

# miR-132 inhibits lung cancer cell migration and invasion by targeting SOX4

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**Background:** Multiple MicroRNAs (miRNAs) have been identified in the development and progression of lung cancer. However, the expression and roles of miR-132 in non-small cell lung cancer (NSCLC) remain largely undefined. The aim of this study is to investigate the biological functions and its molecular mechanisms of miR-132 in human lung cancer cells.

**Methods:** miR-132 expression was measured in human lung cancer cell lines by quantitative real-time PCR (qRT-PCR). The cells migration and invasion ability were measured by wound healing assay and transwell assay. The influence of miR-132 on tumor progression *in vivo* was monitored using NSCLC xenografts in nude mice. The target gene of miR-132 was determined by luciferase assay and western blot.

**Results:** The expression level of miR-132 was dramatically decreased in examined lung cancer cell lines. Then, we found that introduction of miR-132 significantly suppressed the migration and invasion of lung cancer cells *in vitro*. Besides, miR-132 overexpression could also inhibit tumor growth in the nude mice. Further studies indicated that the sex determining region Y-box 4 (SOX4) is a target gene of miR-132. SOX4 re-introduction could reverse the anti-invasion role of miR-132.

**Conclusions:** Our finding provides new insight into the mechanism of NSCLC progression. Therapeutically, miR-132 may serve as a potential target in the treatment of human lung cancer.

**Keywords:** Invasion; lung cancer; miR-132; sex-determining region Y-box 4 (SOX4)

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## Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate gene expression at post-transcription level by target mRNAs degradation or translation repression. Mounting evidence has suggested that deregulation of miRNAs expression is often implicated in variety of disorders associating with kinds of human disease such as metabolic disorders, cardiovascular disease, and particular cancer (1-4). In addition, increasing evidence also indicated miRNAs could be key players in tumor

initiation and progression and affected tumor cell invasion and metastasis (5-7).

miR-132, arising from the miR-212/132 cluster, located in the intron of a non-coding gene on chromosome 17 in humans (8). Studies have shown that the miR-132 is involved in the vascular smooth muscle dysfunction mediated by angiotensin II (Ang-II) (9). In the tumorigenesis, it is reported that downregulation of miR-132 prohibits proliferation, invasion, migration and metastasis in breast cancer by targeting HN1 (10). Additionally, miR-132 inhibits colorectal cancer invasion

and metastasis via targeting ZEB2 (11). However, the mechanism of miR-132 regulated tumor metastasis is still need to be further explored.

The *SOX4* (sex-determining region Y-box 4) gene, a member of the SOX family, has been shown to have important roles in the development and cell fate decision. In tumorigenesis, increasing evidence suggests that *SOX4* was significantly elevated in multiple human cancers, including breast cancer, prostate cancer, liver cancer and lung cancer (12-14). Its overexpression was closely correlated with tumor progression and metastasis (15,16). Besides, *SOX4* was shown to regulate several key signaling pathways in cancer cells. For instance, *SOX4* could regulate  $\beta$ -catenin/T-cell factor activity and act as an agonist of Wnt signaling in colon cancer (17).

In the present study, we found that miR-132 was significantly down-regulated in lung cancer cells, and further revealed that the overexpression of miR-132 could inhibit lung cancer progression *in vitro* and *in vivo*. Additionally, we identified that *SOX4* is a target gene of miR-132. miR-132 is able to inhibit invasion of lung cancer cells by paralyzing the function of *SOX4*.

## Materials and methods

### Cell culture and transfection

The human lung adenocarcinoma cell line A549, Lung squamous carcinoma cell line YTMMLC-9, lung large cell carcinoma cell line H460 and normal human bronchial epithelial (HBE) cells (obtained from Tianjin Key Laboratory of Lung Cancer Metastasis and Tumor Microenvironment, Tianjin Lung Cancer Institute, China) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Gibco, CA, USA) at 37 °C with 5% CO<sub>2</sub> incubator. For transfection, cells were cultured to 80% confluence and transfected with recombinant eukaryotic vector and empty vector using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's recommendation.

### Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to validate the miRNA expression level. qRT-PCR was carried out using SYBR<sup>®</sup>Premix Ex TaqTM (Takara, Japan). PCR were carried out in triplicate and analyzed using the ABI Prism 7900HT fast RT-PCR system (Applied Biosystems, Life technologies, USA). The

relative quantification value for each gene was calculated by the 2<sup>- $\Delta\Delta C_t$</sup>  method using U6 or GAPDH as an internal reference. All primers were shown in *Table S1*.

### Plasmid constructions

The full-length 3'-untranslated region (3'UTR) of *SOX4* was amplified from human genomic DNA, and was cloned into the downstream of the firefly luciferase coding region of pMIR-GLOTM Luciferase vector (Promega, USA). The recombinant vector was named as pMIR-SOX4. Mutations of miR-132 binding sites were introduced by site-directed mutagenesis and the resulted vector was named pMIR-SOX4-Mut. Primers used for the constructions were listed in *Table S1*. All the constructions were confirmed by sequencing.

### Cell migration assay

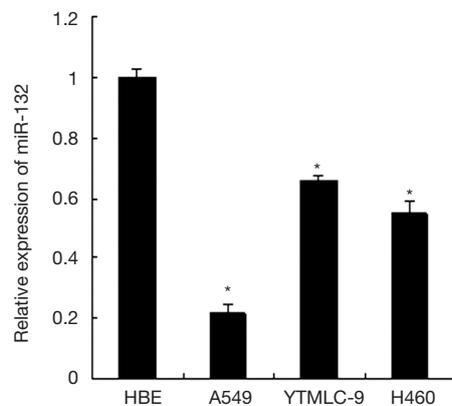
The migration ability was determined using wound-healing assay. The cells were plated into 12-well plates without antibiotics; cells were transfected with miR-132 mimic or mimic control. Twenty-four h later, transfected cells were wounded with a sterile plastic 100  $\mu$ L micropipette tip, the floating debris were washed with PBS and cultured in serum-free medium. Width of the wound was measured at different time points. Three to four different locations were visualized and photographed under a phase-contrast inverted microscope.

### Cell invasion assay

Boyden chamber assay was used to examine cell invasion capability. A549 cells were transfected with miR-132 mimic or mimic control. Sixteen h later, transfected cells were trypsinized and resuspended, 1.0 $\times$ 10<sup>4</sup> cells in 200  $\mu$ L RPMI 1640 medium were placed into the upper chambers (8-mm pore size; Millipore). The lower chambers were filled with 600  $\mu$ L complete medium with 10% FBS. After incubation for 12 h at 37 °C, non-invading cells were removed from the top of the chamber with a cotton swab. The invasion cells on the lower surface of the inserts were fixed and stained with 0.1% crystal violet, and five random fields for each insert were counted at 200 $\times$  magnifications.

### In vivo assay

For the *in vivo* assays, 5 $\times$ 10<sup>5</sup> A549 cells stably expressing miR-132 or negative control (NC) were injected



**Figure 1** Expression levels of miR-132 in lung cancer cells. miR-132 expression was determined by RT-PCR in human lung cancer cell lines and normal human bronchial epithelial (HBE) cell line. (\* $P < 0.01$ , Student's  $t$ -test). RT-PCR, real-time PCR.

subcutaneously to the mouse. The mice were observed over 4 weeks for tumor formation. After the mice were sacrificed, the tumors were recovered and the weight of each tumor was determined.

#### Dual-luciferase reporter assay

Cells were seeded into 24-well plates and cotransfected with 200 ng of pMIR-SOX4 or pMIR-SOX4-Mut vector and 100 ng of miR-132 mimic or mimic control, and the pRL-TK plasmid (Promega, Madison, WI) which was used as internal normalization. After 48 h, cells were lysed using the lysis buffer (Promega). Luciferase reporter gene assay was implemented using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. All experiments were performed at least 3 times.

