# PI3K, AKT, and P-AKT levels in thin endometrium

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**ABSTRACT.** The aim of this study was to explore the expression of PI3K, AKT, and P-AKT, and to investigate the role of PI3K/AKT signaling pathway in thin endometrium. We included 40 women treated in affiliated Shenzhen Nanshan People's Hospital of Guangdong Medical University for endometrial conditions between August 2013 and January 2015, 20 with a normal endometrium, and 20 with thin endometrium. The expression of PI3K, AKT, and P-AKT was evaluated by the immunohistochemical S-P method. The expression of PI3K, AKT, and P-AKT proteins was significantly lower in the thin endometrium group than in the normal endometrium group ($P < 0.05$). The expression of PI3K and AKT was positively correlated with the expression of P-AKT. The expression of PI3K, AKT, and P-AKT proteins in the thin endometrium decreases during the proliferative phase, and this process could be associated with PI3K/AKT signaling.

**Key words:** Thin endometrium; PI3K; AKT

## INTRODUCTION

Thin endometrium refers to endometrium with a thickness <7 mm during the middle luteal phase (6-10 days after ovulation) (Riad and Hak, 2014). The major presentations of thin endometrium is an unexplained low volume of menstrual blood (<30 mL). The menstrual cycle and sexual hormone levels are normal, but hysteroscopy shows a pale, thin, and smooth endometrium without adhesion, and therefore, thin endometrium is also known as "unexplained low menstrual blood volume" (Gleicher et al., 2011). Previous studies have shown that the endometrium should be 8-14 mm thick for intrauterine implantation of zygotes (Aydin et al., 2013). Therefore, thin endometrium could result in repeated miscarriages or infertility, and in a decreased success rate of assisted reproduction (Momeni et al., 2011). The major features of thin endometrium are high uterine blood flow resistance and delayed growth of glandular epithelium, which could inhibit the expression of vascular endothelial growth factor (VEGF), thereby causing poor growth and development of blood vessels, reducing the perfusion of the endometrium (Miwa et al., 2009).

In general, serum estrogen levels are normal in patients with thin endometrium. However, associations between thin endometrium and ERα and ERβ polymorphisms have been suggested (Yuan and Le, 2012; Le et al., 2013). Estrogen-regulatory elements, such as eNOS in the ER, regulate intima proliferation and promote angiogenesis. The NO level in the endometrial glands and in endothelial cells increases significantly during the luteal phase, promoting intima proliferation, blood vessel dilation, and blood perfusion. Progestogen stimulates the expression of eNOS, possibly via the PI3K/AKT signaling pathway (Khorram and Han, 2009). Wortmannin, a PI3K pathway inhibitor, inhibits the phosphorylation of estrogen promoted by AKT. Guzeloglu Kayisli et al. (2004) reported that the PI3K signaling pathway in the endometrium is directly regulated by the phosphorylation of AKT, which is induced by estradiol. In addition, AKT signaling is also directly activated by estrogen, which promotes the proliferation and remodeling of the endometrium (Kazi et al., 2009; Baranda-Avila et al., 2013). Nonetheless, very few studies have examined the involvement of the PI3K/AKT signaling pathway in thin endometrium. Hence, the present study investigates the expression of PI3K, AKT, and P-AKT, and their relationship to thin endometrium.

## MATERIAL AND METHODS

### Patients and samples

Forty women treated for endometrial conditions at the Department of Gynecology, Shenzhen Nanshan Hospital affiliated to Guangdong Medical College between August 2013 and January 2015 were included in this study. The women were divided into two equally large groups: a thin endometrium group and a normal endometrium group. This study was approved by the Ethics Committee of Shenzhen Nanshan Hospital and written informed consent was obtained from every participant.

In the thin endometrium group (thin group), the mean age of the 20 women was 26.41 ± 2.10 years. Patient inclusion depended on the following criteria: 1) menstrual blood volume <30 mL (Hald and Lieng, 2014), 2) normal estrogen levels, 3) hysteroscopy showing thin and smooth endometrium on day 15-25 after menstruation, no adhesion, and visible bilateral oviduct openings, 4) ultrasound showing <7-mm thick endometrium during the middle luteal phase. In addition, we took into account the following exclusion criteria: 1) blood relationship with other study subject, 2) hormone therapy within 3 months before the hysteroscopy, 3) intrauterine device implantation

within 1 year before the hysteroscopy, or 4) diagnosed with endocrine disease, tuberculosis, diabetes, thyroid disease, adrenal disease, surgical disease, or congenital disease.

