



MiR-138-5p suppresses lung adenocarcinoma cell epithelial-mesenchymal transition, proliferation and metastasis by targeting ZEB2

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ABSTRACT

Background: MiR-138-5p is regarded as a tumour suppressor in many cancers. Transforming growth factor beta (TGF- β) often acts as a tumor promotor at the late stages of human cancers. However, the function of miR-138-5p on lung adenocarcinoma cells induced by TGF- β remains to be further confirmed.

Methods: RT-qPCR was used to detect the expression of human lung adenocarcinoma tissues, adjacent normal tissues, and relative cell lines. When the lung adenocarcinoma cells A549 and H1299 were transfected with negative control (NC), miR-138-5p mimics and miR-138-5p inhibitor by lipofectamine3000 and treated with or without TGF- β 1, the lung adenocarcinoma cell function was detected by Immunofluorescence, Western blotting (WB), cell counting Kit-8 (CCK8), colony formation, EdU, Wound-healing and Transwell assays. The relation between miR-138-5p and zinc finger E-box-binding homeobox 2 (ZEB2) was detected by RT-qPCR, WB, and Luciferase reporter assays. When ZEB2 was knocked down, the lung adenocarcinoma cell function was detected by WB, CCK8 and Transwell assays.

Results: The expression of miR-138-5p was decreased in lung adenocarcinoma tissues and cell lines. When treated with or without TGF- β 1, overexpression of miR-138-5p suppressed EMT, proliferation and metastasis of A549 and H1299. ZEB2 was verified as the direct target of miR-138-5p. Downregulation of ZEB2 suppressed EMT, proliferation and metastasis of lung adenocarcinoma cell, which could be reversed by miR-138-5p inhibitor.

Conclusions: MiR-138-5p inhibits epithelial-mesenchymal transition, growth and metastasis of lung adenocarcinoma cells through targeting ZEB2.

1. Introduction

Lung cancer is a major cause of cancer-related deaths globally [1]. The two major subtypes are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [2]. Among them, nearly 80% are NSCLCs, including adenocarcinomas, squamous cell carcinomas, and large cell carcinomas [2]. In most cases, lung cancer develops without symptoms; therefore, more than 40% of patients are diagnosed at an advanced stage [3] and the overall mortality rate of lung cancer remains high, and the 5-year survival rate is lower than 15% [4].

MicroRNAs (miRNAs) are 18–23-nt conserved endogenous non-protein-coding small RNAs. They can bind to the 3'-untranslated region (UTR) regions of target gene transcripts to form a silencing complex, which prevents protein translation [5]. Changes in miRNA expression reportedly may be associated with cancer pathogenesis, apoptosis, and cell growth in various human cancers, and various miRNAs act as oncogenes or tumor suppressors [6]. For example, miR-21 promotes breast cancer cell invasion through HER2/neu signaling [7]. MiR-200 suppresses metastasis by targeting Flt 1 in lung cancer [8].

The expression of miR-138 reportedly is downregulated in various

Abbreviation: CCK8, cell counting Kit-8; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer; TGF- β 1, transforming growth factor beta 1; ZEB2, zinc finger E-box-binding homeobox 2; 3'-UTR, 3'-untranslated region; WB, western blotting

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types of cancers, such as colorectal, thyroid, ovarian, and liver cancers, indicating it is a tumor suppressor [9–12]. Some studies have indicated that miR-138 is downregulated in NSCLC tissues and derived cell lines. miR-138 inhibits NSCLC progression by targeting GIT1 and SEMA4C [13], SIRT-1 [14], LIMK1 [15], and YAP1 [16]. A recent study reported that miR-138 inhibits NSCLC cell growth, invasion, and epithelial-mesenchymal transition (EMT) via a SOX4/p53 feedback loop [17].

EMT refers to the conversion of epithelial into mesenchymal cells, which is a complex, reversible process [18]. EMT endows epithelial cancer cells with migratory and invasive capabilities, which prevents apoptosis and enhances metastatic competence [19]. Therefore, EMT plays a key role in tumor development and invasion. Although EMT can be induced by many extracellular ligands, transforming growth factor beta (TGF- β) and TGF- β -related proteins have emerged as major inducers of this transdifferentiation process in development and cancer [20]. Previous studies have reported that miR-138 can reverse EMT in NSCLC [13,17,21]. TGF- β 1-induced downregulation of miR-138 contributes to EMT in primary lung cancer cells [22].

ZEB2 is a transcription factor that contains a homeodomain and two separate zinc-finger clusters [23]. During EMT, ZEB2 binds to CACCT(G) in the promoter of the E-cadherin gene, thus inhibiting its expression [24]. In addition, ZEB2 activates the vimentin promoter in cooperation with sp1 [25]. In recent years, ZEB2 gene expression has been reported to be affected by the activity of several miRNAs. For example, in hepatocellular carcinoma, miR-141 suppresses cancer progression by targeting ZEB2 [26]. In lung cancer cells, miR-132 targets ZEB2 to inhibit invasion and migration [27].

In the current study, we explored the roles of miR-138-5p in EMT, growth, and metastasis of lung adenocarcinoma cells treated with or without TGF- β 1. Furthermore, we identified candidate targets of miR-138-5p in lung adenocarcinoma cells with the aim to unravel its mechanisms of action.

2. Materials and methods

2.1. Clinical specimen collection

In total, 20 pairs of resected lung adenocarcinoma samples and adjacent tissues were obtained from Shanghai East Hospital affiliated to Tongji University between January 2017 and July 2017. All participants in the study were fully informed and signed informed consent. All tissue samples were confirmed as lung adenocarcinoma at the pathology department and were stored at -80°C after resection. The study was approved by the Ethics Review Board of Shanghai East Hospital at Tongji University (Shanghai, China).

2.2. Cell culture

Human lung cancer cell lines H460, A549, and H1299 were acquired from the cell bank of the Chinese Academy of Science (Shanghai, China). Normal lung epithelial cells (HBE) were supplied by the Xiangya hospital (Changsha, China). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Camarillo, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C in an incubator with 5% CO_2 . When they reached nearly 90% confluence, the cells were passaged. The medium was replaced with fresh DMEM twice or thrice per week.

2.3. RNA isolation and quantitative reverse transcription (RT-q)PCR

All tissue samples were pulverized with a mechanical pulverizer. For cell cultures, A549 and H1299 were seeded in 6-well plates. When the cells reached nearly 90% confluence, total RNA was isolated. Total RNA was isolated with TRIzol (Invitrogen, Camarillo, CA, USA) per the manufacturer's instructions. miRNA was isolated using a miRcute miRNA Isolation Kit (Tiangen, Beijing, China). Total RNA (1 μg) was

reverse-transcribed to cDNA using a First Strand cDNA Synthesis Kit (Thermo, Waltham, MA, USA) and miRNA (1 μg) was prepared using a miRNA First Strand cDNA Synthesis Kit (Tiangen), per the manufacturers' instructions. RT-qPCR for ZEB2 was carried out with Power SYBR Green PCR Master Mix (Thermo) and the cycling conditions were: 40 cycles at 95°C for 15 s and at 60°C for 1 min. GAPDH was used for normalization. The expression of miR-138-5p in the cell lines were determined by RT-qPCR using a miRNA fluorescent quantitative detection kit (Tiangen) and cycling condition were: 40 cycles at 94°C for 20 s and at 60°C for 34 s. U6 was used for normalization. The primer sequences are as follows: miR-138-5p forward: 5'-AGCUGGUGUUGUG AAUCAGGCCG-3'; ZEB2 forward: 5'-GCGATGGTCATGCA-GTCAG-3' and reverse: 5'-CAGGTGGCAGGTCAATTTTCT-3'; GAPDH forward: 5'-GAGTCAACGGATTTGGTCGT-3' and reverse: 5'-TTGATTTGGAGG GATCTCG-3'. Each sample was analyzed in triplicate. Relative target gene expression was calculated by the $2^{-\Delta\Delta\text{CT}}$ method.

