MiR-26b suppresses tumor cell proliferation, migration and invasion by directly targeting COX-2 in lung cancer

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Abstract. – OBJECTIVE: Lung cancer, including non-small cell lung cancer (NSCLC), is the leading cause of cancer-related mortality worldwide. Despite recent advances in clinical and experimental oncology, the prognosis of patients with NSCLC still remains poor and the average survival time of patients suffer from lung cancer is low. Therefore, the potential mechanism accounting for the tumorigenesis of NSCLC is still needed to be explored.

MATERIALS AND METHODS: A lentiviral vector over-expressing miR-26b in A549 lung cancer cells was constructed. Cell proliferation, migration and invasion analysis were measured by cell counting kit (MTT), would healing assay and Transwell assay. Direct target of miR-26b in A549 cells was examined using bioinformatics and Luciferase assay.

RESULTS: Herein, we found that over-expression of miR-26b significantly inhibited the proliferation, migration and invasion of A549 lung cancer cell in vitro and suppressed the growth of established tumors in vivo. By using bioinformatics we found that COX-2 (Cyclooxygenase-2) is one of the potential targets of miR-26b. Moreover, miR-26b was found to negatively regulate COX-2 protein level by directly targeting its 3'UTR. In addition, depletion of endogenous COX-2 by the specific siRNA could mimic the function of miR-26b overexpression.

CONCLUSIONS: Taken together, our results demonstrate that miR-26b could suppress lung cancer cells proliferation, migration and invasion by directly negative regulation of COX-2. MiR-26b could serve as a novel potential marker for NSCLC therapy.

Key words:
NSCLC, miR-26b, COX-2.

Introduction

Lung cancer is the most lethal cancer in men worldwide with an age-standardized rate (ASR) of 33.8 per 100,000 and the fourth most frequent cancer in women (13.5 per 100,000)[1]. Lung cancer is the leading cause of cancer-related mortality among both men and women, which was estimated to cause 160,340 deaths in the United States in 2012, potentially accounting for 28% of all cancer related deaths in the country[2]. Although significant advances have been made with conventional therapies, including surgery, chemotherapy and radiotherapy, the poor prognosis and low overall survival (OS) rate indicates the urgent need for developing novel therapeutic methods[3]. Current studies of lung cancer treatment are mainly focused on molecular therapies, thus the identifying of efficient targets is of great importance.

COX-2 is an important enzyme regulating the conversion of arachidonic acid to the prostaglandin E2 (PGE2)[4]. Some researchers have found that PGE2, which is produced normally by epithelial cells but at very high levels in lung cancer and other malignancies, up-regulates the activity of a type of lymphocytes called T-regulatory cells, which suppress the immune function, resulting in tumor progression and worsened disease outcome[4-8]. Recent study demonstrated that COX-2 as a single molecule could induce tumorigenesis in some transgenic mice without genetic modifications[9]. Masferrer et al[10] suggested that COX-2 was associated with microvascular angiogenesis in tumor tissues and resistance to apoptosis of lung cancer cells. Krzystyniak et al[11] found that COX-2 upregulation affected both angiogenesis and production of specific proteases that were critical to lung cancer growth and metastasis. Based on previous publications, we hypothesis that downregulation of COX-2/PGE2 signaling might be a promising and efficient method in preventing and treating lung cancer.

MicroRNAs (miRNAs), which are a kind of endogenous short non-coding RNAs of ~22 nucleotides, exert a wide range of biological functions in different cellular processes, including proliferation, differentiation, metabolism and apoptosis by direct messenger RNA (mRNA) degradation or protein translation repression[12-14]. Up to now, more than 400 human miRNAs have

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been identified and about 1,000 have been postulated to exist[15]. It is believed that up to 30% of human genes are regulated by miRNAs[16]. Each miRNA regulates a variety of targeting genes, and an individual mRNA may be regulated by distinct miRNAs, giving rise to an extensive regulatory network[17,18]. Recently, Chakrabarty et al[19] revealed that mmu-miR-199a* and mmu-miR-101a were potential regulators of COX-2 expression in the mouse uterus during implantation. More important, miR-26b has been reported to be frequently down-regulated in various tumors, including breast cancer, bladder cancer, gastric cancer, prostate cancer, cervical cancer and liver cancer, etc[20-26]. In addition, by direct targeting COX-2, miR-26b has been shown to inhibit the proliferation, migration, and epithelial-mesenchymal transition of lens epithelial cells[27]. However, different cancer cells have different context, the relationship between miR-26b expression and COX-2 alteration in lung cancer remains unknown.

Therefore, in the present work, we demonstrated that over-expression of miR-26b significantly suppressed A549 cell proliferation, migration and invasion *in vitro* and inhibited the growth of established tumors *in vivo*. In addition, we found COX-2 is a potential target of miR-26b. miR-26b was over-expressed while COX-2 was significantly down-regulated in A549 cells. These results clearly demonstrated that miR-26b is a potential tumor suppressor and may suggest a new therapeutic strategy for lung cancer.

