

MicroRNA-203 inhibits cellular proliferation and invasion by targeting Bmi1 in non-small cell lung cancer

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Abstract. MicroRNAs are proposed to serve vital functions in the regulation of tumor progression and invasion. However, the expression levels of miR-203 in non-small cell lung cancer (NSCLC) and its clinical significance remain unknown. In the present study, the association between B-cell-specific moloney murine leukemia virus insertion site 1 (Bmi1) and miR-203 was investigated. miR-203 was demonstrated to act as a tumor suppressor by regulating the expression of Bmi1. miR-203 expression levels were downregulated in NSCLC tissues while Bmi1 expression was upregulated in NSCLC tissues and cell lines. Furthermore, downregulated Bmi1 or enhanced miR-203 expression inhibited NSCLC cell proliferation and invasion *in vitro*. In addition, a dual-luciferase reporter assay was performed, which identified Bmi1 as a novel target of miR-203. In conclusion, the present study demonstrated that miR-203 functions as a tumor suppressor and is important in inhibiting the proliferation of NSCLC cells through targeting Bmi1. These findings indicate that miR-203 may be useful as a novel potential therapeutic target for NSCLC.

Introduction

Lung cancer is currently the most common type of cancer worldwide in terms of incidence and mortality, and non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases (1). There remains a lack of effective biomarkers or

indicators for diagnosis, which often leads to delayed diagnosis and thus the majority of patients with NSCLC present with an advanced stage. Therefore, the development of novel techniques for early diagnosis of NSCLC is required.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that are ~19-24 nucleotides in length, and they regulate ~20-30% of the genes in the human genome (2). The coding sequences of miRNAs are located in the regions between genes or in the introns (3). Previous studies have demonstrated that miRNAs are involved in a variety of biological processes, including cell proliferation, differentiation, apoptosis and the development and differentiation of tissues and organs (4,5); and pathological processes, including tumorigenesis and tumor progression (6). Such regulation is achieved primarily by their partial recombination with the 3'-untranslated region (3'-UTR) of the target mRNA (7,8).

A previous study revealed that molecular markers of cancer stem-like cells were connected with malignancies by using gene microarray and sequencing analysis (9). Another study demonstrated that miRNAs are critical in the carcinogenesis of lung cancer (10). miR-203 was first identified as a keratinocyte-specific miRNA in the skin, but it is also expressed in the squamous epithelium of the cervix and esophagus (11). miR-203 regulates embryonic epidermal differentiation and has been implicated in skin diseases, but it also serves as a tumor suppressor or oncogene by regulating proliferation, differentiation, invasion, metastasis and apoptosis in certain types of human cancer (12,13). B-cell-specific moloney murine leukemia virus insertion site 1 (Bmi1) is a member of the polycomb repressive complex 1 (PRC1) and is highly expressed in several types of cancer, including lung neoplasm (14,15). Yu *et al* (9) demonstrated that miR-203 inhibited the proliferation of esophageal cancer cells by suppressing Bmi1 directly. However, the level of miR-203 expression and its role in NSCLC remains unclear.

In the present study, the interaction between miR-203 and Bmi1 expression levels was investigated, in addition to the mechanistic role of miR-203 in NSCLC.

Materials and methods

Patient sample collection. A total of 21 paired NSCLC samples were obtained from the First Affiliated Hospital of

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Table I. Primers for RT or amplification of the mature miR-203, U6, Bmi1 and GAPDH mRNA.

Name	Sequence, 5'-3'
RT primers	
U6	CGAGCACAGAATCGCTTCACGAATTTGCGTGTCAT
miR-2031	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGTGGTC
RT-qPCR primers	
U6	F, CGAGCACAGAATCGCTTCA; and R, CTCGCTTCGGCAGCACATAT
miR-203	F, GTATCCAGTGCAGGGTCCGA; and R, CGACGGTGAAATGTTTAG
Bmi1	F, GTGCTTTGTGGAGGGTACTTCAT; and R, TTGGACATCACAAATAGGACAATACTT
GAPDH	F, GAAGGTGAAGGTCGGAGTC; and R, GAAGATGGTGATGGGATTTTC

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Bmi1, B-cell-specific moloney murine leukemia virus insertion site 1.

Soochow University (Suzhou, China) between January 2013 and April 2014. All patients provided written informed consent prior to tissue donation for research purposes. The present study was approved by the ethics committee of Soochow University. All tissues were frozen in liquid nitrogen immediately following the operation and stored at -70°C until required.

Cell culture. The human NSCLC cell lines A549, H1299, H226, H1650, H460 and LTEP- α -2 were obtained from the Cell bank of the Chinese Academy of Sciences (Shanghai, China). The human bronchial epithelial (HBE) cell line was obtained from Bogoo Biological Technology Co. Ltd. (Shanghai, China). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA, USA). All the cells were cultured in humidified sterile conditions containing 5% CO_2 at 37°C .

