Phosphatidic acid induces decidualization by stimulating Akt-PP2A binding in human endometrial stromal cells

So Young Lee1,†, Yun Young Lee1,†, Joong Sub Choi2, Mee-Sup Yoon3 and Joong-Soo Han1,4

1 Department of Biomedical Sciences, Graduate School of Biomedical Science and Engineering, Hanyang University, Seoul, Korea
2 Department of Obstetrics and Gynecology, College of Medicine, Hanyang University, Seoul, Korea
3 Department of Molecular Medicine, School of Medicine, Gachon University, Incheon, Korea
4 Biomedical Research Institute and Department of Biochemistry and Molecular Biology, College of Medicine, Hanyang University, Seoul, Korea

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Correspondence
M.-S. Yoon, Department of Molecular Medicine, Lee Gil Ya Cancer and Diabetes Research Institute, School of Medicine, Gachon University, 7-45 Songdo-Dong, Yeonsu-Gu, Incheon 406-840, Korea
Fax: +82 32 899 5678
Tel: +82 32 899 6067
E-mail: msyoon@gachon.ac.kr
and
J.-S. Han, Biomedical Research Institute and Department of Biochemistry and Molecular Biology, College of Medicine, Hanyang University, Seoul 133-791, Korea
Fax: +82 2 2220 2418
Tel: +82 2 2220 0623
E-mail: jshan@hanyang.ac.kr

†Equal contribution

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Introduction

For a successful pregnancy, the endometrium must grow continuously and mature throughout the menstrual cycle. Decidualization of human endometrial stromal cells (hESCs) is a crucial differentiation event characterized by biochemical and morphological alterations occurring from mid to late secretory phase of the menstrual cycle. During this process, decidual cells become rounded and enlarged, and this morphological

Decidualization of human endometrial stromal cells (hESCs) is crucial for successful uterine implantation and maintaining pregnancy. We previously reported that phospholipase D1 (PLD1) is required for cAMP-induced decidualization of hESCs. However, the mechanism by which phosphatidic acid (PA), the product of PLD1 action, might regulate decidualization is not known. We confirmed that PA induced decidualization of hESCs by observing morphological changes and measuring increased levels of decidualization markers such as IGFBP1 and prolactin transcripts \( P < 0.05 \). Treatment with PA reduced phosphorylation of Akt and consequently that of FoxO1, which led to the increased IGFBP1 and prolactin mRNA levels \( P < 0.05 \). Conversely, PLD1 knockdown rescued Akt phosphorylation. Binding of PP2A and Akt increased in response to cAMP or PA, suggesting that their binding is directly responsible for the inactivation of Akt during decidualization. Consistent with this observation, treatment with okadaic acid, a PP2A inhibitor, also inhibited cAMP-induced decidualization by blocking Akt dephosphorylation.

Abbreviations
AKTi, Akt inhibitor; DAG, diacylglycerol; FIP1, PLD1/2 inhibitor; FoxO1, Forkhead box class O1; hESC, human endometrial stromal cells; IGFBP-1, insulin-like growth factor-binding protein-1; PA, phosphatidic acid; PKB, protein kinase B; PLA2, phospholipase A2; PLD, phospholipase D; PP2A, protein phosphatase 2A; siRNA, small-interfering RNA.
change is accompanied by biochemical changes resulting in increased expression of several marker genes, including insulin-like growth factor-binding protein-1 (IGFBP1) and prolactin [1], and the progesterone level rises and intracellular cAMP increase dramatically. Based on these observations, the cAMP/PKA pathway has been extensively studied as the most likely cause of decidualization [2]. However, the molecular mechanisms underlying the decidualization process are not completely understood.

Phospholipase D (PLD) catalyzes the hydrolysis of phospholipids at the terminal phosphodiester bond. The most abundant phospholipid, phosphatidylcholine (PC), is hydrolyzed to phosphatidic acid (PA) and choline by activated PLD. PA regulates diverse intracellular signaling pathways as a lipid second messenger [3]. Moreover, it is metabolically converted to diacylglycerol (DAG) by lipin, which has phosphatidate phosphatase activity and to lysoPA or arachidonic acid by phospholipase A2 (PLA2). These metabolites also modulate various intracellular signaling processes [1,4]. In mammalian cells, two PLD isoforms (PLD1 and PLD2) have been cloned and characterized. Both have significant roles in processes such as proliferation, differentiation, vesicular trafficking, inflammation, and apoptosis and have been studied in the context of cancer, and neurodegenerative, cardiovascular, and infectious diseases [1,5–8]. Arachidonic acid is involved in the cPLA2a/COX-2 pathway in hESCs [9] and the decidualization of uterine stromal cells [10] in vitro. Additionally, lysoPA regulates male and female reproduction [11], vascularization, and decidualization, which is related to embryo invasion during implantation [12]. However, the role of PLD1 or PA in the differentiation of hESCs is not fully understood.

