PEAK1 promotes invasion and metastasis and confers drug resistance in breast cancer

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Research Article

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Abstract

Background and Aims

Pseudopodium-enriched atypical kinase 1 (PEAK1) has reported to be upregulated in human malignancies and related with poor prognosis. Enhanced PEAK1 expression facilitates tumor cell survival, invasion, metastasis and chemoresistance. However, the role of PEAK1 in breast cancer is not clear. Here, we investigated the PEAK1 expression in breast cancer and analyzed its relation with clinicopathological status and chemotherapy resistance to the neoadjuvant chemotherapy (NAC). We also investigated the role of PEAK1 on breast cancer cells *in vitro* and *in vivo*.

Methods

Immunohistochemistry (IHC) was performed in 112 surgical resected breast cancer tissues. The associations between clinicopathological status, multi-drug resistance and PEAK1 expression were determined. Effect of PEAK1 overexpression or down-expression on proliferation, colony formation, invasion, migration, metastasis and Doxorubicin sensitivity in the MCF-7 cells *in vitro* and *in vivo* was detected.

Results

PEAK1 was overexpressed in breast cancer tissues and NAC -resistant breast cancer tissues. High PEAK1 expression was related with tumor size, high tumor grade, T stage, LN metastasis, recurrence, Ki-67 expression, Her-2 expression and multi-drug resistance. Targeting PEAK1 inhibited cell growth, invasion, metastasis and reversed chemoresistance to Doxorubicin in breast cancer cells in vitro and in vivo.

Conclusion

High PEAK1 expression was associated with invasion, metastasis and chemoresistance of breast cancers. Furthermore, targeting PEAK1 could inhibit cell growth and metastasis, and reverse chemoresistance in breast cancer cells, which provides an effective treatment strategies for breast cancer.

Introduction

Breast cancer metastasis results in poor prognosis and increased mortality, the mechanisms of which are yet to be fully resolved. Tumour resection combined with radiotherapy, endocrine-therapy, and/or chemotherapy is the main way to treat breast cancer, but the development of chemoresistance limits the effectiveness of chemotherapy [1]. Therefore, identifying the molecular mechanisms contributing to breast cancer progression and

chemoresistance would produce new biomarkers for the precise prediction of patient prognosis and for molecular targeted-therapy.

Overexpression of PEAK1 (pseudopodium enriched atypical kinase 1, Sgk269), which was discovered in the pseudopodia of migrating cells [2], is also found in colorectal cancer [3] and pancreatic cancer [4]. PEAK1 expression is associated with metastasis and proliferation in many cancer cells, such as colorectal cancer [3, 5], lung cancer [6] and pancreatic cancer [4]. Enforced PEAK1 expression can cause cell cycle deregulation and resistance to chemotherapy in pancreatic cancer cells [4]. Altering PEAK1 expression can interfere tumor formation and metastasis in pancreatic cancer cells in vivo, indicating that PEAK1 plays an important role in pancreatic cancer growth and metastasis. In breast cancer cells in vivo, enforced PEAK1 expression could induce new blood vessel formation by upregulation of vascular endothelial growth factor receptor-2, which facilitates cell movement and growth [7].

It has recently found that PEAK1 overexpression was significantly associated with advanced clinical stage and poor prognosis in colon cancer [3] and pancreatic cancer [4]. In breast cancer, PEAK1 levels correlate with mesenchymal gene expression, poor cellular differentiation and disease relapse [8]. Croucher et al. [9] reported that PEAK1 overexpression was detected in a subset of basal, HER2-positive, and luminal cancers. However, the relation of PEAK1 expression and clinicopathological status and the relation of PEAK1 expression with chemosensitvity in breast cancer is currently unknown.

In the present study, we examined the PEAK1 protein expression in human breast cancer tissues and explored the relationship between PEAK1 expression and clinical characteristics. Furthermore, we investigated the effect of PEAK1 on breast cancer cell growth, invasion, migration, metastasis and Doxorubicin sensitivity *in vitro* and *in vivo*.

Materials And Methods

Cell culture

The human breast cancer MCF-7 cells were purchased from Institute of Cell Research, Chinese Academy of Sciences (Beijing, China). Doxorubicin (DOX)-resistant MCF-7^{DOX} cells were established by induction with gradient concentrations (0.1-2 μg/mL) of ADR *in vitro*. Cells were cultured in DMEM (Sigma) supplemented with 10% FBS (Gibco), 100 units/ml of penicillin, 100 μg/ml, streptomycin in a 5% CO_2 at 37°C. MCF-7^{DOX} cells were cultured at a final concentration of 2.0 μg/mL.

Patient samples

Breast cancer samples were obtained from Breast disease Center, the affiliated Hospital of Qingdao University. In all, 112 surgical resected tumors from Feb. 2012 to 2018. Pathological diagnosis was verified by two pathologists independently. All human samples were collected with informed consents from the donors according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). The study was performed after approval by the institutional review board (IRB) of the affiliated Hospital of Qingdao University. Written informed consent was obtained from each individual or patient.

Immunohistochemistry of PEAK1

Formalin-fixed paraffin-embedded tissue sections from excised specimens were processed according to standard procedures. Specific primary antibodies against PEAK1 (1:50) was purchased from Sigma-Aldrich (Shanghai, China). IHC staining for PEAK1 was performed as the manufacture's instruction**.** The expression of PEAK1 was positive when 10% of tumor cells showed PEAK1 immunopositivity, and negative when less than 10% of tumor cells showed PEAK1 immunopositivity.