Construction of the luciferase reporter plasmids, transfection and dual-luciferase assay. The psiCHECK-2 dual luciferase vector (Promega Corporation, Madison, WI, USA) was used to construct the plasmid containing the 3'-untranslated region (3'-UTR) of Bmi1. The fragments containing the predicted wild and mutant sites were directly synthesized (Genewiz, Inc., Suzhou, China) and then subcloned into the psiCHECK-2 vector following digestion with *Xho*I and *Not*I restriction enzymes (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) to generate the Bmi1-3'-UTR-wild and Bmi1-3'-UTR-mutant vectors. Subsequently, LTEP- α -2 cells (1×10^5 /well) were seeded into a 24-well plate and co-transfected with 50 ng/well Bmi1-3'-UTR-wild or Bmi1-3'-UTR-mutant vector and 50 nM/well miR-203 mimics (5'-UUGUGCUUGAUC UAACCAUGU-3') or scrambled microRNA negative control (miR-NC; 5'-UUCUCCGAACGUGUCACGUTT-3'). Following culture for 48 h, the LTEP- α -2 cells were collected and the luciferase activities were measured by the Dual-Luciferase Reporter Assay kit (Promega Corporation) on a TD20/20 Luminometer (Turner Designs, Westport, MA, USA). Each experiment was performed in triplicate. The results were expressed as relative *Renilla luciferase* activities, which were obtained following normalization to firefly luciferase activities. All the transient transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA from cell lines and NSCLC tissues was extracted using TRIzol reagent (Invitrogen Life Technologies) and measured on a NanoDrop (ND-2000 spectrophotometer; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) according to the manufacturer's protocol. The synthesis of cDNA was performed using an M-MLV First Strand kit (Invitrogen Life Technologies). The RT primers for mature miR-203 and U6 were designed as RT stem-loop primers (Table I). Quantitative PCR (qPCR) was performed using a SYBR Green PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) and an ABI7500 Real-Time system (Applied Biosystems Life Technologies, Foster City, CA, USA) were used to quantify the expression levels of RNA. U6 small nuclear RNA (snRNA) and GAPDH mRNA were used as endogenous controls to normalize miR-203 and Bmi1 expression levels. The primer sequences for miR-203, U6, Bmi1 and GAPDH detection are listed in Table I. The relative expression levels were calculated using the $\Delta\Delta\text{Ct}$ method.

Western blot analysis. The cells were lysed in RIPA buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) at 72 h post-transfection. The total proteins were separated by 10% SDS-PAGE (Sangon Biotech, Co., Ltd., Shanghai, China) and run at a constant voltage of 110 V for 2 h, and subsequently transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc., Hercules, California, USA). The membranes were blocked using 1% bovine serum albumin (Sigma-Aldrich) for 30 min and incubated with the primary antibody Bmi1 (monoclonal, rabbit anti-human; dilution, 1:3000; catalog no. 5856S; Cell Signaling Technology, Danvers, MA, USA) or GAPDH (polyclonal, rabbit anti-human; dilution, 1:5000; catalog no. AP0063; Bioworld Technology, Minneapolis, MN, USA) with agitation overnight at 4°C . Following 3 washes with Tris-buffered saline and Tween-20 (TBST), the membranes were incubated with secondary antibodies (goat anti-rabbit, dilution, 1:3000; catalog no. sc-2004; Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 2 h and then the washes were repeated. The result was visualized using an ECL detection system (Pierce Biotechnology, Inc., Rockford, IL, USA). The Bmi1 protein expression levels were normalized against the GAPDH protein expression levels

