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ORIGINAL RESEARCH

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Downregulation of PHLDA2 promotes apoptosis and autophagy in endometrial cancer cells while inhibiting their proliferation and metastasis

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Abstract

Background: Pleckstrin homology-like domain family A member 2 (*PHLDA2*) has been implicated as a potential inhibitor of apoptosis and may contribute to tumorigenesis. Here, we report on the function of PHLDA2 in endometrial cancer (EC). **Methods**: Small interfering RNA (siRNA) was utilized to reduce PHLDA2 expression in EC cells. Subsequently, cell proliferation, apoptosis, migration and invasion abilities were evaluated. Autophagy was examined through Microtubule-associated protein 1 light chain 3 beta (LC3B) immunofluorescence staining. Protein expression levels were determined by Western blotting. **Results**: PHLDA2 expression was significantly elevated in EC cell lines, and its knockdown was found to reduce cell proliferation, migration, and invasion while enhancing apoptosis. Moreover, induction of autophagy was observed with PHLDA2 knockdown, marked by elevated LC3B conversion and diminished P62 expression. Additionally, the fall in phosphorylation levels of Phosphatidylinositol 3-kinase (PI3K), Protein kinase B (AKT) and Glycogen synthase kinase-3 beta (GSK-3β) was examined following PHLDA2 knockdown. **Conclusions**: PHLDA2 knockdown can impede the malignant progression of EC cells by promoting apoptosis and autophagy, potentially through inactivation of the PI3K/AKT/GSK-3β pathway.

Keywords

Endometrial cancer; PHLDA2; Apoptosis; Autophagy; PI3K/AKT/GSK-3β

1. Introduction

Endometrial cancer (EC) represents a serious malignany in women [1], with recent data indicating a rising incidence and mortality rate in China, particularly among younger individuals [2, 3]. While patients with early-stage EC generally have a favorable prognosis following comprehensive staging surgery and adjuvant chemoradiation, advanced or recurrent EC remains challenging to treat due to limited therapeutic options, leading to poor prognostic outcomes [4–7]. Recently, the advent of precision medicine has generated increasing interest in targeted therapies, offering a promising avenue for the treatment of advanced and recurrent EC [7–9].

Pleckstrin homology-like domain family A member 2 (*PHLDA2*) is an imprinted gene that is maternally expressed and was the first apoptosis-related imprinted gene identified [10]. It is located on chromosome 11p15.5, a region associated with tumor suppression, and has been implicated in cancer progression [11]. However, the role of *PHLDA2* in malignancies appears to be context-dependent. In osteosarcoma, its expression has been linked to favorable outcomes [12]. In contrast, in colorectal cancer, glioma and liver cancer, *PHLDA2* has been shown to function as an oncogene, promoting tumor growth and metastasis [13–15].

Autophagy is a cellular process that regulates homeostasis by degrading and recycling intracellular components [16]. Its involvement in tumor development is intricate, as it may either advance or suppress cancer growth based on the cellular environment [17]. While autophagy enhances tumor metastasis by increasing cell invasiveness, it can also suppress tumor proliferation by degrading oncogenic proteins through autophagy receptors such as P62 [18–20]. In colorectal cancer and glioma, *PHLDA2* deletion has been shown to trigger autophagy, leading to reduced tumor growth and metastasis [13, 14]. Collectively, *PHLDA2* may influence tumor progression by modulating autophagic activity, though its role in EC remains unclear.

In this study, analysis of The Cancer Genome Atlas (TCGA) database revealed that PHLDA2 expression is elevated in EC tumor tissues. However, direct evidence linking PHLDA2 to EC progression remains lacking. To address this gap, we investigated the impact of PHLDA2 knockdown on EC cells *in vitro* by employing siRNA-mediated suppression of PHLDA2 expression.

2. Materials and methods

2.1 Cell culture and transfection

Normal human endometrial epithelial cells (HEECs) and EC cell lines (HEC-1-B (RRID): CVCL_0294), KLE (CVCL_1329), Ishikawa (CVCL_2529) and RL-952 (CVCL_0505)) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; SH30243.01, HyClone, Beijing, China) supplemented with 10% fetal bovine serum (FBS) at 37 °C.

