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ORIGINAL RESEARCH

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Knockdown of PLAC1 inhibits BCa growth and migration through upregulation of FOXO3a

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Abstract

Placenta-specific protein 1 (PLAC1) is considered to play a pivotal role in cancer progression. Here, we investigated the role of PLAC1 in the growth and motility of bladder cancer (BCa) cells. Database analysis and Immunoblot assays were conducted to determine PLAC1 expression in BCa tissues and its correlation with patient prognosis. Furthermore, wound healing, transwell and tube formation assays were performed to evaluate cell motility and angiogenic potential, and the underlying mechanism *via* which PLAC1 knockdown inhibits BCa progression *in vitro* was investigated. The data revealed that PLAC1 was obviously overexpressed in BCa tissues and was associated with poor patient prognoses. Additionally, silencing PLAC1 led to reduced viability, migratory capacity, invasion potential and angiogenesis of BCa cells, including T24 and UMUC3 cells. Further investigation showed that PLAC1 knockdown modulated the phosphoinositide 3-kinase/protein kinase B/forkhead box O3a (PI3K/Akt/FOXO3a) axis by enhancing the phosphorylation of FOXO3a while suppressing the phosphorylation of PI3K as well as Akt. Moreover, we demonstrated that the inhibition of BCa progression by PLAC1 knockdown was primarily mediated through the targeting of FOXO3a. In summary, these findings confirmed the potential of PLAC1 as a promising target for suppressing BCa growth by elevating FOXO3a levels and modulating the PI3K/Akt/FOXO3a signaling axis.

Keywords

Angiogenesis; PLAC1; BCa; FOXO3a; PI3K/Akt pathway

1. Background

Bladder cancer (BCa), comprising both muscle-invasive and non-muscle-invasive subtypes, primarily originates from urinary tract [1]. In recent decades, there has been a significant increase in the incidence of BCa [2], with approximately 549,000 new cases worldwide [1]. Although the precise etiology and pathophysiology of BCa remain unclear, it has been associated with risk factors [3]. Currently, the therapeutic options for BCa include surgery and chemotherapy. Despite continuous advancements in medical technology and treatment strategies, there is no improvement in the overall survival (OS) of BCa patients [4]. Targeted therapy presents a promising and novel approach to managing BCa. Hence, comprehensive research into the molecular mechanisms governing the onset and progression of BCa is imperative for innovative treatment strategies that can ultimately enhance patient prognosis and quality of life [5].

Placenta-specific protein 1 (PLAC1) primarily localizes to the cell membrane of trophoblasts and plays a pivotal role in their growth and motility. While PLAC1 expression is typically confined to healthy tissues such as the placenta and testes, it has been found to be frequently activated and highly expressed in various human cancers. Notably, it was reported to promote breast cancer metastasis [6], enhance gastric cancer cell proliferation by mediating the Akt/glycogen synthase kinase 3 beta (GSK-3β) axis [7], and facilitate hepatocellular carcinoma cell motility by reducing Akt phosphorylation levels [8]. Moreover, PLAC1 can activate the PI3K/Akt/nuclear factor kappa B (NF-κB) signaling pathway to promote colorectal cancer cell metastasis and angiogenesis [9]. Despite its critical roles in multiple human malignancies, the potential involvement of PLAC1 in BCa remains unexplored.

The FOXO transcription factor is a pivotal regulator of various cellular processes, encompassing cell proliferation, differentiation and apoptosis. It functions as a primary downstream target of the PI3K/Akt axis [10]. Among the FOXO subfamily members, FOXO3a assumes a significant role in regulating cell cycle and apoptosis [11]. Recent investigations have revealed FOXO3a's role as a tumor suppressor in various cancer types, including bladder, colon, liver, breast and prostate cancer [12]. Notably, FOXO3a overexpression was shown to prevent cancer cell growth and impede tumor formation and metastasis *in vivo* [13, 14].

This study aims to elucidate the expression pattern of PLAC1 in BCa tissues, its functional role, and the underlying

mechanism. Collectively, PLAC1 holds promise as a potential target for preventing BCa growth *via* the modulation of FOXO3a and the PI3K/AKT axis.

2. Materials and methods

2.1 Sample collection

Surgical specimens of BCa tissues and adjacent non-cancerous tissues were obtained from 56 patients (preoperative radiotherapy or chemotherapy naive), which were preserved at −80 °C until further analysis.

2.2 Cells culture

The BCa cell lines T24 and UMUC3, as well as human umbilical vein endothelial cells (HUVECs), were purchased from the National Infrastructure of Cell Line Resource (Beijing, China). UMUC3 cells and HUVECs were routinely cultured in RPMI 1640 medium (11320033, Gibco, USA), while T24 cell lines were cultured in Dulbecco's Modified Eagle's Medium (11965092, Gibco, USA). The culture medium was supplemented with 10% fetal bovine serum (FBS, 10099141, Gibco, Waltham, MA, USA) and 1% PS (15070063, Gibco, Waltham, MA, USA). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.3 Bioinformatic analysis

Data on PLAC1 mRNA and protein expression were extracted from various platforms, including the UALCAN database (http://ualcan.path.uab.edu) and the TIMER database (http://cistrome.dfci.harvard.edu/TIMER/). The association between PLAC1 expression and its prognostic significance in BCa was investigated in the following databases: the GEPIA database (http://gepia2.cancer-pku.cn/#analysis) and the Kaplan-Meier Plotter database (https://kmplot.com/analysis/).