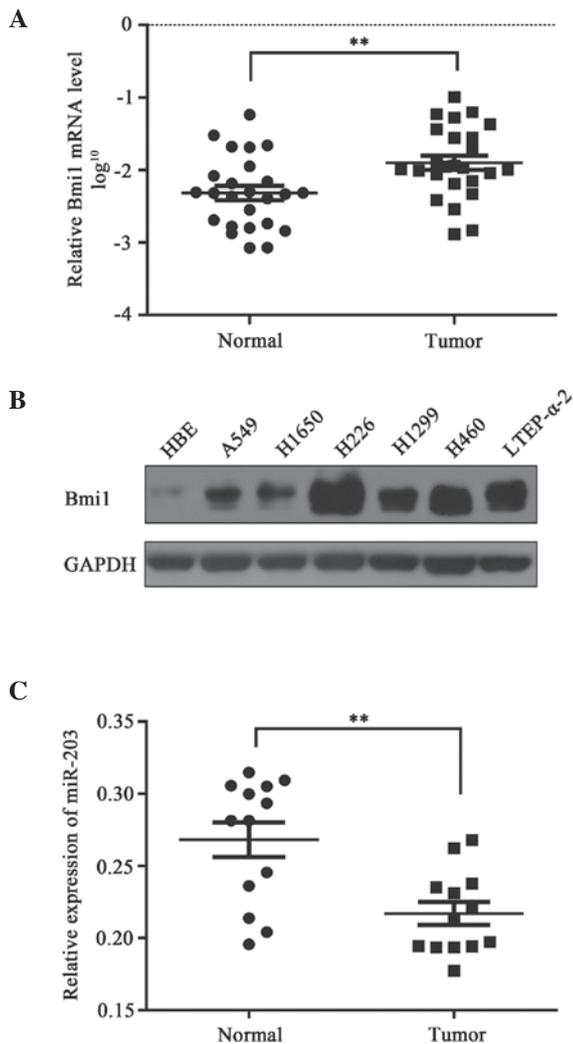


Figure 1. Bmi1 and miR-203 expression in NSCLC tissues and cells quantified by reverse transcription-quantitative polymerase chain reaction. (A) Bmi1 mRNA levels expressed in 25 NSCLC tissues and paired non-tumor lung tissues. (B) Western blot analysis of Bmi1 protein expression levels in HBE cells and NSCLC A549, H1650, H226, H1299, H460 and LTEP- α -2 cells. GAPDH was used as internal control. (C) miR-203 levels expressed in 13 NSCLC tissues and paired non-tumor lung tissues. ** $P < 0.01$. Bmi1, B-cell-specific moloney murine leukemia virus insertion site 1; NSCLC, non-small cell lung cancer; HBE, human bronchial epithelial.

and the density was quantified using Quantity One software, version 4.6 (Bio-Rad Laboratories, Inc.).

Cell proliferation assay. When the cells were in the logarithmic growth phase, they were seeded into 96-well plates at a density of 5×10^3 cells/well. Cell proliferation was detected using the Cell Counting kit-8 (CCK8) and 5-ethynyl-2'-deoxyuridine (EdU) assays. A CCK8 (Beyotime Institute of Biotechnology, Shanghai, China) was used following the manufacturer's instructions. To estimate the number of viable cells, the optical density at a wavelength of 450 nm (OD₄₅₀) was measured daily over 4 consecutive days. The EdU assay (Guangzhou RiboBio, Co., Ltd., Guangzhou, China) was used to label cells undergoing DNA replication. At 48 h post-transfection, the cells were cultured with EdU reagent (Guangzhou RiboBio Co., Ltd.) for 2 h to identify those cells in the S-phase of the cell cycle. The EdU medium mixture was then discarded and the cells were

fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min at room temperature. Following washes with glycine (2 mg/ml; Sigma-Aldrich) and phosphate-buffered saline for 5 min each, 0.5% Triton X-100 (Sigma-Aldrich) was added to each well. Apollo dyeing reaction buffer (Guangzhou RiboBio, Co., Ltd.) was then added to each well and the plate was shaken in the dark for 30 min at room temperature. The DNA was stained by Hoechst 33342 (Guangzhou RiboBio, Co., Ltd.) dye for 30 min and the proportion of nucleated cells coalescent with EdU were observed using fluorescence microscopy (Olympus IX71; Olympus, Tokyo, Japan).

Apoptosis assay. The cells were collected following 48 h of culture post-transfection with miR-203 and the cell density was adjusted to 1×10^6 cells/ml. The cells were then stained using an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The level of apoptosis was assessed by flow cytometry (FC500; Beckman Coulter, Miami, FL, USA).

Transwell invasion assay. At 24 h post-transfection with miR-203 or miR-NC, the cells were harvested. For the invasion assay, the cells were resuspended in RPMI-1640 medium containing 1% FBS and then seeded at a density of 4×10^4 cells/well into the inserts of the Transwell chamber (Corning Incorporated, Corning, NY, USA), which were coated with Matrigel (27.2 $\mu\text{g}/\mu\text{l}$; BD Biosciences, Franklin Lakes, NJ, USA). The inserts were subsequently cultured in wells with 20% FBS-containing medium and the plates were incubated at 37°C for 36 h. Next, the inserts were removed and a cotton tip was used to scrape off the non-invading cells on the upper surface. Cells on the lower surface were then fixed with 4% paraformaldehyde for 30 min at room temperature, and stained with 0.1% crystal violet (Sigma-Aldrich) for a further 30 min. Following fixation, 3 randomly selected images per well were acquired using an inverted microscope (Olympus CKX41; Olympus, Tokyo, Japan) and the average counts were calculated.