PEAK1 shRNA vector construction and transfection

The short-hairpin RNA direct against human PEAK1 gene (PEAK1 shRNA) was synthesized and constructed into the pcDNA3.1 expression vector (Shanghai, China) as the manufacture's instruction. The constructed vectors (PEAK1 shRNA or NC shRNA) were transfected into the MCF-7 cells using Lipofectamine 3000 reagent (Invitrogen, Shanghai, China) as the manufacturer's instruction. The PEAK1 shRNA or NC shRNA transfected MCF-7 cells were selected by puromycin (10 mg/ml) for 5 days. The puromycin-resistant colonies were then picked and expanded. The relative protein was detected by Western blot assay.

Plasmid constructs and transfection

The full-length human PEAK1 adenovirus was constructed as to the manufacturer's instructions using the AdEasy Adenoviral Vector System. Viral particles were produced by GenScript Biotechnology, China. The virus particles containing PEAK1 or control vector were used to infect MCF-7 cells. Transfected cells were selected with G418 (600 μg/ml, Gibco) for 10-12 days. The expression of PEAK1 in stable PEAK1 transfected colonies was detected by western blot analysis.

In vitro doxorubicin sensitivity by MTT assay

MCF-7 cells after transfection with Lv-PEAK1 or PEAK1 shRNA or its control for 48 h were plated in a 96-well plate in triplicate for 24 h, and the cells (300 cells / well) were then exposed to a concentration of 2.0 μg/mL Doxorubicin for 72 h. Subsequently, 20 μL MTT (Sigma-Aldrich) was added to each well and then incubated for 4 hours at 37°C and 5% CO2 humidified atmosphere. The optical density (OD) at 450 nm was measured and considered an indirect index of relative cell viability.

In vitro doxorubicin sensitivity by colony formation assay

MCF-7 cells after transfection with Lv-PEAK1 or PEAK1 shRNA or its control for 48 h were plated in triplicate at 1000 cells per well in 6-well plates with or without 2.0 μg/mL Doxorubicin and cultured for 12 days, then methanol-fixed and Giemsa-stained (GS, Sigma-Aldrich), which was followed by colony counting.

Matrigel invasion assay.

Cell migration and invasion was determined using a Transwell Chamber assay (BD Biosciences) according to the manufacturer's instructions. MCF-7 cells (1×10^5 cells) after transfection with Lv-PEAK1 or PEAK1 shRNA or its control for 24 h were added to the upper compartment (in triplicate), and DMEM plus 10% FCS was added to the lower compartment. Bestatin (Sigma) was added to both compartments. The cells were incubated for 24 h, and the total number of invaded cells was calculated as the manufacture's instruction.

Western Blotting

Cells and tissues were lysed and protein concentration was measured with BCA protein assay reagents (Pierce). The proteins were then resolved on SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore) and probed with the primary antibodies: PEAK1 and a-Tubulin. Band densitometry analysis was performed using ImageJ software (NCI).

In vivo metastasis assay

Female BALB/c nude mice (5-6 weeks of age, 16-18 g) were obtained from National Rodent Shanghai Experimental Branch Center, Chinese Academy of Sciences (Shanghai, China). All experimental procedures involving animals were conducted in accordance with the institutional guidelines by the Affiliated hospital of Qingdao University. The stable Lv-PEAK1 or PEAK1 shRNA or its control transfected MCF-7 cells were cultured to log phase. 2×10^6 cells (n = 6 per group) were injected into mice via tail veins. After 5 weeks, the whole lung tissues were removaled and the numbers of visible nodules on the lung surface were numbered. H&E staining was used to evaluate the tumor metastasis.

In vivo growth and doxorubicin sensitivity assay

A total of stable PEAK1 shRNA transfected MCF-7^{DOX} or stable Lv-PEAK1 transfected MCF-7 cells (5×10^6) were injected s.c. to the left front flank of mice on day 0. From day 3 to day 9, DOX was administered to all groups of mice by i.v. injection (0.1 mL, 10 mg/kg) for a total of 4 times at 2 day intervals. Tumor dimensions were measured in 2 dimensions with microcalipers every other day and tumor volume was calculated by the following formula: tumor volume = (length × width^2)/2. Non-retrospective ethical approval obtained for the animal experiments conducted in the study.

Statistical analysis

Data were shown as mean ± SEM; Data were analyzed using Student *t* test and pearson χ^2 test. Value of $p < 0.05$ were considered significant.

Results

PEAK1 is overexpressed in breast cancer tissues

Using immunohistochemical methods, we detected the expression of PEAK1 in 112 cases of breast cancer tissues and 34 cases of corresponding adjacent tissues (Fig. 1). PEAK1 expression is significantly upregulated in breast cancer tissues 61.6% (69/112) in comparison with adjacent normal tissues 26.4% (9/34) (P=0.033). PEAK1 overexpression is associated with high tumor size, tumor grade, T stage, advanced nodal status, regional recurrence, HER2, Ki-67 and chemotherapy status (Table 1).