Materials and Methods

Cell Culture

Human lung cancer cell lines A549 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA, USA) and maintained at 37°C in an atmosphere of humidified air with 5% CO_2.

Vector Construction

MiR-26b expressed plasmids were constructed using the pRNAT-U6.1/Neo vector (Genscript, Piscataway, NJ, USA), the pre-miR-26b were synthesized and cloned into pRNAT-U6.1/Neo vector, and the empty vector was used as a control. Thereafter, recombinant plasmid pRNAT-U6.1-miR-26b, together with the package vectors psPAX2 and pMD2. G were co-transfected into HEK 293 packaging cells. 72 h later, lentivirus in the supernatant was harvested and concentrated to a final vector titer of 7.8* 10^7TU/ml. Then A549 cells were transducted with concentrated recombinant lentivirus-miR-26b or lentivirus-control using polybrene (10 mg/ml; Sigma-Aldrich, St. Louis, MO, USA). For selection of stable cell lines, MiR-control-A549 and MiR-26b-A549 was cultured in 400 mg/mL neomycin (Gibco, Carlsbad, CA, USA) for 14 days after infection. Real-time qRT-PCR was used to verify the efficiency of transduction. The sequence of the COX2 3'UTR containing the predicted miR-26b binding sites and its mutant of the miR-26b binding sites were synthesized and insert into pGL3-control vector (Promega, Madison, WI, USA).

RNA Isolation and Quantitativ Real Time PCR (qRT-PCR)

Total RNA was reverse transcribed into cDNA by using AMV Reverse Transcriptase (Takara, Otsu, Shiga, Japan) with oligo (dT) 18 or specific RT primer. Equal amounts of the cDNA products were used as templates for subsequent PCR amplification using the Applied Biosystems 7300 Real Time PCR system (Foster City, CA, USA). U6 and belta-actin were used as endogenous control. The 2-△△Ct method was applied to calculate the relative expression levels of miR-26b and COX-2. The following primers were used: miR-26b RT primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCTAT -3'; U6 RT primer, 5'- AAAAATATGGAACGCTCACGAATTTG -3'; miR-26b, 5'- CCAGGCACCGTTCAAGTAATTC-3' (forward); and 5'-CCAGTGCAGGGTCCGAGGTA-3' (reverse). U6, 5'-GTGCTCGCTTCGGCAGCACATATAC-3' (forward) and 5'- AAAAATATGGAACGCTCACGAATTTG-3' (reverse). COX-2, 5'- GTCCCTGAGCATCTACGGTTTG-3' (forward) 5'-CCCATTCAGGATGCTCCTGTT-3' (reverse); beta-Actin, 5'- AAAGACCTGTACGCCAACAC-3' (forward) and 5'- GTCATACTCCTGCTTGCTGAT-3' (reverse).

Dual-luciferase report assay

Dual luciferase assay was performed as previous description[27]. Briefly, cells were plated into a 24-well plate at approximately 80% confluence and pGL3-COX-2-3'UTR, pGL3- COX-2-3'UTR-mut plasmids were transfected into A549 cells combined with miR-26b expressed vector

using lipofectamine 2000 transfection agents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Subsequent to 24 h of transfection, the cells were harvested, lysed and analyzed using Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI, USA). All the experiments were performed in triplicate.

Western blotting

Protein sample of both cells and tissues were prepared with Nuclear and Cytoplasmic Extraction reagents (NE-PER) (Pierce, Rockford, IL, USA), according to the manufacturer's protocol. Resulting protein samples were electrophoresed by 10% SDS-PAGE and transferred to PVDF membrane. After blocking with TBS containing 5% non-fat milk and 0.1% Tween-20 for 2 h, the membrane was incubated with the primary antibody at 4°C overnight. The primary antibodies and dilutions used were as follows: GAPDH and COX-2 were obtained from Cell Signaling Technology Inc (1:2000 dilutions; Beverly, MA, USA). After washing with TBS containing 0.1% Tween-20 three times, the membrane was then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilutions; Beverly, MA, USA) for 1 h at room temperature. Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, USA).

Knockdown of COX-2 by small interfering RNA (siRNA)

SiRNA oligonucleotides for human COX-2 (5'-CACCAAGAGTATAAACCTT-3') and negative control (5'-UUCUCCGAACGUGUCACGU-3') were synthesized from GenePharma Company (Shanghai, China), and were transfected into A549 cells using Lipofectamine® RNAiMAX transfecting reagent (Invitrogen, Carlsbad, CA, USA) in serum-free medium according to the manufacturer‘s instructions.