2.4. Transfection and TGF- β 1 treatment

When A549 and H1299 cells reached nearly 60% confluence, they were transfected with negative control (NC) (20 nM), miR-138-5p mimics (20 nM), or miR-138-5p inhibitor (50 nM) in a culture plate using Lipofectamine 3000 (Invitrogen) per the manufacturer's protocol. Cells were transfected with si-ZEB2, including si-ZEB2-1, si-ZEB2-2, si-ZEB2-3 (20 nM) or si-NC (20 nM) using Lipofectamine 3000. At 24 h after transfection, TGF- β 1 (10 ng/ml; Cell Signaling Technology, Danvers, MA, USA) was added to the wells in low-serum medium (containing 2% FBS) for the indicated periods. Cells incubated without TGF- β 1 in low-serum medium served as a control.

2.5. Analysis of cell morphology

After transfection with NC, miR-138-5p mimics, or miR-138-5p inhibitor and exposure to TGF- β 1 or not for 24 h, A549 cells were examined under an inverted microscope.

2.6. Immunofluorescence analysis

After transfection and exposure to TGF- β 1 or not for 24 h, H1299 and A549 cells were seeded in a 24-well plate and incubated at 37°C overnight. Then, the cells were fixed with 4% paraformaldehyde for 20 min and permeated with 0.2% Triton X-100 for 10 min. After incubation with anti-vimentin (1:250; rabbit monoclonal; cat. no. ab92547; Abcam, Cambridge, MA, USA) overnight at 4°C , the cells were stained with fluorescein isothiocyanate-conjugated secondary antibodies (goat anti-rabbit IgG; 1:1000; cat. no. #A-21428; Thermo, USA). The nuclei were counterstained with DAPI. Six random fields were observed under a fluorescence microscope.

2.7. Cell proliferation assay

After transfection and treatments for 24 h, A549 and H1299 cells were seeded at 3000 cells/well in a 96-well plate ($n = 6$ wells per treatment). Adherent cells in each group were analyzed with the Cell Counting Kit-8 (CCK8; Dojindo, JPN) at 0, 24, 48, and 72 h to evaluate cell proliferation. An EdU imaging kit (RiboBio, Guangzhou, China) was used per the manufacturer's instructions to analyze proliferation. Briefly, cells were incubated with EdU for 2 h. After three washes with PBS, the cells were fixed with 4% polyoxymethylene for 30 min and permeated with 0.5% Triton X-100 for 10 min. Finally, the cells were stained with Apollo and Hoechst and imaged under a fluorescence microscope. To analyze colony formation, after transfection and treatments, 500 cells per treatment were inoculated in 35-mm plates and cultured for 10 days. The cells were stained with Giemsa and colonies were counted. The rate of colony formation was calculated as the number of colonies/500 \times 100%.

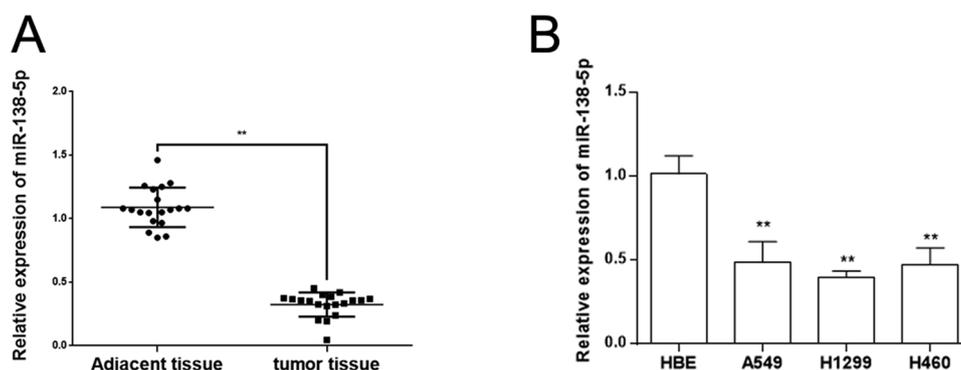


Fig. 1. The expression of miR-138-5p in lung adenocarcinoma tissues and cell lines determined by RT-qPCR. (A) The expression of miR-138-5p in lung adenocarcinoma tissues and adjacent normal tissues. (B) The expression of miR-138-5p in A549, H1299, H460 and HBE cells. U6 was used for normalization. ** $p < 0.05$.

2.8. Wound-healing assay

After transfection and treatments, A549 and H1299 cells were seeded in a 6-well plate at 1×10^6 cells/well, and a scratch was made in the middle of the well with a p200 pipet tip. Cell migration ability was assessed on the basis of images captured at 0 h and 24 h after the scratches were made, with ImageJ. The relative migration area was calculated as: (scratch area at 0 h – scratch area at 24 h)/scratch area at 0 h $\times 100\%$.

2.9. Transwell assay

After transfection and treatments, A549 and H1299 cells in 200 μ l of serum-free DMEM were carefully seeded into the upper chambers of a Matrigel-free or Matrigel-coated Transwell plate (Corning, Cambridge, MA, USA). Complete medium with 10% FBS was added in the lower wells. The next day, adherent cells that had passed through or invaded the filters were fixed with 4% paraformaldehyde and stained with crystal violet. The adherent cells on the lower surface of the membrane were viewed under a light microscope, and cells were counted in four random fields.

2.10. Dual-luciferase reporter assay

H1299 cells were seeded in 24-well plates and incubated for 24 h. Then, the cells were cotransfected with 1 μ g firefly luciferase reporter construct (PGL3-ZEB2-wt or PGL3-ZEB2-mut (Genomeditech, Shanghai) as well as miR-138-5p mimics or NC and PRL-CMV Renilla luciferase reporter plasmid (Genomeditech). The transfection efficiency was normalized to that of the PRL-CMV plasmid. After transfection for 48 h, the H1299 cells were lysed for 15 min and the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to measure relative luciferase activity.