Akt (protein kinase B, PKB) is a serine/threonine protein kinase, and is activated in a variety of cellular processes including apoptosis, proliferation, transcription, and metabolism [8,13,14]. The activity of the PI3K/Akt pathway is correlated with the decidualization progress [14,15] but its role is unknown. The transcription factor Forkhead box, class O1 (FoxO1) is directly regulated by Akt [16]. FoxO1 is a transcription factor, a member of the Forkhead box family [17]. During decidualization, the dephosphorylated form of FoxO1 is responsible for transcribing the IGFBP1 and prolactin genes [17,18]. But, how it is directly regulated in this context is not fully understood.

Protein phosphatase 2A (PP2A) is a conserved serine/threonine phosphatase with broad substrate specificity and diverse cellular functions, comprised of three subunits: a scaffold subunit (A), regulatory subunit (B), and catalytic subunit (C) [19,20]. PP2A in involved many cellular processes but was not known until now to play any role in the decidualization of hESCs.

The purpose of the present study was to see if PA, one of the enzymatic products of PLD1, regulates cAMP-induced decidualization of hESCs, and if so how acts. We found that phosphorylation of Akt and FoxO1 dramatically decreased in cells treated with cAMP or PA. Binding of PP2A to Akt also increased, suggesting that this was responsible for the dephosphorylation of Akt during decidualization. We therefore propose that PA promotes cAMP-induced decidualization in hESCs by stimulating interaction between PP2A and Akt, leading to inactivation of Akt and activation of FoxO1, which in turn is responsible for expressions of the decidualization-related genes.

Results

PLD1 and PA are key regulators of the decidualization of hESCs

Previous studies have shown that PLD1 expression is up-regulated and PLD activity increases during the cAMP-induced decidualization of hESCs [1]. Therefore, we investigated whether loss-of-function of PLD1 affected the decidualization process. We induced decidualization by incubating cells in culture medium containing 0.5 mM 8-Br-cAMP (membrane-permeable cAMP analog) for 2 days; the hESCs developed a decidual-like morphology, becoming rounded with increased amounts of cytoplasm and enlarged nuclei (Fig. 1A). Moreover, the IGFBP1 and prolactin marker genes of decidualization were up-regulated (Fig. 1B), and these changes were abolished by treatment with the PLD inhibitor, FIPI (Fig. 1A,B). Furthermore, depletion of PLD1 transcripts with PLD1 siRNA gave similar results (Fig 1C,D). To see whether phosphatidic acid (PA) induced decidualization, we treated hESCs with PA for 2 days in the absence of cAMP. PA treatment resulted in a decidual-like morphological change (Fig. 1E) as well as increased gene expression of IGFBP1 and prolactin (Fig. 1F). Collectively, these results suggest that PLD1 activation is an important process in decidualization of hESCs, and PA, the product of PLD1, acts as an essential signaling molecule.