Statistical analysis. The results were analyzed using GraphPad Prism Software, version 5.01 (GraphPad Software Inc., La Jolla, CA, USA). All data are presented as the mean \pm standard deviation from 3 independent experiments. A 2-tailed Student's t-test was used for the comparison of the mean between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Upregulation of Bmi1 and downregulation of miR-203 in NSCLC. To determine whether the expression of Bmi1 was increased in NSCLC, the Bmi1 mRNA expression in 25 paired NSCLC tissues and adjacent non-tumor tissues was quantified by RT-qPCR. This analysis revealed that the mRNA expression level of Bmi1 was significantly increased in NSCLC tissues compared with paired non-tumor tissues ($P < 0.01$; Fig. 1A). In addition, Bmi1 protein levels were measured in 7 different cell lines. Compared with HBE cells, Bmi1 protein expression levels were significantly increased in the NSCLC cell lines, including the A549, H1650, H226, H1299, H460 and

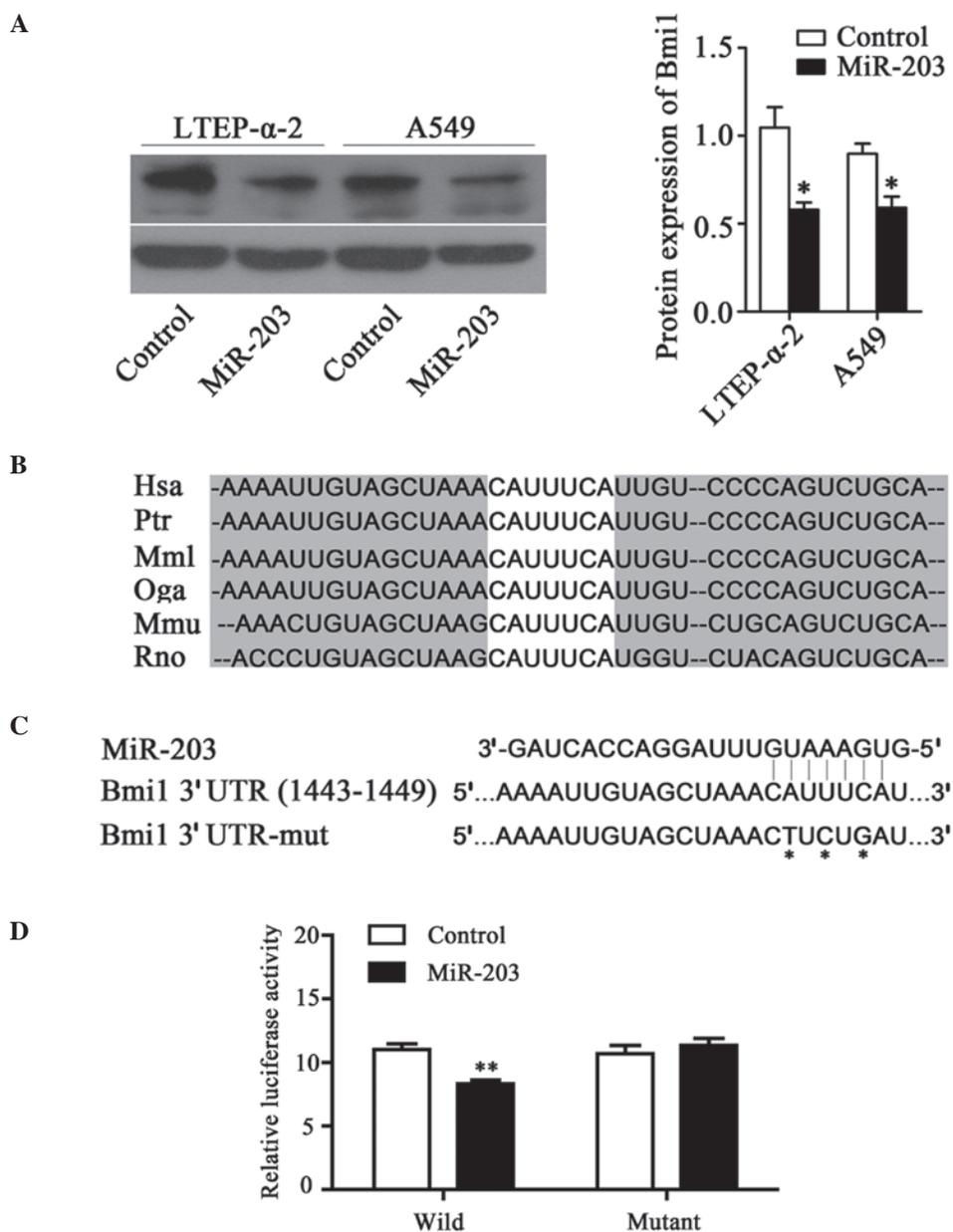


Figure 2. Bmi1 is a direct target of miR-203. (A) Western Blot analysis was performed to detect the expression of Bmi1 in NSCLC LTEP- α -2 and A549 cells transfected with miR-203 mimics or miR-NC (control). (B) Sequence alignment of predicted miR-203 target sites on Bmi1 3'-UTR displaying high conservation between human and other species. (C) Potential association between the predicted miR-203 target sites and miR-203. The asterisks indicate the replaced bases in Bmi1-3'-UTR-mutant vectors. (D) LTEP- α -2 cells were co-transfected with miR-203 mimics or miR-NC and psiCHECK-2 vector with Bmi1 3'-UTR-wild or mutant. *Renilla luciferase* activity was obtained following normalization to firefly luciferase activity. * $P < 0.05$; ** $P < 0.01$ vs. control. Bmi1, B-cell-specific moloney murine leukemia virus insertion site 1; NSCLC, non-small cell lung cancer; UTR, untranslated control.

