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Silencing of Ribosomal Protein L34 (RPL34) Inhibits the Proliferation and Invasion of Esophageal Cancer Cells

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Ribosomal protein L34 (RPL34) belongs to the L34E family of ribosomal proteins and contains a zinc finger motif. Aberrant expression of RPL34 has been reported in several human malignancies. However, the precise role and potential underlying mechanisms of RPL34 in human esophageal cancer remain largely unknown. Thus, the objective of this study was to investigate the role of RPL34 in esophageal cancer progression. Our results showed that the expression of RPL34 at both the mRNA and protein levels was frequently upregulated in esophageal cancer cell lines. Knockdown of RPL34 efficiently inhibited esophageal cancer cell proliferation, migration, and invasion in vitro. Mechanistically, knockdown of RPL34 significantly downregulated the protein expression level of p-PI3K and p-Akt in esophageal cancer cells. Finally, knockdown of RPL34 attenuated tumor growth in nude mice. In conclusion, our study revealed that RPL34 functions as an oncogene that modulates the proliferation and metastasis of esophageal cancer cells, in part, by the inactivation of the PI3K/Akt signaling pathway. Thus, these findings suggest that RPL34 may serve as a potential therapeutic target for the treatment of esophageal cancer.

Key words: Ribosomal protein L34 (RPL34); Esophageal cancer; Proliferation; Invasion; PI3K/Akt pathway

INTRODUCTION

Esophageal cancer is a major cause of death among cancers, and its incidence has continued to increase during the past decades[1,2]. Despite advances in treatment for esophageal cancer, including curative surgical resection, radiotherapy, and chemotherapy[3–5], the 5-year survival rate of esophageal cancer patients is less than 30%[6]. Therefore, exploring the molecular mechanism underlying the development of esophageal cancer is crucial in order to develop new treatment strategies for esophageal cancer.

Ribosomal proteins (RPs) are the main components of ribosomes. Previous studies showed that RPs play an important role in regulating a variety of physiological and pathological processes, such as self-assembly of ribosomes, protein synthesis, cell proliferation, DNA repair, and tumorigenesis[7–9]. Ribosomal protein L34 (RPL34) belongs to the L34E family of RPs and contains a zinc finger motif. Aberrant expression of RPL34 has been reported in several human malignancies, including non-small cell lung cancer (NSCLC), gastric cancer, and osteosarcoma[10–12]. A recent study by Wei et al. confirmed that the expression of RPL34 was upregulated in human pancreatic cancer (PC) tissues and cell lines, and knockdown of RPL34 substantially inhibited the proliferation, colony formation, migration, and drug resistance of PC cells[13]. However, the precise role and potential underlying mechanisms of RPL34 in human esophageal cancer remain largely unknown. Thus, the objective of this study was to investigate the role of RPL34 in esophageal cancer progression. Our data suggest that RPL34 overexpression is associated with esophageal cancer progression and may represent a novel potential prognostic marker for patients with esophageal cancer.

MATERIALS AND METHODS

Cell Culture

The human esophageal cancer cell lines (EC9706, TE13, and ECA109) and esophageal epithelial cell line (HEEC) were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM with 10% fetal bovine serum (FBS), 100 U/ml

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penicillin, and 0.1 mg/ml streptomycin (Sangon Co. Ltd., Shanghai, P.R. China) at 37°C in a 5% CO_2 humidified chamber.

Short Hairpin RNA and Cell Transfection

The short hairpin RNA (shRNA) targeting RPL34 (sh-RPL34) and its negative control (sh-NC) were designed and synthesized by GeneChem Co. Ltd. (Shanghai, P.R. China). ECA109 cells were transfected with sh-RPL34 or sh-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and were used 48 h posttransfection. Depletion of the targeted genes was confirmed with quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized with the PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Japan). qRT-PCR was performed using the SYBR Green Master Mixture on the HT7900 system (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: RPL34, 5′-GTTTGACATACCGACGTAGG C-3′ (forward) and 5′-GCACACATGGAACCACCATA G-3′ (reverse); GAPDH, 5′-TGACTTCAACAGCGACA CCCA-3′ (forward) and 5′-CACCCTGTTGCTGTAGC CAAA-3′ (reverse). The comparative threshold cycle method (ΔΔCt) was used to determine the level of gene expression[14].

