Cell Death & Differentiation

ARTICLE

PAK5 promotes the migration and invasion of cervical cancer cells by phosphorylating SATB1

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Abstract

p21-activated kinase 5 (PAK5) is involved in several oncogenic signaling pathways and its amplification or overexpression has been found in various types of cancer; however, the pathophysiologic role of PAK5 in cervical cancer (CC) remains elusive. This study aims to elucidate the effects of PAK5 on CC metastasis and its specific regulation mechanism. We performed western blotting and immunohistochemistry (IHC) analysis and found that the expression levels of PAK5 were significantly upregulated in CC cells and tissues. In addition, statistical analysis via IHC showed that increased PAK5 significantly correlated with CC progression. Mn^{2+}-Phos-tag SDS-PAGE, western blotting, immunofluorescence and dual luciferase reporter assays were utilized to determine the involvement of SATB1 in PAK5-mediated epithelial–mesenchymal transition (EMT). We found that PAK5-mediated special AT-rich binding protein-1 (SATB1) phosphorylation on Ser47 initiated EMT cascade and promoted migration and invasion of CC cells. Furthermore, overexpression of PAK5 induced lung metastasis of CC cells in xenograft modes. Taken together, we conclude that PAK5 is a novel prognostic indicator and plays an important role in the CC metastasis.

Introduction

Cervical cancer (CC) is one of the most common gynecology tumors and the second leading cause of cancer deaths among women in the world [1], with over 500,000 new diagnosed cases and 260,000 deaths per year [2]. Tumor invasion and distant metastasis are the main causes for treatment failure and death in cancer patients, particularly in CC [3, 4]. Previous studies suggested that epithelial–mesenchymal transition (EMT) stimulated migration and invasion of tumor cells exerts a powerful effect on the process of occurrence, development, invasion, metastasis, and drug resistance of CC [4, 5]. Pelvic lymph node metastasis (LNM) of CC is dismissed as the most significant factor among the numerous high risks leading to unfavorable prognosis for postoperative patients [6]. Five-year survival rate of CC patients is reduced by 20–30% in the presence of LNM [7]. Therefore, there is an unmet clinical need to identify reliable biomarkers and improve therapeutic strategies for CC patients.

p21-activated kinases (PAKs) are highly conserved serine/threonine kinases family and are divided into two subfamilies: group I (PAK1, 2, and 3) and group II (PAK4, 5, and 6) [8, 9]. Unlike other group II PAK, PAK5 contains an autoinhibitory domain that binds with Cdc42-GTP and regulates its activity [10]. PAK5 was initially discovered in neuronal tissue [11], with high expression in the cerebellum, cerebral cortex, and olfactory bulb [9]. Subsequently, it was also detected in the pancreas [11]. Signaling pathways of PAK5 control a variety of cellular processes

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including neuronal outgrowth, cytoskeleton organization and cell proliferation, migration and invasion [10]. Earlier functional studies of PAK5 mainly concentrated in nervous system and suggested that PAK5 facilitates filopodium formation, neurite development, and regeneration of axon in NIE-115 cells [11]. Recently, PAK5 was identified as an oncogene in multiple human malignancies [10], such as colon cancer [12], ovarian cancer [13], osteosarcoma [14], hepatocellular carcinoma [15], breast cancer, and glioma [16, 17]. However, the molecular mechanisms for PAK5 in CC progression remain to be fully elucidated.

Special AT-rich binding protein-1 (SATB1) is a tissue specific DNA-binding protein of nuclear matrix attachment region (MAR). Under physiological conditions, SATB1 predominantly expresses in the thymus, as well as bone marrow and nervous tissues; however, its expression is barely detectable in other tissues [18]. SATB1, as a genome organizer altering more than 1000 genes, specifically regulates temporal and spatial expression of multiple genes via identifying and combining the MAR [19, 20], which is accompanied by recruiting specific chromatin remodeling complexes to its anchorage sites and orchestrating chromatin architecture into loops [21]. In recent years, accumulating data have concluded that up-regulation of SATB1 is observed in many types of cancer, such as cutaneous malignant melanoma [22], prostate cancer [23], nasopharyngeal carcinoma [24], breast cancer and epithelial ovarian cancer [25, 26]. Functional profiling of genes revealed that the underlying mechanisms for SATB1 and chromatin modification affect cellular adhesive molecules and EMT [23].

In the current study, we analyzed PAK5 levels in CC tissues and cell lines and explored the expression relationships of PAK5 with clinicopathological characteristics using immunohistochemistry (IHC) staining. We found that PAK5 positively correlates with the aggressive phenotype of CC. In addition, PAK5 alters the expression of EMT markers and promotes invasion and metastasis of CC cells through phosphorylating SATB1 on Ser47. Our data provided a profound understanding of novel PAK5-SATB1 signaling in promoting CC invasion and metastasis.

Materials and methods

Ethics statement

All experiments involving human participants were approved by the Review Board of the Affiliated Hospital of Xuzhou Medical University. All patients provided a written informed consent prior to the study. Animal experiments were performed in strict accordance with the protocols approved by the Institutional Animal Care and Use Committee of Xuzhou Medical University.

Patients and specimens

We collected tissue specimens, which included 70 normal cervical tissues, 64 cervical intraepithelial neoplasms (CINs) and 163 CCs from the Department of Gynecology and Obstetrics, the Affliated Hospital of Xuzhou Medical University. Patients with CC received laparoscopic radical hysterectomy and pelvic lymphadenectomy. All CCs were staged in accordance with the criteria established by the International Federation of Gynecology and Obstetrics (FIGO). Detailed clinical information of specimen was recorded accurately and completely. All the CC patients were termly followed up for 4 months to 81 months to evaluate the postoperative survival.

IHC staining and evaluation

Paraffin-embedded cervical tissues were sectioned at 4μm thick. After being baked at 60 °C for 2 h, the tissue sections were deparaffinized with dimethylbenzene and rehydrated in different concentrations of alcohol. For retrieving antigen, the slides were heated at 95 °C in 0.01 M citrate buffer (pH = 6.0), and 3% hydrogen peroxide was used to quench peroxidase activity for 20 min. To avoid nonspecific staining, the sections were treated with normal goat serum, followed by incubation overnight with anti-PAK5 antibody (1:100 dilution; Abcam, Cambridge, MA, USA), anti-phospho SATB1 Ser47 (1:100 dilution, Bioss, Beijing, China) and anti-E-cadherin (1:50 dilution, Abcam) at 4 °C. After being rinsed with phosphate buffered saline (PBS), the sections were incubated with a secondary antibody for 1 h and stained with 3, 3′-diaminobenzidine (DAB; Zhongshan biotech, Beijing, China). After hematoxylin counterstain was completed, all the sections were dehydrated and sealed.

Two experienced pathologists independently evaluated the percentage of positive tumor cells and its staining intensity. The values of PAK5 staining intensity were assigned as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The values of percentage of positive tumor cells were scored as follows: (1) (0–25%), (2) (26–50%), (3) (51–75%) and (4) (76–100%). The immunoreactive score (IRS) of each section was calculated by the products of the staining intensity and percentage of tumor cells. According to the IRS, staining patterns were divided into three classes: weak (IRS: 0–3), moderate (IRS: 4–6), and strong (IRS: 8–12).

