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The direct effect of estrogen on cell viability and apoptosis in human gastric cancer cells

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Abstract Epidemiology researches indicated that gastric cancer is a male-predominant disease; both expression level of estrogen and expression pattern of estrogen receptors (ERs) influence its carcinogenesis. But the direct effect of estrogen on gastric cancer cells is still unclear. This study aimed to explore the direct effect of β-estradiol (E2) on gastric cancer cells. SGC7901 and BGC823 were treated with a serial of concentrations of E2. The survival rates of both the cell lines were significantly reduced, and the reduction of viability was due to apoptosis triggered by E2 treatment. Caspase 3 was activated in response to the increasing E2 concentration in both SGC7901 and BGC823. Cleaved Caspase 3 fragments were detected, and the expression levels of Bcl-2 and Bcl-xL were reduced. Apoptosis was further confirmed by flow cytometry. The expression level of PEG10, an androgen receptor target gene, was reduced during E2 treatment. Both ERα and ERβ were expressed in these cell lines, and the result of bioinformatics analysis of gastric cancer from GEO datasets indicated that the expression levels of both ERα and ERβ were significantly higher in noncancerous gastric tissues than in gastric cancer tissues. Our research indicated that estrogen can reduce cell viability and promote apoptosis in gastric cancer cells directly; ERs expression level is associated with gastric cancer. Our research will help to understand the mechanism of gender disparity in gastric cancer.

Keywords Gender disparity · Estrogen · Estrogen receptor · Apoptosis · PEG10

Introduction

Over the last few decades, the incidence and mortality of gastric cancer have substantially declined in a few countries [1]. Nevertheless, it remains the fourth most common cancer and one of the major causes of death from cancer [2]. The results from epidemiological researches reveal that the incidence rate of gastric cancer is about twice higher for men than for women [2, 3]. Delayed menopause, longer fertility life, and hormone therapies are reported to be associated with the decreased risk in gastric cancer [4]. Women treated with tamoxifen (TAM), an antagonist of estrogen signaling, will have a relative high risk of developing gastric cancer [4]. The estrogen therapy for prostate cancer reduces the risk for gastric cancer in humans [5]. These researches suggest that the male predominance of gastric cancer cannot be simply attributed to the differences in lifestyles between men and women, such as smoking and dietary habits, the sex hormone, which especially estrogen, may play an important role in the development of this disease. Fox and coauthors [6, 7] have

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demonstrated in mouse model that 17β-Estradriol can suppress the development of *Helicobacter pylori*-induced gastric cancer, and this protective effect may result from the regulation of estrogen on the immune system. But the evidences of the direct biological effect of estrogen on gastric cancer cells are still insufficient.

Sex hormone receptors always play an important role in the progression of gender-related tumors, such as hepatocellular carcinoma (HCC) which has a striking predominance in males [8, 9]. Androgen receptor (AR), rather than androgen, is reported to be important in the development of HCC [10]. The expression pattern of estrogen receptors (ER) has also been widely studied in gastric cancer, but the results are controversial. Some thought that the expression of ERs is correlated with a bad outcome, while the others are quite the opposite [11–14]. The roles that ERs play in the carcinogenesis of gastric cancer still need to be further studied.

Paternally expressed gene 10 (PEG10) is an AR target gene [15]. The elevated PEG10 expression was found in majority of the human HCC cells and G2/M phase of regenerating mouse liver, which indicated that this gene is related to proliferation [15–17]. The imbalance between the expression of PEG10 and SIAH1, a mediator of apoptosis, may be involved in hepatocarcinogenesis through the inhibition of apoptosis [18]. Elevation of PEG10 expression was also found in B-acute lymphoblastic leukemia $CD23^{+}CD5^{+}$ B cells and related with apoptotic resistance [19]. We previously found that the high expression level of PEG10 can reduce the pro-apoptotic effect of 5-fluorouracil (5-FU), and the expression level of PEG10 was correlated with the expression levels of MMP2 and MMP9 in Raji cells [20]. These researches indicate that PEG10 is an anti-apoptotic protein and plays an oncogenic role in cancer development.

In the present study, we use two cell lines SGC7901 and BGC823 to study the direct effect of estrogen on gastric cancer cells. Our results indicated that estrogen can reduce cell viability, promote apoptosis in both gastric cancer cells directly. The expression level of PEG10, an AR target gene, was reduced during E2 treatment. The primary estrogen receptors ERα and ERβ were both expressed in these cell lines, and the result of our bioinformatics analysis indicated that the expression levels of ERα and ERβ were significantly associated with gastric cancer. Our research will help to understand the mechanism of gender disparity in gastric cancer.

