

## Original Article

# Inhibition of the growth of non-small cell lung cancer by miRNA-1271

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**Abstract:** Non-small cell lung cancer (NSCLC) is a common lung cancer of high incidence. Since NSCLCs often grow rapidly, it is critical to elucidate the underlying mechanisms. Previous studies have shown that microRNAs (miRNAs) play critical roles in the carcinogenesis of NSCLC. Specifically, miR-1271 has been shown to downregulate in NSCLC cells. However, whether the downregulation of miR-1271 relates to carcinogenesis of NSCLC is unknown. Here, we analyzed the levels of miR-1271 and mTor in NSCLC specimens and studied their correlation. We performed bioinformatics analysis and luciferase-reporter assay to study the association of miR-1271 with mTor mRNA. We analyzed miR-1271 levels in NSCLC cells and studied their effects on mTor levels. The growth of miR-1271-modified NSCLC cells was evaluated in vitro in an MTT assay, and in vivo by bioluminescence. We detected significantly higher levels of mTor and significantly lower levels of miR-1271 in the NSCLC specimens, compared to the paired non-tumor lung tissues. We found that miR-1271 bound to the 3'UTR of mTor mRNA to prevent its translation. Overexpression of miR-1271 in NSCLC cells decreased mTor protein levels, whereas inhibition of miR-1271 increased mTor protein levels, without affecting mTor transcripts. Moreover, overexpression of miR-1271 suppressed the NSCLC growth in vitro and in vivo, while inhibition of miR-1271 significantly increased NSCLC growth. Together, these data suggest that in NSCLC, miR-1271 may inhibit the cancer cell growth through mTor suppression.

**Keywords:** Non-small cell lung cancer (NSCLC), mTor, miR-1271

## Introduction

Non-small cell lung cancer (NSCLC) is a prevalent lung cancer of high incidence. NSCLC has been catalogued into three subtypes: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [1-4]. Most NSCLCs are resistant to chemotherapy and radiation therapy, and often grow very quickly [1-5]. Thus, we [3, 4] and others have made great efforts to elucidate the mechanisms that underlie the growth of NSCLC [6-9].

Protein kinase B (PKB), also known as Akt, is a serine/threonine kinase that regulates multiple biological processes including cell survival, proliferation, growth, and metabolism [10-12]. Various growth factors, hormones, and cytokines activate Akt by binding their cognate receptor tyrosine kinase (RTK), cytokine receptor, or GPCR to trigger activation of the lipid kinase Phosphoinositide 3-kinase (PI3K), which

generates PIP3 at the plasma membrane. Akt binds PIP3 through its pleckstrin homology (PH) domain, resulting in translocation of Akt to the membrane [10-12]. Akt is activated through a dual phosphorylation mechanism, and one of the targets of the Akt activation is the mechanistic target of rapamycin (mTor), which is an atypical serine/threonine kinase that is a master growth regulator to control many biological events, like cell survival, proliferation and growth senses [10-12]. Aberrant and over-activated mTor signaling is often involved in many types of cancer [13, 14]. Nevertheless, its regulation appears to cell-type dependent.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs of around 20 nucleotides that regulate various biological processes [15-17]. Interestingly, bioinformatics approaches have predicted one-third of all mammalian genes to be targeted and regulated by miRNAs [15-17]. Previous studies have shown that microRNAs

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(miRNAs) play critical roles in the carcinogenesis of NSCLC [8, 18-22]. In the miRNA family, miR-1271 is a member that was newly discovered [23]. Most recently, the up-regulation of Glypican-3 was associated with a concomitant down-regulation of miR-1271, which appears to facilitate carcinogenesis of Hepatocellular carcinoma [24]. However, whether miR-1271 may be aberrantly expressed in NSCLC and the underlying pathological meanings are unknown.

Here we reported significantly higher levels of mTor and significantly lower levels of miR-1271 in the NSCLC specimen, compared to the paired normal lung tissues. Bioinformatics analysis and luciferase-reporter assay suggest that miR-1271 binds to the 3'UTR of mTor mRNA to prevent its translation. To prove it, we modified miR-1271 levels in NSCLC cells. We found that overexpression of miR-1271 in NSCLC cells decreased mTor protein levels, whereas inhibition of miR-1271 increased mTor protein levels, without affecting mTor mRNA. Moreover, overexpression of miR-1271 suppressed the NSCLC growth in vitro and in vivo, while inhibition of miR-1271 significantly increased NSCLC growth. Taken together, our data demonstrate that miR-1271 may inhibit the growth of NSCLC through mTor.