The mean age of the 20 women in the group with a normal endometrium (normal group) was 28.91 ± 4.60 years. The inclusion criteria for the normal group were: 1) menstrual blood volume >30 mL, 2) hysteroscopy showing smooth and glossy endometrium on day 15-25 after menstruation, and 3) ultrasound showing an endometrium with a thicknes of ≥7 mm at mid-luteal phase.

For all women, 2 mL venous blood was collected before 9 am on day 2-5 after menstruation and the levels of sexual hormones were measured by ELISA.

## Immunohistochemistry

The immunohistochemical S-P method was performed according to the manufacturer's instructions (American Abcam Company). The Image J software was used for the semi-quantitative comparison of the immunohistochemical results. The mean optical densities of the images were obtained by dividing the IntDen (calculated using the software) by the surface area. As a positive control, we stained normal endometrium sections. In the blank control, the primary antibody was replaced by bovine serum albumin.

## Statistical analyses

The SPSS software for Windows 17.0 was used for statistical analysis. Quantitative data are reported as means ± standard deviations (SD). An independent $t$-test was performed to compare the two groups. Spearman correlation analysis was used to investigate the relationships between the different parameters. A P value <0.05 was considered statistically significant.

## RESULTS

## Clinical data

The age, menstrual cycle (Table 1), and the levels of six sex hormones (Table 2) were similar between the patients with thin endometrium and the controls. In contrast, menstrual duration and the thickness of the endometrium were significantly different between the two groups (Table 1).

**Table 1.** Demographic and clinical parameters of study participants (means ± SD).

| Parameter | Thin group | Normal group | $t$ | P |
|---|---|---|---|---|
| Age (years) | 26.41 ± 2.10 | 28.90 ± 4.60 | -2.211 | 0.07 |
| Menstrual cycle (days) | 26.71 ± 1.20 | 27.52 ± 2.30 | -1.379 | 0.176 |
| Menstrual duration (days) | 2.12 ± 1.30 | 5.23 ± 0.60 | -9.370 | <0.001 |
| Endometrium thickness (mm) | 4.80 ± 0.03 | 9.52 ± 0.80 | -26.367 | <0.001 |

**Table 2.** Sex hormone levels (means ± SD).

| Hormone | Thin group | Normal group | $t$ | P |
|---|---|---|---|---|
| E2 (pM) | 204.98 ± 118.07 | 218.16 ± 105.25 | -0.373 | 0.726 |
| P (nM) | 2.25 ± 0.68 | 2.47 ± 1.17 | -0.727 | 0.378 |
| FSH (IU/L) | 56.77 ± 1.99 | 54.47 ± 1.68 | 3.949 | 0,752 |
| LH (IU/L) | 6.52 ± 2.54 | 5.85 ± 2.65 | 0.816 | 0,286 |
| PRL (nM) | 14.25 ± 8.22 | 12.55 ± 6.82 | 0.712 | 0.489 |
| N (nM) | 1.56 ± 0.33 | 1.53 ± 0.45 | 0.240 | 0.861 |

## Histological examinations after hematoxylin and eosin (HE) staining

Histological examinations of the HE-stained tissues in the thin endometrium group showed short cuboidal epithelium and a loose matrix. Glandular epithelium and endometrial epithelial cells were short and flat, with a columnar or cuboidal shape. Mild edema was seen in some parts of the matrix, most areas had sparse blood vessels, and some parts had no cells or only limited cellular structural outlines (Figure 1A-D).

