Aberrant PTEN expression in response to progesterone reduces endometriotic stromal cell apoptosis

JongYeob Choi¹, MinWha Jo², EunYoung Lee¹, Seongsoo Hwang³ and DooSeok Choi¹

¹*Infertility Clinic, Department of Obstetrics and Gynecology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Gangnam-gu, Seoul, Korea,* ²*Center for Clinical Research, Samsung Biomedical Research Institute, Gangnam-gu, Seoul, Korea and* ³*Animal Biotechnology Division, National Institute of Animal Science, Wanju-gun, Jeollabuk-do, Korea*

Correspondence should be addressed to D Choi; Email: dooseok.choi@samsung.com

Abstract

In some human cancer cells, PTEN (phosphatase and tensin homolog deleted on chromosome 10) is known to regulate autophagy induction positively through the inhibition of PI3K/AKT pathway, leading to the activation of mTOR, a major negative regulator of autophagy. Recent studies reported that PTEN expression is abnormally decreased in endometriotic lesions. In endometriosis, abnormal PTEN expression may contribute to the alteration of endometrial cell autophagy, which may affect apoptosis because endometrial cell autophagy is directly involved in the regulation of apoptosis. To test this hypothesis, we evaluated the involvement of PTEN in the regulation of autophagy induction in human normal endometrial stromal cells (NESC). In addition, we sought to determine whether aberrant PTEN expression in endometriotic cyst stromal cells (ECSCs) is associated with autophagy dysregulation, and a subsequent decrease in apoptosis. Our results show that PTEN expression was enhanced by progesterone treatment in NESC. Subsequently, autophagy and apoptosis induction increased through the inhibition of AKT and mTOR activity. This progesterone-induced increase in apoptosis was reversed by the inhibition of autophagy induction using either mifepristone (progesterone receptor modulator) or PTEN inhibitor. In contrast, progesterone had no significant effects on PTEN expression, AKT, mTOR activity, autophagy or apoptosis in ECSCs. Furthermore, in contrast to normal eutopic endometrium, endometriotic tissues have constant PTEN expression, autophagy and apoptosis throughout the menstrual cycle. In conclusion, our results suggest abnormal PTEN expression in response to progesterone was observed in ECSCs, which led to the dysregulation of autophagy induction via AKT/mTOR signalling and a subsequent decrease in apoptosis.

Reproduction (2017) **153** 11–21

Introduction

Endometriosis is characterised by the presence of endometrial tissue outside of the uterine cavity. These lesions typically occur on the ovaries and pelvic peritoneum. Endometriosis is one of the most common causes of chronic pelvic pain, dysmenorrhoea and infertility (Giudice & Kao 2004, Bulun 2009). It affects approximately 5–15% of reproductive-age women and 20–50% of infertile women (Eskenazi & Warner 1997, Pritts & Taylor 2003). The pathogenesis of endometriosis is poorly understood. Unfortunately, the current options for management of endometriosis are limited (Kauppila & Rönnerberg 1985, Vercellini *et al.* 1993, Crosignani *et al.* 2006). To improve its treatment, it is essential to investigate the molecular mechanisms associated with the development of endometriosis.

Apoptosis is a form of programmed cell death (PCD). Accumulating evidence suggests that reduced apoptosis in refluxed endometrial cells might enhance their survival at ectopic sites, leading to the development of

endometriosis (Gebel *et al.* 1998, Dmowski *et al.* 2001). However, apoptosis may not be the only mechanism of endometrial cell death. Autophagy, a nonapoptotic form of PCD, is an intracellular bulk degradation system in which a portion of the cytoplasm is enveloped in double membrane-bound structures called autophagosomes. These autophagosomes undergo maturation, and eventually fuse with lysosomes for degradation (Klionsky & Emr 2000, Levine & Klionsky 2004). Autophagy also plays an important role in promoting cell death by promoting caspase-dependent apoptosis in normal (Choi *et al.* 2011a,b) and cancerous cells (Boya *et al.* 2003, 2005, Wu *et al.* 2009), although it was originally thought to represent a survival response to nutrient deprivation and other forms of cellular stress (Yorimitsu & Klionsky 2005). Furthermore, we recently showed that autophagy induction exerts a proapoptotic effect on normal human endometrial cells (Choi *et al.* 2012). However, autophagy is suppressed in endometriotic cells due to the de-repression of mammalian target

of rapamycin (mTOR), a major negative regulator of autophagy; this results in decreased endometriotic cell apoptosis (Choi *et al.* 2014). These findings suggest a direct role of aberrant autophagy induction in the pathogenesis of endometriosis. However, dysregulated autophagy has never been fully evaluated in the context of endometriosis.

Phosphatase and tensin homolog deleted from chromosome 10 (PTEN) is one of the most frequently mutated tumour suppressor genes in human cancers (Steck *et al.* 1997). PTEN is completely lost or mutated in >50% of primary endometrioid endometrial cancers (Sun *et al.* 2001), and in at least 20% of precancerous endometrial hyperplasias (Levine *et al.* 1998, Sun *et al.* 2001). Furthermore, PTEN loss has also been reported in the malignant transformation of endometriosis (Obata *et al.* 1998, Sato *et al.* 2000a,b). Therefore, the loss and mutation of PTEN may contribute to the pathogenesis of endometrial cancer and endometriosis. This suggests that normal PTEN expression is a key factor in the multistep process that determines the cellular fate (survival vs death) of endometrial cells. The main function of PTEN is to inhibit the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signalling pathway by removing the phosphate from the D3-phosphate group of phosphoinositide-3, 4, 5-triphosphate (PIP3) (Maehama & Dixon 1998, Katso *et al.* 2001). In many normal and cancerous cells, the PI3K/AKT pathway has been shown to negatively regulate autophagy induction by activating mTOR (Corcelle *et al.* 2009, Chen & Karantzis 2011). This finding suggests that inhibiting AKT activity may promote autophagy by preventing mTOR activation. Therefore, PTEN may play an important role in autophagy regulation by controlling the PI3K/AKT pathway. Indeed, the upregulation of PTEN expression has been observed in some human cancer cells. This upregulation decreases AKT activity by inhibiting the PI3K/AKT pathway, thereby leading to mTOR inactivation and subsequent autophagy induction (Arico *et al.* 2001, Degtyarev *et al.* 2008, Fan *et al.* 2010, Nho & Hergert 2014). Furthermore, progesterone has been shown to upregulate PTEN expression (Guzeloglu-Kayisli *et al.* 2003). Increased PTEN expression facilitates the inhibition of AKT activity in human endometrial cells (Mutter *et al.* 2000a,b). 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 PTEN expression may be altered by abnormal responses to progesterone in endometriotic cells. This hypothesis was further supported by previous studies that revealed inappropriate decreases in PTEN expression in endometrial cells from women with endometriosis compared with that in disease-free women (Govatati *et al.* 2014, Zhang *et al.* 2014). However, it is not yet clear whether PTEN is involved in the regulation of autophagy induction in endometrial