2.11. Western blot analysis

After transfection and treatment, A549 and H1299 cells were added to a mixture of RIPA lysis buffer, phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. Proteins were isolated and the concentrations were measured with a BCA protein assay kit (Beyotime, China). The proteins were separated by 8% or 10% SDS-PAGE and then transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk for 1 h. Then, the membranes were incubated with primary antibodies at 4 °C overnight. The following primary antibodies were used: anti-E-cadherin (1:1000; rabbit monoclonal; cat. no. #3195; CST), anti-vimentin (1:3000; rabbit monoclonal; cat. no. ab92547; Abcam), anti-ZEB2 (1:1000; rabbit polyclonal; cat. no. 14026-1-AP; Proteintech, Chicago, IL, USA), anti-N-cadherin (1:2000; rabbit polyclonal; cat. no. 22018-1-AP; Proteintech) and anti-GAPDH (1:1000;

rabbit monoclonal; cat. no. #5174; CST). The next day, after rinsing with TBST, the membranes were incubated with secondary antibody (donkey anti-rabbit IgG; 1:10,000; cat. no. ab186692; Abcam) in the dark for 1 h. The membranes were developed using ECL reagent (Millipore, Billerica, MA, USA). Signals were quantified with ImageJ.

2.12. Statistical analysis

SPSS 20.0 was used for statistical analyses. All data are presented as the mean \pm standard deviation (SD). Experiments were performed three times independently. The *t*-test was used to compare means of two groups, whereas one-way ANOVA was used to compare means of more than two groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. MiR-138-5p is downregulated in lung adenocarcinoma tissues and cell lines

MiR-138-5p expression in lung adenocarcinoma tissues and cell lines (A549, H1299, and H460) was evaluated by RT-qPCR. The result showed that miR-138-5p was significantly downregulated in lung adenocarcinoma tissues when compared with adjacent normal tissues (Fig. 1A). Similar results were observed in the three NSCLC cell lines when compared with normal lung epithelial cells (HBE) (Fig. 1B).

3.2. MiR-138-5p inhibits EMT in lung adenocarcinoma cell lines

Since EMT-like transformation of epithelial cells can be induced by TGF- β 1 in many cell culture models, we established a model of EMT induced by TGF- β 1 to evaluate the mechanism of miR-138-5p in lung adenocarcinoma. First, miR-138-5p transfection efficiency was evaluated by RT-qPCR. MiR-138-5p expression was obviously increased when H1299 and A549 were transfected miR-138-5p mimics, whereas it was decreased after transfection with miR-138-5p inhibitor, in comparison with NC-treated cells (Fig. 2A).

After effective transfection, A549 cells were stimulated with TGF- β 1 and observed for morphological changes. TGF- β 1 can induce cells to take a fusiform shape [28]. Upregulation of miR-138-5p suppressed the induction of the fusiform shape by TGF- β 1. When miR-138-5p was inhibited, cells were more fusiform (Fig. 2B). However, this interesting phenomenon was not observed in H1299 cells. This may be due to the fact that H1299 are mesenchymal cells and thus, morphological changes are difficult to observe [29].

Based on the morphological changes observed, we further investigated the functions of miR-138-5p in EMT of the lung adenocarcinoma cell lines. Upregulation of miR-138-5p suppressed the expression of vimentin as indicated by immunofluorescence analysis and western blotting, and that of N-cadherin as indicated by western

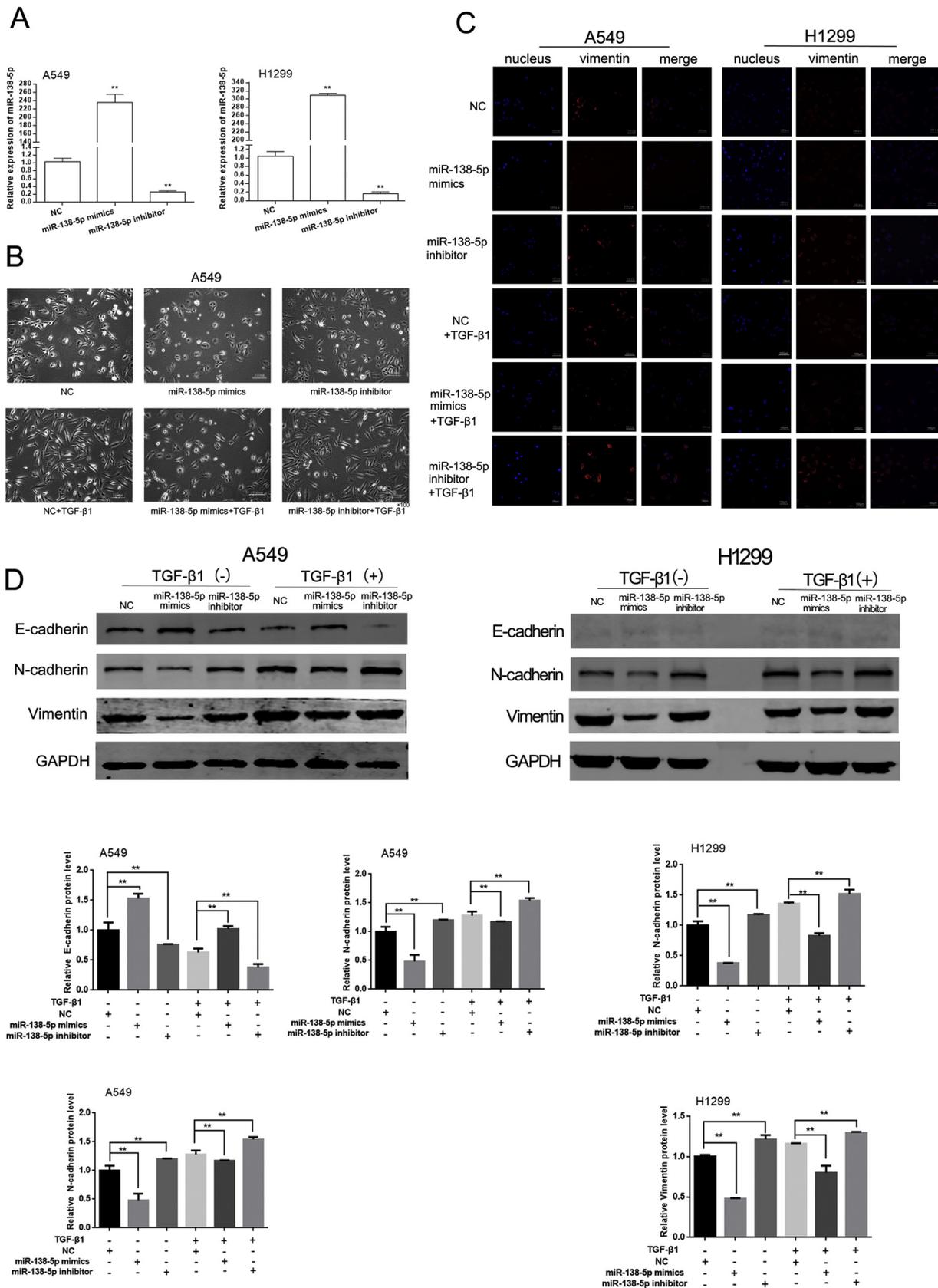


Fig. 2. The effects of miR-138-5p on the EMT of lung adenocarcinoma cells which induced by TGF-β1 or not. (A) The expression of miR-138-5p in A549 and H1299 cells after transfection of NC, miR-138-5p mimics and miR-138-5p inhibitor. (B) the shape of A549 cell lines stimulated by TGF-β1 after different transfection. Original magnification × 100 (C) Immunofluorescence expression of vimentin in A549 and H1299 cells transfected NC, miR-138-5p mimics, and miR-138-5p inhibitor, with or without TGF-β1. Original magnification × 100 (D) The expression of EMT markers in A549 and H1299 cells transfected NC, miR-138-5p mimics, and miR-138-5p inhibitor by western blot, with or without TGF-β1. ** $p < 0.05$.