LTEP- α -2 cell lines (Fig. 1B). However, miR-203 was significantly downregulated in the 13 NSCLC samples compared with paired non-tumor tissues (Fig. 1C).

Bmi1 is a functional target of miR-203 in NSCLC. Bmi1 is a functional target of miR-203 in NSCLC. Using TargetScan software, the potential targets of miR-203 including SNAI2, PKC α , Bmi1, PLD2, LASP1 and c-myc were identified. To evaluate whether miR-203 regulates Bmi1 expression, miR-203 mimics were transiently transfected into A549 and LTEP- α -2 cells. The results demonstrated that the overexpression of miR-203 markedly reduced Bmi1 protein expression levels in LTEP- α -2 and A549 cells ($P < 0.05$; Fig. 2A). In

accordance with these findings, it is possible that miR-203 directly targets Bmi1 to inhibit its expression. To investigate this hypothesis, potential targets of miR-203 were identified using TargetScan software (www.targetscan.org) and a highly conserved predicted target site on the 3'UTR of Bmi1 mRNA was identified (Fig. 2B). This region of the Bmi1 3'UTR, including the predicted miR-203 binding site, was then cloned into the psiCHECK-2 vector (Fig. 2C). The results of the dual-luciferase assay demonstrated that in LTEP- α -2 cells co-transfected with the Bmi1-3'-UTR-wild vector, miR-203 significantly suppressed the luciferase activity compared with the control group. However, this result was not observed with the Bmi1-3'-UTR-mutant vector. These results indicate