cells or whether abnormal PTEN expression in response to progesterone in endometriotic cells contributes to autophagy dysregulation and reduced apoptosis.

Here, we evaluated the involvement of PTEN in the regulation of autophagy and apoptosis induction in normal endometrial stromal cells. In addition, we studied whether abnormal PTEN expression in response to progesterone affects autophagy and apoptosis induction in endometriotic stromal cells, which may contribute to the pathogenesis of endometriosis.

Materials and methods

Tissue collection

Normal endometrial tissues ($n=20$) were obtained from premenopausal women undergoing hysterectomies for uterine leiomyomas or pelvic organ prolapse. Ectopic endometriotic tissues ($n=30$) were obtained from ovarian endometriotic cysts (endometrioma). None of the study participants had taken oral contraceptives or hormonal agents for at least three months before surgery. The average age of the participants was 45.5 ± 3.4 years for eutopic endometrial tissues and 32.0 ± 7.7 years for ectopic endometrial tissues. The endometrial tissue samples were divided into five categories according to the day of the menstrual cycle: early proliferative (days 1–5), mid-to-late proliferative (days 6–14), early secretory (days 15–18), mid-secretory (days 19–23) and late secretory phase (days 24–28). The menstrual cycle day was established based on the patient's menstrual history. Of the 20 eutopic endometrial samples, four each were early proliferative, late proliferative, early secretory, mid-secretory and late secretory, and 6 each of the 30 ectopic endometrial samples were early proliferative, late proliferative, early secretory, mid-secretory and late secretory phase. All samples were snap-frozen in liquid nitrogen and stored at -80°C for western blot analysis. This study was approved by the Ethical Committee of Samsung Medical Center. Written informed consent was obtained from all participants.

Human normal endometrial and endometriotic cyst stromal cell isolation

Normal endometrial stromal cells (NESC) and endometriotic cyst stromal cells (ECSC) in the proliferative phase were dissociated and purified from eutopic endometrial tissues and ovarian endometriotic tissues respectively, using a published procedure (Ryan *et al.* 1994) with minor modifications. Tissues were rinsed with PBS. The endometrial lining was dissected from the myometrium, minced and digested with 2 mg/mL type IV collagenase (Sigma Chemical) at 37°C for 60 min with agitation. Stromal cells were separated from epithelial glands using 70 μm -pore filters, and then a 45 μm -pore nylon mesh. Filtered cells were plated in T75 flasks and allowed to adhere for approximately 30 min. The flasks were washed with PBS to remove blood cells and debris. The stromal cells were cultured in Dulbecco's modified Eagle's/F12 medium (DMEM/F12; Gibco-BRL) supplemented with 10% foetal bovine serum (FBS, Gibco-BRL), 100 U/mL penicillin and 100 mg/mL

streptomycin (Gibco-BRL) in a humidified atmosphere with 5% CO₂ 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. Endometrial stromal cell suspension purity was determined by immunostaining with vimentin stromal cell-specific antibodies.

In vitro experiments

Each experiment with NESCs and ECSCs was performed using cells prepared from eutopic endometrial tissues ($n=4$) and ovarian endometriotic tissues obtained ($n=4$) from 8 different patients respectively, for sex steroid, mifepristone or PTEN inhibitor treatment. Subcultured NESCs 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). Cells were incubated at 37°C in 5% CO₂ in DMEM/F12 supplemented with 10% FBS, glutamine, HEPES, 100 U/mL penicillin and 100 mg/mL streptomycin. At 70–80% confluence, cultures were treated with serum-free Earle's Balanced Salt Solution (EBSS) medium (Sigma) for serum starvation. To mimic physiological hormonal changes, NESCs and ECSCs were also cultured with EBSS medium before hormone treatment. After 24 h of culture, either oestradiol (10 nM, Sigma) alone or oestradiol (10 nM)+progesterone (1 μ M, Sigma) was added for 72 h. For the pharmacological inhibition experiments, NESCs were cultured in EBSS medium supplemented with oestradiol (10 nM) and progesterone (1 μ M) for 72 h. Two hours before the analysis, a progesterone receptor modulator (100 nM, mifepristone, Sigma) or PTEN inhibitor (100 nM, SF1670, Sigma) was added to the culture medium to inhibit the effects of progesterone or PTEN respectively. For the apoptosis experiments, NESCs were cultured in EBSS supplemented with oestradiol (10 nM) and progesterone (1 μ M) for 72 h. Next, mifepristone or PTEN inhibitor was added to the culture medium 6 h before analysis to inhibit the induction of progesterone- or PTEN-mediated autophagy respectively. Treatments were stopped by removing the medium. Cells were then either harvested by scraping to generate protein extracts or fixed for subsequent immunofluorescence and electron microscopy analyses. Apoptosis of the endometrial stromal cells was evaluated using annexin-V/propidium iodide (PI) staining.