### Materials and methods

#### *Patient tissue specimens*

A total of 31 resected specimens from NSCLC patients were collected for this study. NSCLC specimen was compared with the paired normal lung tissue (NLT) from the same patient. All specimens had been histologically and clinically diagnosed at the Department of Lung Tumor Clinical Center, Shanghai Chest Hospital affiliated to Shanghai Jiaotong University from 2007 to 2014. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

#### *Culture of human NSCLC cell line*

A549 is a widely used human NSCLC line purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and was first developed in 1972 by Dr. Giard through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old caucasian

male [25]. A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (Invitrogen).

#### *Animal manipulation*

All animal procedures in the current study have been approved and conducted by the Institutional Review Boards of the Shanghai Chest Hospital affiliated to Shanghai Jiaotong University. Female NOD/SCID mice were purchased from Charles River Laboratories (China), kept in pathogen-free environment, and used for study at 10 weeks of age. Luciferase-carrying, miR-1271-modified A549 cells of  $10^6$  were injected subcutaneously to form tumor in NOD/SCID mice.

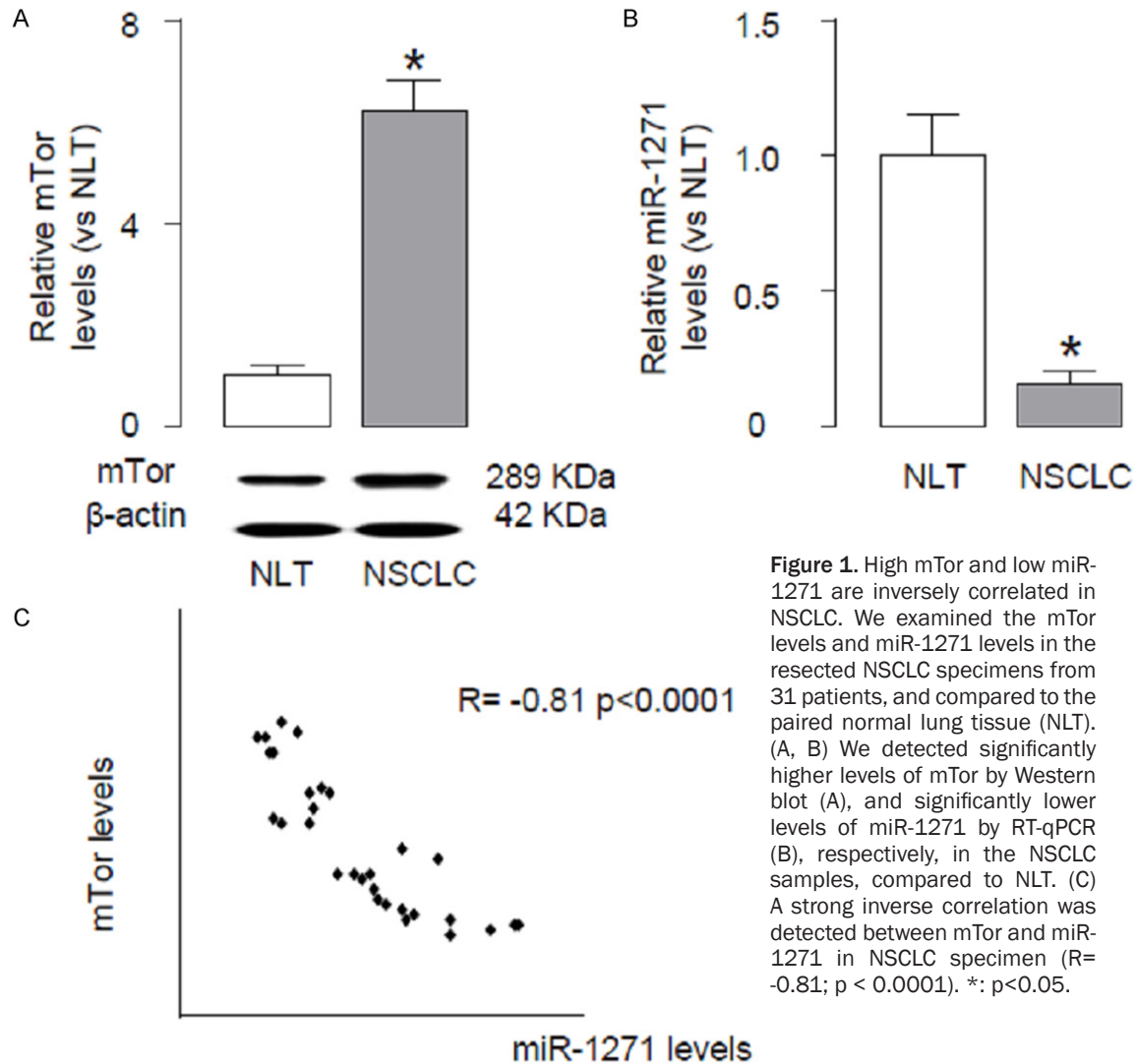
#### *Transduction of A549 cells with adeno-associated viruses (AAVs)*

