ERα increases endometrial cancer cell resistance to cisplatin via upregulation of BAG3

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Abstract. Endometrial cancer is a leading cause of cancer-associated mortality in women and has a poor prognosis in advanced stages. Our previous study revealed that BCL-2-associated athanogene 3 (BAG3) may contribute to enhancing cell viability through downregulation of microRNA (miR)-29b in endometrial cancer cell lines. In addition, a relationship between estrogen receptor α (ERα) and BAG3 was recently reported in several cancer cell types. The present study investigated the relationship between ERα and BAG3 in endometrial cancer cell lines. The results demonstrated that exogenous ERα overexpression enhanced BAG3 expression in the EMTOKA endometrial cancer cell line, which does not endogenously express ERα, but had no effect on BAG3 expression levels in the Ishikawa cell line, which does endogenously express ERα. In addition, ERα overexpression suppressed miR-29b expression and enhanced the expression of Mcl-1, a mediator situated downstream of BAG3, in EMTOKA cells, but not Ishikawa cells. ERα overexpression also enhanced EMTOKA, but not Ishikawa, endometrial cancer cell viability in the presence of cisplatin. These findings suggested that ERα may contribute to enhancing endometrial cancer cell resistance to anticancer agents through BAG3 overexpression.

Introduction

Endometrial cancer is the sixth most commonly diagnosed cancer and the 14th leading cause of cancer death among women worldwide (1). Moreover, the incidence of endometrial cancer has been rising in recent years. Treatments for endometrial cancer include surgery, chemotherapy, radiotherapy, and/or hormone therapy, depending on the disease stage and histologic type. When diagnosed at an early stage, surgery generally entails hysterectomy with or without bilateral salpingo-oophorectomy; at advanced stages, lymph node dissection is also performed. In the past, these surgeries were performed abdominally. In recent years, however, laparoscopic or vaginal surgery, which are less invasive, is often selected for early stage cancers (2). When diagnosed early, endometrial cancer is treatable, but at more advanced stages, it is often fatal. The 5-year survival rate is 95.3% if diagnosed at an early stage, but it is 67.5% when diagnosed at stage III and 16.9% when diagnosed at stage IV (3).

More than 80% of endometrial cancers are estrogen-related (4). This suggests the rising incidence in endometrial cancer may be related to the increasing use of exogenous estrogen as well as to increased exposure of the uterus to endogenous estrogen (nulliparity, fewer pregnancies, earlier age at menarche, and obesity) (5). To exert is effects, estrogen binds to estrogen receptors (ERs) in the nucleus. The ER is a ligand-dependent transcription factor that regulates transcription of target genes after binding estrogen. ERs are encoded by two separate genes, the products of which are ERα and ERβ (6). ERα is known to be highly expressed in certain endometrial and breast cancers, and is thought to play a role in regulating the expression of genes involved in cell proliferation, apoptosis, and differentiation. Activation of ERα promotes cell growth and antagonizes the sensitivity of ovarian cancer cells to chemotherapeutic agents (7).

BAG3 (hsp70 co-chaperone) is a stress-induced anti-apoptotic protein that is reportedly involved in such cell functions as proliferation, apoptosis, adhesion, and migration. We previously showed that in endometrial cancer cell lines, BAG3 enhances cell migration and invasiveness through downregulation of microRNA-29b (miR-29b) (8). Felzen *et al* showed that in human neuroblastoma cell lines, ERα-expressing cells exhibit higher levels of autophagy than cells not expressing ERα, and that this receptor regulates a non-canonical autophagy pathway involving BAG3 (9). In addition, Brendel *et al* showed that ERα-expressing human neuroblastoma cells are more resistant to apoptosis and express higher levels of BAG3 than human neuroblastoma cells not expressing the receptor (10).

MicroRNAs (miRNAs) are small non-coding RNAs that function as negative regulators of gene expression by targeting mRNAs based on their complementarity to the mRNA 3' untranslated region (3'-UTR) (11). Through this action, miRNAs play various roles during carcinogenesis, functioning

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as tumor suppressors or oncogenes (12). As mentioned above, BAG3 enhances the malignant behavior of endometrial cancer cells by suppressing miR-29b expression (8). On the other hand, in other cancer cells, miR-29b contributes to the acquisition of resistance to anticancer drugs and apoptosis through upregulation of Mcl-1, a survival-promoting protein with anti-apoptotic activity (13,14).