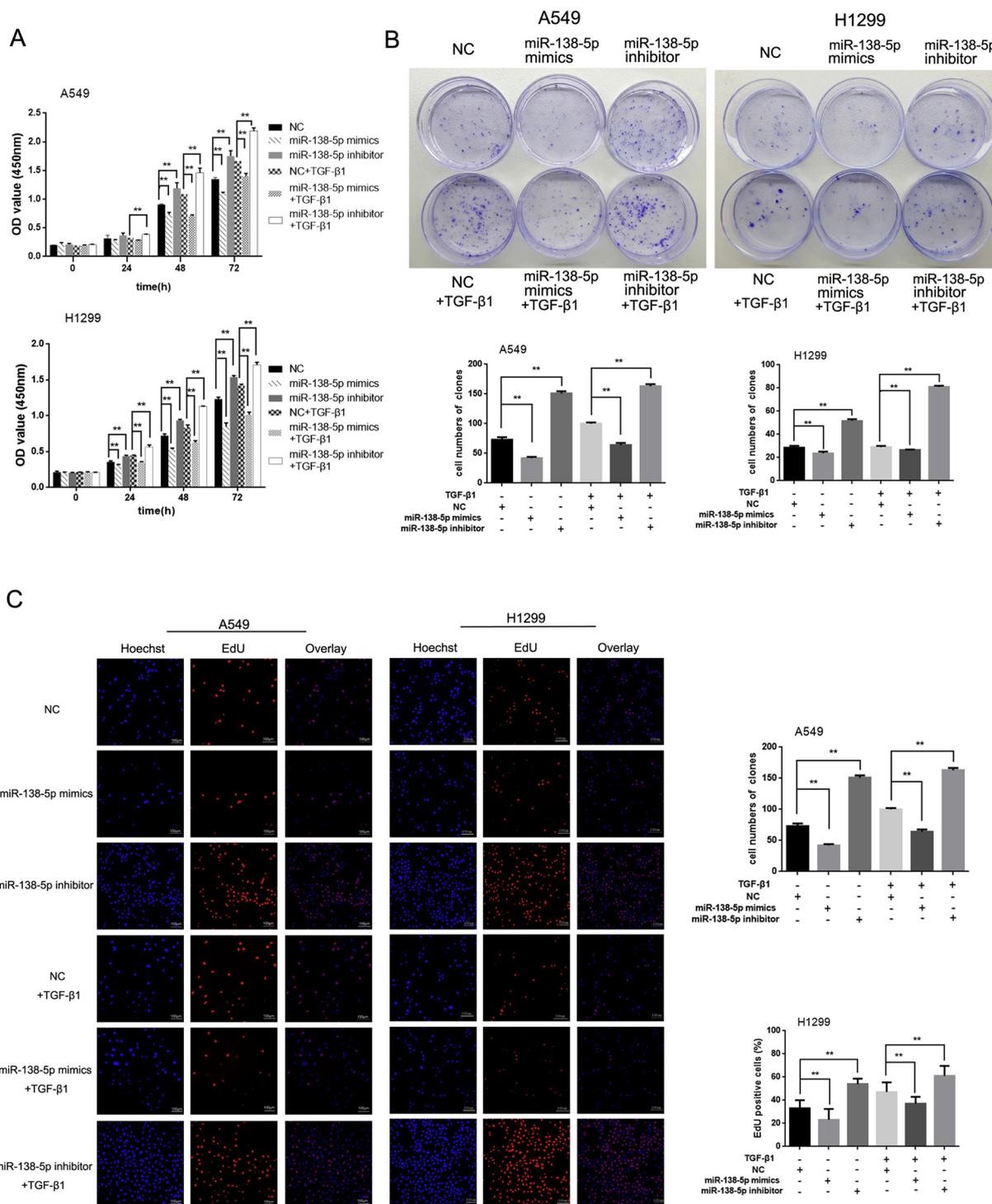


Fig. 3. The effects of miR-138-5p on the proliferation of lung adenocarcinoma cells which induced by TGF-β1 or not. (A) The effects of miR-138-5p on the proliferation of A549 and H1299 cells by CCK8. (B) The effects of miR-138-5p on the proliferation of A549 and H1299 cells by colony formation. (C) The effects of miR-138-5p on the proliferation of A549 and H1299 cells by EdU assays. Original magnification $\times 100$ $** p < 0.05$.

blotting. Overexpression of miR-138-5p promoted the expression of E-cadherin in A549 cells as indicated by western blotting (Fig. 2C, D). However, E-cadherin expression was difficult to detect in H1299 cells, which corroborated that H1299 are mesenchymal cells.

3.3. MiR-138-5p inhibits the proliferation of lung adenocarcinoma cells

The proliferative capacity of the lung adenocarcinoma cell lines was evaluated by CCK8, colony formation, and EdU assays. Upregulation of

miR-138-5p obviously suppressed the growth of A549 and H1299 cells. When the cells were transfected with miR-138-5p inhibitor, more rapid proliferation was observed. Cancer cells transfected with miR-138-5p mimics showed a partial decline in growth rate upon treatment with TGF-β1. When miR-138-5p was inhibited, the cells proliferated more rapidly, which corroborated that miR-138-5p can inhibit growth in both lung adenocarcinoma cell lines (Fig. 3A–C).