Methods and materials

Cell culture and reagents

Human gastric cancer cell lines SGC7901 and BGC823 (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI1640 containing 10 % fetal bovine serum (FBS) in a humidified atmosphere of 5 % CO_2 at 37 °C. Prior to the treatment with β-estradiol (E2, Sigma, E2758), the culture medium was changed into RPMI1640 containing 1 % FBS. E2 was dissolved in ethanol and then added to the medium to a final concentration of 0, 0.1, 1, 10, and 100 nM. After incubated for 24 or 48 h, cells were harvested and analyzed in the following experiments.

Western blotting

After the treatment with E2, the whole-cell extract was prepared by scraping them in RIPA buffer. Proteins were separated by 12 % SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with non-fat milk and incubated with primary antibodies overnight at 4 °C. Secondary antibodies (Goat anti-Rabbit IRDye 800CW, 926-32211, LI-COR Biosciences) were used to detect binding of the primary antibodies by incubation at room temperature for 50 min. The bands were detected using the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE). The primary antibodies used in this study were as follows: β-actin (CST, USA, #4970), Caspase 3 (CST, USA, #9665), phosphory-NF-κB (CST, USA, #3033), Bcl-2 (Boster, China, BA0412), Bcl-xL (Boster, China, BA0414), and MMP9 (Biolegend, USA, 635002).

RNA isolation and reverse transcriptional PCR

The total RNA of each sample was extracted by TRIZOL (Invitrogen, USA) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. RT-PCR was performed using the following conditions: 94 °C for 5 min; 32 cycles at 94 °C for 30 s; annealing at 58 °C for 30 s; elongation at 72 °C for 30 s; and finally at 72 °C for 10 min. PCR products were identified using 1.2 % agarose gel electrophoresis and visualized under ultraviolet light after staining with Ethidium Bromide. Primer pairs for human ERα [21], ERβ, PEG10, MMP9, and β-actin were as follows:

ERα
Sense: 5′-AGACATGAGAGCTGCCAACC-3′;
Antisense: 5′-GCCAGGCACATTCTAGAAGG-3′.
ERβ
Sense: 5′-TCTCCTTTAGTGGTCCATCGC-3′;
Antisense: 5′-AAGTGAGCATCCCTCTTTGAAC-3′.
PEG10
Sense: 5′-CCCATCCTTCCTGTCTTCG-3′;
Antisense: 5′-TCCCTTCTTCGTTCGGTCA-3′.

MMP-9
Sense: 5′-GAGGTTCGACGTGAAGGCGCAGATG-3′;
Antisense: 5′-CATAGGTCACGTAGCCCACTTGGTC-3′
β-actin
Sense: 5′-CCTGGACTTCGAGCAAGAGA-3′;
Antisense: 5′-TGATCTCCTTCTGCATCCTGT-3′.

Cell viability assay

Cell viability assay was performed to analyze the cytotoxic potential of estrogen to gastric cancer cells. Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used for this assay. Briefly, dispense 100 μl of cell suspension (5,000 cells/well) in a 96-well plate and maintained at 37 °C in a humidified incubator. The next day, indicated concentration of E2 was added to the culture medium as mentioned previously. After 24 or 48 h of treatment, 10 μl of the CCK-8 solution was added to each well of the plate. The plate was incubated at 37 °C for 1 h, and the absorbance at 450 nm was measured by microplate reader (VICTOR X, Perkin Elmer) to calculate the number of vital cells in each well. Measurements were performed in triplicate, and the mean [±standard deviation (SD)] optical density (OD) was reported.

Caspase 3 activity assay

Cells were treated with indicated concentration of E2, and the Caspase 3 activity was measured by using Caspase 3 Activity Assay Kit (Beyotime Biotech, China). Cell lysates were prepared by incubating 2×10^6 cells/ml in extraction buffer for 30 min on ice. Then the lysates were centrifuged at $16{,}000g \times 15$ min, the supernatants were collected, and the protein concentration was determined by BCA protein assay. Cellular extracts (30 μg) were incubated with 10 μl Ac-DEVD-*p*NA in a 96-well microplate for 3 h at 37 °C. Activated Caspase 3 catalyzes the substrate Ac-DEVD-*p*NA to generate *p*-nitroaniline (*p*NA), which has strong absorption at 405 nm. Then the absorbance at 405 nm was measured by microplate reader to reflect the activity of Caspase 3. Measurements were performed in triplicate, and the Mean (±SD) OD was reported.

Flow cytometry

The Annexin V/PI apoptosis kit was used for the detection of apoptosis. Briefly, after 24 h treatment with E2 as described above, SGC7901 was double stained with Annexin V-FITC and propidium iodide (PI) and incubated at room temperature for 5 min in dark. Ten thousand cells from each group were analyzed by flow cytometer (FACS Calibur, Becton–Dickinson, USA) at laser emitting excitation light, 488 nm.

Statistical analysis

Experiments were performed and repeated at least three times. The data are expressed as the mean ± SD. Statistical significance differences among the groups were determined by One-way ANOVA using the SPSS program and were reported as * $p < 0.05$ or # $p < 0.01$. A p value of < 0.05 was accepted as statistically significant.

