MiR-185-5p targets RAB35 gene to regulate tumor cell-derived exosomes mediated proliferation, migration and invasion of non-small cell lung cancer cells

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Abstract

Background: Non-small cell lung cancer (NSCLC) is the most common malignant tumor, and its recurrence and metastasis are the main causes of death. Recently, there are evidences that tumor derived exosomes play an important role in the occurrence and development of non-small cell lung cancer.

Material/Methods: First, miR-185-5p and RAB35 expression in tumor tissues, paracancerous healthy tissues, lung cancer cell lines and normal bronchial epithelial cell line were detected. Then, miR-185-5p and RAB35 were over-expressed/knocked down to study their effects on A549 cells and H2170 cells proliferation, migration and invasion . Next, bioinformatics analysis and luciferase reporter gene analysis verified the targeting relationships of miR-185-5p and RAB35 , respectively. Finally, the exosomes secreted by tumor cells with RAB35 gene downregulated or miR-185-5p overexpression were co cultured with their parent cells to verify the regulatory effect of RAB35 on the secretion and function of exosomes.

Results: The miR-185-5p expression was downregulated, while RAB35 expression was prominently upregulated in NSCLC tissues and cell lines. Moreover, miR-185-5p overexpression or RAB35 downregulated suppressed cells proliferation, migration and invasion. Furthermore, we clarified that RAB35 was a direct target of miR-185-5p. Additionally, exosomes derived from tumor cells could restore cells proliferation, migration and invasion, while exosomes secreted by tumor cells with RAB35 downregulated or mR-185-5p overexpression lose the ability to restore cells proliferation, migration and invasion.

Conclusions: Our findings indicate that miR-185-5p targets RAB35 gene to regulate tumor cell-derived exosomes-mediated proliferation, migration and invasion of NSCLC cells.

Background

Lung cancer is the most common malignant tumor in the world, and its mortality and morbidity are the highest in malignant tumors [1]. According to different histological behaviors and pathological manifestations, lung cancer is mainly divided into non-small cell lung cancer and small cell lung cancer (SCLC) [2]. Among them, NSCLC is the most common type of lung cancer, accounting for 80%–85% of all types, mainly including lung adenocarcinoma, squamous cell carcinoma and large cell carcinoma [3].

MicroRNAs (miRNAs) are noncoding single stranded RNA molecules with 18–23 bases encoded by endogenous genes, which regulate gene expression at post transcriptional level [4]. At present, the change of miRNAs expression has been found in a variety of cancers. The abnormal expression of miRNAs plays an important role in the development process of cancer, such as proliferation, differentiation and apoptosis [5,6,7]. It is suggested that miRNAs may be related to the pathogenesis of cancer, tumor growth and metastasis, and play the role of oncogene or tumor suppressor gene. Zhang et al [8] research showed that microRNA-126 inhibits tumor cell invasion and metastasis by downregulating ROCK1 in renal cell carcinoma. Pan et al [9] reported that miRNA-370 acts as a tumor suppressor via the downregulation of PIM1 in hepatocellular carcinoma. Similarly, Yang et al [10] found that miRNA-155 was significantly

increased in glioma tissue and promoted tumor cells proliferation by targeting caudal type homeobox 1 (CXD-1).

RAB GTPases are members of the RAS superfamily of small GTPase proteins [11]. About 70 RAB proteins have been isolated from human cells. RAB GTPases widely exist in many kinds of tissue cells [12]. It regulates many steps of membrane transport by forming active membrane structure and binding corresponding effector proteins, including vesicle formation, vesicle movement along actin and tubulin network, and vesicle membrane fusion [13]. Among them, RAB35 is described as an endosome related protein in different cells [14]. Hsu Chieh et al. [15] found that inhibition of the function of RAB35 led to the accumulation of inner vesicles in oligodendrocytes and impaired the secretion of extracellular bodies, suggesting that RAB35 plays a direct role in the biosynthesis and secretion of exosomes.

Exosomes are small membranous vesicles with a diameter of about 30~100 nm, which are released into extracellular matrix after the fusion of intracellular vesicles and cell membrane [16]. Exosomes come from a wide range of sources and can be secreted by almost all types of cells, including tumor cells [17]. It is also found in almost all body fluids, such as plasma, cerebrospinal fluid, urine, breast milk, saliva and secretions [18,19]. In addition, exosomes contain a variety of proteins, lipids and nucleic acids and participate in many physiological processes such as immune response, antigen presentation, intercellular communication, protein and RNA transport, which is an important tool for intercellular material and information exchange [20,21]. Some of the proteins in exosomes are unique to exosomes, while the four transmembrane proteins such as CD63, CD81 and TSG101 are shared by exosomes, which provide theoretical basis for isolation and identification of exosomes [22].

More and more studies have shown that exosomes derived from tumor cells are related to the occurrence and deterioration of tumor. They are involved in different processes such as tumor microenvironment regulation, tumor invasion, angiogenesis, tumor escape [23,24,25,26]. In conclusion, in this study, we found that miR-185-5p can inhibit the proliferation, migration and invasion of NSCLC cells mediated by exosomes from tumor cells by targeting RAB35 gene. The findings of this study may help to develop a potential treatment for non-small cell lung cancer.

Materials And Methods

Tissue samples

Thirty-five pairs of resected specimens were selected randomly, all of them were non-small cell lung cancer patients with complete clinical data and confirmed by pathological diagnosis. The average age of the patients was (46.2 ± 8.1) years old. All patients had not received any form of anti-cancer treatment three months before the operation. Each specimen included lung cancer tissues and lung tissues more than 5 cm away from the cancer focus. This study protocol was approved by the Ethics Committee of Huaihe Hospital of Henan University (Kaifeng, China), and written informed consent was obtained from each participant.

Cell lines, Transfection and interference

The human NSCLC cell lines A549, SPC-A1, PC9, H2170 and SK-MES-1 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Gibco, Rockville, MD) containing 10 % fetal bovine serum and 100 U/ml penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA) with 5% CO_2 at 37 °C. NC-mimic, miR-185-5p mimic, pcDNA3.1 and pcDNA-RAB35 were purchased from RiboBio Co., Ltd (Guangzhou, China) and transfected using RiboBio Transfection Kit (RiboBio Co., Ltd). Small interfering RNA of RAB35 (siRAB35) and scramble siRNA of RAB35 (Scramble) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All transfection reagent transfected into cells using Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated from osteoarthritis chondrocytes by using the TRIzol (Invitrogen, Carlsbad, CA, USA)). Single-stranded cDNA was synthesized with the PrimeScript Reagent Kit (Promega, USA). Real-time PCR was conducted by using SYBR Premix Ex TaqTM Kit (Applied Biosystems, Foster City, CA, USA). The reaction was run in ABI7500 Real-time PCR system (Applied Biosystems, Carlsbad, CA). GAPDH was used as an endogenous control. The RT PCR cycling conditions consisted of: 95 ˚C for 10 min; then 35 cycle amplification for 20 s at 95 ˚C, 30 s at 55 ˚C, 15 s at 72 ˚C; followed by 1min at 72 ˚C. The primers used in this study were synthesized from Sangon Biotech (Shanghai, China). The level of mRNA was normalized to β-actin expression using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

The A549 cells or H2170 cells cultured for the indicated periods of time (0–4days), and cell viability was detected using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China) and read using a microplate reader (Synergy HT, BioTek), at 450 nm.

Transwell assays

The A549 cells and H2170 cells were maintained in RPMI supplemented with 10% FBS, and 1×10^5 cells were plated on BD BioCoat MATRIGEL Invasion Chambers with Matrigel and control inserts with polyethylene terephthalate membrane (BD Biosciences, San Jose, CA, USA) to assess the cell migratory and invasive abilities as previously described.