In the context of the relationship between ERα and BAG3 in endometrial cancer cell lines, here we also focused on the relationship among ERα, BAG3, miR-29b and Mcl-1, which is situated downstream of BAG3. Our findings provide further insight into the relationship and function of ERα and BAG3 in endometrial cancer cells.

Materials and methods

Cells and cell culture. Four established uterine cancer cell lines and one breast cancer cell line were used in this study. All cells were obtained from National Institutes of Biomedical Innovation, Health, and Nutrition, JCRB cell bank (Tokyo, Japan). Mycoplasma testing was done for all cell lines. The Ishikawa cell line was established from a grade I endometrial carcinoma. The HEC-1-B cell line was established from a grade II endometrial carcinoma, the SNG-II line from an endometrial carcinoma, the EMTOKA line from a carcinosarcoma, and the MCF-7 line from a human breast adenocarcinoma. MCF-7 cells were used as a positive control in western blot analyses. MCF-7, Ishikawa and HEC-1-B cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.), SNG-II cells in Ham's F12 medium (Thermo Fisher Scientific, Inc.), and EMTOKA cells in Roswell Park Memorial Institute (PRMI) medium (Thermo Fisher Scientific). All media were supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.). All cell lines were maintained in a CO_2 incubator (5% CO_2) at 37˚C. Cell culture was performed according to Good Cell Culture Practice (GCCP), paying sufficient attention to infection. This study focused mainly on EMTOKA cells, which is a cell line established from uterine tumors from a 64-year-old Japanese woman who underwent a simple hysterectomy in 1989. Pathologic examination of the cultured material showed papillary and tubular adenocarcinoma (carcinomatous elements) and spindle shaped fiber cells and chondrosarcoma (sarcomatous element). EMTOKA cells show at least five cell types, which include columnar cells, small epithelial cells, moderately sized or large epithelial like cells, malignant tumor giant cells, and spindle cells (15).

ERα overexpression. pcDNA 3.1(+) was obtained from Addgene (Watertown, MA, USA). After cleaving the plasmid with Kpn I (Takara Bio Inc.) and Bam HI (Takara Bio Inc), ERα DNA was inserted using DNA Ligation Kit Mighty Mix (Takara Bio Inc) according to the manufacturer's protocol, yielding pcDNA-ERα. Ishikawa and EMTOKA cells were transfected with the expression vector pcDNA-ERα or with empty pcDNA vector (control) using Lipofectamine 3000 regent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 24 h, the cells were split and allowed to adhere overnight.

Reverse transcription-quantitative PCR (RT-qPCR) for mRNA. Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific), after which cDNA was synthesized from 1 μg of RNA using VILO master mix (Thermo Fisher Scientific, Inc.). RT-qPCR was carried out using Fast SYBR Green Master Mix (Thermo Fisher Scientific) in a StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, Inc.). mRNA levels were standardized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The PCR protocol entailed denaturation at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The following primers were designed and used for RT-qPCR: For BAG3, 5'-TGAGAAGTTTAACCCCGT TGCTTGT-3' (forward) and 5'-CCCCATCTACCCCTCCAG TCCAG-3' (reverse); for ERα, 5'-GTGCCAGGCTTTGTG GATTTG-3' (forward) and 5'-GTTACTCATGTGCCTGAT GTG-3' (reverse); for GAPDH, 5'-TGAACGGGAAGCTCA CTGG-3' (forward) and 5'-TCCACCACCCTGTTGCTG TA-3' (reverse). Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

RT-qPCR for microRNA. Total RNA was extracted using TRIzol reagent, after which reverse transcription was performed with 10 ng of total RNA using a TaqMan® MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) and sequence-specific RT primers from the TaqMan MicroRNA assays (Thermo Fisher Scientific, Inc.). Separate reverse transcription reactions were run for each TaqMan MicroRNA assay with each RNA sample. RT-qPCR was performed with cDNA using inventoried TaqMan MicroRNA assays and TaqMan Universal Master Mix II (Thermo Fisher Scientific, Inc.). The assay was performed in triplicate, and the PCR amplification was performed using a StepOnePlus™ Real-Time PCR system. microRNA levels were standardized to the level of RNU48 small-nucleolar RNA. Primer sequences were as follows: miR-29b (assay ID:00413), 5'-UAGCACCAUUUGAAAUCAGUGUU-3' and RNU48 (assay ID:001006), 5'-GATGACCCCAGGTAACTC TGAGTGTGTCGCTGATGCCATCACCGCAGCGCTCTG ACC-3'. Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method.