Cell culture

Human CC cells SiHa, Caski, C33A, MS751 and HeLa were acquired from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Normal cervical epithelial cells (NCECs) were obtained from endocervical biopsy of patients who had no

underlying endocervical pathology and malignant lesions and had never been subjected to radiotherapy and (or) chemotherapy before operations. C33A, HeLa, and SiHa cells were cultured in DMEM (Gibco, Grand Island, NY, USA), and MS751 and CaSki cells were cultured in RPMI 1640 (Gibco). NCECs were cultured in medium mixed according to the proportion of 75% DMEM (Gibco): 25% MCDB-105 (Sigma, St Louis, MO, USA) in volume [27]. Media were supplemented with 10% FBS (Gibco) and 1% antibiotics. All cells were maintained at 37 °C in a humidified chamber with 5% CO_2.

Transfection and generation of stable cell lines

siRNAs were designed and synthesized by GenePharma (Shanghai, China). HeLa and SiHa cells were transfected with the siRNA control (siCtrl), siRNA PAK5 (siPAK5) and siRNA SATB1 (siSATB1) using siLentFect™ Lipid Reagent (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. The plasmids of pcDNA3.1-control, pcDNA3.1-SATB1, pcDNA3.1-SATB1 Ser47A, pcDNA3.1-SATB1 Ser451A, pcDNA3.1-SATB1 Ser557A, and pcDNA3.1-PAK5 (GenePharma) were transiently transfected into cells using the X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA). After 24 h or 48 h of transfection, cells were harvested for the following experiments. Stable cell lines were established by the infection with PAK5-lentivirus (LV-PAK5) and control-lentivirus (LV-Ctrl, GenePharma) and were selected with 2 µg/ml puromycin (Vicmed, Xuzhou, China) for 15 days.

Protein isolation and western blotting

Protein samples were harvested from cell lysates and the protein concentration was determined by bicinchoninic acid kit (BCA, Beyotime Biotechnology, Beijing, China). The proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in PBS for 1 h at room temperature and then incubated overnight at 4 °C with primary antibody: anti-PAK5, anti-SATB1, anti-Slug, anti-E-Cadherin, anti-N-Cadherin, anti-Vimentin, anti-Twist, anti-Fibronectin (Abcam), anti-phospho SATB1 Ser47 (Bioss), anti-GAPDH (Zhongshan biotech). After been washed with PBS, the membrane was probed with corresponding HRP secondary antibodies for 2 h at room temperature. Finally, the protein signals were detected semiquantitatively with Tanon™ High-sig ECL Western Blotting Substrate (Tanon, Shanghai, China).

Cell proliferation assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) kit (Vicmed). Transfected HeLa and SiHa cells (3×10^3) were seeded into 96-well plates (Corning Incorporated, Corning, NY, USA). 10 µl of CCK-8 solution was then added to each well containing 100 µl of serum-free medium at the indicated time points. The cells were incubated for 2 h at 37 °C. Degree of cell proliferation was measured by the increase in absorbance at 450 nm. All assays were repeated three times.

Colony formation assay

Transfected HeLa and SiHa cells were plated in 6-well plates (Corning Incorporated) at a density of 300 cells per well. After the 2-week incubation, we removed the medium and stained the cells with Giemsa solution for 30 min. Visible colonies (more than 50 cells) were counted under a light microscopy. All assays were repeated three times.

Transwell invasion and migration assay

Cell invasion and migration assays were performed as previously described [28]. For invasion assay, transwell chambers (BD Bioscience, San Jose, CA, USA) containing 8 µm pores were coated with Matrigel in the upper chamber. Briefly, 100 µl of cell suspension (1×10^5 cells) of serum-free medium was plated in the lower chamber, medium with 10% FBS added to the lower chamber. After incubating 14 h for migration and 20 h for invasion, we removed the cells of upper chamber and then stained the invasive cells using Giemsa solution. The CC cells of migration and invasion were calculated and photographed under a light microscopy. All assays were repeated three times.

Wound healing assay

The transfected HeLa and SiHa cells were seeded in 6-well plates (Corning Incorporated). When cell culture reached about 95% confluence, the cell monolayer was slowly scratched with a sterile 200 µl pipette tip. We washed the well with fresh medium to remove cellular debris and cultured cells again. The wound was imaged at 0 and 24 h. All assays were repeated three times.

Immunofluorescence

Briefly, transfected HeLa and SiHa cells were cultured on glass coverslips in 6-well plates (Corning Incorporated). Cells were fixed in cold 4% paraformaldehyde for 20 min at room temperature and were then permeabilized with 0.3% Triton-X100 for 15 min. After being blocked in 5% BSA for 30 min, the cells were incubated with anti-phospho SATB1 Ser47 antibody (dilution 1: 100, Bioss) overnight at 4 °C, followed by the visualization with FITC-conjugated secondary antibody for 60 min. The cells were counterstained

with 4′, 6-Diamidino-2-phenylindole (DAPI) for 5 min at room temperature in the dark. Images were collected and captured by confocal laser scanning microscopy (Zeiss LSM 880).

Mn^{2+}-Phos-tag SDS-PAGE

Phosphate-affinity/Mn^{2+}-Phos-tag SDS-PAGE has been described previously [29]. Briefly, dephosphorylated samples were prepared using cell lysate, $MgCl_2$ and alkaline phosphatase (AP, Thermo Scientific, Waltham, MA, USA). After being incubated at 37 °C for 12 h, the dephosphorylated samples were mixed with 100 μl of 3 × SDS-PAGE loading buffer. For Mn^{2+}-Phos-tag SDS-PAGE, except for conventional reagents, the separating gel comprised acrylamide-pendant Phos-tag ligand and $MnCl_2$. Before being transferred to nitrocellulose membrane, the gel of Mn^{2+}-Phos-tag SDS-PAGE electrophoresis was softly rinsed with EDTA for 10 min. The following steps were consistent with conventional western blotting described above.

Dual luciferase reporter assay

Luciferase assays were performed according to the manufacturer's instruction. Briefly, E-cadherin promoter was subcloned into pGL3-Basic luciferase expression vector (Genepharma). HeLa and SiHa cells were plated into 12-well plates (Corning Incorporated), and each cell was transiently co-transfected with appropriate pcDNA3.1 plasmids together with luciferase plasmids using the X-tremeGENE HP DNA Transfection Reagent (Roche). After 24 h transfection, cell lysates were harvested, and then luciferase activities were measured by the Dual-Luciferase Reporter System (Promega, Fitchburg, WI, USA) on a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). Firefly luciferase activities were normalized to the activities of Renilla luciferase control. The experiment was repeated three times.

Xenograft mouse metastatic model

Twelve female 6-week-old BALB/cA-node mice were purchased from Beijing Huafukang Bioscience (Beijing, China). Stable HeLa cells (LV-PAK5 and LV-Ctrl) were concentrated to $2 \times 10^6/100$ μl PBS and injected into mice via the lateral tail vein. After 5 weeks, all mice were euthanized and lungs were then excised and photographed. The number of metastatic tumors per lung and its weight were counted and recorded. Finally, the lung tissues were harvested, embedded, fixed, and prepared for H&E and IHC staining and western blotting.

