

MicroRNA-214 upregulates HIF-1 α and VEGF by targeting ING4 in lung cancer cells

YUE LI^{1,2}, LONG ZHAO^{1,3}, YAFEI QI¹ and XIANGHONG YANG¹

¹Department of Pathology, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004;

²Department of Pathology, The University of Hong Kong-Shenzhen Hospital, Shenzhen, Guangdong 518053;

³Department of Nuclear Medicine, Shenzhen Hospital, Southern Medical University, Shenzhen, Guangdong 518031, P.R. China

Received July 20, 2017; Accepted September 17, 2018

DOI: 10.3892/mmr.2019.10170

Abstract. Previous reports have indicated a potential link between microRNA (miR)-214 and hypoxia. In the present study, the biological functions and potential mechanisms of miR-214 were determined, as well as its correlation with HIF-1 α signaling in non-small cell lung cancer (NSCLC) cells. Quantitative polymerase chain reaction revealed that miR-214 expression was upregulated in lung cancer tissues compared with adjacent normal tissues. miR-214 mimics were transfected into A549 cells, and MTT, colony formation, invasion and wound healing assays were performed. It was demonstrated that miR-214 mimic transfection promoted the invasion, proliferation and migration of A549 cells. Furthermore, miR-214 inhibitor transfection decreased H1299 cell invasion, proliferation and migration. Next, the association between miR-214 expression and the HIF-1 α signaling cascade was examined. It was demonstrated that miR-214 mimics upregulated the expression of hypoxia-inducible factor (HIF)-1 α , vascular endothelial growth factor (VEGF), adenylate kinase 3 and matrix metalloproteinase (MMP)2, whereas miR-214 inhibitor downregulated the expression of these factors. Using prediction software, it was demonstrated that tumor suppressor ING4 was a target of miR-214. A luciferase reporter assay confirmed that ING4 was a direct target of miR-214. There was a negative correlation between ING4 and miR-214 expression in lung cancer tissues. In addition, ING4 siRNA and plasmid was transfected into cells in order to validate its effect on HIF-1 α , MMP2 and VEGF expression. ING4 overexpression downregulated HIF-1 α and its targets MMP2 and VEGF, while ING4 siRNA upregulated HIF-1 α , MMP2 and VEGF. In conclusion, it was demonstrated that miR-214

targeted ING4 in lung cancer cells, and upregulated the HIF-1 α cascade, leading to MMP2 and VEGF upregulation. This approach may help to clarify the role of miRNA in non-small lung cancer and may be a new therapeutic target for non-small lung cancer.

Introduction

Lung cancers are the most commonly diagnosed and most fatal type of cancers worldwide, of which non-small cell lung cancer (NSCLC) accounts for ~80% of all primary lung cancer cases (1-4). Since it has been reported that the majority of patients with NSCLC are diagnosed at advanced stages, chemotherapeutics are widely applied as the main first-line agents for the treatment of these advanced stage NSCLC patients in addition to surgical resection. Despite efforts to improve the therapeutic efficacy of patients with NSCLC, the total 5-year survival rate is <15% (5). Therefore, it is urgent to uncover the molecular mechanisms involved in NSCLC, which may help to provide new prognostic biomarkers and therapeutic targets for patients with NSCLC.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs which downregulate specific mRNA targets through binding to sequences located in the 3'-untranslated regions (UTRs), leading to reduced gene transcription (6). It has been estimated that miRNAs directly regulate $\geq 30\%$ of human genes. Therefore, miRNAs are involved in several physiological and pathological processes (7-10). In addition, they serve important roles in RNA silencing, and the majority of miRNAs are located at fragile sites, which are frequently dysregulated in human cancers (11,12). In recent decades, the dysregulation of miRNA expression has been identified in numerous human diseases, including cancer (13-15). A previous report revealed that miR-539 was significantly downregulated in cisplatin (DDP)-resistant NSCLC tissues and cells when compared with DDP-sensitive NSCLC tissues and parental NSCLC cells. miR-539 inhibited DDP-resistant NSCLC cell invasion and migration through targeting DCLK1 (16). miR-362 had a greater expression in NSCLC tissues than in adjacent normal tissues. In addition, miR-362 promoted NSCLC cell invasion, migration and colony formation *in vitro* by targeting Semaphorin-3A, which is significantly associated with metastasis (17). In addition, some other miRNAs were involved

Correspondence to: Dr Xianghong Yang, Department of Pathology, Shengjing Hospital of China Medical University, 36 Sanhao Street, Heping, Shenyang, Liaoning 110004, P.R. China
E-mail: yangxianghong_sj@126.com

Key words: lung cancer, microRNA-214, inhibitor of growth family member 4, proliferation, hypoxia-inducible factor-1 α

in non NSCLC, including miR-421 (18), miR-873 (19), miR-21 (20) and miR-486 (21).

Among all known miRNAs, miR-214 has been extensively studied in cancer. It has been reported as a tumor suppressor in gastric, cervical and colorectal cancer (22-24). However, studies have also identified miR-214 as a promoter of growth and metastases in lung cancer (25,26). In addition, miR-214 may mediate hypoxia-induced cell proliferation and apoptosis inhibition in pulmonary artery smooth muscle cells (27). Hypoxia-inducible factor-1 α (HIF-1 α) is overexpressed in several human cancers, and its overexpression promotes tumor growth and metastasis by initiating angiogenesis and regulating metabolism to overcome hypoxia. Additionally, HIF-1 α induces the expression of the angiogenic protein vascular endothelial growth factor (VEGF) (28,29). In the present study, the association between miR-214 with HIF-1 α and VEGF in A549 and H1299 lung cancer cells was explored. The underlying mechanisms were also investigated, which may help to clarify the associations between miR-214 and inhibitor of growth family member 4 (ING4), HIF-1 α , VEGF. Future studies should be performed to test the therapeutic applications of miR-214.

Materials and methods

Clinical specimens. Fresh samples from lung cancer and corresponding normal adjacent tissues were obtained from patients at The First Affiliated and Shengjing Hospitals of China Medical University (Shenyang, China) between May 2013 and December 2015. The present study was conducted with the approval of the Ethics Committee of China Medical University (Shenyang, China). Informed consent was obtained from all patients. The mean patient age was 56.5 years (range, 41 to 77 years). All patients underwent surgical resection without prior chemotherapy or radiation therapy. There were 15 male patients and five female patients. The tumor, node, metastasis (TNM) staging system was used to classify specimens as stage I (n=7), II (n=9) and III (n=4). There were 12 cases of adenocarcinoma and eight cases of squamous cell carcinoma.

