level of Nrf2 and resistance to chemotherapeutic drugs.

Transient silencing of endogenous Nrf2 enhanced the sensitivity of SPEC2 to chemotherapeutic drugs

To further explore the role of Nrf2 in chemoresistance, Nrf2 was knocked down by transient transfection of Nrf2-siRNA into Ishikawa and SPEC2 cell lines. As shown in Figure 3, the protein level of Nrf2 was reduced significantly in both cell types, as well as the levels of NQO1 and γ-GCS (Fig 3A, compare lane 1 with 2; lane 3 with 4). Consistent with the results shown in Fig 2B, the basal level of Nrf2 in SPEC-2 cells is higher than that in Ishikawa cells (Fig 3A, compare lane 1 with 3). Next, the effect of Nrf2 knockdown on cell resistance to therapeutic drugs was assessed. Knockdown of Nrf2 in Ishikawa cells did not significantly affect cell viability in response to cisplatin or paclitaxel (Fig 3B, left two panels). In contrast, silencing of Nrf2 in SPEC-2 cells significantly sensitized SPEC-2 cells to cisplatin and paclitaxel (Fig 3B, right two panels). It is conceivable that the substantial effect of Nrf2 knockdown in SPEC-2 is due to the higher basal level of Nrf2 in this cell line, compared to Ishikawa.

Establishment of a stable SPEC-2 cell line overexpressing Keap1

Transient silencing of Nrf2 sensitized SPEC-2 cells to chemotherapeutic drugs; therefore, SPEC-2 was used to establish a stable cell line, in which Keap1 was overexpressed. Expression of Nrf2 and its downstream genes, as well as their resistance to chemotherapeutic drugs were compared between vector-containing (control) and Keap1-CBD-containing ($Keap1^+$) cell lines. Overexpression of Keap1-CBD did not affect the mRNA level of Nrf2 (Fig 4A, Nrf2 panel), which is consistent with the established notion that Keap1-mediated negative control is primarily at the level of Nrf2 protein stability. Stable incorporation of Keap1 cDNA was confirmed by a 5-fold increase in Keap1-mRNA expression in $Keap1^+$ cells, compared to control cells (Fig. 4A, Keap1 panel). As a consequence of Nrf2 inhibition by Keap1-CBD overexpression, the mRNA level of NQO1, HO-1, MRP2, GCLC, and GCLM was significantly reduced in $Keap1^+$ cells compared to control cells (Fig 4A). Immunoblot analysis also showed a reduction in the protein level of Nrf2, NQO1, and γ-GCS in $Keap1^+$ cells under both basal and induced conditions (Fig. 4B, compare lane 1 with 2; lane 3 with 4). Consistent with a reduction of Nrf2 in $Keap1^+$ cells, NQO1 activity and glutathione levels were also lower in $Keap1^+$ cells than in control cells (Fig 4C and 4D). Collectively, these results demonstrate the successful establishment of a stable cell line with reduced Nrf2 expression. Next, the sensitivity of control and $Keap1^+$ cells to cisplatin or paclitaxel was compared. $Keap1^+$ cells were significantly more sensitive to cisplatin and paclitaxel than control cells (Fig. 4E), indicating that Nrf2 protects cells against the cytotoxic effects of cisplatin and paclitaxel.

Knockdown of Nrf2 increased chemosensitivity of SPEC-2 xenografts to cisplatin

To further confirm the role of Nrf2 in chemoresistance, a human tumor xenograft model in SCID mice was used. As shown in Fig. 5A and 5B, the average tumor size developed after injection of control cells or $Keap1^+$ cells without cisplatin treatment was not much different at the termination of the experiment. In contrast, the $Keap1^+$ xenografts responded better to cisplatin than the control xenografts as evidence by the greater size reduction of $Keap1^+$-tumors than control-tumors in the treated groups (Fig. 5A and 5B). Interestingly, the average tumor weight in the $Keap1^+$ group was less than that in the control group, even in the untreated condition (Fig. 5C). It is worth mentioning that $Keap1^+$-tumors in the untreated group were more cystic and some of them lost fluid during their removal from the mice, resulting in an underestimation of tumor weights in the untreated $Keap1^+$-group. Furthermore, the difference in tumor size between the $Keap1^+$/saline and $Keap1^+$/cisplatin groups is significantly larger than that between control/saline and control/cisplatin, with the smallest tumor size observed in the $Keap1^+$/cisplatin group (Fig. 5D). Collectively, these results indicate that genetic suppression of Nrf2 expression rendered xenografts more susceptible to cisplatin, demonstrating a critical role of Nrf2 in chemoresistance *in vivo*.

