Involvement of endoplasmic reticulum stress in regulation of endometrial stromal cell invasiveness: Possible role in pathogenesis of endometriosis

Running title: Endoplasmic reticulum stress in endometriosis

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ABSTRACT

Endoplasmic reticulum (ER) stress is known to reduce invasiveness in some cancer cells by inhibiting the AKT/mTOR pathway. A previous study from our laboratory suggested that ER stress is promoted by progesterone in human endometrial cells, which suggests that progesterone may inhibit endometrial cell invasiveness by up-regulating ER stress. Therefore, aberrant ER stress in response to progesterone may contribute to the altered invasiveness found in endometriotic tissues. To test this hypothesis, we elucidate whether ER stress is involved in regulation of human endometrial cell invasiveness through the AKT/mTOR pathway and if this involvement is associated with altered invasiveness in endometriotic cells. Specifically, we sought to determine the effects of ER stress on AKT/mTOR pathway by evaluating ER stress-mediated CHOP/TRIB3 signaling, a negative regulator of AKT. We found that ER stress marker GRP78 expression increased with CHOP and TRIB3 expression in normal endometrial stromal cells (NESCs) treated with tunicamycin, and this increase was accompanied by decreased AKT and mTOR activity and cellular invasiveness. Similarly, progesterone increased GRP78, CHOP, and TRIB3 expression in NESCs. Subsequently, inhibition of AKT and mTOR activity decreased cellular invasiveness. This progesterone-induced decrease in cellular invasiveness was reversed by inhibition of ER stress. In contrast, progesterone did not change CHOP, TRIB3, AKT, mTOR, or invasiveness in endometriotic cyst stromal cells. In contrast to normal endometrium, endometriotic tissues showed no changes in CHOP, TRIB3 and invasion-related proteins (MMP2 and MMP9) expression throughout the menstrual cycle. Taken together, our findings indicate that abnormal ER stress response to progesterone increased endometriotic stromal cell invasiveness via the AKT/mTOR pathway.

Keywords: Endometriosis, ER stress, invasiveness, CHOP, mTOR

INTRODUCTION

Endometriosis, characterized by the presence of endometrium-like tissue outside the uterine cavity, typically affects the ovaries and pelvic peritoneum. Although endometriosis is generally considered a benign disease, it shares some biological behavior, such as aggressive migration and invasion, with cancer (Bassi et al., 2009; Vlahos et al., 2010). The migration and invasion of viable endometrial tissues outside the uterine cavity are crucial steps in the progression of endometriosis (Moggio et al., 2012). However, the cellular and molecular mechanisms that underlie migration and invasion of endometriotic cells are not fully elucidated.

The protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway functions to enhance cell invasiveness, and inhibition of this pathway efficiently reduces cell invasiveness in many cancer cells (Tang et al., 2015; Zhang et al., 2015; Wang H et al., 2016). Previous studies have shown that endoplasmic reticulum (ER) stress, a common cellular stress response, can inhibit the AKT/mTOR pathway via CCAAT/enhancer-binding protein homologous protein (CHOP)/ tribbles homolog 3 (TRIB3) signaling (Lin et al., 2017; Xu et al., 2017). Under ER stress conditions, upregulation of CHOP inhibits the AKT/mTOR pathway by increasing expression of TRIB3, a pseudokinase that inhibits AKT (Ohoka et al., 2005). These findings suggest that ER stress-mediated CHOP/TIRB3 signaling may be involved in regulation of cellular invasiveness through the AKT/mTOR pathway. This hypothesis is further supported by previous studies showing that upregulation of CHOP by ER stress inhibits the AKT/mTOR pathway and invasiveness in breast cancer cells. Accordingly, this inhibition is blocked by CHOP deficiency (Yang et al., 2017). Therefore, ER stress has been shown to play a pivotal role in the regulation of cellular invasiveness via the CHOP/TRIB3/AKT/mTOR axis.

According to previous studies, estrogen inhibits ER stress induction in some cell types, including human endometrial cells (Guzel et al., 2011; Kooptiwut et la., 2014; Guo et al., 2014). These findings suggest that ER stress induction may be under the influence of ovarian steroid hormones. Similarly, Choi et al. (14) demonstrated that ER stress in human endometrial cells is suppressed by estrogen. This group also found that ER stress induction significantly increases in endometrial cells during the secretory phase because progesterone prevents the inhibitory effects of estrogen on ER stress. Therefore, it is postulated that progesterone-induced upregulation of ER stress may facilitate suppression of endometrial cell invasiveness during the human endometrial cycle. In contrast, some ectopic and eutopic endometrial stromal cells in women with endometriosis exhibit progesterone resistance (Attia et al., 2000; Bulun et al., 2006; Rizner, 2009). These findings suggest that ER stress induction may be altered by progesterone resistance in endometriotic stromal cells. However, it is not

yet clear whether and how ER stress is involved in regulating the invasiveness of endometrial cells. It is also unknown whether progesterone resistance affects the invasiveness of endometriotic stromal cells.

Here, we evaluated whether ER stress is involved in regulating the invasiveness of normal endometrial stromal cells via the CHOP/TRIB3/AKT/mTOR axis. We also investigated whether progesterone resistance in ovarian endometrioma, the most common type of endometriosis, affects the invasiveness of endometriotic stromal cells.

Materials and Methods

Tissue collection

Normal endometrial stromal cells (NESCs) were obtained from 5 premenopausal patients who had undergone hysterectomies for uterine leiomyoma. Endometriotic cyst stromal cells (ECSCs) were obtained from ovarian endometriotic cysts (endometrioma) from 7 patients undergoing ovarian cystectomy or oophorectomy. All women had a history of regular menstrual cycles and did not take oral contraceptives or hormonal agents for at least 3 months prior to surgery. Samples were kept in room temperature Hank's balanced salt solution and transported to the laboratory for culture within 30 min.