Bioinformatics analysis

GEO datasets of the unpaired normal and cancer samples of gastric cancer (GSE2685) were obtained from NCBI GEO database. The analysis of expression levels of ERα and ERβ in gastric cancer and noncancerous tissues was performed with DNA-chip analyzer and gene set enrichment analysis (GSEA) software. Student's *t* test was used to compare the differences of ERs expression levels between gastric cancer and noncancerous gastric tissues. The significance level for all statistical tests was $p < 0.05$; the significance of enrichment gene sets in GSEA was $p < 0.05$, and false discovery rates < 0.25.

Results

E2 reduced the viability of gastric cancer cells

Cell viability assay is an important and convenient method to examine cell cytotoxicity of substances. In order to understand the effect of estrogen on gastric cells, SGC7901 and BGC823 were treated with E2 at different concentrations for 24 h (with 0, 0.1, 1, 10, and 100 nM) or 48 h (with 0, 0.1, 1, and 10 nM); then CCK-8 was used to analyze whether the viability of these cells were affected by E2. As shown in Fig. 1, when the E2 concentration was lower than 1 nM, the viability of neither of these two cells was affected obviously. The viability began to reduce when SGC7901 was treated with 1 nM E2 for 24 h and BGC823 was treated with 1 nM E2 for 48 h ($p < 0.05$). When E2 concentration reached 10 nM, either the treatment period was 24 or 48 h, the viability of both cells was significantly decreased. The viability of both SGC7901 and BGC823 was decreased by nearly 50 % as compared to the 0 nM E2 group when treated with 100 nM E2 for 24 or 48 h (for both cell lines, $p < 0.01$). These results indicated that E2 could reduce the viability of gastric cancer cells directly.

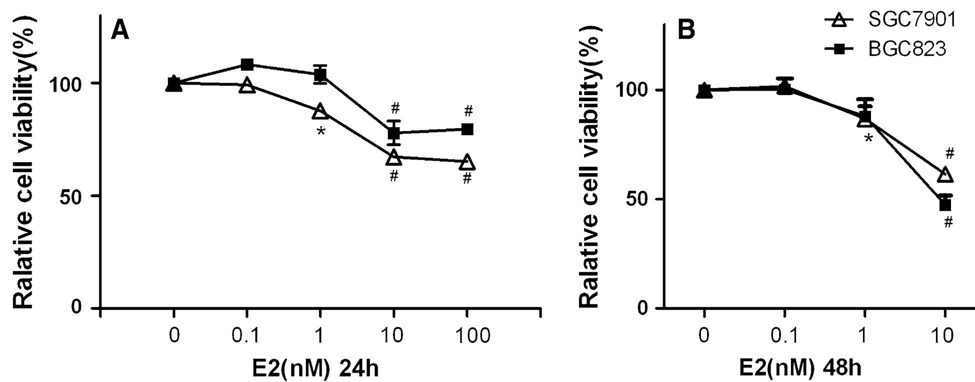

Fig. 1 E2 reduced cell viability in gastric cancer cells. Gastric cancer cells SGC7901 and BGC823 were incubated with serial concentrations of E2 for 24 or 48 h, and then cell viability was analyzed by CCK8 assay. **a** and **b** represents 24 and 48 h E2 treatment, respectively. Data represent the mean values of the relative cell viability as compared with E2 0 nM group (mean ± SD, $n = 3$). * and # indicate statistically significant p values <0.05 and <0.01 as compared with E2 0 nM group, respectively

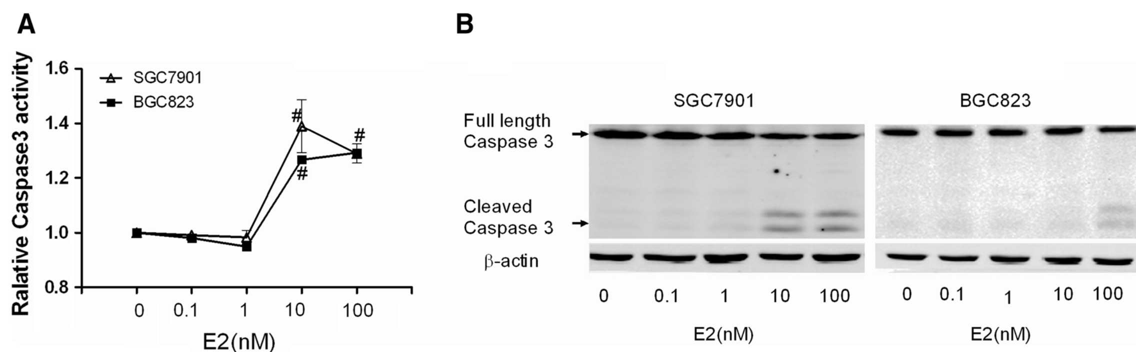

Fig. 2 Caspase 3 was activated in gastric cancer cells by E2 treatment. **a** Gastric cancer cells were harvested after 24 h treatment of various concentrations of E2, and Caspase 3 activities were measured. Data represent the mean values of Caspase 3 activity as compared with E2 0 nM group (mean ± SD, $n = 3$). # indicates statistically significant $p < 0.01$ as compared with E2 0 nM group. **b** Whole-cell lysates were extracted from SGC7901 and BGC823 treated with increasing concentrations of E2 (0, 0.1, 1, 10, and 100 nM) for 24 h was used for Western Blot analysis. When subjected to Western blotting, bands were detected by Caspase 3 and β-actin antibodies