A549 cells were transduced with AAVs carrying a miR-1271 construct, or an antisense (as) of miR-1271, or a scramble sequence (scr). These constructs contained also luciferase and GFP reporter sequences (connected with a 2A sequence), to allow in vivo tracing in mice and purification of transduced cells by flow cytometry, respectively. Briefly, human embryonic kidney 293 cell line (HEK293) was used. MiR-1271 sequence: 5'-CUUGGCACCUAGCAAGCACUCA-3', as-miR-1271 sequence: 5'-UGAGUCUUGCUAGGUGCCAAG-3', scr sequence: 5'-UAAAUGCCGAAAAGUCCGG-3'. We used a pAAV-CMV-LUC-2A-GFP plasmid (Clontech, Mountain View, CA, USA), a packaging plasmid carrying the serotype 8 rep and cap genes, and a helper plasmid carrying the adenovirus helper functions (Applied Viromics, LLC, Fremont, CA, USA) in this study. AAV was prepared by triple transfection of the newly prepared plasmids, R2C8 (containing AAV2 Rep and AAV8 capsid genes) and pAd5 (containing adenovirus helper genes) into HEK293 cells by Lipofectamine 2000 reagent (Invitrogen). The viruses were purified using CsCl density centrifugation and then titered by a quantitative densitometric dot-blot assay. Then, the A549 cells were incubated with AAV at a MOI of 100 for 12 hours.

#### *Luciferase-reporter activity assay*

Luciferase-reporters were successfully constructed using molecular cloning technology.

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**Figure 1.** High mTor and low miR-1271 are inversely correlated in NSCLC. We examined the mTor levels and miR-1271 levels in the resected NSCLC specimens from 31 patients, and compared to the paired normal lung tissue (NLT). (A, B) We detected significantly higher levels of mTor by Western blot (A), and significantly lower levels of miR-1271 by RT-qPCR (B), respectively, in the NSCLC samples, compared to NLT. (C) A strong inverse correlation was detected between mTor and miR-1271 in NSCLC specimen ( $R = -0.81$ ;  $p < 0.0001$ ). \*:  $p < 0.05$ .

Target sequence was inserted into pGL3-Basic vector (Promega, Madison, WI, USA) to obtain pGL3-mTor-3'UTR, which contains the miR-1271 binding sequence (mTor-3'UTR sequence). A549-miR-1271, or A549-scr, or A549-as-miR-1271 cells were seeded in 24-well plates for 24 hours, after which they were transfected with 1  $\mu$ g of Luciferase-reporter plasmids per well using PEI Transfection Reagent. Then luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega), according to the manufacturer's instructions.