For Western blot analysis, normal endometrial tissues and ectopic endometriotic tissues were obtained from premenopausal women diagnosed with uterine leiomyoma (n =10) and ovarian endometrioma (n =14), respectively, at the time of surgical treatment. The average age of participants was 43.8 ± 2.8 years for eutopic endometrial tissues and 28.8 ± 5.4 years for ectopic endometrial tissues. Endometrial tissue samples were divided into two categories according to day of the menstrual cycle: proliferative (days 1–14) and secretory phases (days 15–28). The menstrual cycle day was established based on each patient's menstrual history and was verified by histological examination of the endometrium. Of ten eutopic endometrial samples, five were in the proliferative stage (1 mid and 4 last proliferative) and five were in the secretory (1 mid and 4 last secretory) stage. Of 14 ectopic endometrial samples, 7 were in the proliferative phase (3 mid and 3 last proliferative) and 7 were in the secretory phase (2 mid and 5 last secretory). Tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C until western blot analysis. The Institutional Review Board of Samsung Medical Center (IRB #2012-02-073) approved this study, and written informed consent was obtained from all patients.

Isolation of normal human endometrial and endometriotic cyst stromal cells

NESCs and ECSCs were isolated from eutopic endometrial tissues and ovarian endometriotic tissues in the proliferative phase by enzymatic digestion, as previously described (Ryan et al., 1994). Briefly, tissue samples were minced into small pieces and incubated in 2 mg/ml type IV collagenase (Sigma Chemical Co., St. Louis, MO, USA) in a shaking water bath for 1 hour at 37 °C. The dispersed cells were filtered through a 70-mm nylon mesh to remove the undigested tissue pieces containing glandular epithelium. The filtered fraction was separated further from epithelial cell clumps by differential sedimentation at unit gravity as follows. Cells were resuspended in 2 ml of culture medium and layered slowly over 10 ml of the medium in a centrifuge tube. Sealed tubes were placed in an upright position at 37°C in air with 5% CO_2 for 30 min. After sedimentation, the top 8 ml of medium was collected. The medium containing stromal cells was filtered through a 40-mm nylon mesh. Final purification was achieved by allowing stromal cells to selectively adhere to culture dishes for 30 min at 37°C in 5% CO_2 in air. Non-adhering epithelial cells were removed. Cell purity was assessed by immunocytochemistry using vimentin stromal cell-specific antibodies.

***In vitro* experiments**

NECSs and ECSCs were seeded at 1×10^6 cells/ml in poly-L-lysine-coated nonfluorescent thin-bottom glass culture dishes (MatTek, Ashland, MA, USA). The cells were incubated at 37°C in 5% CO_2 in DMEM/F12 supplemented with 10% (v/v) charcoal-stripped FBS, glutamine, HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin. Upon reaching 70% to 80% confluence, cells were cultured in serum-free, phenol red-free DMEM/F12 with/without 5 μg/ml tunicamycin (Cell Signaling Boston, MA, USA) for 8 hours. This method was conducted to evaluate the effects of ER stress on the CHOP/TRIB3/AKT/mTOR axis, and the invasiveness in NESCs and ECSCs. To evaluate the effects of progesterone on the estrogen-mediated CHOP/TRIB3/AKT/mTOR axis and the invasiveness of NESCS and ECSCs, cells were cultured in serum-free, phenol red-free DMEM/F12 media with 10^{-8} M estrogen (Sigma) alone or with 10^{-8} M estrogen + 10^{-6} M progesterone (Sigma) for 24 hours. In addition, a progesterone receptor modulator (2 μM mifepristone; Sigma) or an ER stress inhibitor (10 μM salubrinal; Selleckchem, Houston, TX, USA) was added to the medium at 6 hours before analysis to block progesterone effects and ER stress induction, respectively. The treatments were

stopped by removing the medium. Cells were harvested by scraping to generate protein extracts or fixed for immunofluorescence or invasion assay. 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 by Western blot analysis as a surrogate for ER stress induction. Because ER stress-mediated CHOP/TRIB3 signaling is a negative regulator of AKT, the expression levels of CHOP and TRIB3 were measured to determine the involved ER stress-mediated regulation mechanism of the AKT/mTOR pathway. The activity of AKT was evaluated by measuring the phosphorylated (i.e., active) form of AKT. The activity of the mTOR pathway was also determined by measuring phosphorylation of ribosomal protein S6 kinase (S6K). Because S6K is a direct substrate of mTOR, the phosphorylation status of S6K can be used as an indicator of mTOR pathway activity (Sarbassov et al., 2005). 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 9 were evaluated to assess *in vitro* cultured endometrial cell invasion. Protein extracts from cultured cells and collected tissues were prepared in ice-cold radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma). Cell lysates were incubated on ice for 30 min to completely solubilize 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, membranes were blocked with 5% (w/v) skim milk. The membranes 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), CHOP (Cell Signaling, #2895, 1:1000 dilution), TRIB3 (Abcam, #ab137526, 1:500 dilution), total (Cell Signaling, #2920, 1:1000 dilution) or phosphorylated AKT (Ser473) (Cell Signaling, #4060, 1:1000 dilution), total (Cell Signaling, #2317, 1:1000 dilution) or phosphorylated S6K (Ser235/236) (Cell Signaling, #4858, 1:1000 dilution), MMP2 (Cell Signaling, #87809, 1:1000 dilution) or MMP9 (Cell Signaling, #13667, 1:1000 dilution). After three consecutive washes with

TBST buffer, the membranes were incubated with an 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 by 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). Expression levels of GRP78, CHOP, TRIB3, MMP2, and MMP9 were normalized to that of β-actin. In contrast, expression of phosphorylated AKT and S6K was normalized to expression of total AKT and S6K, respectively.

siRNA transfection

For siRNA experiments, cells seeded on a 6-well plate were grown to 60%–80% confluence. The cells were transfected with siRNA targeting CHOP (sc-35437), TRIB3 (sc-4426) or a nonspecific control (sc-37007; all Santa Cruz Biotechnology) using Lipofectamine RNAiMax Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The final concentration of siRNA was 25 pmol/L. Total proteins were extracted and Western blot analysis was performed to confirm protein level at 48 hours after transfection.

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 was prepared in serum-free media, and 300 µl of cells was added into the upper chamber. Meanwhile, 500 µl DMEM with 10% FBS was added to the lower chamber. Cells were then 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. Filters were then fixed in 90% alcohol and stained with 0.1% (w/v) crystal violet. The filters were quantitated by dissolving 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.