Caspase 3 was activated by E2 treatment

To figure out whether the reduction of cell viability was due to apoptosis which was triggered by E2 treatment in gastric cancer cells, the activity of Caspase 3 was measured by the expression levels of *p*-nitroanilide cleaved from N–Ac-DEVD-*p*NA (Fig. 2a). When SGC7901 and BGC823 were treated with 10 nM E2 for 24 h, Caspase 3 activities of both the cell lines were significantly increased compared to the 0 nM E2 groups. To further confirm the activation of Caspase 3, Western blotting was performed to detect the cleaved Caspase 3 fragments. Our results showed that when the concentration of E2 was raised to 10 nM, the expression levels of full length Caspase 3 were reduced and the fragments of cleaved Caspase 3 began to be detected, although the bands of BGC823 were weak at this concentration of E2 (Fig. 2b). When the concentration of E2 rose to 100 nM, the bands of cleaved Caspase 3 were strongly detected in both cell lines. The results of Western blotting were consistent with the Caspase 3 activity assay. Our results indicated that Caspase 3 was activated, and apoptosis might be triggered by the treatment of E2.

E2 facilitated apoptosis in gastric cancer cells

We performed Annexin V/PI double staining to assess the apoptotic population of E2-treated SGC7901 by flow cytometry. As shown in Fig. 3, the survival rate decreased (Annexin $V^{-}PI^{-}$ and Annexin $V^{-}PI^{-}$) and apoptotic cells increased in response to the increasing concentrations of E2. The rate of the total apoptotic cells (Annexin $V^{+}PI^{+}$ and Annexin $V^{+}PI^{-}$) was compared among five groups treated with different concentrations of E2. It was noted that when the concentration of E2 rose to 100 nM, the rate of total apoptotic cells was almost three times more than the other groups (Fig. 3). We also examined the expression

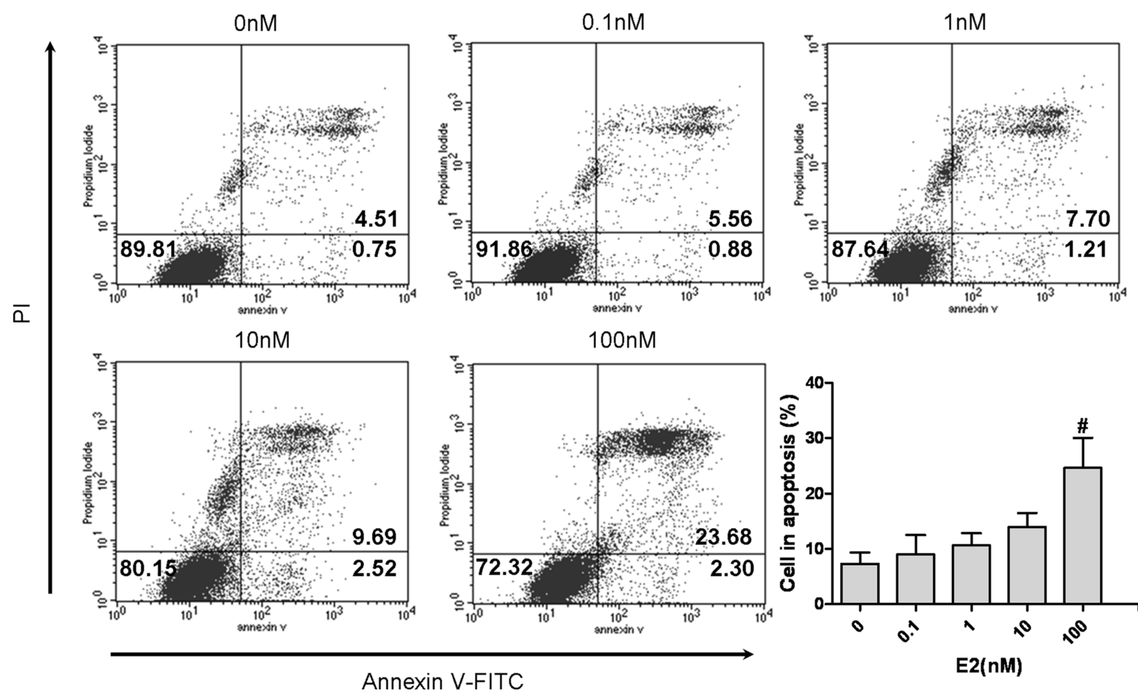

Fig. 3 E2-induced apoptosis in SGC7901. Apoptosis in SGC7901 was assessed after 24 h treatment with the indicated concentrations of E2 by double staining with Annexin V-FITC/PI. *Numbers* indicate the percentage of cells in each quadrant by flow cytometry analysis. The rate of the total apoptotic cells (Annexin V^+PI^- and Annexin V^+PI^+) was compared, and the result was exhibited in the *bar* figure. # indicate statistically significant $p < 0.01$ as compared with E2 0 nM group