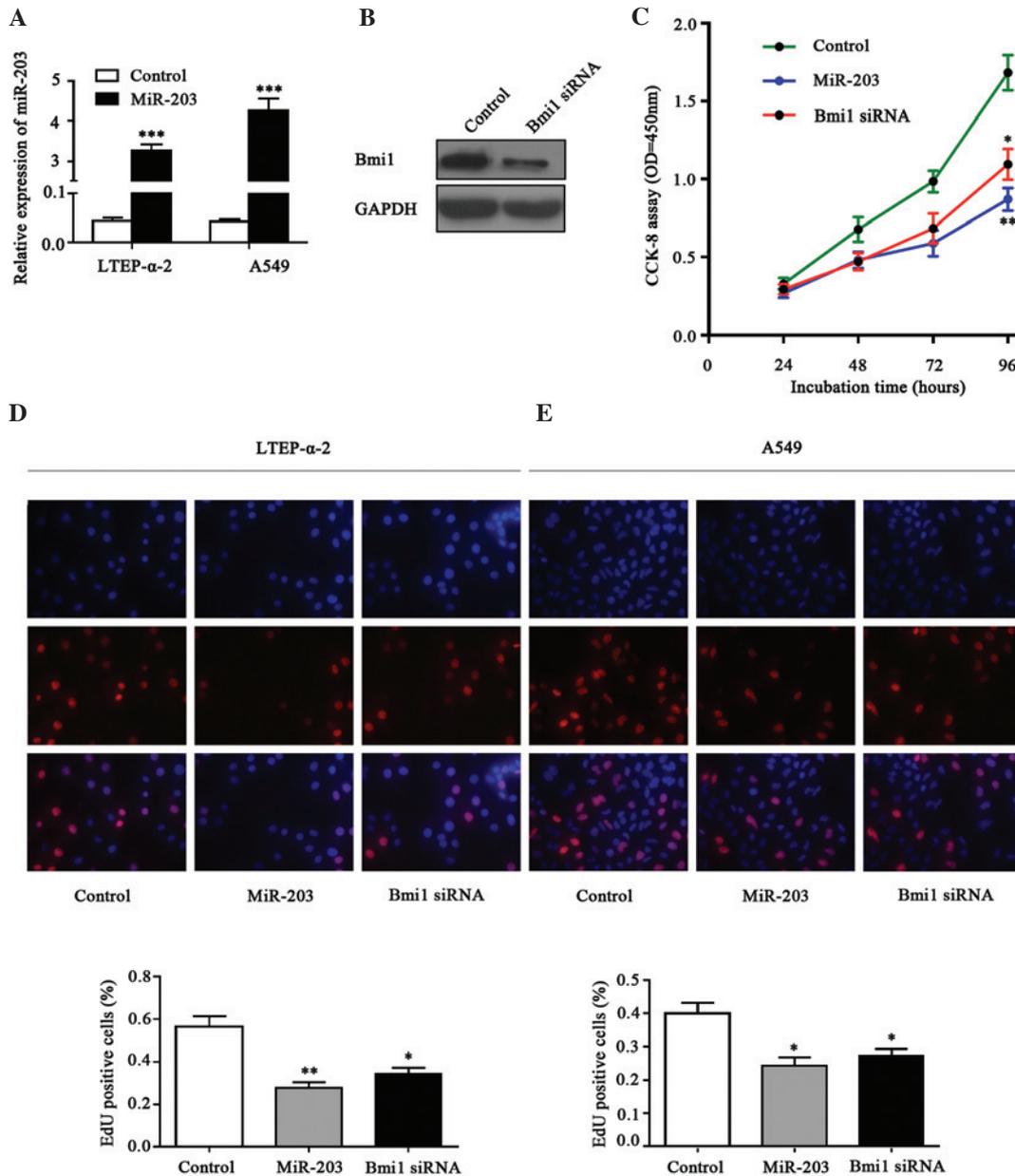


Figure 3. Effects of transfection of miR-203 and Bmi1 siRNA on the proliferation of NSCLC cells. (A) At 48 h post-transfection with miR-203 mimics or miR-NC (control) in LTEP- α -2 and A549 cells, miR-203 expression was quantified by qPCR analysis. (B) Western blot analysis demonstrated that Bmi1 protein expression was knocked down by Bmi1-siRNA transfection. (C) CCK8 analysis demonstrated that cell proliferation in LTEP- α -2 cells was inhibited by transfection with miR-203 or Bmi1-siRNA. At 48 h post-transfection with miR-203 mimics or Bmi1-siRNA EdU assay was applied in (D) LTEP- α -2 and (E) A549 cells. The percentage of EdU-positive cells in each group was calculated and ≥ 200 cells were counted per well. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control. Bmi1, B-cell-specific moloney murine leukemia virus insertion site; NSCLC, non-small cell lung cancer; qPCR, quantitative polymerase chain reaction; EdU, 5-ethynyl-2'-deoxyuridine.

that miR-203 binds to the 3'-UTR of Bmi1 mRNA and thus regulates its expression (Fig. 2D).

miR-203 inhibits cell proliferation in NSCLC cells. The effects of miR-203 expression on malignant phenotypes of NSCLC cells were observed. The efficiency of transfection was determined by RT-qPCR, demonstrating increased expression levels of miR-203 in the mimics-transfected cells compared with the control cells ($P < 0.01$; Fig. 3A). In addition, the protein level of Bmi1 was successfully suppressed

by Bmi1-siRNA, as demonstrated by western blot analysis (Fig. 3B). The results of the CCK8 assays demonstrated that ectopic miR-203 expression significantly inhibited LTEP- α -2 cell growth compared with the control group ($P < 0.01$; Fig. 3C). A similar result was observed following the suppression of Bmi1 expression using Bmi1-siRNA ($P < 0.05$; Fig. 3C). The EdU incorporation assay also indicated that miR-203 inhibited NSCLC cell proliferation. Ectopic expression of miR-203 significantly reduced EdU incorporation in LTEP- α -2 ($P < 0.01$; Fig. 3D) and A549 ($P < 0.05$; Fig. 3E) cells,