Lysate production. Cell lysates were produced from subconfluent cell cultures. After scraping the cells from the dishes, they were lysed by sonication in RIPA buffer (Nacalai Tesque) containing a protease inhibitor cocktail (Thermo Fisher Scientific). The lysates were then centrifuged at 17,000 x g for 15 min at 4˚C to pellet the nuclei, and the supernatant was collected as the cell lysate.

Western blotting. After measuring their protein content, lysates were diluted in 2X sample buffer (Sigma-Aldrich,) and boiled for 5 min at 100˚C. Samples containing 30 μg of protein were then electrophoresed (200 V for 35 min) on 12% SDS polyacrylamide gel, after which the separated proteins were transferred onto PVDF membranes. After blocking with 5% non-fat dry milk in TBS [10 mM sodium phosphate (pH 7.8), 150 mM NaCl and 0.05% Tween-20], the membranes were probed with the following primary antibodies: Rabbit monoclonal anti-BAG3 (1:1,000 dilution;

ab92309; Abcam), mouse monoclonal anti-ERα (1:100 dilution; sc-8002; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Mcl-1 (1:1,000 dilution; ab32087; Abcam) and mouse monoclonal anti-β-actin (1:5,000 dilution; A5441; Sigma-Aldritch). After washing with PBS-T, the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized using ECL Prime Western Blotting Detection Reagent and an ImageQuant LAS 500 (GE Healthcare). Western blot bands were semi-quantified using ImageJ (National Institutes of Health).

Cell viability assay. To test the sensitivity of cells to cisplatin under various culture conditions, cells were plated in 96-well plates (5,000 cells/well) in medium containing 5% serum and incubated at 37˚C under a 5% CO_2 atmosphere. After 24 h, the medium was replaced with medium containing the indicated concentration of cisplatin (Fujifilm Wako Chemical Corporation), and the cells were incubated for an additional 48 h. Cell viability was then assessed using a Cell Proliferation Kit II (XTT; Roche Diagnostics). Following the incubation period, 50 μl of XTT labeling mixture was added to each well, and the cells were incubated for 4 h, after which the absorbance at 492 nm was recorded using an ELISA plate reader.

Statistical analysis. Unpaired Student's t-tests were used for statistical evaluation of the data. Values of $P<0.05$ were considered significant. Two-way ANOVA was used for analysis of cell viability assay results, and one-way ANOVA was used for other statistical comparisons. As post hoc tests, Tukey's multiple comparisons test was used for one-way ANOVA and Bonferroni's multiple comparisons test was used for two-way ANOVA. SPSS 22.0 (IBM Corp.) and GraphPad Prism version 8 (GraphPad Software Inc.) were used for analyses.

Results

Expression of ERα and BAG3 in endometrial cancer cell lines. Ishikawa, HEC-1-B, SNG-II, and EMTOKA cells were used for western blot and RT-qPCR analyses. Among the four cell lines, there was a significant difference in BAG3 mRNA expression between Ishikawa and HEC-1-B ($P=0.0058$), Ishikawa and EMTOKA ($P=0.0006$), and SNG-II and EMTOKA ($P=0.0069$), but no significant difference in expression between other cells (Fig. 1A). On the other hand, expression of BAG3 protein was detected more strongly in HEC-1-B and EMTOKA cells, than in Ishikawa or SNG-II cells (Fig. 1C). Expression of ERα mRNA and protein was detected only in Ishikawa cells ($P<0.0001$) (Fig. 1B). In subsequent experiments, therefore, we used Ishikawa cells as representative of endometrial cancer cells expressing ERα and EMTOKA cells as endometrial cancer cells not expressing ERα.

Effect of ERα overexpression on BAG3 expression. To determine the effect of ERα overexpression, Ishikawa and EMTOKA cells were transfected with pcDNA-ERα. In both cell types, exogenous ERα expression led to upregulated expression of BAG3 mRNA (Fig. 2A and B). ERα overexpression also led to upregulated expression of BAG3 protein in EMTOKA cells, but not in Ishikawa cells (Fig. 2C).

