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ORIGINAL ARTICLE

BMSCs-derived exosomal MiR-126-3p inhibits the viability of NSCLC cells by targeting PTPN9

Jun Chen[1,2]*, Xianzhou Yang[1,2]*, Cheng Ding[1,2]*, Jun Zhao[1,2]

[1]Department of Thoracic Surgery, The First Affiliated Hospital of Soochow University, Medical College of Soochow University, Suzhou, China. [2]Institute of Thoracic Surgery, The First Affiliated Hospital of Soochow University, Suzhou, China.

*Jun Chen, Xianzhou Yang and Cheng Ding contributed equally to this work.

Summary

Purpose: *Recent studies have manifested that bone marrow mesenchymal stem cells (BMSCs) derived exosomes affect the progression of tumors through carrying endogenous molecules. To explore the role of BMSCs-derived exosomes carrying abundant micro ribonucleic acid (miR)-126-3p in non-small-cell lung cancer (NSCLC).*

Methods: *Firstly, A549 cells were transfected with miR-126-3p to detect the role of miR-126-3p in A549 cells. Next, miR-126-3p was transfected into BMSCs, and BMSCs-derived exosomes with over-expressed miR-126-3p (Exo-miR-126-3p) were isolated through ultracentrifugation. After that, A549 cells were co-incubated with Exo-miR-126-3p to determine the effects of Exo-miR-126-3p on cell proliferation, migration, invasion, and apoptosis. Besides, the targeted relationship between miR-126-3p and protein tyrosine phosphatase non-receptor type 9 (PTPN9) was confirmed via bioinformatics analysis and dual-luciferase reporter gene analysis. Western blotting (WB) was employed to measure the expressions of PTPN9 and related proteins in A549 cells. Additionally, the effects of over-expressed miR-126-3p derived from BMSCs exosomes on tumor growth and apoptosis of NSCLC cells were analyzed in connection with lentiviral packaged miR-126-3p in vivo.*

Results: *Overexpressed miR-126-3p suppressed the viability, migration, and invasion of A549 cells in vitro. Based on the results of exosome content analysis, miR-126-3p could mediate the inhibitory effect of exosomes on A549 cells by negative regulation of PTPN9. Notably, over-expressed miR-126-3p derived from BMSCs inhibited the tumor growth and apoptosis in vivo.*

Conclusions: *Taken together, the key finding of the study indicated that over-expressed BMSCs-derived exosomal miR-126-3p can suppress the progression of NSCLC through negatively regulating PTPN9.*

Key words: *BMSCs, exosomal, miR-126-3p, non-small-cell lung cancer*

Introduction

In 2018, about 1.8 million people died of lung cancer, the leading cause of cancer-related deaths in the world, accounting for nearly one-fifth (18.4%) of cancer-related deaths [1]. Histologically, lung cancer can be classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and NSCLC patients account for over 80% of lung cancer cases [2]. Mesenchymal stem cell (MSC) therapy has been widely studied in the fields of tissues and organs in recent years. Current studies have denoted that bone marrow MSCs (BMSCs) can metastasize to tumor tissues [3] and affect the development of tumor tissues through exosomes which they secreted [4]. Besides, BMSCs-derived exosomes can carry functional micro ribonucleic acids (miRNAs) to recipient cells, and more importantly, to tumor cells to affect the proliferation, differentiation and apoptosis of tumor cells [5,6].

Corresponding author: Jun Zhao, MD. Department of Thoracic Surgery, The First Affiliated Hospital of Soochow University, Medical College of Soochow University, No.899 Pinghai Rd, Suzhou 215006, China.
Email: zhaoj0327@126.com; Tel: +8600512-65223637
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For instance, BMSCs-derived exosomal miR-134 targets caspase-8 to suppress the apoptosis of oligodendrocytes [7].

Exosomes, small membrane-encapsulated vesicles (40-150 nm) derived from endocytic pathways [8], are capable of carrying bioactive molecules to recipient cells, and they have a significantly influence on the tumor environment and cancer biology [9]. For example, exosomal PD-L1 enhances the immune escape of cancer cells, leading to resistance to PD-1/PL-L1 blockers [10]. In addition, exosomes are able to carry miRNAs that related to the proliferation, differentiation and apoptosis of cancer cells [11]. These miRNAs act as tumor suppressors or oncogenes to modulate gene expressions after transcription [12]. For instance, miR-126-3p, a subtype of miRNAs, has been proven to serve as a tumor suppressor [13]. It is worth noting that miR-126-3p is a crucial player in the progression of NSCLC and exhibits a notably down-regulated expression level in the case of NSCLC [14,15]. Additionally, miR-126-3p can control the biological characteristics of NSCLC through different mechanisms. However, the specific mechanism of miR-126-3p in the progression of NSCLC needs to be further study.

A study reported that highly expressed protein tyrosine phosphatase non-receptor type 9 (PTPN9) is able to promote the proliferation and invasion of Eca109 cells by negatively regulating miR-126-3p [16]. However, whether BMSCs derived exosomal miR-126-3p is capable of affecting the development and progression of NSCLC by modulating the expression of PTPN9 remains unknown. In this study, therefore, it was assumed that BMSCs-derived exosomes with overexpressed miR-126-3p (Exo-miR-126-3p) can repress the progression of NSCLC by down-regulating PTPN9. In the present study, the role of BMSCs-derived exosomal miR-126-3p in NSCLC was investigated.