2.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated using TRIzol reagent (15596026, Invitrogen, USA), which were then reverse transcribed into complementary DNAs (cDNAs) using the PrimeScript Kit (RR037Q, TaKaRa, Otsu, Japan). The $2^{-\Delta\Delta Cq}$ method was used to quantify. The data, and the primer sequences utilized are listed in Table 1.

TABLE 1. Sequences of primers in the experiment.

Name	Sequence (5′ to 3′)
PLAC1-forward	CCGGACAAAATCCAGTGACTGT
PLAC1-reverse	AACCCAGGCCCAAGTATAACTCA
GAPDH-forward	GAAGGTGAAGGTCGGAGTC
GAPDH-reverse	GAAGATGGTGATGGGATTTC

PLAC1: Placenta-specific protein 1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

2.5 siRNA transfection

T24 and UMUC3 cells were seeded at a density of 5×10^5 cells/mL into separate 6-well plates. For transfection, 100 μL of Opti-MEM® was used to dilute the transfection plasmid (50 pmol/L), and the mixture was gently agitated and then incubated for 5 minutes. In a separate 100 μL of Opti-MEM®, 2 μL of Lipofectamine^{TM} 3000 (Invitrogen, Carlsbad, CA, USA) was mixed. These two dilutions were then combined and incubated for 30 minutes before being added to the cells for transfection for 48 hours. The sequences of the transfection plasmids are provided in Table 2.

TABLE 2. Sequences of primers in the experiment.

Name	Sequence (5′ to 3′)
si-PLAC1#1 sense	CAAACTCCCGCAGACTTCTC
si-PLAC1#1 antisense	GACCAACTGTGGCTGAACCT
si-PLAC1#2 sense	CGCTCTCTGCTCCTCCTGTTC
si-PLAC1#2 antisense	ATCCGTTGACTCCGACCTTCAC
si-NC sense	UUCUCCGAACGUGUCACGU
si-NC antisense	ACGUGACACGUUCGGAGAA

PLAC 1: Placenta-specific protein 1; NC: Negative control.

2.6 Cell Counting Kit-8 (CCK-8) assay

T24 and UMUC3 cells were transfected with si-NC, si-PLAC1#1 and si-PLAC1#2. Subsequently, these cells (3×10^5 cells per well) were seeded in separate 96-well plates. Cell viability was assessed by incubating with CCK-8 reagent (Beyotime, Beijing, China) for 3 hours, and the optical density (OD) 450 value was measured using a microplate reader (Molecular Devices, Silicon Valley, CA, USA).

2.7 Colony formation assay

Cells (5000 cells/well), previously transfected with si-NC, si-PLAC1#1 and si-PLAC1#2, were seeded into 6-well plates for 10 days. The formation of colonies, defined as clusters containing more than 50 cells, was monitored during this time. Subsequently, the cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes and stained using a 0.1% crystal violet solution. The number of colonies was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8 Wound healing assay

T24 and UMUC3 cells were cultured in 6-well plates overnight until reaching confluence. Subsequently, a 200 μL pipette tip was used to create a scratch. A culture medium devoid of FBS was added, and the cells were incubated. After 24 hours, the extent of wound closure was used to assess cell migratory capacity using a light microscope.

2.9 Invasion assay

Invasion assays were performed using cell transwell chambers (351152, Corning, New York, USA). For the invasion assay, a layer of 60 μL Matrigel (356253, Corning, USA) was applied

to the upper chamber and incubated for 2 hours. Subsequently, 5×10^4 cells per well were seeded into the upper chamber, while the lower chamber was filled with medium containing 5% FBS. Invasive cells on the lower surface were fixed with 4% formaldehyde for 15 minutes and then stained using a 0.1% crystal violet solution (94448, Sigma-Aldrich, Burlington, MA, USA).

2.10 Tube formation assay

T24 and UMUC3 cells were cultured in a serum-free medium for 48 hours, then both cells and the medium were collected, centrifuged and filtered to obtain a tumor-conditioned medium (TCM).

For the tube formation assay, T24 and UMUC3 cells were cultured in a serum-depleted medium for 24 hours. Subsequently, 2×10^4 cells were seeded onto Matrigel with reduced growth factors, which had been concentrated 75-fold after being thawed overnight. The tube formation of HUVECs was monitored at various time points using a microscope.

2.11 Immunoblot analysis

The proteins were transferred to polyvinylidene fluoride (PVDF) membranes, followed by blocking in 5% milk diluted with Tris-buffered saline with Tween 20 (TBST) for 1 hour. The membranes were then incubated with primary antibodies: PLAC1 (ab105395, Abcam, Cambridge, UK, 1:500), p-PI3K (ab278545, Abcam, 1:500), Akt (ab8805, Abcam, 1:500), PI3K (ab302958, Abcam, 1:500), p-Akt (ab38449, Abcam, 1:500), p-FOXO3a (ab154786, Abcam, 1:500), FOXO3a (ab279801, Abcam, 1:500), and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (Abcam, 1:1000) overnight at 4 °C. The following day, secondary rabbit anti-rabbit antibodies (1:1000) were applied to the membranes for a 2-hour incubation period, and the immunoreactive bands were quantified using ImageJ software.

