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RESEARCH PAPER

Phosphorylation of small kinetochore-associated protein induced by GSK3β promotes cell migration and invasion in esophageal cancer

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ABSTRACT

Glycogen synthesis kinase-3β (GSK-3β) is a kinase shown to regulate esophageal cancer (EC) progression. However, the significance of GSK-3β in phosphorylation of small kinetochore-associated protein (SKAP) has not been fully characterized. GSK-3β/SKAP expression was analyzed in EC tissues by RT-qPCR. The association between GSK-3β expression and the overall survival was analyzed using the Kaplan–Meier method. Transwell and wound healing assays were performed to assess the effects of GSK-3β/SKAP knockdown on EC cell migration and invasion. By *in vitro* kinase assay, the SKAP T294 site was identified as a phosphorylated target of GSK-3β. Moreover, we established two cell lines expressing either T294D (phosphor-mimic) or T294A (phosphor-deficiency) SKAP to analyze the effect of SKAP phosphorylation on EC cell invasion, migration, and epithelial–mesenchymal transition (EMT) process. GSK-3β was overexpressed and positively correlated with SKAP levels in EC tissues. Increased GSK-3β expression was associated with EC poor prognosis. Both of GSK-3β knockdown and silencing SKAP decreased EC cell migration and invasion. GSK-3β phosphorylated SKAP protein at Thr294 site. Additionally, a T294D mutant SKAP enhanced cell migration, invasion, and EMT process. Conversely, a T294A mutant SKAP inhibited EC cell malignancy. Meanwhile, cell invasion and migration abilities were inhibited after silencing GSK-3β in EC109-WT, EC109-T294A and EC109-T294D cells. Phosphorylation of SKAP induced by GSK-3β promoted EC cell migration and invasion.

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Introduction

Esophageal cancer (EC) is the sixth leading cause of death, with 544,076 cases of new deaths in 2020 [1]. In addition, EC ranks seventh for incidence, with estimated 604,100 new cases, representing 3.1% of all cancer cases in 2020 [1]. It has been known that EC tumor onset is associated with smoking [2], excessive alcohol consumption [3], and mutations of enzymes in oxidative metabolism of ethanol [4]. Surgery is the preferred treatment for the early stage of EC, and surgery combined with adjuvant chemo/radiotherapy is the main treatment for middle and advanced stage of EC patients [5]. Although multi-disciplinary treatment of EC has become the mainstream clinical practice, the 5-year survival rate is still less than 10% [6]. Particularly for EC patients with recurrent and metastasis, their treatment needs are not met, and targeted therapies are urgently needed.

Glycogen synthesis kinase-3β (GSK-3β) is a serine/threonine protein kinase that plays an important role in gene expression and cellular processes [7]. It contains a binding domain that recognizes the substrate and a kinase domain in which Y216 phosphorylation enhances GSK-3β activity [8]. It has been revealed that GSK-3β is involved in some physiological processes, including regulation of cell metabolism, proliferation, migration, and differentiation [9,10]. The protein kinase GSK-3β has recently emerged as a crucial player in the phosphorylation of many substrates thereby exerting essential regulator function in many signaling pathways [7]. Intriguingly, the inhibition of GSK-3β induces a significant therapeutic effect on several cancer types, including

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breast cancer, lung cancer, and colorectal cancer [11–13]. Recently, several studies have reported that the overexpression of GSK-3β is a common prognostic biomarker in human EC tissues and mainly correlated with its kinase activity [14,15]. However, the relevance of GSK-3β to migration and invasion in EC remains poorly understood.

In recent years, small kinetochore associated protein (SKAP, also known as KNSTRN) levels were shown to increase in patients with cancers, e.g. bladder cancer and cutaneous squamous cell carcinoma [16–18]. Previous studies have suggested that SKAP promoted microtubule dynamics and participated in directional cell migration via interacting with the signaling scaffold protein IQGAP1 [19]. These findings indicated that SKAP probably represented as an oncogene in tumorigenesis. Our previous study on HeLa cell mitosis revealed that GSK-3β affects the binding to microtubules depolymerase Kif2b by increasing phosphorylation of Ser232, Ser237, and Thr294 (T294) in SKAP, which ultimately inhibits Kif2b activity. In addition, our preliminary results indicated that SKAP directly interacted with the tubulin CLASP1 [20]. Manning *et al.* have previously demonstrated that the CLASP1–Astrin–Kif2b complex served as a molecular switch and played a regulatory role in spindle assembly checkpoint signaling, motional microtubule stabilization and error correction during the early to metaphase mitosis [21]. However, the role of SKAP T294 phosphorylation on EC tumor progression remains unknown. Based on the previous findings, we hypothesized that phosphorylation of T294 on SKAP by GSK-3β could regulate the invasive and migratory abilities of EC cells.

Materials and methods

Patients and human tissue samples

A total of 36 EC patients from The First Affiliated Hospital of Zhengzhou University were enrolled in this research from 02/2015 until 09/2017. This research was approved by the Ethic Committee of The First Affiliated Hospital of Zhengzhou University. All patients provided their written informed consent. Matched normal and EC tumor tissues were selected during esophagectomy surgery. Overall survival (OS) was defined as the time from diagnosis to death or to the last follow-up date. The Kaplan–Meier method [22] and log-rank test were utilized to assess the OS over 48 months.

Cell culture, treatment, and transfection

The human EC cell line (EC109) was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). EC109 cells were maintained in RPMI 1640 (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and incubated in a 5% carbon dioxide atmosphere at 37°C. For cell treatment, MG132 (Sigma, St. Louis, MO, USA) was used at 20 μM for 4 h to synchronize the cells in metaphase.

To downregulate GSK-3β expression level in EC109 cells, siRNAs targeting GSK-3β (si-GSK-3β-1: 5'-GUAAUCCACCUCUGGCUACTT-3', si-GSK-3β-2: 5'-GGUAUAUCAAGCCAAACUUTT-3') were designed and synthesized by Invitrogen (Carlsbad, CA, USA). Si-NC (5'-UUCUCCGAACGUGUCACGU-3') was used as negative control and non-transfected cells were regarded as control. Cells were transfected with siRNA using Lipofectamine® 2000 (Invitrogen). The shSKAP-expressing lentivirus system was used to knockdown SKAP expression in EC109 cells. The RNA sequence used for sh-SKAP was 5'-AGGCTACAAACCACTGAGTAA-3' (Invitrogen).