Small interfering RNA (siRNA) targeting PHLDA2 (si-PHLDA2) and the negative control siRNA (si-NC) were bought from GenePharma (Shanghai, China). Transfections using 20 nM siRNA were conducted when cells reached 40–50% confluence and after 48 hours, the transfected cells were gathered for further examination.

2.2 Cell counting Kit-8 (CCK-8) assay

The cells (1500 cells/well) were placed into 96-well plates and kept at 37 °C for 48 hours, followed by the addition of 10 μL of CCK-8 reagent (CK04, Dojindo, Shanghai, China) to each well and a further 2-hour incubation. Absorbance at 450 nm (OD450) was recorded. Cell survival rates were calculated by comparing the absorbance of the si-PHLDA2 and si-NC groups with that of the control group.

2.3 Colony formation assay

Cells (1000 cells/well) were seeded in six-well plates and maintained at 37 °C for an estimated two weeks. Colonies were stained with 0.2% crystal violet following fixation with 4% paraformaldehyde. Colonies exceeding 50 cells in number were counted and photographed using a Nikon camera (DS-Fi3, Nikon, Tokyo, Japan).

2.4 Transwell assay

Transwell chambers (8-μm pore size) with or without Matrigel coating in a 24-well plate were employed to assess cell migration and invasion. The upper chamber received 200 μL cell suspension (5×10^4 cells) lacking serum, while the lower chamber was filled with 600 μL of medium containing 10% FBS. After incubation for 24 hours, cells on the upper surface of the membrane were removed, and those that had migrated or invaded were fixed and stained. Five fields chosen at random were imaged and counted.

2.5 Flow cytometry

Cells that underwent transfection were harvested, washed and then stained with Annexin V-Fluorescein Isothiocyanate (Annexin V-FITC) and Propidium Iodide (PI) through the use of the Annexin V-FITC detection kit (C1062S, Beyotime, Shanghai, China). The apoptotic cells were analyzed within one hour using an Epics XL flow cytometer (Beckman Coulter, Brea, CA, USA).

2.6 Immunofluorescence (IF) assay

Intracellular LC3B expression was evaluated using immunofluorescence staining. The cells (1×10^6) were fixed with 4% paraformaldehyde at room temperature for 30 minutes and blocked with 10% goat serum at 4 °C for 15 minutes. They were then incubated overnight at 4 °C with an LC3B primary antibody (ab192890, 1:200, Abcam, Cambridge, MA, USA), followed by incubation with Alexa Fluor@594-conjugated goat anti-rabbit Immunoglobulin G (IgG) H&L (heavy chain/light chain) secondary antibody (ab150080, 1:200, Abcam, Cambridge, MA, USA) at 37 °C for one hour. After counterstaining with 4′,6-Diamidino-2-phenylindole (DAPI) for five minutes, fluorescence images were captured using an Olympus IX71 microscope (Olympus, Tokyo, Japan).

2.7 Western blot

Using Radio Immunoprecipitation Assay (RIPA) buffer, total cellular protein was extracted. After separation by Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE), proteins were moved onto polyvinylidene fluoride (PVDF) membranes. Initially, the membranes were exposed to primary antibodies (see Table 1) and subsequently, secondary antibodies were used. Protein bands were made visible with an Enhanced Chemiluminescence (ECL) detection kit, and ImageJ software was used to quantify the intensity of the bands.

2.8 Statistical analysis

The data are presented as mean $\pm$ standard deviation (SD) based on no fewer than three distinct experiments. For comparing groups, one-way analysis of variance (ANOVA) was conducted, with Tukey's *post-hoc* test applied afterward. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1 PHLDA2 is upregulated in EC

Analysis of the TCGA database revealed that PHLDA2 expression was significantly upregulated in EC tumor tissues (Fig. 1A). Western blot analysis showed that PHLDA2 expression was markedly elevated in HEC-1-B, KLE, Ishikawa and RL-952 cells compared to HEECs (Fig. 1B). Together, these results indicate that PHLDA2 is highly expressed in EC.