Luciferase Reporter Gene Analysis

The putative relationship between miR-185-5p and RAB35 were predicted using TargetScan (http://www.targetscan.org/). The wild type and mutant 3'-UTR sequences of RAB35 were cloned into the pcDNA3.1 (+) vector (Cosmogenetech, Seoul, Korea) containing the luciferase reporter gene, respectively. HEK293 cells were seeded in 24-well plates, and when grown to approximately 70% confluence, co-

transfected with luciferase plasmid and miR-185-5p mimics or NC mimics using Lipofectamine 2000. After 24 hours of transfection, the luciferase reporter activity was measured by Dual-Luciferase Reporter Assay System (Promega, Madison, WI) under the manufacturer's instructions.

Western blotting

Protein homogenates from A549 cells and H2170 cells were extracted as previously described. Briefly, the cells were lysed for 20 min on ice in ice-cold lysis buffer (Roche). The lysates were centrifuged at 12,000 × g for 20 min at 4◦C to obtain a clear lysate. The protein content of each sample was determined using the BCA Protein Assay Kit (Thermo Scientific). Then, equal amounts of proteins(12μg/lane) were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA).The membranes were blocked in 5% (w/v) nonfat dry milk in TBST (Tris-buffered saline–0.1% Tween) at 25 °C for 3 h and then incubated with the following primary antibodies: β-actin (1:800, Abcam, EPR16769), RAB35 (1:700, Abcam, ab152138), TSG101 (1:1500, Abcam, ab125011), CD63 (1:1000, Abcam, EPR21151), HSP70 (1:1000, Abcam, EPR16892). The bands were visualized using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:2,000, Boster) prior to the ECL protocol (Amersham Biosciences, Piscataway, NJ, USA).

Exosome isolation and purification

Exosomes were isolated from the supernatant either of the A549 or H2170 cell culture media. In brief, 2×10^6 cells were plated in a Petri dish with RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. The following day, the medium was changed to exosome-free RPMI supplemented with 10% exosome-depleted FBS. The culture medium was collected until the cells reached 70□80% confluence and exosomes were isolated by ultracentrifugation with a rotor (L8-80M; Beckman, Brea, CA, USA) at the centrifugal force of 110,000 × g for 70 min. Exosomes were re-washed in PBS at 110,000 × g for 70 min to eliminate contaminating proteins, then re-suspended in 100 μl PBS and immediately tested or stored at -80˚C for further analysis.

Exosome enzyme treatment

The exosomes extracted from A549 cells and H2170 cells were treated with 2 μg/ml RNase, which was hydrolyzed at 37 ℃ for 10 min, and then the reaction was terminated with RNase inhibitor (10U/ml). Then, protease K was added and hydrolyzed at 37 ℃ for 60 min. Then the temperature was adjusted to 60 ℃, and the protease was inactivated for 10 minutes. After centrifugation at 110,000 × g for 70 min in 4 ℃, the supernatant was discarded, and the PBS solution was resuspended and precipitated as the exosomes.

Statistical analysis

All statistical analyses were performed using the SPSS software (ver. 13.0; SPSS, Chicago, IL). The quantitative data derived from three independent experiments are expressed as mean ± SEM. Significance was determined by a one-way ANOVA with the Student paired t-test. Values of $P < 0.05$ were considered statistically significant.

Results

Overexpression of miR-185-5p inhibited proliferation, migration and invasion of NSCLC cells.

The expression of miR-185-5p was detected in 35 pairs of NSCLC tissues and paracancerous healthy tissues collected in the early stage. RT-PCR results showed that miR-185-5p was significantly down-regulated in tumor tissues (Fig. 1A). In addition, we found that miR-185-5p is also down-regulated in different lung cancer cell lines (Fig. 1B).

To further explore the regulatory role of miR-185-5p in tumor progression, miR-185-5p mimics were transfected into A549 cells and H2170 cells, respectively. RT-PCR results showed that miR-185-5p mimics significantly increased the expression of miR-185-5p in two kinds of cells (Fig. 1C and 1D). CCK-8 assay results showed that miR-185-5p mimics significantly inhibited the proliferation of A549 cells and H2170 cells (Fig. 2A). Compared with the control group, the migration and invasion of the two kinds of cells were significantly inhibited after miR-185-5p mimics were transfected (Fig. 2B and 2C). These results suggest that miR-185-5p is a tumor suppressor gene in NSCLC.

Knock-down of RAB35 inhibited proliferation, migration and invasion of NSCLC cells

In order to further reveal the regulatory mechanism of miR-185-5p in NSCLC, we found that there was a highly conserved binding sequence between miR-185-5p and RAB35 through online bioinformatics database retrieval, suggesting that miR-185-5p may adsorb RAB35 (Fig. 3A). Furthermore, luciferase reporter gene analysis showed that miR-185-5p mimics significantly reduced the luciferase activity of wild-type RAB35, while the luciferase activity of mutant-RAB35 did not change significantly (Fig. 3B). In addition, RT-qPCR and Western blotting results showed that miR-185-5p mimics significantly inhibited the expression of RAB35 mRNA and protein in the two kinds of cells (Fig. 3C and 3D). These results indicate that RAB35 is a direct regulatory target of miR-185-5p.

In order to further explore the role of RAB35 in NSCLC, we detected 35 pairs of non-small cell lung cancer tumor tissues and paracancerous healthy tissues by RT-PCR, and the results showed that RAB35 expression was significantly up-regulated in tumor tissues (Fig. 4A). Using KM plotter software to analyze the expression and survival of RAB35 gene in 1928 lung cancer patients, we found that the median survival of patients with low expression of RAB35 was 99.43 months, while that of patients with high expression of RAB35 was only 53 months (Fig. 4B). Similarly, the expression of RAB35 was up-regulated in different lung cancer cell lines (Fig. 4C). Next, RAB35 siRNA were transfected into A549 cells and H2170 cells, respectively. Western blotting results showed that RAB35 siRNA significantly inhibited the expression of RAB35 protein (Fig. 4D). CCK-8 assay showed that RAB35 siRNA significantly inhibited cell

proliferation in the two kinds of cells (Fig. 5A). The results of Trans-well assay showed that the migration and invasion of A549 cells and H2170 cells were significantly inhibited by RAB35 siRNA (Fig. 5B and 5C).

MiR-185-5p inhibited proliferation, migration and invasion of NSCLC cells by targeting RAB35

To further verify whether the regulation of miR-185-5p on lung cancer was related to RAB35, the pcDNA-RAB35 was transfected into A549 cells and H2170 cells alone or together with miR-185-5p mimics, respectively. Western blotting results showed that miR-185-5p mimics significantly inhibited RAB35 expression compared with pcDNA-RAB35 transfected alone (Fig. 6A). Moreover, the results of CCK-8 assay indicated that miR-185-5p mimics significantly reversed the promotion of pcDNA-RAB35 on cell proliferation (Fig. 6B). As expected, compared with pcDNA-RAB35 transfected alone, the migration and invasion of A549 cells and H2170 cells were significantly inhibited after transfected together with miR-185-5p mimics (Fig. 6C and 6D). These results confirmed that miR-185-5p inhibited proliferation, migration and invasion of NSCLC cells by targeting RAB35.