#### Western blotting

Cells were transfected with either miR-132 or pCMV-Tag-2b-SOX4. Total cell extracts prepared from cells using RIPA buffer (Beyotime, China), were resolved on 10% gradient SDS-polyacrylamide gel and transferred NC membranes. Membranes were blocked for 1 h in 5% skim milk in TBST and incubated with anti-SOX4 antibody (1:1,000, Santa Cruz) or anti- $\beta$ -actin antibody (1:5,000, CST). Overnight at 4 °C, followed by the incubation with appropriate HRP-conjugated secondary antibody at optimized concentration.

The densitometry of Western blot results was measured using ImageJ software.

#### Statistical analysis

The data were presented as mean  $\pm$  standard deviation (SD).  $T$ -test was used to determine the significant differences between control and treatment groups. Statistical analysis was performed using SPSS15.0 software and  $P < 0.05$  was considered to be a statistically significant difference.

## Results

### miR-132 is down-regulated in non-small cell lung cancer (NSCLC) cells

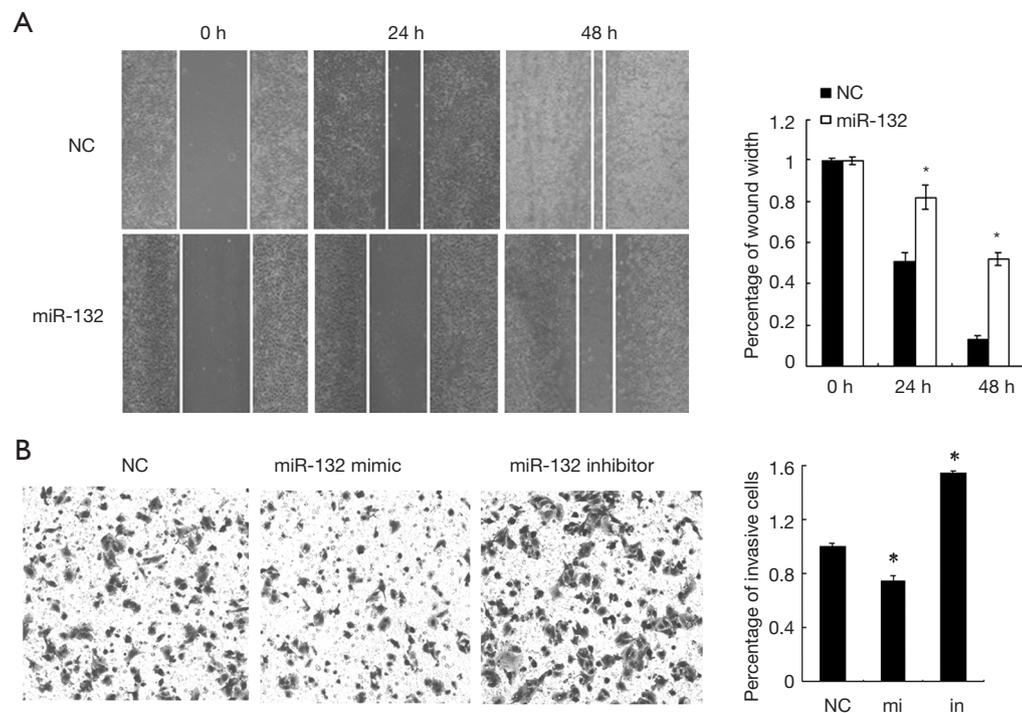
We analyzed the expression of miR-132 in human normal bronchial epithelial cells HBE and three human lung cancer cell lines including A549, YTMLC-9 and H460 by qRT-PCR. The results showed that miR-132 was down-regulated significantly in all the three lung cancer cell lines compared with the HBE cell line (Figure 1). These data indicate that the reduced expression of miR-132 is a frequent event in NSCLC cells, which may be involved in lung cancer progression.