Plasmids

The full-length SKAP mRNA was amplified as previously described [20]. Flag-tagged SKAP full-length and deletion truncations were cloned into pFLAG-CMV-2 (Clontech, Mountain View, CA, USA). Bacterial expression constructs of SKAP were cloned into pGEX-5X-3 (GE Healthcare, Buckinghamshire, UK). Transfection plasmid containing the Flag-tagged wild-type (WT) SKAP with shRNA resistance was modified by site-directed mutation kit (Vazyme Biotech Co., Ltd., Piscataway, NJ, USA) according to the manufacturer's instructions to create the SKAP mutants T294A and T294D. These plasmids,

namely Flag-SKAP-WT, Flag-SKAP-T294A, and Flag-SKAP-T294D. All plasmids used were verified by sequencing (Invitrogen). To establish stable clones of SKAP mutant EC109 cell lines, the Flag-SKAP-WT plasmid was replaced with Flag-T294A or Flag-T294D. The wild-type or mutant-type plasmids were transfected into EC109 cells, and the stable cell clones were selected using 3 ng/mL puromycin. After that, the endogenous SKAP in the three cell lines was knocked down by infection of shRNA-expressing lentivirus.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted employing Eastep® Super Total RNA Extraction Kit (Promega, Madison, WI, USA). Reverse transcription was carried out utilizing the Reverse Transcription System (Promega). qPCR was performed with Eastep® qPCR Master Mix (Promega) on an ABI7500 system (Applied Biosystems, Carlsbad, CA, USA) and analyzed as normalized fold expression using the $2^{-\Delta\Delta Ct}$ method. The gene for GAPDH was used as a reference gene.

Transwell invasion assay

The 8 μM pore insert in the 24-well transwell system was coated with Matrigel (Corning Inc., Coring, NY, USA). A total of 5×10^4 cells in 0.8 mL FBS-free RPMI 1640 medium was added into the upper chamber. The bottom chamber was filled with 0.2 mL RPMI 1640 medium containing 20% FBS. After 48 h, the cells attached to the lower side of the insert were fixed with 4% paraformaldehyde and stained with Giemsa solutions. Five random fields were observed and counted for each sample.

Wound healing assay

The EC cells were plated into a 6-well plate (1×10^5 cells/well) and maintained in a 5% carbon dioxide atmosphere at 37°C for 24 h. The scrape line was generated using a sterile pipette tip, followed by washing with phosphate buffer solution (PBS, Solarbio, Beijing, China). Cells were subjected to incubation at 37°C. Healing was observed at 24 h, and a representative field for each sample was photographed.

In vitro kinase assay

The glutathione-S-transferase-(GST)-tagged SKAP sequence was subcloned into pGEX-6P-1 and expressed in *Escherichia coli*, as described in a previous report [20]. SKAP protein (4 μg) was incubated with 0.1 μg recombinant GSK3β kinase (ab60863, Abcam, Cambridge, MA, USA) on ice for 10 min. Next, the reaction was incubated with 30 μL 1× kinase buffer containing 0.5 μCi [γ-32P] ATP and 0.1 mM ATP at 37°C. 30 min later, the mixture was subjected to western blot analysis for detection of p-SKAP (phospho Thr294), SKAP, and GSK-3β expression level.

Flow cytometry analysis

The Cycle TEST PLUS DNA Reagent Kit (BD Biosciences, San Jose, CA, USA) based on propidium iodide staining of DNA content was utilized to analyze the number of cells in each phase of the cell cycle (G0/G1, S, and G2/M). After 48 h incubation with the analyzed compounds, the indicated cell lines were then analyzed using a BD FACSLyric™ flow cytometry (BD Biosciences).

Protein isolation and western blot

Cell lysate preparation was performed using RIPA Lysis and Extraction Buffer (Thermo Fisher, Carlsbad, CA, USA), and the supernatant was further analyzed with SDS-PAGE. Briefly, 30 μg protein was separated with 12% SDS-PAGE gel on a double plate vertical electrophoresis apparatus (Liuyi biology, Beijing, China). Next, the protein was transblotted onto a PVDF membrane using a wetting transfer method. The membrane was probed with mouse anti-SKAP antibody (1:500, sc-514112, Santa Cruz, Dallas, TX, USA), anti-p-SKAP (phospho Thr294) antibody (Abiocenter, Beijing, China), mouse anti-GSK-3β antibody (1:1000, ab93926, Abcam), mouse anti-E-cadherin antibody (1:500, ab76055, Abcam), mouse anti-N-cadherin antibody (1:1000, ab280375, Abcam), rabbit anti-β-catenin antibody (1:1000, ab223075,

Abcam), rabbit anti-CDK4 antibody (1:1000, ab108357, Abcam), rabbit anti-CDK6 antibody (1:2000, ab124821, Abcam), rabbit anti-Cyclin D1 antibody (1:1000, ab134175, Abcam), and rabbit anti-GAPDH antibody (1:2500, ab9485, Abcam) at 4°C overnight. After washing with the TBST buffer, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5000, ab6721, Abcam) or goat anti-mouse IgG (1:5000, ab6728, Abcam) for 1.5 h at room temperature. Finally, antibody-bound proteins were observed using chemiluminescence reagents (Thermo Fisher) in a Tanon System (Shanghai, China).

Statistical analysis

The experimental results were analyzed using GraphPad software (San Diego, CA, USA) and expressed as mean or mean ± standard deviation (SD). Student's *t*-test with two-tail was utilized for group comparison. $p < 0.05$ was established as statistical significance.

Results

GSK-3β expression was necessary for EC cell migration and invasion

In all, 36 EC patients who underwent tumor excision were included in the study. All the included EC tissues were diagnosed pathologically as esophageal squamous cell carcinoma (ESCC). Firstly, RT-qPCR analysis illustrated that GSK-3β mRNA levels were higher in EC tumor tissues than non-tumor tissues ($p < 0.001$, Figure 1(a)). The mean value of the 36 EC patients' GSK-3ß expression was used as the cutoff value (2.395), 36 EC patients were divided into low GSK-3β expression ($n = 19$) and high GSK-3β expression ($n = 17$) groups. As shown in Table 1, the baseline characteristics of patients with high or low GSK-3β expression were comparable, except for tumor size, TNM stage, and lymph metastasis. The high GSK-3β level was significantly associated with larger tumor size ($p = 0.009$) and advanced TNM stage ($p = 0.008$). Patients with high GSK-3β-expressing tumors underwent lymph node metastasis more than low GSK-3β-expressing patients ($p = 0.029$). Interestingly, the mRNA levels of GSK-3β were highly predictive of poor EC overall survival, as demonstrated by the Kaplan–Meier analysis (log-rank $p = 0.021$, Figure 1(b)). In EC109 cells, silencing GSK-3β was achieved using siRNA technology ($p < 0.001$, Figure 1(c)). Interestingly, silencing GSK-3β significantly inhibited the ability of EC109 cells to invade through the Transwell membrane ($p < 0.001$, Figure 1(d)). Moreover, we also confirmed the negative role of GSK-3β in cell migration using the wound healing assay. GSK-3β-silenced EC109 cells were found to migrate less to the scratched zone than si-NC-transfected cells ($p < 0.001$, Figure 1(e)). We further explored whether downregulation of GSK-3β could influence the expression of cell cycle-related proteins including CDK4, CDK6, and Cyclin D1. The western blot assay proved silencing GSK-3β decreased expression of cell cycle-related proteins ($p < 0.001$, Figure 1(f)). However, most mammalian cells could undergo a doubling within 24 h that contributes to scratch closure. Therefore, to further address the inhibition effect of GSK-3β silencing on EC109 cells scratch closure, cells were treated with MG132 to arrest cell cycle in metaphase. The wound healing assay indicated that MG132 suppressed cell migration ($p < 0.001$) and further silencing GSK-3β promoted the ability of MG132 on migration suppression ($p < 0.001$. Figure 1(g)). These results indicated that GSK-3β might play a key role in the migration, invasion, and cell cycle in EC cells.