Knockdown of RAB35 reduced the number of exosomes from tumor cells and inhibited its function

A large number of studies have shown that tumor derived exosomes play an important role in tumor proliferation, migration and invasion. Therefore, miR-185-5p mimics and RAB35 siRNA were transfected into A549 and H2170 cells, respectively. Next, we collected cell culture medium after 48 h and extracted exosomes. Western blotting results showed that TSG101 protein and CD63 protein were almost not expressed in tumor cells and significantly increased in the exosomes secreted by tumor cells, but the expression of these two proteins were significantly inhibited in A549 cells and H2170 cells with down-regulated of RAB35 (Fig. 7A and 7B). In addition, HSP70 protein, which can be actively released into exosomes from cells under basic or stress induction, was also significantly reduced in exosomes secreted by tumor cells with down-regulated of RAB35 (Fig. 7C). These results indicate that the down-regulation of RAB35 expression inhibits protein synthesis in exosomes and reduces the number of exosomes.

Exosomes from RAB35 down-regulated tumor cells inhibited the proliferation of NSCLC cells

To further study the effect of exosomes secreted by tumor cells on the proliferation of tumor cells, the exosomes were isolated and extracted from A549 and H2170 cells, respectively, and added them to two cell models of RAB35 down-regulation. CCK-8 assay showed that in A549 cells and H2170 cells, the additional exosomes could restore the inhibition of cell proliferation due to the down regulation of RAB35 (Fig. 8A). Further, the exosomes secreted by A549 cells and H2170 cells with down-regulated RAB35 expression were co cultured with RAB35 knockdown cell models to explore whether RAB35 could affect the proliferation of tumor cells by regulating exosomes. Only RAB35 siRNA was transfected in the control group. The results of CCK-8 assay showed that addition of exosomes secreted by RAB35 down-regulated A549 cells and H2170 cells could not reverse the decrease of cell proliferation caused by RAB35 down-regulated (Fig. 8B). These results suggest that the exosomes secreted by tumor cells with down-regulated RAB35 expression lose the function of restoring cell proliferation.

Exosomes from RAB35 down-regulated tumor cells inhibited migration and invasion of NSCLC cells

In order to further explore whether exosomes regulated by RAB35 can affect the migration and invasion of lung cancer cells, the exosomes secreted by A549 cells were treated with or without enzyme, and then co-cultured with A549 cells with RAB35 expression down-regulated. The results of Trans-well assay showed that cell migration and invasion ability were significantly inhibited after removing the protein and RNA components of exosomes (Fig. 9A). The same results were verified in H2170 cells (Fig. 9B). These results suggest that exosomes regulated by RAB35 can increase the migration and invasion of NSCLC cells.

Discussion

Recent studies have shown that miRNA plays an important role in the proliferation and metastasis of a variety of tumor cells, including lung cancer, and is closely related to the clinicopathological characteristics and prognosis of patients. Among these miRNAs, there is emerging evidence that miR-185-5p inhibits cell migration and invasion of hepatocellular carcinoma through CDC42 [27]. In prostate cancer, miR-185-5p targeted inhibition of RNCR3 expression inhibited tumor cell proliferation and promoted apoptosis [28]. In this study, we found that miR-185-5p was down regulated in non-small cell lung cancer tissues and cell lines, promoting the proliferation, migration and invasion of lung cancer cells. In addition, luciferase reporter gene analysis confirmed the targeting relationship between miR-185-5p and RAB35, and the expression of RAB35 was up-regulated in non-small cell lung cancer tissues and cell lines. After the RAB35 plasmid with the miR-185-5p binding site removed was overexpressed, the inhibition of miR-185-5p on the proliferation of lung cancer cells was eliminated.

More and more evidences show that RAB35 expression is increased in various types of malignant tumors, which is accompanied by the increase of malignant degree and poor prognosis [29,30] . Zhu et al [31] found that RAB35 expression increased in MCF-7 breast cancer cells, and significantly inhibited Wnt5a-induced cell proliferation after knocking down RAB35 expression. Duan et al [32] reported that RAB35 silencing significantly reduced the stability and phosphorylation of GIT2, as well as the polarization and migration of NSCLC cells. In addition, Yang et al. [33] found that in hepatocellular carcinoma, long non coding RNA HOTAIR was positively regulating the expression of RAB35 to promote tumor development, and they also found that exosomes secreted related genes enriched in the HOTAIR high expression group. Villagomez et al [34] showed that RAB35 promoted the invasion, metastasis and immune escape of leukemia. Therefore, RAB35 may be a new molecular target for NSCLC.

As an important tool of intercellular communication, tumor derived exosomes can change tumor microenvironment by participating in angiogenesis, regulating stromal cells and changing extracellular matrix, so as to promote tumor proliferation and invasion through various ways [35,36]. Not only tumor derived exosomes, but also normal cells of the body, such as platelets, stromal cells and fibroblasts, can enter tumor cells and release corresponding contents to participate in tumor proliferation and invasion [37]. There was evidence that the exosomes secreted by colorectal cancer cells could induce the

morphological and functional changes of mesenchymal stem cells (MSCs), stimulate the proliferation, migration and invasion of tumor cells [38]. Wu [39] et al suggest that gastric cancer cells derived exosomes stimulate the activation of NF-κB pathway in macrophages to promote cancer progression. In breast cancer cells, Metastasis-associated protein 1 (MTA1) is transferred by exosomes and contributes to the regulation of hypoxia and estrogen signaling [40]. As an important part of tumor microenvironment, exosomes play an important role in the process of drug resistance. Qu et al. [41] found that exosomes derived from HCC cells increase drug resistance *in vivo* or *in vitro* by inhibiting apoptosis induced by sorafenib, leading to poor prognosis. In this study, we found that the exosome fluorescence signals secreted by A549 cells and H2170 cells were decreased after RAB35 gene was knocked down or miR-185-5p was overexpressed, which significantly inhibited the proliferation, migration and invasion of lung cancer cells. After culturing the exosomes with the parent cells, the growth ability of tumor cells was restored. These results further proved the role of RAB35 in the secretion and function of exosomes. The exosomes could affect the receptor cells through its components, and played a key role in the proliferation and metastasis of non-small cell lung cancer.

Conclusion

In conclusion, our study demonstrated that RAB35 gene is targeted by miR-185-5p, which regulates the synthesis and secretion of exosomes to mediate the proliferation, migration and invasion of NSCLC cells. Therefore, RAB35 may be a new molecular target for NSCLC treatment.

Abbreviations

NSCLC, Non-small cell lung cancer

miRNAs, MicroRNAs

PVDF, polyvinylidenedifluoride

Declarations

Conflict of interest

Authors declare that there are no conflicts of interest.

Ethics approval and consent to participate

This study protocol was approved by the Ethics Committee of Huaihe Hospital of Henan University (Kaifeng, China), and written informed consent was obtained from each participant.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Authors' contributions

Dan Wang and Shufang Yu analyzed the data. Dan Wang and Sichan Liu wrote the manuscript. Shasha Yi supervised the study and reviewed the manuscript.