3.2 Knockdown of PHLDA2 suppresses EC cell progression

To investigate the functional role of PHLDA2, HEC-1-B and Ishikawa cells were transfected with siRNA targeting PHLDA2 (si-PHLDA2). PHLDA2 expression was effectively reduced in si-PHLDA2-transfected cells, as confirmed by western blot (Fig. 2A). Significant reductions in cell viability and clonogenic potential were observed in CCK-8 and colony formation following PHLDA2 knockdown significantly decreased cell viability and clonogenic potential (Fig. 2B,C). Additionally, Transwell assays revealed that the number of migrating and invading cells was significantly

TABLE 1. Antibodies used for western blot.

Antigen	Catalog no.	Working dilution	Supplier
PHLDA2	#73901	1:1000	Cell signaling Technology
Bax	ab270742	1:1000	Abcam
Bcl-2	ab241548	1:1000	Abcam
Cleaved-caspase-3	ab32351	1:5000	Abcam
LC3B	ab63817	1:1000	Abcam
P62	ab240635	1:1000	Abcam
p-PI3K (Tyr458)	#4228	1:500	Cell signaling Technology
PI3K	sc-23962	1:2000	Cell signaling Technology
p-AKT	Ser473	1:2000	Cell signaling Technology
AKT	#4691	1:1000	Cell signaling Technology
p-GSK-3β	ab131097	1:500	Abcam
GSK-3β	ab280376	1:1000	Abcam
GAPDH	ab59164	1:2000	Abcam

PHLDA2: Pleckstrin homology-like domain family A member 2; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; LC3B: Microtubule-associated protein 1 light chain 3 beta; p-PI3K (Tyr458): phosphorylated phosphatidylinositol 3-kinase; PI3K: Phosphatidylinositol 3-kinase; p-AKT: phosphorylated protein kinase B; p-GSK-3β: phosphorylated glycogen synthase kinase-3β; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