Silencing SKAP inhibited EC cell migration and invasion

In order to assess the role of SKAP in EC, SKAP mRNA expression was validated in EC tissues utilizing qPCR. As shown in Figure 2(a), SKAP mRNA expression was significantly higher in EC tissues compared with that in non-tumor samples ($p < 0.001$). We also found that the induction of GSK-3β was highly correlated with SKAP expression in EC tissues (Spearman's $r = 0.7579$, $p < 0.001$, Figure 2(b)). Moreover, we silenced GSK-3β in EC109 cells and observed that silencing GSK-3β resulted in a significant reduction of SKAP phosphorylation level as expected ($p < 0.001$, Figure 2(c)). To elucidate whether SKAP affects the phenotype of EC cells, we inhibited SKAP mRNA expression level in EC109 cells by RNA interference ($p < 0.001$, Figure 2(d)). The transwell

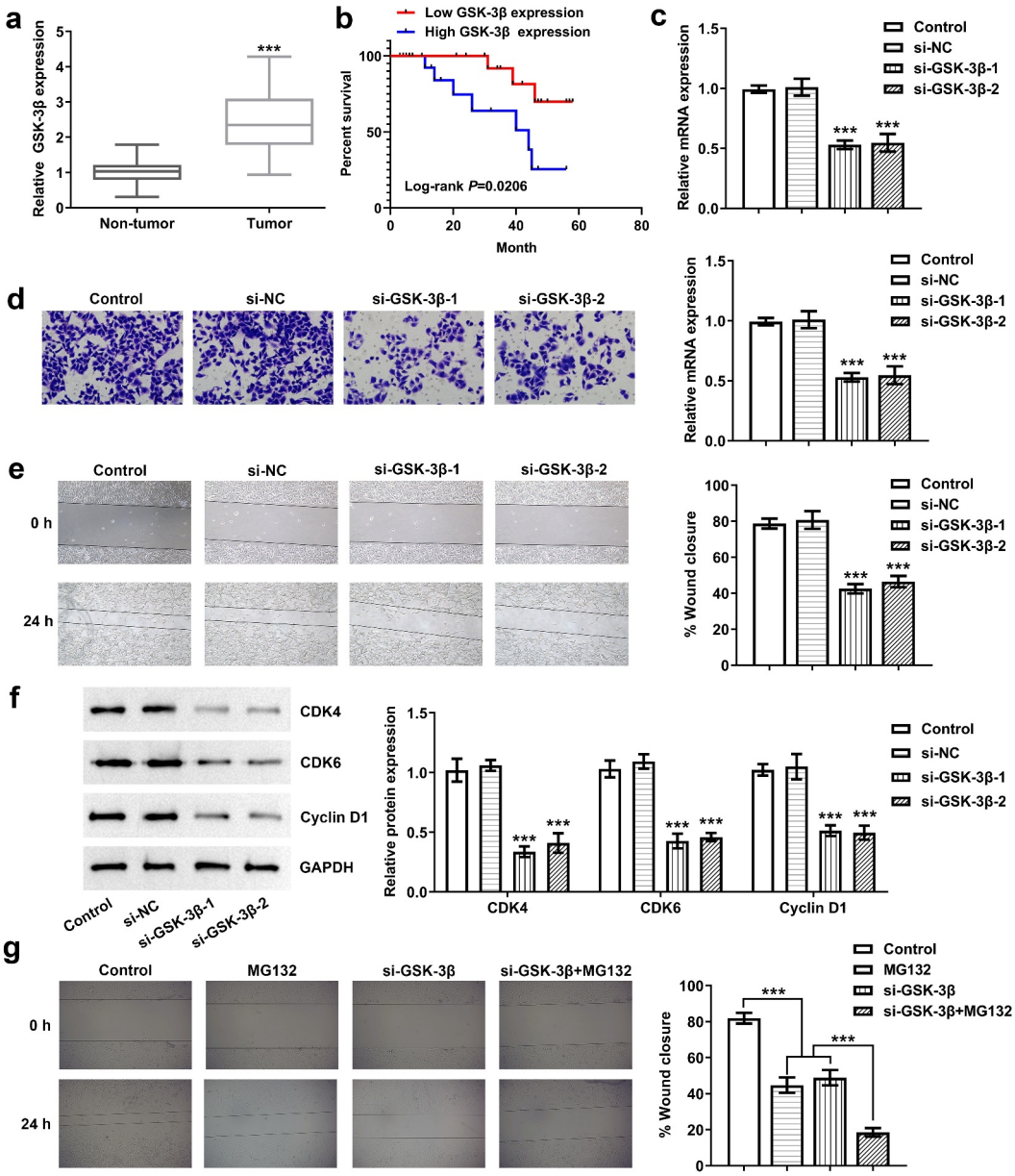

Figure 1. GSK-3β expression was necessary for EC cell migration and invasion. (a) GSK-3β mRNA expression levels in EC tumor ($n = 36$) and non-tumor tissues. (b) Kaplan–Meier analysis of overall survival in EC patients. (c) GSK-3β expression was knocked down in EC109 cells by transfection of si-GSK-3β. (d) Transwell cell invasion assay of GSK-3β-silenced EC109 cells. (e) Wound healing cell migration assay of GSK-3β-silenced EC109 cells. (f) The western blot analysis of CDK4, CDK6, and Cyclin D1 expression level in GSK-3β-silenced EC109 cells. (g) The wound healing analysis of cell migration in MG132-treated or/and GSK-3β-silenced EC109 cells. ***$p < 0.001$.

assay confirmed that SKAP knockdown significantly suppressed the invasion ratio in EC109 cells ($p < 0.001$, Figure 2(e)). In addition, the knockdown of SKAP resulted in a significant inhibition of wound closure rate compared with the sh-NC group, indicating a EC109 cell migration reduction ($p < 0.001$, Figure 2(f)). Also, MG132 treatment suppressed EC109 cell migration as well ($p < 0.001$); moreover, sh-SKAP transfection further enhanced the inhibitory effect of MG132 on cell migration ($p < 0.01$, Figure 2(g)).