Keap1+ xenografts had an increase in apoptosis and a decrease in proliferation

The excised tumors were used to confirm reduced expression of Nrf2 in $Keap1^+$ tumors. Three tumors excised from three different mice in each group were used for immunoblot analysis. Overexpression of Keap1-CBD was confirmed by immunoblot analysis with an anti-CBD antibody (Fig 6A, Keap1-CBD panel). Consequently, the protein level of Nrf2 was significantly lower in $Keap1^+$-tumors, compared to that in control-tumors in both untreated and treated groups (Fig. 6A, compare lane 1–3 to lane 7–9; lane 4–6 to lane 10–12, and the bar graph). IHC data further confirmed inhibition of Nrf2 expression in $Keap1^+$ xenografts, as shown by negative staining of Nrf2 in $Keap1^+$-tumors in both untreated and treated groups (Fig. 5B). Next, cellular proliferation in tumor tissues was assessed by IHC with an anti-Ki67 antibody. Cisplatin treatment significantly inhibited proliferation in both control- and $Keap1^+$-tumors (Fig 6C). Furthermore, there were less proliferative cells in $Keap1^+$-tumors, compared to control-tumors following cisplatin treatment (Fig 6C). On the other hand,

Keap1^{+}-tumors showed a marked increase in the number of apoptotic cells as detected by TUNEL analysis (Fig 6D). Taken together, these results clearly demonstrate that suppression of Nrf2 expression results in a reduction in tumor cell proliferation and enhanced apoptosis of tumor cells in response to cisplatin treatment, which is likely the basis for sensitization of cancer cells to chemotherapeutic drugs when Nrf2 is inhibited.

DISCUSSION

Endometrial serous carcinoma is the most common gynecological cancer in developed countries and is one of the leading causes of death in woman. Despite the fact that many new chemotherapeutic drugs have been developed and many aggressive treatment options are available, the overall survival of patients has not been significantly improved because many patients with type II endometrial cancer are either refractory to chemotherapeutic treatments or develop resistance to chemotherapy (38,39). Thus, resistance of cancer cells to chemotherapeutic treatment represents a major obstacle in cancer treatment.

In this study, we have investigated the possible contribution of Nrf2 on the poor prognosis of type II endometrial cancer. Tissue sections from over one hundred patients with different types of endometrial lesions were obtained. The expression of Nrf2 in these tissues was measured using IHC staining. The results are striking: Nrf2 was not detected in non-malignant endometrial lesions and it was marginally expressed in type I endometrial tumors; however, a large percentage of type II endometrial tumors had a high level of Nrf2 (Table 2 and Fig. 1). In addition, the functional ability of overexpressed Nrf2 was confirmed by elevated HO-1 expression in a consecutive tissue section (Fig. 1). To our knowledge, it is the first large-scale study of Nrf2 expression in human endometrial cancer. To further investigate the possible role of Nrf2 in the resistance of type II endometrial cancer to therapeutic treatments, we utilized two cell lines derived from type I and type II endometrial cancer, Ishikawa and SPEC-2, respectively. Our data clearly indicate that Nrf2 was overexpressed and transcription of its target genes was elevated in SPEC-2 cells, which correlated with the resistance of SPEC-2 to cisplatin or paclitaxel treatment (Fig. 2). In previous reports, high basal level of Nrf2 expression in certain cancer tissues or cancer cell lines was attributed to somatic mutations in Nrf2 or Keap1 that disrupt interaction, thus, resulting in stabilization of Nrf2 (27–30). To our surprise, no mutations were detected in the interaction domains of Nrf2 or Keap1 in SPEC-2 (data not shown). However, a 30% reduction in Keap1 mRNA expression and a 20% decrease in the Keap1 protein level, compared to Ishikawa cells, were detected. We believe that one of the molecular bases for Nrf2 overexpression in SPEC-2 is due to the reduced expression of Keap1, which leads to the stabilization and higher expression of Nrf2 (Fig. 2A and 2B). Next, downregulation of Nrf2 by transient transfection of Nrf2-siRNA or stable overexpression of Keap1 rendered SPEC-2 cells more susceptible to cisplatin and paclitaxel (Fig. 3 and Fig. 4). More importantly, the notion that blockage of Nrf2 expression enhances tumor response to chemotherapeutic drugs is demonstrated *in vivo* using SCID mice. Keap1^{+}-tumors in the cisplatin-treated group had the smallest tumor size and weight among all the groups (Fig. 5), implicating Nrf2 as an important determinant of tumor response to chemotherapy. Obviously, coordinated upregulation of many Nrf2 target genes are responsible for protecting cancer cells to anti-caner drugs. However, the contribution of individual Nrf2 target genes in chemoresistance may be different. Prompt recovery of glutathione levels by upregulating the Nrf2-target genes, GCLC and GCLM, following glutathione depletion may be important. In a recent report, Hayes and colleagues demonstrated that sulforaphane-mediated protection against the cytotoxic effects of chlorambucil, not menadione, was lost when cells were co-treated with BSO, an inhibitor of glutathione synthesis (40). Based on this finding, we speculate that inhibition of glutathione synthesis may have comparable effects as Nrf2 inhibition in sensitizing SPEC-2 to cisplatin treatment.