Cell culture and transfection. A549, H1299, H2228, H292, H3255 and H358 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂.

miR-214 mimics (cat. no. miR10000271; 100 nM)/mimic control (cat. no. miR 01101; 100 nM) and miR-214 inhibitor (cat. no. miR20000271; 150 nM)/inhibitor mimic (cat. no. miR 021011; 50 nM) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China; all sequences are not commercially available). ING4 small interfering (si)RNA (cat. no. SR309575; 50 nM) and non-targeting siRNA (cat. no. SR30004; 50 nM) were obtained from OriGene Technologies, Inc. (Rockville, MD, USA). Cells were transfected with miR-214 mimics, inhibitor or ING4 siRNA using DharmaFECT 1 transfection reagent (GE Healthcare Dharmacon, Inc., Lafayette, CO, USA) according to the manufacturer's protocol; cells were used for subsequent experimentation at 48 h post-transfection.

pCMV6-ING4 plasmid and pCMV6 empty plasmid were purchased from OriGene Technologies, Inc. Lipofectamine® 3000 transfection reagent was used for plasmid transfection (Invitrogen; Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from fresh tissue samples and cells with TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Total RNA was reversed transcribed to cDNA using PrimeScript RT Master mix (Takara Biotechnology Co., Ltd., Dalian, China). In brief, reverse-transcription reaction solution (10 μ l) was prepared, which contained 2 μ l 5X RT Master mix, 500 ng RNA and DEPC H₂O. Reverse transcription was performed at 37°C for 15 min and 85°C for 5 sec using the PrimerScript RT Reagent Mix kit (cat. no. RR037A; Takara Biotechnology Co., Ltd.). Bulge-Loop™ miRNA RT-qPCR primer sets for miR-214 and U6 were purchased from Guangzhou RiboBio Co., Ltd. qPCR analyses were performed in an ABI 7500 Real-time PCR system (Thermo Fisher Scientific, Inc.) using the SYBR Green Master mix (cat. no. a25778; Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were 50°C for 2 min, 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 40 sec. Relative gene expression was determined with the following formula: $\Delta Cq = Cq_{\text{gene}} - Cq_{\text{reference}}$. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Cq}$ method (30). Primer sequences for mRNA PCR were as follows: ING4 forward, 5'-GCCCGTTTTGAG GCTGAT-3' and reverse, 5'-CACGAGCAGCTTTCTTCT CCT-3'; β -actin forward, 5'-ATAGCACAGCCTGGATAG CAACGTAC-3' and reverse, 5'-CACCTTCTACAATGAGCT GCGTGTG-3'; miR-214 forward, 5'-TATACATCAAACAGC AGGCACA-3', and reverse, 5'-CATTCGATCTTCTCCACA GTCTC-3'; U6 forward, 5'-CTGGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The ratio (>1.5) of miR-214 in cancer tissues and the corresponding normal tissue was defined as miR-214 upregulation.

Western blot analysis. Total proteins were extracted using Radioimmunoprecipitation Assay lysis and extraction buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.). Proteins concentration was determined using the Bradford method. Proteins (50 μ g/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with primary antibodies against ING4 (1:800; cat. no. 10617-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), adenylate kinase 3 (AK3; 1:800; cat. no. 12562-1-AP; ProteinTech Group, Inc.), matrix metalloproteinase 2 (MMP2; 1:800; cat. no. 10373-2-AP; ProteinTech Group, Inc.), HIF-1 α (1:1,000; cat. no. ab51608; Abcam, Cambridge, MA, USA), VEGF (1:1,000; cat. no. ab53465; Abcam) and GAPDH (1:2,000; cat. no. 2118; Cell Signaling Technology, Inc., Danvers, MA, USA). Following incubation with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (cat. nos. #7076 and #7074, respectively; 1:2,000; Cell Signaling Technology, Inc.) at 37°C for 2 h, target proteins were visualized using enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). Images were captured using a MicroChemi (DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel). The relative intensity of blotted proteins was

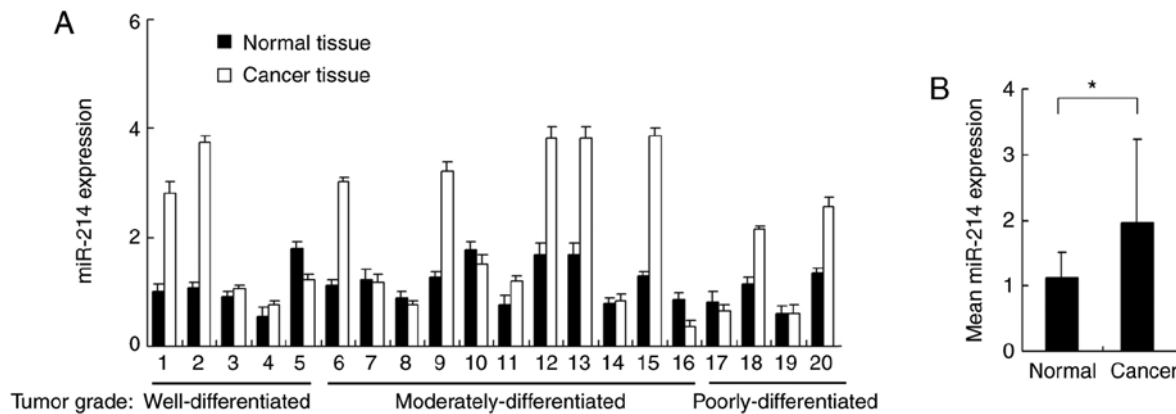


Figure 1. Expression of miR-214 in lung cancer tissues. (A) Relative miR-214 expression levels in 20 lung cancer and paired adjacent normal tissue samples. Samples were divided into well/moderately/poorly differentiated groups according to their tumor grade. (B) Mean miR-214 expression levels in cancer tissues were higher compared with corresponding normal tissues (Student's t-test). * $P < 0.05$ vs. normal group. miR-214, microRNA-214.

determined using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Matrigel invasion assay. The cell invasion assay was performed in a 24-well Transwell chamber (pore size, 8 μ m), and the inserts were coated with 20 μ l Matrigel (1:6 dilution; dilution with serum-free medium; BD Biosciences; Becton, Dickinson and Company, Franklin, Lakes, NJ, USA). The transfected cells were cultured for 48 h following transfection at 37°C with 5% CO₂. Then the cells were trypsinized, transferred to the upper Matrigel chamber in 100 μ l serum-free medium (1 \times 10⁵ cells/ml) and incubated at 37°C for 18 h. Medium supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) was added to the lower chamber. Following this, non-invaded cells on the upper membrane surface were removed and cells that invaded through the filter were fixed in 4% paraformaldehyde at room temperature for 20 min and stained with hematoxylin (Fuzhou Maixin Biotech Co, Ltd., Fuzhou China) at room temperature for 5 min. The cells were counted under a light microscope (magnification, x200; Olympus CX22LED).

MTT assay. Cells (10⁵/well) were plated in 96-well plates and cultured overnight at 37°C. MTT (20 μ l; 5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) solution was added to each well. Following a 4 h incubation, the supernatant was removed and 150 μ l dimethyl sulfoxide was added to dissolve the formazan crystals. Absorbance was measured at 490 nm and the data were obtained from triplicate wells.

Colony formation assay. Cells (10²/dish) were seeded into three 6 cm cell culture dishes and incubated for ~2 weeks. Following this, plates were washed with PBS and stained with hematoxylin (Fuzhou Maixin Biotech Co, Ltd.) at room temperature for 10 min. Colony numbers were manually counted.

Wound healing assay. After 24 h of culture, cells were seeded into 6-well plates until 70-90% confluence was reached. The monolayer was gently scratched using a 1 ml pipette tip.

Detached cells were washed away with PBS and the plates were incubated at 37°C for 24 h. Photos of the stained monolayer were taken using a light microscope (magnification, x200; Olympus CX22LED).

Validation of target gene. A reporter vector (pmiR-RB) (Promega Corporation, Madison, Wisconsin, USA) was used for the luciferase assay. TargetScan 7.1 software was used to predict potential binding sites (31). The wild-type miR-214 target site in ING43'-UTR was CCUGCUG. The mutant miR-214 target site was CCCCCUG. Transfection of reporter plasmid was performed using Attractene reagent (Qiagen, Inc., Valencia, CA, USA). Luciferase activity was measured in cellular extracts using a dual luciferase reporter gene assay kit (Promega Corporation) at 36 h post-transfection. The relative activity of the reporter gene was normalized to *Renilla* luciferase activity.