GSK-3β modulated SKAP phosphorylation at Thr294 site

Our previous study has reported that GSK-3β phosphorylated SKAP at Ser232, Ser237, and Thr294 [20]. Figure 3(a) showed a schematic

Table 1. Correlation between GSK-3β expression and clinic pathologic characteristics.

Characteristic		Cases (n = 36)	GSK-3β		Chi-square	p Value
			Low (n = 19)	High (n = 17)		
Age	<60	8	3	5	0.963	0.326
	≥60	28	16	12		
Gender	Male	26	12	14	1.648	0.199
	Female	10	7	3		
Tumor size	<3	12	10	2	6.743	0.009
	≥3	24	9	15		
TNM stage	I–II	19	14	5	7.056	0.008
	III–IV	17	5	12		
Lymph node metastasis	Yes	23	9	14	4.760	0.029
	No	13	10	3		

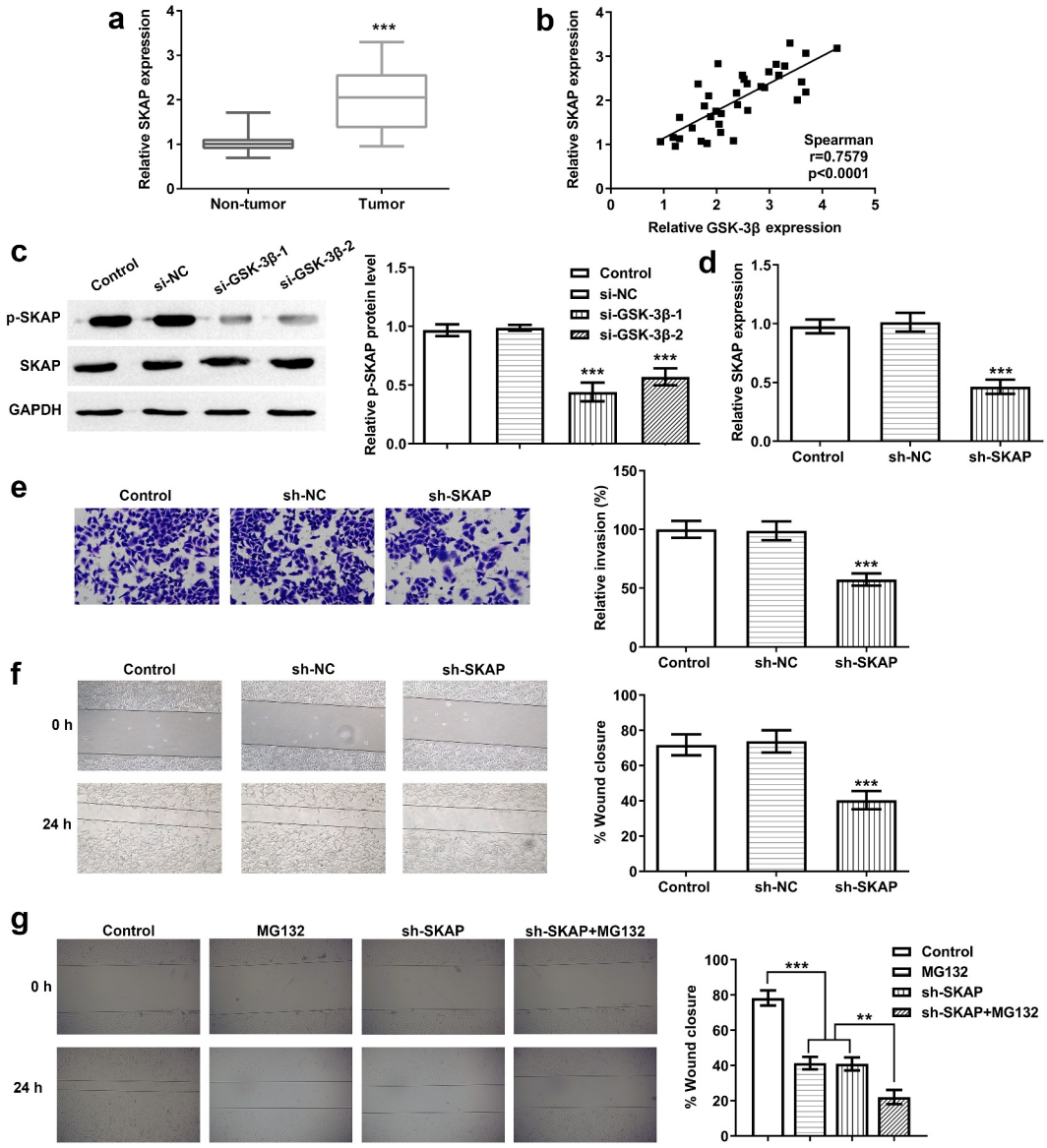

Figure 2. Silencing SKAP inhibited EC cell migration and invasion. (a) SKAP mRNA expression levels in EC tumor (n = 36) and non-tumor tissues. (b) Correlation analysis of GSK-3β expression and SKAP expression. GSK-3β expression data plotted on x-axis and SKAP expression data on y-axis for EC tissues. The p-value was derived from Spearman's correlation testing (Spearman's r = 0.7579). (c) EC109 cells were transiently transfected with si-GSK-3β. Total protein was extracted and the expression levels of phosphorylation (p)-SKAP and SKAP were measured by western blot. (d) SKAP expression was knocked down in EC109 cells by transfection of sh-SKAP. (e) Transwell cell invasion assay of SKAP silencing EC109 cells. (f) Wound healing cell migration assay of GSK-3β silencing EC109 cells. (g) The wound healing analysis of cell migration in MG132-treated or/and SKAP-silenced EC109 cells. $^{**}p < 0.01$, $^{***}p < 0.001$.

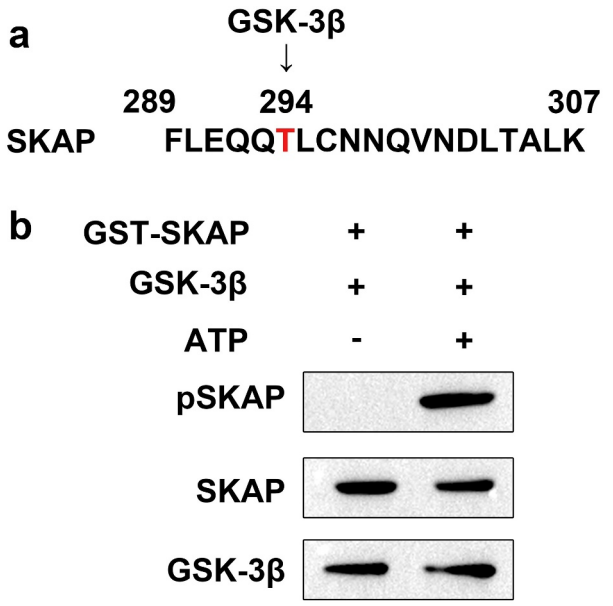

Figure 3. GSK-3β modulated SKAP phosphorylation at Thr294 site. (a) Potential phosphorylated Thr294 site of SKAP and GSK-3β was predicted using Mass Spectrometry. (b) *In vitro* kinase assay was utilized to the SKAP phosphorylation induced by GSK-3β.

diagram of SKAP phosphorylation site (Thr294) being targeted by GSK-3β. To investigate whether GSK-3β protein regulated the phosphorylation of SKAP, we generated a GST-SKAP plasmid and constructed a GSK-3β overexpressing vector. The *in vitro* kinase assay showed that GSK-3β, based on the kinase level, directly phosphorylates SKAP in the presence of ATP (Figure 3(b)). The result indicated that GSK-3β was required for SKAP phosphorylation at the Thr294 site.