Histological examinations of the normal group showed short cuboidal epithelium, a loose matrix, and an abundance of glands and blood vessels. The glands were tube-shaped, and the glandular surface epithelia were columnar- and cuboidal-shaped. The matrix cells were spindle- or oval-shaped, and the nuclei were oval-shaped. Abundant spiral arteries were found in the deep endometrium (Figure 1E-H).
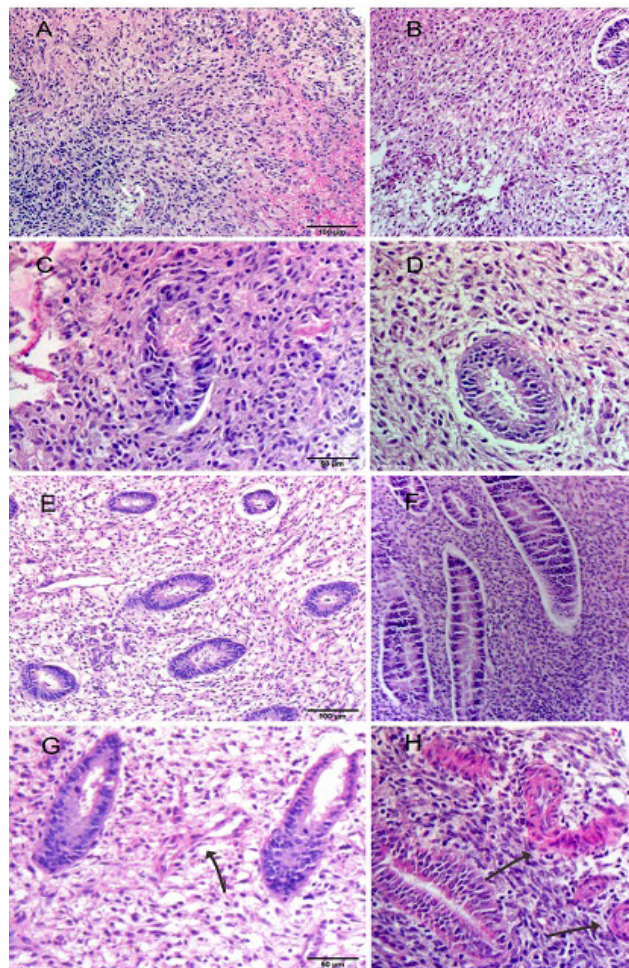


**Figure 1.** HE staining of endometrial tissues (**A. B. E.** and **F.**: 20X magnification; **C. D. G.** and **H.**: 40X magnification). The arrows indicate spiral small arteries.

## Expression of PI3K, AKT, and P-AKT

PI3K was mainly expressed in the glandular epithelial cells, luminal epithelial cells, and interstitial cell membrane, while low PI3K expression levels were detected in the nuclei (Figure 2). The expression of the PI3K protein was significantly lower in the thin group than in the normal group ($P < 0.05$; Table 3).

AKT was mainly expressed in the interstitial cell cytoplasm and in the glandular epithelial cell cytoplasm, while low AKT expression levels were detected in the nuclei (Figure 3). The expression of the AKT protein was significantly lower in the thin group than in the normal group ($P < 0.05$; Table 4).