Statistical analysis

All statistical analyses were determined using the SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Quantitative data were presented as means ± SD. Statistical significance of Student's t-test was used for two-group comparisons. The χ^2 or Fisher's exact test was applied to analyze the correlation between PAK5 expression and the clinicopathological parameters of CC patients. Survival analysis was estimated by the Kaplan–Meier method and the log-rank test. $P < 0.05$ was considered significant.

Results

Elevated expression of PAK5 is correlated with the clinicopathological features of CC

Western blotting results revealed that the expression of PAK5 was significantly up-regulated in diverse CC cells including SiHa, CaSki, C33A, MS751, and HeLa, as compared with NCECs (Fig. 1a, $P < 0.05$). To further explore the correlation between PAK5 expression and CC progression, we conducted IHC staining in cervical specimens of 70 normal cervical tissues, 64 CINs and 163 CCs (Fig. 1b, c). Tissue specimens were classified as low (IRS: 0–4) and high (IRS: 6–12) PAK5 expression via quantitative analysis of protein staining. PAK5 increased significantly with malignant progression of cervical tissues (Fig. 1b, d, $P < 0.05$). High expression of PAK5 was observed in 98 of 163 (60.1%) CC tissues, 21 of 64 (32.8%) CINs and 0 of 70 (0.0%) normal cervical tissues (Table 1). These results suggested that PAK5 expression was upregulated in both CC tissues and cells.

The clinical relationship between PAK5 expression and clinicopathological parameters in CC was further analyzed to explore the importance of PAK5 expression. Statistical analysis suggested that high level of PAK5 was strongly correlated with some clinicopathological parameters of CC (Table 1), including high-risk HPV infection ($P = 0.001$), FIGO Stage ($P = 0.003$), LNM ($P = 0.009$), and recurrence ($P = 0.039$), whereas we did not find the compelling correlations of PAK5 expression with patient's age, histological type, tumor size, pathological grade, type of tumor growth, vaginal involvement, parametrial infiltration in CC.

To further understand the impact of PAK5 on clinical outcome of CC patients, Kaplan–Meier survival analysis and the log-rank test were performed based on expression level of PAK5. Our results indicated that PAK5 overexpression negatively correlated with five-year overall survival rate (Fig. 1e, $P < 0.05$), which suggested that PAK5 might serve as a prognostic marker for CC patients.
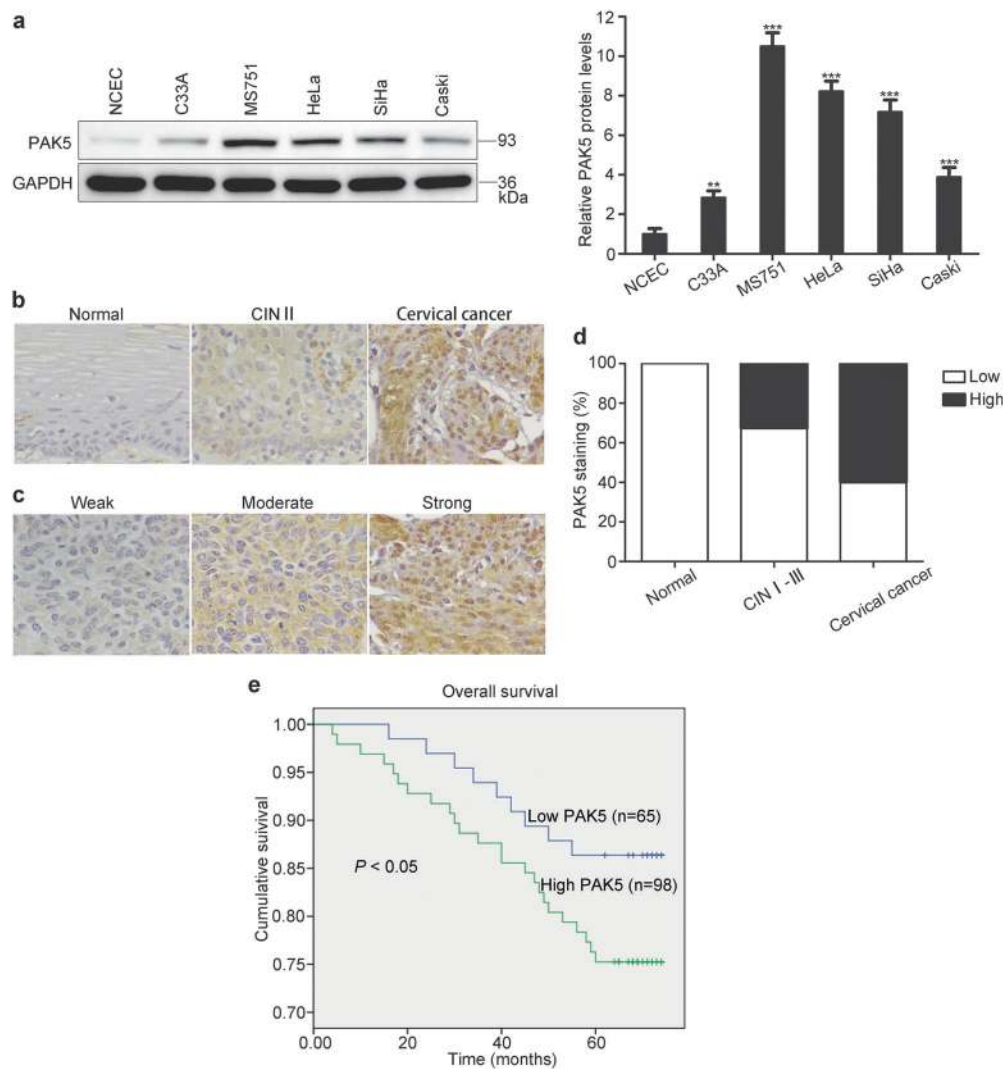
Fig. 1 Expression of PAK5 is up-regulated in CC cells and tissues and associated with 5-year overall survival in CC patients. **a** Protein expression of PAK5 in normal NCECs and human CC cells (SiHa, CaSki, C33A, MS751, HeLa) determined by western blotting. **b** IHC assay of PAK5 protein expression in normal cervical tissues, CIN II tissues and CC tissues. Original magnifications, ×400 for b. **c** Representative images of IHC staining for PAK5 in CC tissues. Original magnifications, ×400 for c. **d** PAK5 expression increased progressively with malignant progression of cervical tissues ($P < 0.05$). **e** Kaplan–Meier survival analysis of 163 cervical patients with high or low PAK5 expression ($P < 0.05$, log-rank test). **, $P < 0.01$; ***, $P < 0.001$. *NCECs* normal cervical epithelial cells, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *CC* cervical cancer, *CIN II* cervical intraepithelial neoplasia, *IHC* immunohistochemistry

The effects of PAK5 on proliferation of CC cells

The above results showed that increased PAK5 expression is associated with malignant progression and an unfavorable prognosis of CC. We thus wanted to determine whether overexpression of PAK5 promotes the growth of CC cells in vitro. HeLa and SiHa cells transfected with PAK5 plasmid were used for proliferation assay. Results from CCK-8 assay suggested that enforced PAK5 markedly accelerated cellular growth in both HeLa and SiHa cells (Fig. 2a), compared with the vector. Inversely, inhibiting the endogenous expression of PAK5 by siRNA remarkably suppressed proliferation of HeLa and SiHa cells in comparison with siCtrl (Fig. 2b). Clone forming assays were then performed to validate the effect of PAK5 on the malignant growth of CC cells. As expected, the colony forming efficiency of HeLa and SiHa cells with PAK5 overexpression was significantly more than that in vector cells (Fig. 2c). Conversely, rate of clone formation HeLa and SiHa cells decreased upon endogenous PAK5 knockdown (Fig. 2d). These findings indicated that PAK5 potentiated CC cell proliferation and survival ability.