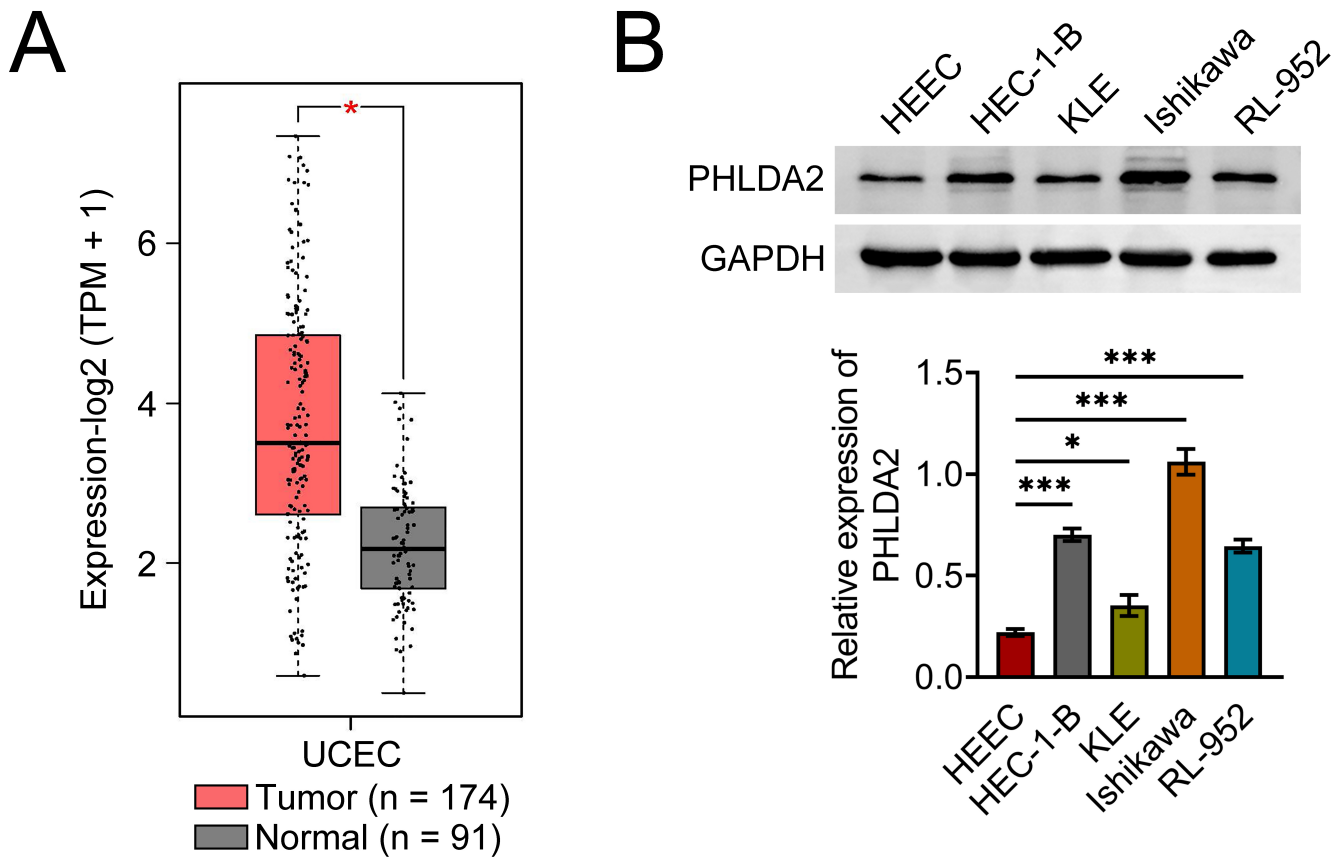

FIGURE 1. PHLDA2 is highly expressed in endometrial cancer (EC). (A) PHLDA2 expression levels in Uterine Corpus Endometrial Carcinoma (UCEC) tumor tissues were analyzed using The Cancer Genome Atlas (TCGA) database. (B) Western blot analysis of PHLDA2 protein expression in EC cell lines compared to normal human endometrial epithelial cells (HEECs). *$p < 0.05$, *$p < 0.05$, ***$p < 0.001$. PHLDA2: Pleckstrin homology-like domain family A member 2; TPM: Transcripts Per Million; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