P-AKT is mainly present in the cytoplasm of glandular epithelial cells and stromal cells. P-AKT was also partially located in the nucleus (Figure 4). The expression of the P-AKT protein was significantly lower in the thin group than in the normal group ($P < 0.05$; Table 5).
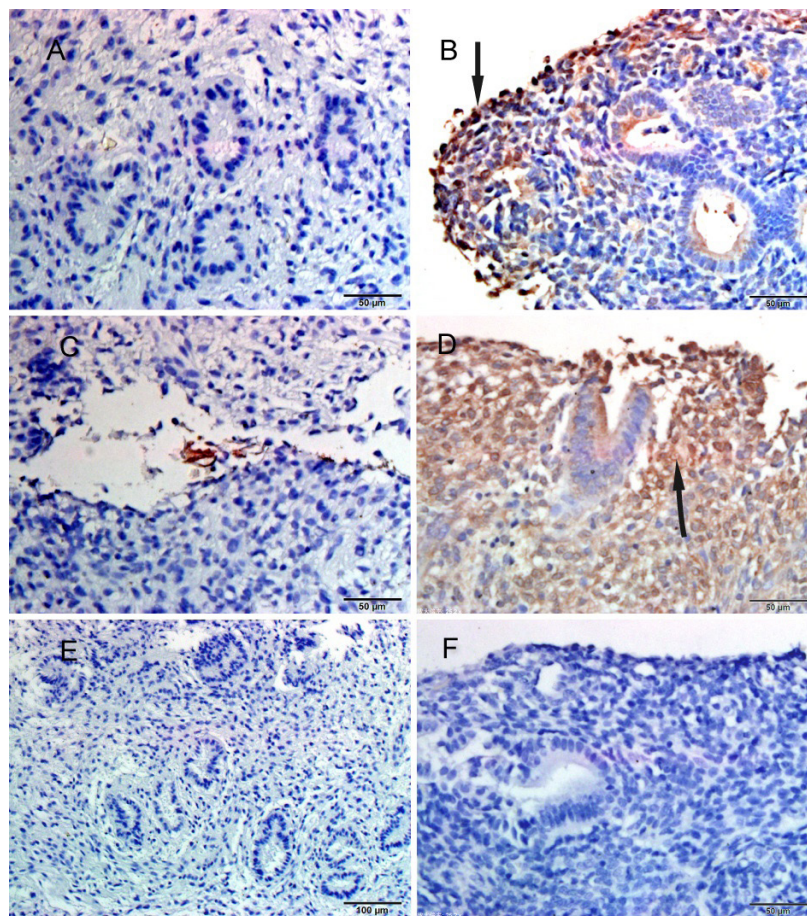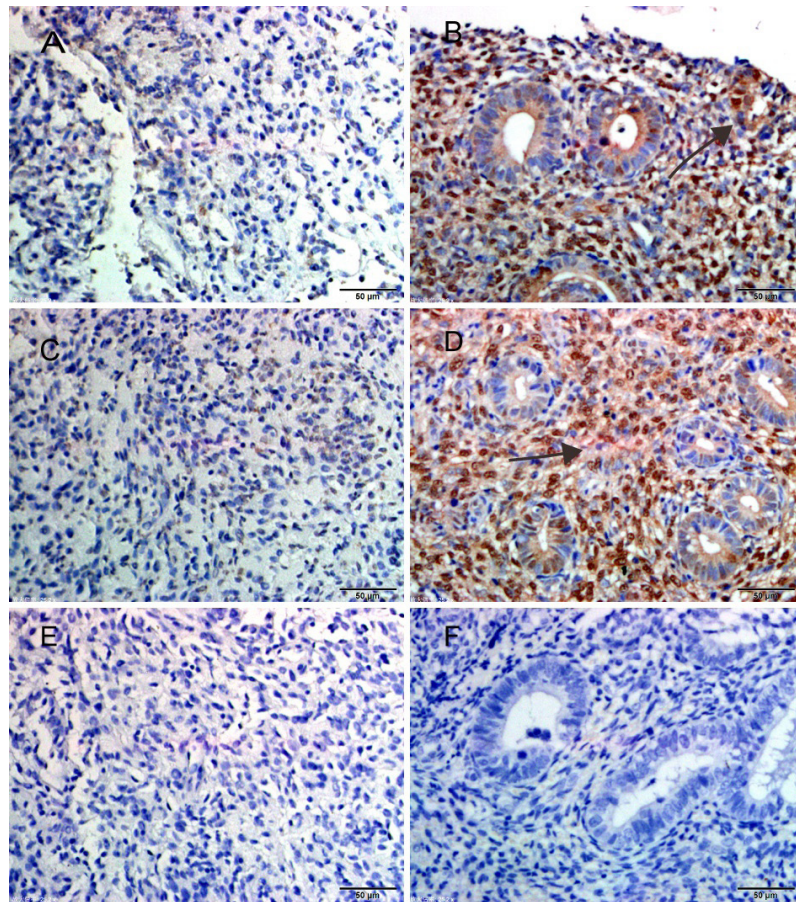


**Figure 2.** PI3K expression (immunohistochemistry, E: 20X magnification, other panels: 40X magnificiation). **A. C.** and **E.** Thin group; **B. D.** and **F.** normal group; **E. F.** blank control. The arrows indicate a positive signal for PI3K expression.

**Table 3.** PI3K protein average optical density (means ± SD).

| Groups | Average optical density | t | P |
|---|---|---|---|
| Thin group | 0.15320 ± 0.00765 | -13.260 | <0.001 |
| Normal group | 0.32670 ± 0.01062 | | |



**Figure 3.** AKT expression (immunohistochemistry, 40X magnification). **A. C.** and **E.** Thin group; **B. D.** and **F.** normal group; **E. F.** blank control. The arrows indicate a positive signal for AKT expression.