2.12 Statistical analyses

Data analysis was conducted using the SAS 9.0 software (SAS Institute Inc., Cary, NC, USA). Log-rank test was used to assess differences and determine statistical significance. Student's *t*-test was used for further statistical analysis, with a significance level set at $p < 0.05$.

3. Results

3.1 High expression of PLAC1 was associated with the prognosis of BCa

To elucidate the role of PLAC1 in BCa, we conducted a comparative analysis of PLAC1 levels between BCa and normal tissues using the UALCAN, GEPIA and TIMER databases, and the data demonstrate significant upregulation of PLAC1 in BCa tissues compared to normal (Fig. 1A–C). We further examined PLAC1 mRNA as well as protein expression in BCa and adjacent tissues in all patients and observed that both mRNA levels as well as protein expression of PLAC1 were obviously elevated in BCa tissues, consistent with the database findings (Fig. 1D,E). To assess the potential of PLAC1 as a biomarker for BCa, the GEPIA and KMplot web tools were used, and the data indicated a clear association between PLAC1 overexpression and a shorter OS rate in BCa patients (Fig. 1F,G). Collectively, these findings highlight the elevated expression of PLAC1 in BCa tissues and correlated with prognosis.

3.2 Knockdown of PLAC1 could inhibit the growth of BCa cells

Subsequent experiments were conducted to investigate the biological effects of PLAC1 in BCa cells. To assess the functional role of PLAC1 in BCa cells, we used two types of siRNAs targeting PLAC1, siPLAC1#1 and si-PLAC1#2, to down-regulate PLAC1 expression in T24 as well as UMUC3 cells. The efficiency of PLAC1 knockdown was confirmed through Immunoblot analysis (Fig. 2A). Silencing PLAC1 led to an inhibition of BCa cell growth (Fig. 2B), and colony formation assay revealed a reduction in the number of colonies in both the si-PLAC1#1 and si-PLAC1#2 groups of T24 and UMUC3 cells (Fig. 2C). Collectively, these findings indicate that PLAC1 plays a critical role in regulating the viability of BCa cells.

3.3 Knockdown of PLAC1 could inhibit migration and invasion of BCa cells

The data above confirmed that PLAC1 knockdown was associated with decreased cell viability of BCa. Subsequently, we assessed the influence of PLAC1 on cell motility. In the wound healing assay, PLAC1 silencing was found to obviously reduce the migratory ability of T24 and UMUC3 cells (Fig. 3A). Furthermore, the transwell assay indicated that PLAC1 depletion obviously suppressed the invasive potential of both T24 and UMUC3 cells (Fig. 3B). Overall, silencing PLAC1 could inhibit the motility of BCa cells.

3.4 Knockdown of PLAC1 could inhibit angiogenesis of BCa

Considering that angiogenesis is a crucial process for the development and growth of solid tumors [15], we investigated whether PLAC1 knockdown could impede angiogenesis in BCa. As shown in Fig. 4, the TCM obtained from the si-PLAC1#1 and si-PLAC1#2 groups of T24 and UMUC3 cells induced HUVECs to form fewer and smaller tubes compared to the control or si-NC groups, indicating the inhibitory effects of PLAC1 on angiogenesis in BCa.

3.5 Depletion of PLAC1 suppressed the PI3K/Akt/FOXO3a pathway in BCa cells

The above results collectively demonstrate that knocking down PLAC1 effectively inhibited the growth, motility and angiogenesis of BCa cells. To investigate the underlying mechanism *via* which PLAC1 silencing exerts its suppressive effects on BCa, we conducted Immunoblot assays. Specifically, we observed a significant reduction in the phosphorylation levels of PI3K and Akt following PLAC1 depletion compared to both the control and the si-NC group, indicating inhibition of the PI3K/AKT pathway (Fig. 5). Additionally, the phos-