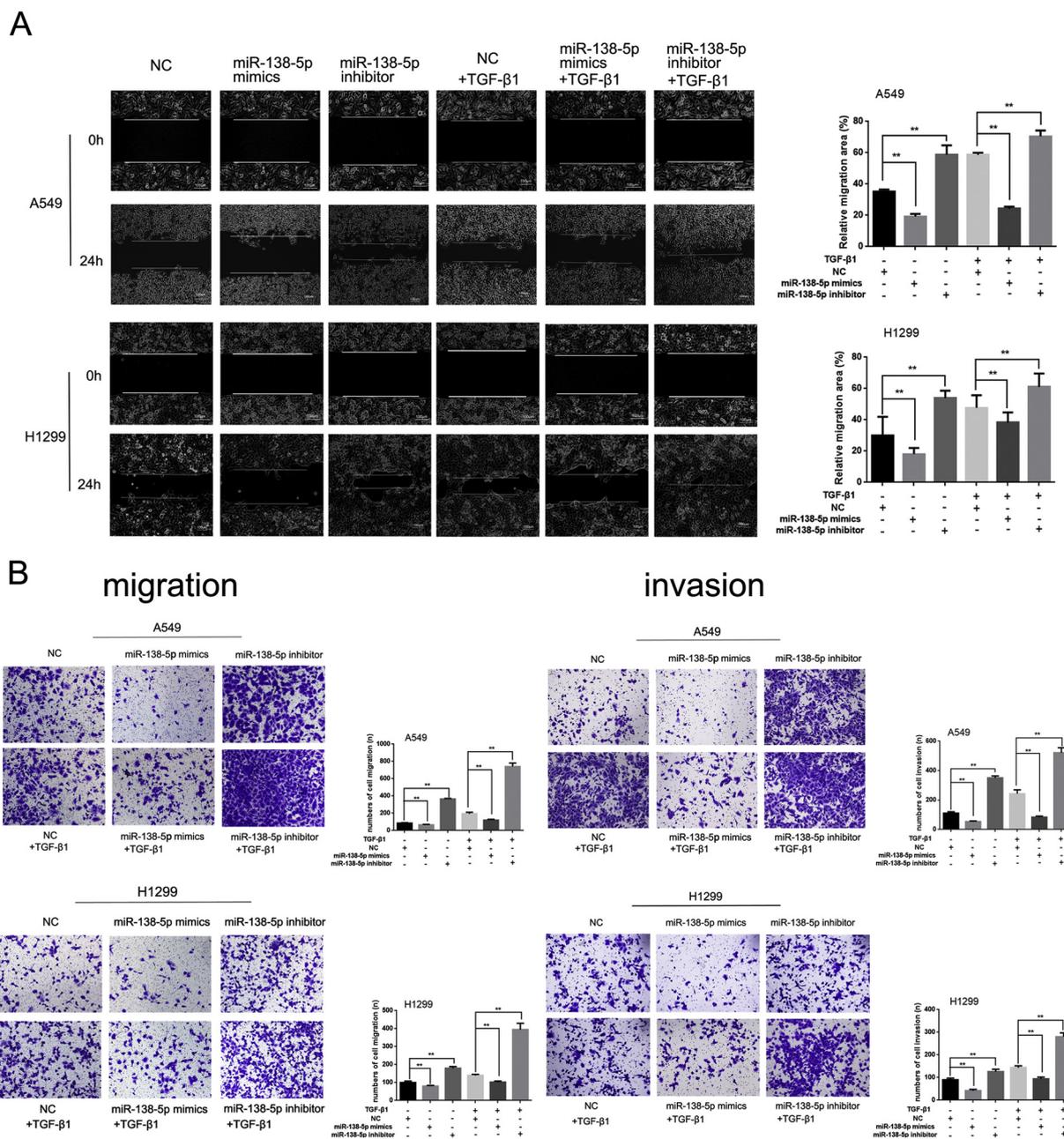


Fig. 4. The effects of miR-138-5p on the migration and invasion of lung adenocarcinoma cells which induced by TGF- β 1 or not. (A) The effects of miR-138-5p on the migration of A549 and H1299 cells by Wound-healing assay. Original magnification $\times 100$ (B) The effects of miR-138-5p on the migration and invasion of A549 and H1299 cells by Transwell assay. Original magnification $\times 100$ ** $p < 0.05$.

3.4. miR-138-5p inhibits the migration and invasion of lung adenocarcinoma cells

The migrative and invasive capacities of the lung adenocarcinoma cell lines were evaluated by wound-healing and Transwell assays. The wound-healing assay results showed that upregulation of miR-138-5p resulted in slower healing. Meanwhile, migration of the cancer cells was significantly increased when miR-138-5p was inhibited (Fig. 4A). Similarly, the Transwell assay results suggested that miR-138-5p inhibited the migratory and invasive abilities of the lung adenocarcinoma cells (Fig. 4B).

3.5. ZEB2 is a direct target of miR-138-5p

The functions of miRNAs in tumor progression are related to those

of their target genes [30]. Target genes of miR-138-5p were predicted from the comprehensive database miRWalk 2.0 (<http://zmf.um-m.uni-heidelberg.de/apps/zmf/mirwalk2>). miR-138-5p was predicted to bind to the 3'-UTR region of ZEB2. Firstly, the expression of ZEB2 in lung adenocarcinoma tissues was evaluated by RT-qPCR. The results revealed that ZEB2 expression was increased in lung adenocarcinoma tissues compared with adjacent normal tissues (Fig. 5A). Furthermore, a significant negative correlation was found between ZEB2 and miR-138-5p expression ($r = -0.641$; $p < 0.01$; Fig. 5B). Similar results were obtained in the cell lines. Compared with HBE cells, ZEB2 mRNA and protein expression were obviously increased in A549, H1299, and H460 cells (Fig. 5C, D). Next, ZEB2 expression was evaluated in adenocarcinoma cells transfected NC, miR-138-5p mimics, or miR-138-5p inhibitor. ZEB2 mRNA and protein expression were significantly decreased in cells transfected with miR-138-5p mimics and increased in