**Table 4.** AKT protein average optical density (means ± SD).

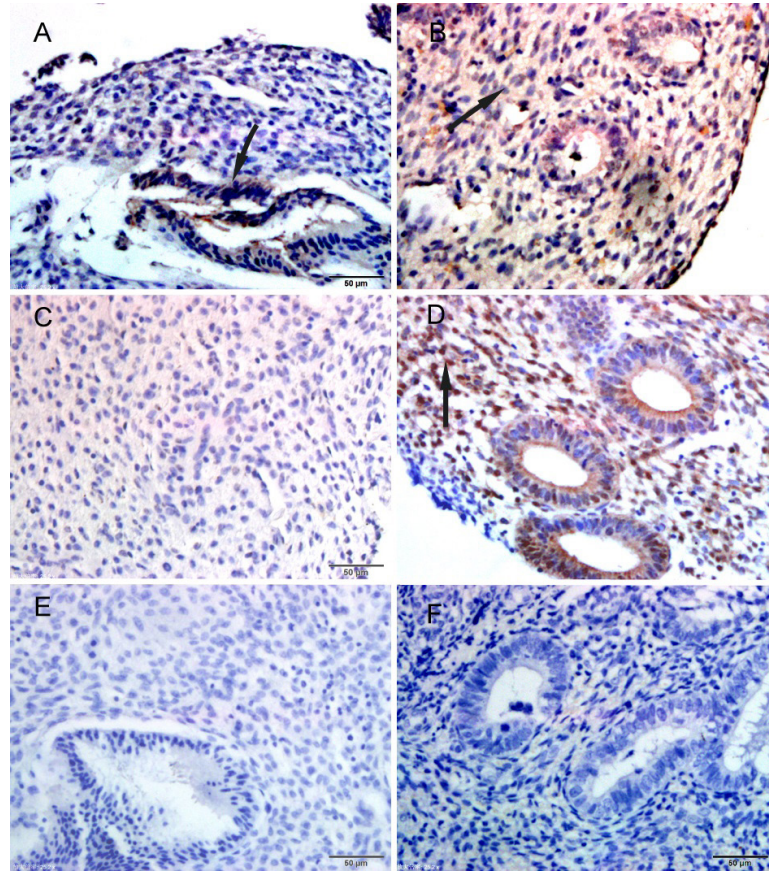| Groups | Average optical density | t | P |
|---|---|---|---|
| Thin group | 0.20700 ± 0.00854 | -38.550 | <0.001 |
| Normal group | 0.68880 ± 0.00913 | | |

**Figure 4.** P-AKT expression (immunohistochemistry, 40X magnification). **A. C.** and **E.** Thin group; **B. D.** and **F.** normal group; **E. F.** blank control. The arrows indicate a positive signal for P-AKT expression.

**Table 5.** P-AKT protein average optical density (means ± SD).

| Groups | Average optical density | t | P |
|---|---|---|---|
| Thin group | 0.37110 ± 0.00865 | -8.167 | <0.001 |
| Normal group | 0.85980 ± 0.05922 | | |

## Correlation between PI3K, AKT, and P-AKT protein levels

Spearman correlation analysis showed that PI3K and AKT levels were positively correlated with P-AKT protein levels in both thin and normal groups ($P < 0.05$) (Tables 6 and 7).

**Table 6.** Correlations between PI3K, AKT, and P-AKT protein levels in the thin group.

| | PI3K | P-AKT | AKT |
|---|---|---|---|
| PI3K | 1 | | |
| P-AKT | 0.708 | 1 | |
| AKT | 0.651 | 0.650 | 1 |

**Table 7.** Correlations between PI3K, AKT, and P-AKT proteins levels in the normal group.

|        | PI3K  | P-AKT | AKT |
|--------|-------|-------|-----|
| PI3K   | 1     |       |     |
| P-AKT  | 0.632 | 1     |     |
| AKT    | 0.611 | 0.623 | 1   |