Cell Proliferation Assays

Cell proliferation was determined by the 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT, Roche, Mannheim, Germany) assay as previous descriptions[15]. Briefly, cells were seeded in 96-well plates at a density of 1,000 cells per well. At various time points, 20 µl of 5 mg/ml solution of MTT dissolved in PBS was added to each well and incubated at 37˚C for 4 h. The generated formazan was dissolved in dimethyl sulfoxide (DMSO) and the absorbance of the solution was measured at 490 nm to determine the cell viability.

Wound Healing Assays

Cells were grown to confluence in 6-well plates. A linear wound was made by scraping a non-opening Pasteur pipette across the confluence cell layer and the cells were washed twice to remove detached cells and debris. Cells that migrated into the wound were measured at two different time points (0 and 48 hours) from the central of the filter by a light microscope at 100 × magnification. Then, we subtracted it from the wound width at time 0. The values were expressed as a migration percentage (setting the gap width at 0 hour as 0%).

Transwell assays

Cell migration assessment was performed using 24-well Transwell plates (Millipore, Billerica, MA, USA) as previous descriptions[28]. Briefly, the under surface of the membrane was coated with fibronectin (10 mg/ml) in PBS at 37°C for 2 hours. The lower chamber was filled with 0.6 ml of DMEM supplemented with 10% FBS. Before experiment, cells were serum-starved overnight (DMEM plus 0.5% bovine serum albumin (BSA); then, 1×10^6 cells in a volume of 0.1 ml migration medium (DMEM plus 0.5% BSA) were added to the upper chamber. After incubation at 37°C for 48 h, cells on the upper surface of the membrane were removed. The migrant cells attached to the lower surface were fixed in 10% formalin and stained with a solution containing 1% crystal violet and 2% ethanol in 100 mmol/L borate buffer (pH=9.0). The number of migrated cells on the lower surface of the membrane was counted under a light microscope.

in vivo *tumor growth assays*

Four-week-old female nude mice were purchased from Shanghai Experimental Animal Center of the Chinese Academic of Sciences (Shanghai, China). Mice were housed in specific pathogen-free (SPF) conditions and treated in accordance with guidelines of the Committee on Animals of Jingling Hospital (Nanjing, China). Localized human lung cancer Xeno-transplant models were established as previous studies[29-30]. Briefly, miR-26b-A549 cells and the control cells were injected subcutaneously into nude mice, respectively. The tumor size was measured in two perpendicular diameters with precision calipers twice a week and calculated by the following equation: Volume = $(\text{Length}\times\text{Width}^2/2)$[30].

Statistical Analysis

The statistical analysis involving two groups was performed by means of Student's *t*-test, whereas analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used in order to compare more than two groups. All data were processed with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Over-expression of miR-26b inhibits proliferation, migration and invasion of A549 cells.

To explore the effects of miR-26b on lung cancer cell, A549 cell lines were infected with a lentivirus encoding the mature sequence of miR-26b. The infected cells produced high levels of miR-26b in A549 cells (Figure 1A). First, we used the MTT assay to examine the effect of miR-26b on cell proliferation. As shown in Figure 1B, compared with the miR-control cells, the miR-26b-overexpressing cells led to growth inhibition in A549 cells as early as 3 days post-transfection, persisting for 7 days ($p < 0.01$), indicating that miR-26b could significantly inhibit the proliferation of lung cancer cells. In addition, we explored the potential impact of miR-26b on lung cancer cell migration and invasion using wound-healing assay and Transwell assay. The wound healing assay showed that overexpression of miR-26b slowed the scratch wounds closure and suppressed the migration rate of A549 cells compared with the controls remarkably ($p < 0.01$, Figure 2A). Furthermore, we also found that ectopic expression of miR-26b significantly suppressed the invasion of A549 cells in Transwell assay after 48h ($p < 0.05$, Figure 2B). Taken together, these results indicate that miR-26b has the ability to inhibit proliferation, migration and invasion of lung cancer cells.

Over-expression of miR-26b inhibits tumor growth in vivo

To determine whether miR-26b regulates lung cancer carcinogenesis, we further assessed the effects of miR-26b over-expression on tumor growth *in vivo*. MiR-26b-A549 cells and their respective control cells were implanted into the right and left flanks ($5.0*10^6$ cells per flank) of 4-week old female nude mice by subcutaneous injection. The development of established tumors was monitored every 7 days in a range of 7 weeks. The host mice displayed visible tumors within 7 days after injection and over-expression of miR-26b in A549 suppressed tumor growth and decreased the volume *in vivo* compared with the controls ($p < 0.05$,Figure 3A). At the end of the observation (42 days), the average weight of tumors expressing miR-26b was significantly lighter than those in control groups ($p < 0.01$, Figure 3B). These results show that over-expression of miR-26b can result in significant retardation of tumor growth *in vivo*, which might be a useful treatment to overcome lung cancer.