Immunofluorescence staining

NESCs were plated on sterile glass coverslips and fixed with 4% (v/v) paraformaldehyde. Nonspecific

binding sites were blocked with 0.1% (w/v) bovine serum albumin, and fixed cells were then incubated with anti-TRIB3 rabbit polyclonal antibody (Abcam, ab137526, 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-phosphorylated S6K (Ser235/236) rabbit monoclonal antibody (Alexa Fluor® 488 Conjugate) (Cell Signaling, #4803, 1:500 dilution). Finally, slides were mounted in mounting media (Vector Laboratories), and images were captured with a confocal microscope (Bio-Rad).

Statistical analysis

The results are expressed as means ± standard errors based on four independent experiments. ANOVA and post hoc Tukey test for pairwise comparisons were used for statistical analysis of the Western blot and invading analyses data regarding the effects of steroid hormones. A Student's t-test was used to compare in vitro and in vivo data from the two groups. Statistical analyses were executed using SAS version 9.4 (SAS Institute, Cary, NC, USA). Statistical significance was inferred at $P < 0.05$.

RESULTS

ER stress-induced CHOP and TRIB3 expression inhibits cell invasion via the AKT/mTOR pathway

The role that ER stress plays in regulating the AKT/mTOR pathway in normal endometrial cells was studied through controlling CHOP and TRIB3 expression. The effects of tunicamycin, an ER stress inducer, on the levels of ER stress markers GRP78, CHOP, TRIB3, phosphorylated AKT, and S6K was examined in cultured NECSs. Tunicamycin treatment significantly increased expression of GRP78, CHOP, and TRIB3 in the control group ($P < 0.05$) (Fig. 1A). In contrast, expression of phosphorylated AKT and S6K was significantly lower after tunicamycin treatment ($P < 0.05$). Endogenous TRIB3 and phosphorylated S6K expression was further examined using immunofluorescence staining to confirm these findings. The expression of endogenous TRIB3 and phosphorylated S6K was readily detected in cultured NESCs as red and green fluorescent areas in the nucleus and cytoplasm, respectively (Fig. 1B). In the absence of tunicamycin, cultured NESCs stained weakly for the TRIB3 protein (Fig. 2B, I) but had intense phosphorylated S6K immunoreactivity (Fig. 2B, II). After the addition of tunicamycin, NESCs developed intense TRIB3 immunoreactivity (Fig. 2B, III) with very weak

staining for phosphorylated S6K (Fig. 2B, VI).

The effects of ER stress-induced inhibition of the AKT/mTOR pathway on invasiveness was next evaluated by measuring invasion-related protein (MMP2 and MMP9) expression and cell invasion in cultured NESCs. Tunicamycin treatment significantly decreased MMP2 and MMP9 expression in the control group ($P < 0.05$) (Fig. 1C). Cell invasion was also significantly reduced in tunicamycin-treated NESCs ($P < 0.05$) compared to the control group (Fig.1D).

Involvement of CHOP/TRIB3 signaling in regulation of endometrial cell invasion via the AKT/mTOR pathway

ER stress induced upregulation of CHOP and TRIB3 expression, with subsequent reduction in cellular invasion through inhibition of the AKT/mTOR pathway. We further examined whether CHOP or TRIB3 was involved in regulating the invasiveness of endometrial cells. NESCs were transfected with CHOP siRNA, TRIB3 siRNA, or nonspecific control siRNA. Transfection with CHOP siRNA led to a decrease in CHOP expression to 24.1±8.0 ($P < 0.05$) in NESCs compared to that of cells transfected with nonspecific control siRNA (Fig. 2A-a and b). After transfection with CHOP siRNA, TRIB3 expression was significantly lower in NESCs compared to those transfected with nonspecific control siRNA. In contrast, AKT and S6K phosphorylation were enhanced after transfection with CHOP siRNA ($P < 0.05$). Furthermore, the proportion of invading cells increased significantly in NESCs transfected with CHOP siRNA (Fig. 2A-c, $P < 0.05$). Similar results were observed in the TRIB3 siRNA experiments. TRIB3 expression decreased significantly to 24.1±8.0 ($P < 0.05$) without changing CHOP expression after transfection with TRIB3 siRNA. This transfection led to enhanced AKT and S6K phosphorylation (Fig. 2B-a and b, $P < 0.05$). Cell invasion also increased significantly after transfection with TRIB3 siRNA (Fig. 2B-c, $P < 0.05$).

Progesterone-induced ER stress inhibits cell invasion through the CHOP/TRIB3/AKT/mTOR axis in NESCs

To evaluate the effects of ovarian steroids on ER stress-induced CHOP/TRIB3 signaling and its downstream AKT/mTOR pathway, the effects of estrogen and progesterone on expression of GRP78, CHOP, TRIB3, phosphorylated AKT, and phosphorylated S6K was characterized in cultured NECSs. There were significantly

higher expression levels of GRP78, CHOP, and TRIB3, and decreased phosphorylated AKT and S6K expression in NESCs that were cultured with estrogen and progesterone compared to those cultured with estrogen alone (Fig. 3A, $P < 0.05$). However, progesterone-stimulated GRP78 expression was significantly inhibited by the addition of both a progesterone receptor modulator (mifepristone) and an ER stress inhibitor (salubrinal). This inhibition was accompanied by decreased CHOP and TRIB3 expression as well as increased AKT and S6K phosphorylation (Fig. 3A, $P < 0.05$).

We also studied whether progesterone-induced upregulation of ER stress affected the invasiveness of NESCs. MMP2 and MMP9 expression levels and cell invasion decreased significantly in NESCs cultured with the addition of progesterone compared to respective levels in NESCs cultured with estrogen alone ($P < 0.05$) (Fig. 3B and C). This suppression was reversed by the addition of either mifepristone or salubrinal (Fig. 3B and C, $P < 0.05$).

Progesterone treatment does not influence ER stress-induced CHOP and TRIB3 expression, the AKT/mTOR pathway, or cell invasion in ECSCs

Next, we investigated whether the effects of ovarian steroids on ER stress and the CHOP/TRIB3/AKT/mTOR axis differed between NESCs and ECSCs. We also studied whether this difference affected ECSC invasiveness. To do so, the effects of estrogen and/or progesterone on expression of GRP78, CHOP, TRIB3, phosphorylated AKT, and phosphorylated S6K in cultured ECSCs was characterized. In estrogen-treated ECSCs, the addition of progesterone did not change expression of GPR78 CHOP, TRIB3, phosphorylated AKT, S6K, MMP2, or MMP9, or cell invasion (Fig. 4A, B and C). However, there was significantly higher GRP78, CHOP, and TRIB3 expression in cells cultured with tunicamycin compared to that of the control group (Fig. 4D, $P < 0.05$). Furthermore, there was significantly less ECSC invasion in cells cultured with tunicamycin compared to that of the control group (Fig. 4E, $P < 0.05$).