Methods

Cell lines

Human NSCLC A549 cells were bought from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and they were cultured in F12 complete medium (Gibco, Grand Island, NY, USA). Upon reaching 90% confluence, the cells were normally sub-cultured. Next, they were separately transfected with small interfering (si)-PTPN9, si-negative control (NC), miR-126-3p mimic and NC-mimic using Lipofectamine 2000 (Thermo, Waltham, MA, USA) according to the instructions. Human BMSCs purchased from GuYan Biotech Co., Ltd. (Shanghai, China) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA). Then, the BMSCs in the logarithmic growth phase were collected and transfected with miR-126-3p and miR-126-3p-NC, respectively. All plasmids were provided by Hanbio Biotechnology Co., Ltd. (Shanghai, China).

Isolation and identification of exosomes

Fetal bovine serum (FBS, Thermo, Waltham, MA, USA) was centrifuged at 120,000 g for 18 h to remove exosomes. Then, BMSCs were cultured in exosome-free complete DMEM containing 10% FBS. Next, the supernatant of cell culture was collected and centrifuged at 300 g for 10 min, 2,000 g for 15 min, and 10,000 g for 30 min to remove cells and cell debris. Afterwards, the supernatant was collected and filtration with a 0.22 μm filter membrane, the filtrate was centrifuged at 120,000 g for 90 min. After that, the supernatant was discarded, and the pellet was re-suspended with an appropriate volume of phosphate-buffered saline (PBS) and centrifuged at 120,000 g for 90 min again. Next, the pellet was collected.

Exosomes (10 μL) were added to a grid in drops using a pipette, followed by precipitation for 1 min. Next, excess liquid was absorbed using filter papers, and negative staining was conducted with 10 μL of 2% uranyl acetate. Following drying at room temperature, the morphology of exosomes was observed with a transmission electron microscope (TEM, Libra 120, Zeiss, Oberkochen, Germany). Western blotting (WB) was applied to measure the expressions of exosome-specific proteins [cluster of differentiation 63 (CD63), tumor susceptibility gene 101 (TSG101) and apoptosis-linked gene 2-interacting protein X (ALIX)] (CST, 1:1000, Danvers, MA, USA). In addition, the size of exosomes was detected using nanoparticle tracking analysis (NTA, Malvern, Worcestershire, UK).

Uptake of Exosomes

A PKH67 Fluorescent Cell Linker Kit provided by Sigma (Louis, MO, USA) was used for labeling exosomes. Specifically, 4 μL of PKH67 was mixed with 500 μL of Diluent C and incubated at room temperature in a dark place for 5 min. Then, 2 mL of 10% FBS was added to terminate staining, and the labeled exosomes were centrifuged at 120,000 g for 2 h to remove residual dye, and stored at -80°C for later use.

For the uptake of exosomes, A549 cells were carried out as follows. A549 cells were seeded in a 6-well plate, and added with PKH-67 labeled exosomes when the confluence reached 70%. Next, they were incubated for 12 h and the cells were washed twice with PBS. Thereafter, the cells were fixed with 4% paraformaldehyde for 20 min, and the nuclei were stain with Hoechst (Beyotime, Shanghai, China). Finally, the uptake was observed using a laser scanning confocal microscope.

Cell counting kit-8 (CCK-8) assay

A total of 3.5×10^4 A549 cells were seeded in a 96-well plate. After attachment, the cells were grouped according to the requirements of the assay. The plates were incubated with 5% CO_2 at 37°C for 24 h, 48 h, 72 h and 96 h, respectively. Then, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well, and cultured in the incubator for 3 h. Next, the supernatant was discarded, and the optical density (OD) at 570 nm was read using a microplate reader.

Transwell assay

Transwell migration assay was conducted as follows. The A549 cells in the logarithmic growth phase were trypsinized, and re-suspended with serum-free medium to prepare a cell suspension. Next, 2×10^5 cells were seeded in the upper chamber of a 24-well Transwell chamber with a pore size of 8 μm (Millipore, Millipore, Billerica, MA, USA). Besides, complete medium containing 10% FBS was added to the lower chamber to induce cell migration. After 48 h of culture, the chamber was taken out, and the cells were fixed with methanol for 30 min and stained with crystal violet for 20 min. Thereafter, the cells were photographed and counted in 5 fields randomly selected under an inverted fluorescence microscope.

Transwell invasion assay was performed as follows. Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) was thawed at 4°C overnight and diluted with pre-cooled serum-free DMEM. Then, 100 μL of diluted Matrigel was added to the upper chamber. The remaining procedures were the same as those in Transwell migration assay.

Dual-luciferase reporter gene assay

The A549 cells (6×10^4) in the logarithmic growth phase were harvested and inoculated into a 24-well plate. Next, they were divided into three groups, namely, control group, PTPN9 3' untranslated region (3'-UTR) wild-type (Wt-PTPN9) plasmid group, and PTPN9 3'-UTR mutant (Mut-PTPN9) plasmid group *as per* the instructions. 48 h later, the cells were collected, and their luciferase activity was determined using a luciferase reporter gene assay kit (Promega, Madison, WI, USA).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from cells and exosomes containing miRNAs according to the instructions of TRIzol (Invitrogen, Carlsbad, CA, USA). The expressions of miR-126-3p and PTPN9 messenger RNA (mRNA) were measured via qRT-PCR. Primers were shown in Table 1. CDNAs were synthesized in accordance with the instructions and used for qRT-PCR. U6 and GAPDH were used as controls for miR-126-3p and PTPN9, respectively. QRT-PCR results were analyzed with $2^{-\Delta\Delta Ct}$ method.