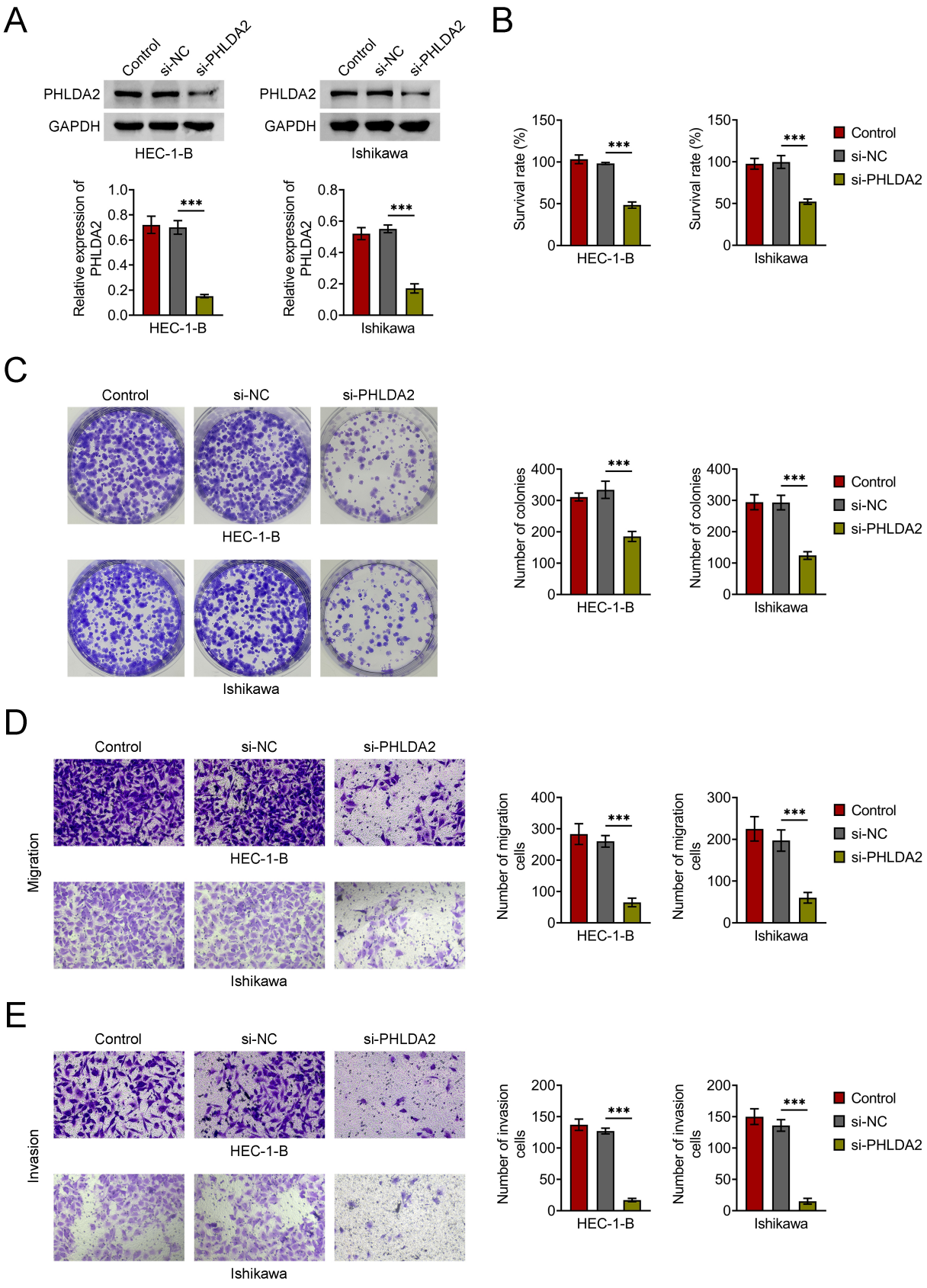

FIGURE 2. Knockdown of PHLDA2 suppresses EC cell proliferation, migration and invasion. (A) Western blot analysis confirming PHLDA2 knockdown in EC cells transfected with si-PHLDA2. (B) Cell viability was assessed using the CCK-8 assay. (C) Clonogenic potential was evaluated using the colony formation assay. (D,E) Transwell assays were performed to assess cell migration and invasion. ***$p < 0.001$. PHLDA2: Pleckstrin homology-like domain family A member 2; si-NC: small interfering RNA negative control; si-PHLDA2: small interfering RNA targeting PHLDA2; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

lower in si-PHLDA2-transfected cells (Fig. 2D,E). These findings suggest that PHLDA2 knockdown inhibits EC cell proliferation, migration and invasion.

3.3 Knockdown of PHLDA2 promotes EC cell apoptosis

Flow cytometry analysis demonstrated a significant elevation in apoptosis following PHLDA2 knockdown (Fig. 3A). Western blot analysis further confirmed that apoptosis-related proteins Bax and Cleaved-caspase-3 were upregulated, while B-cell lymphoma-2 (Bcl-2) expression was reduced in si-PHLDA2-transfected cells (Fig. 3B). Overall, these results imply that PHLDA2 knockdown enhances apoptosis in EC cells.

3.4 Knockdown of PHLDA2 induces autophagy in EC cells

Next, the results of the western blot showed that LC3-II/LC3-I protein levels increased, while P62 expression decreased following PHLDA2 knockdown (Fig. 4A), suggesting enhanced autophagic activity. Immunofluorescence analysis further confirmed increased LC3B expression in si-PHLDA2-transfected cells (Fig. 4B), suggesting that PHLDA2 knockdown could promote autophagy in EC cells.