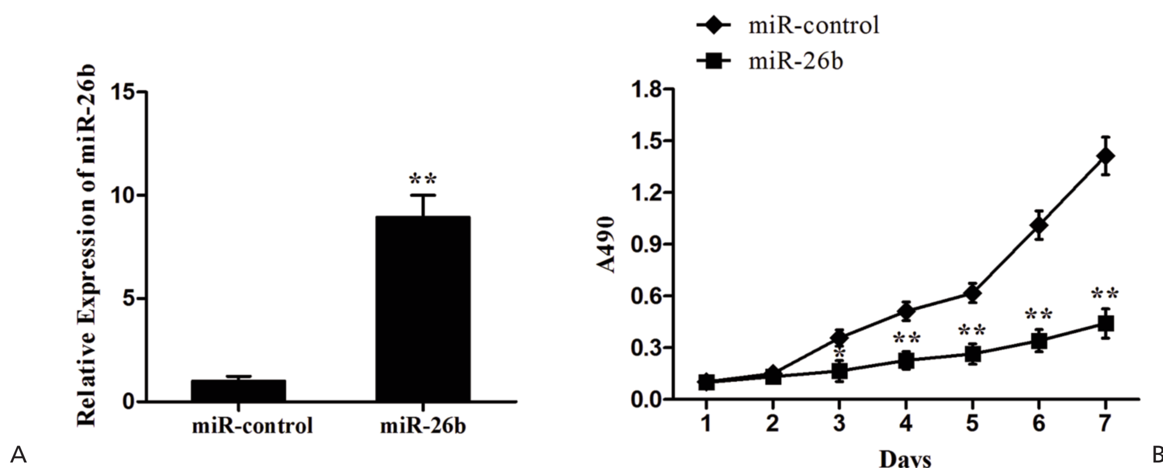

Figure 1. Over-expression of miR-26b inhibits A549 cell proliferation in vitro. **A**, qRT-PCR analysis detected the miR-26b expression in MiR-control-A549 and MiR-26b-A549 cells. The average miR-26b expression was normalized by U6 expression. **B**, The rates of cell proliferation were measured using a MTT assay at various time points (1, 2, 3, 4, 5, 6 and 7 days). The absorbance at 490 nm was measured. Data are shown as mean ± SEM (n=3), $**p < 0.01$.