Detection of protein expression using Western blotting

The cells were harvested and added with 1 mL of radio immunoprecipitation assay (RIPA, Thermo, Waltham, MA, USA) lysis buffer to extract total proteins. Next, the concentration was determined by bicinchoninic acid (BCA, Thermo, Waltham, MA, USA) assay. Afterwards, the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF, Millipore, Billerica, MA, USA) membrane. Then, the membrane was blocked and incubated with primary antibodies at 4°C overnight. After that, The proteins were then again incubated with secondary antibodies at room temperature for 1 h and washed with tris-buffered saline tween (TBST), 5 min/3 times. This was followed by image development using enhanced chemiluminescence (ECL, Thermo, Waltham, MA, USA). The gray value of the protein band was analyzed using ImageJ.

Xenograft in nude mice

After anesthesia, the Male BALB/c nude mice (aged 4-6 weeks; weight 18-24 g) were inoculated with cells. A549 in logarithmic growth phase were re-suspended in DMEM, with 3×10^6 cells/mL. Then 0.2 mL single-cell suspension (6×10^5 cells) was subcutaneously injected into the right armpit of nude mice. After the tumor volume about 100 mm3, the plasmids of Exo-miR-NC or Exo-miR-126-3p were injected into the nude mice by i. v. The tumor volume was calculated as ($L\times W^2$), where L was tumor length and W denoted tumor width. Nude mice were euthanized after six rounds treatment, and tumor was collected and weighted.

Statistical analysis

All assays were repeated at least 3 times. Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was used for statistical analysis. Measurements were expressed as mean ± standard deviation. t-test was employed for analyzing the difference between two groups of data, and analysis of variance (ANOVA) was used for comparing the differences between groups. $p<0.05$ indicated that the difference was statistically significant.

Results

Overexpressed miR-126-3p inhibited the proliferation, migration and invasion and promoted the apoptosis of A549 cells

In order to explore the effect of miR-126-3p on the biological function of A549 cells. MiR-126-3p was overexpressed in NSCLC cell line A549

Table 1. The primer sequence of gene

List	*F: primer (5'-3')*	*R: primer (5'-3')*
miR-126-3p	GCTGGCGACGGGACATTA	CGCATTATATACTCACGGAAGG
PTPN9	ATGTGCTCCGTGCCATAGAATTG	GAGGATCTGAGAACGAAGAGGTTCC
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCAGAATTTGCGTGTCAT
GAPDH	CGACCACTTTGTCAAGCTCA	ACTGAGTGTGGCAGGGACTC