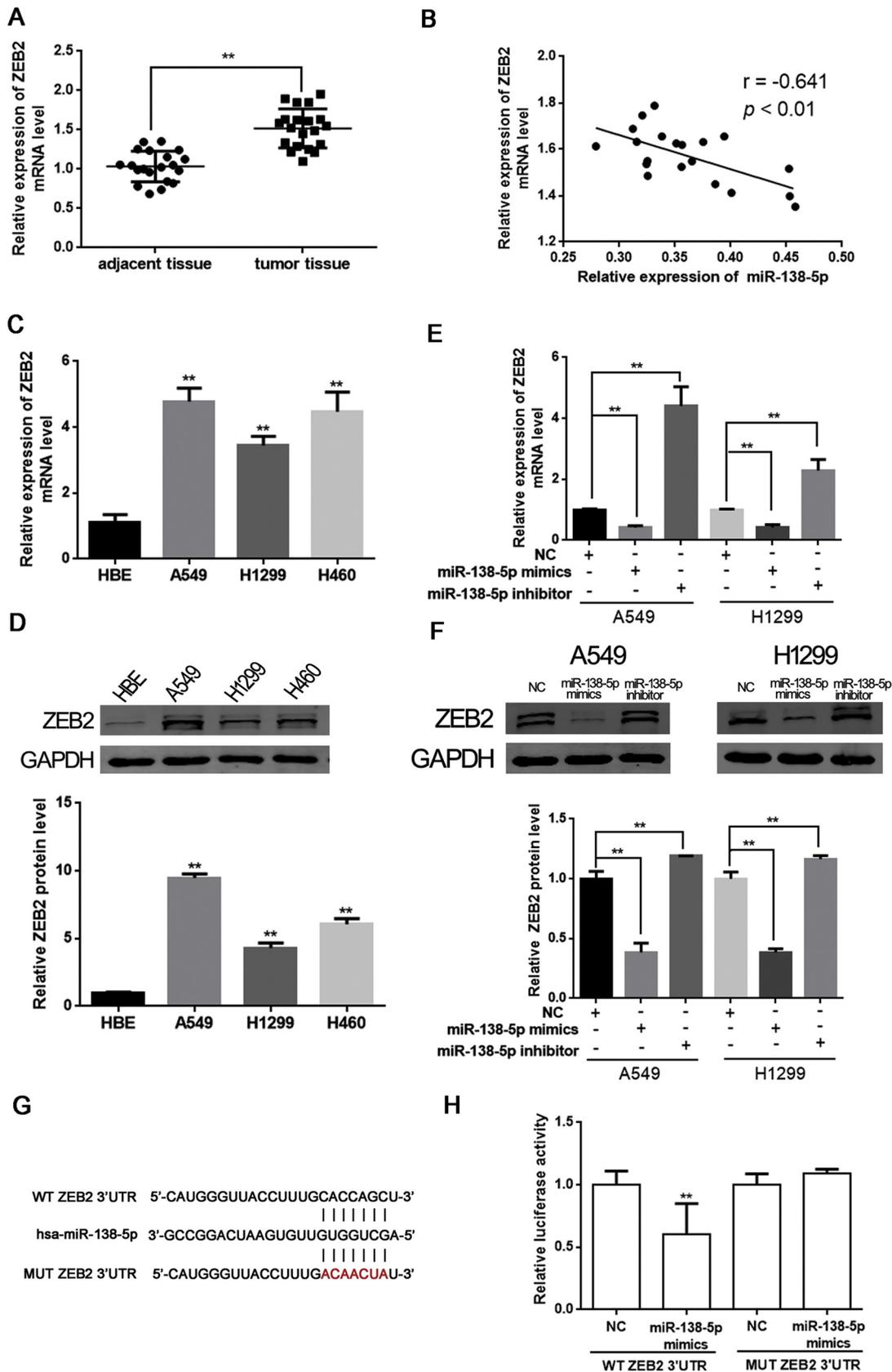


Fig. 5. Identification ZEB2 as the target gene of miR-138-5p. (A) The expression of ZEB2 on mRNA level in lung adenocarcinoma tissues and adjacent normal tissues. (B) Negative correlation between ZEB2 and miR-138-5p expression ($r = -0.641$; $p < 0.01$). (C) The expression of ZEB2 on mRNA level in A549, H1299, H460, and HBE. (D) The expression of ZEB2 on protein level in A549, H1299, H460, and HBE. (E) The expression of ZEB2 on mRNA level after transfection of NC, miR-138-5p mimics, and miR-138-5p inhibitor in A549 and H1299. (F) The expression of ZEB2 on protein level after transfection of NC, miR-138-5p mimics, and miR-138-5p inhibitor in A549 and H1299. (G) 3'-UTR fragment of wildtype (WT) and mutant type (MUT) ZEB2 which disrupted interaction with miR-138-5p. (H) The wildtype or mutated reporter plasmid was co-transfected into lung adenocarcinoma cells with miR-138-5p mimics or NC to detect relative luciferase activity, renilla plasmid as an internal reference. $** p < 0.05$.

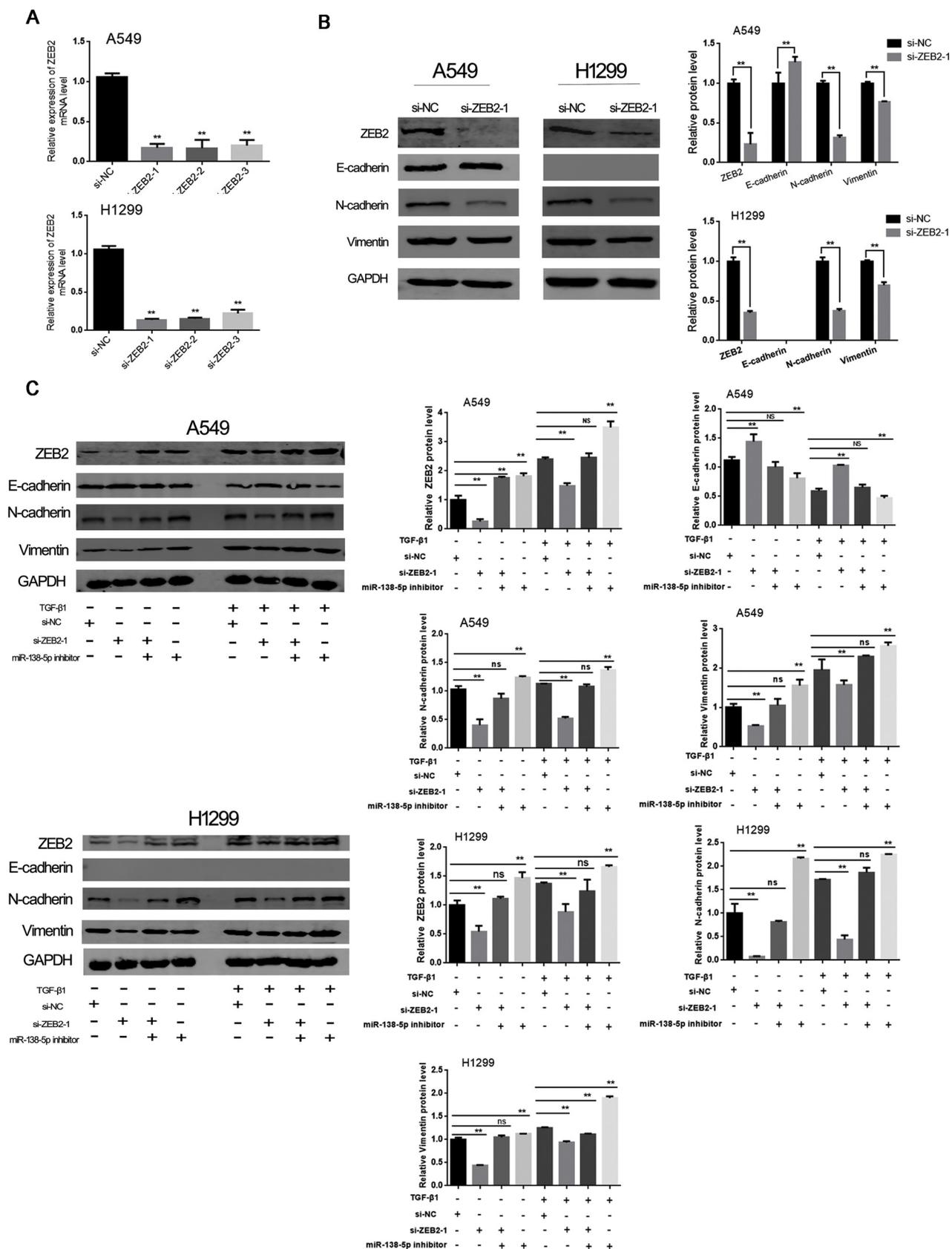


Fig. 6. The effect of si-ZEB2 on EMT in lung adenocarcinoma cells. (A) The expression of si-ZEB2-1, si-ZEB2-2, si-ZEB2-3, and si-NC on mRNA level. (B) The expression of E-cadherin on protein level in A549 cells and the expression of N-cadherin and vimentin in A549 and H1299 cells between si-NC and si-ZEB2-1 group. (C) E-cadherin, N-cadherin and vimentin expression on protein level in A549 and H1299 cells when the cells transfected with si-NC, si-ZEB2-1, miR-138-5p inhibitor + si-ZEB2-1, miR-138-5p inhibitor and treated with TGF-β1 or not. ** $p < 0.05$. ns: no significance.

cells transfected with miR-138-5p inhibitor (Fig. 5E, F). Finally, ZEB2 with a wild-type or a mutant 3'-UTR was cloned into the PGL3 reporter vector (Fig. 5G). A dual-luciferase reporter assay showed that the luciferase activity obviously declined when the wild-type reporter plasmid was cotransfected with the miR-138-5p mimics in H1299 cells. In contrast, there was no change in luciferase activity when the mutant reporter plasmid was cotransfected with the miR-138-5p mimics (Fig. 5H). Based on the above results, it was concluded that ZEB2 is targeted by miR-138-5p.