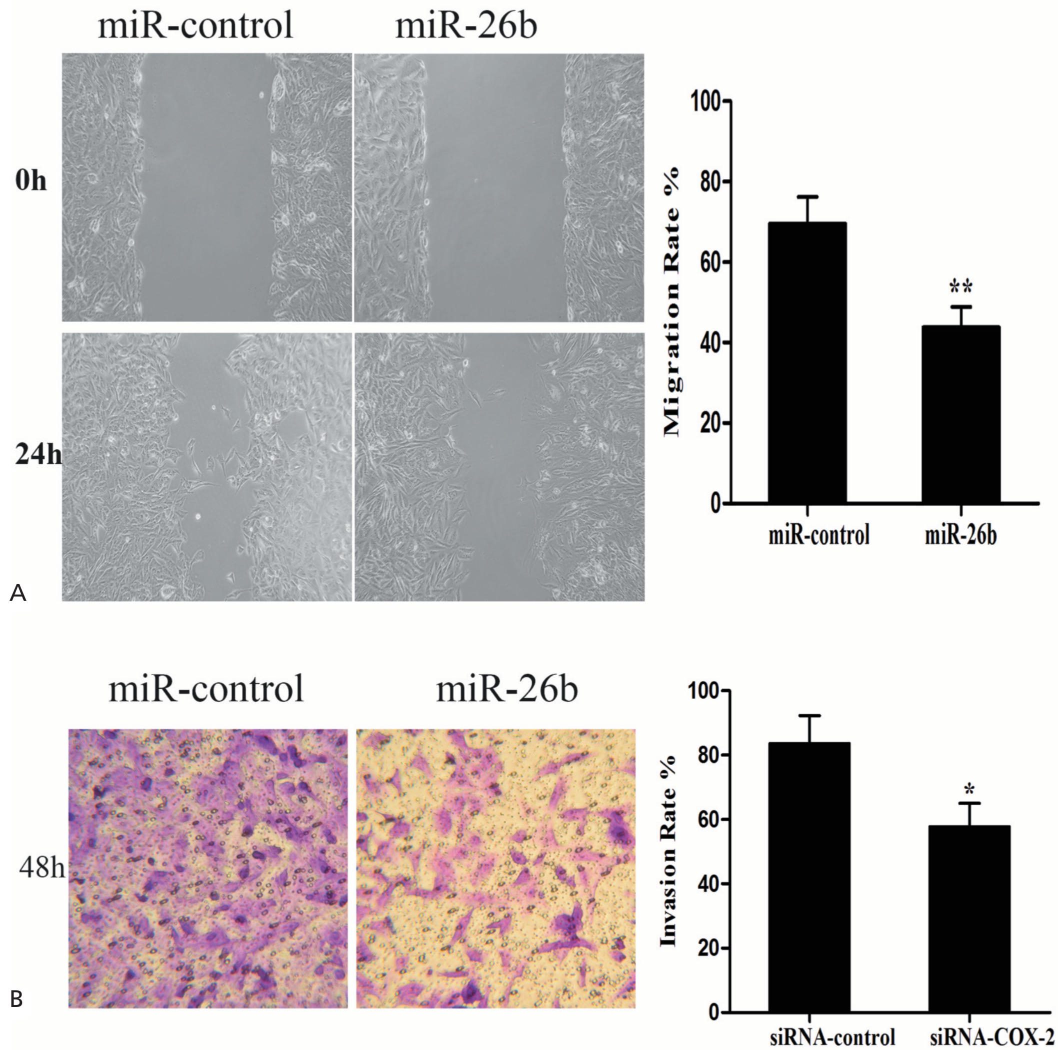

Figure 2. miR-26b suppress migration and invasion in A549 cells. **A**, Cell migration was evaluated by wound healing assay in A549 cells. Representative images were obtained at time point 0 and 24 h. Migration rate was quantified by measuring gap distance. **B**, Cell invasion ability was evaluated with matrigel transwell assay. Data are shown as mean ± SEM (n=3), $*p < 0.05$, $**p < 0.01$.

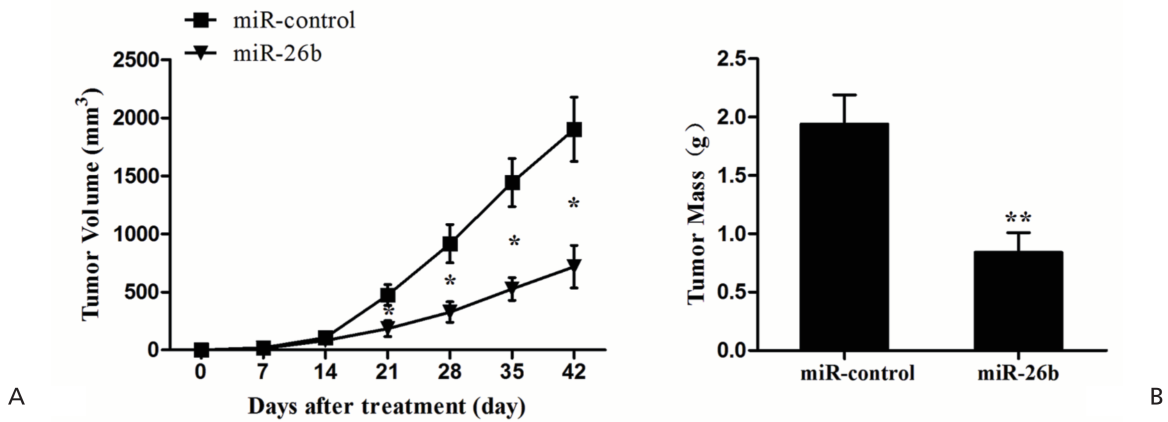

Figure 3. Up-regulation of miR-26b decreases the growth of established tumors in vivo. **A**, MiR-control-A549 and MiR-26b-A549 cells were injected subcutaneously into BAL B/c nude mice. The development of tumors was recorded in a range of 6 weeks. **B**, Tumor bearing mice were sacrificed and the tumors were weighted. Data are shown as mean ± SEM (n=3), $*p < 0.05$, $**p < 0.01$.

COX-2 is identified as a direct target of miR-26b in lung cancer cells

Two algorithm programs (PicTar and TargetScan Release) were used to predict the potential targets of miR-26b. The two algorithm programs showed that the 3'-UTR of COX-2 has predicted binding sites of miR-26b (Figure 4A). To prove that miR-26b can directly target to COX-2, a luciferase activity assay was performed. The results demonstrated that overexpression of miR-26b inhibited the luciferase activity in A549 cells. However, the luciferase activity with the mutated 3'UTR was not influenced by the up-regulation of miR-26b (Figure 4B). These results suggest that miR-26b can specifically bind to the 3'-UTR of COX-2. To directly assess the effect of miR-26b on COX-2 expression, qRT-PCR and western blot analysis were performed. As seen in Figure 4C and 4D, up-regulated of miR-26b markedly decreased COX-2 mRNA and protein levels in A549 cells. Taken together, these results indicate COX-2 is a direct downstream target of miR-26b in lung cancer cells.

Depletion of COX-2 inhibits proliferation, migration and invasion of A549 cells.

In order to determine the impact of COX-2 on the proliferation and invasion of lung cancer cells, an RNAi approach was used to suppress COX-2 gene expression. The results indicated that the COX-2 siRNA could significantly reduce endogenous COX-2 expression in mRNA and protein levels (Figure 5A and 5B). Inhibition of COX-2 expression decreased A549 cells growth as compared with the control group (Figure 5C). Furthermore, cells with reduced levels of COX-2 inhibited A549 cells migration and invasion (Figure 6A and 6B). These results are consistent with the finding that miR-26b over-expression can suppress cell growth, migration and invasion *in vitro*. Collectively, our data provides further evidence that COX-2 is involved in the miR-26b-mediated suppression of lung cancer.