## DISCUSSION

PI3Ks are a group of lipid and serine/threonine protein kinases, which consist of a catalytic subunit p110 and a regulatory subunit p85. Activation of G-protein-coupled receptors or tyrosine kinases specifically catalyzes phosphatidylinositol. After binding to platelet-drived growth factor or epidermal growth factor, PI3K catalyzes tyrosine phosphorylation. The binding of p85 and p110 causes the activation of PI3K. The catalytic subunit p110, a 124-kDa protein encoded by PIK3Cab, has phosphatidylinositol kinase activity, and catalyzes the phosphorylation of the 3'-position of the inositol ring to convert PIP2 to PIP3 (Rodon et al., 2013; Fruman and Rommel, 2014). AKT is a serine/threonine protein kinase, which is also called protein kinase B, and its kinase domain is homologous to the domains in protein kinases A and C. AKT consists of 480 amino acids and includes a PH domain at its N-terminus, a middle kinase domain, and a regulatory domain at the C-terminus (Warfel and Kraft, 2015). After the PH-regulatory domain of AKT binds to PI3K, AKT is activated and translocates from the cytoplasm to the membrane, and consequently mediates the activation of multiple downstream genes. The kinase domain contains a threonine at position 308 that is essential for its activation, and a serine at position 473 that could maximize activation (Yang et al., 2015). The PI3K/AKT pathway plays an important role in the phosphorylation of AKT, which consequently changes the extracellular environment to intracellular responses. It plays an important role in cellular metabolism and survival, as well as in the inhibition of cell apoptosis (Zhou et al., 2014). The activation of the PI3K/AKT pathway could induce the migration of endothelial cells and angiogenesis through the stimulation of cyclooxygenase-2. The activation of AKT is triggered by several cytokines and prevents apoptosis by inhibiting pro-apoptotic gene expression. Stimulation of angiopoietin-I and VEGF activates AKT, and thus inhibits apoptosis of endothelial cells. In addition, activated AKT activates eNOS, which in turn accelerates the migration of endothelial cells induced by VEGF, and therefore also promotes angiogenesis (Viglietto et al., 2011).

The expression of AKT, P-AKT, and PI3K was significantly lower in the thin endometrium group than in the normal endometrium group. In addition, Spearman correlation analysis showed that the expression levels of AKT, P-AKT, and PI3K were positively correlated in both the thin and normal endometrium group. Previous studies have shown that AKT and P-AKT are involved in increasing endometrial receptivity, by regulating proliferation, differentiation, and migration of endometrial cells (Veillette et al., 2013; Liu et al., 2014). While the PI3K/AKT signaling pathway is active in both normal and thin endometrium, we found significantly lower levels of P-AKT in thin endometrium than in normal endometrium. We speculate that the activity of the PI3K/AKT pathway is also lower in thin endometrium due to the relatively lower levels of P-AKT. Thus, the regulatory effects on proliferation, differentiation and apoptosis of endometrial cells could also be lower.

The endometrium undergoes a continuous cycle of change that involves the remodeling and replacement of the intra- and extra-cellular matrix. P-AKT is a highly specific molecular biomarker for this process, which is essential for endometrium decidualization and the implantation of fertilized eggs. In women with a normal menstrual cycle, P-AKT is present in the epithelial

layer and in the nuclei of the functional cell layers in the proliferative phase, while its levels in the cytoplasm and nuclei are significantly lower during the secretory phase. In contrast, P-AKT levels in the cytoplasm and the nuclei of interstitial cells increase in the secretory phase, a process that is positively associated with a high degree of endometrium decidualization (Toyofuku et al., 2006). High P-AKT levels in the interstitial cells in the functional cell layers could be associated with tissue remodeling in the menstrual phase or during implantation. However, the P-AKT levels in the interstitial cells at the endometrial basal layer are generally low at any phase of the menstrual cycle (Khorram and Han, 2009). In accordance with previous studies, P-AKT was mainly detected in the cytoplasm and in the nuclei. Estrogen promotes the proliferation of endometrial epithelial cells, while progestogen promotes the proliferation of interstitial cells. Endometrium decidualization and the implantation of fertilized eggs require a switch from epithelial cell proliferation to interstitial cell proliferation (Vallejo et al., 2005). Previous studies have reported that during cellular proliferation induced by estrogen and progestogen, the PI3K/AKT signaling pathway is also associated with follicular implantation (Haynes et al., 2003). The expression of AKT increases correspondingly during embryo implantation in mice. AKT is mainly distributed in the luminal and glandular epithelial cells of the uterus in mice before follicular implantation. However, AKT was also detected in decidualized matrix cells (Herington and Bany, 2009). These findings indicate that activation of AKT is involved in the remodeling of endometrium and egg implantation, while miscarriage and infertility in women with thin endometrium could be associated with decreased expression of PI3K and AKT. Taken together the previous findings and our own results, we suggest that thin endometrium could be associated with perturbed PI3K/AKT signaling. Further *in vivo* and *in vitro* studies are needed to validate this hypothesis.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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