Statistical analysis. SPSS version 16 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Data was presented as the mean \pm standard deviation. Student's t-test or one way analysis of variance with Tukey's post hoc test was used to compare differences between control and treatment groups. Linear regression was used to estimate the correlation between miR-214 and ING4 expression in tissues. All P-values were based on a two-sided statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-214 expression is upregulated in lung cancer tissues. The expression of miR-214 was examined in 20 cases of lung carcinoma tissues and paired adjacent normal tissues using RT-qPCR (Fig. 1A). The mean value of miR-214 expression was higher in cancer tissues compared with normal tissues (Fig. 1B). A cancer/normal ratio of >1.5 was defined as miR-214 upregulation, which was observed in 10 out of 20 paired tissues (Fig. 1A). The percentage of miR-214 upregulation in tissues with different tumor grades was also examined. miR-214 upregulation in well/moderately/poorly differentiated tumors was present in 40% (2/5), 36.3% (5/11), and 50% (2/4) of

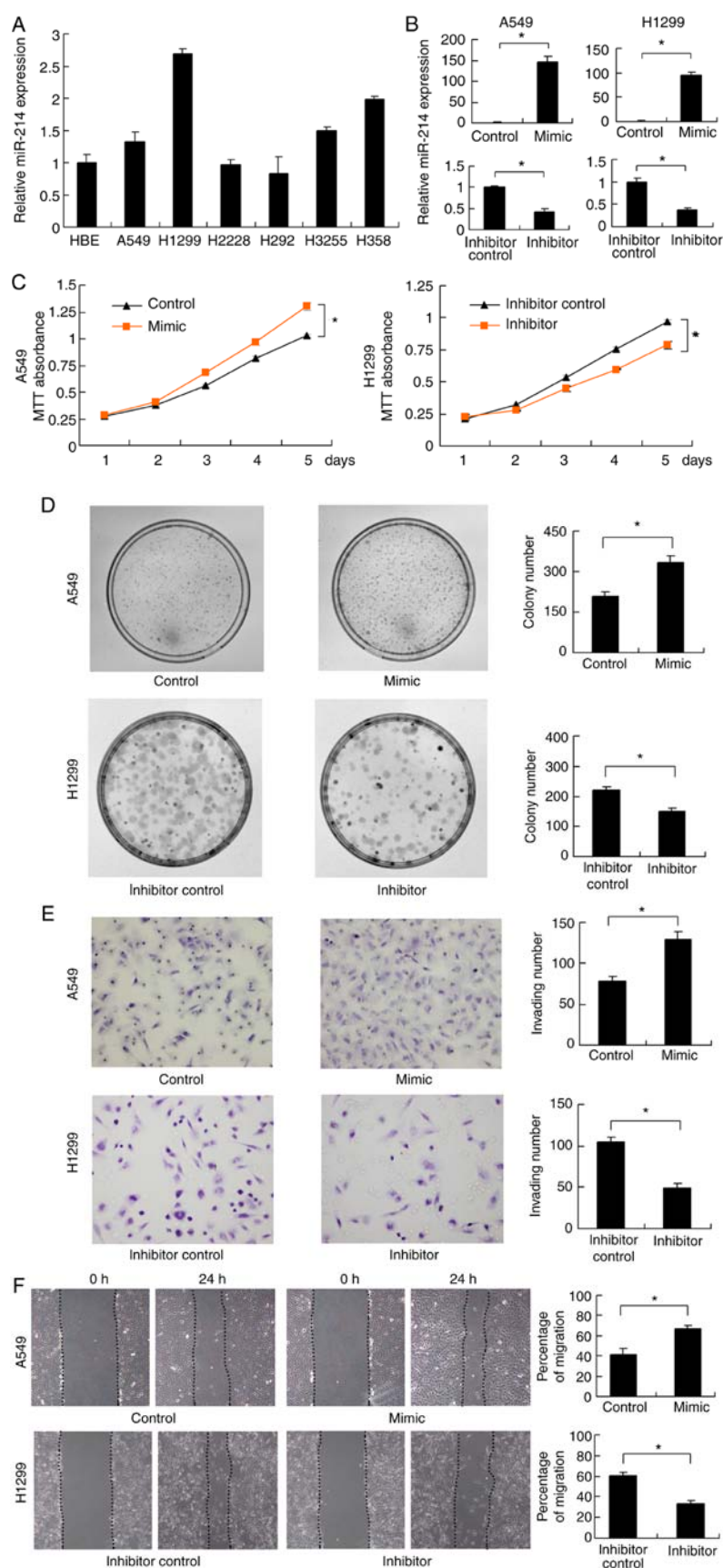


Figure 2. miR-214 regulates lung cancer cell proliferation, invasion and migration. (A) miR-214 expression was examined in lung cancer cell lines (A549, H1299, H2228, H292, H3255, H358) and the normal bronchial epithelium cell line HBE. (B) The transfection efficiency of miR-214 in the A549 and H1299 cell lines was determined by RT-qPCR. (C) MTT assay results demonstrated that the miR-214 mimics promoted cell growth rate, whereas miR-214 inhibitor decreased cell growth rate. (D) The colony formation assay revealed that miR-214 mimics increased A549 cell colony number. miR-214 inhibitor decreased H1299 cell colony number. (E) The Matrigel invasion assay demonstrated that the number of invading A549 cells increased when transfected with miR-214 mimic. The number of invading H1299 cells decreased when transfected with miR-214 inhibitor. (F) The wound healing assay determined that miR-214 mimics upregulated A549 migration, whereas miR-214 inhibitor downregulated H1299 migration. * $P < 0.05$ vs. corresponding control group. miR-214, microRNA-214.