### miR-132 overexpression inhibits the aggressiveness of NSCLC cells in vitro

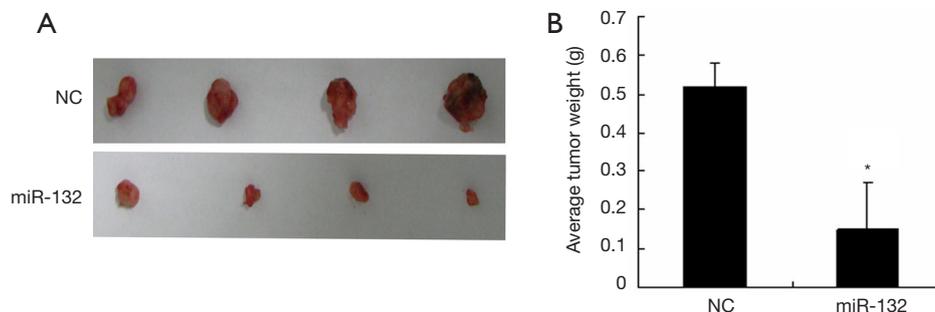
Next, we tested the role of miR-132 in NSCLC cells, its mimics or NCs were transfected into A549 cells. Wound healing assay showed that the ectopic expression of miR-132 in A549 cells significantly inhibited cell migration, compared to the control group (Figure 2A). In agreement, Boyden chamber assay revealed that miR-132 overexpressing cells showed decreased invasion ability, compared to NC cells (Figure 2B). Taken together, our results indicate that miR-132 is able to suppress the migration and invasion of NSCLC cells *in vitro*.

### miR-132 overexpression suppresses NSCLC progression in vivo

To further determine the roles of miR-132, A549 cells with stable overexpression of miR-132 were generated and injected subcutaneously to the nude mice. The tumor growth was closely monitored for another 4 weeks. As a result, the tumor size (Figure 3A) and weight (Figure 3B) were markedly reduced in miR-132-overexpressed group,



**Figure 2** miR-132 inhibits lung cancer cell migration and invasion. (A) The wound healing assay was used to detect the migration ability of A549 cells. The cells were transfected with miR-132 mimics or negative control (NC); (B) Boyden chamber assay was employed to examine the invasion ability of A549 cells. The cells were transfected with miR-132 mimics or miR-132 inhibitor for 24 h. The results were from three independent experiments. The migratory cell number in each group was normalized to the control. (\* $P < 0.05$ , Student's  $t$ -test).



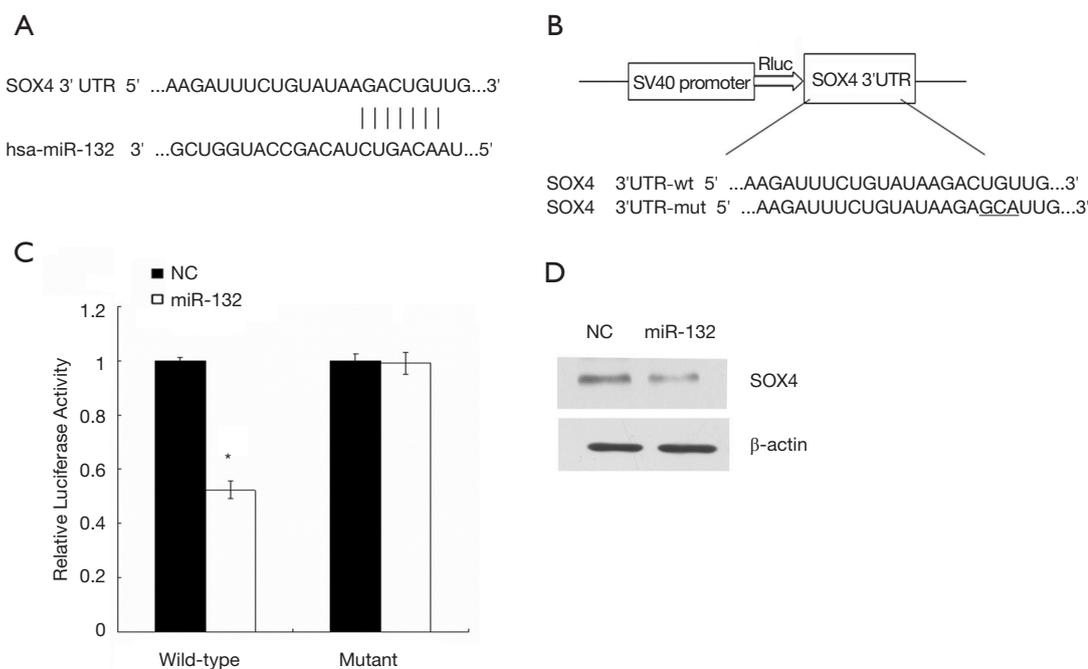
**Figure 3** miR-132 inhibits tumor growth *in vivo*. A549 cells stably transfected with miR-132 or negative controls (NCs) were injected into nude mice ( $n=4$  for each group) and followed up for tumorigenesis. Representative images (A) and tumor weights (B) were taken 4 weeks after injection. (\* $P < 0.05$  between two groups).