levels of anti-apoptotic proteins (Bcl-2 and Bcl-xL) by Western blotting and found that the expression levels of both Bcl-2 and Bcl-xL were strongly reduced when E2 concentrations increased (Fig. 4). The expression levels of Bcl-2 and Bcl-xL begun to decrease in 10 nM group when E2 treatment period expanded from 24 to 48 h. The interaction between anti-apoptotic and pro-apoptotic proteins of Bcl-2 family is critical for intrinsic apoptosis pathway. Our result suggested that E2 could reduce cell viability by inducing apoptosis in gastric cancer cells directly, and intrinsic apoptotic pathway regulated by Bcl-2 might be responsible for this effect.

Expression levels of PEG10 and MMP9 were reduced

Our and other groups' previous researches have proved that PEG10 is closely related to apoptosis [18]. We also demonstrated that PEG10 has the potential to regulate invasion by affecting the expression levels of MMP2 and MMP9 in Raji cells [20]. To figure out whether PEG10 was involved in the apoptosis induced by E2, we analyzed the mRNA expression levels of PEG10 in E2-treated gastric cancer cells. The results showed that PEG10 was expressed in both SGC7901 and BGC823 cells, and its expression levels were gradually declined as the concentration of E2 increased (Fig. 5). In addition, we measured the expression levels of MMP9. The results showed that both mRNA and protein levels of MMP9 were declined in a dose-dependent manner in both cell lines. MMP-9 functioned as one of the most important enzymes that degrade the extracellular matrix structures of tissue and blood vessels and was thought to be associated with tumor transmigration [22, 23]. In the present research, we demonstrated that E2 treatment has the potential to reduce the expression level of MMP9, which suggested that E2 might reduce the invasion ability of gastric cancer cells, and PEG10 might play a role in the promotion of apoptosis and down-regulation of MMP9 by E2 treatment.

Estrogen receptors' expressions are related with gastric cancer progression

There are two primary forms of ERs, usually referred to as α and β. In the present research, we examined the mRNA expression level of ERα and ERβ on E2-treated SGC7901 and BGC823. We found that the two receptors of estrogen were consistently expressed in both the cell lines without influence on the expression level by E2 treatment (Fig. 6a). To further investigate the relationships between ERs and the carcinogenesis and progression of gastric cancer, the bioinformatics analysis was performed, and the microarray database obtained from NCBI