3.6. Downregulation of ZEB2 inhibits EMT in lung adenocarcinoma cells

As ZEB2 was predicted as a target gene of miR-138-5p, we knocked down its expression using siRNA to determine its function in lung adenocarcinoma cells. siRNA-NC and siRNA-ZEB2-1, -2, and -3 were transfected into H1299 and A549 cells. The transfection efficiency was determined by RT-qPCR; ZEB2 expression was inhibited significantly in cells transfected with si-ZEB2 (Fig. 6A). We selected si-ZEB2-1 for subsequent experiments because it showed the best efficiency. Protein expression of E-cadherin, N-cadherin, and vimentin was determined by western blot analysis. Obviously, when ZEB2 was knocked down, E-cadherin expression was upregulated in A549 cells and vimentin and N-cadherin were downregulated in A549 and H1299 cells as compared with si-NC-treated cells. E-cadherin was not detected in H1299 cells by western blotting (Fig. 6B).

Since miR-138-5p can target ZEB2, the lung adenocarcinoma cells were cotransfected with miR-138-5p inhibitor and si-ZEB2-1 to evaluate whether miR-138-5p inhibition could reverse the effect of si-ZEB2. Indeed, regardless of the presence of TGF- β 1, when cells were cotransfected with miR-138-5p inhibitor and si-ZEB2-1, compared with transfection with si-ZEB2-1 alone, E-cadherin expression was decreased whereas vimentin and N-cadherin were increased. When compared with cells transfected with miR-138-5p inhibitor, E-cadherin expression was increased and that of vimentin and N-cadherin was decreased (Fig. 6C). Based on these findings, it was inferred that the EMT process promoted by miR-138-5p inhibitor in lung adenocarcinoma cell lines was reversed upon ZEB2 knockdown.

3.7. Downregulation of ZEB2 inhibits the proliferation of lung adenocarcinoma cells

After A549 and H1299 cells were transfected si-NC, si-ZEB2-1, si-ZEB2-1 + miR-138-5p inhibitor, and miR-138-5p inhibitor respectively, the proliferative capacity of the lung adenocarcinoma cell lines was evaluated by CCK8 assay. Regardless of the presence of TGF- β 1, downregulation of ZEB2 obviously suppressed the growth of A549 and H1299 cells when compared with si-NC cells. However, in the si-ZEB2-1 + miR-138-5p inhibitor group, proliferation was faster than in the si-ZEB2-1 group. Moreover, the proliferation in the miR-138-5p inhibitor group was the fastest among the four groups (Fig. 7).

3.8. Downregulation of ZEB2 inhibits the migration and invasion of lung adenocarcinoma cells

The migrative and invasive capacities of the lung adenocarcinoma cell lines after ZEB2 knockdown were evaluated by Transwell assay. The results showed that knockdown of ZEB2 inhibited the migratory and invasive abilities when compared with si-NC treatment. When cotransfected with si-ZEB2-1 and miR-138-5p inhibitor, cell migration and invasion were increased when compared with transfection with si-ZEB2-1 alone. Meanwhile, cell migration and invasion were obviously increased when miR-138-5p was inhibited. The above results showed that miR-138-5p inhibition could reverse the effect of si-ZEB2 (Fig. 8).

4. Discussion

In this study, we found that miR-138-5p is expressed at a lower level in lung adenocarcinoma tissues and cell lines. *In-vitro* experiments showed that miR-138-5p suppressed EMT and the proliferative, migratory, and invasive abilities of lung adenocarcinoma cell lines and thus, acts as a tumor suppressor. When the lung adenocarcinoma cells were stimulated with TGF- β 1, miR-138-5p partly suppressed their EMT, growth and metastasis. Furthermore, it was confirmed that ZEB2 was targeted by miR-138-5p. Downregulation of ZEB2 suppressed EMT, proliferation, and metastasis of the lung adenocarcinoma cells.

Previous studies have suggested that miR-138-5p is downregulated in various tumors and might act as a tumor suppressor. For example, upregulation of miR-138-5p enhanced osteosarcoma cell chemosensitivity to cisplatin via targeting EZH2 [31]. Elevated miR-138-5p inhibited gallbladder carcinoma cell proliferation by directly targeting Bag-1 [32]. In lung cancer, some studies have reported that the migratory and invasive abilities of NSCLC cells are inhibited by miR-138-5p via targeting SIRT-1, LIMK1, and YAP1 [14–16]. The above studies suggested that miR-138-5p can suppress tumor growth and metastasis.

We found that miR-138-5p was downregulated in lung adenocarcinoma tissues and cell lines. After successfully establishing a TGF- β 1-induced EMT model in A549 and H1299 adenocarcinoma cells, we found that the EMT process was partly reversed through upregulation of miR-138-5p in these cells. In addition, miR-138-5p inhibited TGF- β 1-induced cell proliferation in the two cell lines. Further, the migratory and invasive abilities of the cell lines were inhibited by miR-138-5p. Taken together, these results indicate that miR-138-5p functions as a cancer suppressor by inhibiting EMT, proliferation, and metastasis of lung adenocarcinoma cancer cells. Moreover, in A549 cells, the mesenchymal cell morphology induced by TGF- β 1 was reversed by overexpression of miR-138-5p. EMT morphology and E-cadherin expression were not observed in H1299 cells because this cell line originated from mesenchymal cells [29]. However, vimentin expression in H1299 cells was similar to that in A549 cells.

ZEB2, an EMT regulator, is closely associated with carcinogenesis, cancer progression and response to chemotherapy [33]. For example, in ovarian cancer, ZEB2 promotes EMT and the acquisition of an aggressive phenotype [34]. In hepatocellular carcinoma, ZEB2 promotes vasculogenic mimicry when EMT is induced by TGF- β 1 [35]. In lung cancer, ZEB2 expression is high and is targeted by several miRNAs. For instance, miR-598 [36] and miR-205-5p [37] target ZEB2 to inhibit NSCLC proliferation and invasion. MiR-218 [38], miR-203 [39], miR-154 [40] inhibit NSCLC metastasis by targeting ZEB2. MiR-200b contributes to multidrug resistance of SCLC by regulating ZEB2 [41]. In the current study, miR-138-5p targeted and downregulated ZEB2. Knocking down of ZEB2 inhibited EMT, proliferation and metastasis in A549 and H1299 cells, regardless of treatment with TGF- β 1. Moreover, when miR-138-5p inhibitor and si-ZEB2 were cotransfected into lung adenocarcinoma cells, the inhibitory effect of si-ZEB2 was significantly reversed by miR-138-5p inhibitor. These results indicated that miR-138-5p suppresses EMT, growth and metastasis of lung adenocarcinoma cells, at least in part, through targeting ZEB2.

One limitation of this study is that we did not assess the function of miR-138-5p *in vivo*. Further, advanced human lung cancer tissues to compare the expression of miR-138-5p between metastatic and non-metastatic NSCLC tissues were not obtained. We plan to investigate the role of miR-138-5p in a mouse model and to evaluate miR-138-5p expression in the blood of early- and late-stage patients to compare the expression levels between metastatic and non-metastatic NSCLC in a future study.