compared to control group, suggesting that miR-132 could also suppress NSCLC progression *in vivo*.

#### miR-132 directly targets SOX4 in NSCLC cells

To detect the molecular mechanism by which miR-132 suppresses the metastasis of lung cancer cells, we predicted

the putative target genes of miR-132 in human cells using the tool miRanda, PicTar and TargetScans. Among which, we found that the gene encoding SOX4 harbored a potential miR-132 binding site (Figure 4A). Therefore, the wild type or mutant 3'UTR of SOX4 gene was cloned and inserted into pMIR reporter vector (Figure 4B). Overexpression of miR-132 led to a reduction of luciferase activity carrying the



**Figure 4** miR-132 directly inhibits the expression of SOX4 through its 3'UTR. (A) The miR-132 binding site predicted in the 3'UTR of SOX4; (B) Mutant was generated at the seed region of SOX4 3'UTR as indicated by the underline. A 3'UTR fragment of SOX4 mRNA containing wild-type or mutant of the miR-132 binding sequence was cloned into the downstream of the luciferase gene in pMIR vector; (C) A549 cells were transfected with pMIR reporter vectors containing either wild-type or mutant SOX4 3'UTR (indicated as Wild-type and Mutant) with either NC or miR-132 mimic. Luciferase activity was determined 48 h after transfection; (D) The protein levels of SOX4 was examined by Western blot in cells transfected with NC or miR-132 mimic. Data are reported as mean  $\pm$  SD. ( $P < 0.01$ , Student's *t*-test). SOX4, sex-determining region Y-box 4; 3'UTR, 3'-untranslated region; SD, standard deviation.

wild-type 3'UTR. However, mutation of the potential miR-132 binding site abolished the inhibitory roles of miR-132 (Figure 4C). Moreover, transfection of miR-132 mimics in lung cancer cells resulted in a reduced SOX4 expression (Figure 4D). Therefore, our results suggest that SOX4 might be a target of miR-132 in NSCLC cells.

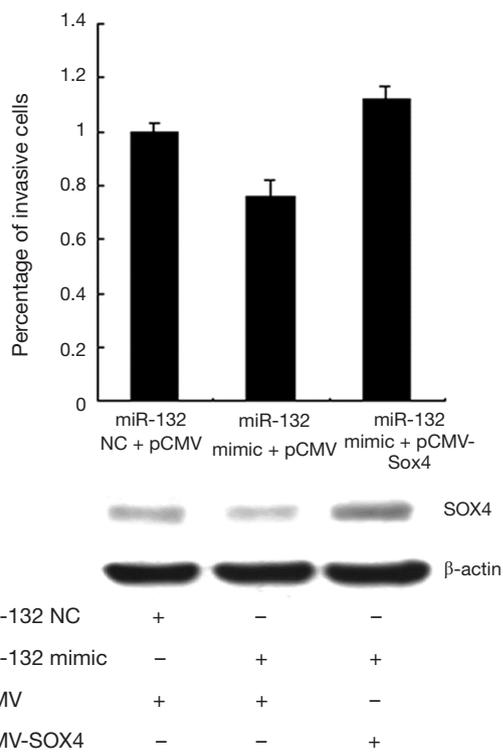
#### *SOX4 contributes to miR-132 suppressed invasion of NSCLC cells*