Phosphorylation of SKAP was involved in EC cell invasion, migration, and EMT

We next investigated the role of GSK-3β-mediated SKAP T294 phosphorylation in EC cells. Firstly, we established two stable clones of the EC109 cell line expressing shRNA-resistant and T294 mutant SKAP plasmids. Briefly, EC109 cells were firstly infected by a lentivirus that carried both shRNA-resistant and T294-mutated SKAP sequences, including the Flag-SKAP-T294A or the Flag-SKAP-T294D. The western blot assay confirmed the exogenous expression of SKAP in EC109 cells (Figure 4(a), left panel). In addition, in EC109 cells transfected with Flag-WT, Flag-T294A SKAP, or Flag-T294D SKAP, lentivirus-based sh-SKAP significantly knocked down endogenous SKAP expression, but expressed exogenous shRNA-resistant SKAP wild-type (Flag-SKAP-WT) or T294-mutated (T294A or T294D) (Figure 4(a), right panel). The resulting cell lines were named as EC109-WT, EC109-T294A, and EC109-T294D cells, respectively. The transwell assay demonstrated that EC109-T294D cells had the highest invasive ability among the three cell lines ($p < 0.001$, Figure 4(b)). Remarkably, the EC109-T294A cells exhibited the lowest invasion ratio, implying that SKAP T294 phosphorylation might promote EC cell invasion ($p < 0.05$, Figure 4(b)). The wound healing scratch assay was also performed to examine whether phosphorylation of SKAP could enhance EC109 cell migration. As shown in Figures 4(c), 24 h after the scratch, EC109-T294D cells migrated into and largely covered the original wound area ($p < 0.01$), whereas those EC109-T294A cells failed to cover a substantial portion of the wound ($p < 0.01$). To address whether phosphorylation of SKAP affects the metastasis-related EMT process in EC cells, we detected the mRNA and protein levels of EMT-related markers. As shown in Figure 4(d,e), compared with EC109-WT cells, both mRNA and protein expression of E-cadherin and β-catenin were significantly upregulated in EC109-T294A cells, while phosphor-deficient SKAP inhibited N-cadherin expression ($p < 0.05$, $p < 0.01$, $p < 0.001$). In addition, phosphor-mimic (T294D) inhibited the E-cadherin and β-catenin expression and enhanced the N-cadherin level in EC cells ($p < 0.05$, $p < 0.01$, $p < 0.001$). These data demonstrated that phosphorylation of SKAP is coupled not only with EC cell invasion and migration but also with the EMT process.

Phosphorylation of SKAP was involved in cell cycle of EC cells

Furthermore, to investigate the effect of SKAP phosphorylation on the cell cycle, flow cytometry analysis was conducted and the results revealed that SKAP T294 phosphorylation reduced the percentage of cells in the G2/M phase of the cell cycle and increased the percentage of cells in the G0/G1 phase ($p < 0.001$, Figure 5(a)). Additionally, CDK4,

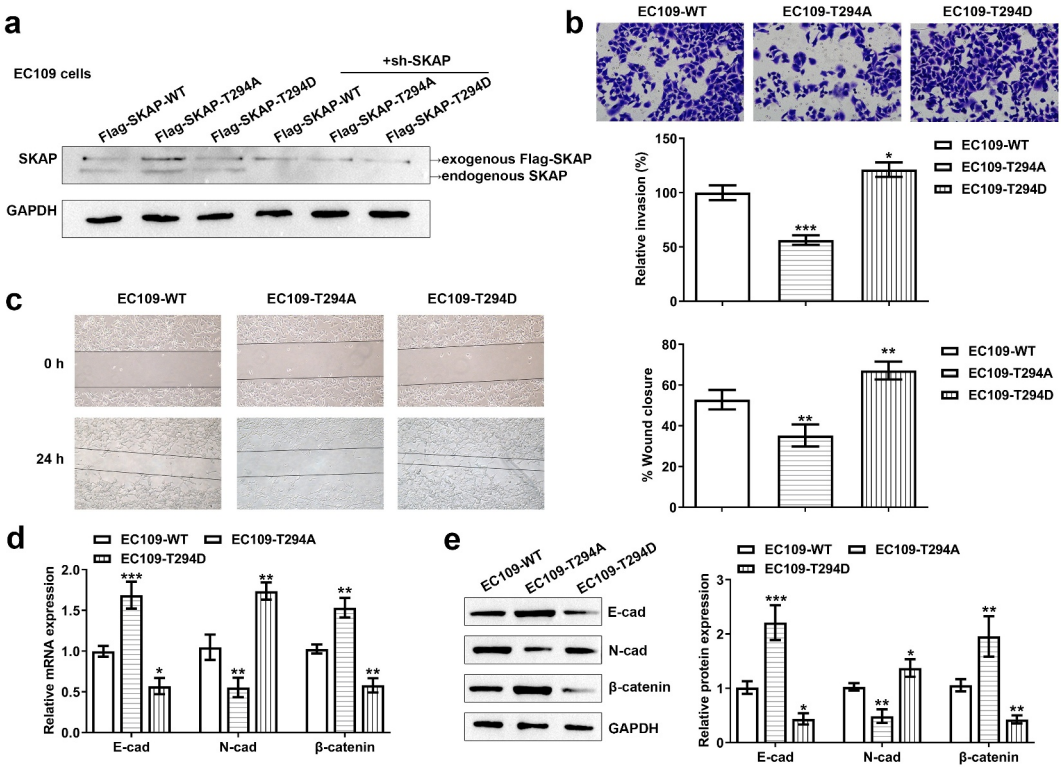

Figure 4. Phosphorylation of SKAP was involved in EC cell invasion, migration and EMT process. (a) Establishment of EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cell lines. EC109 cells were firstly infected with lentivirus which carried both shRNA-resistant and T294-mutated SKAP sequence (Flag-SKAP-WT or Flag-SKAP-T294A or Flag-SKAP-T294D), respectively. Next, knockdown of endogenous SKAP was performed using shRNA. Western blot was performed to detect the expression levels of exogenous and endogenous SKAP. (b) Transwell cell invasion assay of EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cells. (c) Wound healing cell migration assay of EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cells. (d) RT-qPCR and (e) western blot analysis for E-cad, N-cad, and β-catenin expression levels in EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CDK6, and Cyclin D1 protein expression were decreased in EC109-T294D cells ($p < 0.05$ or $p < 0.001$), while increased in EC109-T294A cells ($p < 0.05$, $p < 0.01$ or $p < 0.001$, Figure 5(b)). Moreover, MG132 treatment suppressed cell migration in EC109-WT, EC109-T294A and EC109-T294D cells ($p < 0.001$, Figure 5(c)). These results suggested that T294 phosphorylation-mimic SKAP (T294D) promoted EC109 cell cycle and migration, while T294 phosphorylation-defective SKAP (T294A) conversely inhibited EC cell motility.