Western Blot

Total protein was extracted using RIPA lysis buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerol phosphate, 2 mM EDTA, 0.2 mM Na_3VO_4, 1% Triton X-100, and protease inhibitor cocktail, pH 7.4) according to the manufacturer's instructions, and protein concentrations were measured using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Berkeley, CA, USA). Forty micrograms of protein from each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Sigma-Aldrich, St. Louis, MO, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h followed by incubation in the primary antibodies (anti-RPL34, anti-E-cadherin, anti-N-cadherin, anti-PI3K, anti-p-PI3K, anti-Akt, anti-p-Akt, and anti-GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After washing with TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Finally, the signals were detected by enhanced chemiluminescence reagent.

Cell Proliferation Assay

Cell proliferation was measured using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. In brief, ECA109 cells at a density of 1×10^5 cells per well were seeded onto a 96-well cell culture plate. After 24, 48, 72, or 96 h, 5 mM MTT was added into each well for 4 h. Next, cells were resuspended in 100 μl of dimethyl sulfoxide (DMSO), and the optical density (OD) of the solution was determined using a spectrophotometer at 490 nm.

Cell Invasion and Migration Assays

For the Transwell invasion assay, ECA109 cells at a density of 1×10^5 cells per well were plated into the upper chamber. Cells were allowed to invade through the Matrigel-coated inserts at 37°C for 16 h. For the migration assay, ECA109 cells at a density of 1×10^5 cells per well were plated into the upper chamber without Matrigel for 16 h. For both assays, 600 μl of DMEM supplemented with 10% FBS was added to the lower chamber. The cells on the lower surface of the membrane were fixed with 95% ethanol and stained with 0.1% crystal violet, and then counted under a light microscope (Olympus Corp., Tokyo, Japan).

Xenograft Tumor Model

BALB/c-nu mice (4–5 weeks of age, 18–20 g) were purchased from the Center of Experimental Animal of The First Affiliated Hospital of Zhengzhou University (P.R. China). ECA109 cells transfected with sh-RPL34 and the corresponding control cells (5×10^6) were suspended in 200 μl of PBS and then injected subcutaneously into the right flank of mice ($n=6$/group). Tumor size was evaluated by caliper measurements and calculated with the formula: volume = $(\text{width})^2\times\text{length}/2$ every 5 days. Mice were sacrificed after 20 days, and the tumors were dissected and weighed. All experimental procedures were approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Zhengzhou University.

Statistical Analysis

All experiments were performed at least in triplicate. Data are presented as mean ± SD. Statistical comparisons were performed using one-way analysis of variance followed by the Student's *t*-test. A value of $p<0.05$ was considered significant.

RESULTS

RPL34 Was Upregulated in Esophageal Cancer Cell Lines

We first investigated the gene expression levels of RPL34 in human esophageal cancer cell lines using

qRT-PCR analysis. The mRNA expression of RPL34 was remarkably higher in human esophageal cancer cell lines (EC9706, TE13, and ECA109) compared to the control cell line HEEC (Fig. 1a). Similarly, Western blot analysis revealed significantly increased RPL34 protein expression in human esophageal cancer cell lines compared to the HEEC cell line (Fig. 1b).