To further verify the functional effect of SOX4 on miR-132-mediated regulation of invasion. A549 cells were transfected with SOX4 expression plasmid after transfection of miR-132 mimics. As shown in Figure 5, transfection of miR-132 mimic into A549 cells led to a decrease of cell invasion, whereas SOX4 re-introduction reversed the anti-invasion role of miR-132, underlining the specific importance of the SOX4 for miR-132 action in the cell invasion. Additionally, the overexpression efficiency of SOX4 was examined by Western.

#### Discussion

Advances in diagnostic techniques and therapeutic means have improved the early detection and reduced the mortality rate of lung cancer; however, it still is the first leading cause of cancer-related deaths worldwide and is responsible for more than 1 million deaths every year. The primary reason for its mortality and relapse is that lung cancer cells have a powerful ability to metastasis. Although the great progresses has been made in recent decades and it has been suggested that the tumor metastasis is a complex process including multiple sequential steps, the molecular mechanisms that regulate metastasis in lung cancer cells are still poorly understood.

In our study, we found that miR-132 was markedly decreased in lung cancer cells. Ectopic expression miR-132 was able to inhibit migration and invasion and of NSCLC cells *in vitro*. Furthermore, the anti-tumorigenesis role of miR-132 was performed *in vivo*. The data show that miR-



**Figure 5** SOX4 re-introduction reverses the anti-invasion roles of miR-132. Cells were pre-transfected with miR-132 mimics or negative control (NC) for 24 h, and then transfected with expression plasmids for SOX4 (pCMV-SOX4) or empty vector (pCMV) or another 24 h. The SOX4 protein expression was determined by western blot in A549 cells (down) and the cell invasion ability was determined by Boyden chamber assay (up). (\* $P < 0.01$ ). SOX4, sex-determining region Y-box 4.

132 may be a tumor suppressor in the development and progression of lung cancer. This result is similar with our previous report (18). However, the mechanism of miR-132 regulated migration and invasion of NSCLC still need to be further investigated.

At the molecular level, Zhao *et al.* found that miR-132 could suppress the G1/S phase transition of the cell cycle and the epithelial to mesenchymal transition in cervical cancer cells by targeting SMAD2 (19). Lei *et al.* found that miR-132 inhibits proliferation of hepatic carcinoma cells by targeting YAP (20). Additionally, You *et al.* found that miR-132 could suppress the migration and invasion of lung cancer cells via targeting the EMT

regulator ZEB2 (18). Our present study found that SOX4 was a functional target of miR-132 by luciferase reporter gene assays and western blot analysis method. SOX4 is a transcription factor required for tissue development and differentiation in vertebrates (21). Recently, overexpression of SOX4 has been reported in many cancers including NSCLC (15,22-25). Overexpression of SOX4 was correlated with poor prognosis in patients with NSCLC. Zhou *et al.* demonstrated that SOX4 could regulate lung cancer cell metastasis (26). So we thought that SOX4 may be involved in miR-132 mediated lung cancer cell metastasis. In our study, we found that SOX4 could reverse the anti-invasion role of miR-132.

In summary, we investigated the role of miR-132 in NSCLC development. Our finding suggests that miR-132 may be a novel tumor suppressor miRNA. miR-132 blocks the migration and invasion of NSCLC cells through targeting SOX4. Our data provide new insight into the mechanism responsible for the development of human NSCLC. Additionally, miR-132 may serve as a potential therapeutic candidate in the treatment of NSCLC.

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## Footnote

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

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