PEAK1 promotes cell growth ,invasion and migration *in vitro*

The Lv-PEAK1 or empty vector was transfected into MCF-7 cells. PEAK1 expression was significantly increased in MCF-7/Lv-PEAK1 cells compared to the MCF-7/ empty vector cells (Fig. 2A). Cell growth was significantly increased in MCF-7/Lv-PEAK1 cells compared to the MCF-7/ empty vector cells by MTT assay (Fig. 2B). To confirm the MTT data, we carried out in vitro colony formation assays. Representative pictures and their quantification confirm that PEAK1 overexpression significantly promoted MCF-7 cells growth (Fig. 2C).

The cell migration and invasion of transfected cells in vitro was observed by transwell assays. As shown in Fig. 2D, the invasive and migrative cells were significantly increased in MCF-7/Lv-PEAK1 cells compared to the MCF-7/ empty vector cells.

Targeting PEAK1 inhibits cell growth ,invasion and migration *in vitro*

The Lv-PEAK1 shRNA or empty vector was transfected into MCF-7 cells. PEAK1 expression was significantly decreased in MCF-7/ PEAK1 shRNA cells compared to the MCF-7/ control shRNA cells (Fig.2A). Cell growth was significantly decreased with PEAK1 depletion in the MCF-7/ PEAK1 shRNA cells by MTT assay (Fig. 3A) and colony formation assays (Fig. 3B). As shown in Fig. 3C, the invasive and migrative cells were significantly decreased in MCF-7/ PEAK1 shRNA cells compared to the MCF-7/control shRNA cells.

PEAK1 regulated doxorubicin sensitivity in MCF-7 cells

The Lv-PEAK1 or empty vector was transfected into MCF-7 cells, then treated with 2.0 μg/mL doxorubicin for 72 h. The results confirmed that PEAK1 overexpression reduced doxorubicin-induced cytotoxicity **(Fig. 4A-4B).**

Similarly, the MCF-7^{DOX} cells were transfected with PEAK1 shRNA, then treated with 2.0 μg/mL doxorubicin for 72 h. The results confirmed that PEAK1 depletionincreased doxorubicin-induced cytotoxicity **(Fig. 4A-4B).** These data suggest that targeting PEAK1 reverses **doxorubicin** resistance in doxorubicin-resistant breast cancer cells.

PEAK1 depelation increases the sensitivity of MCF-7cells to doxorubicin *in vivo*

To determine whether the effect of PEAK1 on chemosensitivity in vitro also extended to tumors growing in vivo, we injected female BALB/c mice with 5×10^5 MCF-7^{DOX} or MCF-7 cells and then

evaluated their response to doxorubicin treatment. The results showed that MCF-7^{DOX} or MCF-7cells with PEAK1 knockdown had a markedly reduced growth rate following doxorubicin treatment (Fig. 5A-5B). These data suggest that targeting PEAK1 inhibited tumor growth *in vito* and sensitized MGF-7 cells to doxorubicin treatment.

PEAK1 depelation inhibited lung metastasis of MCF-7 cells *in vivo*

Finally, we investigated the role of PEAK1 in mediating breast cancer cells metastasis *in vivo*. The stable PEAK1 shRNA or Lv-PEAK1 transfected MCF-7 cells were injected into the female nude mice by the tail vein. After 8 weeks, the whole lung tissues were removaled and the numbers of visible nodules on the lung surface were numbered. H&E staining was used to evaluate the tumor metastasis. The results showed that the PEAK1 shRNA transfected MCF-7 cells has fewer tumor nodes (Fig. 5C) and the Lv-PEAK1 transfected MCF-7 cells has more tumor nodes (Fig. 5C). These data suggest that targeting PEAK1 inhibited lung metastasis *in vivo*.

Discussion

Breast cancer is the commonest female cancer worldwide and its propensity to metastasize negatively impacts on therapeutic outcome. Several clinicopathological parameters with prognostic/predictive significance have been associated with metastatic suppressor expression levels.

PEAK1 is a newly described tyrosine kinase and scaffold protein that transmits integrin-mediated extracellular matrix (ECM) signals to facilitate cell movement and growth. It has reported to be upregulated in human malignancies and related with poor prognosis [3, 4]. In the present study, we performed immunohistochemistry (IHC) of PEAK1 in 112 surgical resected breast cancer tissues and 43 adjacent non-tumor breast tissues. PEAK1 is localized in the cytoplasm, membrane and nuclear, and predominately cytoplasm staining. These observations were consistent with the IHC staining results in colorectal cancer [3] and pancreatic cancer [4]. We also found that PEAK1 is overexpressed in breast cancer tissues at significantly higher levels than adjacent non-tumor breast tissues.