Interestingly, Keap1$^+$-xenografts in the untreated group had tumor sizes slightly smaller than control xenografts, but the difference was not statistically significant (Fig. 5). However, the average tumor weight in this group was significantly less than that in control group (Fig. 5C). We observed liquid discharge during isolation of the tumors from this group. At this point, the watery nature of the Keap1$^+$-tumors is not clear. The proliferation and apoptosis status measured by IHC-Ki67 and TUNEL did not show any difference in the tumors between these two untreated groups (Fig 6C and 6D). This is consistent with the results shown in figure 5D, which demonstrate that inhibition of Nrf2 did not significantly affect cancer cell growth, but sensitized them to chemotherapeutic drugs. Interestingly, Biswal's group reported that inhibition of Nrf2 in A549 cells by stable incorporation of Nrf2-shRNA completely eliminated their growth in soft agar and completely inhibited tumor formation in nude mice without any treatment of chemotherapeutic drugs (34). They also showed that suppression of Nrf2 expression eliminated soft agar growth of H460 cells, while tumors developed in nude mice; however, the size was significantly reduced (34). The reason underlying this discrepancy is not clear.

In conclusion, we show that Nrf2 is overexpressed in human type II endometrial cancer compared to other types of endometrial cancer, suggesting that Nrf2 may play a role in the aggressiveness, resistance, and poor prognosis of type II endometrial cancer. Studies using type I- and type II-derived cell lines further confirmed the higher expression of Nrf2 in type II endometrial cancer. Stable knockdown of Nrf2 by Keap1 overexpression significantly sensitized both SPEC-2 cells and xenografts to chemotherapeutic drugs, suggesting that combined therapy with an Nrf2 inhibitor may represent a new avenue in cancer treatment.

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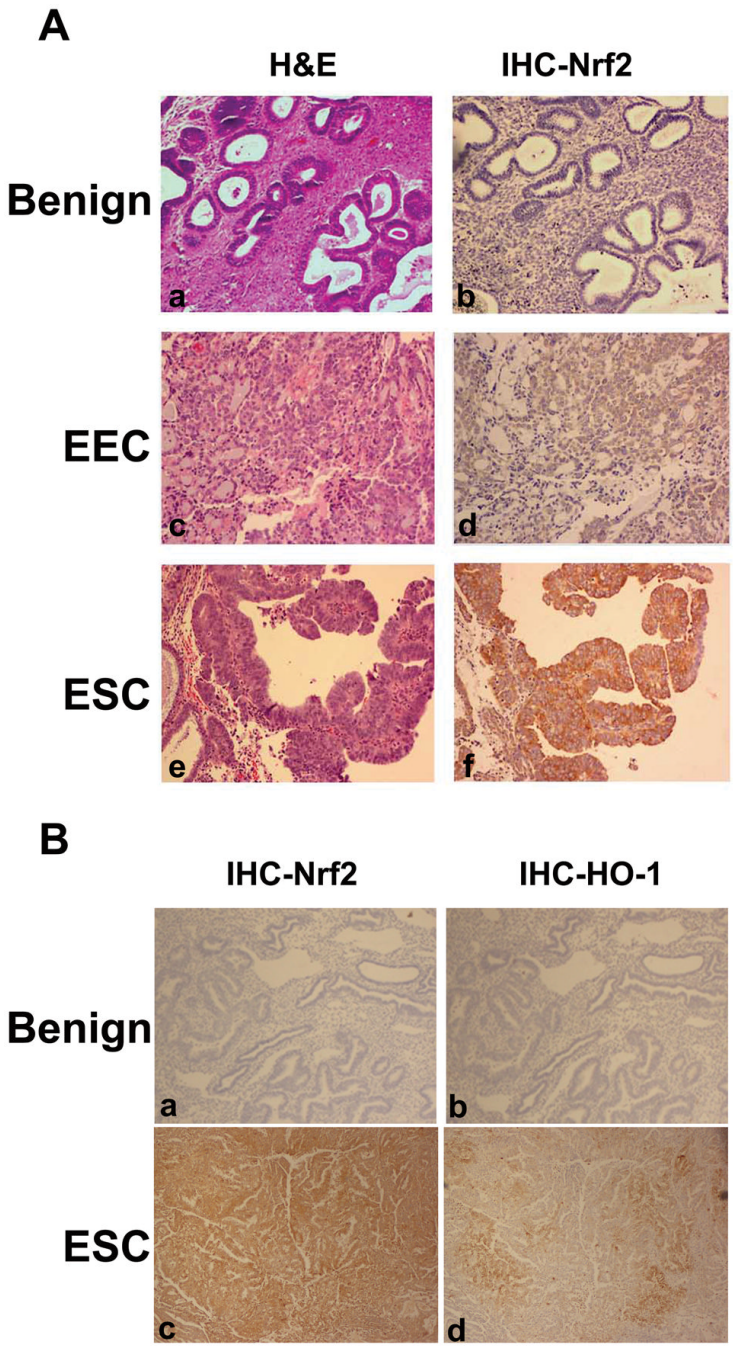