The effects of PAK5 on migration and invasion of CC cells

Given that there are significant correlations between increased PAK5 expression and aggressive features in

Table 1 The correlation between PAK5 expression and clinicopathologic characteristics in IHC analysis

Variables	Number (n=297)	PAK5 expression		
		Low (%)	High (%)	P-value
Diagnostic category				<.001
Normal	70	70 (100)	0 (0.0)	
CIN II-III	64	43 (67.2)	21 (32.8)	
Cervical cancer	163	65 (39.9)	98 (60.1)	
Age (years)				0.735
<55	118	48 (40.7)	70 (59.3)	
≥55	45	17 (37.8)	28 (62.2)	
High-risk HPV infection				0.001
Yes	101	30 (29.7)	71 (70.3)	
No	62	35 (56.5)	27 (43.5)	
Histological type				0.490[a]
Squamous carcinoma	136	52 (38.2)	84 (61.8)	
Adenocarinoma	20	9 (45.0)	11 (55.0)	
Others	7	4 (57.1)	3 (42.9)	
FIGO Stage				0.003
I	102	50 (49.0)	52 (51.0)	
II	50	14 (28.0)	36 (72.0)	
III	11	1 (9.1)	10 (90.9)	
Tumor size (cm)				0.670
≤4	106	41 (38.7)	65 (61.3)	
>4	57	24 (42.1)	33 (57.9)	
Pathological grade				0.121
G1	43	22 (51.2)	21 (48.8)	
G2	79	31 (39.2)	48 (60.8)	
G3	41	12 (29.3)	29 (70.7)	
Types of tumor growth				0.852
Exophytic	82	33 (40.2)	49 (59.8)	
Endophytic	45	19 (42.2)	26 (57.8)	
Ulcerative	36	13 (36.1)	23 (63.9)	
Vaginal involvement				0.615
Yes	28	12 (42.9)	16 (57.1)	
No	135	51 (37.8)	84 (62.2)	
Parametrial infiltration				0.282
Yes	38	18 (47.4)	20 (52.6)	
No	125	47 (37.6)	78 (62.4)	
Lymph node metastases				0.009
Yes	60	16 (26.7)	44 (73.3)	
No	103	49 (47.6)	54 (52.4)	
Recurrence				0.039
Yes	24	5 (20.8)	19 (70.9)	
No	139	60 (43.2)	79 (56.8)	

CIN cervical intraepithelial neoplasia, *FIGO* the International Federation of Gynecology and Obstetrics, *LNM* lymph node metastasis

[a]Fisher's exact test

CC, which indicates that PAK5 facilitates tumor progression and transferring of CC. It is well-accepted acknowledged that malignant cells are characterized by possessing the capabilities and properties of adhesion and invasion. Hence, we assessed the effect of PAK5 on motility of CC cells. Wound-healing assays were utilized to examine the effect of PAK5 on cell migration. Our results showed that overexpression of PAK5 increased the capacity for HeLa and SiHa cell motility. HeLa and SiHa cells over-expressing PAK5 led to the faster closing of scratch wound compared with controls (Fig. 2e). Similarly, the wound filling was obviously retarded in HeLa and SiHa cells with siPAK5 (Fig. 2f). Next, quantification analysis through transwell assays showed that enforced expression of PAK5 dramatically enhanced the migration and invasion compared with negative control in both HeLa and SiHa cells (Fig. 2g). Knockdown of PAK5 effectively reduced the number of invaded cells in these two cell lines (Fig. 2h). Overall, these results suggested that PAK5 promotes the migration and invasion in CC cells in vitro.