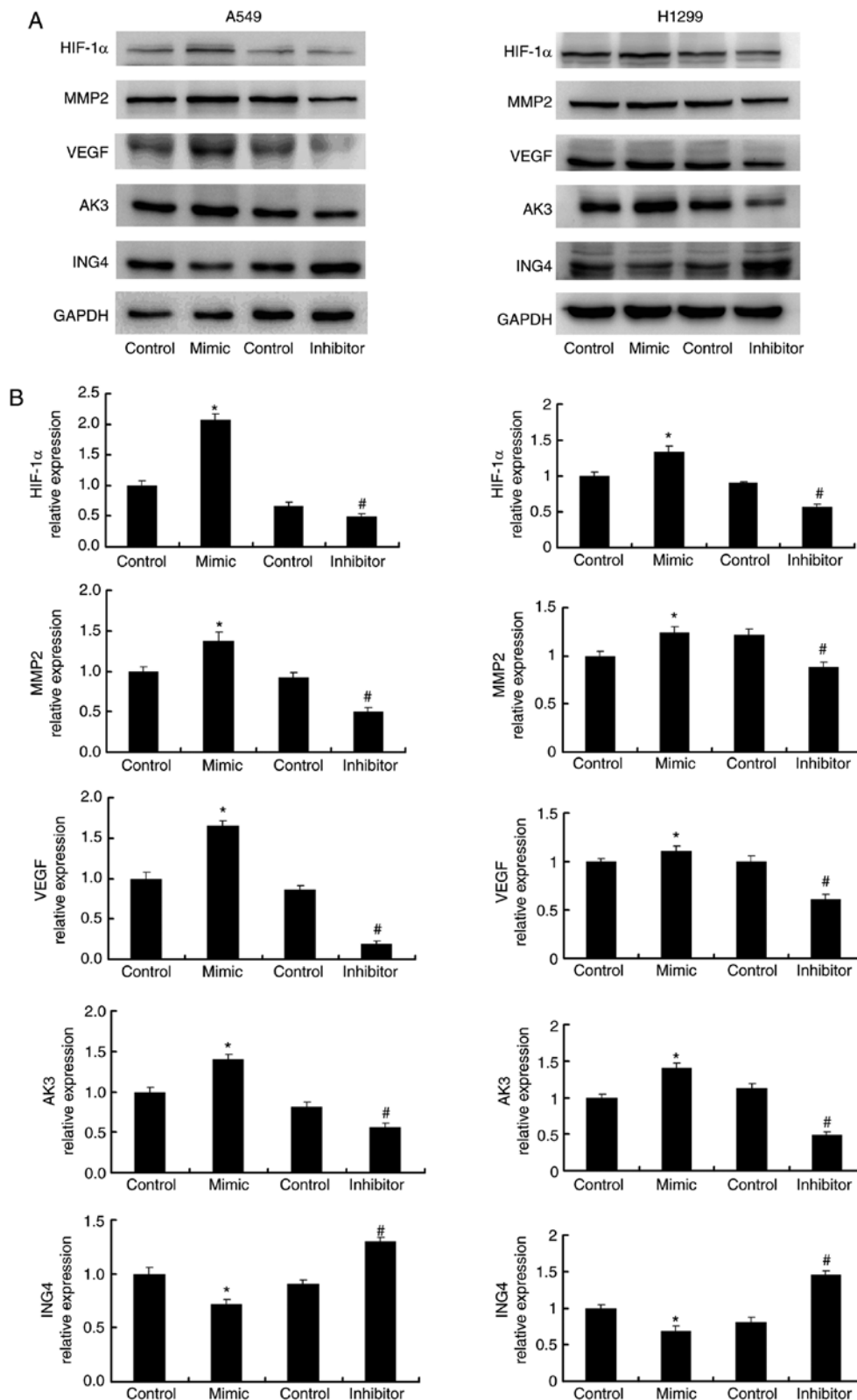


Figure 3. miR-214 regulates ING4, HIF-1 α , VEGF, AK3 and MMP2 protein expression. (A) Western blotting showed that miR-214 mimic transfection significantly increased HIF-1 α , VEGF, AK3 and MMP2 protein expression levels, and significantly decreased ING4 expression in A549 and H1299 cell lines. HIF-1 α , MMP2, AK3 and VEGF expressions levels decreased, and ING4 expression increased in A549 and H1299 cells transfected with miR-214 inhibitor. (B) Quantification of the western blotting results. *P<0.05 vs. mimic control; #P<0.05 vs. inhibitor control. ING4, inhibitor of growth family member 4; HIF-1 α , hypoxia-inducible factor 1 α ; AK3, adenylate kinase 3; VEGF, vascular endothelial growth factor; MMP2, matrix metalloproteinase 2; miR-214, microRNA-214.

cases, respectively. There was no obvious correlation between miR-214 overexpression and tumor grade.

miR-214 promotes lung cancer cell proliferation, invasion and migration. miR-214 expression levels were examined in

several lung cancer cell lines (A549, H1299, H2228, H292, H3255 and H358) and normal bronchial epithelium cell line HBE (Fig. 2A). High miR-214 expression was detected in H1299, H3255 and H358 cell lines. Relatively low miR-214 expression was detected in HBE, A549, H2228 and H292 cell lines. The A549 and H1299 cell lines were subsequently selected for miR-214 mimic and inhibitor transfection; the A549 cell line was used as it has high transfection efficiency and is widely used in literature (32). Transfection efficiency was confirmed by RT-qPCR. miR-214 mimics significantly upregulated miR-214 expression, whereas miR-214 inhibitor downregulated its expression in both cell lines (Fig. 2B). A MTT assay was performed for 5 days to examine the cell growth curves. As presented in Fig. 2C, miR-214 mimics promoted cell growth rate, whereas miR-214 inhibitor decreased the cell growth rate. The colony formation assay demonstrated that miR-214 mimics promoted cell colony formation ability, whereas miR-214 inhibitor decreased this ability (Fig. 2D). Similarly, the Matrigel invasion assay demonstrated that miR-214 mimics promoted A549 cell invasion, whereas miR-214 inhibitor downregulated H1299 invasion (Fig. 2E). To assess cell migration alterations, a wound healing assay was performed. The results revealed that miR-214 mimics facilitated A549 migration, while the miR-214 inhibitor prevented H1299 cell invasion (Fig. 2F).

miR-214 regulates HIF-1 α , VEGF, MMP2 and AK3 expression.

To investigate the mechanisms underlying cell proliferation and invasion regulation by miR-214, and to explore the potential association between miR-214 and the hypoxia induced effects, A549 and H1299 cells were transfected with miR-214 mimics and inhibitors, and the expression of relevant proteins was detected by western blotting (Fig. 3A). It was demonstrated that miR-214 upregulated the expression of MMP-2, HIF-1 α , as well as its target proteins VEGF and AK3, compared with the corresponding control group. Transfection of miR-214 inhibitor exhibited the opposite effects by downregulating MMP2, HIF-1 α , VEGF and AK3 expression, compared with the corresponding inhibitor control (Fig. 3B).

miR-214 targets and downregulates ING4 in A549 and H1299 cell lines. Using the online target prediction database TargetScan 7.1, it was determined that miR-214 directly bound to the 3'UTR of ING4, which has previously been reported to be a tumor suppressor and inhibitor of HIF-1 α . To determine their exact relationship, ING4 expression was examined following transfection with miR-214 mimics or inhibitor. miR-214 mimics significantly downregulated ING4 expression, whereas miR-214 inhibitor transfection upregulated ING4 expression at the mRNA and protein level (Figs. 3A and 4A). To determine if ING4 was a direct target of miR-214, luciferase reporter assays were performed. Reporter plasmids with wild-type (CCUGCUG) or mutant (CCCCCUG) 3'-UTR binding sites for ING4 were transfected in A549 and H1299 cells together with miR-214 mimic. miR-214 mimics significantly suppressed luciferase activity in cells transfected with the wild-type vector (Fig. 4B). No marked alteration was detected in cells with the mutant site plasmid, suggesting that miR-214 bound to the ING4 3'-UTR and thus downregulated ING4 expression. To validate their

association in lung cancer tissues, the mRNA expression of both miR-214 and ING4 was examined. Linear regression analyses demonstrated that there was an inverse correlation between miR-214 and ING4 mRNA expression (Fig. 4C; $P=0.008$).

The effects of miR-214 on HIF-1 α , MMP2 and VEGF are dependent on ING4. ING4 has been reported as a tumor suppressor which inhibits MMP2 and HIF-1 α expression (33-35). To confirm the involvement of ING4 in miR-214-mediated regulation of MMP2, VEGF and HIF-1 α , ING4 plasmid was transfected into A549 and H1299 cells together with the miR-214 mimic. Western blotting demonstrated the success of ING4 plasmid transfection in the two cell lines (Fig. 5A). ING4 overexpression was demonstrated to abolish the miR-214-induced upregulation of MMP2, VEGF and HIF-1 α expression (Fig. 5B and C).

In addition, ING4 siRNA was introduced into A549 and H1299 cells in combination with miR-214 inhibitor. ING4 expression was markedly reduced by siRNA transfection, compared with the control (Fig. 6A). As presented in Fig. 6B, ING4 siRNA upregulated MMP2, VEGF and HIF-1 α expression. These data indicated that miR-214 induced the expression of MMP2, VEGF and HIF-1 α by targeting tumor suppressor ING4.