Western blot

During autophagy induction, LC3 was converted from LC3-I to LC3-II. The LC3-II localises to isolated membranes and autophagosomes (Kabeya *et al.* 2000, 2004). The level of LC3-II expression has been shown to correlate with the autophagosome number (Nara *et al.* 2002). Therefore, the level of LC3-II protein was assessed using western blotting as a surrogate measure of endometrial cell autophagy. The level of autophagy is also reflected by the expression level of autophagy-related 5 (ATG5), which is a protein specifically required for autophagy induction. AKT activity was determined by quantifying the phosphorylated (i.e., active) form of AKT. The activity of the mTOR pathway was determined by measuring the phosphorylation of ribosomal protein S6

kinase (S6K). S6K is a direct substrate of mTOR. Therefore, the phosphorylation status of S6K reflects the activity of the mTOR pathway (Sarbasov *et al.* 2005). The extent of endometrial cell apoptosis was determined by measuring the level of cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3, the final mediator of apoptosis (Porter & Janicke 1999). Endometrial tissues, endometriotic tissues or cultured stromal cells were lysed with an ice-cold radioimmunoprecipitation assay buffer that was supplemented with a protease inhibitor cocktail (Sigma). For complete solubilisation of cellular proteins, lysates were incubated on ice for 30 min and then centrifuged at 13,000g at 4°C for 30 min. Proteins in whole cell lysates (20 μ g/lane) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad). After blocking nonspecific binding sites with 5% skim milk, membranes were incubated with rabbit polyclonal antibodies against PTEN (1:1000; Cell Signaling Technology), total and phosphorylated AKT (Ser473) (1:500 and 1:500 respectively; Cell Signaling Technology), total and phosphorylated S6K (Ser235/236) (1:1000 each; Cell Signaling Technology), ATG5 (1:1000; Cell Signaling Technology), LC3 (1:1000; Cell Signaling Technology) or cleaved caspase-3 (1:1000; Cell Signaling Technology) overnight at 4°C. Membranes were washed 3 times for 15 min in wash buffer (PBS containing 0.1% Tween 20) and incubated with the appropriate secondary antibodies (1:5000; Santa Cruz Biotechnology) at room temperature for 1 h. After three 15-min washes in wash buffer at room temperature, immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Bands were quantified using NIH ImageJ software (NIH Image Processing and Analysis in Java). The expression levels of LC3-II and cleaved caspase-3 were normalised to that of β -actin. In contrast, the expression levels of phosphorylated AKT and S6K were normalized to those of total AKT and S6K respectively.

Immunofluorescence

Endometrial stromal cells were cultured on sterilised glass coverslips, fixed with 4% paraformaldehyde and blocked with 0.1% bovine serum albumin in PBS. Cells were incubated with anti-LC3 mouse polyclonal antibody (1:500) and PTEN rabbit polyclonal antibody (1:500) in PBS. Next, the cells were incubated with Alexa 488- and 568-conjugated secondary antibodies (1:5000; Vector Laboratories, Burlingame, CA, USA). The slides were mounted in mounting media (Vector Laboratories) with 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. Images were captured with a confocal microscope (Bio-Rad).

Transmission electron microscopy

To identify autophagosomes at the ultrastructural level, endometrial stromal cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 45 min at 4°C. The cells were then rinsed in cacodylate buffer, post-fixed in 1% OsO₄ in cacodylate buffer, dehydrated and embedded in Eponate. Ultra-thin sections were briefly contrasted with

uranyl acetate and photographed with a transmission electron microscope (Hitachi 7100).

Assessment of human endometrial stromal cell apoptosis

Apoptotic cell percentages were determined with an annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, after drug treatment, 1×10^5 cells were pelleted and washed once with PBS. Cells were then resuspended in 100 μ L binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM potassium chloride, 1 mM $MgCl_2$, and 2 mM calcium chloride). Subsequently, 5 μ L annexin-V and PI were added and the cells were incubated for 15 min at room temperature in the darkness. After incubation, 400 μ L binding buffer was added, and cells were analysed using a FACSaria flow cytometry (BD Biosciences, Heidelberg, Germany). At least 10,000 cells were analysed before treatment. Data analysis was conducted using CellQuest software.

Statistical analysis

Relative protein expression levels and proportions of apoptotic cells are reported as means $\pm$ standard errors. All statistical analyses were performed using ANOVA. Significant differences between the treatment groups were identified using Duncan's multiple range test. P values <0.05 were considered statistically significant.

Results

Upregulated PTEN expression increases endometrial cell autophagy by inhibiting AKT activity, thereby inactivating mTOR

We sought to determine the involvement of PTEN in the regulation of autophagy induction in endometrial cells through its control of AKT and mTOR activity. To do so, we characterised the effects of oestrogen and progesterone on the levels of PTEN, phosphorylated AKT, S6K, ATG5 and LC3-II expression in cultured NESC. Progesterone treatment significantly increased PTEN, LC3-II and ATG5 expression in oestrogen-treated NESC ($P < 0.05$) (Fig. 1A and B). In contrast, AKT and S6K phosphorylation were both significantly decreased after progesterone treatment ($P < 0.05$). To block the effects of progesterone, we used pharmacological inhibitors of progesterone and PTEN and measured the levels of phosphorylated AKT, S6K and LC3-II expression. Progesterone-stimulated PTEN expression was suppressed by the addition of both progesterone receptor modulator (mifepristone) and PTEN inhibitor (SF1670). This suppression was accompanied by increased AKT and S6K phosphorylation, and decreased LC3-II and ATG5 expression ($P < 0.05$) (Fig. 1A and B).