### Imaging of the implanted tumor by bioluminescence

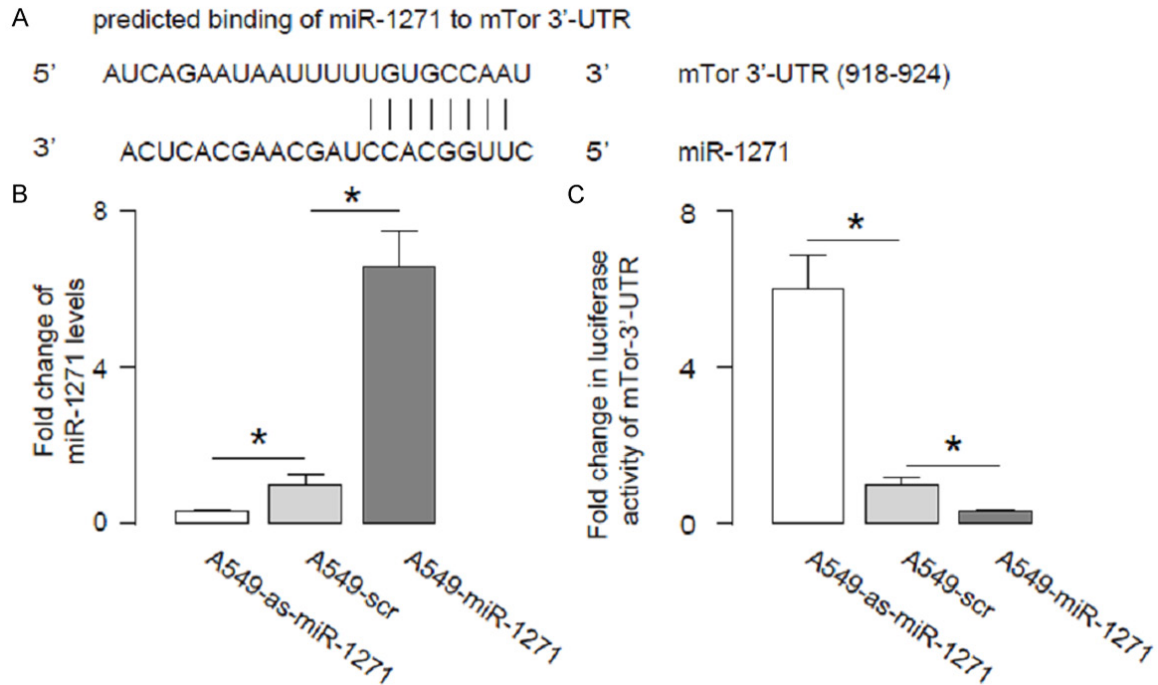
The tumor growth in the living animals was monitored and quantified by luminescence levels. Bioluminescence was measured with the

IVIS imaging system (Xenogen Corp., Alameda, CA, USA). All of the images were taken 10 minutes after intraperitoneal injection of luciferin (Sigma-Aldrich, St. Louis, MO, USA) of 150 mg/kg body weight, as a 60-second acquisition and 10 of binning. During image acquisition, mice were sedated continuously via inhalation of 3% isoflurane. Image analysis and bioluminescent quantification was performed using Living Image software (Xenogen Corp).

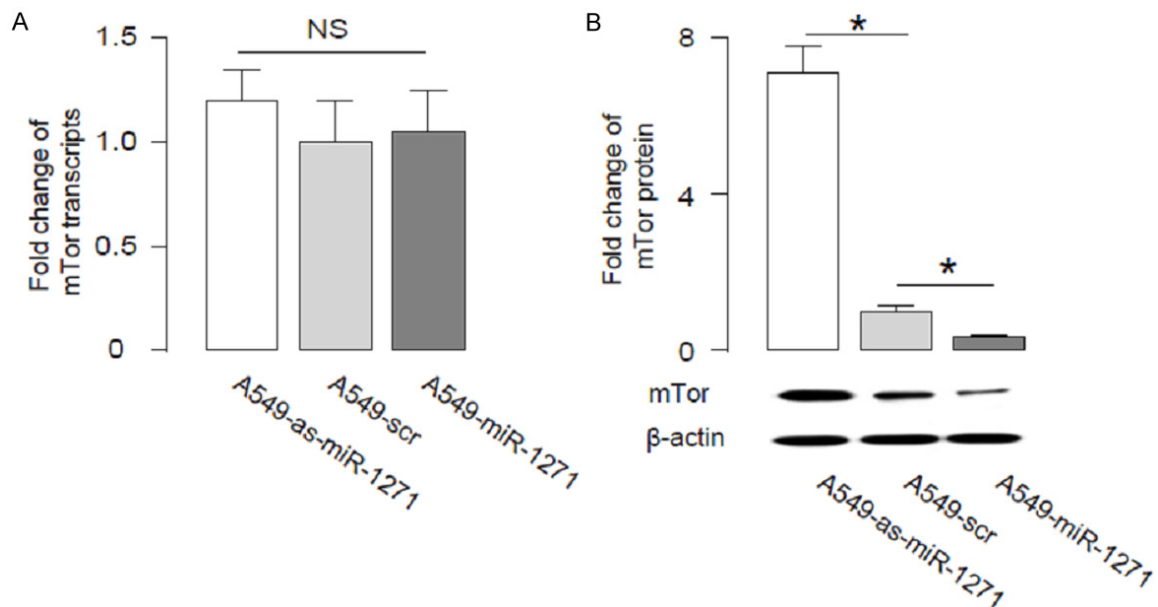
### Quantitative PCR (RT-qPCR)

