Dienogest regulates apoptosis, proliferation, and invasiveness of endometriotic cyst stromal cells via endoplasmic reticulum stress induction

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ABSTRACT

Dienogest, a specific progesterone receptor agonist, is used in the treatment of endometriosis. However, it is still unclear as to the mechanisms of therapeutic effects on endometriosis. Our recent study showed that endometriosis may be the result of aberrant endoplasmic reticulum (ER) stress induction due to progesterone resistance. This finding suggests that the regulation of ER stress induction may play a key role in treatment of endometriosis. Therefore, the anti-endometriotic effects of dienogest may be mediated by regulation of ER stress. To test this hypothesis, we elucidate whether dienogest affects endometriotic stromal cell apoptosis, proliferation, and invasiveness by modulating ER stress-induced CCAAT/enhancer-binding protein homologous protein (CHOP) expression. Specifically, PRKR-like ER kinase (PERK)/eukaryotic initiation factor 2α (eIF2α)/activating transcription factor 4 (ATF4), inositol-requiring kinase 1 (IRE1)/TNF receptor-associated factor 2 (TRAF2)/apoptosis signal-regulating kinase 1 (ASK1)/c-Jun N-terminal kinase (JNK) signaling, and downstream CHOP were evaluated to determine the involved ER stress-mediated regulation mechanism of CHOP expression. Our results show that progesterone treatment did not have any significant effects on ER stress, apoptosis, proliferation, and invasion in estrogen-treated endometriotic cyst stromal cells (ECSCs). However, dienogest treatment upregulated the induction of ER stress. It also led to increased apoptosis, and decreased proliferation and invasiveness. These dienogest-induced changes in apoptosis, proliferation and invasiveness were reversed by the ER stress inhibitor salubrinal. Furthermore, dienogest-induced ER stress increased CHOP expression through activation of both PERK/elf2α/ATF4 and IRE1/TRAF2/ASK1/JNK signaling. This upregulation was blocked by transfection with PERK and IRE1 siRNA, which decreased apoptosis, and increased the proliferation and invasiveness of dienogest-treated ECSCs. Taken together, our findings indicate that dienogest enhances ER stress induction in endometriotic stromal cells, which affects apoptosis, proliferation, and invasiveness via CHOP upregulation.

Keywords: Endometriosis, Dienogest, ER stress, CHOP, Apoptosis, Proliferation, Invasiveness

INTRODUCTION

Endometriosis is a common benign gynecological disease that is defined by the presence of endometrial tissue outside the uterine cavity. Although its etiology and pathophysiology remain unclear, endometriosis is characterized by the enhanced survival, proliferation, adhesiveness, and invasiveness of endometrial cells after retrograde menstruation. Dienogest is a selective progesterone receptor agonist that is widely used to treat endometriosis (Harada and Taniguchi, 2010; McCormack, 2010; Andres et al., 2015). Dienogest suppresses serum estrogen levels by preventing ovulation, and thereby indirectly controls endometriosis. It also directly controls endometriosis by promoting apoptosis and reducing the proliferation of endometriotic cells (Shimizu et al., 2009; Miyashita et al., 2014; Prechapanich et al., 2014). Therefore, dienogest has become a first-line treatment for endometriosis. Regardless, there remains a need for further clinical studies to demonstrate that dienogest can efficiently relieve the pain and symptoms associated with endometriosis, reduce endometriotic cyst volume, and prevent cyst recurrence (Momoeda et al., 2009; Angioni et al., 2015; Ota et al., 2015; Adachi et al., 2016). The exact mechanism(s) by which dienogest exerts its effects on endometriotic cells must also be clarified.

The endoplasmic reticulum (ER) is a vital organelle in eukaryotic cells (Díaz-Villanueva et al., 2015). A number of intracellular and extracellular factors can disturb the homeostasis of the ER, leading to ER stress (Schröder, 2008). Growing evidence suggests that ER stress is directly involved in apoptosis induction. Apoptosis induction is predominantly mediated by upregulation of the CCAAT/enhancer-binding protein homologous protein (CHOP), which is a major ER stress pro-apoptotic transcription factor (Oyadomari and Mori, 2004). Previous studies have shown that ER stress-induced CHOP expression is positively regulated by unfolded protein response (UPR) signaling pathways such as PRKR-like ER kinase (PERK) and inositol-requiring kinase 1 (IRE1) (Ron and Walter, 2007; Lin et al., 2008; Kim et al., 2018). Under ER stress conditions, the activation of PERK and IRE1 signaling enhances apoptosis induction by increasing CHOP expression (Chen et al., 2011; Rozpedek et al., 2016; Xu et al., 2016). These findings suggest that ER stress is important in apoptosis induction via UPR signaling pathways, which lead to CHOP upregulation. Recent studies have also shown that ER stress-mediated CHOP upregulation can inhibit the proliferation and invasiveness of some cell types. This inhibition is blocked by CHOP deficiency, which suggests that ER stress-induced CHOP has an inhibitory effect on cellular proliferation and invasiveness (Yang et al., 2017; Choi et al., 2019). Therefore, ER

stress is not only directly involved in the regulation of apoptosis induction, but also in cellular proliferation and invasiveness through controlling CHOP expression.