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Figures

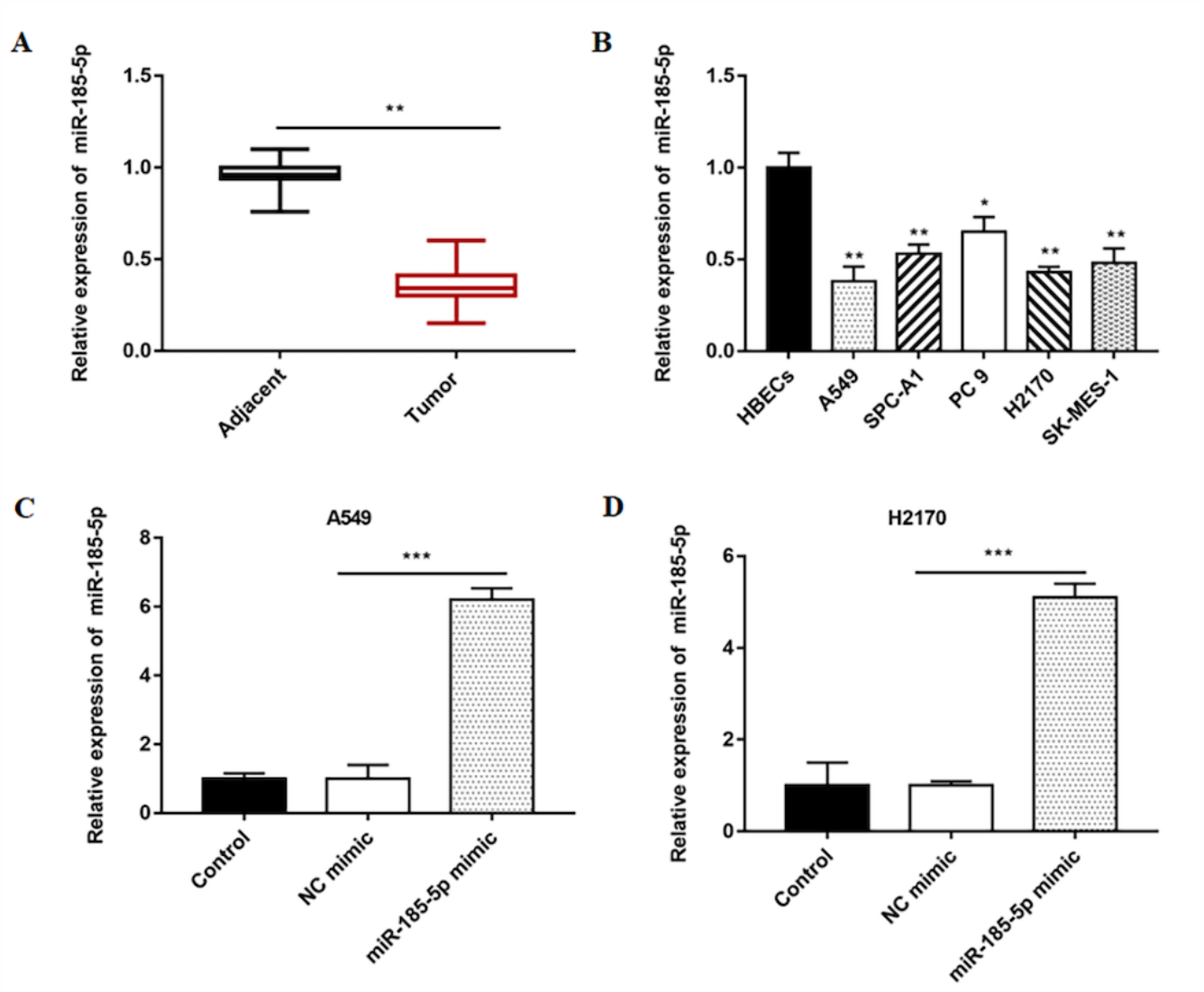

Figure 1

MiR-185-5p was down regulated in lung cancer tissues and cell lines. The non-small cell lung cancer tissues and tissues larger than 5 cm around the tumor were collected (N=70, 35 tumor tissues and 35 adjacent healthy tissues). A. RT-PCR was used to detect the relative expression of miR-185-5p in tumor tissues and adjacent healthy tissues. B. Relative expression of miR-185-5p in lung cancer cell lines and normal bronchial epithelial cell line were detected by RT-PCR assay. The miR-185-5p mimics were

transfected into A549 cells and H2170 cells, respectively. C-D. Relative expression of miR-185-5p in A549 cells and H2170 cells were analyzed by RT-qPCR assay. N=3, * P<0.05, ** P<0.01.

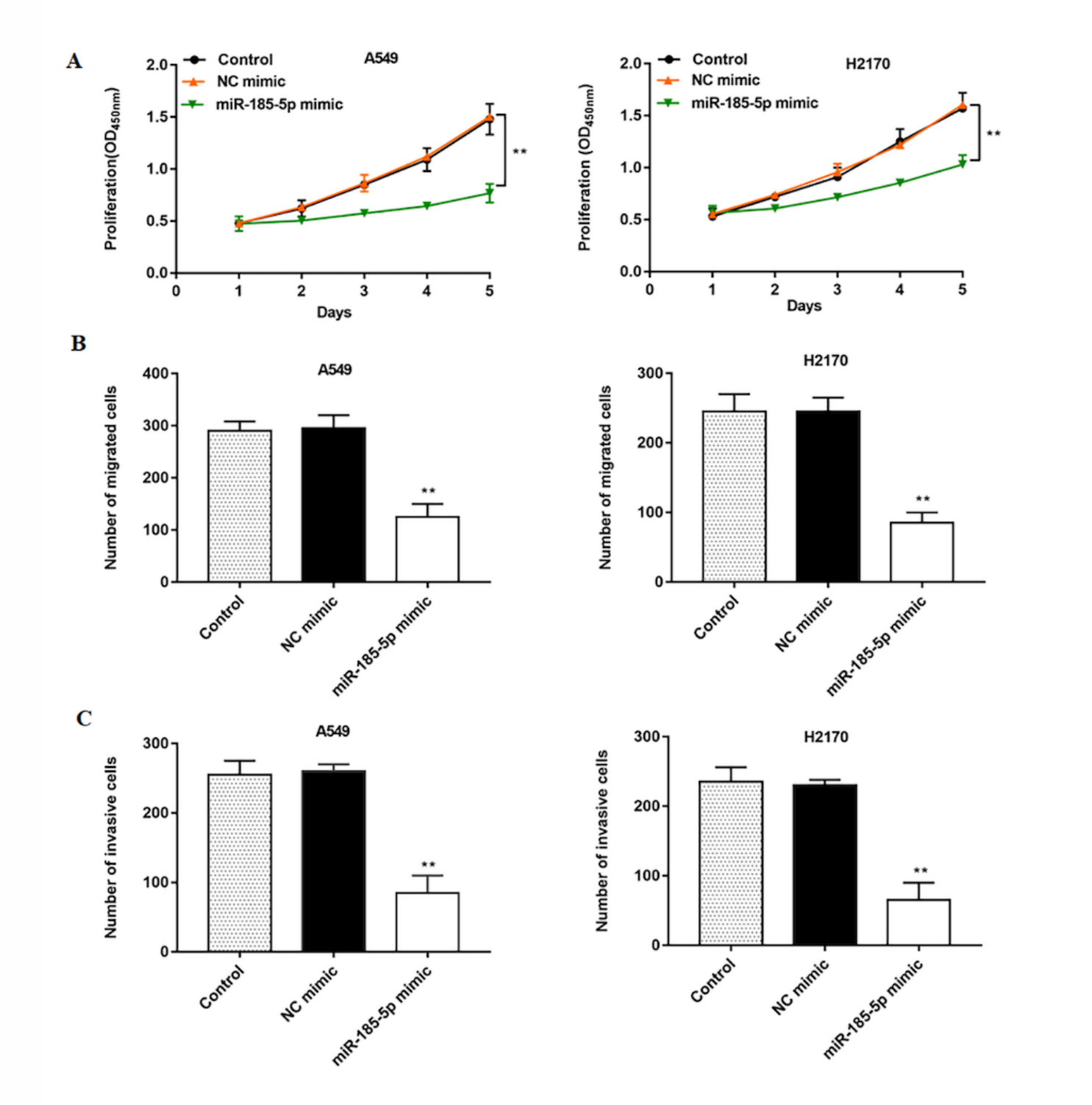

Figure 2

Overexpression of miR-185-5p inhibited proliferation, migration and invasion of NSCLC cells. A. A549 cells and H2170 cells proliferation were analyzed by CCK-8 assay. B. The migration of A549 cells and H2170 cells were detected by Trans-well assay. C. A549 cells and H2170 cells invasion were analyzed by Trans-well assay. N=3, * P<0.05, ** P<0.01.