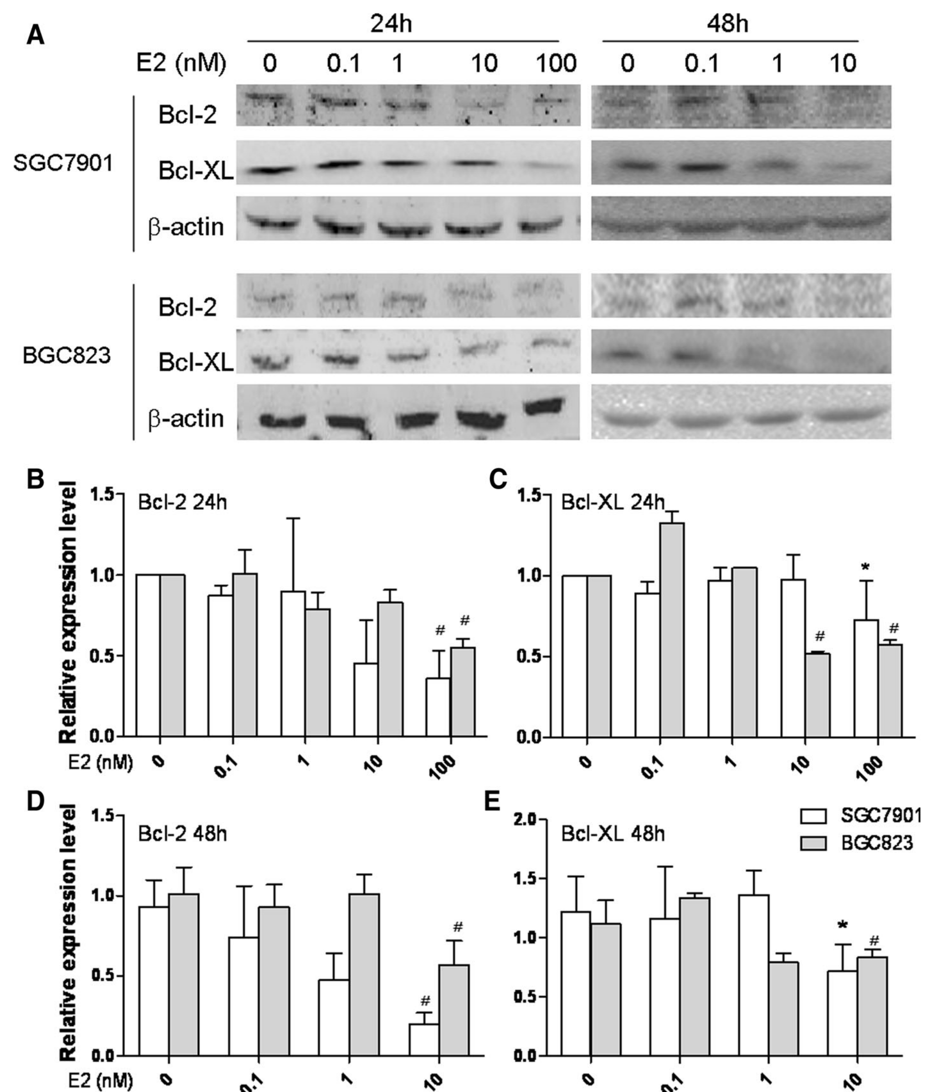

Fig. 4 Expression levels of Bcl-2 and Bcl-xL were reduced. **a** Total extracts from SGC7901 and BGC823 treated with increasing concentrations of E2 for 24 or 48 h were used for Western Blot analysis. The expression levels of Bcl-2 and Bcl-xL of were examined. **b–e** The *bars* represented the relative expression levels of Bcl-2 and Bcl-xL, and the values were normalized to β-actin. * and # indicate statistically significant *p* values <0.05 and <0.01 as compared with E2 0 nM group, respectively. For all *bar* figures, *white bars* represented SGC7901 and *gray bars* represented BGC823

GEO datasets (GSE2685) was analyzed. Twenty-two gastric cancer advanced tissues and 8 noncancerous tissues were analyzed in this microarray, and some of the cancer tissues were diagnosed to accompany lymph node metastasis. The result of our bioinformatics analysis revealed that the expression levels of both ERα and ERβ in gastric cancer tissues were significantly lower than that in noncancerous gastric tissues (for ERα and ERβ, $p < 0.01$, Fig. 6). Previous research reported that ERs may take effect by negatively interfering with NF-κB transcriptional activity [24], and transcription activity of NF-κB was mainly controlled by phosphorylation of p65, such as Ser-536 [25, 26]. We found that the expression level of phosphorylated p65 (Ser-536) was declined after 24 h E2 treatment. These results indicated that the expression level of ERs is closely associated with gastric cancer, and the activation of ERs might have the protective effect against gastric cancer progression.

Discussion

It has been proved that Estradiol can rapidly increase proliferation and attenuated apoptosis by activating signaling transduction pathways of ERs in cancer cells, such as breast cancer cells [27–30]. In this article, we focused on the anti-proliferative and pro-apoptotic effects of E2 in human gastric cancer cells. Epidemiology researches suggest that sex hormone, especially estrogen, may play an important role in the pathology of gastric cancer [2–5]. Recently, Fox et al. [6, 7] have demonstrated in mouse model that estrogen plays a protective role in gastric cancer development by regulating the function of immune system. The direct effect of estrogen in gastric cancer is still unclear. In this study, we propose that E2 has the potential ability to attenuate tumor malignancy by inducing apoptosis and reducing cell viability in gastric cancer cells directly.