Prior literature suggests that endometrial progesterone resistance, which is characterized by alterations in progesterone responsive gene and protein expression, is a central element in the pathophysiology of endometriosis (Attia et al., 2000; Burney et al., 2007; Al-Sabbagh et al., 2012). In particular, one study found that an aberrant ER stress response to progesterone decreased the CHOP expression of endometriotic stromal cells compared to that of normal endometrial stromal cells. These expression differences ultimately led to an increase in the invasiveness found in endometriotic lesions (Choi et al., 2019). These findings suggest that abnormal ER stress response to progesterone may be involved in the pathogenesis of endometriosis by affecting the apoptosis, proliferation, and invasiveness of endometriotic cells. In contrast, previous studies have reported that dienogest exerts progestational activity, which directly suppresses proliferation and cytokine production in endometriotic stromal cells (Fu et al., 2008; Yamanaka et al., 2012). Therefore, dienogest may increase the ER stress induction in endometriotic cells. However, it is not yet known whether ER stress is involved in the therapeutic effects of dienogest in endometriotic stromal cells, or if it is associated with endometriotic cell apoptosis, proliferation, and invasiveness.

In this study, we evaluated the effects of dienogest on ER stress induction in endometriotic cells. Specifically, we investigated whether dienogest-mediated ER stress is involved in the regulation of apoptosis, proliferation, and invasiveness of endometriotic stromal cells via UPR signaling pathways.

MATERIALS AND METHODS

Human endometriotic cyst stromal cell isolation

Endometriotic cyst stromal cells (ECSCs) were obtained from the ovarian endometriotic cysts (endometrioma) of sixteen patients who underwent ovarian cystectomy or oophorectomy. All of the women had a history of regular menstrual cycles, and did not take oral contraceptives or hormonal agents for at least three months prior to surgery. The average participant age was 31.5 ± 5.7 years. ECSCs were isolated from the ovarian endometriotic tissues in the proliferative phase using enzymatic digestion, as previously described (Ryan et al., 1994). The fresh endometriotic lesion collected in sterile medium was dissected free from the underlying parenchyma, and digested with 2 mg/ml type IV collagenase (Sigma Chemical Co., St. Louis, MO, USA) at

37°C for 60 min with agitation. The stromal cells were separated from the epithelial glands by 70 mm-pore filters, followed by 45 mm-pore nylon mesh. The filtered cells were plated in T75 flasks and allowed to adhere for 30 min. Next, the flasks were washed with PBS to remove blood cells and debris. Stromal cells were cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL) in a humidified atmosphere with 5% CO_2 at 37°C. The medium was changed every other day. At confluence, the cells were subcultured in 24-well culture plates using 1 ml of culture medium. We evaluated endometrial stromal cell suspension purity using immunostaining with vimentin stromal cell-specific antibodies. This study was approved by the Ethical Committee of Samsung Medical Center. Written informed consent was obtained from all participants.

***In vitro* experiments**

Subcultured ECSCs were seeded at 1×10^6 cells/ml in poly-L-lysine-coated nonfluorescent thin-bottom glass culture dishes (MatTek, Ashland, MA, USA). Cells were incubated at 37°C in 5% CO_2 in DMEM/F12 supplemented with 10% charcoal-stripped FBS, glutamine (Gibco-BRL), HEPES (Gibco-BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin. Upon reaching 70–80% confluence, the cultures were serum starved in serum-free Earle's Balanced Salt Solution (EBSS) medium (Sigma). In order to evaluate the effects of progesterone and dienogest on ER stress, PERK and IRE1 signaling, apoptosis, proliferation, and invasiveness in ECSCs, the cells were cultured in EBSS medium before hormone treatment. After 24 h of culture, estradiol (10 nM, Sigma) alone, estradiol (10 nM) + progesterone (1 μM, Sigma), or estradiol (10 nM) + dienogest (10 uM, Abcam, Cambridge, MA, USA) were added to the cell cultures for 24 h. In addition, an ER stress inhibitor (10 μM salubrinal; Selleckchem, Houston, TX, USA) was added to the medium 6 h before the analysis was performed to block ER stress induction. The treatments were stopped by removing the medium. Cells were harvested by scraping to generate protein extracts, or fixed for immunofluorescence or invasion assay. Apoptotic ECSCs were evaluated using annexin V/propidium iodide (PI, BD biosciences, San Jose, CA, USA) staining. In addition, the conditioned medium from each treatment was separately collected, pooled and concentrated using a centricon (Millipore, Bedford, MA, USA). The protein concentrations were analyzed using the Bio-Rad (Hercules, CA, USA) system.

Western blot analysis

The ER chaperones glucose-regulated protein 78 (GRP78) and GRP94 were highly expressed under ER stress conditions (Kozutsumi et al., 1988). Therefore, GRP78 and GRP94 levels were measured using Western blot analysis as a surrogate for ER stress induction. Apoptosis was determined by measuring cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP). Proliferation cell nuclear antigen (PCNA) was also evaluated as a marker of cell proliferation, as it is universally expressed throughout the G1/S-phase interface and reaches a plateau during G2 (Xiong et al., 1992). Matrix metalloproteinase (MMP) production can be regulated at the level of secretion (Taraboletti et al., 2000). Therefore, the levels of invasion-related proteins MMP2 and MMP9 were evaluated to assess *in vitro* cultured endometriotic cell invasion. ER stress-induced PERK and IRE1 positively regulate CHOP expression via downstream eukaryotic initiation factor 2α (eIF2α)/activating transcription factor 4 (ATF4) and Tumor necrosis factor receptor-associated factor 2 (TRAF2)/apoptosis signal-regulating kinase 1 (ASK1)/c-Jun N-terminal kinase (JNK), respectively (Ron and Walter, 2007; Lin et al., 2008; Kim et al., 2018). Therefore, we measured the expression levels of CHOP, PERK, phosphorylated eIF2α, ATF4, IRE1, phosphorylated TRAF2, phosphorylated ASK1, and phosphorylated JNK to determine the involved ER stress-mediated regulation mechanism of the CHOP expression. Protein extracts from cultured cells and collected tissues were prepared in ice-cold radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma). The cell lysates were incubated on ice for 30 min to completely solubilize the cellular proteins. This was followed by centrifugation (13000 x g, 4 °C, 30 min). Equal amounts of whole cell lysates (20 μg/lane) or conditioned media (10 μg/lane) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA, USA). Before incubation with the primary antibody, the membranes were cut into multiple strips according to the size of each target protein identified by a color size marker and blocked with 5% (w/v) skim milk. The strips were subsequently incubated overnight at 4°C with the following primary antibodies: GRP78 (Cell Signaling, #3177, 1:1000 dilution), GRP94 (Cell Signaling, #2104, 1:1000 dilution), cleaved caspase-3 (Cell Signaling, #9661, 1:1000 dilution), cleaved PARP (Cell Signaling, #5625, 1:1000 dilution), PCNA (Cell Signaling, #13110, 1:1000 dilution), MMP2 (Cell Signaling, #87809, 1:1000 dilution), MMP9 (Cell Signaling, #13667, 1:1000 dilution), PERK (Cell Signaling, #5683, 1:1000 dilution), total elf2α (Cell Signaling, #9722, 1:1000 dilution) or phosphorylated elf2α (Cell Signaling, #3398, 1:1000 dilution), CHOP (Cell Signaling, #2895, 1:1000 dilution), ATF4 (Cell Signaling, #11815, 1:1000 dilution), IRE1 (Cell Signaling,