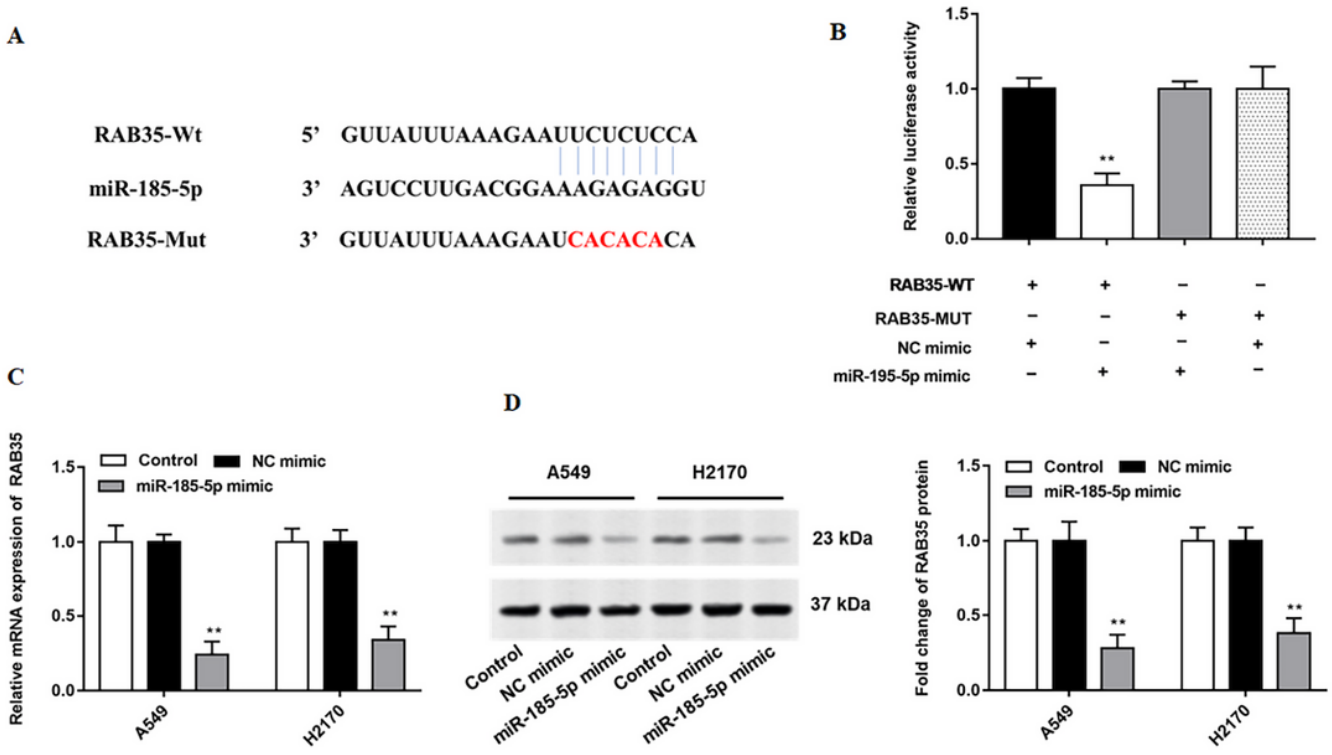

Figure 3

MiR-185-5p directly targeted 3'UTR of RAB35. A. TargetScan 7.1 was used to predicts the targeting site of miR-185-5p in RAB35 mRNA 3'UTR. B. The relative luciferase activity was tested with wild-type and mutant-type RAB35, respectively. C. The mRNA expression of RAB35 in A549 cells and H2170 cells transfected with miR-185-5p mimics were analyzed by RT-qPCR. D. Fold change of RAB35 protein in A549 cells and H2170 cells transfected with miR-185-5p mimics were analyzed by Western blotting. β-actin was used as an invariant internal control for calculating protein-fold changes. N=3, ** P<0.01.

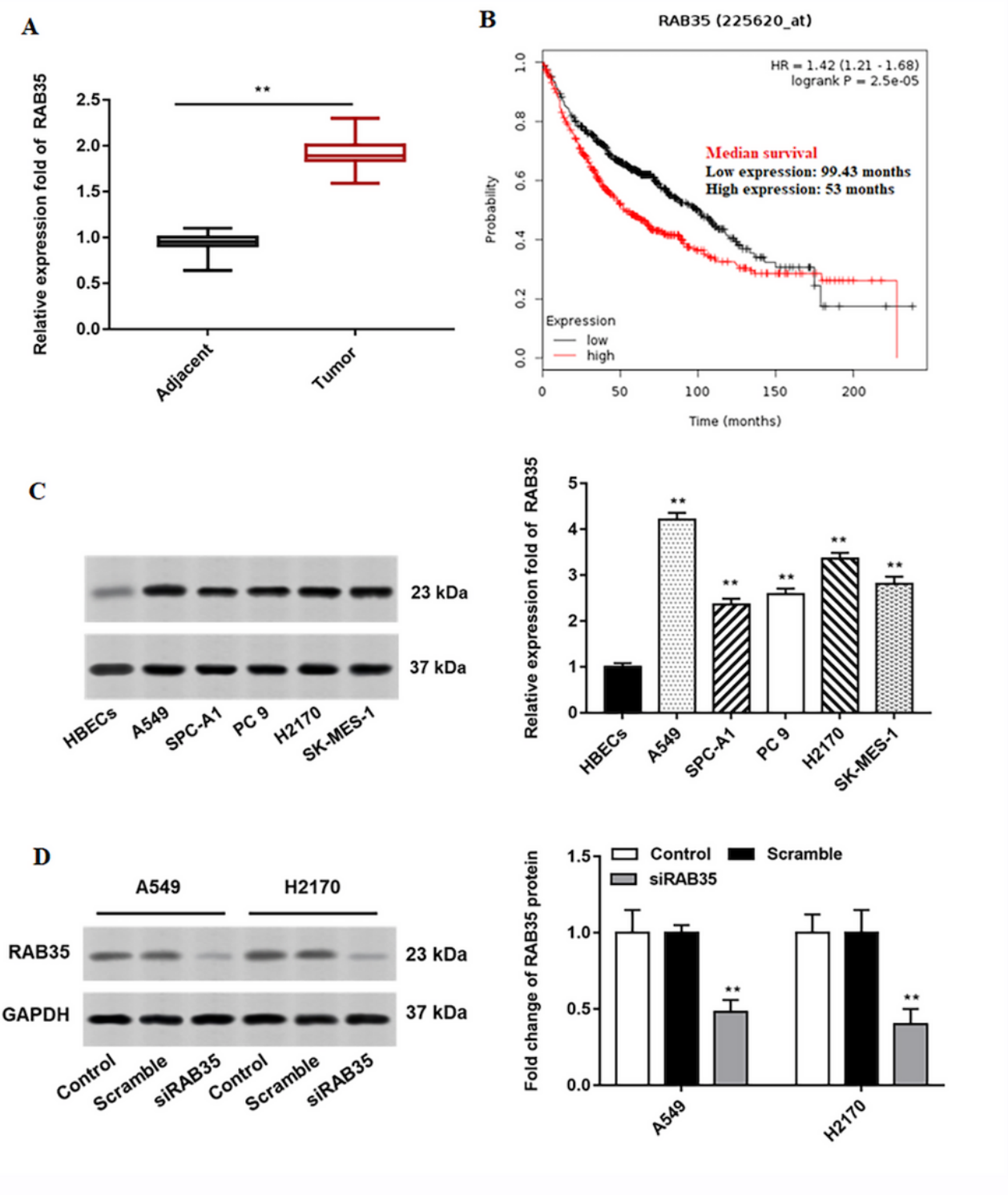

Figure 4

Up-regulation of RAB35 expression in lung cancer tissues and cell lines A. RT-PCR was used to detect the relative expression of RAB35 in tumor tissues and adjacent healthy tissues. B. KM plotter software was used to analyze the expression and survival of RAB35 gene in 1928 lung cancer patients. C. Western blotting was used to detect the relative expression of RAB35 in lung cancer cell lines and normal bronchial epithelial cell line. D. The RAB35 siRNA were transfected into A549 cells and H2170 cells,

respectively. The protein expression of RAB35 were analyzed by Western botting. β-actin was used as an internal reference. N=3, ** P<0.01.