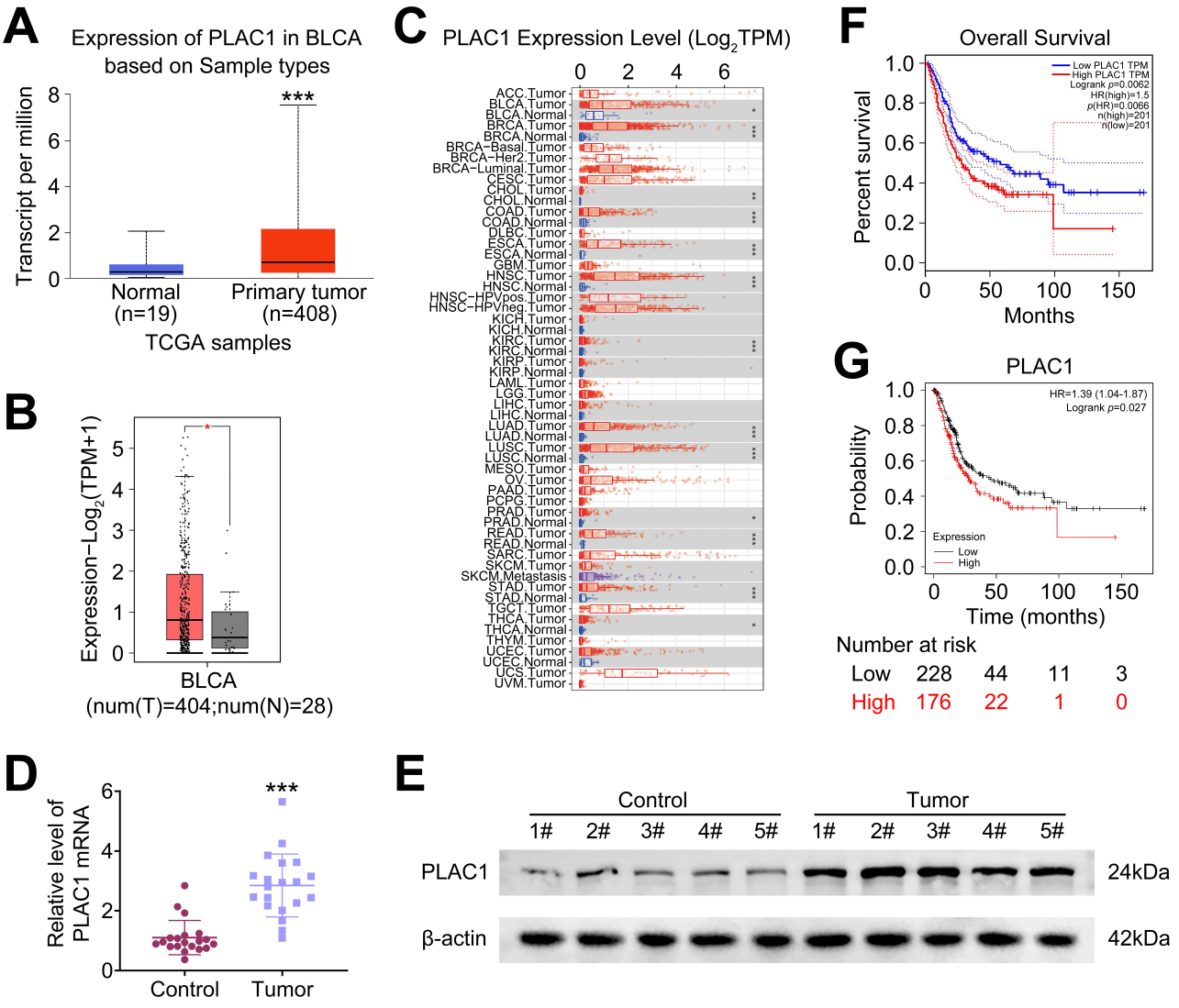

FIGURE 1. Expression of PLAC1 in BCas and its association with the prognosis of patients with BCa. (A) Differential expression analysis reveals PLAC1 transcripts per million (TPM) in tumor and normal tissues in the UALCAN dataset. (B) GEPIA displays PLAC1 expression differences between tumor and normal tissues. (C) TIMER shows the differential expression of PLAC1 in tumor and normal tissues. (D) qRT-PCR assays depict RNA expression levels of PLAC1 in adjacent nontumor bladder tissues and BCa tissues. (E) Immunoblot analysis of PLAC1 expression in 5 representative adjacent nontumor bladder tissues and BCa tissues. (F) Kaplan-Meier survival curve illustrating the correlation between PLAC1 expression and BCa prognosis. (G) Kaplan-Meier survival analysis demonstrating the survival outcomes of BCa patients in relation to PLAC1 expression, as confirmed by the KMplot database. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. TPM: transcripts per million.

phorylation levels of FOXO3a were also markedly decreased upon PLAC1 depletion (Fig. 5). These findings collectively indicate that silencing PLAC1 suppresses the activation of the PI3K/Akt/FOXO3a axis.

3.6 Knockdown of PLAC1 inhibited BCa progression through FOXO3a

To investigate the potential mechanism through which PLAC1 knockdown suppresses BCa progression *in vitro*, we conducted additional experiments involving the silencing of FOXO3a in T24 cells and observed that co-knockdown of both PLAC1 and FOXO3a resulted in further suppression of BCa cell growth compared to PLAC1 depletion alone (Fig. 6A). Similarly, co-knockdown of PLAC1 and FOXO3a obviously mitigated the inhibitory effects of PLAC1 depletion on cell motility, as confirmed by wound healing and transwell assays (Fig. 6B,C). Moreover, the TCM obtained from the co-knockdown of PLAC1 and FOXO3a group exhibited a more pronounced suppression of angiogenesis compared to PLAC1 depletion alone (Fig. 6D). Collectively, FOXO3a serves as a direct downstream target of PLAC1 in modulating the progression of BCa, based on which we hypothesize that the inhibition of BCa progression achieved through PLAC1 knockdown is mediated by FOXO3a.