PEAK1 is upregulated in multiple human malignancies and has been associated with tumor invasion and metastasis [3, 4]. However, PEAK1 was downexpressed in gastric cancers, higher PEAK1 expression was related with non-lymph node metastases and good prognosis [10]. Here we found that PEAK1 overexpression was related with larger tumor size, higher tumor stage, tumor grade, T stage, LN metastasis and recurrence, suggesting that PEAK1 overexpression might promote the malignant potential of breast cancer. Therefore, PEAK1 might act as a valuable marker for prediction of breast cancer invasion, and also play an important role in prognosis prediction. Ki-67 expression was reported to be positively correlated with a higher incidence of lymphovascular invasion and lymph node metastasis in breast cancer [11, 12]. In the study, PEAK1 expression was correlated with lymph node metastasis and Ki-67 expression, further confirming that PEAK1 may be used as a diagnostic marker for breast cancer invasion and prognosis. In clinical settings, low-proliferative tumors are less sensitive to chemotherapy

[13]. Nowadays immunohistochemistry for Ki-67 assessment is used for estimation of cell proliferation and guiding the decision on adjuvant treatment choice and predicting of the neoadjuvant treatment response in breast cancer [14]. Immunohistochemistry for ER, PR and HER2 assessment is also used to predict sensitivity to drugs and to determine the application and types of systemic therapy [15]. In the present study, PEAK1 expression was related with both Ki-67 expression and HER2 expression in the breast cancer, suggesting that PEAK1 expression may predict chemosensitivity in breast cancer. In addition, PEAK1 was overexpressed in 112 cases of breast cancer patients accepting chemotherapy. However, the difference in PEAK1 expression in groups of radiation and hormonal treatments and without radiation and hormonal treatments did not show significance. To confirm our observations, we tested PEAK1 expression in 53 cases of breast cancer patients accepting neoadjuvant chemotherapy (NAC) treatment, and found that PEAK1 expression was significantly enhanced in chemoresistant breast cancer.

In the laboratory, targeting PEAK1 expression inhibited cell proliferation and metastasis *in vitro* and *in vivo*, and vice verse [2–5, 7, 16]. Croucher et al. [9] reported that PEAK1 was overexpressed in luminal, HER2, primary basal breast cancers and cell lines by western blot assay. Furthermore, enhanced PEAK1 promoted acinar growth and cell invasion in vitro. Abu-Thuraia et al.[17] reported that PEAK1 is required for both tumor growth and metastasis in a TNBC cellular model. In the present study, the blockade of PEAK1 expression inhibited cell growth, invasion and migration *in vitro*, and inhibited tumor growth and lung metastasis *in vivo*, suggesting that PEAK1 is the target gene for breast cancer gene therapy.

To further confirm whether PEAK1 expression regulates the chemosensitivity of breast cancer cells to therapeutic drug, the PEAK1 knockdown and overexpresssing MCF-7 cells were treated with doxorubicin. As might be predicted from prior studies of doxorubicin, a remarkably decreased cell or tumor growth was observed in MCF-7 cells with suppressed PEAK1 expression, whereas more cell growth were detected in MCF-7 cells overexpressing PEAK1 *in vitro* and *in vivo*. These data indicated that enhanced PEAK1 expression promoted the development of chemoresistance, and vice versa.

Conclusion

In conclusion, PEAK1 is overexpressed in breast cancer and chemoresistant breast cancers. PEAK1 overexpression is related with clinicopathological parameters. Monitoring PEAK1 level and PEAK1 depletion may be utilized to predict and reverse the chemoresistance of breast cancers. PEAK1 may be as a useful prognostic biomarker and a potential therapeutic target for patients with breast cancer.

Declarations

Competing interests

The authors declare that they have no competing interests.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Author Contribution

Yu Wang and Xingang Wang designed the experiments, analysed the data and prepared the manuscript. Yan Zheng selected the materials. All authors read and approved the final manuscript.

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Tables

Table 1. PEAK1 expression and clinicopathological status in 112 patients with breast cancer			
	PEAK1 expression		
Variable	Low expression(n=43)	High expression(n=69)	p-value
Age(year)			0.547
≤50	15	28	
>50	28	41	
Tumor size (cm)			0.032
≤2	27	29	
>2	16	40	
Tumor grade			0.049
1	6	11	
2	26	26	
3	11	32	
T stage			0.012
T1	26	24	
T2	17	40	
T3	0	5	
LN metastasis			0.036
Negative	12	33	
Positive	31	36	
Distant metastasis			0.073
No	30	58	
Yes	13	11	
ER			0.074
positive	28	33	
negative	15	36	
PR			0.262
positive	24	31	
negative	19	38	

HER-2			0.0218
positive	9	29	
negative	34	40	
Triple-negative			0.180
positive	9	8	
negative	34	61	
Chemotherapy			0.0135
No	29	30	
Yes	14	39	
Radiation therapy			0.208
No	34	47	
Yes	9	22	
Endocrine therapy			0.269
No	30	41	
Yes	13	28	
Local recurrence			0.243
No	28	52	
Yes	15	17	
Regional recurrence			0.0473
No	37	48	
Yes	6	21	
Ki-67			0.0366
positive	25	53	
negative	18	16	