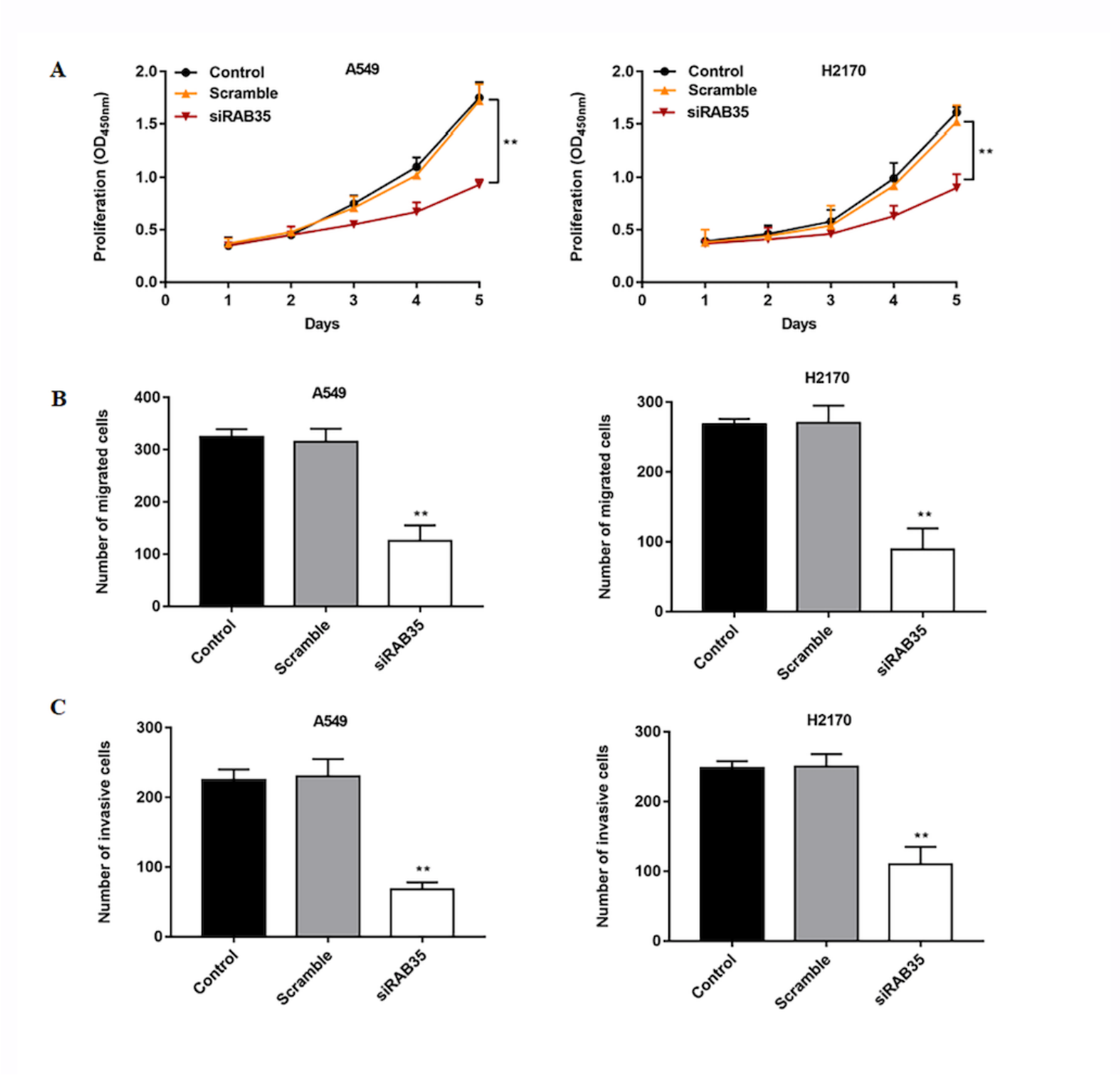

Figure 5

Knock-down of RAB35 inhibited proliferation, migration and invasion of NSCLC cells. A. CCK-8 assay was used to measure the cell proliferation. C. The migration of A549 cells and H2170 cells were detected by Trans-well assay. C. A549 cells and H2170 cells invasion were analyzed by Trans-well assay. β-actin was used as the loading control. N=3, ** P<0.01.