Discussion

In the past 100 years, lung cancer has been transformed from a rare disease into a global

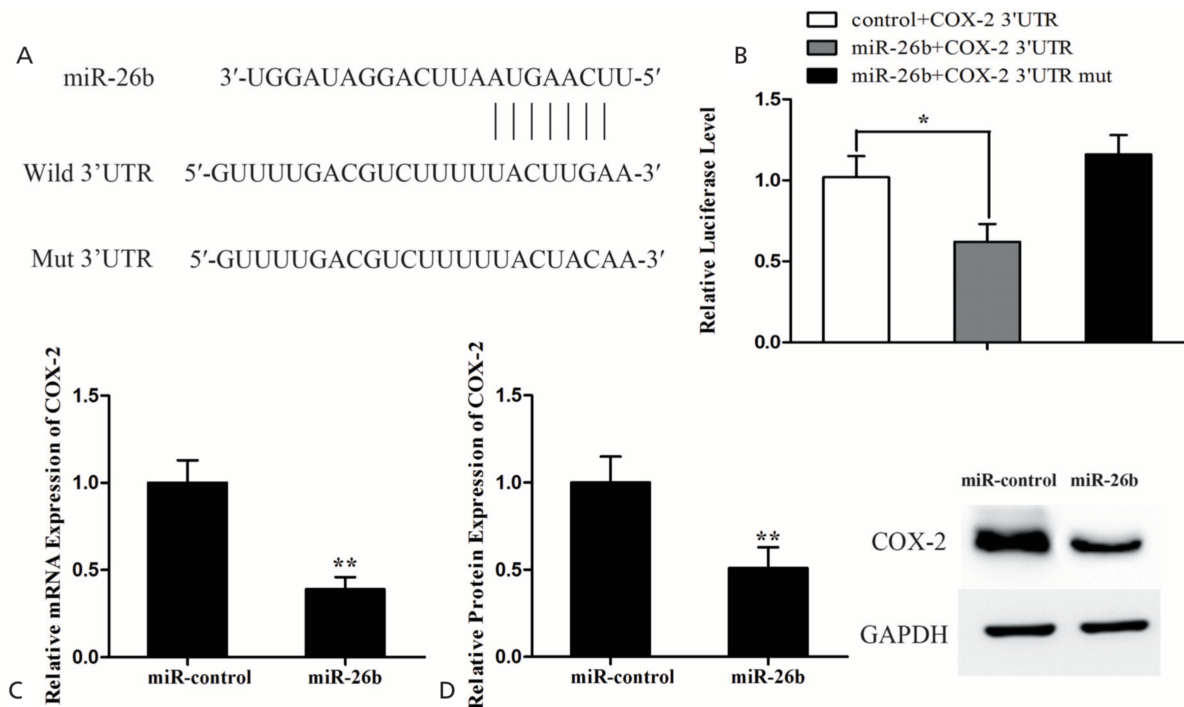

Figure 4. COX-2 is a direct target of miR-26b in vitro. **A**, Predicted miR-26b target sequences in 3'UTR of COX-2 and mutated nucleotides in 3'UTR of COX-2. **B**, A549 cells were co-transfected with miR-26b and luciferase reporters containing the predicted miRNA target site in COX-2 3'UTR or its corresponding mutant form, which was used as the positive control. **C-D**, The mRNA and protein expression level of COX-2 was analyzed by qRT-PCR and Western blot in A549 cells. Data are shown as mean ± SEM (n=3), $^*p < 0.05$, $^{**}p < 0.01$.