Fig 1.

Type II endometrial cancer had elevated Nrf2 expression. (A) The left panels represent H&E staining and the right panels represent IHC staining with a monoclonal Nrf2 antibody. No Nrf2 expression was detected in benign tumors or in endometrial endometrioid carcinoma (EEC). However, Nrf2 staining was observed in endometrial serous carcinoma (ESC). (B) Two consecutive tissue sections were analyzed by IHC for Nrf2 (left panels) and HO-1 (right panels).

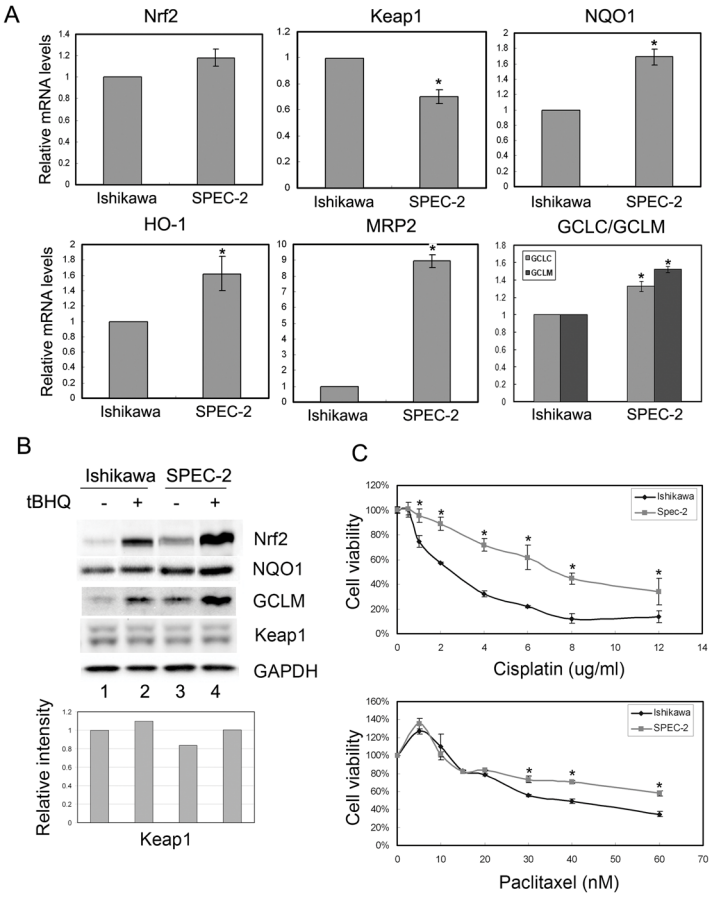

Fig 2.

SPEC-2 cells express a higher level of Nrf2 than Ishikawa and are more resistant to chemotherapeutic drugs. (A) mRNA levels of Nrf2, Keap1, NQO1, HO-1, MRP2, GCLC, and GCLM were compared between Ishikawa and SPEC-2 cells using real-time RT-PCR. The data presented were normalized to GAPDH. (B) The protein levels of Nrf2, NQO1, and γ-GCS were compared between Ishikawa and SPEC-2 cells. (C) Ishikawa and SPEC-2 cells were treated with the indicated doses of cisplatin and paclitaxel for 48 h. Cell viability was determined by MTT assay. The data are presented as means ± SD, *P < 0.05.