MiRNA and total RNA were extracted from tissue specimen or from cultured cells with miR-Neasy mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. Quantitative PCR was performed in duplicates with QuantiTect SYBR

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**Figure 2.** MiR-1271 targets 3'UTR of mTor to inhibit its expression. A. Bioinformatics analyses of mTor target sequence show that the miR-1271 binds to 3'UTR of mTor mRNA. B, C. We overexpressed or inhibited miR-1271 levels by AAVs in a human NSCLC cell line, A549, to obtain A549-miR-1271 and A549-as-miR-1271 cells, respectively. The cells were also transfected with an AAV that carries a control scramble sequence (A549-scr). B. RT-qPCR on miR-1271. C. A549-miR-1271, A549-scr and A549-as-miR-1271 cells were transfected with 1  $\mu$ g of mTor-3'UTR luciferase-reporter plasmid. The luciferase activities in these cells were then evaluated. \*:  $p < 0.05$ .

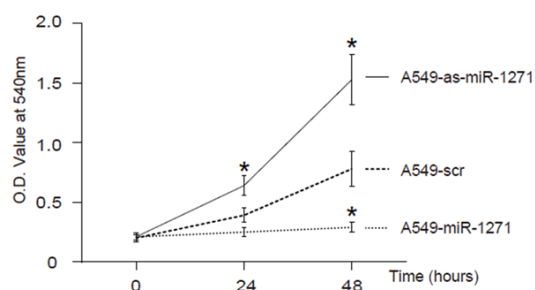


**Figure 3.** MiR-1271 decreases mTor levels in NSCLC cells. A. RT-qPCR on mTor. B. Western blot images and quantification for mTor. \*:  $p < 0.05$ . NS: Non-significant.

Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Values of genes were nor-

malized against  $\beta$ -actin, and then compared to controls.

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**Figure 4.** MiR-1271 inhibits NSCLC growth in vitro. In an MTT assay, the increases in cell number of A549-as-miR-1271 cells were significantly greater than the control A549-scr cells, while the luciferase activities in A549-miR-1271 cells were significantly lower than the control A549-scr cells. \*:  $p < 0.05$ .

### Western blot

The protein was extracted from the resected NSCLC specimen or adjacent normal lung tissue (NLT), or cultured cells. Primary antibodies were anti-mTor and anti- $\beta$ -actin (Cell Signaling, San Jose, CA, USA).  $\beta$ -actin was used as a protein loading control. Secondary antibodies were HRP-conjugated anti-rabbit, and were all purchased from Jackson ImmunoResearch Labs (West Grove, PA, USA). The protein levels were first normalized to  $\beta$ -actin, and then normalized to control, quantified by NIH ImageJ software (Bethesda, MA, USA).

### Statistical analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software package. All values are depicted as mean  $\pm$  standard deviation and are considered significant if  $p < 0.05$ . All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test to compare two groups. Bivariate correlations were calculated by Spearman's rank correlation coefficients.

## Results

### High levels of mTor and low levels of miR-1271 are inversely correlated in NSCLC

We examined the mTor levels by Western blot and miR-1271 levels by RT-qPCR in the resected NSCLC specimens from 31 patients, and compared to the paired normal lung tissue (NLT). We detected significantly higher levels of mTor (**Figure 1A**), and significantly lower levels

of miR-1271 (**Figure 1B**), respectively, in the NSCLC samples, compared to NLT. Moreover, a strong inverse correlation was detected between mTor and miR-1271 levels in NSCLC specimen (**Figure 1C**,  $R = -0.81$ ;  $p < 0.0001$ ), suggesting a causal relationship.