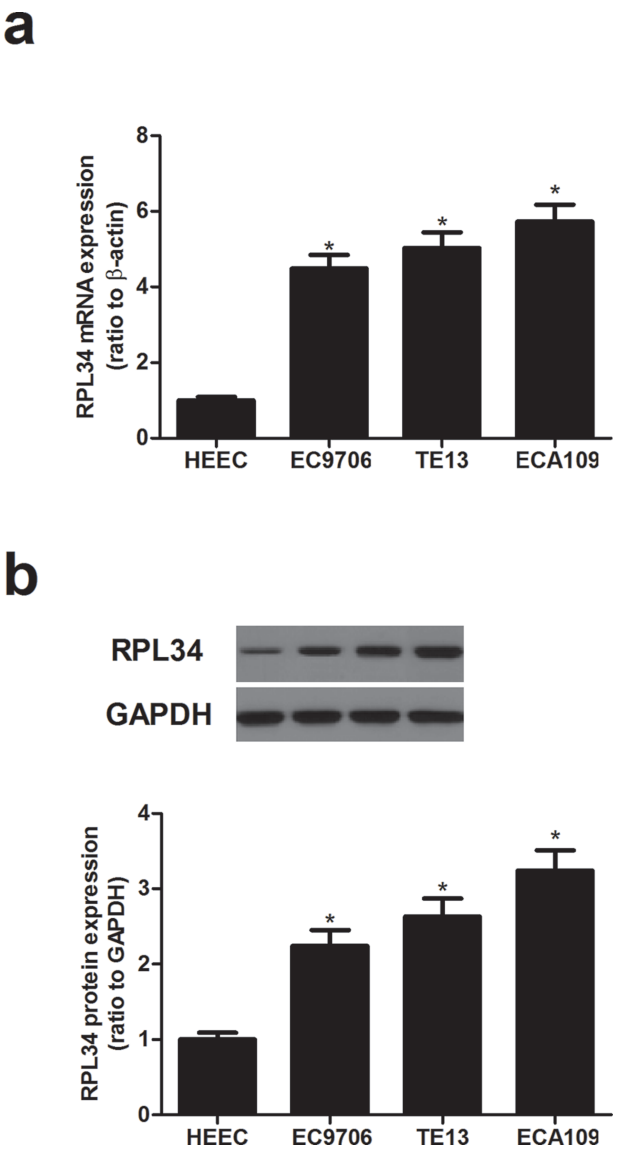

Figure 1. RPL34 was upregulated in esophageal cancer cell lines. (a) The mRNA expression of RPL34 was evaluated in human esophageal cancer cell lines using quantitative real-time polymerase chain reaction (qRT-PCR) analysis. (b) The protein expression of RPL34 was measured in human esophageal cancer cell lines using Western blot analysis. Experiments were performed in triplicate. *$p < 0.05$ versus HEEC group.

Knockdown of RPL34 Inhibited Esophageal Cancer Cell Proliferation In Vitro

To examine the biological role of RPL34 in esophageal cancer progression, we employed shRNA against RPL34 to stably knock down RPL34 in ECA109 cells. The expression of RPL34 at both the mRNA and protein levels was significantly downregulated in ECA109 cells transfected with sh-RPL34, compared with the sh-NC group (Fig. 2a and b). Furthermore, we investigated the effect of RPL34 on esophageal cancer cell proliferation by the MTT assay. The results showed that downregulation of RPL34 expression obviously suppressed the proliferation of ECA109 cells in a time-dependent manner (Fig. 2c).

Knockdown of RPL34 Inhibited Esophageal Cancer Cell Invasion and Migration In Vitro

Next, we examined the effects of RPL34 on esophageal cancer cell invasion and migration using Matrigel invasion and Transwell migration assays. The number of ECA109 cells passing through the Transwell with Matrigel was significantly lower in the sh-RPL34 group than those in the sh-NC group (Fig. 3a). Similarly, we observed that downregulation of RPL34 expression sharply inhibited the migration of ECA109 cells (Fig. 3b). Furthermore, we analyzed the protein level of several epithelial–mesenchymal transition (EMT) markers in RPL34-silenced ECA109 cells. The results of the Western blot analysis indicated that knockdown of RPL34 sharply upregulated E-cadherin expression and downregulated N-cadherin in ECA109 cells (Fig. 3c).