Next, we used immunofluorescence staining to determine the subcellular localisations of endogenous

PTEN and LC3. The distributions of endogenous PTEN and LC3 were readily detected in the cultured NESC as green and red fluorescent areas respectively (Fig. 1C). In NESC cultured with oestrogen alone, there was weak PTEN staining and a few punctate LC3-II structures were detected (Fig. 1C, Est). The addition of progesterone resulted in intense PTEN immunoreactivity and the cytoplasmic accumulation of punctate LC3-II structures (Fig. 1C, Est + Prog). This change could be prevented by the addition of either mifepristone (Fig. 1C, Est + Prog + Mife) or a PTEN inhibitor (Fig. 1C, Est + Prog + PTEN In). To confirm these observations on an ultrastructural level, we analysed the autophagosome formation using transmission electron microscopy. Autophagic structures are characterised by multiple autophagosomes, which are double-membraned vacuoles containing engulfed cytoplasmic material. In the NESC cultured with oestrogen, there was an accumulation of autophagic vacuoles (indicative of autophagy induction) after the addition of progesterone. However, this accumulation could also be suppressed by the addition of either mifepristone or a PTEN inhibitor (Fig. 1D).

Endometrial cell apoptosis is induced after increased PTEN expression

To determine whether PTEN-mediated autophagy induction in NESC drives apoptosis, we evaluated the effects of progesterone, mifepristone and SF1670 on apoptosis in oestrogen-treated NESC. The NESC treated with progesterone exhibited significantly higher expression of cleaved PARP and cleaved caspase-3 than did the NESC cultured in oestrogen alone (Fig. 2A and B). Progesterone-mediated upregulation of cleaved PARP and cleaved caspase-3 expression was decreased by the addition of mifepristone and by the addition of SF1670. We also conducted flow cytometry assays using annexin-V and PI to determine the proportion of apoptotic cells. The proportion of apoptotic NESC induced by treatment with oestrogen alone increased significantly (by 2.23-fold) in the presence of progesterone (Fig. 2C and D). In contrast, the proportion of apoptotic progesterone-treated NESC decreased by ~42% and ~30% after the addition of mifepristone and SF1670 respectively ($P < 0.05$).

The effects of oestrogen and progesterone on PTEN expression, AKT levels, mTOR activity, autophagy and apoptosis in NESC and ECSCs in vitro

We sought to determine whether the effects of ovarian steroids on PTEN expression, AKT and mTOR activity are different in NESC and ECSC. In addition, we studied whether this differential expression affects autophagy and apoptosis in ECSC. To do so, we characterised the effects of oestrogen and progesterone on the levels of PTEN, phosphorylated AKT, S6K, ATG5, LC3-II and

Figure 1 Effects of oestrogen and progesterone on the levels of PTEN, phosphorylated AKT, phosphorylated S6K, ATG5 and LC3-II in NESC. (A) Representative immunoblots of PTEN, AKT, S6K, ATG5 and LC3 in protein extracts from *in vitro*-cultured NESC. (B) Densitometric quantification of PTEN, phosphorylated AKT (p-AKT), p-S6K, ATG5 and LC3-II protein contents. Experiments were repeated four times and data are expressed as means $\pm$ s.e.s. * $P < 0.05$ compared with the oestrogen-only group. ** $P < 0.05$ compared with the oestrogen + progesterone group. Est, oestrogen; Prog, progesterone; Mife, mifepristone. (C) Double-immunofluorescence staining for PTEN and LC3 in cultured HECs. PTEN and LC3 were stained with red and green fluorophores, respectively. LC3-I exhibited a diffuse cytoplasmic distribution. LC3-II was found in punctate structures. (D) Transmission electron microscope images of NESC cultured with oestrogen alone, oestrogen + progesterone, oestrogen + progesterone + mifepristone or oestrogen + progesterone + SF1670. Arrows indicate representative autophagosomes. Scale bars: 2 μ m. N, nucleus.

cleaved caspase-3 expression in cultured NESC and ECSC. There was significantly higher PTEN expression and decreased phosphorylation of AKT and S6K in NESC

cultured with oestrogen and progesterone compared with that of those cultured with oestrogen alone (Fig. 3A and B, $P < 0.05$). After incubation with oestrogen and

Figure 2 PTEN-mediated autophagy induction increases apoptotic cell death in NESC. (A and B) Representative immunoblots of cleaved PARP and cleaved caspase 3 in protein extracts from *in vitro* cultured NESC. Experiments were repeated four times and data are expressed as means $\pm$ standard errors (s.e.s). * $P < 0.05$ compared with the oestrogen-only group. ** $P < 0.05$ compared with the oestrogen + progesterone group. Est, oestrogen; Prog, progesterone; Mife, mifepristone. (C) Representative flow cytometry plots from the oestrogen alone, oestrogen + progesterone, oestrogen + progesterone + mifepristone and oestrogen + progesterone + SF1670 groups. Lower right quadrant, annexin V+/PI-; upper right quadrant, annexin V+/PI+ (considered apoptotic). (D) Percentages of apoptotic cells from flow cytometry. Bar graphs display means $\pm$ standard errors (s.e.s). * $P < 0.05$ compared with the oestrogen-only group. ** $P < 0.05$ compared with the oestrogen + progesterone group.

progesterone, the removal of both hormones from the media for 24 h also increased PTEN expression and decreased phosphorylated AKT and S6K expression ($P < 0.05$). Furthermore, the expression levels of LC3-II and cleaved caspase-3 increased significantly in NESC cultured with the addition of progesterone or withdrawal

of both oestrogen and progesterone compared with these respective levels in NESC cultured with oestrogen alone ($P < 0.05$). In ECSC, however, the oestrogen and progesterone treatment did not influence the expression of PTEN, phosphorylated AKT, S6, LC3-II or cleaved caspase-3 (Fig. 3C).