#3294, 1:1000 dilution), total TRAF2 (Cell Signaling, #4724, 1:1000 dilution) or phosphorylated TRAF2 (Cell Signaling, #13908, 1:1000 dilution), total ASK1 (Cell Signaling, #8662, 1:1000 dilution) or phosphorylated ASK1 (Cell Signaling, #3764, 1:1000 dilution) or total JNK (Cell Signaling, #9252, 1:1000 dilution) or phosphorylated JNK (Cell Signaling, #4668, 1:1000 dilution). After three consecutive washes with tris buffered saline with tween 20 (TBST) buffer, the membranes were incubated with the appropriate secondary antibody IgG (SC-2004 or SC-2005; Santa Cruz Biotechnology) at room temperature for 1 hour at a dilution of 1:2000. The proteins were visualized using the enhanced chemiluminescence method (Millipore) according to the manufacturer's recommendations and the signal of band intensities was quantitated using NIH ImageJ software (NIH Image Processing and Analysis in Java). The expression levels of GRP78, CHOP, TRIB3, MMP2, and MMP9 were normalized to that of *beta*-actin. In contrast, the expressions of phosphorylated elf2α, TRAF2, ASK1, and JNK were normalized to those of total elf2α, TRAF2, ASK1, and JNK, respectively.

siRNA transfection

For the siRNA experiments, cells that were seeded on a 6-well plate were grown to 60%–80% confluence. The cells were transfected with an siRNA targeting PERK (sc-36213), IRE1 (sc-40705), or a nonspecific control (sc-37007; all Santa Cruz Biotechnology) using the Lipofectamine RNAiMax Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The final concentration of siRNA was 25 pmol/L. The total proteins were extracted, and Western blot analysis was performed to confirm the protein level 48 hours after transfection. On the next day, the cells were treated with dienogest (10 uM) for 8 h. The treatments were stopped by removing the medium. The cells were harvested by scraping to generate the protein extracts, or fixed for immunofluorescence. Apoptosis was evaluated using annexin V/propidium iodide (PI) staining. The invasiveness of the ECSCs was evaluated using a cell invasion assay.

Assessment of human endometriotic stromal cell apoptosis

The apoptotic cell percentages were determined using annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. After drug treatment, 1×10^5 cells were pelleted, washed with PBS, resuspended in 100 μL binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM potassium chloride, 1 mM $MgCl_2$ and 2 mM calcium chloride), and incubated

with 5 μL annexin V and PI for 15 min at room temperature in the dark. After the binding buffer (400 μL) was added, the cells were analyzed using FACSAria flow cytometry (BD Biosciences, Heidelberg, Germany). At least 10,000 cells were analyzed per treatment. Data analysis was performed using CellQuest software.

Immunofluorescence staining

The ECSCs were plated on sterile glass coverslips, fixed with 4% paraformaldehyde and blocked with 0.1% bovine serum albumin in PBS. In order to detect ER stress induction, the cells were incubated with anti-GRP78 rabbit polyclonal antibody (1:50; Abcam; ab32618) in PBS, and then Alexa 488-conjugated secondary antibodies (1:1000; Abcam; ab15007). The cells were also incubated with anti-CHOP rabbit polyclonal antibody (Cell Signaling, #2895, 1:500 dilution). After this, cells were incubated with Alexa 568-conjugated secondary antibody (Invitrogen, a21069, 1:1000 dilution). Next, cells were incubated with anti-Ki-16 rabbit monoclonal antibody (Alexa Fluor® 488 Conjugate) (Cell Signaling, #11882, 1:500 dilution) to detect proliferating cells. Finally, the slides were mounted in mounting media (Vector Laboratories). Images were captured using a confocal microscope (Bio-Rad).

Cell invasion assay

Transwell chambers (BD Bioscience, San Jose, CA, USA) were used for the invasion analysis. A total of 5×10^5 silenced cells were prepared in serum-free media. A volume of 300 μl of cells was added into the upper chamber. Meanwhile, 500 μl DMEM with 10% FBS was added to the lower chamber. The cells were incubated at 37°C for 24 h. A cotton-tipped swab was used to carefully wipe off the cells that did not invade through the pores. The filters were then fixed in 90% (v/v) alcohol and stained with 0.1% (w/v) crystal violet. The filters were quantified by dissolving the stained cells in 10% (w/v) acetic acid. A consistent amount of the dye/solute mixture was transferred to a plate for colorimetric reading of the OD at 560 nm.