PAK5 phosphorylates SATB1 on serine 47

It is reported that PAK5 could promotes the tumorigenic features of tumor cells through regulation transcription factor [16, 17]. Since SATB1 is characterized by regulating various gene transcriptions, we wondered whether there are potential regulation mechanisms between PAK5 and SATB1. Western blotting results showed that increased PAK5 had no significant effect on the total SATB1 expression (Fig. 3a). The Mn^{2+}-Phos-tag SDS-PAGE assays confirmed that PAK5 led to the upregulation of phosphorylated SATB1 in HeLa cells, which could be reversed by AP that preserves protein samples in the forms of non-phosphorylation (Fig. 3b). To further determine specific PAK5 phosphorylation site in SATB1, we calculated and predicted three possible phosphorylation sites (Ser47, Ser451, and Ser557) of SATB1 according to the principles and guidelines of PAKs phosphorylating substrates [30]. Three single-site mutation plasmids (described Ser47A, Ser451A and Ser557A) which were derived from candidate phosphorylation sites replaced by alanine were, respectively, co-transfected with PAK5 plasmids into HeLa cells. The results of Mn^{2+}-Phos-tag SDS-PAGE assay showed that Ser47A (Ser47 to alanine) abrogated the phosphorylated status of SATB1 while accompanying with the elevation of unphosphorylated SATB1 in HeLa cells, while other mutations did not (Fig. 3c, d), suggesting that SATB1 Ser47 might be the major phosphorylation site by PAK5. Consistently, the observations from immunofluorescence staining found that the phospho-SATB1 Ser47 signal (red) that predominantly occurred in the nucleus was strikingly enhanced in HeLa and SiHa cells over-expressing PAK5 (Fig. 3e), which fully validated that PAK5 increased the expression of phospho-SATB1 in CC cells. Collectively, SATB1 is a phosphorylated substrate of PAK5.
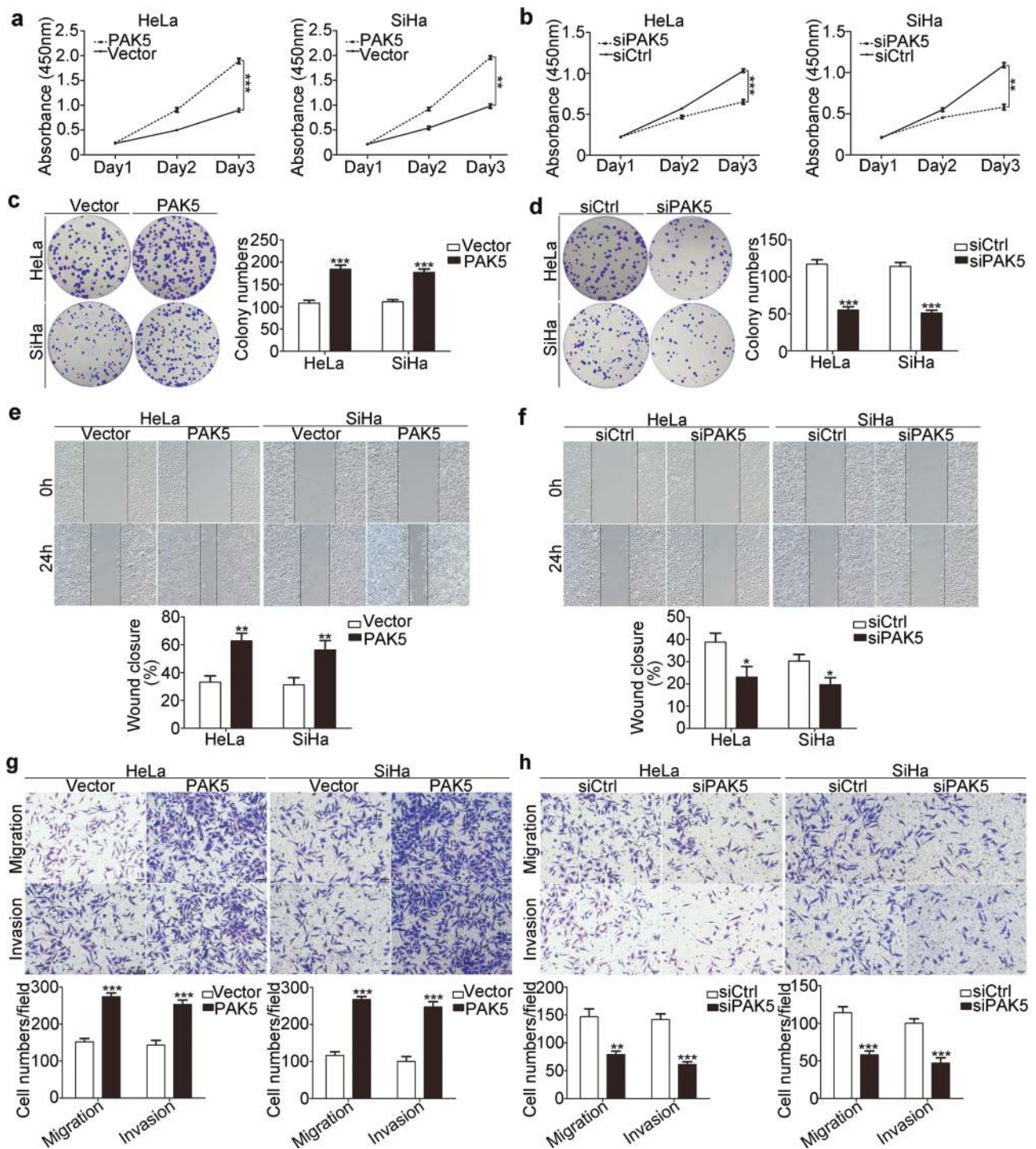
Fig. 2 PAK5 promotes proliferation, migration and invasion of CC cells in vitro. **a**, **b** CCK-8 assays were performed to determine the effects of PAK5 overexpression and knockdown on the proliferation of HeLa and SiHa cells. **c**, **d** The effects of PAK5 overexpression and knockdown on the clone formation of HeLa and SiHa cells. **e**, **f** The HeLa and SiHa cell motilities were measured through testing the wound closure after PAK5 overexpression and knockdown in HeLa and SiHa cells, respectively. Original magnifications, ×400 for e and f. **g**, **h** Transwell assays were used to detect the migration and invasion abilities after PAK5 overexpression and knockdown in HeLa and SiHa cells, respectively. Scale bar, 100 μm for g and h. Data are showed as mean ± SD for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *CCK-8* Cell Counting Kit-8, *siCtrl* siRNA control, *siPAK5* siRNA PAK5
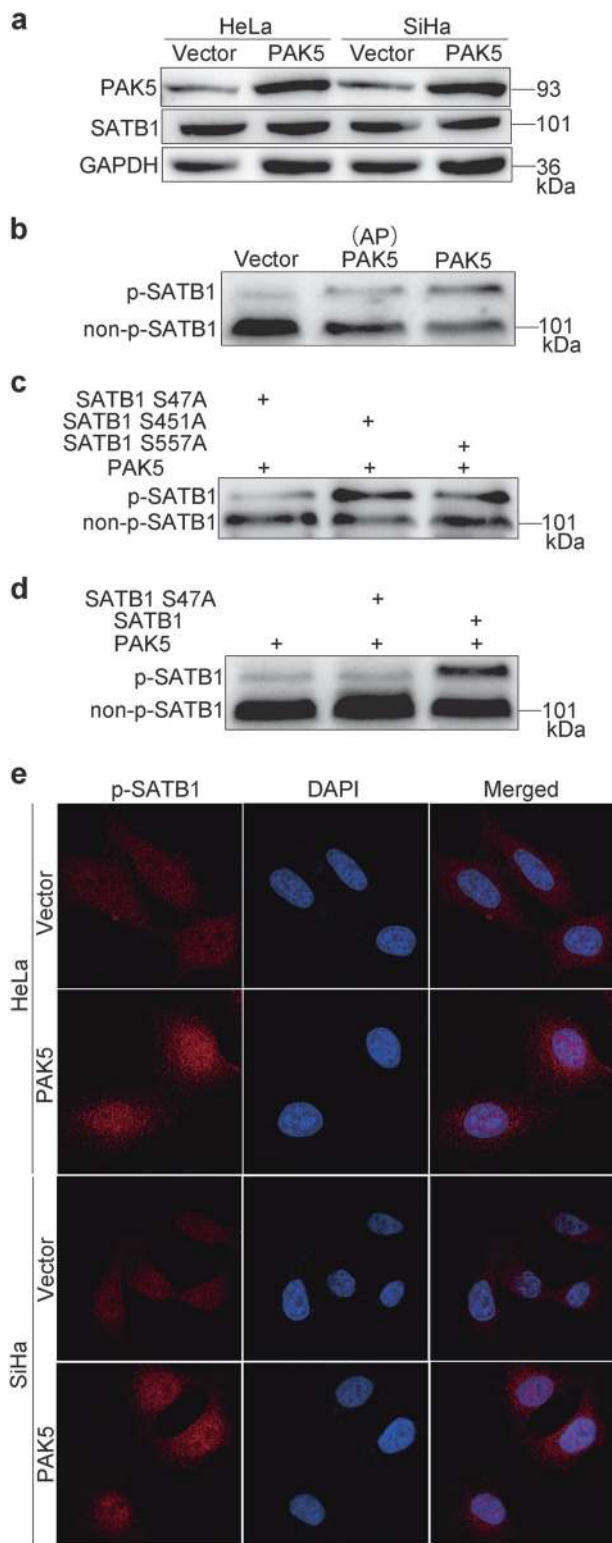
Fig. 3 PAK5 phosphorylates SATB1 on Ser47. **a** Western blotting analysis of total SATB1 expression after PAK5 overexpression in HeLa and SiHa cells. **b** Lysates from HeLa cells transfected with control and PAK5 plasmids were used for Mn^{2+}-Phos-tag SDS-PAGE assay with SATB1 antibodies. Lysates of middle lane were treated with AP. Shift bands (top) represented p-SATB1; bottom bands represented non-p-SATB1. **c** PAK5 plasmids and three single-site mutation (Ser47A, 451 A and 557 A) of SATB1 plasmids were respectively co-transfected into HeLa cells and lysates were subjected to Mn^{2+}-Phos-tag SDS-PAGE assay to determine the specific phosphorylation site of SATB1 by PAK5. **d** PAK5 plasmids were respectively co-transfected with SATB1 and SATB1 Ser47A plasmids into HeLa cells to confirm the phosphorylation of SATB1 on Ser47 by PAK5. **e** Immunofluorescence staining of p-SATB1 Ser47 (red signal) was analyzed by confocal microscopy and the change of red fluorescence signal mainly occurred in the nucleus (blue signal) of HeLa and SiHa cells after overexpressing PAK5. Original magnifications, ×400 for e. *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *AP* alkaline phosphatase, *p-SATB1* phosphorylated SATB1, *non-p-SATB1* non-phosphorylation SATB1, *DAP*I 4′,6-diamidino-2-phenylindole