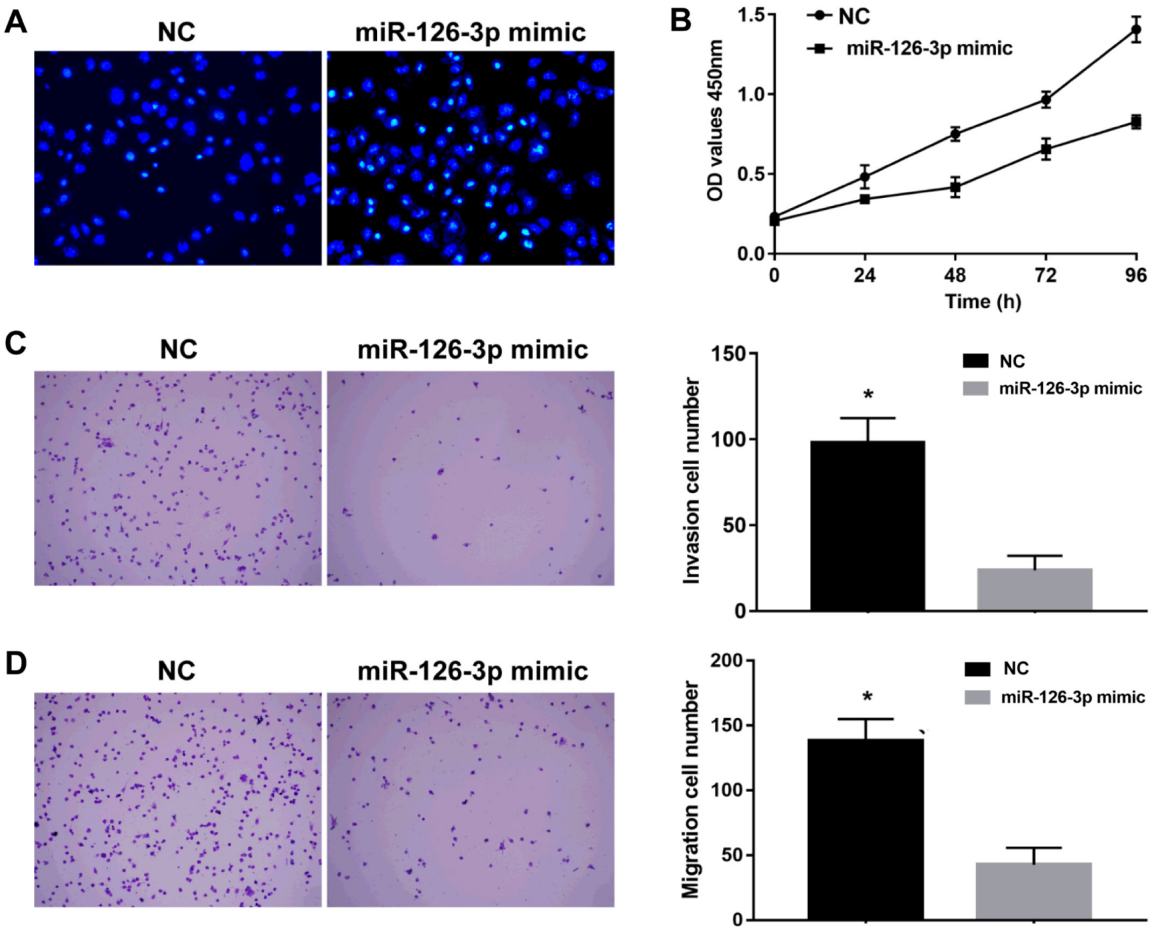

Figure 1. Overexpressed miR-126-3p inhibited the proliferation, migration and invasion and promoted the apoptosis of A549 cells. **(A)** The cells apoptosis were detected by DAPI; **(B)** The cells viability were determined with CCK-8 assay; **(C, D)** The migration and invasion of cells was conducted through transwell assay (*p<0.05).

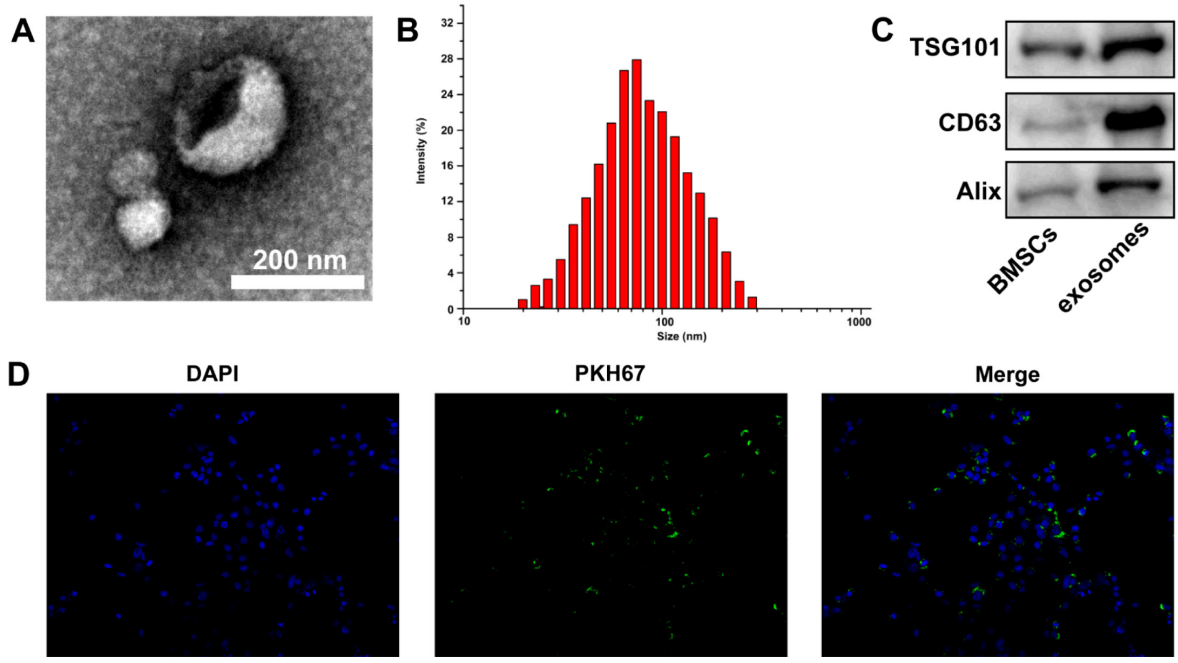

Figure 2. Identification and internalization results of exosomes. **(A)** The ultrastructure of exosomes observed by TEM; **(B)** Size distribution of exosomes with NanoSight particle size analysis. **(C)** Western blot detected the special marker of exosomes (TSG101, CD63 and Alix). **(D)** The exosomes absorbed by A549 cells with confocal fluorescence microscope.

to detect the apoptosis, proliferation, migration, and invasion of A549. It was observed that, when compared with the A549 cells transfected with NC group, those transfected miR-126-3p mimic, the viability, migration, and invasion were decreased and apoptosis was increased. (Figure 1A-1D).

Identification and internalization results of exosomes

Under the TEM (Figure 2A), it was observed that BMSCs-derived exosomes were round or elliptical, with a diameter of about 100 nm. In addition, it results of NTA shown that multimodal distribution was observed in the suspension, the size of the particles was about (102.6 ± 42.3) nm, and the concentration was about 2.3×10^{12} particles/L (Figure 2B). For further verification, WB was employed to detect exosomal surface-specific proteins. The results showed that BMSCs-derived exosomes had positively expressed CD63, TSG101 and ALIX (Figure 2C). According to Figure 2D, green fluorescence was clearly observed in A549 cells, suggesting that exosomes can be taken up by A549 cells.