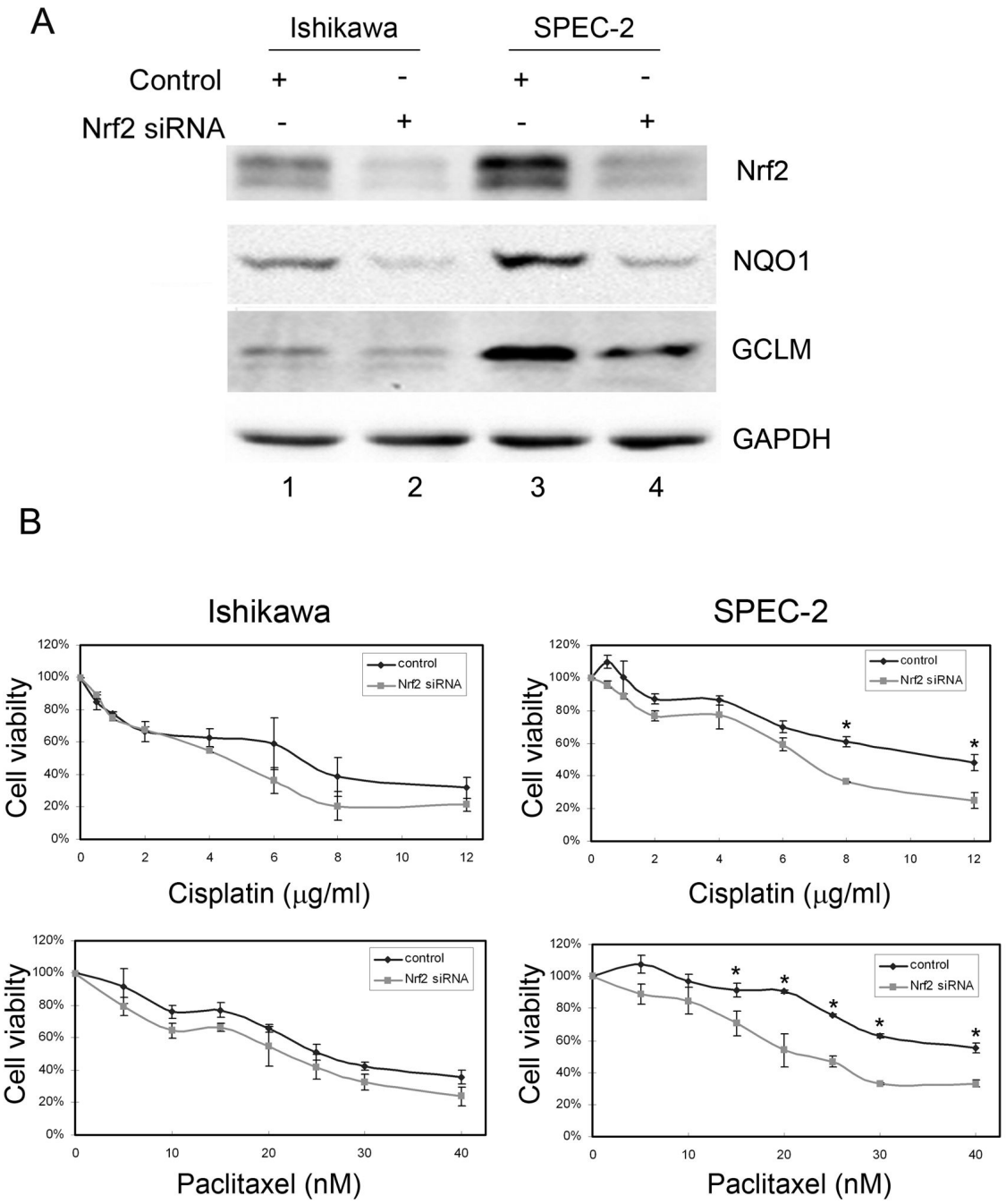

Fig 3.

Transient knockdown of Nrf2 expression by Nrf2-siRNA sensitized SPEC-2 cells to chemotherapeutic drugs. (A) The protein levels of Nrf2, NQO1, and γ-GCS were compared between Ishikawa and SPEC-2 cells transfected with Nrf2-siRNA or control siRNA. (B) Ishikawa and SPEC-2 cells were transfected with Nrf2-siRNA or control siRNA for 24 h, and then treated with different doses of cisplatin or paclitaxel for another 48 h. Cell viability was assessed by MTT assay. The data are presented as means ± SD, *P < 0.05.