Figures

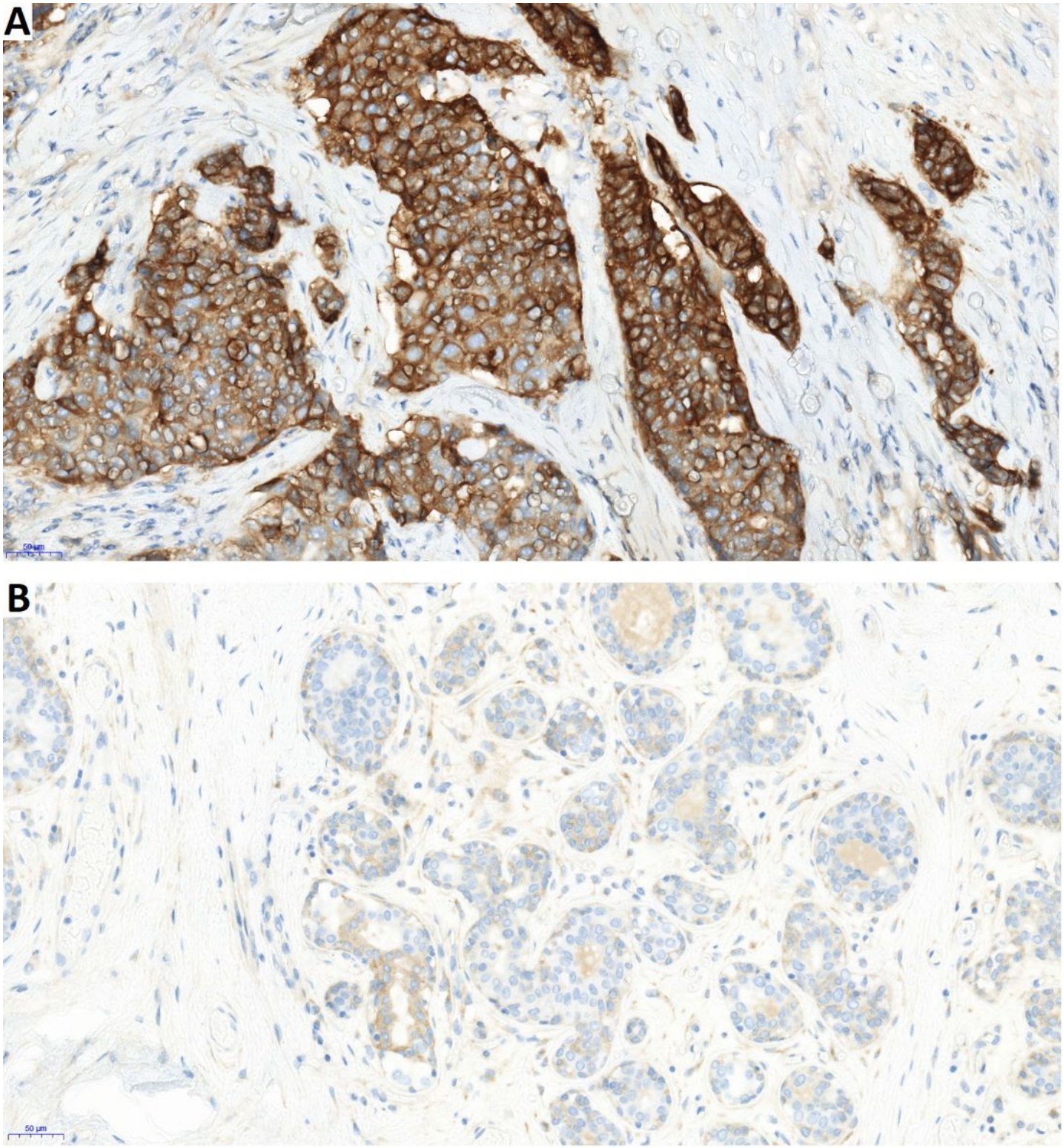

Figure 1

PEAK1 expression in breast cancer tissues and adjacent tissues by immunohistochemistry. A, High PEAK1 expression in breast cancer tissues; B, Low PEAK1 expression in the adjacent tissues of A.