In conclusion, our study revealed that miR-138-5p can inhibit tumor EMT, proliferation, and metastasis through targeting ZEB2, regardless of stimulation with TGF- β 1. MiR-138-5p is a potential therapeutic target that is worth further preclinical and clinical testing in the near future.

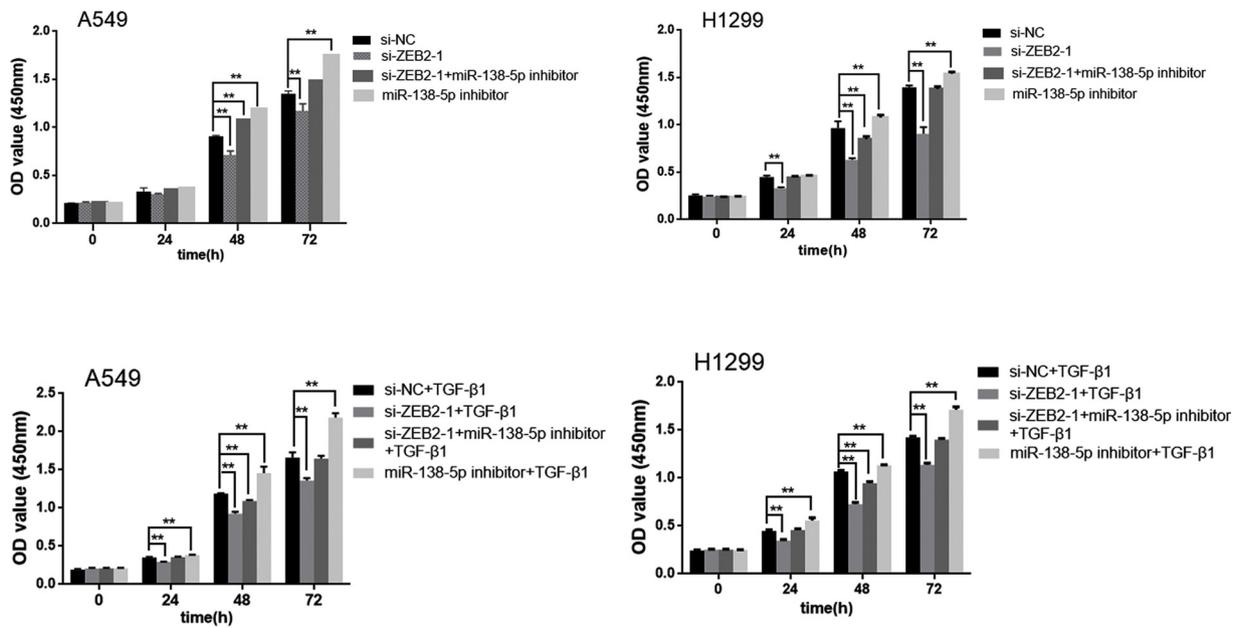


Fig. 7. The effect of si-ZEB2 on the proliferation in lung adenocarcinoma cells when the cells transfected with si-NC, si-ZEB2-1, miR-138-5p inhibitor + si-ZEB2-1, miR-138-5p inhibitor and treated with TGF-β1 or not. ** $p < 0.05$.

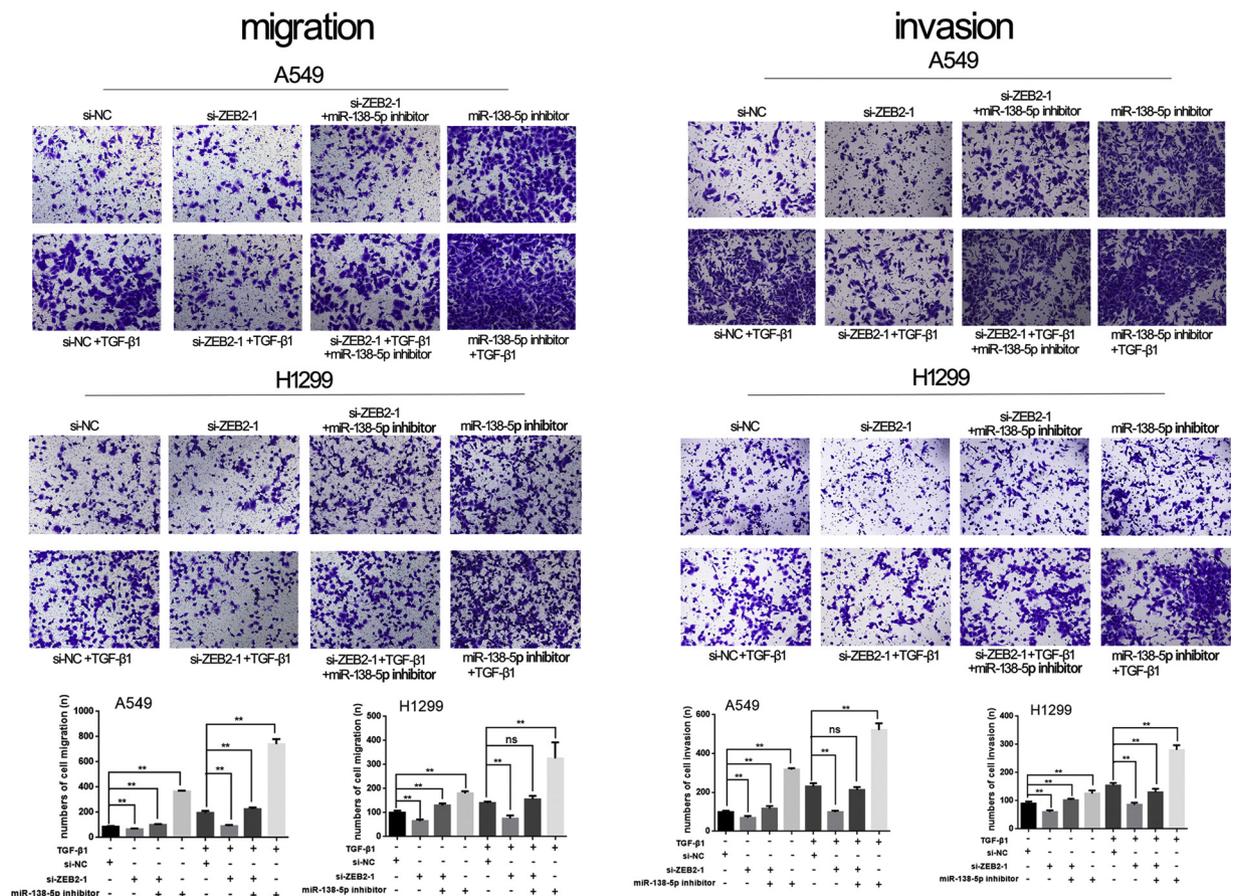


Fig. 8. The effect on the migration and invasion of cancer cells when the cells transfected with si-NC, si-ZEB2-1, miR-138-5p inhibitor + si-ZEB2-1, miR-138-5p inhibitor and treated with TGF-β1 or not. Original magnification $\times 100$ ** $p < 0.05$. ns: no significance.

Authors' contributions

TR and QCL designed and monitored the research. DYZ, LG and WJJ conducted the experiments. DYZ and ZXL have contributed to data analysis. The manuscript was written by DYZ. All authors have read and approved the final manuscript.

Declaration of interest

The authors declare that they have no competing interests

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