3.5 Knockdown of PHLDA2 inhibits the phosphatidylinositol-3-kinases/AKT/glycogen synthase kinase-3β (PI3K/AKT/GSK-3β) pathway

Lastly, PHLDA2 knockdown reduced the phosphorylation of PI3K, AKT and GSK-3β, while total protein levels remained unchanged, as shown by western blot (Fig. 5). These findings suggest that PHLDA2 knockdown disrupts PI3K/AKT/GSK-3β signaling in EC cells.

4. Discussion

EC poses a significant threat to women's health due to its complex pathogenesis and poor prognosis [21–23]. Thus, gaining a more profound insight into the molecular mechanisms driving EC progression is crucial for developing effective therapeutic strategies. Herein, we demonstrated that PHLDA2 expression was elevated in EC cells, and its knockdown suppressed tumor-like behaviors while promoting apoptosis and autophagy. These findings suggest that PHLDA2 plays a role in EC malignancy and may serve as a potential therapeutic target to slow disease progression and improve patient outcomes.

Autophagy, an evolutionarily conserved cellular process, is responsible for breaking down and recycling intracellular elements [24], involving key steps such as phagophore formation, autophagosome-lysosome fusion and autophagolysosomal degradation [25, 26], and its dysregulation has been strongly linked to tumor development [17]. Studies have

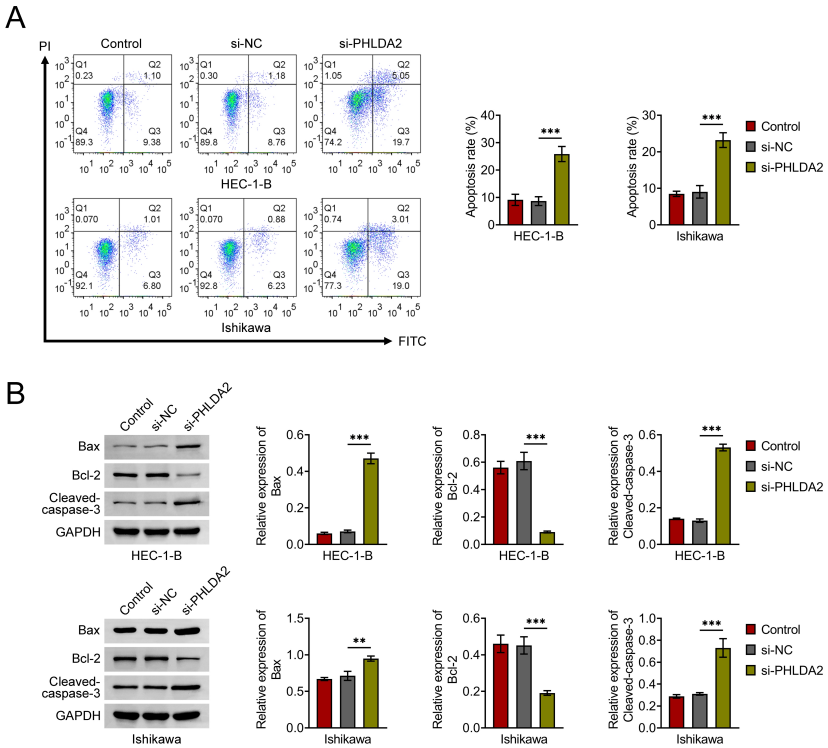

FIGURE 3. PHLDA2 knockdown promotes apoptosis in EC cells. (A) Flow cytometry analysis of apoptosis in PHLDA2-knockdown and control EC cells. (B) Western blot analysis of apoptosis-related proteins Bax, Bcl-2 and Cleaved-caspase-3 in EC cells following PHLDA2 knockdown. $^{**}p < 0.01$, $^{***}p < 0.001$. si-NC: small interfering RNA negative control; si-PHLDA2: small interfering RNA targeting PHLDA2; HEC-1-B: PI: Propidium Iodide; FITC: Fluorescein Isothiocyanate; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