Discussion

Evidence indicates that miRNAs participate in cancer development. miR-214 has been reported to be dysregulated in a variety of human diseases, including cancer (36-38). Downregulation of miR-214 has been implicated in several cancers including gastric, cervical, esophageal and colorectal cancer (22-24). However, studies have also identified miR-214 as a promoter of growth and invasion in non-small cell lung cancer (25,39). Thus, the biological roles of miR-214 in human cancer are contradicting. In the present study, it was confirmed that miR-214 expression was upregulated in lung cancer tissues and cell lines. Using MTT, colony formation, Matrigel invasion and migration assays, it was demonstrated that miR-214 served as a promoter of cell growth invasion and migration in NSCLC cells. As miR-214 has been reported to mediate hypoxia-induced cell proliferation in pulmonary artery smooth muscle cells (27), the expression of HIF-1 α and its downstream factors were measured. It was revealed that miR-214 upregulated HIF-1 α , while miR-214 inhibitor downregulated HIF-1 α .

HIF-1 α is involved in adaptation to hypoxia and angiogenesis during the development of various cancers (40). HIF-1 α has been reported to be overexpressed in human NSCLCs, and is associated with angiogenesis, invasion, epithelial-mesenchymal transition and chemoresistance (41-43). VEGF is a downstream protein of HIF-1 α , which has been implicated as an angiogenesis stimulating protein during cancer progression (44-46). AK3 is a target protein of HIF-1 α (47), which is located exclusively in the mitochondrial matrix (48). AK3 may participate in the high-energy phosphate transfer process (49). The results of the present study demonstrated that miR-214 induced HIF-1 α and its target VEGF, which may have promoted tumor angiogenesis and cell survival.

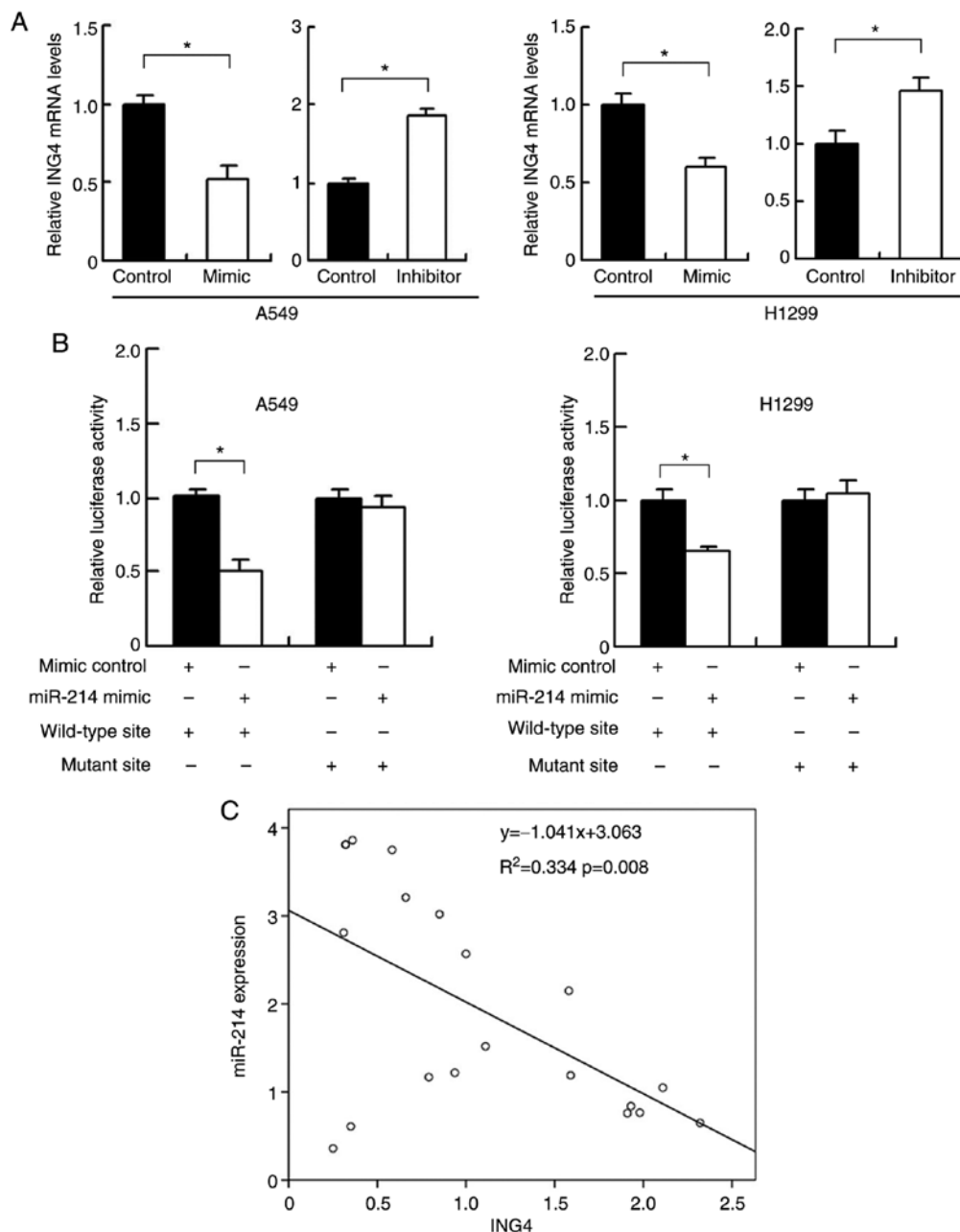


Figure 4. ING4 is a target of miR-214. (A) RT-qPCR demonstrated that miR-214 mimics downregulated the mRNA expression of ING4 mRNA, whereas miR-214 inhibitor had the opposite effect. (B) The luciferase reporter assay showed that in cells transfected with the wild-type reporter, miR-214 mimics significantly inhibited luciferase activity. In cells transfected with the mutant site reporter plasmid, no significant alteration was detected. (C) ING4 expression was examined by RT-qPCR in 20 lung cancer cases. Linear regression analyses demonstrated that there was an inverse correlation between miR-214 and ING4 mRNA expression levels. * $P < 0.05$ vs. corresponding control. ING4, inhibitor of growth family member 4; miR-214, microRNA-214; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Next, the underlying mechanisms were explored. Prediction software and reporter assays were used, which demonstrated that miR-214 suppressed the mRNA and protein expression of ING4, a well-defined tumor suppressor. ING4 has been reported to inhibit tumor invasion by suppression of the MMP family of proteins in osteosarcoma, gastric and lung cancer (50,51). ING4 may also suppress HIF-1 α and its downstream signaling proteins (52,53). Thus, it was postulated that the biological effects of miR-214 may be dependent on its regulation of ING4 expression. To confirm this hypothesis, ING4 plasmid and siRNA were co-transfected with miR-214 mimics or inhibitor. The results demonstrated that ING4

siRNA prevented the effects of the miR-214 inhibitor by upregulating MMP2, VEGF and HIF-1 α expression, whereas ING4 plasmid suppressed the expression of these proteins. Collectively, these data demonstrated that miR-214 induced MMP2 and HIF-1 α signaling in lung cancer cells by targeting tumor suppressor ING4.

In conclusion, the present study demonstrated that miR-214 functioned as an oncogene in lung cancer cells. miR-214 targeted tumor suppressor ING4, which in turn inhibited HIF-1 α , VEGF and MMP2 expression. Therefore, miR-214 may serve as a potential therapeutic target in non-small cell lung cancer.