Knockdown of RPL34 Inhibited the PI3K/Akt Signaling Pathway in Esophageal Cancer Cells

To determine the mechanism by which sh-RPL34 inhibited esophageal cancer cell proliferation and invasion, we examined the effect of RPL34 deregulation on the protein expression of p-PI3K and p-Akt by Western blot. Compared with the sh-NC group, knockdown of RPL34 significantly downregulated the protein expression levels of p-PI3K and p-Akt in ECA109 cells (Fig. 4a). We then examined the effect of the PI3K inhibitor (LY294002) on ECA109 cell proliferation and invasion. We observed that treatment of 15 μM LY294002 markedly decreased cell viability; thus, 10 μM LY294002 was chosen for additional experiments (Fig. 4c). In addition, cell proliferation (Fig. 4d) and invasion (Fig. 4e) of ECA109 cells were significantly suppressed by LY294002 treatment.

Knockdown of RPL34 Inhibited Esophageal Cancer Progression In Vivo

We used a xenograft tumor model to examine the role of RPL34 in esophageal cancer progression in vivo.

Tumors formed by RPL34-silenced ECA109 cells were smaller in size than the tumors formed by control cells (Fig. 5a). In addition, downregulation of RPL34 expression in ECA109 cells strikingly reduced the weight of tumors compared to control mice (Fig. 5b).

DISCUSSION

To the best of our knowledge, this study is the first to report that RPL34 may play an important role in the development and progression of esophageal cancer. The expression of RPL34 at both mRNA and protein levels was frequently upregulated in human esophageal cancer cell lines. Knockdown of RPL34 efficiently inhibited esophageal cancer cell proliferation, migration, and invasion in vitro. Mechanistically, knockdown of RPL34 significantly downregulated the protein expression level of p-PI3K and p-Akt in esophageal cancer cells. Finally, knockdown of RPL34 attenuated tumor growth in nude mice.

Mounting evidence suggests that deregulation of RPL34 is associated with various types of human cancer. A recent study showed that the expression of RPL34 was significantly upregulated in NSCLC tissues compared to adjacent normal tissues. Lentivirus-mediated shRNA knockdown of RPL34 resulted in an obvious decrease in proliferation in the NSCLC cell line[11]. RPL34 has also been reported to be overexpressed in gastric cancer cell lines, and knockdown of RPL34 expression significantly suppressed cell proliferation and increased apoptosis in human gastric cancer cells[10]. In accordance with these findings, herein we observed that the expression of RPL34 at both the mRNA and protein levels was significantly increased in human esophageal cancer cell lines, and knockdown of RPL34 expression inhibited the tumorigenicity of esophageal cancer cells, suggesting that abnormal expression of RPL34 may contribute to tumorigenesis in esophageal cancer cells.

Tumor metastasis is the result of cancer cell migration and invasion[15]. EMT plays a pivotal role in driving esophageal cancer metastasis[16]. Decreased expression of E-cadherin is a critical molecular event of EMT, which endows the epithelial cells with fibroblast-like properties and induces migration and invasion[17]. In addition, a prior study reported that the expression of E-cadherin was greatly decreased in esophageal cancer tissues, and the reduction of E-cadherin was closely associated with tumor dedifferentiation, infiltrative growth, and lymph node metastasis[18]. In the current study, we observed that knockdown of RPL34 efficiently inhibited esophageal cancer cell migration and invasion in vitro. Moreover, we observed that knockdown of RPL34 sharply upregulated E-cadherin expression and downregulated N-cadherin in ECA109 cells. These results are in line with that from PC cells and partly support our data from esophageal