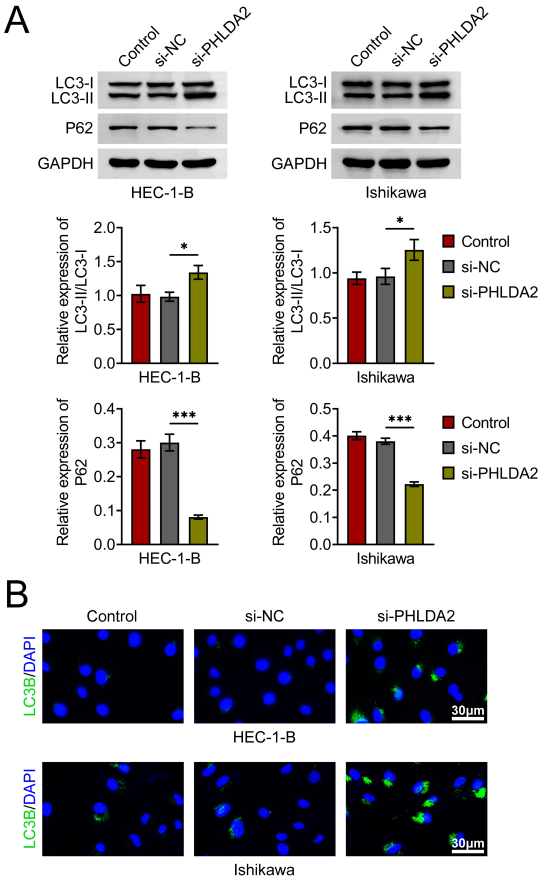

FIGURE 4. PHLDA2 knockdown induces autophagy in EC cells. (A) Western blot analysis of autophagy-related proteins LC3-I, LC3-II and P62 following PHLDA2 knockdown. (B) Immunofluorescence (IF) staining of LC3B expression in EC cells with PHLDA2 knockdown. $*p < 0.05$, $***p < 0.001$. si-NC: small interfering RNA negative control; si-PHLDA2: small interfering RNA targeting PHLDA2; LC3: Microtubule-associated protein 1 light chain 3; GAPHD: Glyceraldehyde-3-Phosphate Dehydrogenase. LC3B: Microtubule-associated protein 1 light chain 3 beta; DAPI: 4′,6-Diamidino-2-Phenylindole.