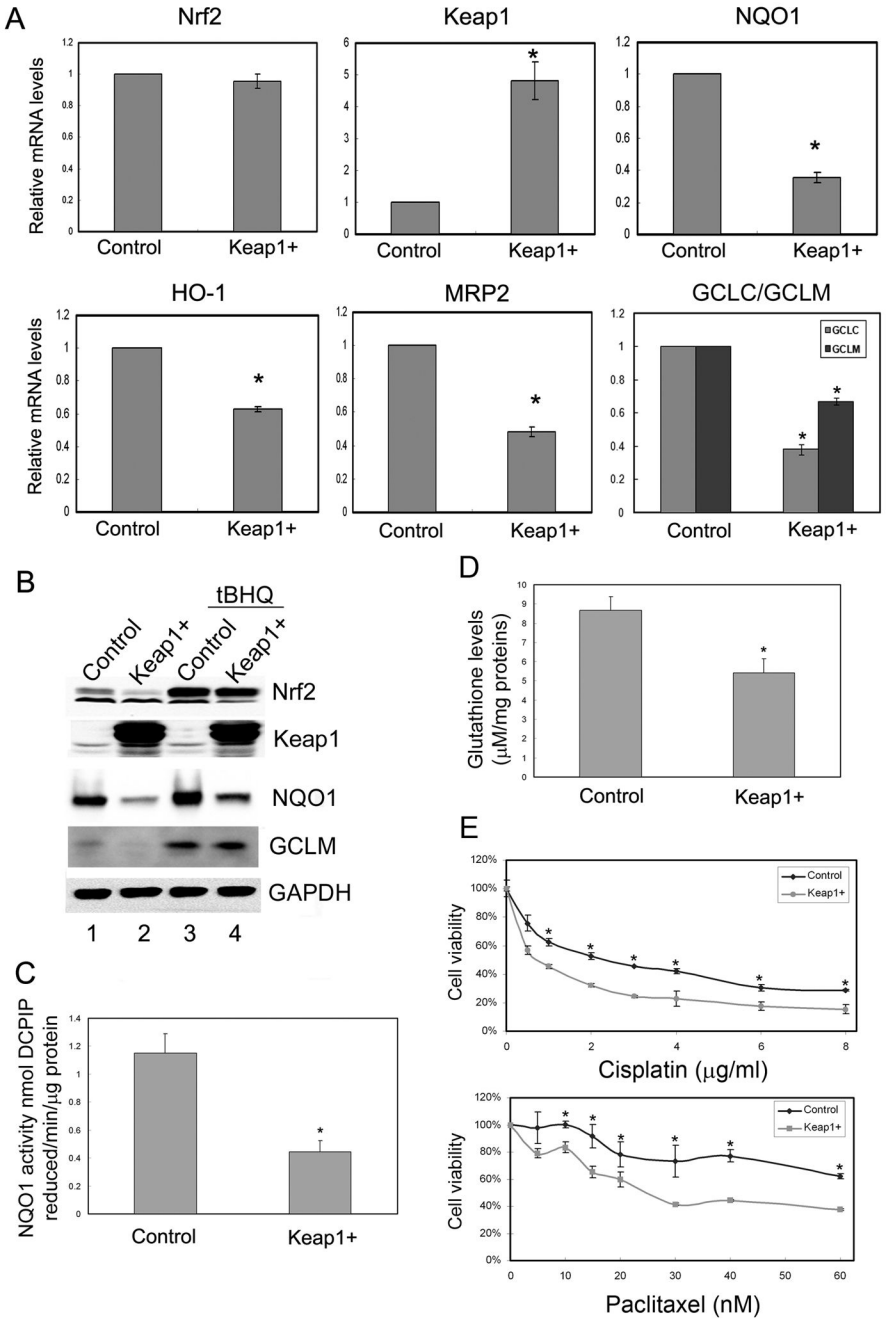

Fig 4.

Stable knockdown of Nrf2 by overexpression of Keap1 increased the susceptibility of SPEC-2 cells to chemotherapeutic drugs. (A) Two SPEC-2-derived cell lines, control and Keap1^{+}, stably expressing the control vector or Keap1-CBD were established using a retrovirus-based system. mRNA extracted from these two cell lines were subjected to real-time RT-PCR. (B) Cell lysates from these two cell lines were subjected to immunoblot analysis with antibodies against Nrf2, CBD (for detection of Keap1), NQO1, γ-GCS, and GAPDH. (C) The NQO1 enzymatic activity was measured by reduction of DCPIP. (D) The intracellular glutathione level was measured by using the QuantiChrom Glutathione Assay Kit (E) Cell viability was

assessed by the MTT assay following 48 h treatment with the indicated doses of cisplatin and paclitaxel. The data are presented as means ± SD, *P < 0.05.