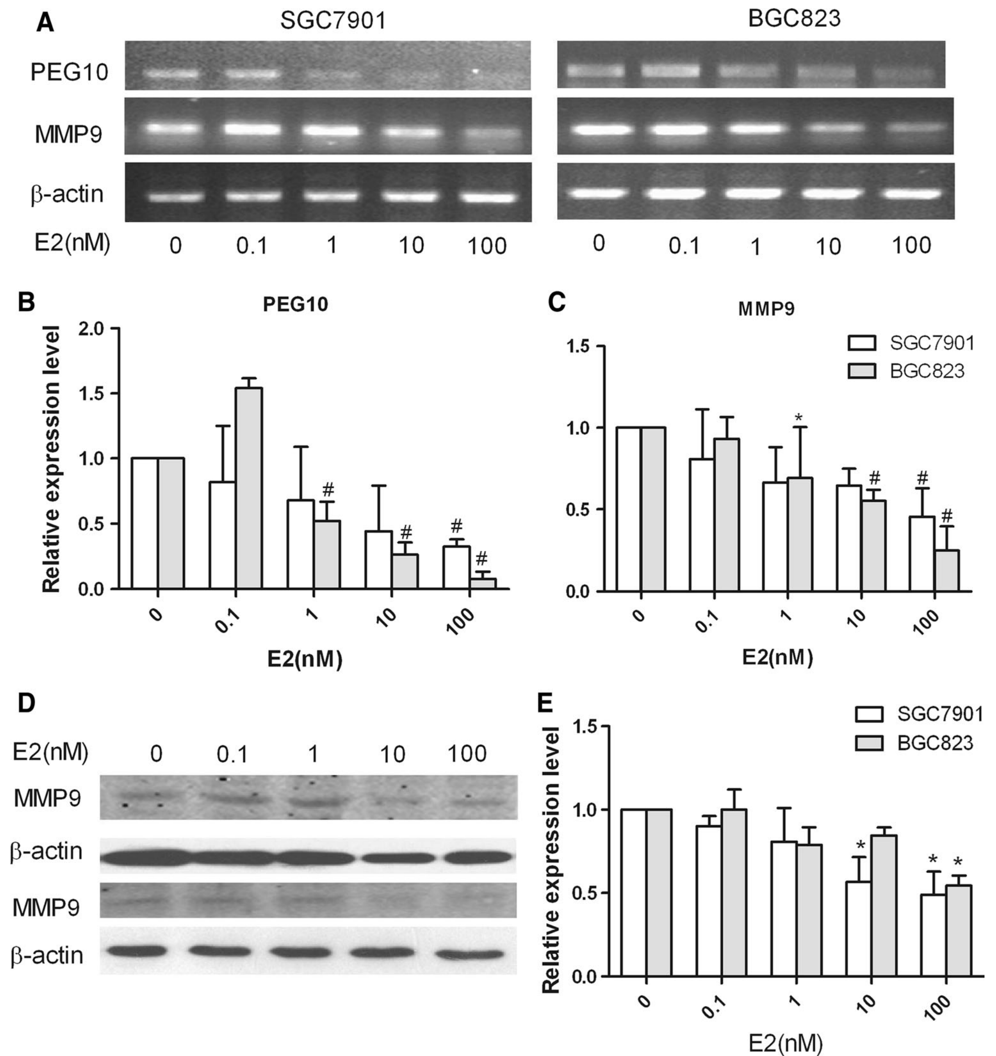

Fig. 5 E2 attenuates expression levels of PEG10 and MMP9 in gastric cancer cells. **a** After 24 h treatment of serial concentrations of E2, total mRNA was extracted from both SGC7901 and BGC823 cell. The mRNA levels of PEG10 and MMP9 were examined. **b** and **c** The bars represented the relative mRNA expression levels of PEG10 and MMP9 normalized to β-actin. **d** Western Blot analysis of MMP9 expression level after 24 h treatment of serial concentrations of E2, the *upper two panels* and the *lower two panels* represented SGC7901 and BGC823, respectively. **e** The *bars* represented the relative expression level of MMP9 normalized to β-actin. * and # indicate statistically significant p values <0.05 and <0.01 as compared with E2 0 nM group, respectively. For all *bar* figures, *white bars* represented SGC7901 and *gray bars* represented BGC823

The results of cell viability assay indicated that E2 decreased the survival rate in both SGC7901 and BGC823 cell lines. In the following experiments, we demonstrated that the decreased survival rate was due to apoptosis triggered by E2. Caspase 3, a pivotal protease in apoptosis, was activated by the treatment of E2. Previous researches have proved that when Caspase 3 is activated, a serious irreversible events leading to cell apoptosis will be set on [31]. Flow cytometry showed that the total rate of early apoptosis (Annexin V^{+}/PI^{-}) and late apoptosis (Annexin V^{+}/PI^{+}) increased in a dose-dependent manner in E2-treated SGC7901. Decreased expression levels of anti-apoptotic proteins Bcl-2 and Bcl-xL were observed in both the cell lines. Collectively, these results revealed that estrogen could reduce cell viability and induce apoptosis in gastric cancer cells directly, and the intrinsic mitochondrial pathway regulated by Bcl-2 might be involved in this effect. The expression level of the phosphorylated p65 was reduced by E2 treatment, which could decrease the transcriptional activity of NF-κB p65 on its target genes such as Bcl-2, Bcl-xL, and MMP9 [32, 33].