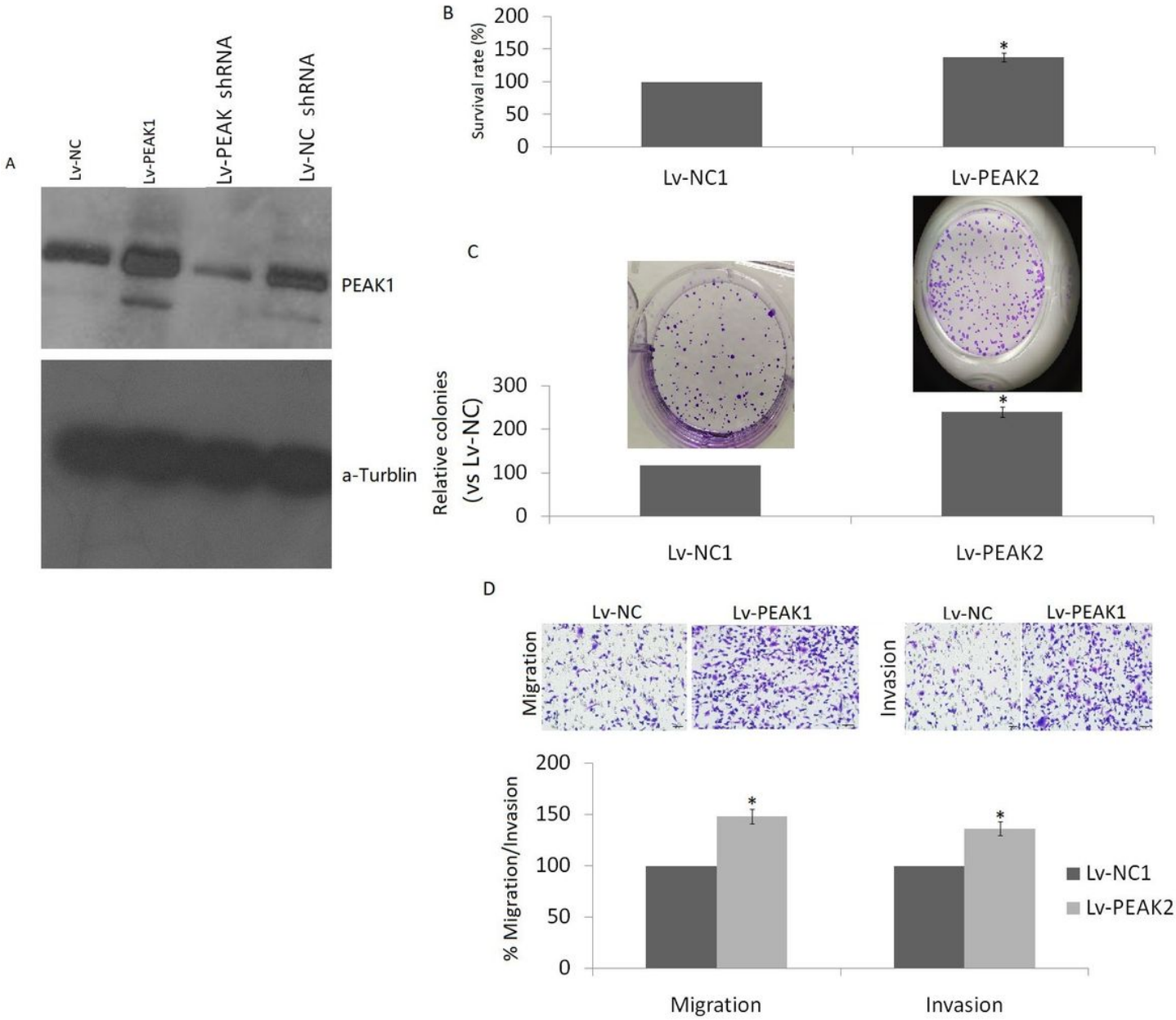

Figure 2

Effect of PEAK1 overexpression on cell growth, invasion and migration in vitro. A, PEAK1 expression was detected in MCF-7/Lv-PEAK1 or MCF-7/Lv-PEAK1 shRNA and MCF-7/ empty vector cells by western blot assay. B, cell growth was detected by MTT assay in MCF-7/Lv-PEAK1 and MCF-7/ empty vector cells. C, Cell growth was detected by colony formation assay in MCF-7/Lv-PEAK1 and MCF-7/ empty vector cells. D, The MCF-7 cells invasion and migration abilities were measured by transwell after PEAK1 overexpression. VS control, *p<0.01.