Expression of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 in normal endometrial and endometriotic tissues during the menstrual cycle

We evaluated whether ER stress and cell invasion were induced differently throughout the menstrual cycle in normal endometrial and endometriotic tissues. In normal endometrial tissues, GRP78, GRP94, CHOP, and

TRIB3 expression increased during the secretory phase compared to the proliferative phase (Fig. 5A and B), while MMP2 and MMP9 expression decreased significantly ($P < 0.05$). In contrast, GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 expression did not change during the secretory phase (Fig. 5C) in endometriotic tissues.

We also compared expression levels of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 during the late secretory phase of the menstrual cycle. The expression levels of GRP78, GRP94, CHOP, and TRIB3 were significantly lower while MMP2 and MMP9 levels were significantly higher in endometriotic tissues than they were in normal endometrial tissues ($P < 0.05$) (Fig. 5D and E).

DISCUSSION

The ER is a highly dynamic organelle responsible for the synthesis, folding, and assembly of almost all secreted and transmembrane proteins. Extracellular or intracellular stimuli that perturb ER function lead to accumulation and aggregation of unfolded and/or misfolded proteins in the ER, resulting in ER stress (Xu and Bailly-Maitre, 2005). Growing evidence suggests that ER stress negatively regulates the AKT/mTOR pathway through activation of CHOP/TRIB3 signaling (Lin et al., 2017; Xu et al., 2017). Recent studies have shown that mTOR inhibition could suppress cancer cell invasion (Chandrika et al., 2016; Song et al., 2016). These findings suggest that increased ER stress inhibits cell invasion by preventing mTOR activation. There is increased ER stress in human endometrial cells during the secretory phase of the menstrual cycle (Guzel et al., 2011; Choi et al., 2018); however, its precise role in regulation of endometrial cell invasiveness remains unclear. Here, ER stress conditions were induced using tunicamycin, an agent commonly used for this purpose (Ozcan et al., 2004). We evaluated whether ER stress is involved in human endometrial cell invasiveness through modulation of the AKT/mTOR pathway. We observed that tunicamycin-induced ER stress increased CHOP and TRIB3 expression in NESCs, which led to a decrease in AKT and mTOR activity. Immunofluorescence analysis also revealed that ER stress decreased mTOR activity through TRIB3 regulation. Furthermore, we found that this inhibition was accompanied by decreased expression levels of MMP2 and MMP9. Both MMP2 and MMP9 are known to accelerate tumor migration and invasion (Li et al., 2013; Yang et al., 2014; Yuan et al., 2014), as well as the proportion of invading endometrial cells. Therefore, ER stress reduces endometrial cell invasiveness via upregulation of CHOP and TRIB3, which inhibit the AKT/mTOR pathway. To confirm this pathway, the role of ER stress-mediated CHOP/TRIB3 signaling in endometrial cell invasiveness was evaluated using CHOP and

TRIB3 siRNA. siRNA-mediated downregulation of CHOP and TRIB3 increased AKT and mTOR activity and subsequently promoted cell invasion in NESCs. This finding suggests that ER stress suppresses endometrial cell invasiveness via CHOP/TRIB3 signaling-dependent inhibition of the AKT/mTOR pathway, implicating ER stress as an inhibitor of endometrial cell invasiveness.

According to previous studies, physiologic ER stress induction (during the menstrual cycle) is mediated by estrogen and progesterone in endometrial cells. Other studies have also independently demonstrated that estrogen inhibits ER stress in endometrial cells (Guzel et al., 2011; Choi et al., 2018). In contrast, progesterone, the main hormone during the secretory phase of the menstrual cycle, reverses this inhibition (Choi et al., 2018). Similarly, our *in vitro* experiments show that expression of GRP78, CHOP, and TRIB3 in NESCs treated with estrogen alone (proliferative phase) increased with the addition of progesterone (secretory phase). Subsequently, MMP expression and cell invasion decreased when AKT and mTOR activity was inhibited. These results suggest that progesterone-induced ER stress decreases endometrial cell invasiveness by enhancing CHOP and TRIB3 expression, thereby inhibiting the AKT/mTOR pathway. This hypothesis is also supported by the observation that these progesterone-induced effects were reversed when CHOP and TRIB3 expression was blocked by inhibiting ER stress using mifepristone or salubrinal. Therefore, these results suggest that progesterone inhibits endometrial stromal cell invasiveness through ER stress upregulation. This finding indicates that ER stress is an important mediator of progesterone action. However, endometriosis is well known to be associated with progesterone resistance (Attia et al., 2000; Bulun et al., 2006; Rizner, 2009). Accordingly, progesterone is thought to inhibit AKT and mTOR activity in normal endometrial cells, but not in ectopic endometriotic cells (Choi et al., 2014; 2017). This study further demonstrated that, in addition to AKT and mTOR activity, progesterone had no significant effect on ER stress and CHOP/TRIB3 signaling and therefore does not play a significant role in ECSC invasiveness. Accordingly, progesterone does not decrease endometriotic stromal cell invasiveness during the menstrual cycle. In contrast, upregulation of ER stress by tunicamycin significantly decreased the invasiveness of ECSCs by inhibiting AKT and mTOR activity.

These findings were also supported by *in vivo* evaluation of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 expression during the menstrual cycle. In contrast to normal endometrial tissues, the constant level of ER stress in endometriotic tissues disinhibits cell invasiveness, as evidenced by a lack of change in the expression of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 throughout the menstrual cycle. Recent study also reported that GRP78 expression was significantly lower in the endometriotic ovarian cysts compared with that in the normal endometrium (Ciavattini et al., 2018), which suggest that ER stress abnormally

decreased in endometriotic lesions. In this study, furthermore, GRP78, GRP94, CHOP, and TRIB3 expression were significantly lower in endometriotic tissues, while expression of MMP2 and MMP9 were higher during the late secretory phase than in normal tissues. Consistent with these findings, previous studies have also demonstrated inappropriately high MMP2 and MMP9 expression in endometriotic tissues. This expression pattern is associated with increased cell invasiveness (Bruner et al., 1997; Collette et al., 2006). These results suggest that an aberrant ER stress response to progesterone increases the invasiveness of endometriotic tissues compared to that of normal tissues.