### MiR-1271 targets 3'UTR of mTor to inhibit its expression

Based on our findings in the patients' samples, we performed bioinformatics analysis of mTor target sequence, which show that miR-1271 binds to 3'UTR of mTor mRNA at 918<sup>th</sup>-924<sup>th</sup> base site (**Figure 2A**). To prove that the binding of miR-1271 to mTor mRNA may be functional, we modified the levels of miR-1271 in a human NSCLC cell line, A549. We transduced A549 cells with AAVs carrying either miR-1271, or antisense for miR-1271 (as-miR-1271), or a scramble sequence as a control (scr). These viral constructs also contained both luciferase and GFP reporters. Modulation of miR-1271 levels in A549 cells was confirmed by RT-qPCR (**Figure 2B**). Then, these A549-miR-1271, A549-scr and A549-as-miR-1271 cells were transfected with 1  $\mu$ g of mTor-3'UTR Luciferase-reporter plasmid. We found that the luciferase activities in A549-as-miR-1271 cells were significantly higher than the control, while the luciferase activities in A549-miR-1271 cells were significantly lower than the control (**Figure 2C**). These data suggest that miR-1271 targets 3'UTR of mTor to inhibit its translation.

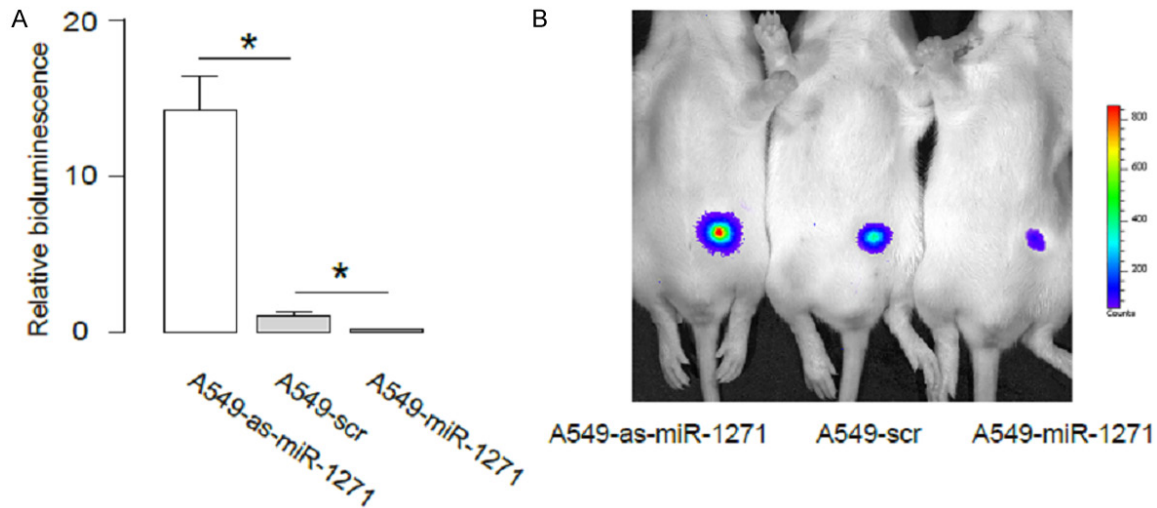
### MiR-1271 decreases mTor protein without altering its transcription

Since mTor is well-known to promote tumor growth in NSCLC, we were thus prompted to evaluate whether mTor may be regulated by miR-1271 in NSCLC cells. We found that although the mTor transcripts did not change with miR-1271 levels (**Figure 3A**), the protein levels of mTor in miR-1271-overexpressing A549 cells was significantly decreased, while the protein levels of mTor in miR-1271-depleted A549 cells was significantly increased (**Figure 3B**). These data suggest that the translation of mTor in NSCLC cells is regulated by miR-1271.

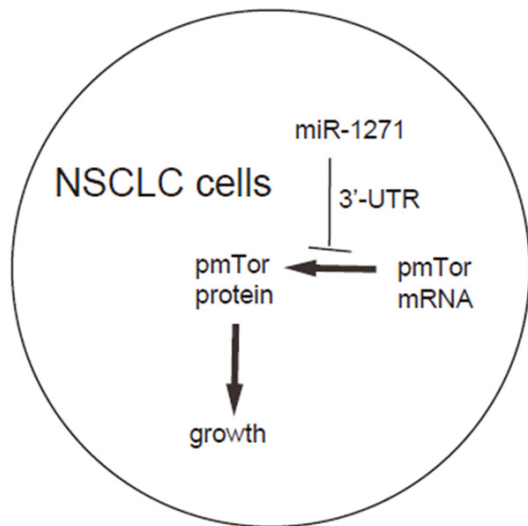
### MiR-1271 inhibits NSCLC growth in vitro

Then we examined whether the effects of miR-1271 on mTor may result in changes in cell

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**Figure 5.** MiR-1271 inhibits NSCLC growth in vivo. A, B. We used 30 female NOD/SCID mice that were randomly separated into 3 groups of 10 each. Each group of the mice received subcutaneous injection of  $10^6$  A549-miR-1271, or A549-scr, or A549-as-miR-1271 cells to form tumor. One month later, the tumor growth was monitored and quantified by bioluminescence levels. A. Quantification of bioluminescence. B. Representative images at 4 weeks after transplantation. \* $p < 0.05$ .