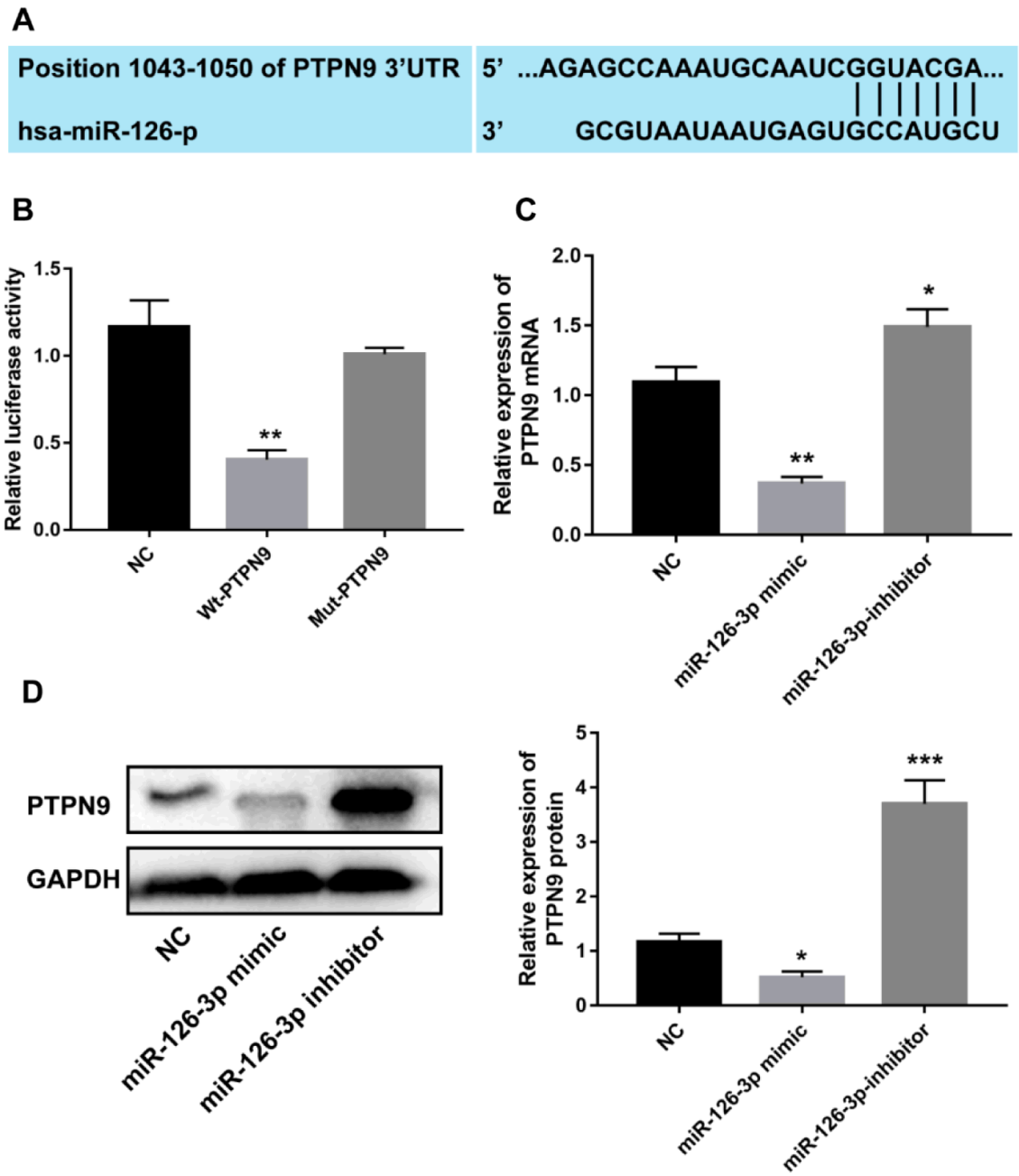

Figure 3. MiR-126-3p targeted PTPN9. **(A)** The binding site of miR-126-3p on the 3'-UTR of PTPN9; **(B)** Dual-luciferase assay detected the target relation between miR-126-3p and PTPN9; **(C, D)** The expression of PTPN9 were determined in A549 cells treatment with miR-126-3p mimics and miR-126-3p inhibitor (*p<0.05; **p<0.01; ***p<0.001).

MiR-126-3p targeted PTPN9

Based on the prediction using the TargetScan website (http://www.targetscan.org/vert_71/), miR-126-3p had a specific binding region to PTPN9 in its 3'-UTR (Figure 3A). The dual-luciferase reporter gene assay was conducted to verify the above prediction. It was discovered that miR-126-3p mimic reduced the luciferase activity of Wt- PTPN9 and had no effect on that of Mut- PTPN9 (Figure 3B). Besides, the mRNA and protein expressions of PTPN9 declined in A549 cells after overexpressing miR-126-3p, whereas opposite results were observed in the treatment of miR-126-3p inhibitor (Figure 3C and 3D).

BMSCs-derived exosomes carried miR-126-3p

The level of miR-126-3p was measured in BMSCs, BMSCs-derived exosomes and co-culture system A549 through qRT-PCR. The results (Figure 4A-4C) shown that the miR-126-3p expression was significantly higher in BMSCs, exosomes, and co-cultured A549 cells when compared with NC group ($p<0.05$), which suggested that BMSCs-derived exosomes carried miR-126-3p. The level of PTPN9 expression was further detected and the results demonstrated there to be diminished levels of PTPN9 after the A549 cells had absorbed the exosomal miR-26-3p (Figure 4D-4E, $p<0.05$).