We could not use endometrium from absolutely disease-free controls in this study due to ethical reasons. Instead, we recruited the patients with leiomyoma, assuming that their endometrium was normal. In addition, although we have shown the potential effect of ER stress in endometriosis progression using endometriotic stromal cells obtained from only ovarian endometrioma, the results would be much more persuasive if we used different endometriotic stromal cells obtained from all types of endometriosis. Despite these limitations, the present study has shown for the first time that ER stress negatively regulates the cellular invasiveness of normal endometrial stromal cells through the CHOP/TRIB3/AKT/mTOR axis during the menstrual cycle and suggested that an abnormal ER stress response to progesterone increases the invasiveness of endometriotic stromal cells, which may be associated with disease progression.

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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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440

Figure Legends

Figure 1. Effects of tunicamycin on CHOP/TRIB3/AKT/mTOR axis and invasiveness in NESCs. A: Representative immunoblots (left) and densitometric quantification (right) of GRP78, CHOP, TRIB3, phosphorylated AKT, and phosphorylated S6K from cultured NESCs. B: Double-immunofluorescence staining for TRIB3 and p-S6K in NESCS cultured without tunicamycin (I and III) or with tunicamycin (II and IV) . TRIB3 and p-S6K were stained with red and green fluorophores in the nucleus and cytoplasm, respectively. C: Representative immunoblots (left) and densitometric quantification (right) of MMP2 and MMP9 from conditioned media. D: Percentages of invading NESCs, as determined by invasion assay. Experiments were repeated four times. Data are expressed as percentages, and control groups are normalized to 100%. *Significant differences ($P < 0.05$) compared with the control group.

Figure 2. Downregulation of CHOP and TRIB3 expression increase AKT activity, mTOR activity, and cellular invasiveness in NESCs. A and B: Representative immunoblots (a), densitometric quantification (b) of CHOP, TRIB3, phosphorylated AKT and phosphorylated S6K, and percentages of cell invasion (c) after transfection of NESCs with CHOP siRNA (A) and TRIB3 siRNA (B). Experiments were repeated four times. Data are expressed as percentages, and cells transfected with nonspecific control siRNA are normalized to 100%. *$P <$ 0.05 compared with control siRNA group.

Figure 3. Progesterone-induced ER stress decreases cellular invasiveness by inhibiting AKT and mTOR activity via CHOP/TRIB3 signaling in NESCs. A: Representative immunoblots (left) and densitometric quantification (right) of GRP78, CHOP, TRIB3, phosphorylated AKT, and phosphorylated S6K from cultured NESCs. B: Representative immunoblots (left) and densitometric quantification (right) of MMP2 and MMP9 from conditioned media. C: Percentages of invading NESCs, as determined by invasion assay. Experiments were repeated four times. Data are expressed as percentages, and cells treated with estrogen alone are normalized to 100% (*$P < 0.05$ by post hoc Tukey test). Est, estrogen; Pro, progesterone; Mife, mifepristone; Sal, salubrinal.

Figure 4. Progesterone does not induce ER stress or inhibit cellular invasiveness in ECSCs. A: Representative immunoblots (left) and densitometric quantification (right) of GRP78, CHOP, TRIB3, phosphorylated AKT, and phosphorylated S6K from ECSCs cultured with estrogen and/or progesterone. B: Representative

immunoblots (top) and densitometric quantification (bottom) of MMP2 and MMP9 from conditioned media. C: Percentages of invading NESCs cultured with estrogen and/or progesterone, as determined by invasion assay. Experiments were repeated four times. Data are expressed as percentages, and cells treated with estrogen alone are normalized to 100%. *$P < 0.05$ compared with control estrogen-alone group. D: Representative immunoblots (left) and densitometric quantification (right) of GRP78, CHOP, and TRIB3 from ECSCs cultured with/without tunicamycin. E: Percentages of invading ECSCs cultured with/without tunicamycin, as determined by invasion assay. Experiments were repeated four times. Data are expressed as percentages, and control groups are normalized to 100%. *Significant differences ($P < 0.05$) compared with control group.

Figure 5. Expression of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 in normal eutopic endometrial and ectopic endometriotic tissues during menstrual cycle. A: Representative immunoblots of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 in eutopic endometrial (left) and ectopic endometriotic tissues (right). B and C: Densitometric quantification of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 protein contents in eutopic endometrial (B) and ectopic endometriotic tissues (C). Pro, proliferative phase; Sec, secretory phase. Experiments were repeated five times. Data are expressed as percentages, and endometrial tissues in proliferative phase are normalized to 100%. *$P < 0.05$ compared with proliferative tissues. D and E: Comparison of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9 in late secretory normal eutopic endometrial (NEET) and ectopic endometriotic tissues (EET). Representative immunoblots (D) and densitometric quantification (E) of GRP78, GRP94, CHOP, TRIB3, MMP2, and MMP9. Experiments were repeated four times. Data are expressed as percentages, and eutopic endometrial tissues are normalized to 100%. *$P < 0.05$ compared with eutopic endometrial tissues.

Figure 1.