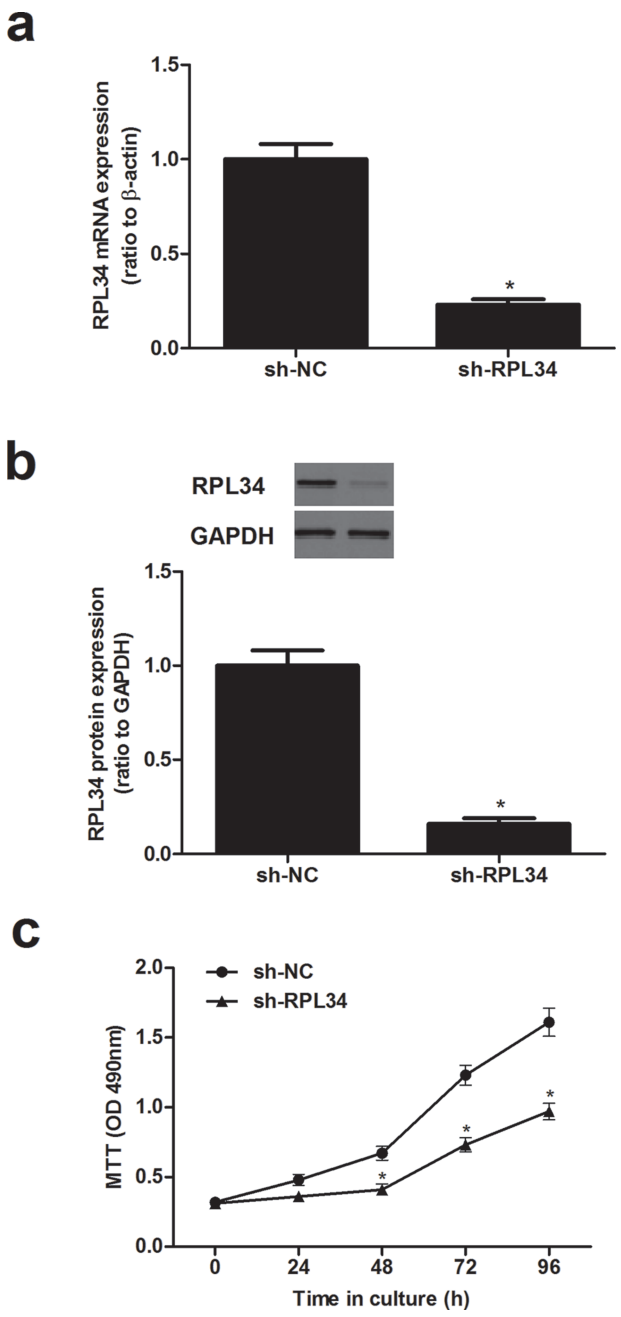

Figure 2. Knockdown of RPL34 inhibited esophageal cancer cell proliferation in vitro. ECA109 cells were transfected with sh-RPL34 or sh-NC for 48 h, respectively. (a) The mRNA expression of RPL34 was evaluated in ECA109 cells using qRT-PCR analysis. (b) The protein expression of RPL34 was detected in ECA109 cells using Western blot analysis. (c) The effect of RPL34 on esophageal cancer cell proliferation was measured by the MTT assay. Experiments were performed in triplicate. *$p < 0.05$ versus sh-NC group.

cancer cell lines showing that depletion of RPL34 is sufficient to suppress the EMT phenotype, resulting in the inhibition of migration and invasion of esophageal cancer cells.

There is growing evidence that the PI3K/Akt signaling pathway is important in the development and progression of esophageal cancer[19–21]. PI3K/Akt is constitutively activated in human esophageal tumor tissues[22]. Akt encodes a serine/threonine kinase and is activated through phosphorylation, which mediates the activation of target genes, thus regulating cell proliferation, angiogenesis, invasion, metastasis, and survival[23]. Several studies have reported that specific inhibitors of PI3K/Akt significantly suppress esophageal cancer growth in vitro and in vivo[24–26]. In the present study, we observed that knockdown of RPL34 significantly downregulated the protein expression levels of p-PI3K and p-Akt in esophageal cancer cells. These data suggest that knockdown of RPL34