Statistical analysis

The results are expressed as means ± standard errors based on four independent experiments. ANOVA and post hoc Tukey test were used for pairwise comparisons of the Western blot analyses data regarding the effects

of steroid hormones and dienogest. A Student's t-test was used to compare the in vitro findings from the two groups. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA). *P* values < 0.05 were considered statistically significant.

RESULTS

Dienogest-induced ER stress enhances apoptosis and inhibits proliferation and invasiveness in ECSCs

We used Western blotting to measure the levels of GRP78 and GRP94 and investigate whether progesterone or dienogest induce ER stress in ECSCs cultured with estrogen. As shown in Figure 1A, the addition of progesterone had no effect on the expression level of GRP78 or GRP94 in ECSCs cultured with estrogen. In contrast, the expression levels of these proteins were significantly increased by the addition of dienogest ($P < 0.05$). We conducted immunofluorescence to determine endogenous GRP78 expression. Endogenous GRP 78 was readily detected in cultured ECSCs (Fig. 1B). In ECSCs cultured with estrogen alone, GRP78 staining was weakly detected in the cytoplasm (Fig. 1B, left). Although the immunoreactivity for GRP78 did not change with the addition of progesterone (Fig. 1B, middle), GRP78 was intensely stained in the cytoplasm upon the addition of dienogest (Fig. 1B, right).

The effects of dienogest-induced ER stress on apoptosis, proliferation, and invasiveness was next evaluated by measuring cleaved caspase-3, PARP, PCNA, MMP2, and MMP9 expression in ECSCs cultured with estrogen. As shown in Figure 1C and D, the addition of progesterone had no effect on the expression level of GRP78, cleaved caspase-3, cleaved PARP, PCNA, MMP2, or MMP9 in the ECSCs cultured with estrogen. In contrast, there were significantly higher expression levels of GRP78, cleaved caspase-3, and cleaved PARP, and decreased expression levels of PCNA, MMP2, and MMP9 in ECSCs that were cultured with estrogen and dienogest compared to those cultured with estrogen alone (Fig. 1C and D, $P < 0.05$). However, dienogest-stimulated GRP78 expression was significantly inhibited by the addition of an ER stress inhibitor (salubrinal). This inhibition was accompanied by decreased cleaved caspase-3 and cleaved PARP expression, as well as increased PCNA, MMP2, and MMP9 expression (Fig. 1C and D, $P < 0.05$).

Dienogest-induced ER stress increases CHOP by activating PERK/elfFα/ATF4 and IRE1/TRIF2/ASK1/JNK stress signaling in ECSCs

We next evaluated the effects of progesterone and dienogest on CHOP expression and its upstream PERK/elF2α/ATF4 and IRE1/TRAF2/ASK1/JNK signaling pathways. These experiments were conducted to determine if dienogest-induced ER stress upregulates CHOP expression. As shown in Figure 2A, progesterone did not affect PERK, phosphorylated elF2α, ATF4 or CHOP expression. In contrast, dienogest significantly increased the expression levels of these proteins in estrogen-treated ECSCs (Fig. 2A, $P < 0.05$). Dienogest treatment also significantly increased the expression levels of IRE1, phosphorylated TRAF2, phosphorylated ASK1, phosphorylated JNK, and CHOP in estrogen-treated ECSCs (Fig. 2B, $P < 0.05$), although progesterone did not.

Dienogest induced endometriotic stromal cell apoptosis by activation of activating PERK/elfFα/ATF4 signaling, leading to upregulation of CHOP

We found that activation of PERK/elF2α/ATF4 signaling by dienogest increased CHOP expression. We next investigated whether PERK/elF2α/ATF4 signaling is involved in the regulation of apoptosis, proliferation, and invasiveness through CHOP upregulation in dienogest-treated ECSCs. In order to do so, dienogest-treated ECSCs were transfected with PERK siRNA and nonspecific control siRNA. Transfection with PERK siRNA led to a decrease in PERK expression to 28.8 ± 4.4 ($P < 0.05$) in the ECSCs compared to that of cells transfected with non-specific control siRNA (Fig. 3A). After transfection with PERK siRNA, CHOP, cleaved caspase-3, and PARP expression was significantly lower in ECSCs than in those transfected with non-specific control siRNA (Fig 3A, $P <0.05$). In contrast, PCNA, MMP2, and MMP9 expressions were enhanced after transfection with PERK siRNA (Fig 3 B, $P <0.05$).

We also conducted flow cytometry assays using annexin V and PI to determine the proportion of apoptotic cells. The proportion of apoptotic dienogest-treated ECSCs decreased by 49.8% after transfection with PERK siRNA (Fig. 3C, $P < 0.05$). In order to further characterize the proliferative activity in ECSCs, immunofluorescence staining was performed to examine the subcellular localization of endogenous CHOP and Ki-16. The expression of endogenous CHOP and Ki-16 was readily detected in cultured NESCs as red and green fluorescent areas in the cytoplasm and nucleus, respectively (Fig. 3D). In cells that were transfected using nonspecific control siRNA, the dienogest-treated ECSCs were stained intensely for the CHOP protein (Fig. 3D, I) but had negative or very weak Ki-16 immunoreactivity (Fig. 3D, II). After transfection PERK siRNA,

however, the CHOP staining (Fig. 3D, III) decreased in the cytoplasm, while the immunoreactivity for Ki-16 (Fig. 3D, IV) increased markedly in the nucleus. In addition, the proportion of invading cells increased significantly in ECSCs transfected with PERK siRNA (Fig. 3E, $P < 0.05$).