FoxO1 is dephosphorylated during PA-induced decidualization of hESCs

Next, we investigated how PA regulates the decidualization process in hESCs. Several transcription factors...
such as FoxO1, ETS1, p53, STAT5, and C/EBPβ are activated during decidualization [18,21,22]. FoxO1 is required for the expression of IGFBP-1 and prolactin [17,23–26]. Under normal conditions, FoxO1 exists in a phosphorylated form and is present in the cytoplasm. When it is dephosphorylated by various factors, FoxO1 is translocated to the nucleus where it acts as a transcription factor [27,28]. As shown in Fig. 2A, a large percentage of FoxO1 protein existed in the phosphorylated form in control cells; the
amount of the phosphorylated form was reduced by cAMP treatment and this reduction was antagonized by treatment with the PLD inhibitor FIPI (Fig. 2A), or transfection of PLD1 siRNA (Fig. 2B). Moreover PA had the same effect as cAMP on FoxO1 dephosphorylation; when we added 50 μM or 100 μM PA for 40 min in the absence of cAMP, much of the FoxO1 became dephosphorylated, representing PA has same effect on the dephosphorylation of FoxO1 with cAMP (Fig. 2C). We also investigated whether the FoxO1 depletion affected decidualization (Fig. 3A–C). As shown in Fig. 3B, the cAMP-induced decidual-like morphological change was completely disappeared by FoxO1 depletion using siRNA transfection. Decidualization marker genes (IGFBP1 and prolactin) were also drastically reduced by FoxO1 siRNA transfection (Fig. 3C), which were consistent with previous studies [23–26]. Hence, these results indicate that PA, the product of PLD1, promotes FoxO1 dephosphorylation during cAMP-induced decidualization of hESCs.

PA induces Akt dephosphorylation during decidualization of hESCs

Many studies suggest that Akt is an upstream kinase in the phosphorylation of FoxO1 [29,30]. Moreover, Akt is dephosphorylated during decidualization [8]. We therefore tested the idea that PLD1 activation induces Akt dephosphorylation (and so renders it inactive) during decidualization thus protecting FoxO1 from being inactivated by phosphorylation by Akt. As expected, we found that phosphorylation of Akt on Thr308 during decidualization was decreased by cAMP, and PLD inhibition completely prevented the dephosphorylation of Akt in the presence of cAMP (Fig. 4A), as did down-regulation of PLD1 with PLD1 siRNA (Fig. 4B). Next, to address the impact of PLD1 activation on Akt dephosphorylation at Thr308 during decidualization, we assessed whether PA mimics the cAMP-induced Akt dephosphorylation. Treatment with 50 μM or 100 μM PA for 40 min in the absence of cAMP mimicked cAMP in completely repressing Akt phosphorylation (Fig. 4C). Because Akt protein is activated by dual phosphorylation of Thr308 and Ser473, we also investigated the phosphorylation level of Akt on Ser473 during the decidualization. The pattern of phosphorylation on Ser473 was the same as that on Thr308 (data not shown). Taken together, these results demonstrate that PLD1 and its enzymatic product, PA, induce Akt dephosphorylation leading to inactivation of Akt during decidualization of hESCs.

PA induces FoxO1 dephosphorylation by inactivating Akt during decidualization

To see whether Akt inactivation is responsible for the dephosphorylation of FoxO1, we inhibited the Akt activity with the specific inhibitor, AKTi (Akt inhibitor) [31]. hESCs were incubated in culture medium containing 8-Br-cAMP or AKTi for 40 min. AKTi completely blocked the phosphorylation of FoxO1 showing the same effect on FoxO1 as cAMP alone (Fig. 5A). These results indicate that Akt is the kinase responsible for FoxO1 phosphorylation in hESCs.

Next, we examined whether Akt inhibition promoted decidualization in the absence of cAMP. As expected, hESCs treated with AKTi developed a decidua-like morphology reminiscent of the phenotypic response to cAMP (Fig. 5B). They also produced higher levels of IGFBP1 and prolactin transcripts (Fig. 5C). Taken together, these findings indicate that PLD1 activation leads to dephosphorylation of Akt, which in turn inhibits phosphorylation of FoxO1 and so activates it.

PP2A binding to Akt is responsible for the dephosphorylation of Akt/FoxO1 during decidualization

Akt inactivation is regulated by several phosphatases, such as PP2A and PHLPP. The former is a major serine/threonine phosphatase; it is regulated by interaction between its catalytic and regulatory subunits, and modulates the activity of various protein kinases such as Akt [19,32,33]. Moreover, PP2A is known to bind to PA in plant models [34,35]. Accordingly, we used the PP2A inhibitor, okadaic acid [19,36], to identify any effects of PP2A on PA-induced decidualization. Cells were pretreated with okadaic acid for 1 h and then differentiated with PA for 40 min. As shown in Fig. 6A, PA-induced Akt dephosphorylation at Thr308 was reversed by okadaic acid and phosphorylation of FoxO1 was increased (Fig. 6A). Furthermore, the development of decidua-like morphology was abolished (Fig. 6B), and expression of IGFBP1 and prolactin was also substantially decreased (Fig. 6C). This shows that inhibition of PP2A increases accumulation of phosphorylated Akt and the active Akt phosphorylates FoxO1, so inactivating it. Thus, PP2A seems to have an important role in decidualization by controlling Akt inactivation.