HeLa and SiHa cells. Up-regulation of wildtype (WT) SATB1 markedly strengthened the migration and invasion ability of CC cells compared with vector cells (Fig. 4a, b), whereas this was reversed and abated in cells transfected with SATB1 Ser47A (Fig. 4a, b). EMT is implicated in CC progression and metastasis [4, 5], and loss of the epithelial phenotype E-cadherin is a vital hallmark of EMT. We next elucidated the transcriptional regulation of PAK5 kinase on E-cadherin gene via luciferase reporter constructs. As shown in Fig. 4c, PAK5 overexpression significantly inhibited the transcriptional activity of E-cadherin in HeLa and SiHa cells. In addition, it was WT SATB1 rather than SATB1 Ser47A that notably inhibited the transcriptional stimulation of E-cadherin promoter activity in HeLa and SiHa cells. This observation confirmed that phosphorylation of SATB1 on Ser47 by PAK5 repressed the E-cadherin expression at the transcriptional level in CC cells. All the data suggested that PAK5 is an upstream signal molecule of SATB1 and induces migration and invasion of CC cells.

PAK5-mediated STAB1 phosphorylation contributes to the alternation of EMT markers in CC cells

As an oncogene in tumorigenesis, PAK5 has been reported to play a decisive role in promoting tumor progression and metastasizes [12, 16, 17, 31, 32]. We next determined the possible roles of SATB1 in PAK5-induced migration and invasion of CC cells. Western blotting showed that increased PAK5 enhanced the expressions of N-cadherin, Fibronectin, Slug, Twist, and Vimentin and decreased the expression of E-cadherin and ZO-1. Silencing endogenous SATB1 by siSATB1 abolished PAK5-induced changes in E-cadherin, ZO-1, N-cadherin, Fibronectin, Slug, Twist and Vimentin in HeLa cells (Fig. 4d). Meanwhile, PAK5 knockdown resulted in the up-regulation of E-cadherin and

Phosphorylation of SATB1 promotes the migration and invasion of CC cells

To evaluate the potential function of phospho-SATB1 Ser47 in cell motility, we performed transwell assays in

Fig. 4 PAK5-mediated phosphorylation promotes EMT in cervical cancer cells. **a**, **b** Effects of SATB1 and SATB1 Ser47A on the migration and invasion ability in HeLa and SiHa cells. Scale bar, 100 μm for a and b. **c** HeLa and SiHa cells were transfected with pGL3-E, together with the indicated expression plasmids for Dual luciferase reporter assays. **d** Effects of SATB1 knockdown on EMT markers were analyzed by western blotting in HeLa cells overexpressing PAK5. **e** Effects of SATB1 overexpression on EMT markers in HeLa cells silencing PAK5. Data are showed as mean ± SD for three independent experiments. **, $P < 0.01$; ***, $P < 0.001$. pGL3-E *pGL3-E*-cadherin promoter, siCtrl siRNA control, siPAK5 *siRNA* PAK5, ns not significant, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase
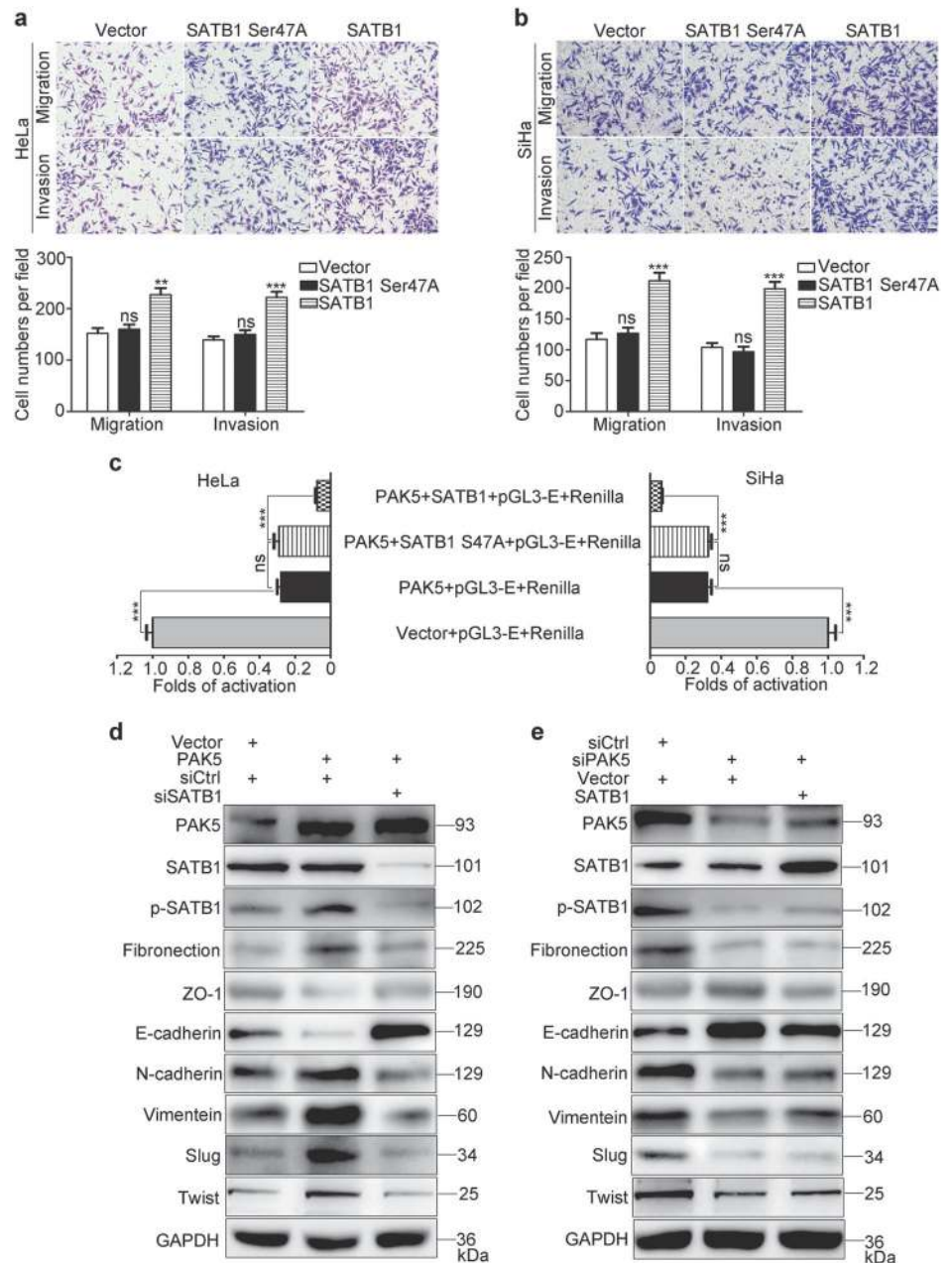
ZO-1 and the down-regulaton of N-cadherin, Fibronectin, Slug, Twist and Vimentin in HeLa cells, on this foundation, those EMT markers did not alter significantly after over-expressing SATB1 (Fig. 4e). These data were consistent with the results of the transwell assays displayed in Fig. 4a–c. Taken together, these results indicated that SATB1 works as a physiological substrate of PAK5 to promote EMT in CC cells.