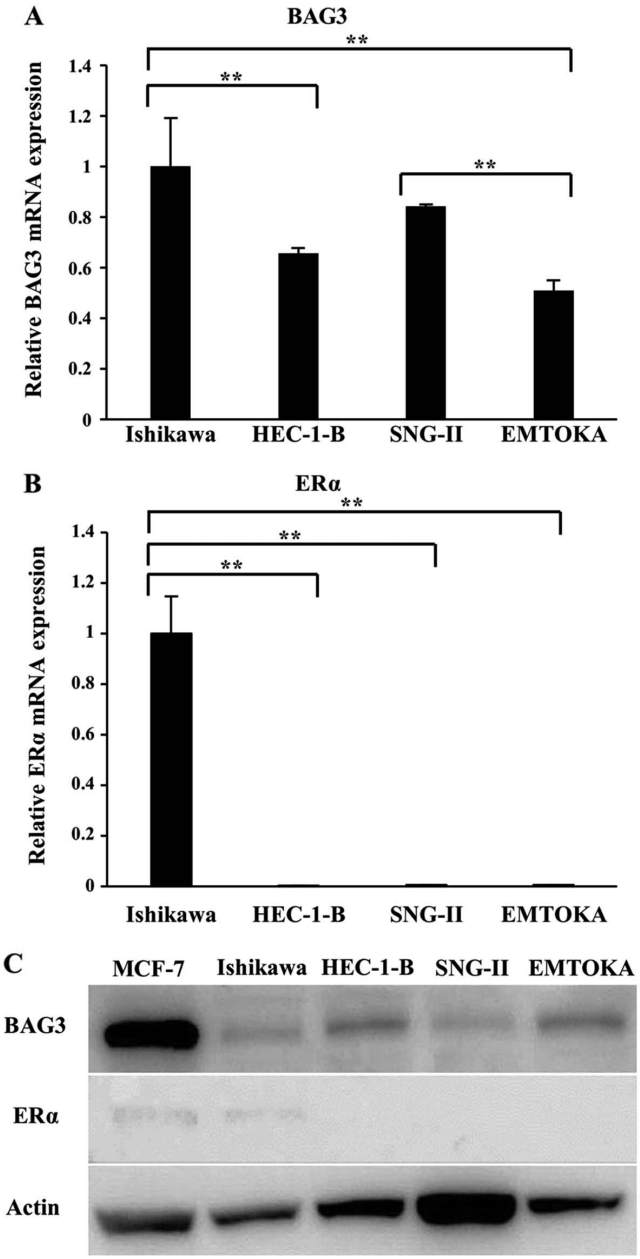

Figure 1. Expression of BAG3 and ERα. RT-qPCR analysis of (A) BAG3 and (B) ERα expression in Ishikawa, HEC-1-B, SNG-2 and EMTOKA cells. Levels of BAG3 and ERα mRNA were determined using real-time RT-qPCR. Bars depict the relative mRNA levels normalized to the level of GAPDH mRNA. The results are presented as means ± SD; $^{**}P<0.01$. (C) Western blot analysis of BAG3, ERα and actin expression in MCF-7, Ishikawa, HEC-1-B, SNG-2 and EMTOKA cells. Blots were probed using a rabbit monoclonal anti-BAG3 or mouse monoclonal anti ERα antibody. As a loading control, the blots were probed using mouse monoclonal anti-actin antibody. MCF-7 cells were used as a positive control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ERα, estrogen receptor α; BAG3, BCL-2-associated athanogene 3.

Effect of ERα overexpression on miR-29b levels. RT-qPCR analysis revealed that in Ishikawa cells, ERα overexpression had no effect on miR-29b expression (Fig. 3A). In EMTOKA