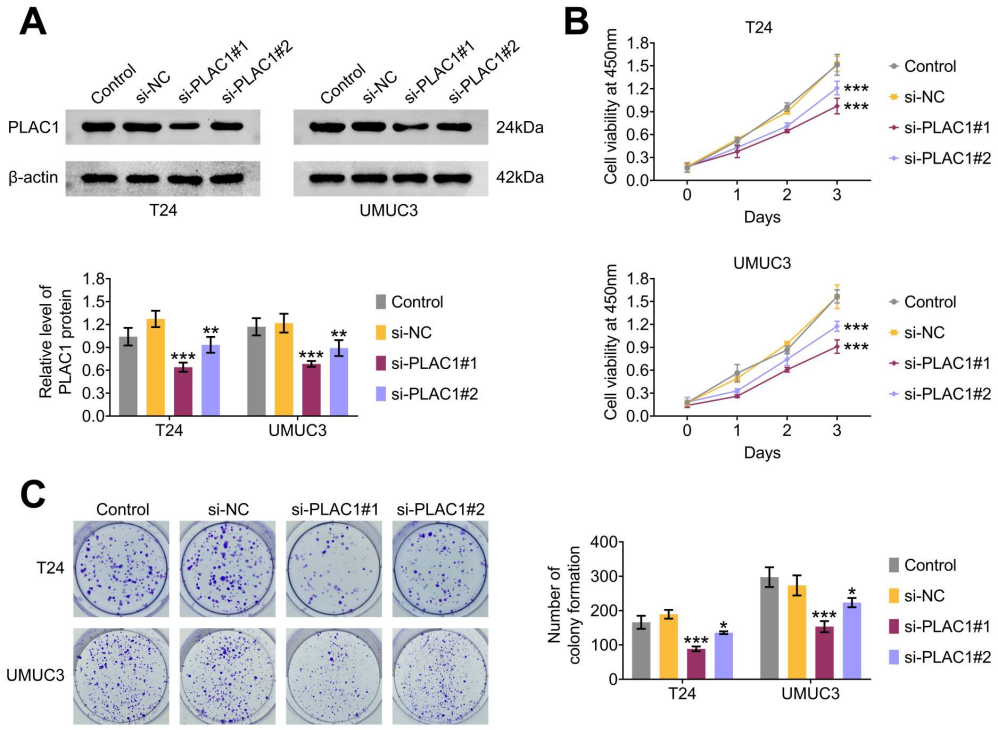

FIGURE 2. Impact of PLAC1 knockdown on viability and proliferation in T24 and UMUC3 cells. (A) Immunoblot analysis revealing PLAC1 expression levels in T24 and UMUC3 cells after transfections. (B) CCK-8 assays illustrating the viability of T24 and UMUC3 cells at 0, 24h, 48h and 72h post-transfection. OD values were detected at 450 nm *via* CCK-8 assays. (C) Colony formation assay demonstrating the proliferation ability of T24 and UMUC3 cells after the indicated transfections. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, siPLAC1 *vs.* siNC (n = 3). NC: negative control; PLAC1: Placenta-specific protein 1.

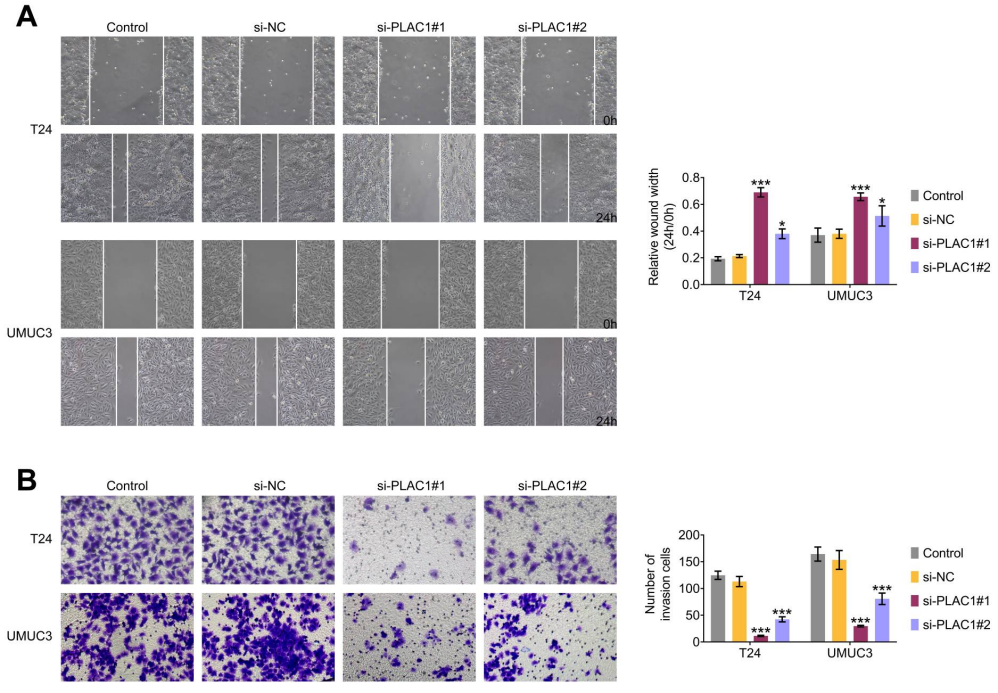

FIGURE 3. Significance of PLAC1 knockdown on the migration and invasion of BCa cells. (A) Wound healing assay confirming the effect of PLAC1 on the migration of T24 and UMUC3 cells. (B) Transwell assay demonstrating the influence of PLAC1 on the migration of T24 and UMUC3 cells. Cells that migrated to the lower chamber of the filter after 24 hours of incubation were stained and counted under an optical microscope. *$p < 0.05$, ***$p < 0.001$, siPLAC1 *vs.* siNC (n = 3). NC: negative control; PLAC1: Placenta-specific protein 1.