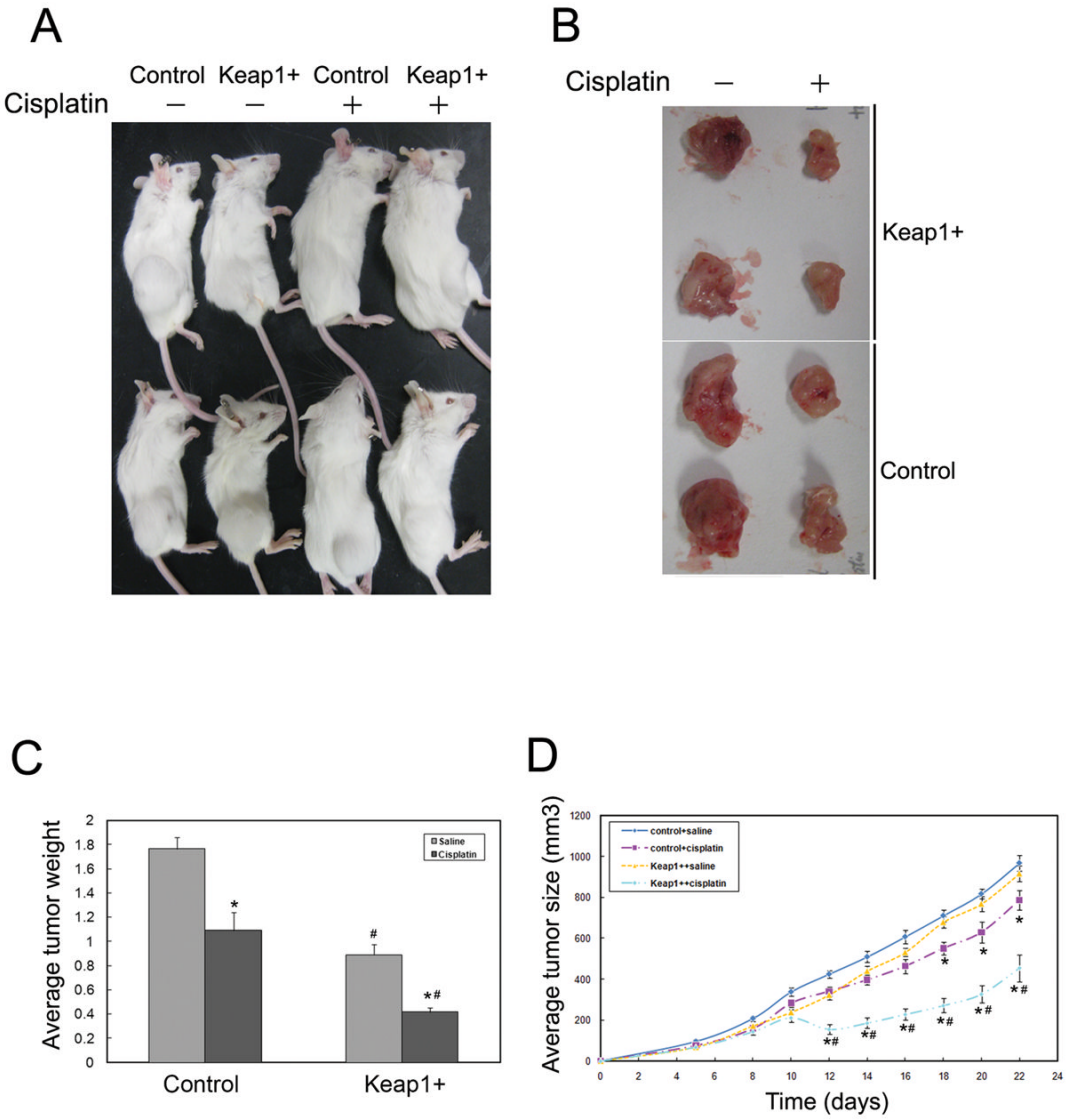

Fig 5.

Keap1$^+$-tumors had a substantial reduction in tumor volume following cisplatin treatment. A total of 32 female SCID mice were divided into four groups (8 mice per group): (i) mice were injected with control cells and treated with saline only; (ii) mice were injected with control cells and treated with cisplatin; (iii) mice were injected with Keap1$^+$ cells and treated with saline; and (iv) mice were injected with Keap1$^+$ cells and treated with cisplatin. (A and B) tumors developed in four groups of mice before and after dissection of tumors. Two mice from each group were shown. (C) Tumor weight of SCID mice at the termination of the experiments. (D) Tumor volume in SCID mice was measured every other day.