Dienogest-mediated ECSC apoptosis, proliferation, and invasiveness is associated with IRE1/TRIF2/ASK1/JNK signaling-induced upregulation of CHOP

We next transfected dienogest-treated ECS with IRE1 siRNA and nonspecific control siRNA. This experiment was performed to determine whether activation of IRE1/TRIF2/ASK1/JNK signaling increases CHOP expression, leading to apoptosis induction and inhibition of proliferation and invasiveness in dienogest-treated ECSCs. Figure 4A shows that IRE1 expression decreased significantly to 34.5 ± 3.7 ($P < 0.05$) after transfection with IRE1 siRNA. After transfection with IRE1 siRNA, CHOP expression was significantly lower in ECSCs compared to those transfected with non-specific control siRNA ($P < 0.05$). This effect was accompanied by decreased cleaved caspase-3 and PARP expression, as well as increased PCNA, MMM2, and MMP9 expression (Fig. 4A and B, $P < 0.05$). Furthermore, the proportion of apoptotic cells decreased significantly in dienogest-treated ECSCs transfected with IRE1 siRNA (Fig. 4C, $P < 0.05$). Immunofluorescence images also showed that the immunoreactivity for CHOP decreased, and Ki-16 staining increased after transfection with IRE1 siRNA (Fig. 4D-I, II, III and IV). In addition, transfection with IRE1 siRNA led to enhanced cell invasion (Fig. 4E, $P < 0.05$)

DISCUSSION

Upregulation of ER stress is known to efficiently trigger apoptosis in various cell types (Iurlaro and Muñoz-Pinedo, 2016). Previous studies have demonstrated that ER stress is directly involved in the inhibition of cellular proliferation and invasiveness (Nami et al., 2016; Hou et al., 2018; You et al., 2018). Therefore, upregulation of ER stress is a crucial event in the regulation of apoptosis, proliferation, and invasiveness. Our group previously found that ER stress in normal endometrial cells is upregulated by progesterone, which leads to an increase in apoptosis and a decrease in invasiveness (Choi et al., 2018; 2019). These findings suggest that ER stress is a signaling component of progesterone action in endometrial cells, and is an important mediator of the pro-apoptotic and anti-invasive function of progesterone. However, endometriosis is associated with a reduced response to progesterone. It is also reported that the resistance of endometriotic tissue to progesterone, evident

in both laboratory and clinical observations, can be explained by dysregulation of progesterone target genes (Osteen et al., 2005, Bulun et al., 2006). Furthermore, in contrast to normal endometrial cells, our recent study showed that progesterone did not increase ER stress in endometriotic stromal cells (Choi et al., 2019). Therefore, it is postulated that endometriotic cells respond abnormally to progesterone, which contributes to the dysregulation of ER stress in these cells. Consistent with this finding, we showed that progesterone treatment did not affect expression of the ER stress marker protein GRP78 and GRP94 in estrogen-treated ECSCs. This result suggests that ER stress induction is altered due to progesterone resistance. In contrast, dienogest treatment significantly increased ER stress induction in estrogen-treated ECSCs. These findings indicate that dienogest enhances ER stress induction in endometriotic stromal cells through progesterone action, as dienogest is known to exert progestogenic effects on estrogen-primed endometrium (Schweppe, 2001). Therefore, dienogest-mediated upregulation of ER stress may affect apoptosis, proliferation, and invasiveness in endometriotic stromal cells. This hypothesis is supported by our western blot results, which showed that dienogest treatment increased cleaved caspase-3 and PARP and decreased PCNA, MMP2 and MMP9 expression in estrogen-treated ECSCs. In contrast, these changes were not present with progesterone treatment. Furthermore, the dienogest-induced change in the expression of these marker proteins was reversed by the ER stress inhibitor salubrinal. These results suggest that dienogest upregulates ER stress in endometriotic stromal cells. This upregulation is associated with the change in apoptosis, proliferation, and invasiveness.

According to previous studies, CHOP, an ER stress-inducible protein, is expressed at low levels under physiological conditions. However, CHOP is dramatically upregulated during severe and prolonged ER stress. It plays a crucial role in cell arrest and apoptosis induction. Therefore, CHOP is a crucial mediator of ER stress-induced apoptosis (Oyadomari and Mori, 2004; Tajiri et al., 2004). Recent studies found that ER stress-induced CHOP expression can inhibit the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway (Lin et al., 2017; Xu et al., 2017). It is also well known that the AKT/mTOR pathway enhances cell proliferation and invasiveness; inhibition of this pathway efficiently reduces cellular proliferation and invasiveness in many cancer cells (Zhang et al., 2016; Wu et al, 2018). These findings suggest that ER stress may reduce proliferation and invasiveness through inactivation of the AKT/mTOR pathway by CHOP. Upregulation of CHOP expression inhibits proliferation and invasiveness through inhibition of mTOR activity in ovarian cancer and endometrial cells (Yang et al., 2017; Choi et al., 2019). In this study, therefore, we evaluated the effects of dienogest on CHOP expression to determine whether dienogest-mediated ER stress was associated with endometriotic cell apoptosis, proliferation, and invasiveness. In many cell types, ER stress triggers CHOP expression, which is

mainly mediated by PERK/elf2α/ATF4 signaling (Lin et al., 2008). When ER stress is upregulated, PERK activation leads to the phosphorylation of eIF2a and selectively induces ATF4, which is a transcription factor that enhances CHOP expression. Several recent studies have also shown that under ER stress conditions, IRE1 activation recruits phosphorylation of TRAF2 and ASK1, which then activates JNK and induces CHOP expression (Ron and Walter, 2007; Kim et al., 2018). This signaling suggests IRE1/TRAF4/ASK1/JNK signaling-dependent CHOP expression. These findings indicate that both PERK/elf2α/ATF4 and IRE1/TRAF4/ASK1/JNK signaling pathways are involved in the regulation of ER stress-induced CHOP expression. We also found that dienogest upregulates CHOP expression through activation of PERK/elf2α/ATF4 and IRE1/TRAF4/ASK1/JNK signaling in estrogen-treated ECSCs. In contrast, progesterone does not upregulate CHOP expression. Therefore, these results suggest that dienogest-induced ER stress is associated with endometriotic cell apoptosis, proliferation, and invasiveness through CHOP upregulation that is mediated by the PERK/elf2α/ATF4 and IRE1/TRAF4/ASK1/JNK signaling pathways.