GSK-3β regulated EC cell invasion and migration through SKAP phosphorylation

To address GSK-3β-mediated SKAP phosphorylation acted to regulate EC cell invasion and migration. EC109-WT, EC109-T294A, and EC109-T294D cells were transfected with si-GSK-3β; transwell and wound healing assays showed that cell invasion and migration abilities were inhibited after silencing GSK-3β in both EC109-WT and EC109-T294A cells ($p < 0.01$ or $p < 0.001$, Figure 6(a,b)). It is noted here that the depletion of GSK-3β suppressed EC109-T294D cell invasion ($p < 0.001$, Figure 6(a)) and migration ($p < 0.001$, Figure 6(b)) as well, suggesting that GSK-3β may mediate phosphorylation of other substrates to regulate migration and invasion; therefore, further experiments are needed to verify in the future. Meanwhile, in comparison with GSK-3β-silenced EC109-WT cells, GSK-3β-silenced EC109-T294A cells show a decreased invasion ($p < 0.01$, Figure 6(a)) and migration ($p < 0.05$, Figure 6(b)) abilities, while EC109-T294D cell invasion ($p < 0.05$, Figure 6(a)) and migration ($p < 0.05$, Figure 6(b)) was promoted. Therefore, we preliminary verified that GSK3β-mediated phosphorylation of SKAP promoted EC cell invasion and migration.

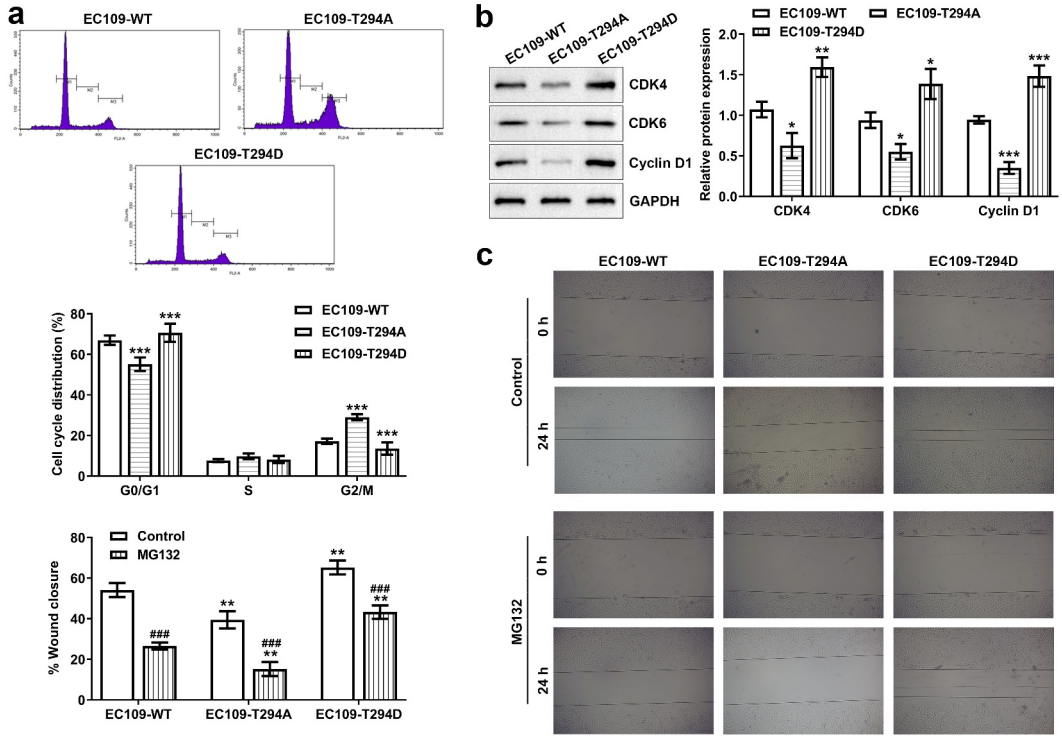

Figure 5. Phosphorylation of SKAP was involved in cell cycle of EC cells. (a) EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cells were stained with propidium iodide and analyzed by flow cytometry. (b) CDK4, CDK6, and Cyclin D1 expression were detected by western blot in EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cells. (c) The wound healing analysis of cell migration in MG132-treated EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cells. EC109-WT *vs* EC109-T294A or EC109-T294D, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Control *vs* MG132, ###$p < 0.001$.

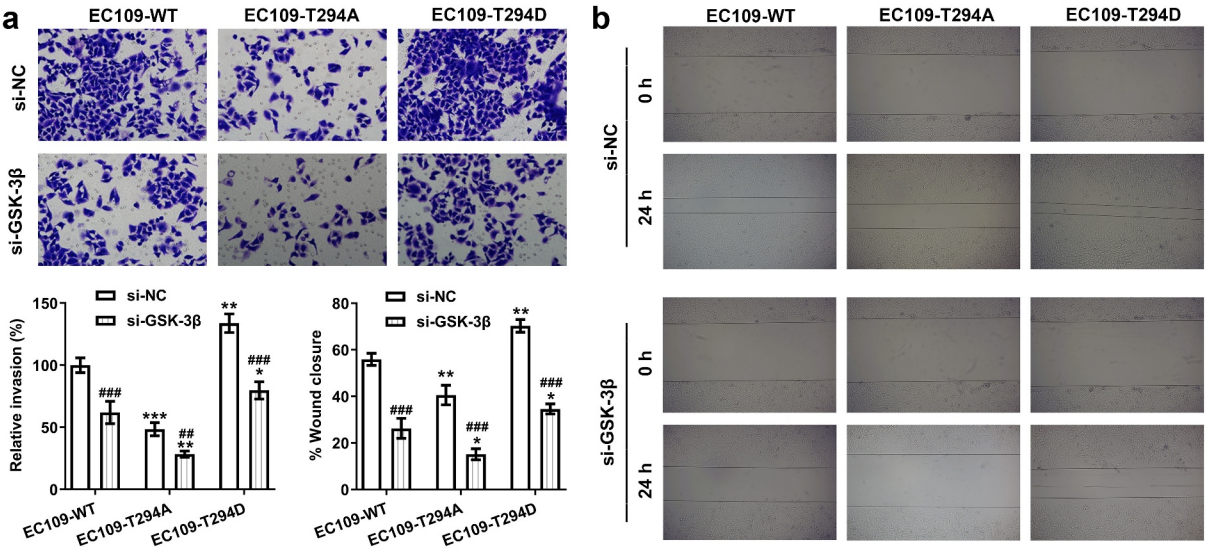

Figure 6. GSK-3β-mediated SKAP phosphorylation regulated EC cell invasion and migration. In si-GSK-3β-transfected EC109-SKAP-WT, EC109-SKAP-T294A, and EC109-SKAP-T294D cells, (a) transwell and (b) wound healing assays were conducted to assess cell invasion and migration abilities. EC109-WT *vs* EC109-T294A or EC109-T294D, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; si-NC *vs* si-GSK-3β, ##$p < 0.01$, ###$p < 0.001$.