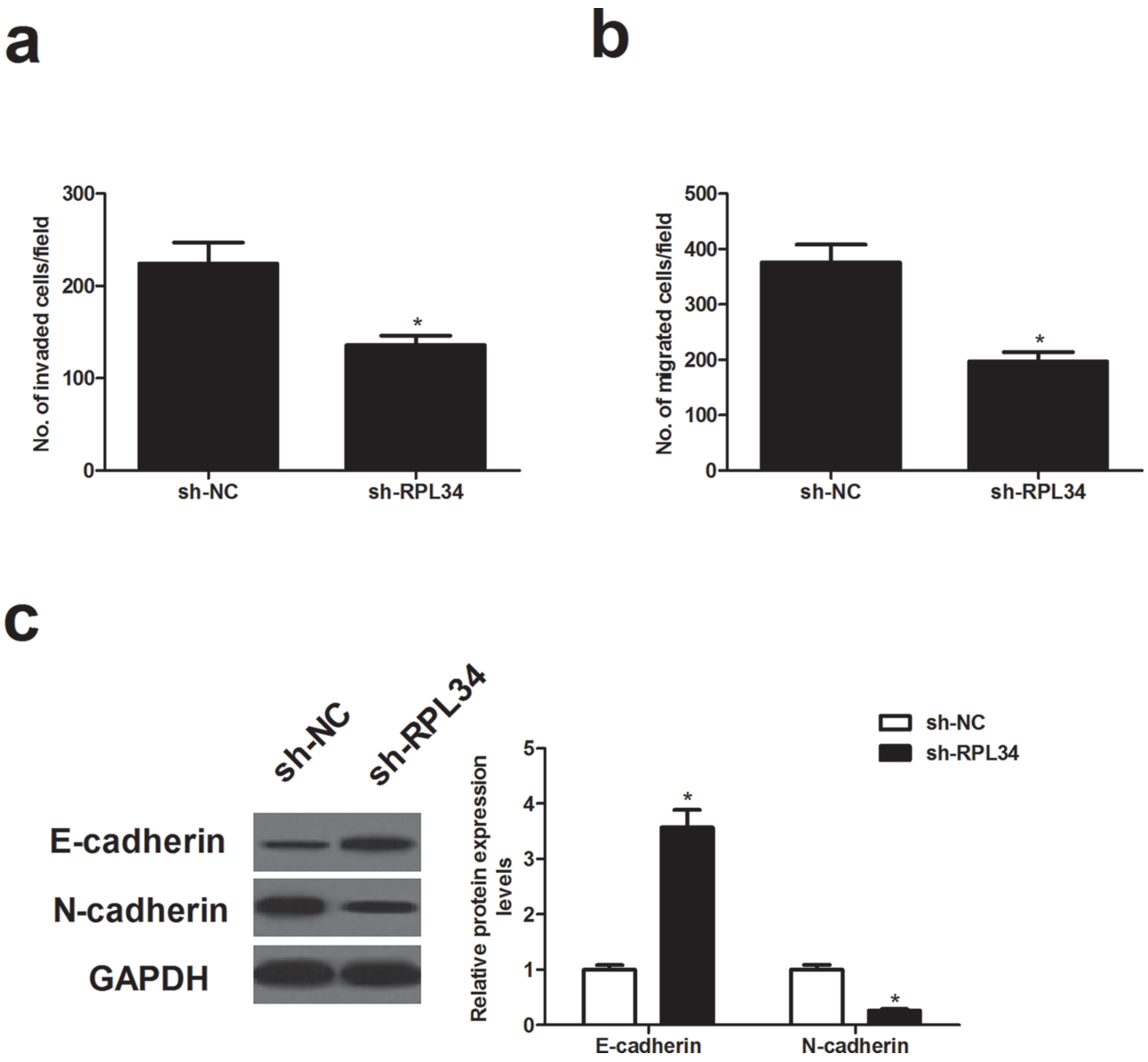

Figure 3. Knockdown of RPL34 inhibited esophageal cancer cell invasion and migration in vitro. (a) Matrigel invasion assay was used to evaluate the effect of RPL34 on cell invasion in ECA109 cells transfected with sh-RPL34. (b) Transwell migration assay was used to evaluate the effect of RPL34 on cell migration in ECA109 cells transfected with sh-RPL34. (c) The protein levels of E-cadherin and N-cadherin were measured by Western blot in RPL34-silenced ECA109 cells. Experiments were performed in triplicate. *$p < 0.05$ versus sh-NC group.