There is evidence that PP2A dephosphorylates Akt by binding to it [36]. To determine whether PP2A binds to Akt during decidualization, we performed pull-down assays after inducing decidualization by
8-Br-cAMP or PA. When cells were stimulated with 8-Br-cAMP or PA, PP2A was coimmunoprecipitated with Akt, indicating that the affinity of PP2A to Akt was increased by treatment with 8-Br-cAMP or PA (Fig. 6D). Taken together, these results suggest that during decidualization, PA promotes the formation of an Akt-PP2A binding complex, and Akt dephosphorylation then leads to increased dephosphorylation (and activation) of FoxO1 (Fig. 6E).

Discussion

Decidualization is a process characterized by dramatic changes in the biochemical properties as well as morphological appearance of hESCs required to establish implantation and to maintain pregnancy [37,38]. Progesterone and cAMP are decidualization inducers [39,40]. Previously, we showed that PLD1 activity and expression increase during cAMP-induced decidualization [1,41]. Additionally, we found that PLD1 expression is up-regulated by the progesterone receptor, which acts as a transcription factor for PLD1 during decidualization [42]. However, the mechanisms by which PLD1 regulates decidualization remained unknown. In the present work, we confirmed the role of PLD1 in decidualization; cellular transformation into a decidual-like morphology by cAMP was completely inhibited by PLD1 inhibition or mRNA
depletion (Fig. 1A,C). Similarly, increased expressions of IGFBP1 and prolactin during decidualization were significantly repressed by loss-of-function of PLD1 (Fig. 1B,D). Furthermore, we showed that PA, an enzymatic product of PLD1, mimics the effects of PLD1 on decidualization.

FoxO1 plays a major role as a transcription factor in IGFBP-1 and prolactin gene expression during decidualization of hESCs [17]. In mammalian cells, FoxO1 activity is inhibited by phosphorylation of Ser256 by Akt, and phosphorylated FoxO1 translocates from the nucleus to the cytosol [16]. The F-box protein, SKP2, and E3 ubiquitin ligases recognize phosphorylated FoxO1 and FoxO1 is slowly degraded by the ubiquitin-proteasomal system [43]. We found that FoxO1 activation (dephosphorylation) was elevated when we treated hESCs with cAMP or PA (Fig. 2), implicating that it acts as a transcription factor in cAMP- or PA-induced decidualization. Furthermore, several reports have suggested that Akt activity is regulated by progesterone and/or cAMP treatment [8,14]. Accordingly, we found that Akt activity was regulated by cAMP or PA (Fig. 4), and was responsible for FoxO1 phosphorylation in hESCs (Fig. 5).

PP2A is a representative serine/threonine phosphatase, and targets various kinases, like Akt [44]. We demonstrated that PA promotes cAMP-induced decidualization in hESCs by inactivating Akt via an Akt-PP2A complex (Fig. 6). However, as shown in Fig. 6, PA-induced decidualization was not completely inhibited by the PP2A inhibitor, okadaic acid. This indicates that not only PP2A but also some other phosphatase(s) is involved in the PA-induced Akt inactivation. Indeed, PHLPP, another major serine/threonine phosphatase, selectively dephosphorylates Ser473 in the hydrophobic motif at the C terminus of Akt [33,45]. Therefore, we suspect that PHLPP also induces Akt dephosphorylation on Ser473 in differentiating hESCs.

In addition to these phosphatases, ribosomal protein S6 kinase 1 (S6K1) and insulin receptor substrate 1 (IRS1) also regulate Akt phosphorylation. Insulin binds to the insulin receptor on the plasma membrane and triggers an increase in tyrosine phosphorylation of IRS1 [46]. Moreover, the IRS1-associated class 1 PI3K recruits Akt to the plasma membrane by increasing PIP3 production [47]. In this case, Akt is phosphorylated at Thr308 by PDK1. The mammalian target of rapamycin (mTOR)/S6K1 pathway mediates several biological effects of nutrients, insulin, and energy, and is activated by insulin. The rapamycin-insensitive companion of mTOR (rictor) complex 2 (mTORC2) mediates Akt phosphorylation at Ser473. Akt is simultaneously phosphorylated by mTORC2 and PDK1 at Ser473 and Thr308 [48]. On the other hand, the rapamycin-sensitive companion of mTOR (raptor) complex 1 (mTORC1) regulates S6K1 phosphorylation at a number of residues in an Akt-independent pathway [49]. Interestingly, under nutrient overload conditions, mTORC1/S6K signaling is negatively regulated in insulin signaling through inhibition of IRS1 transcription and IRS1 phosphorylation at a serine residue [46].
Moreover, the mTORC2/S6K1 pathway represses IRS1, and Akt is subsequently inactivated by this negative pathway [46]. These reports indicate that PA may also control mTOR/S6K1 signaling by inactivating Akt. Accordingly, we suggest that the PP2A and the IRS1 pathway through S6K1 may jointly mediate Akt dephosphorylation in decidualization models.