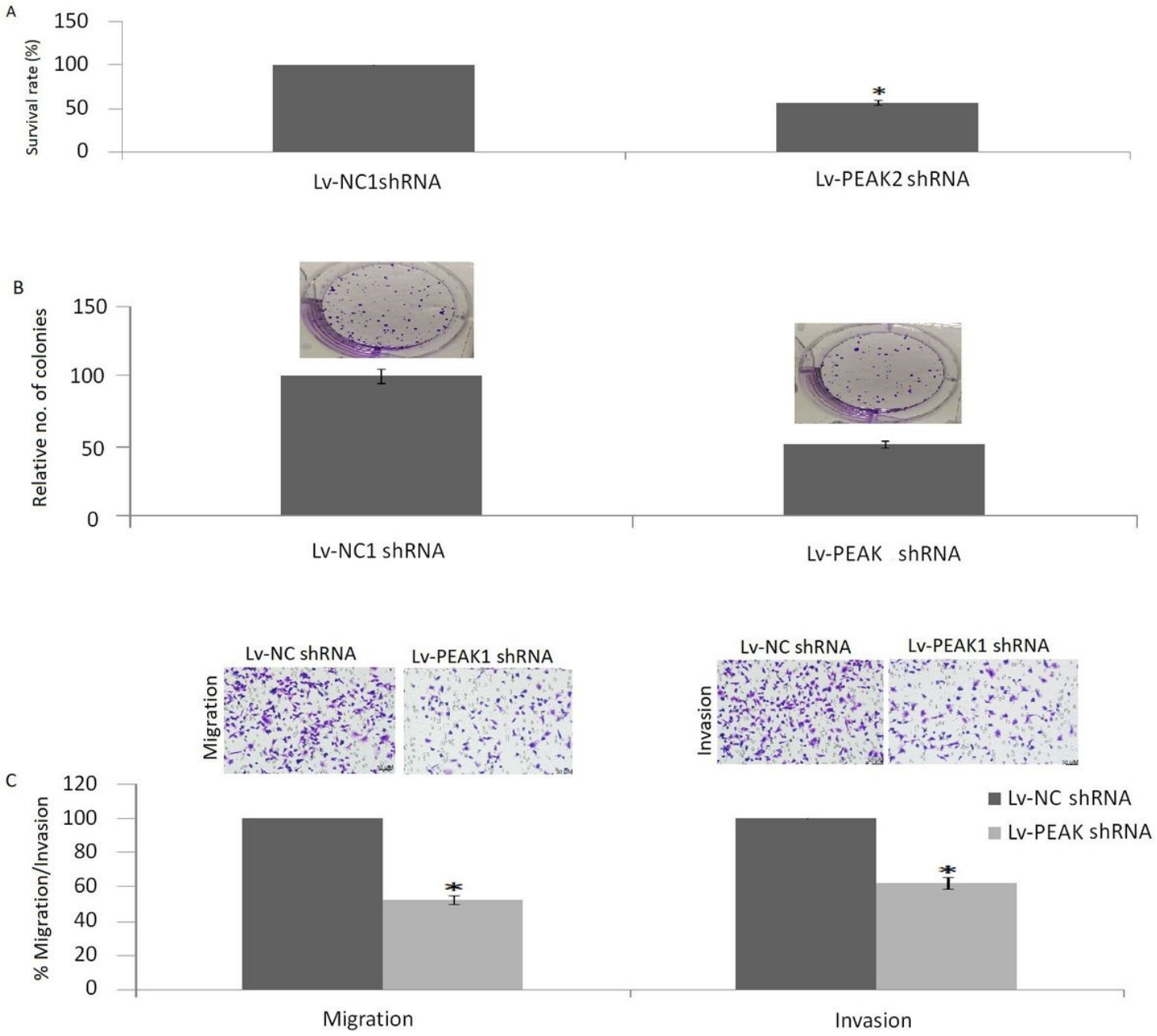

Figure 3

Effect of PEAK1 depletion on cell growth, invasion and migration in vitro. A, cell growth was detected by MTT assay in MCF-7/Lv-PEAK1 shRNA and MCF-7/empty vector cells. B, Cell growth was detected by colony formation assay in MCF-7/Lv-PEAK1 shRNA and MCF-7/ empty vector cells. C, The MCF-7 cells invasion and migration abilities were measured by transwell after PEAK1 depletion. VS control, *p<0.01.

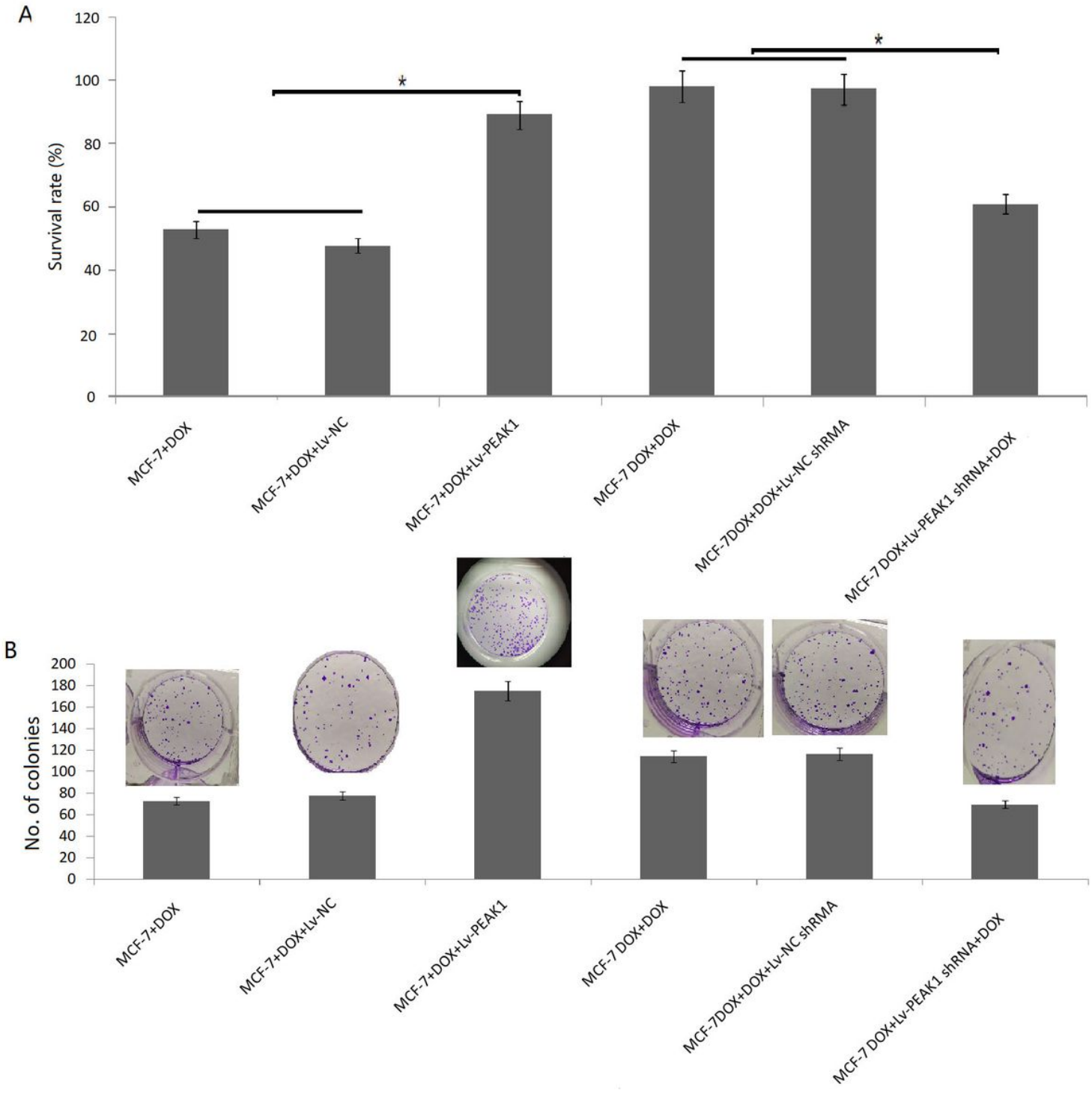

Figure 4

Effect of PEAK1 depletion or overexpression on doxorubicin sensitivity in MCF-7 cells. MCF-7/Lv-PEAK1 and MCF-7/ empty vector cells were treated with 2.0 μg/mL Doxorubicin for 72 h, MCF-7 DOX/Lv-PEAK1 shRNA and MCF-7 DOX / empty vector cells were treated with 2.0 μg/mL Doxorubicin for 72 h. Cell growth was detected by MTT assay (A) and colony formation assay(B). *p<0.01.