We used PERK and IRE1 siRNA to confirm the role of ER stress-mediated PERK/elf2α/ATF4 and IRE1/TRAF4/ASK1/JNK signaling in endometriotic cell apoptosis, proliferation, and invasiveness. Downregulation of PERK and IRE1 by siRNA inhibited CHOP expression in dienogest-treated ECSCs. This inhibition was accompanied by decreased expression of apoptotic marker proteins and the proportion of apoptotic cells observed by flow cytometry. This finding suggests that dienogest promotes endometriotic cell apoptosis through upregulation of CHOP expression by ER stress induction. Our results also showed that siRNA-mediated downregulation of PERK and IRE significantly increased the expression of proliferation marker and invasion marker proteins through CHOP inhibition in dienogest-treated ECSCs. This result is further confirmed by decreased immunoreactivity for Ki-16 and the proportion of invading ECSCs. These results indicate that dienogest-induced ER stress suppresses proliferation and invasiveness in endometriotic stromal cells via PERK/elf2α/ATF4 and IRE1/TRAF4/ASK1/JNK signaling, which is dependent on CHOP. Therefore, ER stress is an important mediator of the pro-apoptotic, anti-proliferative and anti-invasive effects of dienogest on endometriotic stromal cells.

In conclusion, this study has shown for the first time that dienogest upregulates ER stress induction in endometriotic stromal cells. This upregulation results in CHOP upregulation, which ultimately increases apoptosis, and decreases cell proliferation and invasiveness. Therefore, dienogest may suppress the progression of endometriosis through ER stress induction. However, further studies such as *in-vivo* analysis using

endometriotic tissues obtained from patients with endometriosis who received dienogest are necessary to expand the potential clinical relevance of our findings.

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Authors' roles

J.C. designed the study, interpreted data, and drafted the manuscript. M.J. performed all experiments, interpreted data, and provided critical discussion. E.L. and D.Y.L. were involved in sample recruitment and data interpretation. D.C. made substantial contributions to conception and design, interpretation of results and discussion, critical review, and editing the final version of the manuscript.

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Conflicts of interest

None declared.

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Figure 1. Effects of progesterone and dienogest on ER stress induction, cleaved cas-pase-3, PARP, PCNA, MMP2, and MMP9 expression on ECSCs. A: Representative immunoblots (left) and densitometric quantification (right) of GRP78 and GRP94. B: Immunofluorescence of GRP78 stained with green fluorophore in cultured ECSCs. Bars =10 μm. C: Representative immunoblots (left) and densitometric quantification (right) of cleaved, caspase-3, PARP and PCAN from cultured ECSCs. D: Representative immunoblots (left) and densitometric quantification (right) of MMP2 and MMP9 from conditioned media. Experiments were repeated four times. Data are expressed as percentages. Bar graphs display the mean ± SEM. Cells treated with estrogen alone are normalized to 100% (*P < 0.05 by post hoc Tukey test). PARP, poly (ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; MMP, matrix metallopeptidase; ECSCs, Endometriotic cyst stromal cells; Est, estrogen; Pro, progesterone; Die, dienogest; Sal, salubrinal. ■ , Est; □ , Est+Pro; ▥ , Est+Die; ▨ , Est+Die+Sal.