We also considered the possibility that cAMP-regulated guanine nucleotide exchange factors (Epac) might be involved in FoxO1 dephosphorylation in our system. cAMP stimulates Epac by directly binding to PA in the plasma membrane [50]. Moreover, the Epac/Rap1 signaling pathway is activated during decidualization of hESCs, and FoxO1 phosphorylation is regulated through the Epac/ERK pathway in the same models [51]. Thus, these reports suggest that the association between PA and Epac can increase FoxO1 activity during decidualization in hESCs.

Aberrant decidualization elicits physiological dysfunction of the endometrium and is associated with pathologies such as endometriosis, deciduosis, and endometrial cancer [52–54]. Endometrial decidualization converts the normal endometrium into a specialized uterine lining adequate for optimal accommodation of the gestation [55]. The presence of endometrial gland and stroma outside of endometrial cavity induces endometriosis [56], which affects 10–15% of women at reproductive age and is associated with pelvic pain and infertility [57]. Moreover, endometriotic stromal cells lose their capacity for cellular differentiation in the

Fig. 4. PLD1 and its product PA induce dephosphorylation of Akt during decidualization. (A) hESCs were pretreated with 10 μM, or 20 μM FIP1 for 1 h and then treated with 0.5 mM 8-Br-cAMP for 40 min. Cells were lysed and analyzed by western blotting with anti-phospho-Akt (Thr308), anti-Akt, and anti-GAPDH. Bar graph presents the ratio of phospho-Akt versus total Akt (Akt) by densitometric analysis (n ≥ 3) (IMAGEJ; National Institutes of Health). (B) hESCs were transfected with control siRNA or 200 nM PLD1 siRNA for 48 h and then treated with 0.5 mM 8-Br-cAMP for 40 min to induce decidualization. Lysates were analyzed by western blotting with anti-phospho-Akt (Thr308), anti-Akt, and anti-GAPDH. The ratio of phospho-Akt versus total Akt was calculated based on band intensities measured by densitometry (IMAGEJ; National Institutes of Health) (n ≥ 3). (C) hESCs were stimulated with 50 μM or 100 μM PA for 40 min in the absence of 8-Br-cAMP. Proteins were isolated and subjected to western blotting with anti-phospho-Akt (Thr308), anti-Akt, and anti-GAPDH. The results of five independent experiments present densitometry analysis of phospho-Akt versus total Akt (IMAGEJ; National Institutes of Health). Data are expressed as means ± SEM of at least five independent experiments. Differences were assessed by Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001.
ectopic environment [58]. Furthermore, decidualized tissue can grow in ectopic sites during pregnancy, resulting in decidualis with a gross appearance that macroscopically mimics a malignant tumor [53]. In present study, we found that PA controls decidualization by dampening Akt-FoxO1 signaling pathway, which was observed in endometriosis and endometrial cancer patients group [59–62]. In this context, we are tempting to conclude that PA might be implicated in various endometrial disorders, such as deciduosis and endometriosis and subsequently fertility.

In spite of the clinical importance of decidualization, the mechanisms of decidualization are not clearly defined in vivo due to its complexity. Hence, in vitro systems of decidualization have been well established to overcome some of the inherent limitations of studying [63]. Nevertheless, several factors of in vitro system restricts the importance of studies, such as concern of artificial system, cellular variation of isolated stromal cells, and effect of cultural environment on in vitro decidualization [64,65]. Future study will need to examine the role of PA in in vivo decidualization systems using either pregnant or pseudopregnant mice through uterine injection of oil or sepharose beads or mechanical stimulation [66,67].