PAK5 promotes CC metastasis in vivo

To further determine whether PAK5 promotes lung colo-nization of disseminated CC cells in vivo, we developed metastasis models of murine xenograft. HeLa cells were transfected by lentivirus encoding PAK5. Next, equal numbers of LV-PAK5 and LV-Ctrl HeLa cells were intra-venously injected into two groups of 6-week-old BALB/cA-node mice each via tail respectively. Five weeks after implantation, all mice were sacrificed, and the metastatic nodules on the lung surfaces were recorded. There was a higher incidence of lung metastasis in mice inoculated with LV-PAK5 group compared with LV-Ctrl group. Enforced expression of PAK5 promoted HeLa cells colonization to the lung and increased the numbers of metastasis nodules in mice (Fig. 5a, b, $P < 0.05$). Additionally, the lung weights in LV-PAK5 group were higher than that in LV-Ctrl group
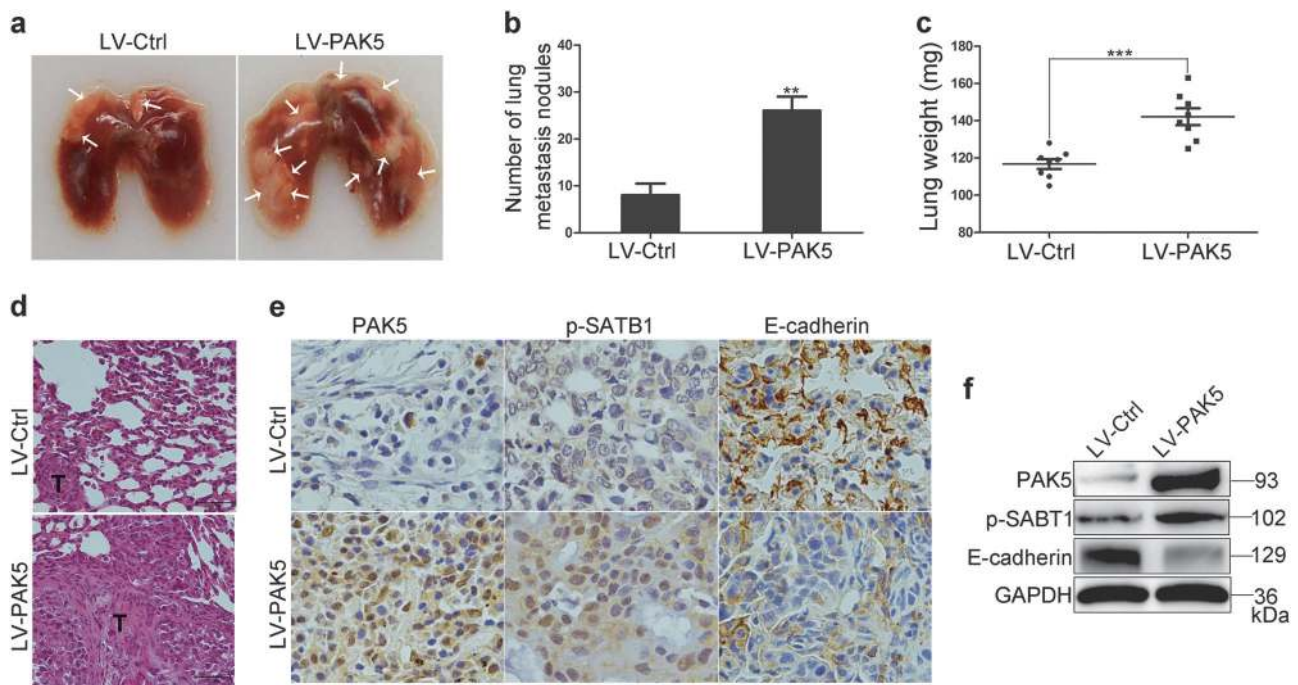
Fig. 5 PAK5 promotes CC cell metastasis in vivo. **a** Lungs originated from mice injected with lentivirus-mediated HeLa cells. The white arrow marked metastatic nodules. **b** Numbers of lung metastasis nodules from each mouse were counted. **c** Weights of lung were counted. **d** H&E staining of lung sections. Scale bar, 100μm for d. **e** Representative images of IHC staining for PAK5, p-SATB1 Ser47 and E-cadherin in lung tissues. Original magnifications, ×400 for e. **f** Western blotting detected the protein expressions of PAK5, phospho-SATB1 Ser47 and E-cadherin in lung tissues. Data are showed as mean ± SD. **, $P < 0.01$; ***, $P < 0.001$. *LV-Ctrl* control-lentivirus, *LV-PAK5* PAK5-lentivirus, *p-SATB1 Ser47* phosphorylated SATB1 Ser47, *H&E* hematoxylin-eosin staining, *IHC* immunohistochemistry, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

(Fig. 5c, $P < 0.05$). H&E staining confirmed that the nidi in mice lungs were metastatic tumors (Fig. 5d). Furthermore, IHC assay on these metastasis nodules excised from two groups of mice revealed that nodules with overexpressed PAK5 showed the enhanced expression of phospho-SATB1 Ser47 and lower expression of E-cadherin compared with control tumors (Fig. 5e). In accordance with this, western blotting showed that treatment with PAK5 increased phospho-SATB1 Ser47 protein and decreased E-cadherin protein in lungs (Fig. 5f). Taken together, these results indicated that PAK5 overexpression enhanced the metastatic capability of CC cells through up-regulating phosphorylated SATB1 resulting in the low expression of E-cadherin in vivo.

Discussion

PAKs, as the best characterized effector proteins of small GTPases Rac and Cdc42, have been shown to be involved in tumor progression [8, 33, 34]. The Rho GTPases plays an important role in oncogenic transformation and affects cellular processes from cell metastasis to cell proliferation through their interaction with downstream effector proteins [35]. The effects of aberrant expression or activation of PAKs on downstream signaling cascades result in phenotypic changes related to tumorigenesis and progression [8, 36]. Previous studies suggested that PAK5 promotes the invasion and metastasis of breast cancer and glioma cells via regulating Egr1/MMP2 signal axis [16, 17]. Gong et al confirmed that the high expression level of PAK5 is significantly associated with tumor progression in colon cancer and might act as a prognostic factor of worse outcome [32]. Apart from tumorigenesis and progression, PAK5 exerts considerable influence on attenuating chemosensitivity of anti-cancer drugs [13, 37], such as cisplatin in hepatocellular carcinoma, paclitaxel in ovarian cancer and gemcitabine in pancreatic cancer. The existing researches indicate that the roles of PAK5 in tumor development and progression are mainly embodied in the followings: promotion of tumor cells migration, diffusion and anti-apoptosis and accelerating of tumor cells proliferation. However, the effects of PAK5 on CC tumorigenesis and metastasis have never been reported by others.