BMSCs-derived exosomal miR-126-3p suppressed the proliferation, migration and invasion of A549 cells and increased their apoptosis by down-regulating the expression of PTPN9

To investigate the effects of Exo-miR-126-3p on the functions of A549 cells, A549 cells were treated with Exo-miR-126-3p. The results demonstrated that the proliferation, migration and invasion of A549 cells treated with Exo-miR-126-3p were significantly reduced, while their apoptosis was markedly increased (Figure 5A-5D). WB results further revealed that after treatment with Exo-miR-126-3p, the expression of apoptosis-related proteins in A549 cells increased significantly, whereas that of PTPN9 was decreased (Figure 5E, $p<0.05$). These above results implied that exosomal miR-126-3p is able to suppress the proliferation, migration, and invasion of A549 cells while promote their apoptosis of A549 cells by inhibiting the expression of PTPN9.

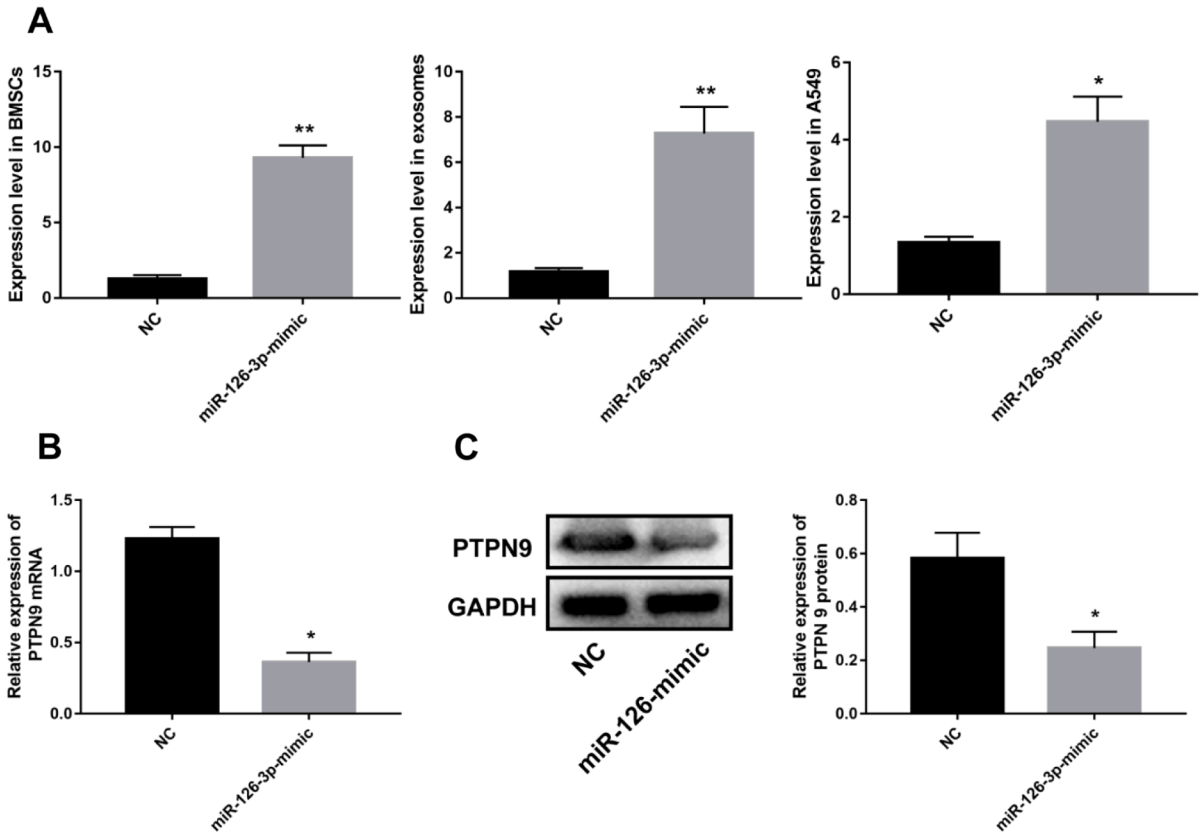

Figure 4. BMSCs-derived exosomes carried miR-126-3p. **(A)** RT-PCR detected the expression of miR-126-3p in BMSCs, exosomes, and co-cultured A549 cells. **(B)** The mRNA expression of PTPN9 in A549 cells; **(C)** Western blot analysis the protein expression of PTPN9 (*$p<0.05$; **$p<0.01$).