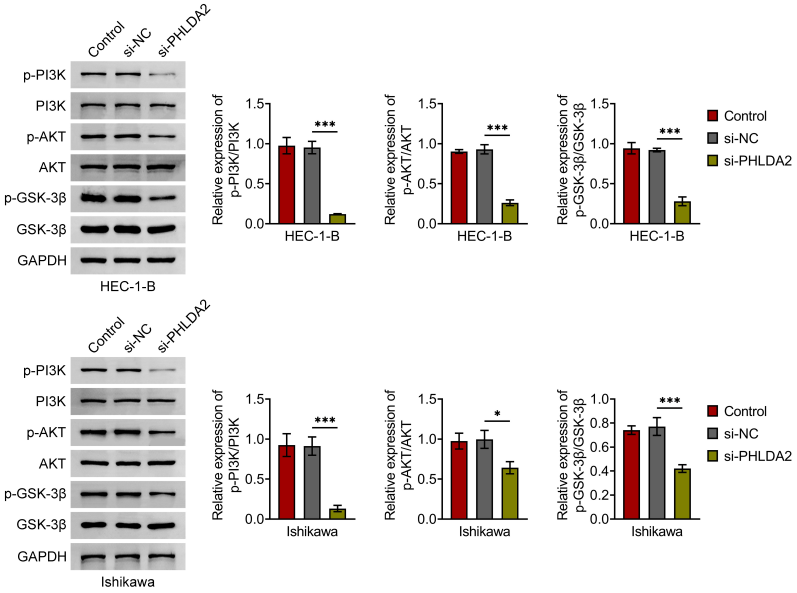

FIGURE 5. PHLDA2 knockdown inactivates the PI3K/AKT/GSK-3β pathway. Western blot analysis of phosphorylated and total PI3K, AKT and GSK-3β protein levels in EC cells following PHLDA2 knockdown. $*p < 0.05$, $***p < 0.001$. si-NC: small interfering RNA negative control; si-PHLDA2: small interfering RNA targeting PHLDA2; p-PI3K: phosphorylated phosphatidylinositol 3-kinase; p-AKT: phosphorylated protein kinase B; p-GSK-3β: phosphorylated glycogen synthase kinase-3β; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

identified multiple autophagy-related gene mutations in EC, which are associated with reduced tumor aggressiveness [27–29]. In addition, autophagy has been implicated in regulating apoptosis and necrosis, ultimately inhibiting tumor cell growth [30, 31]. Thus, enhancing autophagy may provide a therapeutic advantage in EC.

In this study, PHLDA2 knockdown was found to inhibit EC cell proliferation, migration and invasion while promoting apoptosis. Moreover, LC3-II/LC3-I protein expression increased and P62 expression decreased following PHLDA2 knockdown. Immunofluorescence staining further confirmed an increase in LC3B expression in PHLDA2-silenced cells. LC3 is an important regulator of autophagosome formation, and its conversion from cytoplasmic LC3-I to membrane-bound LC3-II marks the initiation of autophagy [32, 33]. In this regard, increased LC3-II levels could serve as a reliable indicator of autophagy activation. Additionally, P62, a selective autophagy substrate that links LC3 to polyubiquitinated proteins, is degraded during autophagic flux [33, 34]. Therefore, reduced P62 expression is indicative of enhanced autophagic activity. These findings suggest that PHLDA2 knockdown promotes autophagy in EC cells, potentially contributing to its tumor-suppressive effects.

PHLDA2 encodes a structural protein containing a pleckstrin homology (PH) domain, which has a high affinity for phosphoinositides [35]. Previous studies have suggested that PHLDA2 may interfere with the PI3K/AKT signaling pathway [13, 36]. Interestingly, this pathway is critical for cell survival and apoptosis, and it also regulates epithelial-mesenchymal transition (EMT) by modulating GSK-3β, thereby influencing migration and invasion [37, 38]. Moreover, the PI3K/AKT pathway is recognized as a key regulator of autophagy in tumor cells [39]. A previous study demonstrated that PHLDA2 inhibited apoptosis in gastric cancer cells via the PI3K/AKT pathway [40]. Similarly, another study reported that PHLDA2 knockdown suppressed tumor growth and PI3K activity while boosting autophagy and deminishing EMT through the PI3K/AKT/ Mammalian Target of Rapamycin (mTOR) and PI3K/AKT/GSK-3β pathways in colorectal cancer [13]. In line with these results, our research revealed that PHLDA2 knockdown inactivated the PI3K/AKT/GSK-3β pathway in EC cells, suggesting that this signaling axis may be one of the key mechanisms by which PHLDA2 exerts its effects in EC.

5. Conclusions

In conclusion, our findings demonstrate that PHLDA2 knockdown induces apoptosis and autophagy while inhibiting EC cell growth and metastasis, and that these effects may be mediated by the suppression of the PI3K/AKT/GSK-3β pathway. Nevertheless, our limitation of this research is the absence of direct validation of the PI3K/AKT/GSK-3β pathway as the mechanistic link between PHLDA2 and EC progression, along with absence in vivo evidence. Upcoming research might aim to overcome these limitations to offer a more thorough insight into the therapeutic potential of PHLDA2 in EC.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

MPG, MYL—designed the study and carried them out; prepared the manuscript for publication and reviewed the draft of the manuscript. MPG, ZYW, MYL—supervised the data collection; analyzed the data; interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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