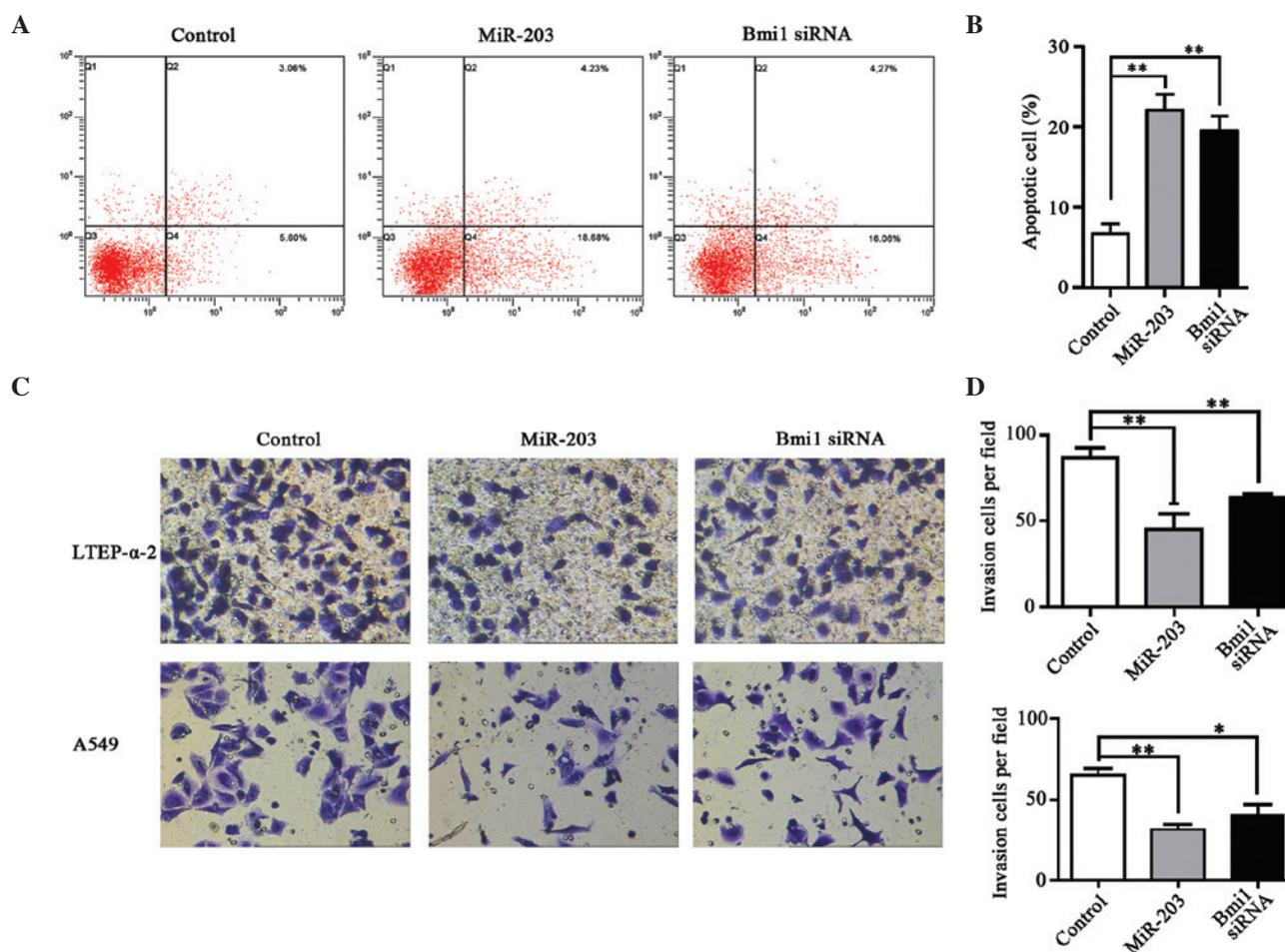


Figure 4. (A) Flow cytometric analysis of LTEP- α -2 cells following transfection with miR-203 or Bmi1 siRNA. Q2 + Q4 represents the total apoptosis rate. (B) Percentage of apoptotic cells. Each experiment was performed at least in triplicate. Following transient transfection with miR-203 or Bmi1-siRNA, Transwell invasion assay was performed in LTEP- α -2 and A549 cells. (C) Crystal violet staining and (D) quantification of cell invasion. * $P < 0.05$; ** $P < 0.01$. Bmi1; B-cell-specific moloney murine leukemia virus insertion site 1.

which is in accordance with the reduction in EdU incorporation induced by attenuated expression of Bmi1 with specific siRNA in LTEP- α -2 ($P < 0.05$; Fig. 3D) and A549 ($P < 0.05$; Fig. 3E) cells. These results indicate that forced expression of miR-203 resulted in significant inhibition of cell growth in NSCLC cells *in vitro*.

Restoration of miR-203 induces apoptosis in NSCLC cells.