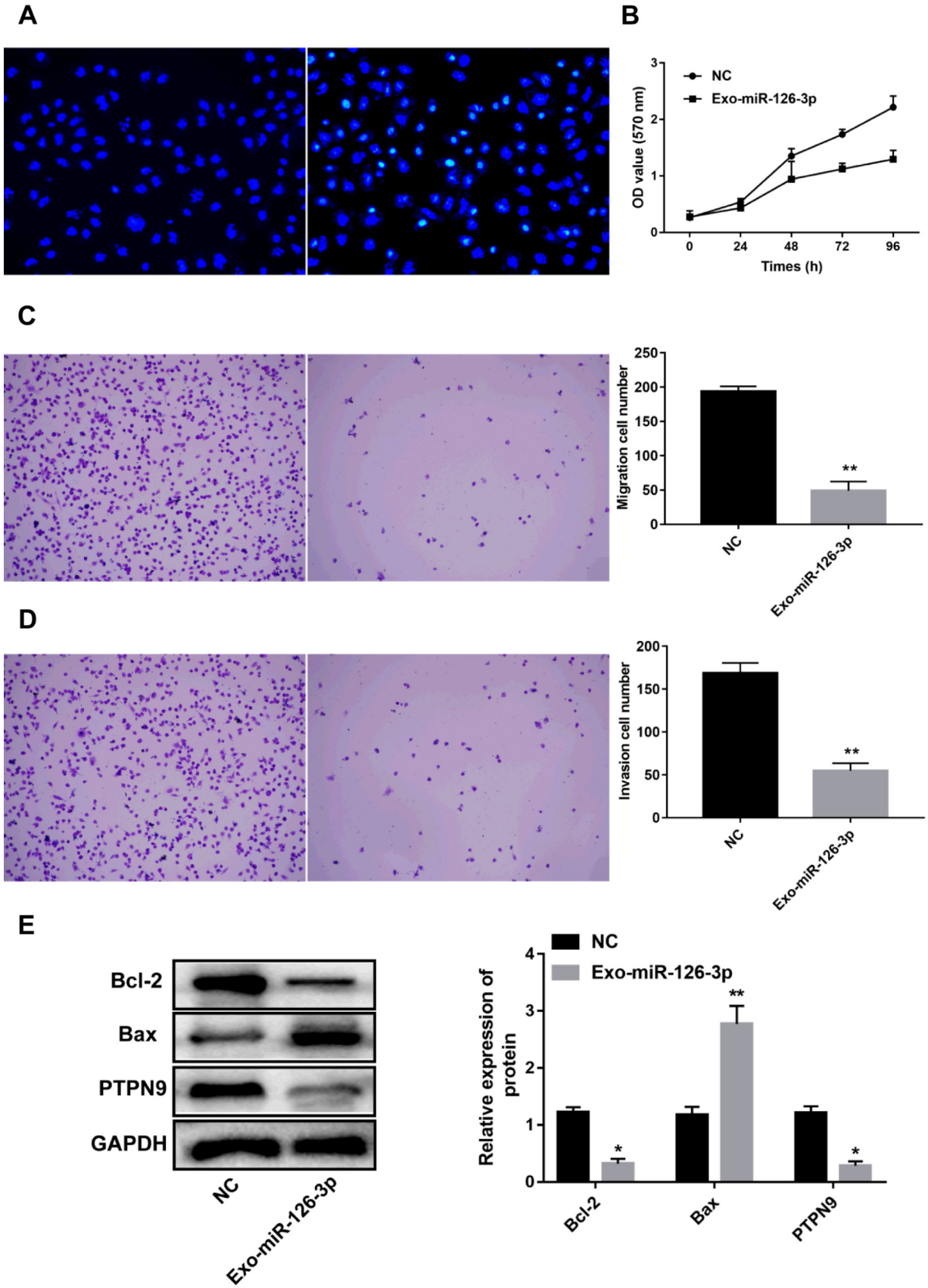

Figure 5. BMSCs-derived exosomal miR-126-3p suppressed the proliferation, migration and invasion of A549 cells and increased their apoptosis by down-regulating the expression of PTPN9. **(A)** The cells apoptosis were detected by DAPI; **(B)** The cells viability were determined with CCK-8 assay; **(C, D)** The migration and invasion of cells was conducted through transwell assay; **(E)** The expression of cell apoptosis protein and PTPN9 was detected by Western blot (*p<0.05; **p<0.01).