In the present study, we investigated PAK5 expression in cervical tissues and CC cells. The protein level of PAK5 was up-regulated in CC cells compared with NCECs. Strong PAK5 staining is detected in CC tissues, whereas absent or low expression is showed in most normal cervical tissues and CIN, which indicates that PAK5 may display

vital role in the carcinogenesis and progression of CC. We further evaluated the correlations between PAK5 expression and clinicopathological features as well as prognostic survival in CC. Our findings showed that elevated PAK5 expression was significantly associated with the high-risk HPV infection, histological type, FIGO stage, LNM and recurrence. High levels of PAK5 protein strongly reduced the overall survival among CC patients and PAK5 might therefore serve as a prognostic parameter for CC. Subsequently, we investigated the roles of PAK5 in CC cells by specifically interfering or overexpressing PAK5. PAK5 overexpression conferred on cells tumorigenic properties and contributed to proliferation, migration and invasion of CC cells in vitro, PAK5 knockdown resulted in a reverse trend. These data evidence supported that PAK5 is involved in tumorigenesis and progression of CC, which is consistent with previous studies to support cancer-promoting characters of PAK5 [15–17, 31, 32].

SATB1, a MAR-binding transcription factor, plays a vital role in reprograming chromatin architecture and gene regulation, and its changes are affecting many gene expressions [19, 20], with transcriptional activation or repression. This suggests that SATB1 upstream should have one or more mechanisms which affect its transcriptional activity. PAK5 signaling has been proved to modulate diverse transcription factors such as GATA1 [38], egr1 [16, 17], RAF1, and NF-κB-p65 [39, 40]. However, PAK5-SATB1 signaling pathway in CC has yet to be determined. A growing number of studies have focused on the relationship between SATB1 expression and the occurrence and development of human cancers in the past decade [22, 23, 25, 41, 42], and its biological functions in various tumors have been explored in detail. For example, Mir R et al reported that SATB1 reprograms tumor related genes to facilitate tumorigenesis and progression of colorectal cancer via Wnt/β-catenin signaling [43]. Han HJ et al. revealed that SATB1 potentiates the invasive behavior and metastatic phenotype of breast cancer and serves as a novel biomarker for breast cancer progression and distant metastasis [25]. Beyond that, aberrant expression of SATB1 is also associated with pathological parameters and poorer outcome in solid tumors, such as cutaneous malignant melanoma [22], prostate cancer [23], nasopharyngeal carcinoma [24], and epithelial ovarian cancer [26]. In this paper, we specifically focused on exploring and understanding the mechanisms by which PAK5 governs SATB1 and affect the migration and invasion of CC cells.

Our western blotting results revealed that total SATB1 protein was unaltered upon PAK5 overexpression. It has been documented that SATB1 is directly phosphorylated by PKC at serine residue (s) via phosphorylation assays [44]. As an analog, PAK5 may phosphorylate SATB1. Our findings confirmed that SATB1 was a novel substrate

protein of PAK5, and PAK5 primarily phosphorylated SATB1 on Ser47 in CC cells. PAK5 overexpression inhibited the level of E-cadherin and promoted migration and invasion in CC cells. Animal model of CC metastases confirmed that PAK5 enhanced lung metastases in vivo. It has been reported that PAK5 is characterized by remodeling cytoskeleton, somehow regulating cell mobility through modulating cytoskeleton shape [10]. PAK5-mediatd phosphorylation animates Raf-1 [39], which take part in the activation mechanism of Raf-1 and partly affects cell invasion and migration via ERK/MAPK pathway [10]. All these well documented that PAK5 may play essential roles in initiating the metastatic cascade of CC. Malignancy invasion and distant metastasis is a multi-factor, multi-step dynamic processes which are usually initiated by the EMT during which epithelial cells are subjected to morphological changes, loss of adhesion and gain the property of invasion and migration [45, 46]. Transcription factor activation, such as Slug and Twist are the causative agents to suppress epithelial marker, E-cadherin and ZO-1, and increase mesenchymal markers, Fibronectin, Vimentin, and N-cadherin [45, 46]. Consistent with this, PAK5-mediated SATB1 phosphorylation led to the alteration of several EMT markers, including Snail, Slug, Twist and ZO-1, Fibronectin, Vimentin and N-cadherin, which induced cellular dispersion and metastatic cascade in CC. Metastasis models of PAK5 overexpression showed higher level of phospho-SATB1 Ser47 and lower level of E-cadherin. Those are also in line with the previous discoveries that PAK overexpression or hyperactivation is often responsible for triggering EMT event [47]. Overall, PAK5-mediated SATB1 phosphorylation contributed to the downregulation of E-cadherin, consequently decreased intercellular cohesion and induced motility and metastasis of CC cells.

Our research investigated the role of SATB1 in PAK5 inducing migration and invasion of CC cells. However, there still are some deficiencies in current studies, due to the complexity of mechanism related to SATB1 signaling in carcinogenesis. In recent years, SATB1 has been proved to be implicated in the occurrence and development of diverse tumors, whereas there is no unified explanation and exact conclusion on the roles of SATB1 in tumor. A few reports showed that SATB1 is low expressed or absent and is scarcely relevant to tumor progression in certain human tumors, such as colorectal cancer and lung cancer [48, 49]. Han HJ et al. confirmed that overexpression of SATB1 has a poor prognosis and could serve as an independent prognostic factor [25]. Different from the above perspective, Iorns E et al. specifically proposed that SATB1 cannot be used as a prognostic marker for breast cancer patients through statistic and analysis of 2058 cases [42]. Moreover, the co-expression of PAK5 and phosphorylated SATB1 and their correlations are still needed to the further testify in CC

tissues. The effects of phosphorylated SATB1 on proliferation of CC cells are also uncertain. In this paper, we first reported that increased PAK5 is closely related to aggressive phenotype in CC and it promotes the adhesion, migration and invasion of CC cells via phosphorylating SATB1 on Ser47, which shed light on understanding PAK5 activity in CC pathophysiology.

Conclusion

In summary, our results provide an evidence for the emerging connections among PAK5, EMT markers and CC metastasis mediated by SATB1, despite some controversy concerning SATB1. PAK5 expression is increased in CC tissues and cell lines. High expression of PAK5 is significantly linked to the progression of CC and it could act as valid prognostic factor of CC. PAK5 induces EMT and enhances metastasis of CC by phosphorylating SATB1 on Ser47. Therefore, PAK5-SATB1 signals provide potential therapeutic avenues for therapeutic intervention in CC.

Author contributions F.C.H., Y.J.P. performed the experiments and wrote the paper; T.T.L. analyzed and data and partially participated in the experiments; J.M., D.S.P. designed and supervised the project.

Compliance with ethical standards

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