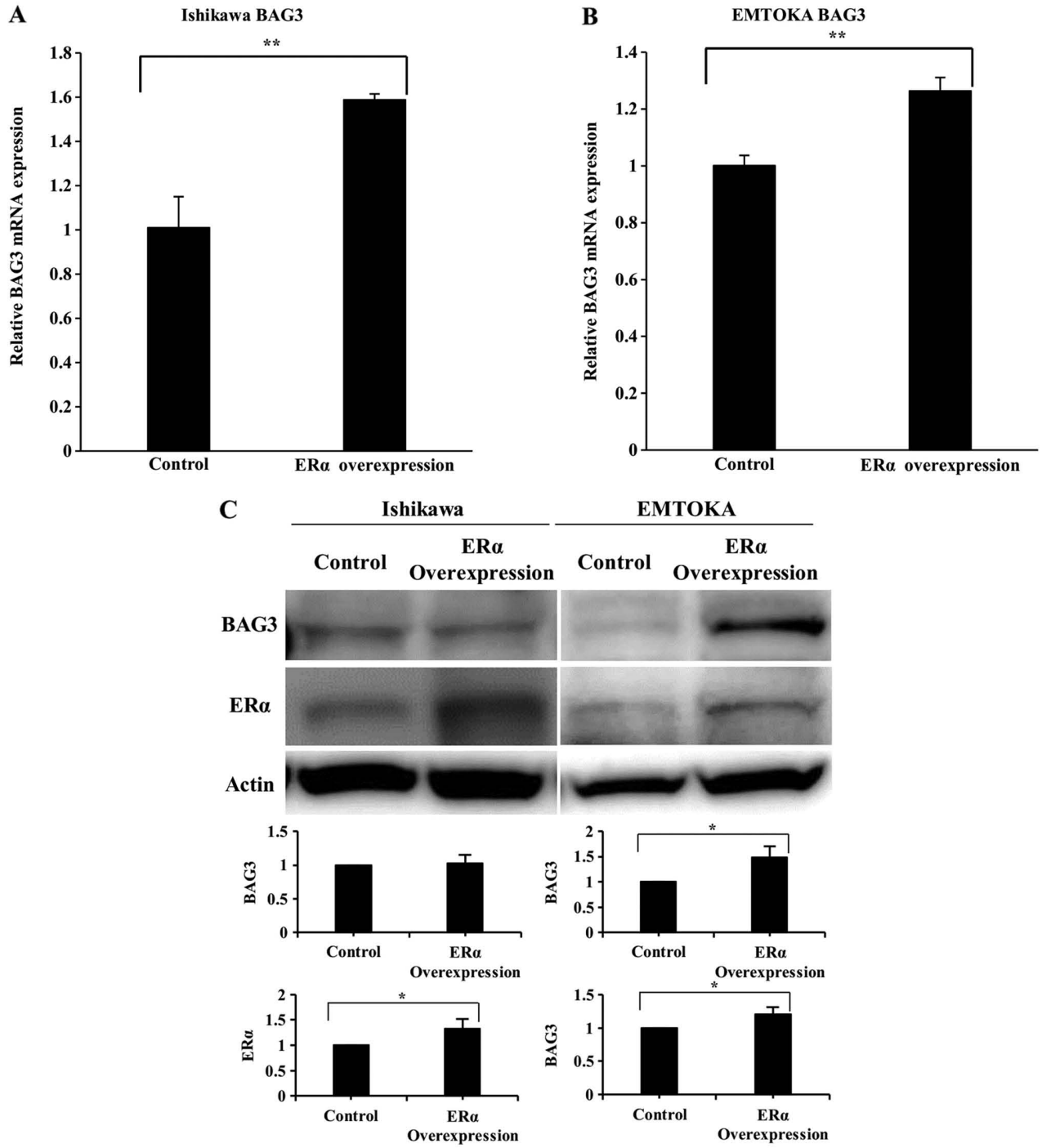

Figure 2. Effect of ERα overexpression on BAG3 levels. (A) Ishikawa and (B) EMTOKA cells were transfected with empty vector (Control) or pcDNA-ERα (ERα overexpression). BAG3 levels were analyzed using reverse transcription-quantitative PCR. Bars depict relative mRNA levels normalized to the level of GAPDH mRNA. (C) Western blot analysis of BAG3 and ERα expression. Total protein lysates from Ishikawa and EMTOKA cells transfected with empty vector (Control) or pcDNA-ERα (ERα overexpression) were analyzed for BAG3 and ERα. Actin served as a loading control. Bands were semi-quantified using ImageJ and actin was used as a control to represent relative protein expression. The results are presented as means ± SD; *P<0.05, **P<0.01. ERα, estrogen receptor α; BAG3, BCL-2-associated athanogene 3.

cells, by contrast, ERα overexpression led to downregulation of miR-29b (Fig. 3B).

Effect of ERα overexpression on expression Mcl-1 protein. In EMTOKA cells, overexpression of ERα led to upregulation of Mcl-1, a mediator situated downstream of BAG3 and miR-29b. In Ishikawa cells, however, overexpression of ERα had no effect on Mcl-1 expression (Fig. 4).

Effect of ERα overexpression on chemosensitivity to cisplatin. Finally, we investigated the effect of ERα overexpression on the viability of cells exposed to cisplatin. We found that after exposure to cisplatin for 48 h, the numbers of viable ERα-overexpressing EMTOKA cells was significantly higher than the number of control cells. On the other hand, ERα overexpression had no effect on Ishikawa cell viability in the presence of cisplatin (Fig. 5).