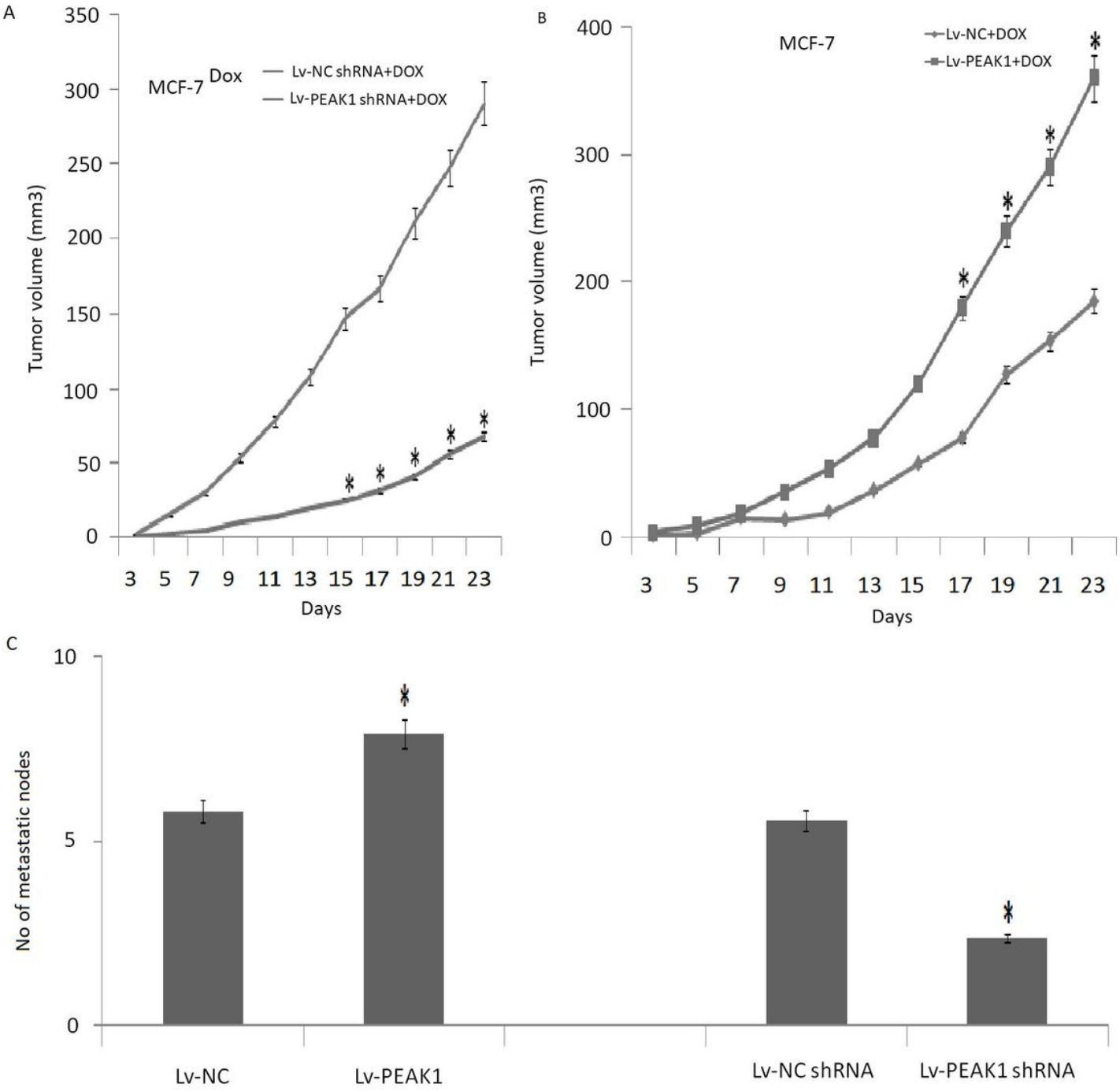

Figure 5

PEAK1 expression influences metastasis and chemosensitivity of MCF-7 cells in vivo. MCF-7DOX cells (A) or MCF-7 cells (B) with varying PEAK1 status, were injected s.c. in the left front flank of female BALB/c mice (n = 4/group) on day 0. All mice were treated with DOX by i.v. injection (day 3-9). When tumor growth became visible, tumor volume was monitored and results were graphically displayed. MCF-7 / PEAK1 or NC cells□MCF-7 /Lv-NC shRNA or MCF-7/Lv-PEAK1 shRNA cells were inoculated

subcutaneously into nude mice for 35 days. Lung metastatic nodes were counted in the lungs (C).*, $P < 0.05$, versus control group.