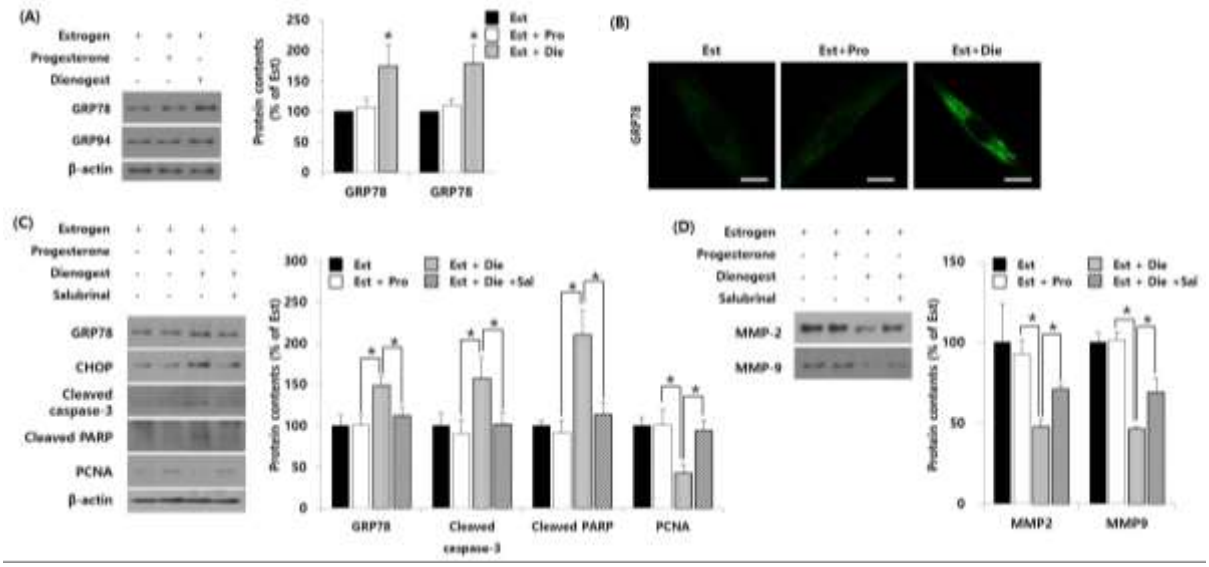

Figure 2. Dienogest-induced ER stress increases CHOP expression by activating the PERK/elF2α/ATF4 and IRE1/TRAF2/ASK1/JNK signaling pathways in ECSCs. A: Representative immunoblots (left) and densitometric quantification (right) of PERK, phosphorylated elF2α, ATF4 and CHOP from cultured ECSCs. B: Representative immunoblots (left) and densitometric quantification (right) of IRE1, phosphorylated TRAF2, phosphorylated ASK, phosphorylated JNK and CHOP from cultured ECSCs. Experiments were repeated four times. Data are expressed as percentages. Bar graphs display the mean ± SEM. Cells treated with estrogen alone are normalized to 100% (*$P < 0.05$ by *post hoc* Tukey test). CHOP, CCAAT/enhancer-binding protein homologous protein; PERK, PRKR-like ER kinase; elf2α, eukaryotic initiation factor 2α; AKT4, activating transcription factor 4; IRE1, inositol-requiring kinase 1; TRAF2, TNF receptor-associated factor 2; ASK1, apoptosis signal-regulating kinase 1; JNKc-Jun N-terminal kinase; Est, estrogen; Pro, progesterone; Die: dienogest. ■, Est; □ , Est + Pro; ▨, Est + Die.