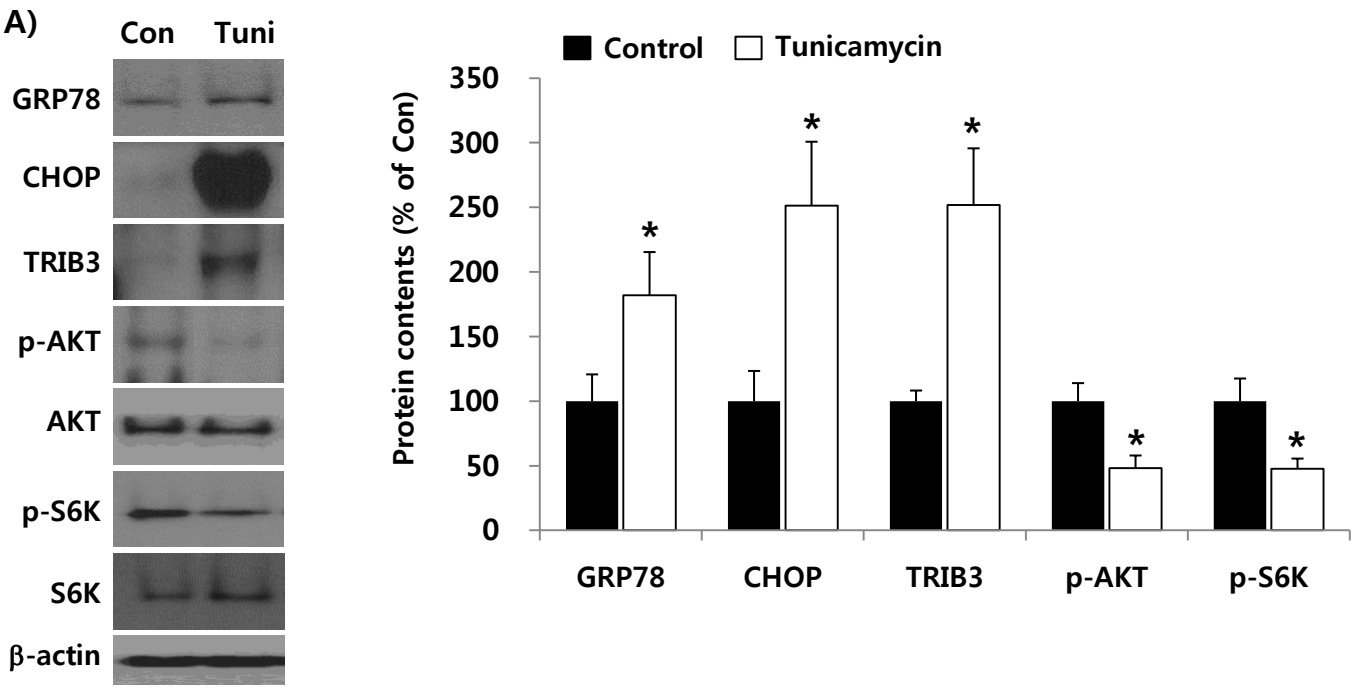

Figure 2.

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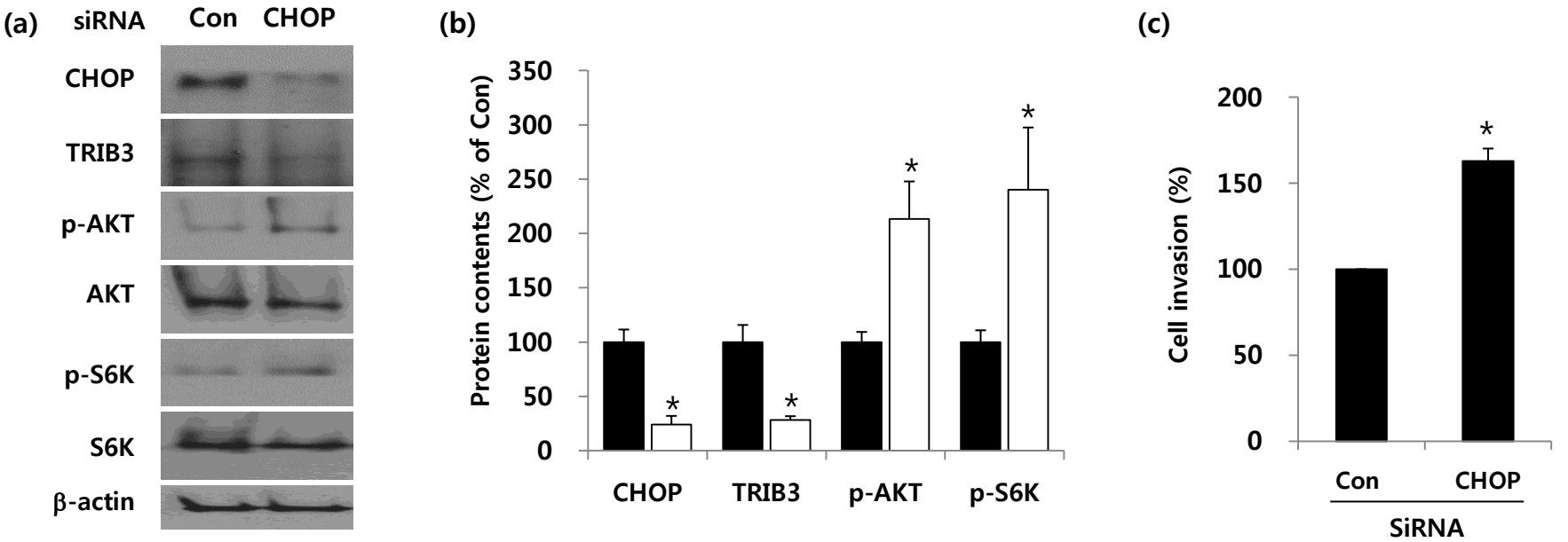

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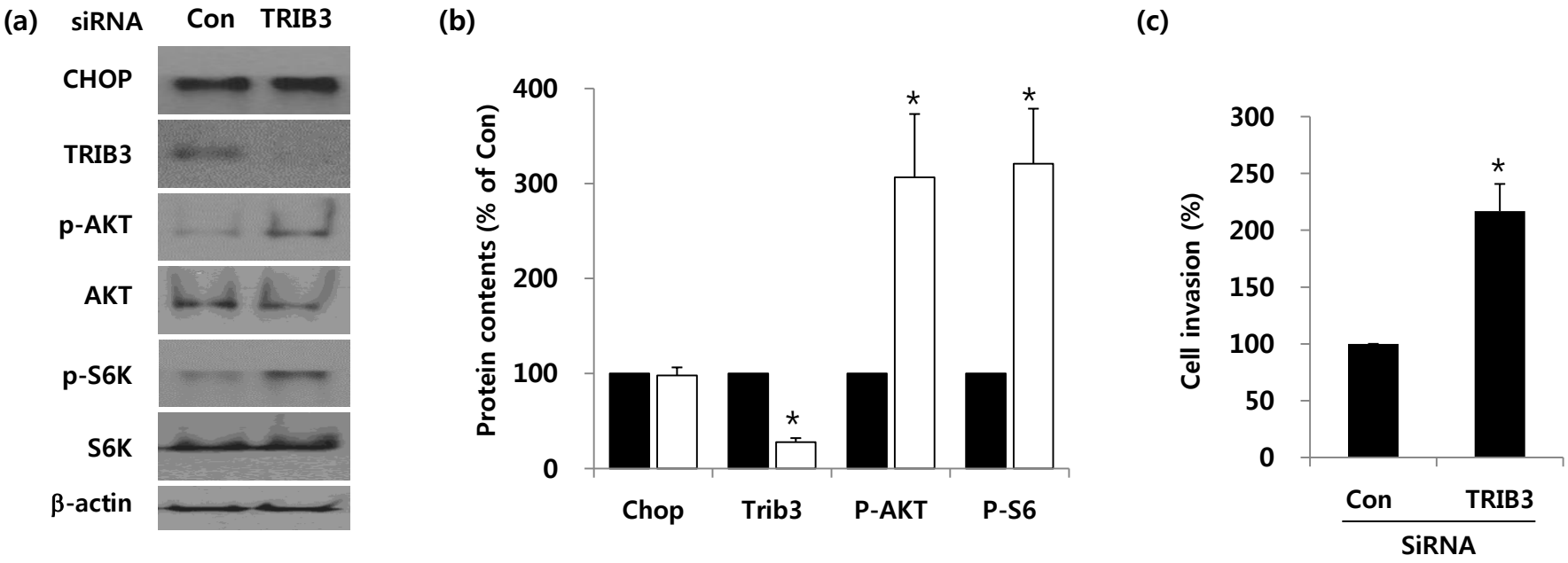