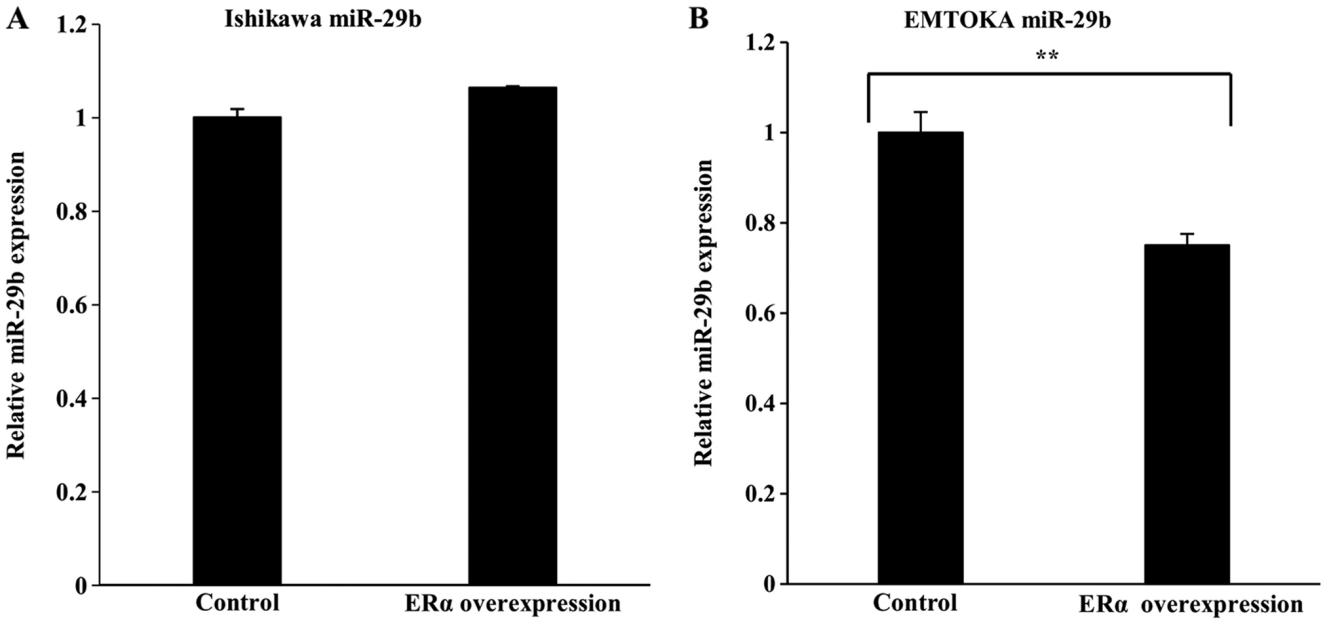

Figure 3. Expression of miR-29b in Ishikawa and EMTOKA cells. Reverse transcription-quantitative PCR analysis of miR-29b expression in (A) Ishikawa and (B) EMTOKA cells transfected with empty vector (Control) or pcDNA-ERα (ERα overexpression). Bars depict relative miRNA levels normalized to the RNU 48 level (internal control). The results are presented as means ± SD; **P<0.01. ERα, estrogen receptor α; miRNA/miR, microRNA.