**Figure 6.** Schematic of the model. MiR-1271 inhibits growth of NSCLC through mTor suppression.

growth in NSCLC cells. In an MTT assay, the increases in cell number of A549-as-miR-1271 cells were significantly greater than the control A549-scr cells, while the increases in cell number of A549-miR-1271 cells were significantly lower than the control A549-scr cells (**Figure 4**). These data suggest that miR-1271 inhibits NSCLC growth in vitro.

### *MiR-1271 inhibits NSCLC cell growth in vivo*

Same number of miR-1271-modified A549 cells were then subcutaneously transplanted into

NOD/SCID mice. The presence of luciferase in these cells allowed quantification of tumor size in living animals. We used 30 female NOD/SCID mice that were randomly separated into 3 groups of 10 each. Each group of the mice received subcutaneous injection of  $10^6$  A549-miR-1271, or A549-scr, or A549-as-miR-1271 cells to form tumor. One month later, the tumor growth was monitored and quantified by luminescence levels. We detected significant greater tumor growth in AAV-as-miR-1271-injected mice, and significant lower tumor growth in AAV-miR-1271-injected mice, compared to controls, shown by quantification (**Figure 5A**), and by representative images (**Figure 5B**), suggesting that inhibition of miR-1271 suppresses NSCLC cell growth in vivo. Together, our study thus demonstrates that miR-1271 inhibits the growth of NSCLC through mTor suppression in NSCLC cells (**Figure 6**).

### Discussion

Previous studies have demonstrated an essential role of miRNAs in tumor growth, whereas a role of miR-1271 in NSCLC has not been determined. In this study, we aimed to understand the molecular mechanisms that underlie the regulation of the tumor growth of NSCLC by miR-1271. Then we used 5 human NSCLC cell lines (including A549, H157, H460, Calu-3 and SKLU-1) for this study. We obtained similar results from these NSCLC lines. Thus, only data from A549 cells were shown here.

## MiR-1271 inhibits growth of NSCLC

Of note, we detected strong inverse correlation between mTor and miR-1271 in NSCLC specimens, suggesting presence of a regulatory relationship. Since mTor plays a critical role in cell growth and has been well-known to express in NSCLC cells, we thus examined whether miR-1271 may inhibit the expression of mTor. We either overexpressed or inhibited miR-1271 expression in NSCLC cells, which decreased or increased the protein levels of mTor, respectively, without affecting mTor transcript levels. These data suggest that mTor translational controls in NSCLC cells may be regulated by miR-1271, which is consistent with the function of miRNAs. Moreover, using luciferase reporter assay, we identified a binding sites of miR-1271 at 3'UTR on mTor mRNA. Taken together, these findings strongly demonstrate a critical role of miR-1271 in regulating mTor translation, rather than in regulating the degradation of mTor mRNA.

In addition, we found that the modification of miR-1271 levels in NSCLC cells significantly altered cell growth in vitro and in vivo. These data are consistent with a well-known role of mTor in the cell-cycle activation. The best-characterized downstream effectors of mTor include two signaling pathways that act in parallel to control mRNA translation: the 70-kDa ribosomal protein S6 kinase 1 (p70S6K1 or S6K1) pathway and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1)/eIF4E pathway. mTOR-dependent signals, in cooperation with PI3K-dependent signals, mediate phosphorylation and activation of S6K1 and phosphorylation and inactivation of 4E-BP1, which is a repressor of translation initiation. S6K1 directly phosphorylates the 40S ribosomal protein S6, which is thought to increase the translation of mRNA species.

Together, our findings thus highlight miR-1271/mTor axis as a novel therapeutic target for inhibiting the growth of NSCLC. In future, experiments should be designed to determine the other possible targets of miR-1271 to completely understand its role in the carcinogenesis of NSCLC.

### Acknowledgements

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### Disclosure of conflict of interest

The authors have declared that no competing interests exist.

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