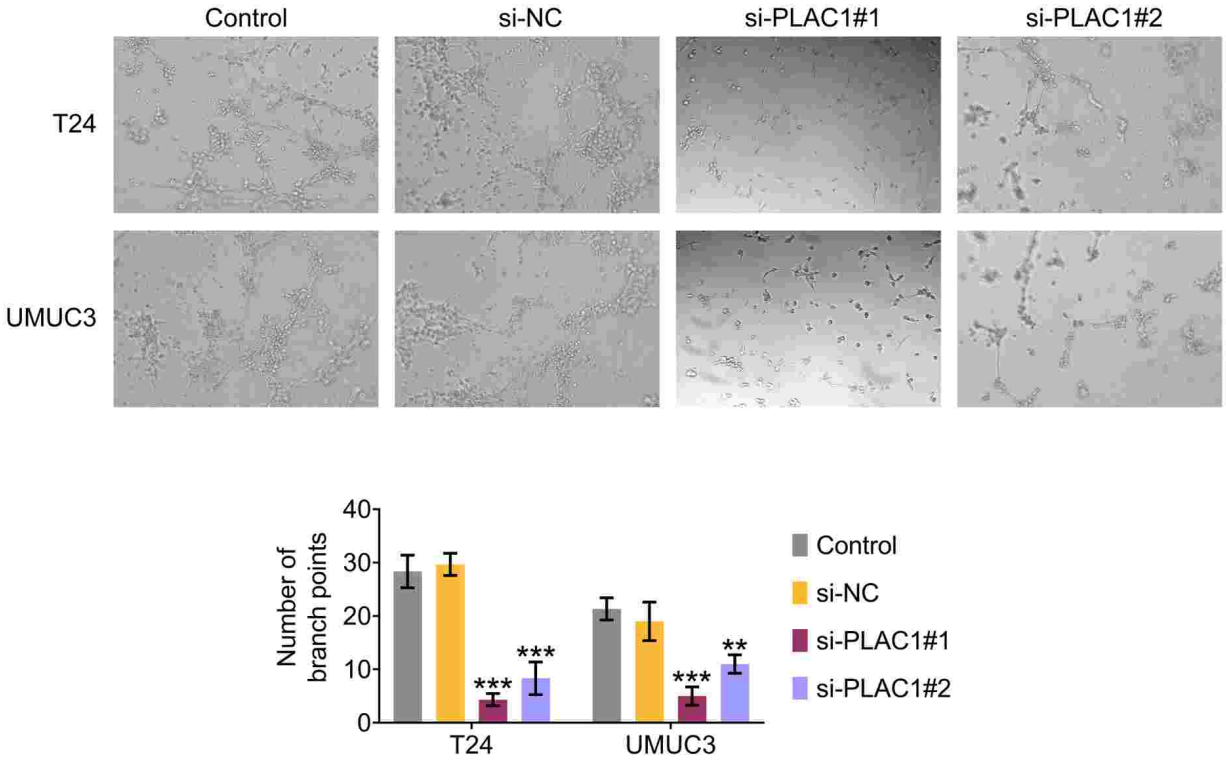

FIGURE 4. Effects of PLAC1 knockdown in BCa cells on HUVECs angiogenesis. The tube formation ability of HUVECs was assessed after 6 hours of cultivation in the tumor-conditioned media (TCMs) from the indicated groups using tube formation assays, and the number of branch points in each field was counted. $^{**}p < 0.01$, $^{***}p < 0.001$, siPLAC1 *vs.* siNC. NC: negative control; PLAC1: Placenta-specific protein 1.