Figure 3 Differential expression of PTEN, autophagy and apoptosis in NESCs and ECSCs following treatment with oestrogen and/or progesterone. (A) Representative immunoblots of PTEN, phosphorylated AKT and S6K, ATG5, LC3 and cleaved caspase-3 in NESCs (left) and ECSCs (right). (B and C) Densitometric quantification of PTEN, phosphorylated AKT and S6K, ATG5, LC3-II and cleaved caspase-3 protein in NESCs (B) and ECSCs (C). Experiments were repeated four times, and data are expressed as mean ($\pm$ s.e.). *Significant differences ($P < 0.05$) when compared with the oestrogen alone group. W, withdrawal.

PTEN expression, AKT levels, mTOR activity, autophagy and apoptosis induction in normal eutopic endometrium and ectopic endometriotic tissues during the menstrual cycle

We investigated whether PTEN, AKT and mTOR activity are expressed differently during the menstrual cycle in normal eutopic endometrium and ectopic endometriotic tissues from ovarian endometriotic cysts. There PTEN expression was higher during the late proliferative phase than it was during the early proliferative phase, although the difference was not significant (Fig. 4A and B). The PTEN expression was significantly higher in the early (1.54-fold), mid- (1.65-fold) and late- (1.79-fold) secretory endometrium than it was in the early proliferative endometrium. In contrast, the levels of phosphorylated AKT and S6K were significantly lower in the secretory phase than they were in the early proliferative phase ($P < 0.05$). We also investigated whether PTEN-mediated endometrial AKT and mTOR activity is associated with autophagy and apoptosis induction during the menstrual cycle. To do so, we evaluated the expression patterns of Atg5, LC3-II and cleaved caspase-3. In the endometrial cells, the expression levels of Atg5, LC3-II and cleaved caspase-3 increase significantly during the secretory phase, peaking during the late secretory phase ($P < 0.05$; Fig. 4A and B).

In ectopic endometriotic tissues, however, the expression levels of PTEN, phosphorylated AKT, S6K, LC3-II and cleaved caspase-3 did not change during the secretory phase, instead remaining constant throughout the menstrual cycle (Fig. 4C). We also compared the expression levels of PTEN, phosphorylated AKT, S6K, LC3-II and cleaved caspase-3 during the late secretory phase of the menstrual cycle. The expression levels of PTEN, LC3-II and cleaved caspase-3 were significantly lower in the ectopic endometriotic tissues than they were in the normal endometrium; in contrast, the levels of phosphorylated AKT and S6K were significantly higher ($P < 0.05$) (Fig. 4D and E).

Discussion

In some human cell lines, the tumour suppressor PTEN positively regulates autophagy by inhibiting the PI3K/AKT/mTOR pathway (Arico *et al.* 2001, Degtyarev *et al.* 2008). Although PTEN has been reported to inhibit the PI3K/AKT pathway in human endometrial cells (Mutter *et al.* 2000a,b), its precise role in endometrial cell autophagy has remained unclear. Here, we evaluated whether PTEN is involved in autophagy induction by controlling the PI3K/AKT/mTOR pathway in human endometrial cells. In human endometrial cells, PTEN

Figure 4 Expression of PTEN, phosphorylated AKT and S6K, ATG5, LC3-II and cleaved caspase-3 in normal eutopic endometrial and ectopic endometriotic tissues during the menstrual cycle. (A) Representative immunoblots of PTEN, phosphorylated AKT and S6K, ATG5, LC3 and cleaved caspase-3 in eutopic endometrial (left) and ectopic endometriotic tissues (right). (B and C) Densitometric quantification of PTEN, phosphorylated AKT and S6K, ATG5, LC3 and cleaved caspase-3 protein contents in eutopic endometrial (B) and ectopic endometriotic tissues (C). EP, early proliferative phase; ES, early secretory phase; LP, mid- to late-proliferative phase; LS, late-secretory phase; MS, mid-secretory phase. Data are mean $\pm$ S.E. * $P < 0.05$ compared with EP. ** $P < 0.05$ compared with ES. (D and E) Comparison of PTEN, phosphorylated AKT and S6K, ATG5, LC3 and cleaved caspase-3 in late secretory normal eutopic endometrial (NEET) and ectopic endometriotic tissues (EET). Representative immunoblots (D) and densitometric quantification (E) of PTEN, phosphorylated AKT and S6K, ATG5, LC3 and cleaved caspase-3. Experiments were repeated four times, and data are expressed as means $\pm$ S.E.s. * $P < 0.05$ compared with normal.

expression (Guzeloglu-Kayisli *et al.* 2003) and autophagy induction (Choi *et al.* 2014) have been described to display a progesterone-dependent expression pattern. Similarly, our *in vitro* experiments showed that oestrogen treatment of NESC results in increased expression of PTEN and LC3-II. After the addition of progesterone, there is downregulation of phosphorylated AKT and S6K. These findings suggest that progesterone promotes autophagy induction in endometrial cells by increasing PTEN expression, which then inhibits AKT and mTOR activity. Our pharmacological data support this finding. Specifically, treatment with

mifepristone (a potent progesterone receptor modulator) or a PTEN inhibitor downregulates the expression of PTEN and LC3-II and promotes AKT and mTOR activity. Both mifepristone and PTEN inhibitors block the effects of progesterone treatment. These results indicate that progesterone-mediated PTEN expression increases autophagy induction in NESC via the AKT/mTOR signalling. Immunofluorescence analysis also supported the hypothesis that progesterone enhances autophagy induction by regulating PTEN expression in NESC. Specifically, the accumulation of LC3-II was accompanied by increased PTEN expression.

Furthermore, our TEM images provided direct evidence that autophagy induction in progesterone-treated NESCs decreased by the addition of mifepristone and by the addition of a PTEN inhibitor. Specifically, ultrastructural changes typical of autophagy, such as decreased numbers of autophagosomes within cells, were evident. These results suggest that progesterone increases PTEN expression, which then is directly involved in autophagy induction through the regulation of AKT and mTOR activity in human endometrial stromal cells.