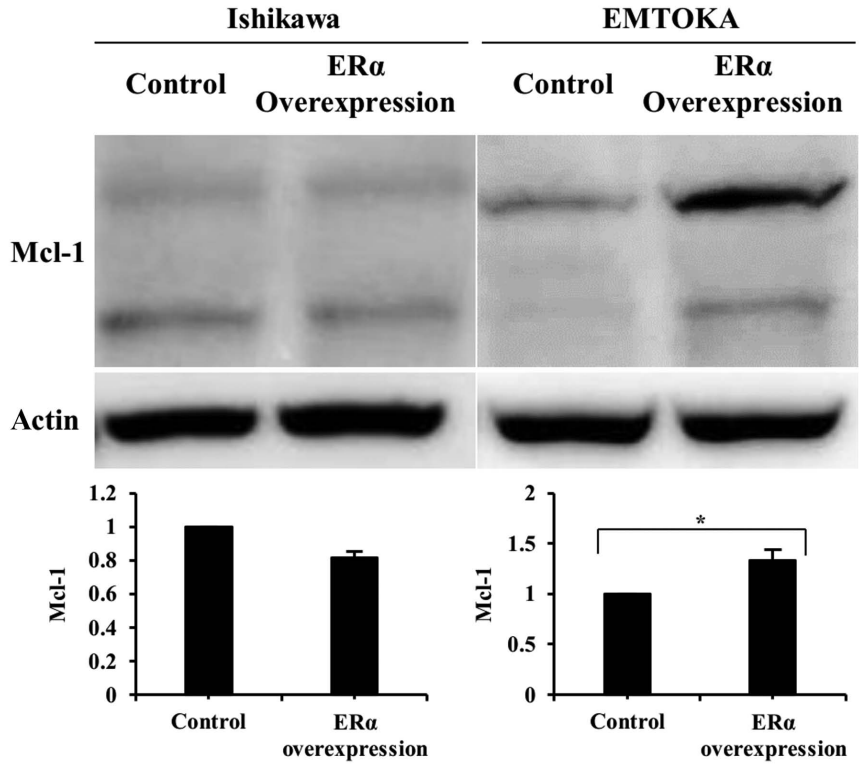

Figure 4. Effect of ERα overexpression on Mcl-1 protein levels. Western blot analysis of Mcl-1. Total protein lysates from Ishikawa and EMTOKA cells transfected with empty vector (Control) or pcDNA-ERα (ERα overexpression) were analyzed for Mcl-1. Actin served as a loading control. Bands were semi-quantified using ImageJ and actin was used as a control to represent relative protein expression. The results are presented as means ± SD; *P<0.05. ERα, estrogen receptor α.

Discussion

Estrogen is known to be associated with carcinogenesis and to promote the progression of endometrial cancer (17). For example, ERα expression on macrophages from endometrial cancer patients correlates positively with cancer progression (18). In addition, in ovarian cancer cells, activation of ERα by estrogen and cisplatin can induce platinum-resistance by increasing expression of an anti-apoptotic protein (7). Our results suggest that ERα expression in EMTOKA human

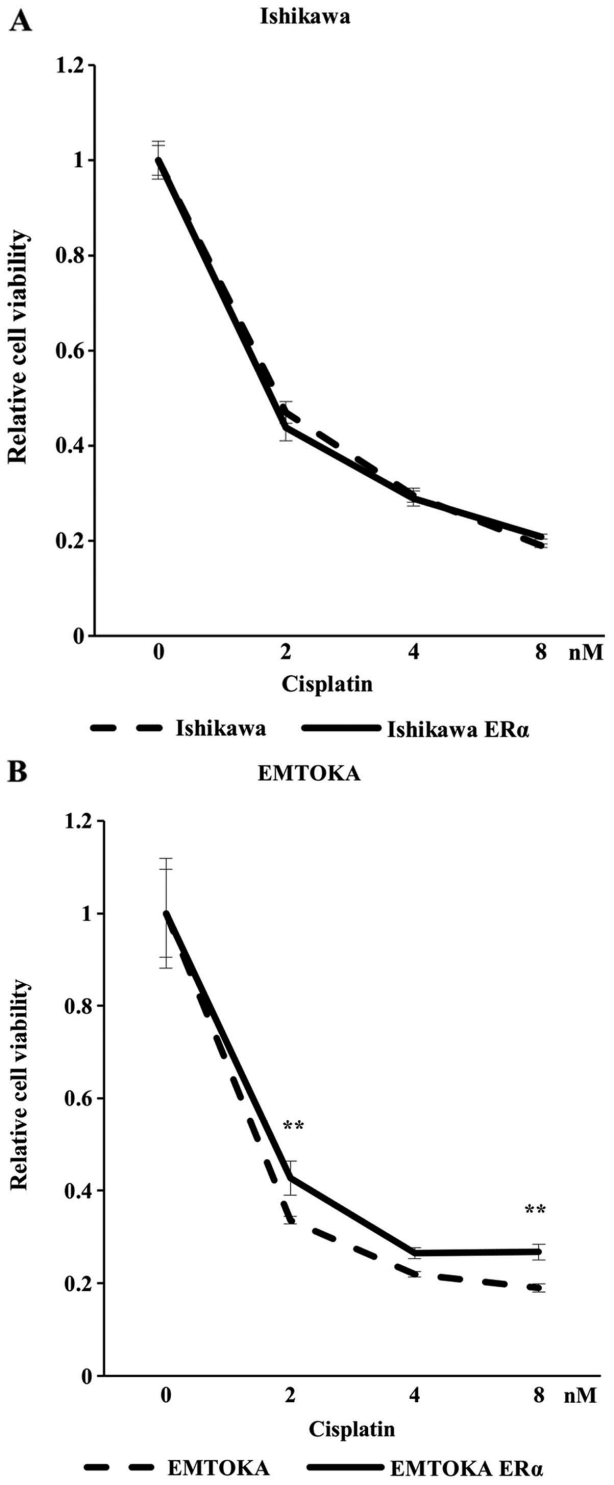

Figure 5. Overexpression of ERα increases chemoresistance to cisplatin. Viability of (A) Ishikawa and (B) EMTOKA cells assessed after 48 h of cisplatin (0, 2, 4 and 8 nM) treatment in XTT assays. Ishikawa and EMTOKA cells were transfected with empty vector or pcDNA-ERα (ERα overexpression). The results are presented as means ± SD; **P<0.01. ERα, estrogen receptor α.