In conclusion, the present study demonstrated that PA is a critical mediator in cAMP-induced decidualization of hESCs through regulating direct binding between PP2A and Akt.

Materials and methods

Ethics statement

Samples were collected under protocols approved by the Institutional Review Board of Hanyang University Hospital, and written informed consent was obtained from all participants.

Materials

Materials for cell culture including fetal bovine serum (FBS), penicillin–streptomycin, and Dulbecco’s modified Eagle medium, low glucose (DMEM) were obtained from Wisent Inc. (St. Bruno, QC, Canada). 8-Br-cAMP was purchased from Sigma-Aldrich (St. Louis, MI, USA). FIP1 (PLD inhibitor) and AKTi were obtained from Calbiochem (San Diego, CA, USA). Okadaic acid was from Enzo (Farmingdale, NY, USA) and phosphatidic acid was from Avanti Polar Lipid Inc. (Alabaster, AL, USA).

Isolation and culture of hESCs

Human endometria were obtained by hysterectomy from 25 premenopausal women, aged 40–45 years, who underwent...
Fig. 6. PA induces Akt dephosphorylation via Akt-PP2A binding. (A) hESCs were pretreated with 30 nM okadaic acid (a PP2A inhibitor) for 1 h, and then treated with 0.5 mM 8-Br-cAMP or 100 μM PA for 40 min. Proteins were analyzed by western blotting with anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-FoxO1 (Ser256), anti-FoxO1, and anti-GAPDH. The ratio of phospho-Akt versus total Akt and phospho-FoxO1 versus total FoxO1 was calculated based on band intensities measured by densitometry (IMAGEJ; National Institutes of Health) (n ≥ 5). Data are expressed as means ± SEM of at least five independent experiments. Differences were assessed by Student’s t-test. **P < 0.01; ***P < 0.001. (B) hESCs was pretreated with 30 nM okadaic acid for 1 h, and then treated with 100 μM PA for 2 days. hESCs were photographed under a microscope (original magnification 200×). (C) Total RNA isolated from the hESCs was analyzed by RT real-time PCR for IGFBP1 and prolactin mRNA expression levels. Data are expressed as means ± SEM of at least five independent experiments. Differences were assessed by Student’s t-test. *P < 0.05; **P < 0.01. (D) Cells were treated with 0.5 mM 8-Br-cAMP or 100 μM PA for 40 min. Immunoprecipitated with anti-PP2A were analyzed by western blotting with anti-PP2A and anti-Akt. Western blots of immunoprecipitates (IP) were incubated with anti-Akt, anti-PP2A, and anti-phospho-Akt (Thr308). (E) Scheme for the role of PA in cAMP-induced decidualization in hESCs.
surgery for nonendometrial abnormalities in Hanyang University Hospital between September 2008 and September 2014. Each endometrial specimen obtained was examined histologically. hESCs were isolated as described previously. Briefly, tissue samples were collected in DMEM containing 100 IU·mL⁻¹ penicillin, 100 μg·mL⁻¹ streptomycin, 2 mM L-glutamine, and 10% (v/v) FBS. After cleaning and trimming to remove blood clots and mucus, the specimens were minced to fragments of less than 1 mm in size under a laminar flow hood and digested at 37 °C for 60 min with 0.25% collagenase I. The cell suspension was filtered twice through a 40 μm pore-size sieve (BD Falcon, Bedford, MA, USA). After enzymatic digestion, most of the stromal cells were present as single cells or small aggregates. The purity of the stromal cells obtained by this method was typically > 90%, as determined by immunocytochemical staining for vimentin, a stromal cell marker. The purified stromal cells were washed, and viable cells were counted by dye exclusion using trypan blue. The viability of the isolated cells was at least 90% in each experiment. hESCs were cultured to confluence in 100-mm-diameter culture dishes at 37 °C in DMEM supplemented with 10% (v/v) FBS, 100 IU·mL⁻¹ penicillin, and 100 μg·mL⁻¹ streptomycin in a humidified atmosphere of 5% CO₂ in air. To induce in vitro decidualization, cells were exposed to 0.5 mM 8-Br-cAMP for 3 days. Phase-contrast microscopy was used to verify the morphological changes associated with differentiation.