According to previous studies, autophagy is an important cellular mechanism responsible for apoptosis induction in some systems (Saiki *et al.* 2011, Chow *et al.* 2012, Singh *et al.* 2012). Consistent with this finding, we have shown that autophagy induction is closely related to the induction of apoptosis in both endometrial and endometriotic cells (Choi *et al.* 2014). Indeed, autophagy induction promoted apoptosis; its pro-apoptotic effect was reversed by autophagy inhibition with 3-methyladenine (3-MA, autophagy inhibitor). Therefore, the high expression levels of PTEN in the progesterone-treated endometrial cells in this study may promote apoptotic cell death by enhancing autophagy induction. This hypothesis is supported by the observation that the progesterone-induced increase in apoptosis was prevented by mifepristone or a PTEN inhibitor. These results indicate that progesterone-induced PTEN expression plays a pivotal role in the regulation of apoptosis by controlling autophagy induction in endometrial cells. In contrast, it is well known that endometriosis is associated with progesterone resistance (Attia *et al.* 2000, Bulun *et al.* 2006, Rizner 2009). Indeed, recent reports have suggested that endometriotic cells have an abnormal response to ovarian steroids, which suppresses autophagy and apoptosis induction during the secretory phase of the menstrual cycle (Choi *et al.* 2014). Therefore, it is possible that abnormal PTEN response to progesterone in endometriotic cells may cause autophagy dysregulation, and subsequently decreased apoptosis during the menstrual cycle.

To confirm this hypothesis, we cultured NESCs and ECSCs *in vitro* with or without the ovarian steroids oestrogen and progesterone to mimic physiologic hormonal changes. We previously reported that these ovarian steroids control autophagy in endometrial cells during the menstrual cycle. These steroids are the two central balancing factors in the human endometrium (Choi *et al.* 2014). Indeed, autophagy induction in endometrial cells treated with oestrogen alone (the proliferative phase) increased after the addition of progesterone (early-to-mid secretory phase) or with the removal of oestrogen and progesterone (late secretory and menstrual phase). This study further demonstrated that PTEN expression increased with the downregulation of AKT and mTOR activity in oestrogen-treated NESCs by the addition of progesterone and the removal of oestrogen and progesterone. In contrast, progesterone treatment

did not affect the PTEN expression in oestrogen-treated ECSCs. This suggests that aberrant PTEN expression in endometriotic cells may be associated with an abnormal response to progesterone, called progesterone resistance. Furthermore, constant PTEN expression was accompanied by constant levels of AKT, mTOR activity, autophagy and apoptosis in ECSCs after ovarian steroid treatment. Accordingly, endometriotic cell autophagy combined with apoptosis may not be induced due to an abnormal PTEN response to progesterone during the menstrual cycle.

These findings were further demonstrated by our *in vivo* experiment, which evaluated whether PTEN expression, AKT and mTOR activity are expressed differently in human normal endometrium and endometriotic tissues according to the phases of the menstrual cycle. In human endometrial cells, PTEN displays a cycle-dependent expression pattern (Guzeloglu-Kayisli *et al.* 2003). Specifically, PTEN expression increases as the menstrual cycle progresses, reaching a maximum during the late secretory phase. In this study, we confirmed that PTEN expression increases as the menstrual cycle progresses in endometrial tissues. We also showed that this pattern is inversely correlated with AKT and S6K phosphorylation. Furthermore, we showed that the cyclic change of LC3-II and cleaved caspase-3 expression coincides with that of PTEN. These results indicate that the increased PTEN expression enhances endometrial cell autophagy. This occurs through the inhibition of AKT/mTOR signalling, followed by an increase in apoptosis during the secretory phase of the human endometrial cycle. In endometriotic tissues, however, our results also demonstrated that no cyclic changes were observed in the expression patterns of PTEN, phosphorylated AKT, S6K, LC3-II and cleaved caspase-3 throughout the menstrual cycle. Therefore, the constant level of PTEN expression in endometriotic tissues contributes to the dis-inhibition of AKT and mTOR activity, which is related to decreased autophagy and apoptosis. Indeed, PTEN expression, autophagy and apoptosis induction are significantly lower in endometriotic tissues and the activity of AKT and mTOR are higher when comparing the normal endometrium with endometriotic tissues during the late secretory phase. These *in vivo* results also suggest that endometriotic cell autophagy is suppressed by abnormal PTEN expression during the menstrual cycle, followed by a decrease in apoptosis. Therefore, it can be postulated that altered induction of endometriotic cell autophagy by aberrant PTEN expression facilitates the decreased apoptosis found in endometriotic tissues compared with that in normal tissues.

In conclusion, PTEN is directly involved in autophagy induction via control of AKT/mTOR signalling in normal endometrial cells during the menstrual cycle. However, endometriotic stromal cells exhibit an abnormal response to progesterone, which leads to suppression of PTEN. This suppression results in autophagy inhibition

via AKT/mTOR signalling, ultimately leading to decreased apoptosis. This pathway may contribute to the pathogenesis of endometriosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by a grant from Samsung Biomedical Research Institute.