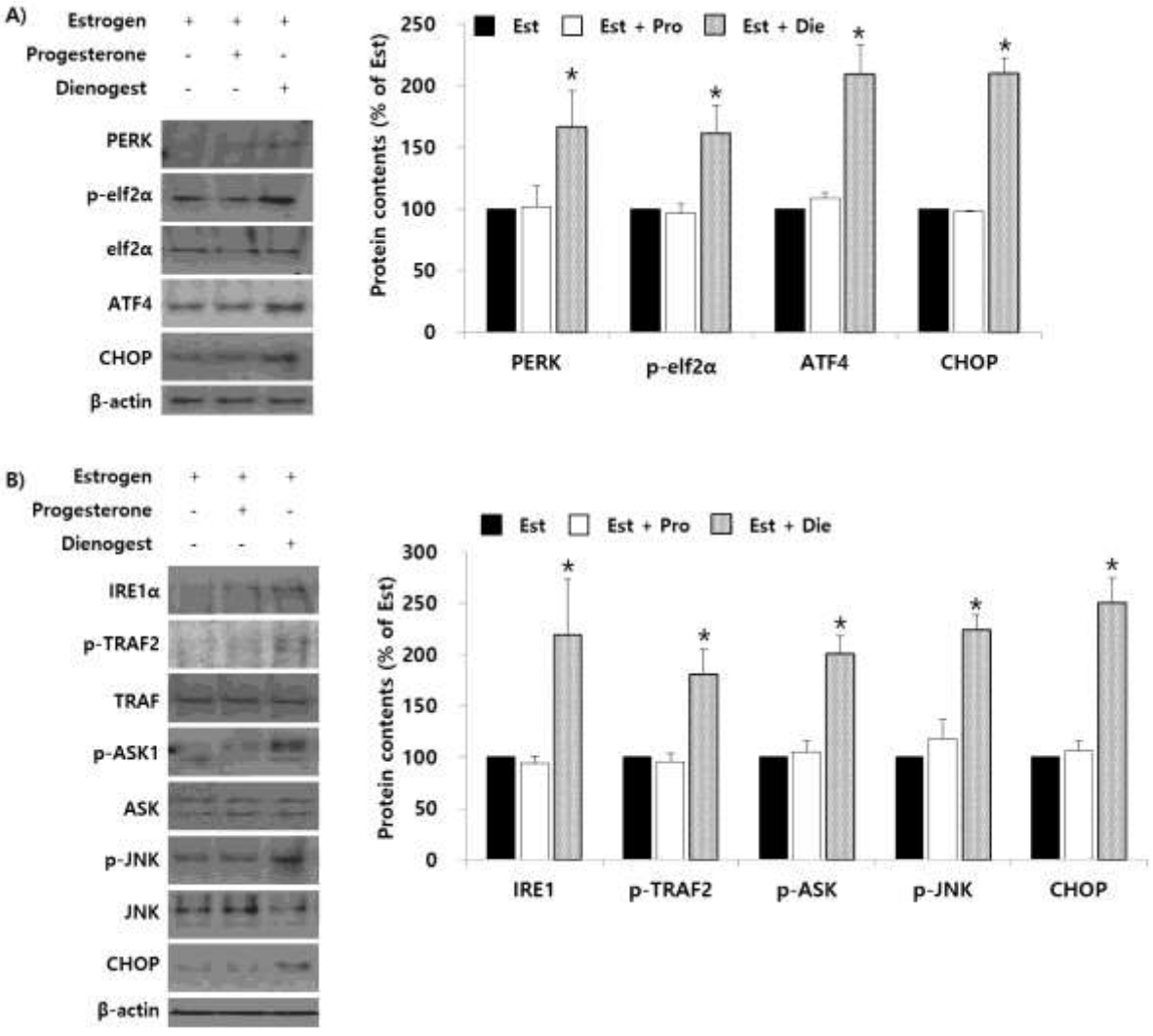

Figure 3. The effects of PERK downregulation on CHOP expression, apoptosis, proliferation, and invasiveness in dienogest-treated ECSCs. A: Representative immunoblots (left) and densitometric quantification (right) of PERK, CHOP, cleaved, caspase-3, PARP and PCAN from dienogest-treated ECSCs after transfection with PERK siRNA. B: Representative immunoblots (left) and densitometric quantification (right) of MMP2 and MMP9 from conditioned media ECSCs after transfection with PERK siRNA. Experiments were repeated four times. Data are expressed as percentages. Bar graphs display the mean ± SEM. Cells transfected with nonspecific control siRNA are normalized to 100%. *$P < 0.05$ compared with control siRNA group. C: Representative flow cytometry plots (left) and percentages of apoptotic cells from flow cytometry (right) on dienogest-treated cultured ECSCs transfected with PERK siRNA. Lower right quadrant, annexin V+/PI−; upper right quadrant, annexin V+/PI+ (apoptotic). Bar graphs display mean ± SEM. (*$P < 0.05$). D: Double-immunofluorescence staining for CHOP and Ki-16 in dienogest-treated ECSCs transfected with control siRNA and PERK siRNA. CHOP and Ki-16 were stained with red and green fluorophores in the cytoplasm and nucleus, respectively. Bars =10 μm. E: Percentages of invading dienogest-treated ECSCs after transfection PERK siRNA, as determined by invasion assay. Bar graphs display mean ± SEM (*$P < 0.05$). ■, control siRNA; □ , PREK siRNA.

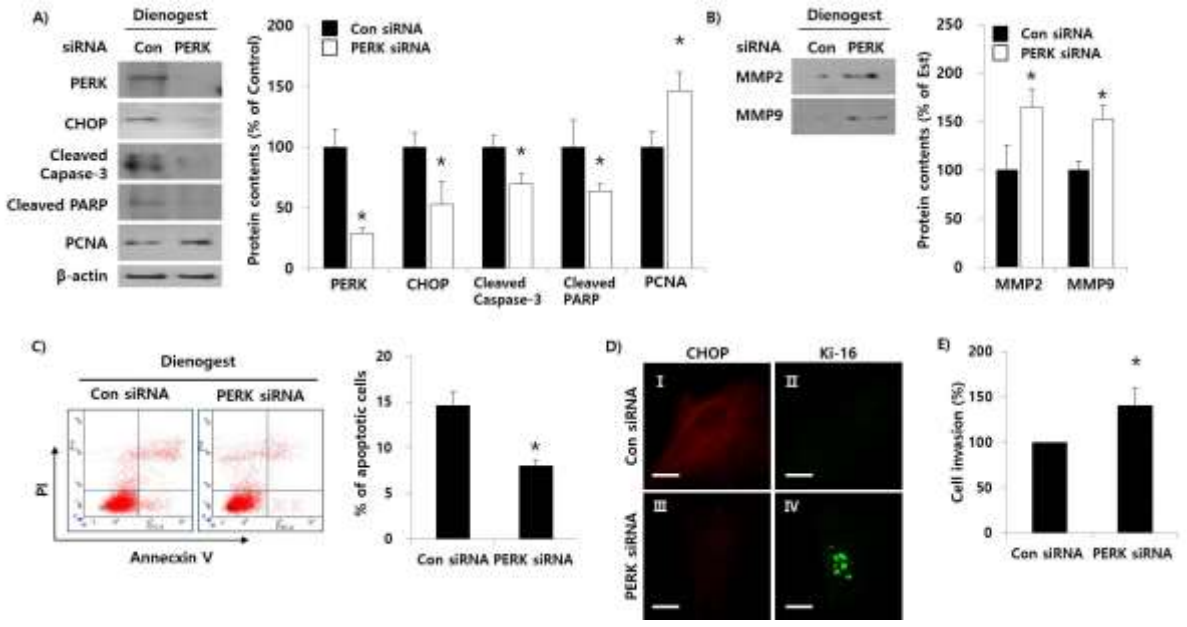

Figure 4. Downregulation of IRE1 decreased apoptosis induction and increased proliferation and invasiveness through CHOP inhibition in dienogest-treated ECSCs. A: Representative immunoblots (left) and densitometric quantification (right) of IRE1, CHOP, cleaved, caspase-3, PARP, and PCAN from dienogest-treated ECSCs after transfection with IRE1 siRNA. B: Representative immunoblots (left) and densitometric quantification (right) of MMP2 and MMP9 from conditioned media ECSCs after transfection with IRE1siRNA. Experiments were repeated four times. Data are expressed as percentages, and cells transfected with nonspecific control siRNA are normalized to 100%. Bar graphs display the mean ± SEM. **P* < 0.05 compared with control siRNA group. C: Representative flow cytometry plots (left) and percentages of apoptotic cells from flow cytometry (right) on dienogest-treated cultured ECSCs transfected with IRE1siRNA. Bar graphs display mean ± SEM (**P* < 0.05). D: Double-immunofluorescence staining for CHOP and Ki-16 in dienogest-treated ECSCs transfected with control siRNA and IRE1 siRNA. CHOP and Ki-16 were stained with red and green fluorophores in the cytoplasm and nucleus, respectively. Bars =10 μm. E: Percentages of invading dienogest-treated ECSCs after transfection IRE1 siRNA, as determined by invasion assay. Bar graphs display mean ± SEM (**P* < 0.05). ■, control siRNA; □ , IRE1 siRNA.