An apoptosis assay was performed in LTEP- α -2 cells with increased miR-203 expression or silenced Bmi1 (Fig. 4A). The percentage of apoptotic cells in the miR-203 transfection group was significantly increased compared with the control group (21.7 vs. 8.7%, $P < 0.01$; Fig. 4B). Similar to the results obtained from the miR-203 transfection, comparable results were obtained from downregulation of Bmi1, in which the percentage of apoptotic cells was also significantly increased compared with the control group (20.3 vs. 8.7%, $P < 0.01$; Fig. 4B). The flow cytometry results demonstrated that ectopic expression of miR-203 and downregulation of Bmi1 by siRNA induced cell apoptosis.

Upregulation of miR-203 inhibited metastatic ability of NSCLC cells. To demonstrate whether miR-203 expression affects the metastatic capability of NSCLC cells, a Transwell

invasion assay was performed (Fig. 4C). The quantity of LTEP- α -2 and A549 cells that invaded the filter in the miR-203 transfected group was markedly reduced compared with the control group in the two cell types ($P < 0.01$; Fig. 4D). Similarly, cell invasion was significantly reduced following transfection of Bmi1-specific siRNA ($P < 0.01$ and $P < 0.05$ in LTEP- α -2 and A549 cells, respectively; Fig. 4D).

Discussion

MicroRNAs are a class of small noncoding RNAs that serve important functions in the regulation of the biological processes of cells, including differentiation, proliferation and apoptosis (16). Previous studies have demonstrated that miRNA dysregulation occurs in various types of human cancer and these aberrantly expressed miRNAs are considered to serve pivotal roles in carcinogenesis (17-19). Whether miRNAs act as oncogenes or tumor suppressor genes depends on the context. For example, miR-203 behaves as a tumor suppressor and is downregulated in pancreatic, glioma and esophageal cancer, while it is upregulated in epithelial ovarian cancer and acts as an oncogene (9,20-22).

The present study demonstrated that miR-203 was downregulated in NSCLC specimens, which is in agreement with a

previous study (23). The inhibitory effect of miR-203 expression on the proliferation and invasion of NSCLC cells that was observed in the present study indicates that miR-203 may be involved in the tumorigenesis of lung cancer. Future studies should aim to investigate whether miR-203 expression affects cell cycle progression and can induce cell apoptosis in NSCLC.

A previous study demonstrated that downregulated miR-203 levels were significantly associated with lymph node metastasis in laryngeal squamous cell carcinoma (24). However, to the best of our knowledge, no studies have yet compared the association between miR-203 and lymph node metastasis in lung cancer. The ability of tumor cells to migrate and invade is associated with their cell adhesion properties (25,26). The results of the present study indicate that the restoration of miR-203 expression may serve an important role in the progress of NSCLC treatment through inhibiting cell invasion, though further *in vivo* studies are required to confirm this.

To understand the mechanisms by which miR-203 suppresses cell proliferation, TargetScan software was used to predict the target genes of miR-203. SNAI2, PKC α , Bmi1, PLD2, LASP1 and *c-myc* were identified in the present study as potential targets. A proportion of these targets have already been demonstrated to be directly regulated by miRNAs, for example SNAI2 expression is reported to be repressed by miR-203 in prostate cells (27). SNAI2 is a transcription factor that belongs to the E-box-motif family. It inhibits apoptosis by repressing p53-mediated transcription and promotes epithelial-mesenchymal transition by directly repressing E-cadherin (28,29). However, it is not clear whether there is an interaction between miR-203 and Bmi1 in NSCLC. Bmi1, a polycomb gene family member, is expressed in almost all tissues and is upregulated in various types of human cancer indicating that Bmi1 may be involved in tumor progression (14). A previous study demonstrated that Bmi1 is a direct target of miR-128 in glioma and that it serves an important function in glioma proliferation and self-renewal (30). Another study demonstrated that Bmi1 was overexpressed in lung cancer (15), which is in accordance with the results of the present study. The present study also demonstrated Bmi1 protein expression levels were significantly increased in the NSCLC cell lines, A549, H1299, H226, H1650, H460 and LTEP- α -2, compared with healthy HBE cells.

To assess whether miR-203 directly represses Bmi1 expression, western blot analysis and a luciferase assay were performed. These analyses corroborated that miR-203 specifically downregulated endogenous Bmi1 expression at the post-transcriptional level.

There are numerous genes predicted to be targets of miR-203, and Bmi1 was the only one confirmed by the present study. There may be other potential targets of miR-203 that function in the tumorigenesis of lung cancer, such as *c-myc*, therefore further studies focusing on these novel target genes are required. In addition, the sample size in the present study was small, and further studies from different specimens of NSCLC patients are required to strengthen the findings of the present study.

In conclusion, the present study demonstrated that miR-203 represses Bmi1 protein expression by directly targeting the Bmi1 3'UTR, which may reduce the proliferative and invasive abilities of NSCLC cells. These findings indicate

that miR-203 may be a novel target for the diagnosis and therapy of NSCLC.

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