endometrial cancer cells increases cell viability in the presence of cisplatin through upregulation of BAG3, which plays important roles in the regulation of apoptosis, autophagy, and cell differentiation. Notably, this effect of exogenous ERα upregulation was not seen in Ishikawa cells, which endogenously express ERα. The effect of exogenous ERα upregulation was only seen in EMTOKA cells, which do not endogenously express ERα. ERα is expressed in brain, mammary gland, ovary (thecal cells), uterus, bone, and testis (19,20). The ERα expression rates among endometrial cancer patients are 50-60%, 30-40%, and 5-15% in endometrioid cancer grades 1, 2, and 3, respectively, but it is nearly absent in serous and clear cell cancers (21,22). Felzen *et al* showed that in human neuroblastoma cell lines, upregulation of ERα increased autophagic activity by enhancing BAG3 expression, but in the MCF7 ERα-expressing human breast cancer cells line, ERα knockdown did not alter BAG3 levels or autophagic activity (9). Our results also show that the level of BAG3 expression is unaffected by ERα knockdown in the Ishikawa ERα-expressing human endometrial cancer cell line. This suggests that expression of a small amount of ERα is sufficient to enhance expression downstream mediators (e.g., BAG3 and Mcl-1) in the ERα signaling pathway, and that higher levels of ERα do not further enhance expression of those proteins.

Previous studies indicate that miR-29b acts as a tumor suppressor (23,24) and that it is associated with differentiation, proliferation, invasiveness and metastasis of lung cancer, breast cancer, cholangiocarcinoma, and leukemia cells (25-28). miR-29b downregulates Mcl-1, thereby promoting cell apoptosis. Correspondingly, downregulation of miR-29b correlates with more aggressive forms of cancer and with recurrence. In the present study, we demonstrated that ERα overexpression leads to decreased miR-29b expression and thus increased Mcl-1 expression.

Mcl-1 is an antiapoptotic Bcl-2 family member that modulates apoptosis-related signaling pathways and promotes cell survival. Mcl-1 also appears to be an important factor mediating resistance to cancer chemotherapy, and its downregulation has proved effective for inducing apoptosis (29-31). Consistent with those findings, we observed here that suppression of miR-29b through overexpression of ERα increased Mcl-1 levels and induced resistance to cisplatin in EMTOKA endometrial cancer cells.

In an earlier study, we found that upregulation of BAG3 increased tumor cell motility and invasiveness through downregulation of miR-29b and subsequent upregulation of MMP-2 (8). We also previously reported that BAG3 upregulates Mcl-1 by suppressing miR-29b and induces anticancer drug resistance in ovarian cancer cell lines (13). Consistent with those earlier observations, our results in the present study show that ERα likely contributes to the acquisition of resistance to anticancer drugs by endometrial cancer cells via an ERα-BAG3-miR-29b-Mcl-1 pathway. However, several issues remain to be addressed by future research. First, the relationship between ERα and the Bcl-2 family does not indicate a direct relationship between ERα and apoptosis. To investigate the direct relationship, it will be necessary to examine the relationship between ERα and caspase activity. Second, because this report describes a basic study using endometrial cancer cell lines, our findings will need to be verified and extended through investigation of protein expression in human endometrial cancer tissue. We anticipate the results of those studies will deepen our understanding of the relationship between ERα and chemoresistance and apoptosis, and shed light on whether ERα can serve as an effective therapeutic target.

Although there are some challenges, these results suggest that ERα is a key determinant of the responsiveness of some endometrial cancer cells to cisplatin, and that ERα is a potentially useful therapeutic target for the treatment of some types of endometrial cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SA and MI designed and completed the experiments together. SH provided guidance on the overall experimental technique and also performed RT-qPCR along with SA. TM performed statistical analysis. MT and MM revised the article and also performed western blotting along with SA. SS performed cell culture and cell viability assays along with MI and SA. TS oversaw the composition of the manuscript and the overall experiments, and also performed RT-qPCR. All authors read and approved the final manuscript, and each author believes that the manuscript represents honest work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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