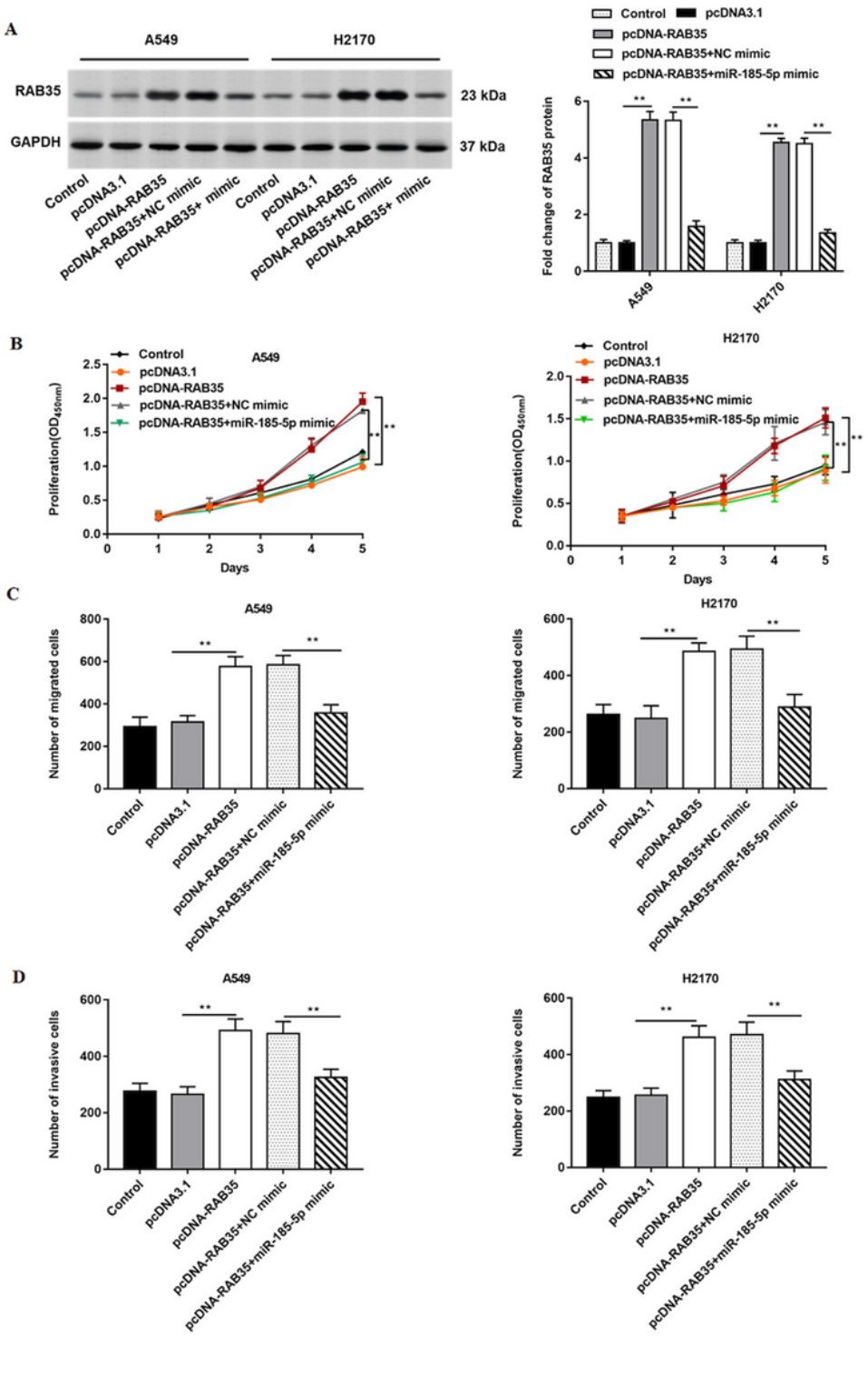

Figure 6

MiR-185-5p inhibited proliferation, migration and invasion of NSCLC cells by targeting RAB35. The pcDNA-RAB35 was transfected into A549 cells and H2170 cells alone or together with miR-185-5p mimics, respectively. A. Fold change of RAB35 protein was analyzed by Western blotting. B. The cell proliferation was measure by CCK-8 assay. C. Trans-well assay was used to measure the migration of

A549 cells and H2170 cells. D. The cell invasion was detected by Trans-well assay. β-actin was used as an internal reference. N=3, ** P<0.01.

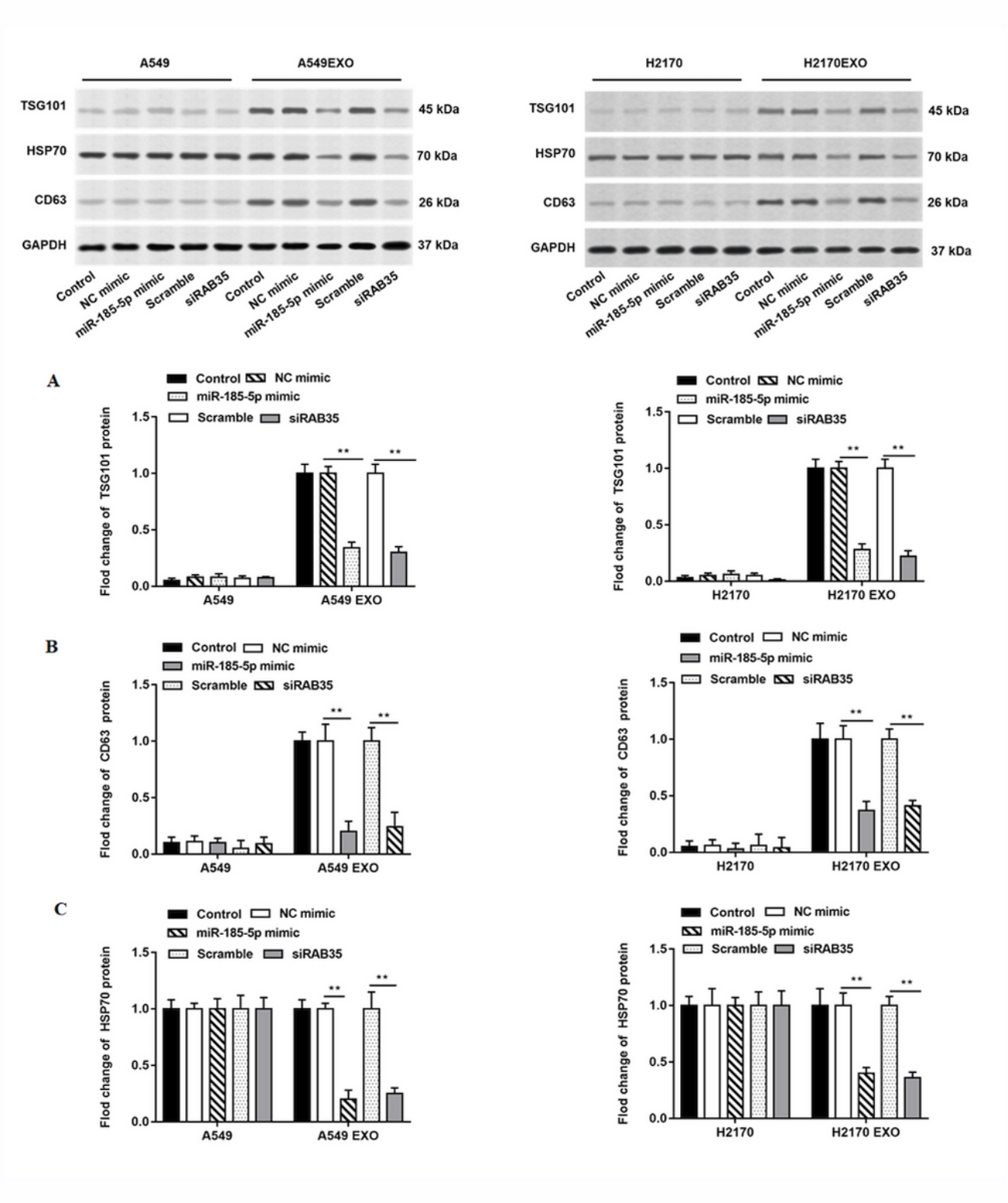

Figure 7

Knockdown of RAB35 reduced the number of exosomes from tumor cells and inhibited its function. The miR-185-5p mimics and RAB35 siRNA were transfected into A549 and H2170 cells, respectively. Next, the cell culture medium was collected after 48 hours and extracted exosomes. A. Western blotting was used

to detect the relative expression of TSG101 protein in A549 cells, H2170 cells and their secreted exosomes. B. Relative protein expression of CD63 in A549 cells, H2170 cells and their secreted exosomes was detected by Western blotting. C. The relative expression of HSP70 protein was also measured by Western blotting. β-actin was used as an invariant internal control for calculating protein-fold changes. N=3, ** P<0.01.