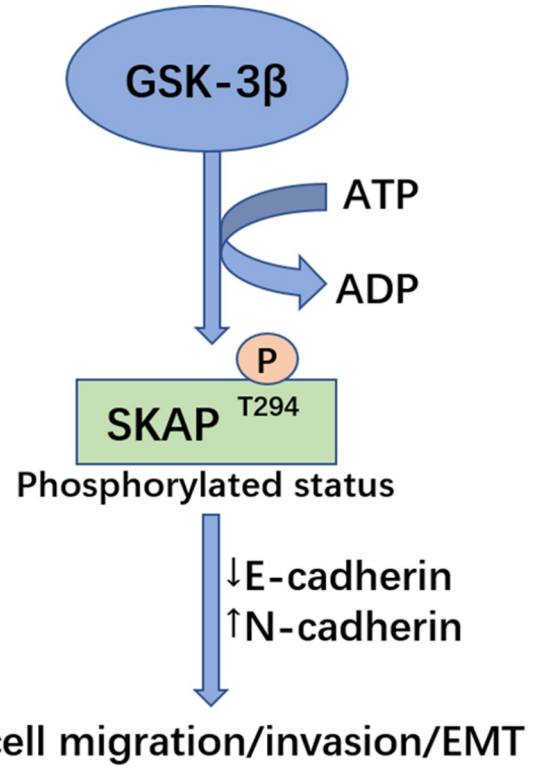

Figure 7. A model of GSK3β-SKAP axis in maintaining EC cellular processes.

Discussion

It was previously reported that GSK-3β is a critical regulator of cancer cell proliferation and differentiation abnormality [23]. However, the underlying molecular mechanisms of how GSK-3β governs the metastasis of EC cells are poorly understood. Herein, we identified the threonine-specific kinase GSK-3β as an upstream regulator of SKAP function and modulated the ability of SKAP to confer migrated and invasive properties *in vitro*.

GSK-3β has been previously implicated in the regulation of tumor progression with different functions. On the one hand, GSK-3β is regarded as a tumor suppressor gene. For example, GSK-3β promotes the degradation of proto-oncogenes, including c-Jun, β-catenin, cyclin D1, and c-Myc [24,25]. However, a growing number of studies have shown that GSK-3β had a strong oncogenic effect, exhibiting gene mutation, abnormal protein expression, and altered localization in tumors. As far as molecular mechanism, GSK-3β promotes the binding between NF-κB p65/p50 and promoter region of the downstream target gene, enhancing tumor cell survival and promoting ESCC progression [14]. A recent study has indicated that GSK-3β was highly expressed in ESCC tissues and cells, inhibition of GSK-3β attenuated tumor cell survival and proliferation and induced apoptosis in ESCC cells [26]. Which was consistent with our resultsthat GSK-3β expression was significantly enhanced in ESCC specimens and high expression of GSK-3β was associated withpoor prognosis of ESCC patients. Bolidong *et al.* [26] also demonstrated that inhibition of GSK-3β decreased the expression of cyclin D1 and CDK4, which was consistent with our results, suggesting GSK-3β mediated cell cycle regulation. Furthermore, our results also proved that silencing GSK-3β inhibited EC cell migration and invasion and GSK-3β-mediated regulation of cell cycle progression was responsible for EC109 cell migration.

SKAP is believed to be responsible for spindle localization and chromosome segregation [27]. Importantly, SKAP was proved to directly bind to motor protein CENP-E and regulate its binding to microtubules [16]. In addition, SKAP links the outer kinetochore KNL1-MIS12-NDC80 complex via directly bounding to the kinetochore protein MIS13 to orchestrate accurate interaction between kinetochore and dynamic spindle microtubules [28]. More importantly, cell migration was orchestrated by dynamic interaction of microtubules with the plasma membrane cortex, which suggested that SKAP may play a role in tumor cell migration. Cao *et al.* [19] found silencing SKAP inhibited cell migration in breast cancer MDA-MB -231 cells. In the mechanism, the N-terminal of SKAP binds to EB1, and its C terminus binds to IQGAP1 in order to orchestrate directional cell migration, via coupling dynamic microtubule plus-ends, to the cell cortex. Likewise, our results also show that SKAP inhibition decreased cell invasion and migration abilities. Besides, our data clearly provided evidence that the SKAP expression was up-regulated and positively correlated with GSK-3β expression in EC tissues.

An increasing number of findings suggests that the dynamic localization of SKAP in the HeLa cell mitosis may be regulated by the protein kinase Aurora B [29,30]. Interestingly, GSK-3β as a serine/threonine protein kinase could serve as a key mitotic kinase for regulating metaphase spindle dynamics [31]. Indeed, GSK3-β can

phosphorylate Astrin, a spindle- and kinetochore-associated protein required for proper chromosome alignment in metaphase, moreover, Astrin can form a complex with SKAP [32]. Therefore, GSK-3β may also phosphorylate SKAP to regulate its cellular function. Our previous study has reported that GSK-3β phosphorylated SKAP at Ser232, Ser237, and Thr294 [20], to investigate the function of GSK-3β-modulated SKAP Thr294 phosphorylation on EC cell progress, in the present study, we established two EC109 cell lines in which the endogenous SKAP was replaced with either T294D or T294A SKAP, respectively. Notably, we found that a phosphorylation-mimic T294D mutant of SKAP promoted cell invasion and migration, conversely, a phosphorylation-defective T294A mutant suppressed invasion and migration. Additionally, the depletion of GSK-3β in EC109-T294A cells further suppressed cell invasion and migration. However, GSK-3β silencing decreased the migration and invasion of EC109-T294D cells as well, suggesting that in addition to SKAP, GSK-3β could regulate cell migration and invasion by phosphorylating other substrates. Furthermore, in comparison with GSK-3β-silenced EC109-WT cells, GSK-3β-silenced EC109-T294A cells showed further decreased invasion and migration abilities while EC109-T294D cell invasion and migration were promoted. Therefore, we demonstrated GSK-3β-mediated SKAP Thr294 phosphorylation promoted EC cell migration and invasion.