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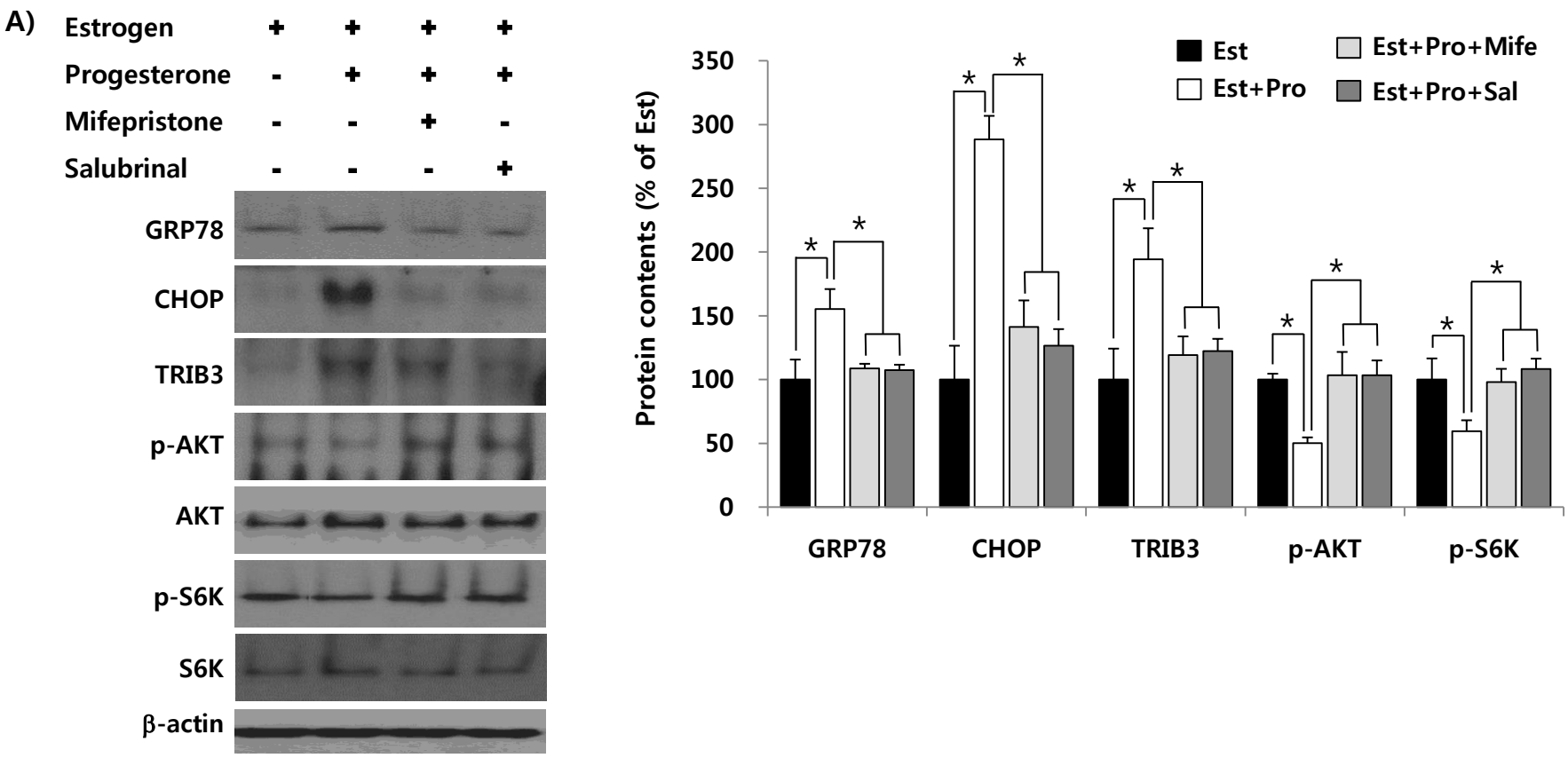
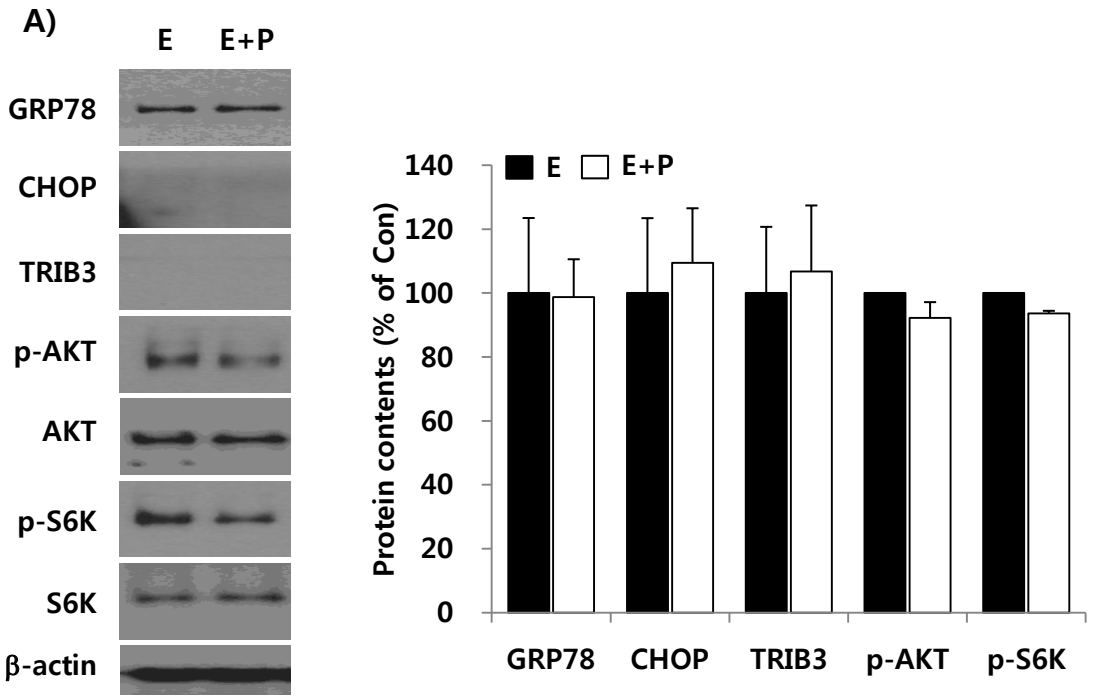