References

- Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P & Ogier-Denis E 2001 The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *Journal of Biological Chemistry* **276** 35243–35246. (doi:10.1074/jbc.C100319200)
- Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR & Bulun SE 2000 Progesterone receptor isoform A but not B is expressed in endometriosis. *Journal of Clinical Endocrinology and Metabolism* **85** 2897–2902. (doi:10.1210/jc.85.8.2897)
- Boya P, Gonzalez-Polo RA, Poncet D, Andreau K, Vieira HL, Roumier T, Perfettini JL & Kroemer G 2003 Mitochondrial membrane permeabilization is a critical step of lysosome-initiated apoptosis induced by hydroxychloroquine. *Oncogene* **22** 3927–3936. (doi:10.1038/sj.onc.1206622)
- Boya P, Gonzalez-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Métévier D, Meley D, Souquere S, Yoshimori T *et al.* 2005 Inhibition of macroautophagy triggers apoptosis. *Molecular and Cellular Biology* **25** 1025–1040. (doi:10.1128/MCB.25.3.1025-1040.2005)
- Bulun SE, Cheng YH, Yin P, Imir G, Utsunomiya H, Attar E, Innes J & Julie Kim J 2006 Progesterone resistance in endometriosis: link to failure to metabolize estradiol. *Molecular and Cellular Endocrinology* **248** 94–103. (doi:10.1016/j.mce.2005.11.041)
- Bulun SE 2009 Endometriosis. *New England Journal of Medicine* **360** 268–279. (doi:10.1056/NEJMr0804690)
- Chen N & Karantza V 2011 Autophagy as a therapeutic target in cancer. *Cancer Biology and Therapy* **11** 157–168. (doi:10.4161/cbt.11.2.14622)
- Choi J, Jo M, Lee E & Choi D 2011a Induction of apoptotic cell death via accumulation of autophagosomes in rat granulosa cells. *Fertility and Sterility* **95** 1482–1486. (doi:10.1016/j.fertnstert.2010.06.006)
- Choi J, Jo M, Lee E & Choi D 2011b The role of autophagy in corpus luteum regression in the rat. *Biology of Reproduction* **85** 465–472. (doi:10.1095/biolreprod.111.091314)
- Choi J, Jo M, Lee E, Oh YK & Choi D 2012 The role of autophagy in human endometrium. *Biology of Reproduction* **86** 1–10. (doi:10.1095/biolreprod.111.096206)
- Choi J, Jo M, Lee E, Kim HJ & Choi D 2014 Differential induction of autophagy by mTOR is associated with abnormal apoptosis in ovarian endometriotic cysts. *Molecular Human Reproduction* **20** 309–317. (doi:10.1093/molehr/gat091)
- Chow SE, Chen YW, Liang CA, Huang YK & Wang JS 2012 Wogonin induces cross-regulation between autophagy and apoptosis via a variety of Akt pathway in human nasopharyngeal carcinoma cells. *Journal of Cellular Biochemistry* **113** 3476–3485. (doi:10.1002/jcb.24224)
- Corcelle EA, Puustinen P & Jaattela M 2009 Apoptosis and autophagy: targeting autophagy signalling in cancer cells – ‘trick or treats’? *FEBS Journal* **276** 6084–6096. (doi:10.1111/j.1742-4658.2009.07332.x)
- Crosignani P, Olive D, Bergqvist A & Luciano A 2006 Advances in the management of endometriosis: an update for clinicians. *Human Reproduction Update* **12** 179–189. (doi:10.1093/humupd/dmi049)
- Degtyarev M, De Mazière A, Orr C, Lin J, Lee BB, Tien JY, Prior WW, van Dijk S, Wu H, Gray DC *et al.* 2008 Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents. *Journal of Cell Biology* **183** 101–116. (doi:10.1083/jcb.200801099)
- Dmowski WP, Ding J, Shen J, Rana N, Fernandez BB & Braun DP 2001 Apoptosis in endometrial glandular and stromal cells in women with and without endometriosis. *Human Reproduction* **16** 1802–1808. (doi:10.1093/humrep/16.9.1802)
- Eskenazi B & Warner ML 1997 Epidemiology of endometriosis. *Obstetrics and Gynecology Clinics of North America* **24** 235–258. (doi:10.1016/S0889-8545(05)70302-8)
- Fan QW, Cheng C, Hackett C, Feldman M, Houseman BT, Nicolaides T, Haas-Kogan D, James CD, Oakes SA, Debnath J *et al.* 2010 Akt and autophagy cooperate to promote survival of drug-resistant glioma. *Science Signaling* **3** ra81. (doi:10.1126/scisignal.2001017)
- Gebel HM, Braun DP, Tambur A, Frame D, Rana N & Dmowski WP 1998 Spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis. *Fertility and Sterility* **69** 1042–1047. (doi:10.1016/S0015-0282(98)00073-9)
- Giudice LC & Kao LC 2004 Endometriosis. *Lancet* **364** 1789–1799. (doi:10.1016/S0140-6736(04)17403-5)
- Govatati S, Kodati VL, Deenadayal M, Chakravarty B, Shivaji S & Bhanoori M 2014 Mutations in the PTEN tumor gene and risk of endometriosis: a case-control study. *Human Reproduction* **29** 324–336. (doi:10.1093/humrep/det387)
- Guzeloglu-Kayisli O, Kayisli UA, Al-Rejjal R, Zheng W, Luleci G & Arici A 2003 Regulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10) expression by estradiol and progesterone in human endometrium. *Journal of Clinical Endocrinology and Metabolism* **880** 5017–5026. (doi:10.1210/jc.2003-030414)
- Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y & Yoshimori T 2000 LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosomal membranes after processing. *EMBO Journal* **19** 5720–5728. (doi:10.1093/emboj/19.21.5720)
- Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y & Yoshimori T 2004 LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *Journal of Cell Science* **117** 2805–2812. (doi:10.1242/jcs.01131)
- Katso R, Okkenhaug K, Ahmadi K, White S, Timms J & Waterfield MD 2001 Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annual Review of Cell and Developmental Biology* **17** 615–675. (doi:10.1146/annurev.cellbio.17.1.615)
- Kaupilla A & Rönnberg L 1985 Naproxen sodium in dysmenorrhea secondary to endometriosis. *Obstetrics and Gynecology* **65** 379–383.
- Klionsky DJ & Emr SD 2000 Autophagy as a regulated pathway of cellular degradation. *Science* **290** 1717–1721. (doi:10.1126/science.290.5497.1717)
- Levine B & Klionsky DJ 2004 Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Developmental Cell* **6** 463–477. (doi:10.1016/S1534-5807(04)00099-1)
- Levine RL, Cargile CB, Blazes MS, van Rees B, Kurman RJ & Ellenson LH. 1998 PTEN mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma. *Cancer Research* **58** 3254–3258.
- Maehama T & Dixon JE 1998 The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *Journal of Biological Chemistry* **273** 13375–13378. (doi:10.1074/jbc.273.22.13375)
- Mutter GL, Lin MC, Fitzgerald JT, Kum JB & Eng C 2000a Changes in endometrial PTEN expression throughout the human menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **85** 2334–2338. (doi:10.1210/jcem.85.6.6652)
- Mutter GL, Lin MC, Fitzgerald JT, Kum JB, Baak JP, Lees JA, Weng LP & Eng C 2000b Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *Journal of the National Cancer Institute* **92** 924–930. (doi:10.1093/jnci/92.11.924)
- Nara A, Mizushima N, Yamamoto A, Kabeya Y, Ohsumi Y & Yoshimori T 2002 SKD1 AAA ATPase-dependent endosomal transport is involved in autolysosome formation. *Cell Structure and Function* **27** 29–37. (doi:10.1247/csf.27.29)
- Nho RS & Hergert P 2014 IPF fibroblasts are desensitized to type I collagen matrix-induced cell death by suppressing low autophagy via