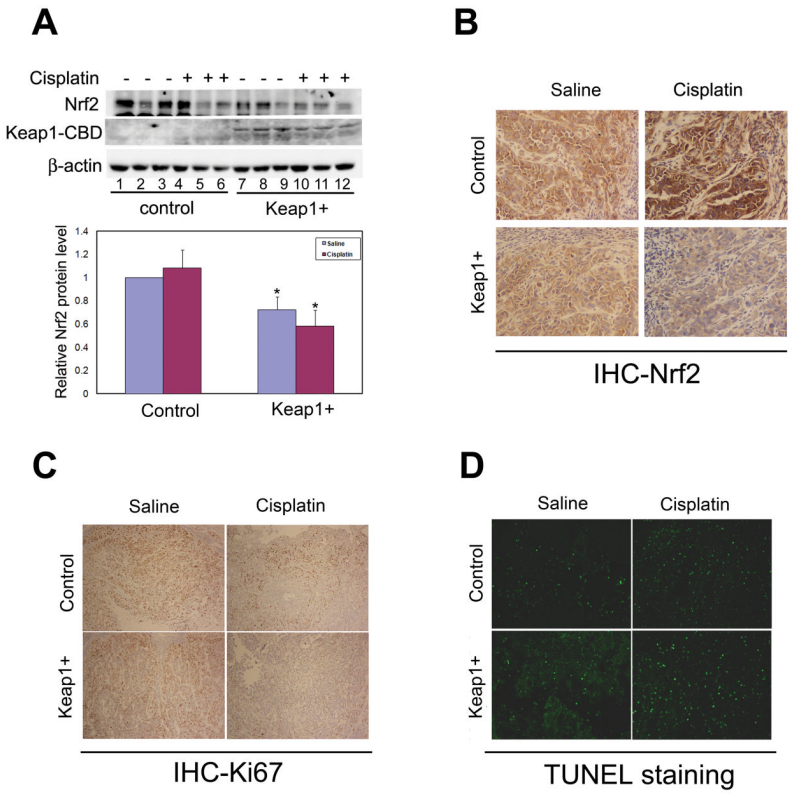

Fig 6.

Keap1$^+$-tumors had a reduction in cell proliferation and an induction of apoptosis in response to cisplatin treatment. (A) Protein levels of Nrf2 and Keap1-CBD in tumor tissues. Each lane contained the tumor tissue lysate extracted from individual mouse. Three tumor tissues per group were used for the immunoblot analysis. (B) Reduced Nrf2 expression in Keap1$^+$-tumors was confirmed by IHC staining. (C) Keap1$^+$ tumor tissues had reduced proliferation in response to cisplatin treatment, as measured by IHC-Ki67. (D) Keap1$^+$ tumor tissues had reduced proliferation in response to cisplatin treatment, as measured by TUNEL staining.

Table 1

Primers used for real-time PCR

Primers	Sequences
hNrf2	forward (acacggtccacagctcatc), reverse (tgtcaatcaaatccatgtcctg);
hKeap1	forward (attggctgtgtgtggagttgc), reverse (caggttgaagaactcctcttgc);
hNQO1	forward (atgtatgacaaaggacccttcc), reverse (tcccttgcagagagtacatgg);
hHO-1	forward (aactttcagaagggccaggt), reverse (ctgggctctccttgttgc);
hMRP2	forward (tgagcatgcttcccatgat), reverse (cttctctagccgctctgtgg);
hGCLC	forward (gacaaaacacagttggaacagc), reverse (cagtcaaatctggtggcatc);
hGCLM	forward (ggatgatgctaatgagtctgacc), reverse (tctactctccatccaatgtctgag);
hGAPDH	forward (ctgacttcaacagcgacacc), reverse (tgctgtagccaaattcgttgt);

Table 2

Comparison of Nrf2 protein expression in different types of endometrial lesions.

	# of cases	Positive (+)	Percentage (%)
Benign *	20	0	0
EEC	51	14	27.5
ESC	46	41	89.1‡
Total	117	55	47

‡P<0.0001 Compared with EEC.

*Consists of resting endometrium, proliferative endometrium and endometrial polyps.

Abbreviations: EEC, endometrial endometrioid carcinoma; ESC, endometrial serous carcinoma.