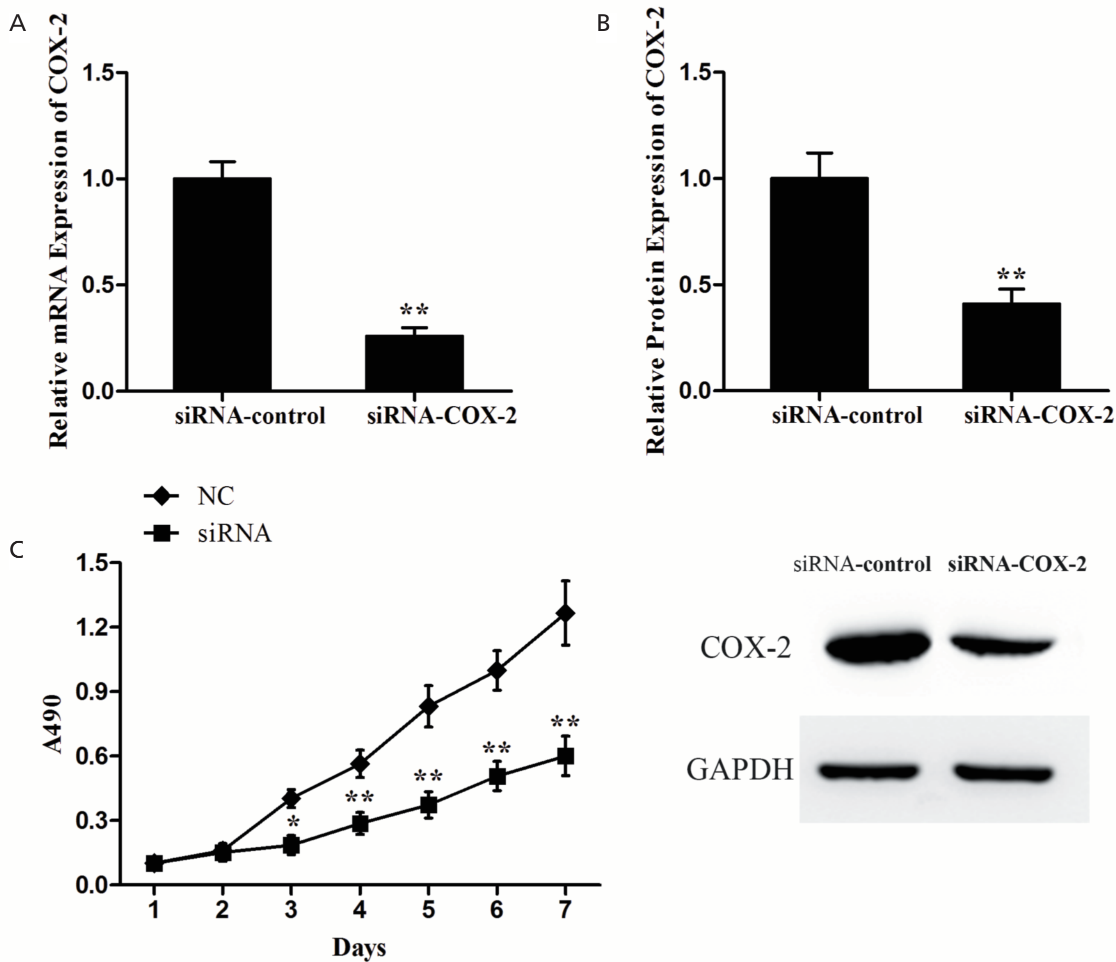

Figure 5. Knockdown of cox-2 inhibits A549 cell proliferation. ***A-B***, COX-2 RNA and protein expression levels in A549 cells transfected with siRNA-COX-2 or siRNA-control were detected by qRT-PCR and Western blotting assay. (C) The functional role of COX-2 in A549 cell proliferation was analyzed by MTT assay. Data are shown as mean ± SEM (n=3), $*p < 0.05$, $**p < 0.01$.

problem[1,2]. Although significant advances have been made with conventional therapies, lung cancer still remains the leading cause of cancer-related mortality among both men and women, the fact of which calls for improvements in the diagnosis and treatment of the disease.

Growing evidences have demonstrated that up-/down-regulation of miRNAs is involved in the initiation and progression of different malignancies through target oncogenes or tumor suppressor genes[12-14]. Recent study indicated that miRNAs may function as tumor suppressors (let-7, miR-126, miR-145, miR-200 and miR-34) or oncogenes (miR-17-92, miR-21, miR-31, miR-221 and miR-222) in the biogenesis and maintenance of numerous kind of cancers[15-18]. In addition, miRNAs also show potentials as biomarkers for the diagnosis and prognosis of lung cancer which can complement and improve upon other techniques. Furthermore, increasing results have revealed the possibility of using miRNAs as treatments in lung cancer[31]. miR-26b has been reported to be frequently down-regulated in various tumors and has an evident effect on tumor growth, migration or metastasis through target different genes[20-26]. Each miRNA can potentially interact with several mRNA targets via perfect or imperfect base pairing, primarily in the 3-UTR portion. Whether COX-2 can be proved to be regulated by miR-26b in lung cancer may enlighten the new therapeutic target.

Cyclooxygenase (COX), a key enzyme involved in the production of prostaglandins and other eicosanoids from arachidonic acid is the best known target of non-steroidal anti-inflammatory drugs (NSAIDs)[32]. The COX exists as two isoforms, COX-1 and COX-2. COX-1 is considered a constitutively expressed housekeep-