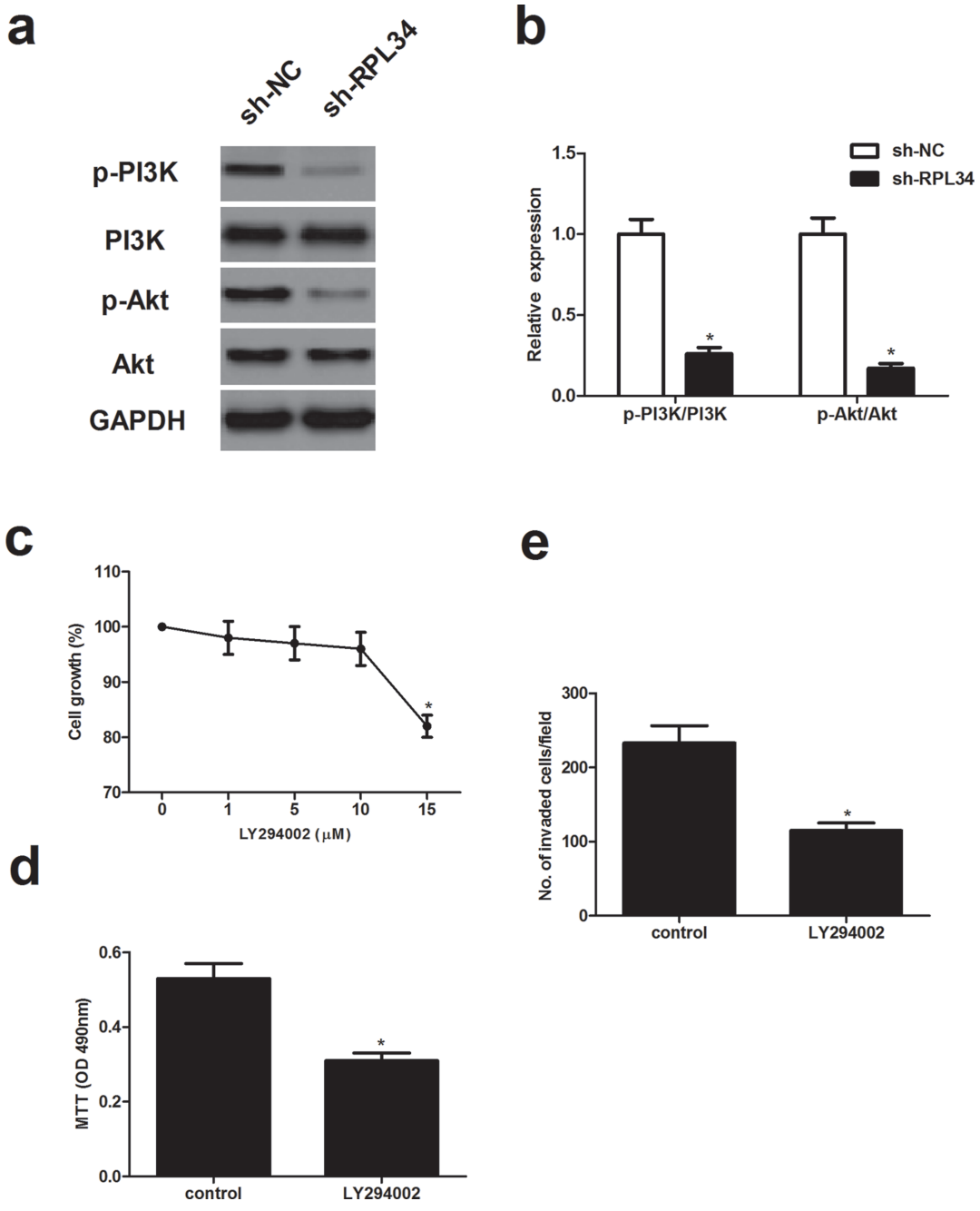

Figure 4. Knockdown of RPL34 inhibited the PI3K/Akt signaling pathway in esophageal cancer cells. ECA109 cells were transfected with sh-RPL34 or sh-NC for 48 h, respectively. (a) The protein expression of p-PI3K and p-Akt was detected in ECA109 cells using Western blot analysis. (b) The relative protein expression levels of p-PI3K and p-Akt were quantified using the Image-Pro Plus 6.0 software and normalized to GAPDH. *$p<0.05$ versus sh-NC group. (c) ECA109 cells were treated with various concentrations of LY294002 for 24 h, and cell growth was measured using the MTT assay. ECA109 cells were treated with 10 μM LY294002 for 24 h. (d) Cell proliferation was measured by the MTT assay. (e) Cell invasion was measured by the Matrigel invasion assay. Experiments were performed in triplicate. *$p<0.05$ versus control group.

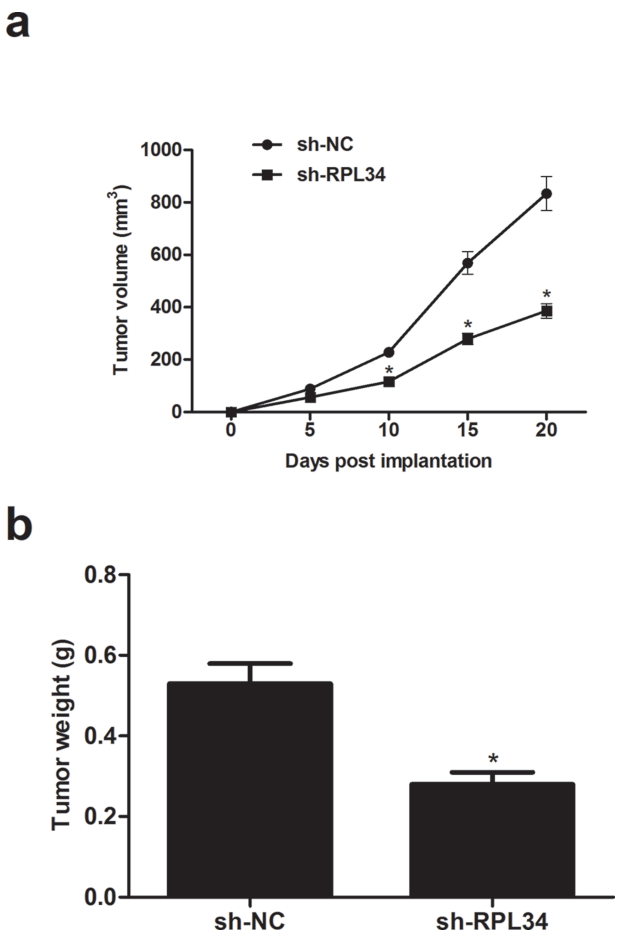

Figure 5. Knockdown of RPL34 inhibited esophageal cancer progression in vivo. ECA109 cells transfected with sh-RPL34 and the corresponding control cells (5×10^6) were suspended in 200 µl of PBS and then injected subcutaneously into the right flank of mice. (a) The volume of tumors was monitored every 5 days. (b) Mice were sacrificed after 20 days, and the tumors were dissected and weighed. Experiments were performed in triplicate. *$p<0.05$ versus sh-NC group.

suppressed the proliferation and metastasis of esophageal cancer cells, likely through inactivation of the PI3K/Akt signaling pathway.

In conclusion, our study revealed that RPL34 functions as an oncogene that modulates the proliferation and metastasis of esophageal cancer cells in part through the inactivation of the PI3K/Akt signaling pathway. Thus, these findings suggest that RPL34 may serve as a potential therapeutic target for the treatment of esophageal cancer.

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