Reverse transcription (RT) and real-time PCR

Total RNA was extracted from hESC culture with TRI Reagent (Takara, Otsu, Japan). For reverse transcription, cDNA was synthesized from 1 μg of total RNA using a GoScript™ Reverse Transcriptase Kit (Promega, Madison, WI, USA) and used as template in real-time. Primer sequences (sense and antisense) were as follows: IGFBP1, 5'-TTGGGACGCCATCAGTACCTA-3' and 5'-TTGGGCT AAACCTCTACGACTCT-3'; prolactin, 5'-TGACCTCTCTCACCTTGGGACTGTTTG-3') and the negative control (5'-GGUGGGACGAACAAUGA-3' and 5'-GGUGGGACGACAAUGA-3'); GAPDH, 5'-CATGAGAAGTATGACAACAGCCT-3' and 5'-GA PDH-3'; FOXO1; 5'-CTTGCTTCTTGTCTCCGG G-3'; FoxO1, 5'-TTCCGTGTGCAGAATGAAGGA-3'; PLD1, 5'-CATGAGAATATGACAACAGCCT-3' and 5'- AGTCTCTCAGATAACAAAGT-3'. Transcripts were detected with IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Thermocycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 61 °C for 1 min. Input cDNA was normalized with GAPDH.

Western blot analysis and immunoprecipitation

hESCs cell lysates were washed once with cold PBS and lysed in mild lysis buffer [pH 7.5, 10 mM Tris, 2 mM EDTA, 100 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM Na₂VO₄, and protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA)] and were collected after centrifugation at 13 000 g for 10 min at 4 °C. For immunoprecipitation, these cell lysates were incubated with protein A/G plus-agarose immunoprecipitation reagent (Santa Cruz Biotechnology) at 4 °C for 1 h, followed by coupled with PP2A C subunit antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. Protein A/G plus-agarose beads were incubated with cell lysates bound to PP2A C subunit antibody at 4 °C for 1 h. Immunoprecipitates were washed with mild lysis buffer five times (9000 r.p.m.; 1 min 4 °C) and used for further analyses. Rabbit IgG was used as a negative control.

For western blotting, proteins were resolved by 6% or 8% SDS/PAGE and transferred onto poly(vinylidene difluoride) membranes (GE Healthcare Life Science, Piscataway, NJ, USA). The membranes were blocked for 1 h with Tris-buffered saline with 0.01% (v/v) Tween-20 containing 5% (w/v) phosphoBLOCKER Blocking Reagent (Cell Biolabs Inc., San Diego, CA, USA), and then incubated overnight at 4 °C with primary antibodies (1 μg·mL⁻¹), followed by HRP (horse radish peroxidase)-conjugated secondary antibody (1: 2000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and specific bands were detected by ECL (Thermo Fisher Scientific, Rockford, IL, USA). The following primary antibodies were purchased from Cell Signaling Technology: anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-FoxO1 (Ser256), anti-FoxO1 (C29H4), anti-PP2A C subunit (52F8) and anti-PLD1. Anti-GAPDH was from Bioworld Technology (St. Louis Park, MN, USA).

PLD1 and FoxO1 transcript depletion

The siRNA for PLD1 (5'-GGUGGGAGCAACUGA GCA-3') and the negative control (5'-CCUAGCCACCA AUUUGU-3') were purchased from Bioneer (Daejeon, Daeaeok, South Korea). FoxO1 siRNA treatments were performed with Dharmacon (Lafayette, CO, USA) ONTARGETplus SMART pool siRNA (L-000306-00-0005). PLD1 siRNA 200 nM and FoxO1 siRNA 200 nM were transfected into hESCs using Lipofectamine® 2000 (Invitrogen™, Carlsbad, CA, USA) for 48 h. After transfection, the cells were then treated with 0.5 μg mL⁻¹ 8-BrcAMP for 40 min or 2–3 days. Gene silencing was assayed by RT-PCR or western blotting.

Statistical analysis

Data are expressed as means ± SEM of at least five independent experiments; P < 0.05 was considered statistically significant. Comparisons between groups were made using unpaired Student’s t-tests using the statistical package GRAPHPAD PRISM 6 (GraphPad Software Incorporated, La Jolla, CA, USA).
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Author contributions

SYL, YYL, MSY, and JSH designed the study and edited the manuscript. SYL and YYL performed the research and SYL wrote the draft of manuscript. JSC supplied the hESCs for experiments.

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