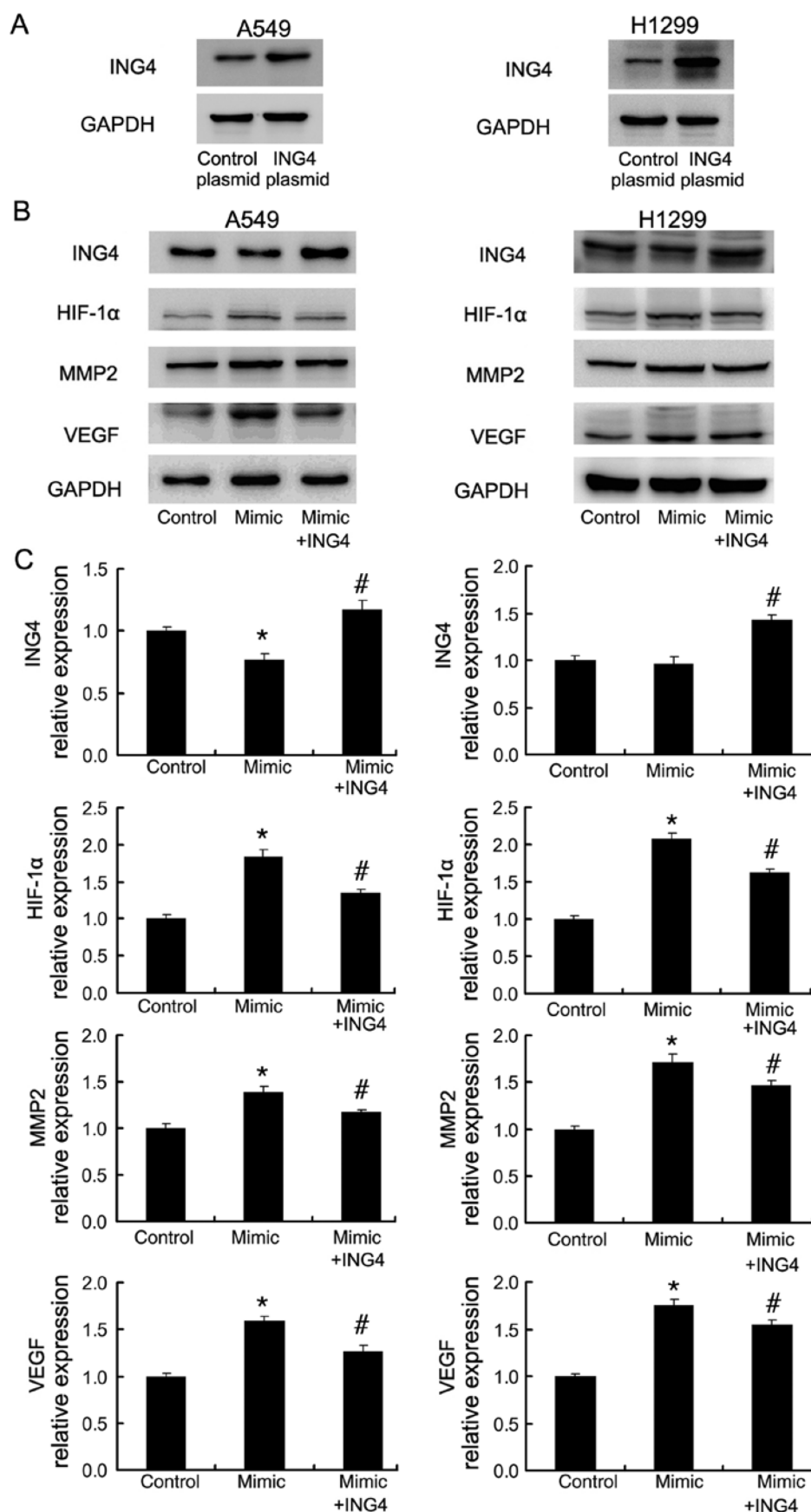


Figure 5. ING4 overexpression upregulates MMP2, HIF-1 α and VEGF expression. (A) Western blotting confirmed that ING4 plasmid transfection upregulated ING4 protein expression in A549 and H1299 cells. (B) A549 and H1299 cells were transfected with the mimic control, miR-214 mimics alone or together with the ING4 plasmid. ING4 plasmid re-constituted its endogenous expression and downregulated MMP2, HIF-1 α and VEGF expression when compared with the groups transfected with miR-214 mimic alone in A549 and H1299 cell lines. (C) Quantification of western blotting results. These experiments were performed in triplicate. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. mimic group. ING4, inhibitor of growth family member 4; HIF-1 α , hypoxia-inducible factor 1 α ; AK3, adenylate kinase 3; VEGF, vascular endothelial growth factor; MMP2, matrix metalloproteinase 2; miR-214, microRNA-214.