In summary, in this research, GSK-3β was proved to be a critical positive regulator of SKAP phosphorylation, which contributed to the migration and invasion of EC cells (Figure 7). Inhibition of SKAP suppressed the abilities of EC metastasis. Importantly, we generated SKAP-mutant EC cell lines (EC109-T294A and EC109-T294D) to confirm the effects of SKAP phosphorylation on EC cell migration and invasion. Our results provided novel insight into the option of potential target (GSK-3β and SKAP) therapy for EC.

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This research was approved by the Ethic Committee of The First Affiliated Hospital of Zhengzhou University.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable requests.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Informed consent

All patients provided their written informed consent.

References

[1] Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–249.
[2] Short MW, Burgers KG, Fry VT. Esophageal cancer. Am Fam Physician. 2017;95(1):22–28.
[3] Abnet CC, Arnold M, Wei WQ. Epidemiology of esophageal squamous cell carcinoma. Gastroenterology. 2018;154(2):360–373.
[4] Lewis SJ, Smith GD. Alcohol, ALDH2, and esophageal cancer: a meta-analysis which illustrates the potentials and limitations of a Mendelian randomization approach. Cancer Epidemiol Biomarkers Prev. 2005;14(8):1967–1971.
[5] Watanabe M, Otake R, Kozuki R, et al. Recent progress in multidisciplinary treatment for patients with esophageal cancer. Surg Today. 2020;50(1):12–20.
[6] Huang FL, Yu SJ. Esophageal cancer: risk factors, genetic association, and treatment. Asian J Surg. 2018;41(3):210–215.
[7] Lin J, Song T, Li C, et al. GSK-3β in DNA repair, apoptosis, and resistance of chemotherapy, radiotherapy of cancer. Biochim Biophys Acta Mol Cell Res. 2020;1867(5):118659.

[8] Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. Pharmacol Ther. 2015;148:114–131.
[9] Mancinelli R, Carpino G, Petrungaro S, et al. Multifaceted roles of GSK-3 in cancer and autophagy-related diseases. Oxid Med Cell Longev. 2017;2017:4629495.
[10] Jellusova J, Cato MH, Apgar JR, et al. Gsk3 is a metabolic checkpoint regulator in B cells. Nat Immunol. 2017;18(3):303–312.
[11] Domoto T, Uehara M, Bolidong D, et al. Glycogen synthase kinase 3beta in cancer biology and treatment. Cells. 2020;9(6):1388.
[12] Vijay GV, Zhao N, Den Hollander P, et al. GSK3beta regulates epithelial-mesenchymal transition and cancer stem cell properties in triple-negative breast cancer. BCR. 2019;21:37.
[13] Sahin I, Eturi A, De Souza A, et al. Glycogen synthase kinase-3 beta inhibitors as novel cancer treatments and modulators of antitumor immune responses. Cancer Biol Ther. 2019;20(8):1047–1056.
[14] Gao S, Li S, Duan X, et al. Inhibition of glycogen synthase kinase 3 beta (GSK3beta) suppresses the progression of esophageal squamous cell carcinoma by modifying STAT3 activity. Mol Carcinog. 2017;56 (10):2301–2316.
[15] He H, Ding F, Li Y, et al. Migfilin regulates esophageal cancer cell motility through promoting GSK-3β-mediated degradation of β-catenin. Mol Cancer Res. 2012;10(3):273–281.
[16] Huang Y, Wang W, Yao P, et al. CENP-E kinesin interacts with SKAP protein to orchestrate accurate chromosome segregation in mitosis. J Biol Chem. 2012;287(2):1500–1509.
[17] Xiong Y, Ju L, Yuan L, et al. KNSTRN promotes tumorigenesis and gemcitabine resistance by activating AKT in bladder cancer. Oncogene. 2021;40(9):1595–1608.
[18] Lee CS, Bhaduri A, Mah A, et al. Recurrent point mutations in the kinetochore gene KNSTRN in cutaneous squamous cell carcinoma. Nat Genet. 2014;46 (10):1060–1062.
[19] Cao D, Su Z, Wang W, et al. Signaling scaffold protein IQGAP1 interacts with microtubule plus-end tracking protein SKAP and links dynamic microtubule plus-end to steer cell migration. J Biol Chem. 2015;290 (39):23766–23780.
[20] Qin B, Cao D, Wu H, et al. Phosphorylation of SKAP by GSK3β ensures chromosome segregation by a temporal inhibition of Kif2b activity. Sci Rep. 2016;6(1):38791.
[21] Manning AL, Bakhoum SF, Maffini S, et al. CLASP1, astrin and Kif2b form a molecular switch that regulates kinetochore-microtubule dynamics to promote mitotic progression and fidelity. EMBO J. 2010;29 (20):3531–3543.
[22] Bland JM, Altman DG. Survival probabilities (the Kaplan-Meier method). BMJ. 1998;317(7172):1572.
[23] Ahmad F, Woodgett JR. Emerging roles of GSK-3α in pathophysiology: emphasis on cardio-metabolic disorders. Biochim Biophys Acta Mol Cell Res. 2020;1867:118616.
[24] de Groot RP, Auwerx J, Bourouis M, et al. Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the Drosophila kinase shaggy. Oncogene. 1993;8:841–847.
[25] Sears R, Nuckolls F, Haura E, et al. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev. 2000;14 (19):2501–2514.
[26] Bolidong D, Domoto T, Uehara M, et al. Potential therapeutic effect of targeting glycogen synthase kinase 3β in esophageal squamous cell carcinoma. Sci Rep. 2020;10(1):11807.
[27] Kern DM, Nicholls PK, Page DC, et al. A mitotic SKAP isoform regulates spindle positioning at astral microtubule plus ends. J Cell Biol. 2016;213(3):315–328.
[28] Wang X, Zhuang X, Cao D, et al. Mitotic regulator SKAP forms a link between kinetochore core complex KMN and dynamic spindle microtubules. J Biol Chem. 2012;287(47):39380–39390.
[29] Shrestha RL, Conti D, Tamura N, et al. Aurora-B kinase pathway controls the lateral to end-on conversion of kinetochore-microtubule attachments in human cells. Nat Commun. 2017;8(1):1–3.
[30] Draviam VM, Schmidt JC, Kiyomitsu T, et al. Aurora B kinase controls the targeting of the Astrin-SKAP complex to bioriented kinetochores. Nat Commun. 2010;191:269–280.
[31] Tighe A, Ray-Sinha A, Staples OD, et al. GSK-3 inhibitors induce chromosome instability. BMC Cell Biol. 2007;8(1):34.
[32] Xu W, Ge Y, Liu Z, et al. Glycogen synthase kinase 3β orchestrates microtubule remodeling in compensatory glomerular adaptation to podocyte depletion. J Biol Chem. 2015;290(3):1348–1363.