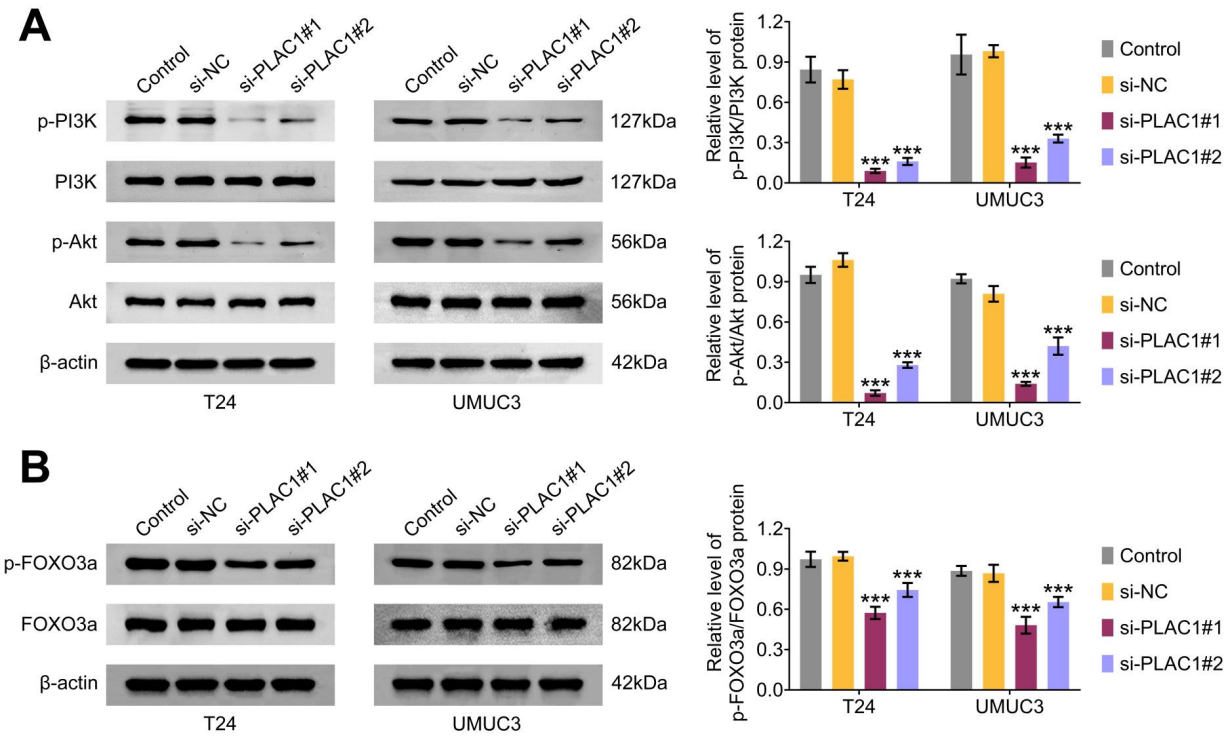

FIGURE 5. Suppression of the PI3K/Akt/FOXO3a pathway by PLAC1 in BCa cells. Immunoblot analysis of the expression and phosphorylation levels of PI3K, Akt and FOXO3a in T24 and UMUC3 cells following transfection with the indicated siRNAs. $^{***}p < 0.001$, siPLAC1 *vs.* siNC. NC: negative control; PLAC1: Placenta-specific protein 1; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; FOXO3a: forkhead box O3a.

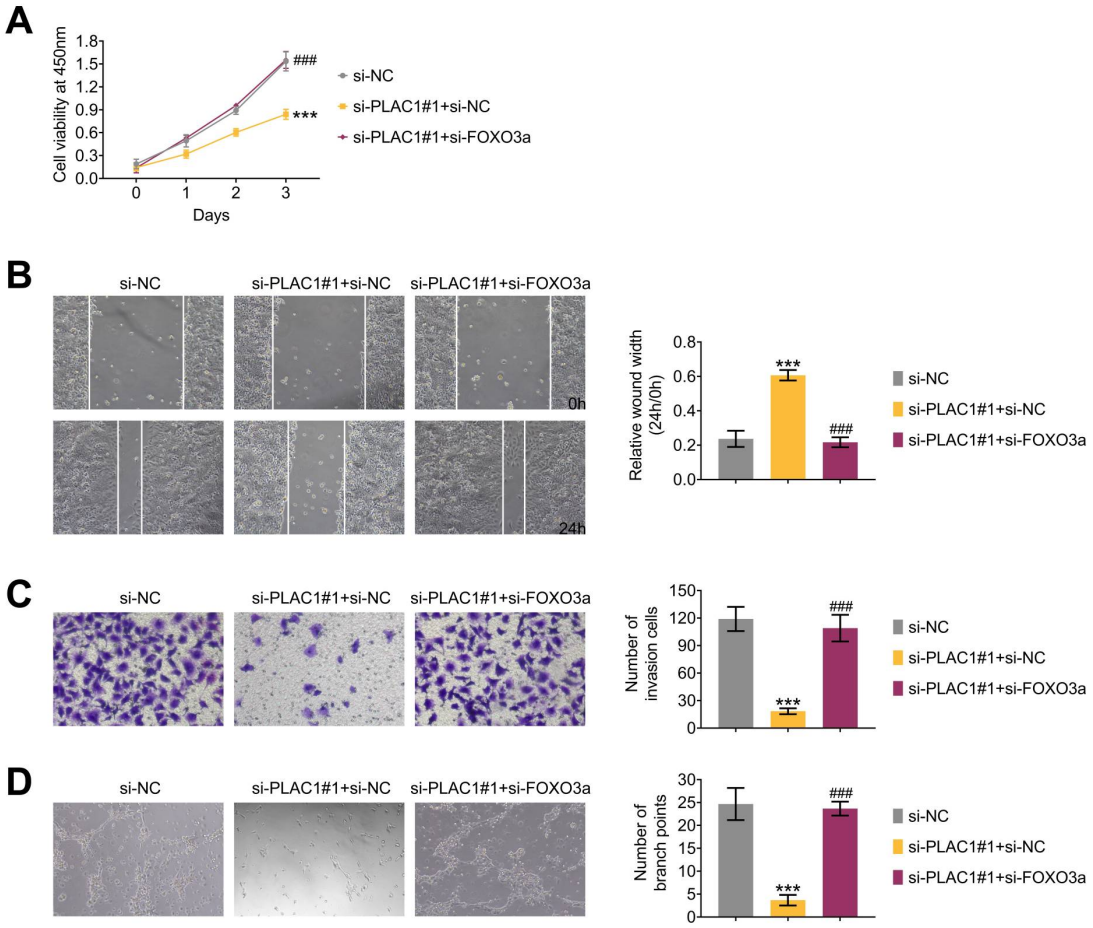

FIGURE 6. Knockdown of PLAC1 inhibits BCa progression through FOXO3a. (A) Viability of T24 cells following the indicated transfections assessed by CCK-8 assays. (B) Migration of T24 cells upon the indicated transfections evaluated by wound healing assay. (C) Migration of T24 cells following the indicated transfections confirmed by transwell assay. (D) Tube formation ability of HUVECs cultivated for 6 hours in the tumor-conditioned media (TCMs) from the indicated groups assessed through tube formation assays. ***$p < 0.001$ siPLAC1 *vs*. siNC, ###$p < 0.001$ siPLAC1 + siFOXO3a *vs*. siPLAC1 (n = 3). NC: negative control; PLAC1: Placenta-specific protein 1; PI3K: phosphoinositide 3-kinase; FOXO3a: forkhead box O3a.