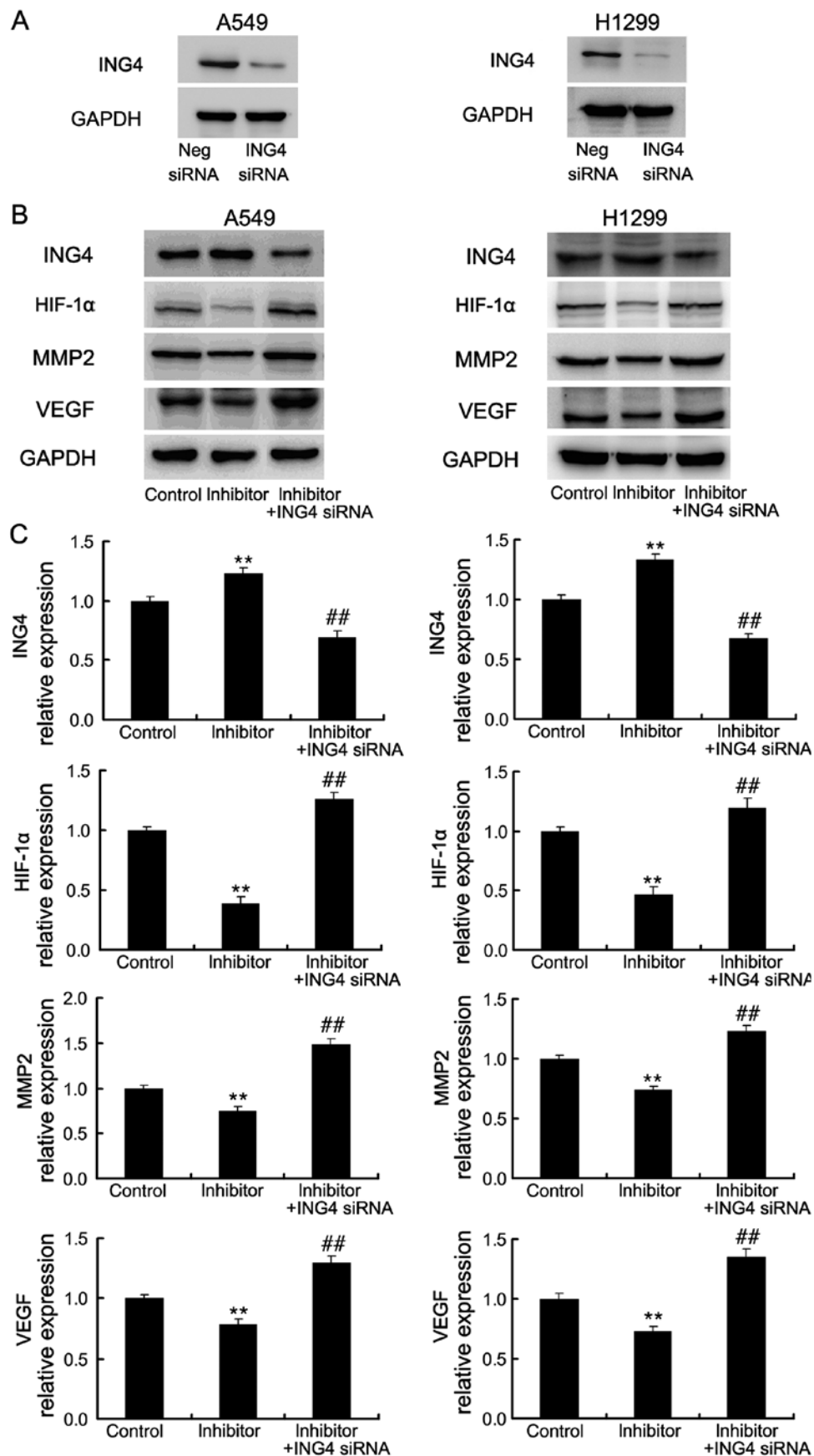


Figure 6. ING4 siRNA transfection downregulates MMP2, HIF-1α and VEGF expression. (A) Western blotting confirmed that ING4 siRNA transfection downregulated ING4 protein expression in A549 and H1299 cells. (B) A549 and H1299 cells were transfected with the inhibitor control, miR-214 inhibitor or miR-214 inhibitor together with ING4 siRNA. ING4 siRNA downregulated MMP2, HIF-1α and VEGF, which were induced by miR-214. (C) Quantification of western blotting results. These experiments were performed in triplicate. **P<0.01 vs. control group; ##P<0.01 vs. mimic group. ING4, inhibitor of growth family member 4; HIF-1α, hypoxia-inducible factor 1α; AK3, adenylate kinase 3; VEGF, vascular endothelial growth factor; MMP2, matrix metalloproteinase 2; miR-214, microRNA-214.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author's contributions

YL and LZ performed the experiments, evaluated the data, wrote the manuscript and prepared the figures. YL designed the experiments. YL and YQ evaluated the data and wrote the manuscript. XY conceived and designed the study. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was conducted with the approval of the Ethics Committee of China Medical University (Shenyang, China). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Gharibvand L, Beeson WL, Shavlik D, Knutsen R, Ghamsary M, Soret S and Knutsen SF: The association between ambient fine particulate matter and incident adenocarcinoma subtype of lung cancer. *Environ Health* 16: 71, 2017.
- Sartorius B and Sartorius K: How much incident lung cancer was missed globally in 2012? An ecological country-level study. *Geospat Health* 11: 396, 2016.
- Bailey C, Hewison A, Karasouli E, Staniszewska S and Munday D: Hospital care following emergency admission: A critical incident case study of the experiences of patients with advanced lung cancer and chronic obstructive pulmonary disease. *J Clin Nurs* 25: 2168-2179, 2016.
- Tual S, Silverman DT, Koutros S, Blair A, Sandler DP, Lebaillly P, Andreotti G, Hoppin JA and Freeman LE: Use of dieselized farm equipment and incident lung cancer: Findings from the Agricultural Health Study Cohort. *Environ Health Perspect* 124: 611-618, 2016.
- Akamatsu H, Mori K, Naito T, Imai H, Ono A, Shukuya T, Taira T, Kenmotsu H, Murakami H, Endo M, *et al*: Progression-free survival at 2 years is a reliable surrogate marker for the 5-year survival rate in patients with locally advanced non-small cell lung cancer treated with chemoradiotherapy. *BMC Cancer* 14: 18, 2014.
- Tian T, Wang J and Zhou X: A review: MicroRNA detection methods. *Org Biomol Chem* 13: 2226-2238, 2015.
- Silva BOD, Lima KF, Gonçalves LR, Silveira MBD and Moraes KCM: Correction: MicroRNA profiling of the effect of the heptapeptide angiotensin-(1-7) in A549 lung tumor cells reveals a role for miRNA149-3p in cellular migration processes. *PLoS One* 12: e0190204, 2017.
- Pastorkova Z, Skarda J and Andel J: The role of microRNA in metastatic processes of non-small cell lung carcinoma. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 160: 343-357, 2016.
- Zhang HM, Kuang S, Xiong X, Gao T, Liu C and Guo AY: Transcription factor and microRNA co-regulatory loops: Important regulatory motifs in biological processes and diseases. *Brief Bioinform* 16: 45-58, 2015.
- Kraemer A, Anastasov N, Angermeier M, Winkler K, Atkinson MJ and Moertl S: MicroRNA-mediated processes are essential for the cellular radiation response. *Radiat Res* 176: 575-586, 2011.
- Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, *et al*: MicroRNA expression profiles classify human cancers. *Nature* 435: 834-838, 2005.
- Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, *et al*: Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 64: 3753-3756, 2004.
- Zhang DQ, Zhou CK, Jiang XW, Chen J and Shi BK: Increased expression of miR-222 is associated with poor prognosis in bladder cancer. *World J Surg Oncol* 12: 241, 2014.
- Köhler CU, Bryk O, Meier S, Lang K, Rozynek P, Brüning T and Kafferlein HU: Analyses in human urothelial cells identify methylation of miR-152, miR-200b and miR-10a genes as candidate bladder cancer biomarkers. *Biochem Biophys Res Commun* 438: 48-53, 2013.
- Puerta-Gil P, García-Baquero R, Jia AY, Ocaña S, Alvarez-Múgica M, Alvarez-Ossorio JL, Cordon-Cardo C, Cava F and Sánchez-Carbajo M: miR-143, miR-222, and miR-452 are useful as tumor stratification and noninvasive diagnostic biomarkers for bladder cancer. *Am J Pathol* 180: 1808-1815, 2012.
- Deng H, Qianqian G, Ting J and Aimin Y: miR-539 enhances chemosensitivity to cisplatin in non-small cell lung cancer by targeting DCLK1. *Biomed Pharmacother* 106: 1072-1081, 2018.
- Luo D, Zhang Z, Zhang Z, Li JY, Cui J, Shi WP, Dong XW, Yuan L, Lin P, Chen ZN, *et al*: Aberrant expression of miR-362 promotes lung cancer metastasis through downregulation of Sema3A. *J Immunol Res* 2018: 1687097, 2018.
- Li Y, Cui X, Li Y, Zhang T and Li S: Upregulated expression of miR-421 is associated with poor prognosis in non-small-cell lung cancer. *Cancer Manag Res* 10: 2627-2633, 2018.
- Jin S, He J, Li J, Guo R, Shu Y and Liu P: MiR-873 inhibition enhances gefitinib resistance in non-small cell lung cancer cells by targeting glioma-associated oncogene homolog 1. *Thorac Cancer* 9: 1262-1270, 2018.
- Zheng W, Zhao J, Tao Y, Guo M, Ya Z, Chen C, Qin N, Zheng J, Luo J and Xu L: MicroRNA-21: A promising biomarker for the prognosis and diagnosis of non-small cell lung cancer. *Oncol Lett* 16: 2777-2782, 2018.
- Yu S, Geng S and Hu Y: miR-486-5p inhibits cell proliferation and invasion through repressing GAB2 in non-small cell lung cancer. *Oncol Lett* 16: 3525-3530, 2018.
- Yang TS, Yang XH, Wang XD, Wang YL, Zhou B and Song ZS: MiR-214 regulate gastric cancer cell proliferation, migration and invasion by targeting PTEN. *Cancer Cell Int* 13: 68, 2013.
- Wen Z, Lei Z, Jin-An M, Xue-Zhen L, Xing-Nan Z and Xiu-Wen D: The inhibitory role of miR-214 in cervical cancer cells through directly targeting mitochondrial transcription factor A (TFAM). *Eur J Gynaecol Oncol* 35: 676-682, 2014.
- Cristóbal I, Caramés C, Madoz-Gúrpide J, Rojo F, Aguilera O and García-Foncillas J: Downregulation of miR-214 is specific of liver metastasis in colorectal cancer and could play a role determining the metastatic niche. *Int J Colorectal Dis* 29: 885, 2014.
- Long H, Wang Z, Chen J, Xiang T, Li Q, Diao X and Zhu B: microRNA-214 promotes epithelial-mesenchymal transition and metastasis in lung adenocarcinoma by targeting the suppressor-of-fused protein (Sufu). *Oncotarget* 6: 38705-38718, 2015.
- Liao J, Lin J, Lin D, Zou C, Kurata J, Lin R, He Z and Su Y: Down-regulation of miR-214 reverses erlotinib resistance in non-small-cell lung cancer through up-regulating LHX6 expression. *Sci Rep* 7: 781, 2017.
- Liu H, Tao Y, Chen M, Yu J, Li WJ, Tao L, Li Y and Li F: Upregulation of microRNA-214 contributes to the development of vascular remodeling in hypoxia-induced pulmonary hypertension via targeting CCN12. *Sci Rep* 6: 24661, 2016.