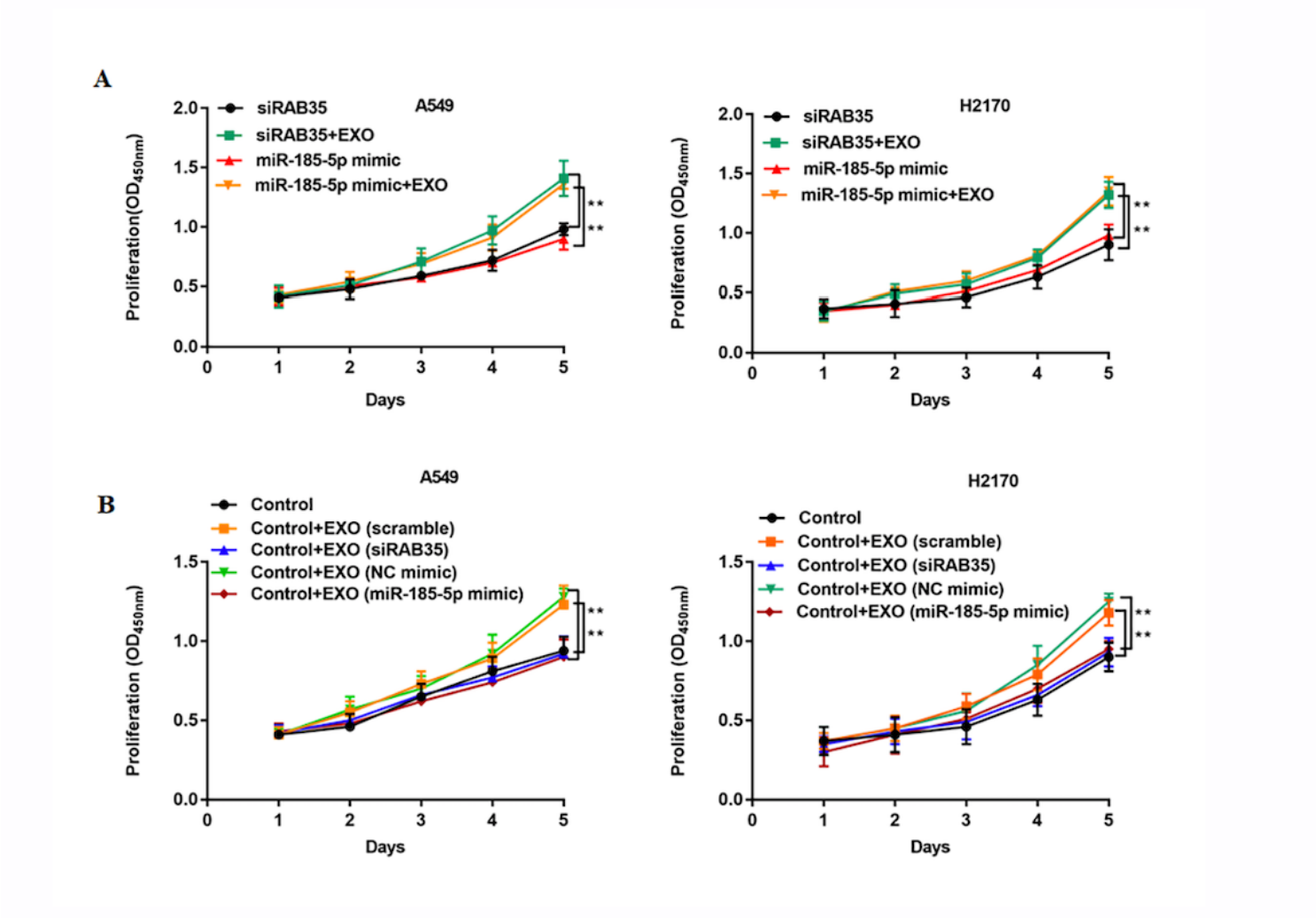

Figure 8

Exosomes from tumor cells promoted cell proliferation. A. The exosomes secreted by A549 cells and H2170 cells were added to two kings of cell models with downregulation of RAB35. CCK-8 assay was used to measure the proliferation of A549 cells and H2170 cells. B. RAB35 siRNA was transfected as negative control group and co-cultured with the exosomes secreted by RAB35 downregulated or miR-185-5p overexpression A549 cells and H2170 cells, respectively. The cell proliferation was measure by CCK-8 assay. N=3, ** P<0.01.

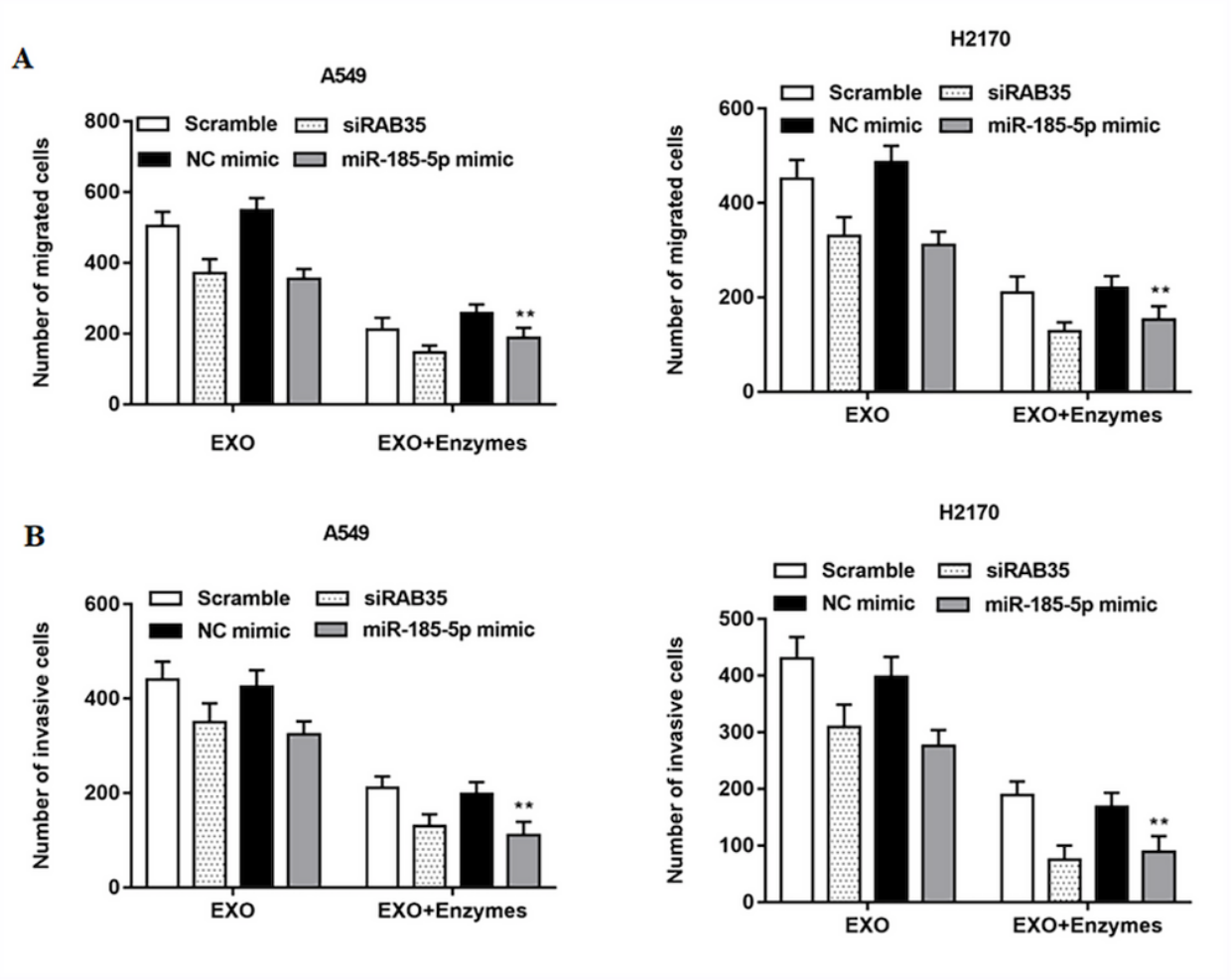

Figure 9

Inactivated exosomes lose the ability to promote tumor cells migration and invasion after enzyme treatment. The exosomes secreted by A549 cells and H2170 cells were treated with enzyme and then added into the two kinds of cell models with downregulation of RAB35. A. Trans-well assay was used to measure the migration of A549 cells and H2170 cells. B. The cell invasion was detected by Trans-well assay. N=3, ** P<0.01.