Figure 4.



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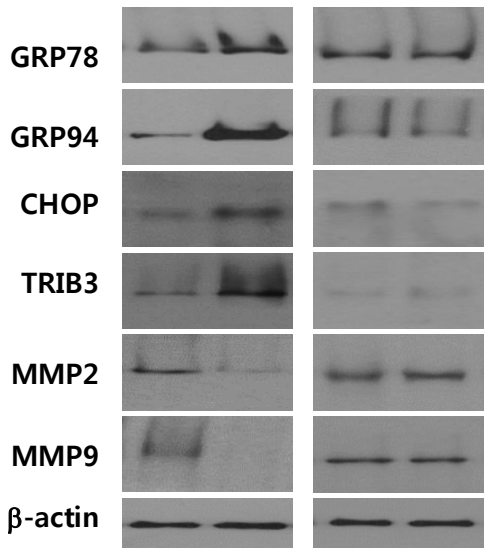

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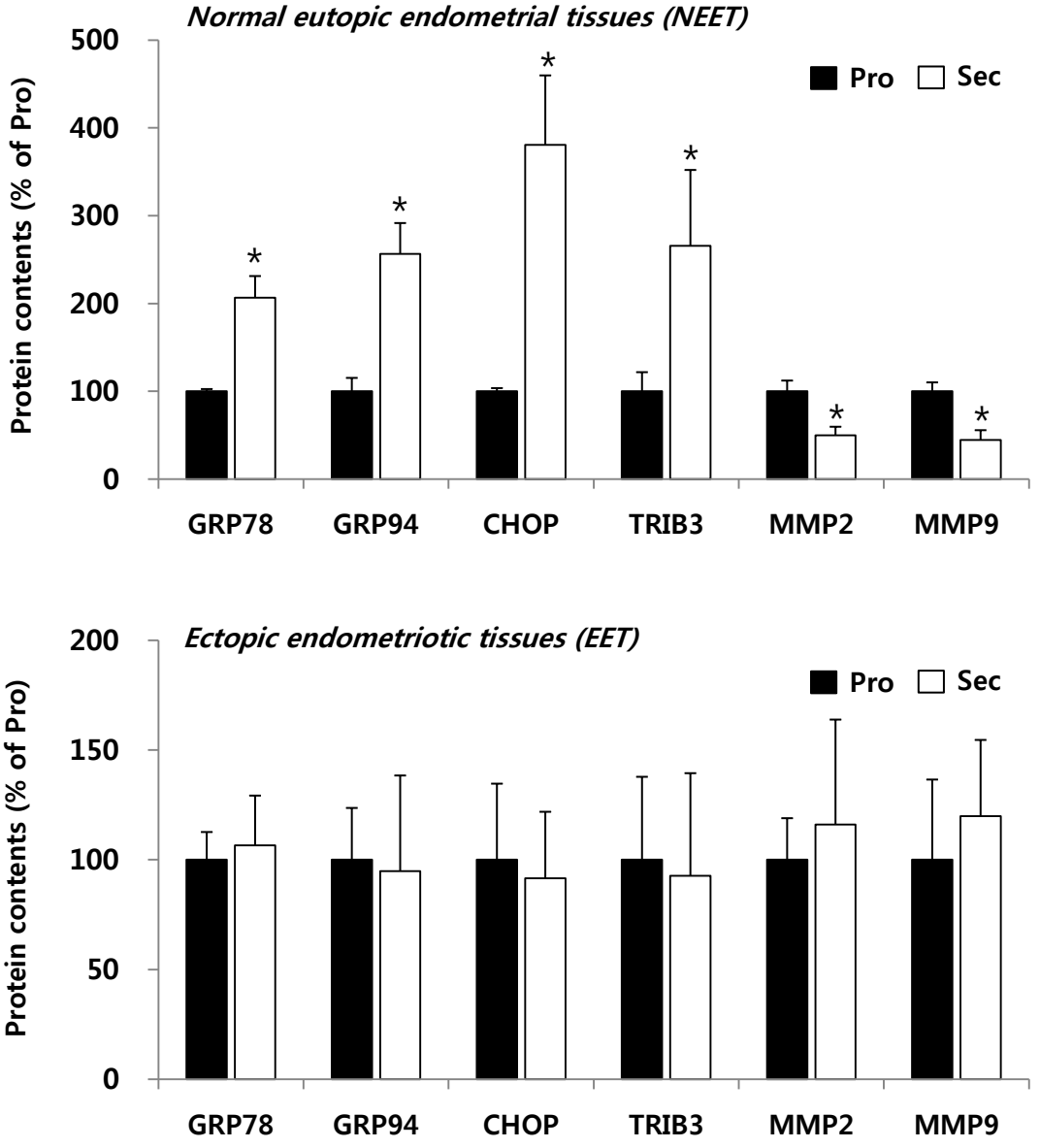

C)

D)