28. Shenoy SK, Han S, Zhao YL, Hara MR, Oliver T, Cao Y and Dewhirst MW: β -arrestin1 mediates metastatic growth of breast cancer cells by facilitating HIF-1-dependent VEGF expression. *Oncogene* 31: 282-292, 2012.
29. Wang Y, Zhao Q, Ma S, Yang F, Gong Y and Ke C: Sirolimus inhibits human pancreatic carcinoma cell proliferation by a mechanism linked to the targeting of mTOR/HIF-1 α /VEGF signaling. *IUBMB Life* 59: 717-721, 2007.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
31. Agarwal V, Bell GW, Nam JW and Bartel DP: Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 4, 2015.
32. Grelet S, Link LA, Howley B, Obellianne C, Palanisamy V, Gangaraju VK, Diehl JA and Howe PH: A regulated PNUTS mRNA to lncRNA splice switch mediates EMT and tumour progression. *Nat Cell Biol* 19: 1105-1115, 2017.
33. Chen Y, Huang Y, Hou P, Zhang Z, Zhang Y, Wang W, Sun G, Xu L, Zhou J, Bai J and Zheng J: ING4 suppresses tumor angiogenesis and functions as a prognostic marker in human colorectal cancer. *Oncotarget* 7: 79017-79031, 2016.
34. Wang CJ, Yang D and Luo YW: Recombinant ING4 suppresses the migration of SW579 thyroid cancer cells via epithelial to mesenchymal transition. *Exp Ther Med* 10: 603-607, 2015.
35. Wang QS, Li M, Zhang LY, Jin Y, Tong DD, Yu Y, Bai J, Huang Q, Liu FL, Liu A, *et al*: Down-regulation of ING4 is associated with initiation and progression of lung cancer. *Histopathology* 57: 271-281, 2010.
36. Peng R, Men J, Ma R, Wang Q, Wang Y, Sun Y and Ren J: miR-214 down-regulates ARL2 and suppresses growth and invasion of cervical cancer cells. *Biochem Biophys Res Commun* 484: 623-630, 2017.
37. Liu J, Li D, Dang L, Liang C, Guo B, Lu C, He X, Cheung HY, He B, Liu B, *et al*: Osteoclastic miR-214 targets TRAF3 to contribute to osteolytic bone metastasis of breast cancer. *Sci Rep* 7: 40487, 2017.
38. Xu CX, Xu M, Tan L, Yang H, Permuth-Wey J, Kruk PA, Wenham RM, Nicosia SV, Lancaster JM, Sellers TA and Cheng JQ: MicroRNA miR-214 regulates ovarian cancer cell stemness by targeting p53/Nanog. *J Biol Chem* 291: 22851, 2016.
39. Wang C, Ding M, Xia M, Chen S, Van Le A, Soto-Gil R, Shen Y, Wang N, Wang J, Gu W, *et al*: A five-miRNA panel identified from a multicentric case-control study serves as a novel diagnostic tool for ethnically diverse non-small-cell lung cancer patients. *EBioMedicine* 2: 1377-1385, 2015.
40. Semenza GL: Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3: 721-732, 2003.
41. Ding G, Huang G, Liu HD, Liang HX, Ni YF, Ding ZH, Ni GY and Hua HW: MiR-199a suppresses the hypoxia-induced proliferation of non-small cell lung cancer cells through targeting HIF1 α . *Mol Cell Biochem* 384: 173-180, 2013.
42. Liu Y, Bernauer AM, Yingling CM and Belinsky SA: HIF1 α regulated expression of XPA contributes to cisplatin resistance in lung cancer. *Carcinogenesis* 33: 1187-1192, 2012.
43. Eleftheriadis SG, Sivridis E, Koutsopoulos A, Galatoudis ZG, Chloropoulou PA, Koukourakis MI, Didilis VN, Bougioukas GI and Giatromanolaki A: One-lung ventilation and HIF1 α expression in lung cancer and pneumothorax. *Anticancer Res* 30: 1143-1148, 2010.
44. Carbajo-Pescador S, Ordoñez R, Benet M, Jover R, García-Palomo A, Mauriz JL and González-Gallego J: Inhibition of VEGF expression through blockade of Hif1 α and STAT3 signalling mediates the anti-angiogenic effect of melatonin in HepG2 liver cancer cells. *Br J Cancer* 109: 83-91, 2013.
45. Kafousi M, Vrekoussis T, Tsenteliero E, Pavlakakis K, Navrozoglou I, Dousias V, Sanidas E, Tsiftsis D, Georgoulas V and Stathopoulos EN: Immunohistochemical study of the angiogenic network of VEGF, HIF1 α , VEGFR-2 and endothelial nitric oxide synthase (eNOS) in human breast cancer. *Pathol Oncol Res* 18: 33-41, 2012.
46. Giatromanolaki A, Koukourakis MI, Turley H, Sivridis E, Harris AL and Gatter KC: Tumour and Angiogenesis Research Group: Phosphorylated KDR expression in endometrial cancer cells relates to HIF1 α /VEGF pathway and unfavourable prognosis. *Mod Pathol* 19: 701-707, 2006.
47. O'Rourke JF, Pugh CW, Bartlett SM and Ratcliffe PJ: Identification of hypoxically inducible mRNAs in HeLa cells using differential-display PCR. Role of hypoxia-inducible factor-1. *Eur J Biochem* 241: 403-410, 1996.
48. Nobumoto M, Yamada M, Song S, Inouye S and Nakazawa A: Mechanism of mitochondrial import of adenylate kinase isozymes. *J Biochem* 123: 128-135, 1998.
49. Chang X, Ravi R, Pham V, Bedi A, Chatterjee A and Sidransky D: Adenylate kinase 3 sensitizes cells to cigarette smoke condensate vapor induced cisplatin resistance. *PLoS One* 6: e20806, 2011.
50. Li M, Zhu Y, Zhang H, Li L, He P, Xia H, Zhang Y and Mao C: Delivery of inhibitor of growth 4 (ING4) gene significantly inhibits proliferation and invasion and promotes apoptosis of human osteosarcoma cells. *Sci Rep* 4: 7380, 2014.
51. Li S, Fan T, Liu H, Chen J, Qin C and Ren X: Tumor suppressor ING4 overexpression contributes to proliferation and invasion inhibition in gastric carcinoma by suppressing the NF- κ B signaling pathway. *Mol Biol Rep* 40: 5723-5732, 2013.
52. Colla S, Tagliaferri S, Morandi F, Lunghi P, Donofrio G, Martorana D, Mancini C, Lazzaretti M, Mazzeri L, Ravanetti L, *et al*: The new tumor-suppressor gene inhibitor of growth family member 4 (ING4) regulates the production of proangiogenic molecules by myeloma cells and suppresses hypoxia-inducible factor-1 α (HIF-1 α) activity: Involvement in myeloma-induced angiogenesis. *Blood* 110: 4464-4475, 2007.
53. Ozer A, Wu LC and Bruick RK: The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). *Proc Natl Acad Sci USA* 102: 7481-7486, 2005.