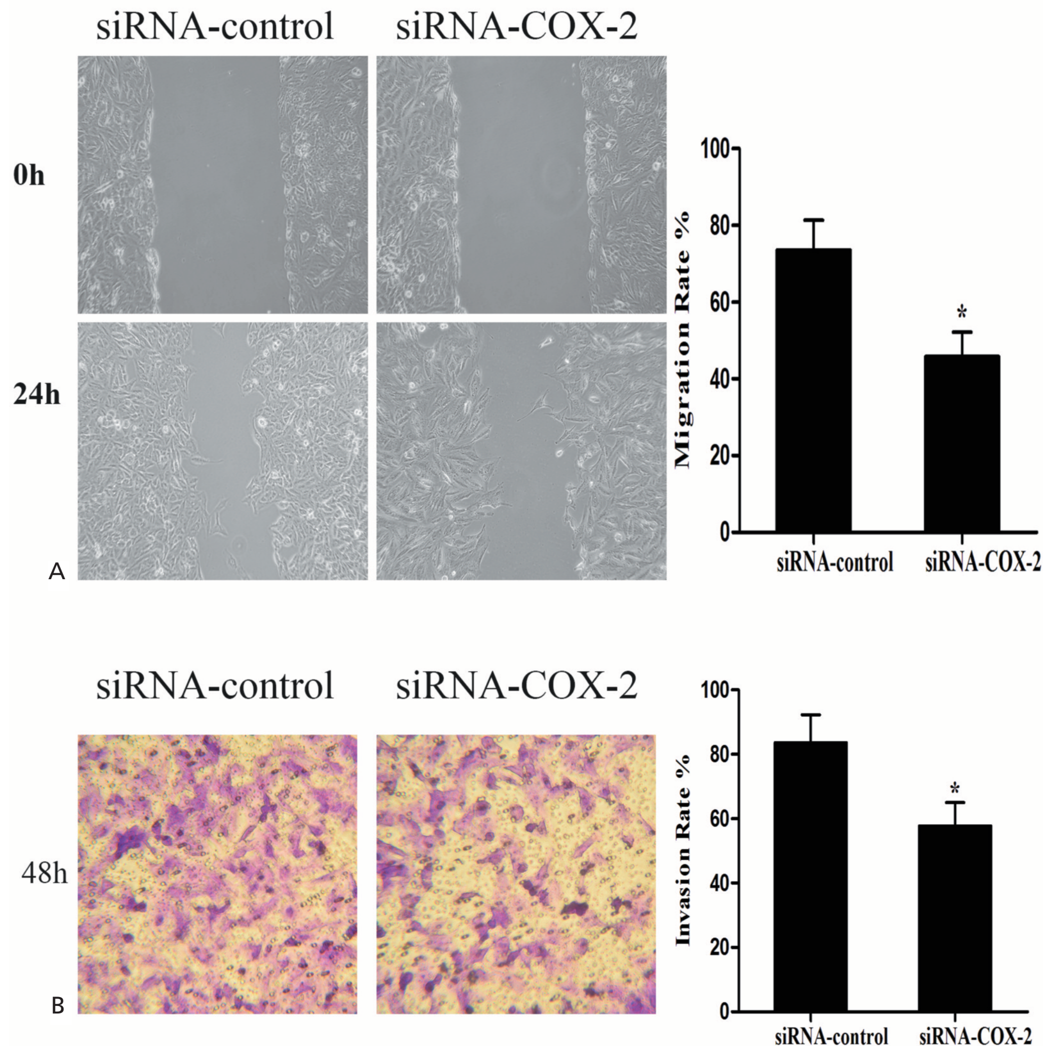

Figure 6. Knockdown of cox-2 inhibits A549 cell migration and invasion. ***A-B***, Influence of COX-2 on migration and invasion in A549 cells was monitored by wound healing assay and Transwell assay. Data are shown as mean ± SEM (n=3), $*p < 0.05$.

ing gene and plays a central role in many normal physiological processes. COX-2 is a highly inducible gene, activated by cytokines, growth factors, phorbol esters, oncogenes and chemical carcinogens[32]. Moreover, COX-2 participates in numerous aspects of biological processes involved in tumor tumorigenesis, including cell proliferation, differentiation, migration and the regulation of anti-tumor immunity[4,5,8-11]. Importantly, COX-2 has a detrimental effect on survival in stage I NSCLC [33].

In our recent study, we confirmed that overexpression of miR-26b could significantly suppressed lung cancer cell proliferation, migration and invasion *in vitro* and inhibited the growth of established tumors *in vivo*. The luciferase reporter assay was applied and the data indicated that miR-26b could bind efficiently to the 3'-UTR of COX-2. Up-regulation of miR-26b was associated with down-regulation of COX-2 in mRNA and protein levels. Furthermore, knockdown of COX-2 suppresses A549 cell proliferation migration and invasion. These results clearly demonstrated that miR-26b is a potential tumor suppressor and may suggest a new therapeutic strategy for lung cancer.

Conclusions

Our study provides new evidences that miR-26b directly down-regulates COX-2 and inhibits lung cancer cell proliferation, migration and invasion. These results indicate that miR-26b may serve as a tumor suppressor gene involved in lung cancer pathogenesis.

Conflict of Interest
The Authors declare that there are no conflicts of interests

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