4. Discussion

BCa is characterized by its invasive nature, propensity for metastasis, and frequent recurrence [16]. Understanding the aberrant changes that occur during BCa is of paramount importance to improve its diagnosis, treatment and patient prognosis [17]. Notably, patients with gastric cancer exhibiting low PLAC1 expression were shown to have a longer survival compared to those with higher PLAC1 expression [18]. Furthermore, several studies have established a correlation between PLAC1 levels and clinical parameters in various cancers [6]. We observed a significant increase in PLAC1 expression in BCa tissues, high levels of PLAC1 were associated with an unfavorable prognosis, and both mRNA and protein levels of PLAC1 were obviously elevated in BCa tissues.

The functional role of PLAC1 is well-established in various cancers. For instance, it obviously enhances the motility of nasopharyngeal carcinoma cells [19]. In osteosarcoma, PLAC1 promotes disease progression by stimulating cell proliferation. Furthermore, PLAC1's function has been implicated in driving metastasis in breast cancer cells through the Furin/Notch Intracellular Domain (NICD)/phosphatase and tensin homolog (PTEN) pathway [20]. Initially identified as a protein specifically expressed in the placenta and other cells originating from the trophoblast lineage during embryonic development, PLAC1 has been discovered to be ectopically expressed in a wide array of human malignancies, with breast cancers being a prominent example.

Notably, PLAC1 is recognized as a membrane-associated protein believed to function in a receptor-like capacity, potentially modulating unique cell-cell or ligand-receptor interactions specific to the maternal-placental interface [20]. Reduced expression of PLAC1 has been associated with decreased levels of cyclin D1 and a reduction in Akt kinase expression. This is noteworthy, especially considering that PLAC1 is expressed on the surface of cancer cells [20]. However, our findings indicate that the knockdown of PLAC1 inhibited proliferation, migration and invasion in BCa cells through the regulation of the PI3K/Akt/FOXO3a pathway, and further investigation is required to determine the precise underlying mechanism.

The FOXO transcription factor family consists of FOXO1, FOXO3a, FOXO4 and FOXO6 [21]. Among these, FOXO3a has been extensively investigated compared to the other transcription factors, and it was revealed to play a pivotal role

in regulating the expression of numerous genes involved in cell proliferation, cell cycle and apoptosis [22]. Reduced expression and activity of FOXO3a have been implicated in the onset, progression and drug resistance of various cancers, including breast cancer, pancreatic cancer and liver cancer [23]. Furthermore, decreased FOXO3a expression is associated with the malignant biological characteristics of BCa [24]. In this study, we observed that PLAC1 knockdown enhanced FOXO3a expression, and subsequent experiments confirmed that PLAC1 functions by regulating FOXO3a.

A limitation of this study is that the complete molecular mechanism by which PLAC1 promotes BCa has not been fully elucidated. Further investigations in the future, combined with multidimensional omics analyses, are warranted to provide a more comprehensive understanding.

5. Conclusions

In conclusion, this research work demonstrated that PLAC1 knockdown effectively inhibited cell viability, migration, invasion and angiogenesis by suppressing the PI3K/Akt/FOXO3a axis in T24 and UMUC3 cells (Fig. 7).

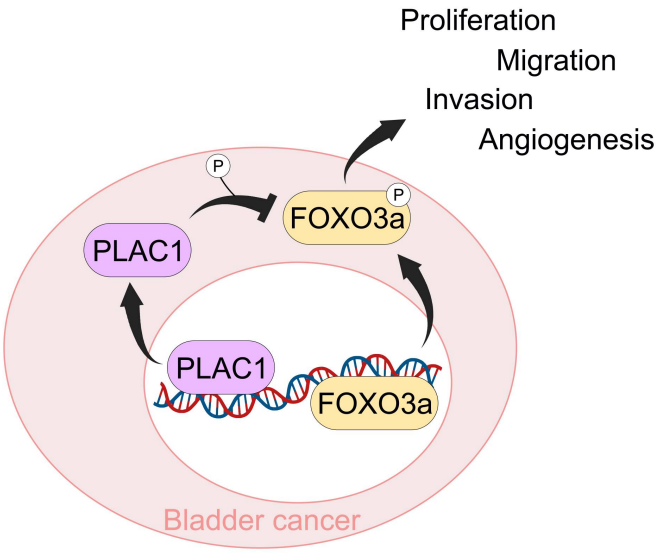

FIGURE 7. Mechanism model underlying PLAC1 depletion in blocking the viability, migration, invasion and angiogenesis of BCa cells by suppressing the PI3K/Akt/FOXO3a axis. PLAC1: Placenta-specific protein 1; FOXO3a: forkhead box O3a.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

TTJ—performed material preparation and the experiments. MJW and WFH—performed data collection and analysis. YJL—written the first draft of the manuscript. And all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. All authors contributed to the study conception and design.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of the Fourth Hospital of Hebei Medical University (Approval No. 2022KY420). Written informed consent was obtained from a legally authorized representative for anonymized patient information to be published in this article.

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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