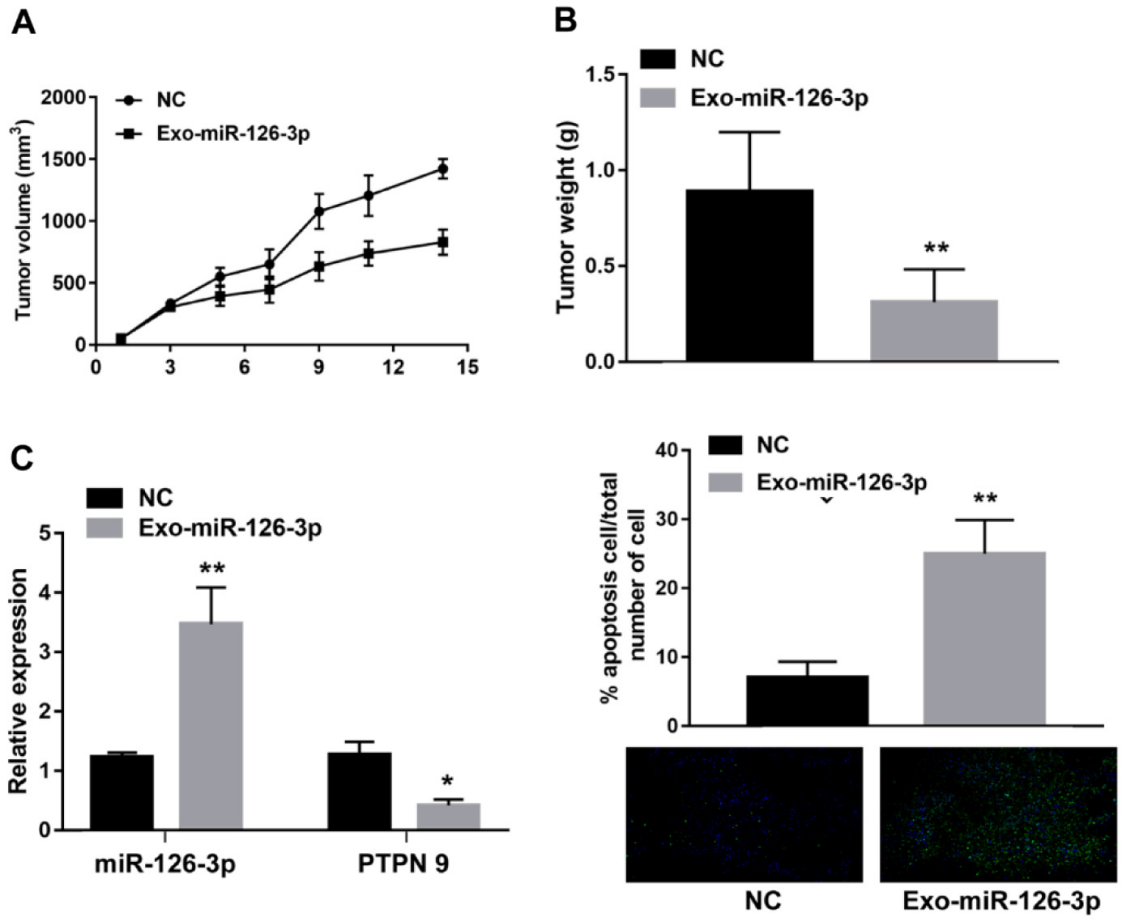

Figure 6. Exosomal-miR-126-3p derived from BMSCs inhibits tumor growth. **(A)** The effect of Exo-miR-126-3p on the growth of the tumor analyzed with xenograft in nude mice; **(B)** Tumor weight after Exo-miR-126-3p treatment. **(C)** The mRNA expression of miR-126-3p and PTPN9 after Exo-miR-126-3p treatment; **(D)** TUNEL detected the percent apoptosis cells (*p<0.05; **p<0.01).

Exosomal-miR-126-3p derived from BMSCs inhibits tumor growth

In order to explore the effect of exosomal-miR-126-3p on tumor growth, over-expressed miR-126-3p in BMSCs was determined by nude mice. As shown in Figure 6A-6B, compared with NC group, the exosomal-miR-126-3p up-regulation decreased tumor growth, tumor volume, and tumor weight. The expression of miR-126-3p and PTPN9 was detected with qRT-PCR. Compared with NC group, the expression of miR-126-3p in exosomal-miR-126-3p was increased significantly, while PTPN9 was decreased markedly (Figure 6C). Moreover, the TUNEL results demonstrated that exosomal-miR-126-3p up-regulation promote the apoptosis of tumor cells (Figure 6D).

Discussion

As the most common tumor, NSCLC accounts for over 80% of all diagnosed malignant lung cancers [17]. Numerous research has manifested that the content of exosomal miRNAs in cancer patients is different from that in healthy people, and the content and amount of exosomal miRNAs have correlations with the development stage of cancers and the regulation of malignant phenotypes [18]. In this study, the role and mechanism of BMSCs-derived exosomes in NSCLC through the delivery of miR-126-3p were evaluated. The results showed that BMSCs-derived exosomes could deliver miR-126-3p to down-regulate the expression of PTPN9, thereby inhibiting the proliferation, invasion and metastasis of NSCLC cells and promoting their apoptosis. Hence, they are expected to become potential biomarkers for the treatment of NSCLC.

It was found in the present study that BMSCs-derived exosomal miR-126-3p could be absorbed by A549 cells, thus inhibiting the proliferation, migration, and invasion of A549 cells. Besides, natural killer cells can impede the abnormal proliferation of melanoma cells through exosomes secreted, thereby prolonging the survival time of mice with melanoma [19]. More importantly, researchers have

confirmed that BMSCs-derived exosomes can repress the proliferation, migration, and invasion of pancreatic cancer cells by carrying miRNAs [20]. In this study, it was discovered that overexpressed or exosomal miR-126-3p inhibited the proliferation, migration and invasion of A549 cells. According to the results of a previous study, miR-126-3p can suppress the proliferation and invasion of ovarian cancer cells [21]. Moreover, Wang et al. [22] confirmed that miR-126-3p exerts an inhibitory effect on human breast cancer by directly targeting metalloproteinase-9. Furthermore, the high expression of miR-126-3p is a promising positive factor for the treatment of NSCLC patients [23]. The results of this study revealed that BMSCs-derived exosomes suppressed the proliferation, migration and invasion of NSCLC cells via delivering miR-126-3p.

Importantly, it was also found in this study that PTPN9, a target gene of miR-126-3p, had a down-regulated expression after treatment with exosomeal miR-126-3p inhibitor. MiR-126-3p has been reported to inhibit the growth and metastasis of bladder cancer cells by targeting ADAM9. Additionally, there is a specific binding site between miR-126-3p and the 3'-UTR of PTPN9, and highly expressed PTPN9 facilitates the proliferation and invasion of Eca109 cells through negative regulation of miR-126-3p [24], verifying the targeted relationship between PTPN9 and miR-126-3p. Furthermore, this study found that BMSCs-derived exosomes restrained the proliferation, migration, and invasion of A549 cells by transmitting miR-126, and also promoting the apoptosis of NSCLC cells by down-regulating PTPN9.

Conclusions

In this research, for the first time, exosomal miR-126-3p secreted from BMSCs cells was demonstrated for NSCLC. Exosomal miR-126-3p could mediate the inhibitory effect of exosomes on A549 cells via negative regulating of PTPN9. This suggested that miR-126-3p may be a potential target for the treatment and diagnosis of NSCLC. However, there were some shortcomings in the present study. For example, only A549 cell line was studied. In the future, therefore, multiple cell lines and animal experiments should be selected and conducted, to improve the reliability, reproducibility, and implication of results.

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Conflict of interests

The authors declare no conflict of interests.

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