- aberrant Akt/mTOR kinases. *PLoS ONE* **9** e94616. (doi:10.1371/journal.pone.0094616)
- Obata K1, Morland SJ, Watson RH, Hitchcock A, Chenevix-Trench G, Thomas EJ & Campbell IG 1998 Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Research* **58** 2095–2097.
- Porter AG & Janicke RU 1999 Emerging roles of caspase-3 in apoptosis. *Cell Death and Different* **6** 99–104. (doi:10.1038/sj.cdd.4400476)
- Pritts EA & Taylor RN 2003 An evidence-based evaluation of endometriosis associated infertility. *Endocrinology Metabolism Clinics of North America* **32** 653–667. (doi:10.1016/S0889-8529(03)00045-8)
- Rizner TL 2009 Estrogen metabolism and action in endometriosis. *Molecular and Cellular Endocrinology* **307** 8–18. (doi:10.1016/j.mce.2009.03.022)
- Ryan IP, Schriock ED & Taylor RN 1994 Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro. *Journal of Clinical Endocrinology and Metabolism* **78** 642–649. (doi:10.1210/jcem.78.3.8126136)
- Saiki S, Sasazawa Y, Imamichi Y, Kawajiri S, Fujimaki T, Tanida I, Kobayashi H, Sato F, Sato S, Ishikawa K *et al.* 2011 Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition. *Autophagy* **7** 176–178. (doi:10.4161/auto.7.2.14074)
- Sarbassov DD, Ali SM & Sabatini DM 2005 Growing roles for the mTOR pathway. *Current Opinion in Cell Biology* **17** 596–603. (doi:10.1016/j.ceb.2005.09.009)
- Sato N, Tsunoda H, Nishida M, Morishita Y, Takimoto Y, Kubo T & Noguchi M 2000a Loss of heterozygosity on 10q23.3 and mutation of the tumor suppressor gene PTEN in benign endometrial cyst of the ovary: possible sequence progression from benign endometrial cyst to endometrioid carcinoma and clear cell carcinoma of the ovary. *Cancer Research* **60** 7052–7056.
- Sato N, Nishida M & Noguchi M 2000b An approach to early genetic alterations in precancerous cells. *Human Cell* **13** 103–108.
- Singh BN, Kumar D, Shankar S & Srivastava RK 2012 Rottlerin induces autophagy which leads to apoptotic cell death through inhibition of PI3K/Akt/mTOR pathway in human pancreatic cancer stem cells. *Biochemical Pharmacology* **84** 1154–1163. (doi:10.1016/j.bcp.2012.08.007)
- Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T *et al.* 1997 Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nature Genetics* **15** 356–362. (doi:10.1038/ng0497-356)
- Sun H, Enomoto T, Fujita M, Wada H, Yoshino K, Ozaki K, Nakamura T & Murata Y 2001 Mutational analysis of the PTEN gene in endometrial carcinoma and hyperplasia. *American Journal of Clinical Pathology* **115** 32–38. (doi:10.1309/7JX6-B9U9-3P0R-EQNY)
- Vercellini P, Trespidi L, Colombo A, Vendola N, Marchini M & Crosignani PG 1993 A gonadotropin-releasing hormone agonist versus a low-dose oral contraceptive for pelvic pain associated with endometriosis. *Fertility and Sterility* **60** 75–79. (doi:10.1016/S0015-0282(16)56039-7)
- Wu YC, Wu WK, Li Y, Yu L, Li ZJ, Wong CC, Li HT, Sung JJ & Cho CH 2009 Inhibition of macroautophagy by bafilomycin A1 lowers proliferation and induces apoptosis in colon cancer cells. *Biochemical and Biophysical Research Communications* **382** 451–456. (doi:10.1016/j.bbrc.2009.03.051)
- Yorimitsu T & Klionsky DJ 2005 Autophagy: molecular machinery for self-eating. *Cell Death and Differentiation* **12** (Supplement 2) 1542–1552. (doi:10.1038/sj.cdd.4401765)
- Zhang G, Hou X, Li Y & Zhao M 2014 MiR-205 inhibits cell apoptosis by targeting phosphatase and tensin homolog deleted on chromosome ten in endometrial cancer Ishikawa cells. *BMC Cancer* **14** 440. (doi:10.1186/1471-2407-14-440).

Received 14 June 2016

First decision 23 July 2016

Revised manuscript received 19 September 2016

Accepted 10 October 2016