Since the activation of ERs is important for the signal transduction of E2, lots of researches have been done on the relationship between expression pattern of ERs and gastric cancer progression. It has been reported that overexpression of ERα could effectively inhibit cell growth and cancer progression in gastric cancer [34]. On the other hand, the presence of ERβ in gastric cancer seems to be correlated with a better 3-year survival rate [11, 13, 14]. In the present research, our bioinformatics analysis revealed that expression levels of both ERα and ERβ were relatively higher in noncancerous tissues than in cancer tissues. This result indicated that higher ERs expression levels might have protect effects in gastric cancer progression. It is regret that microarray databases about gastric cancer were limited, and in database GSE2685, there were only 22 samples; the sample size was relatively small. Our bioinformatics analysis needs larger sample size of gastric

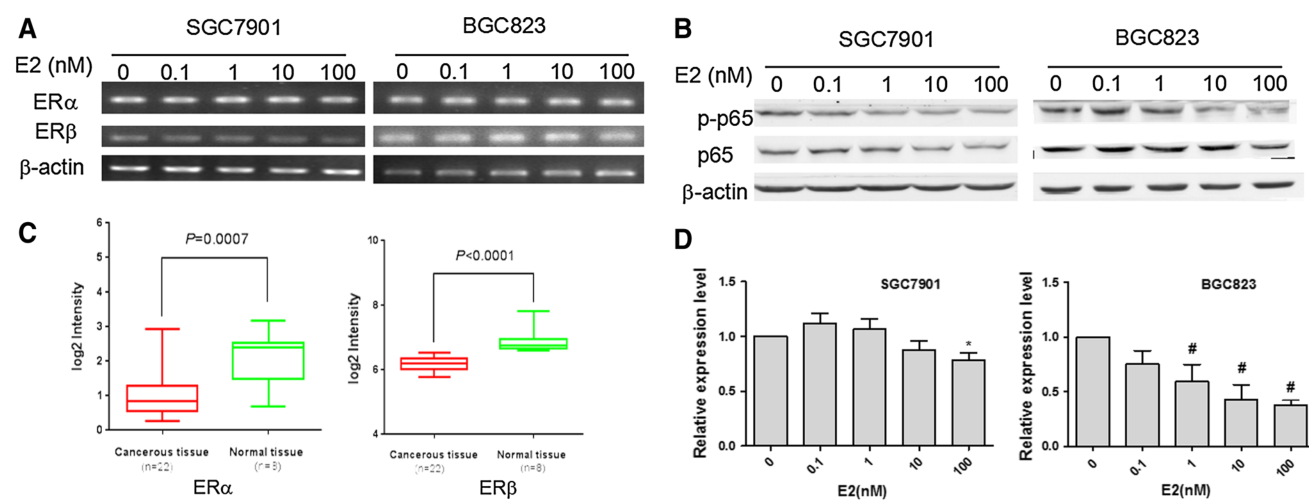

Fig. 6 Estrogen receptors expressions are related with gastric cancer progression. **a** Total RNA was extracted from each cell lines after 24 h of treatment of indicated concentrations of E2 and mRNA expression level of ERα and ERβ were examined. ERα and ERβ were expressed in both SGC7901 and BGC 823 cells. **b** Whole-cell lysates of gastric cancer cells after 24 h treatment of serial concentrations of E2 were used for Western Blot analysis. Phosphory-p65 was detected. **c** Bioinformatics analysis was performed to analyze the association of the expression level of ERα and ERβ in gastric cancer (data from GSE2685, unpaired samples). The expression levels of both ERα and ERβ were significantly associated with gastric cancer. **d** The *bars* represented the relative expression level of phosphorylated p65 and the values were normalized to β-actin. * and # indicate statistically significant p values <0.05 and <0.01 as compared with E2 0 nM group, respectively

cancer to confirm. More importantly, ERα and ERβ are the primary plasma receptors of estrogen; there are variants of ERα, such as ERα36, which also play an important role that cannot be despised. Whether and which ERs were associated with gastric cancer progression still needs further investigation, such as functional analysis. A detailed investigation of the roles of ERα, ERβ, and all of their variants will be critical for interpreting gender disparity in gastric cancer.

The pro-apoptotic effect of E2 is also reported in HCC [35], which has a higher incidence and worse prognosis in males than in females, too [8, 9]. It has been reported that the activation of AR could promote HCC development [10], while our and other groups' researches suggested that E2 has the potential to attenuate malignancy phenotype in gastric cancer and HCC [2–5]. PEG10 was reported to be an oncogene in HCC development by promoting proliferation and apoptosis resistance [16–18]. It is interesting to notice that AR and estrogen regulated PEG10 expression in an opposite direction. AR could increase PEG10 expression level by directly binding to PEG10 promotor region [15], and in the present research we demonstrated that E2 had the potential ability to reduce PEG10 expression in both the cell lines. It will be important to find out what role PEG10 plays in gender disparity in both liver and gastric cancer development. We suggest that a further investigation of the relationships between PEG10 and sex hormones will be helpful for understanding the development of gender-related cancers.

In conclusion, our research provides a preliminary evidence of the important role of estrogen in gastric cancer progression. In this research, we observed that E2 could directly reduce cell viability and induce apoptosis in gastric cancer cells. The levels of E2 and ERs expression might affect gastric cancer progression. The decreased expression level of PEG10, an AR-targeted oncogene, was an important event in this process. Our study will help to further understand human gastric carcinogenesis.

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