

Original Article

MicroRNA-365 inhibits growth, invasion and metastasis of lung cancer by targeting NRP1 expression

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Abstract: Objective: The role of miR-365 in cancer cells seemed controversial in previous studies. We thereby in this article aimed to define the role of miR-365 in lung cancer pathogenesis. Methods: We detected miR-365 expression in lung cancer cell lines and then investigated the effects of miR-365 on the metastasis and malignancy of lung cancer cells. The correlation between miR-365 level and NRP1 (neuropilin1) was further investigated in clinical lung adenocarcinoma specimens. Results: MiR-365 was strongly down-regulated in lung cancer (LC) tissues and cell lines, and its expression levels were associated with lymph node metastasis and clinical stage, as well as overall survival and relapse-free survival of LC. We also found that ectopic expression of miR-365 inhibited LC cell proliferation and LC metastasis *in vitro* and *in vivo*. We further identified a novel mechanism of miR-365 to suppress LC growth and metastasis. NRP1 was proved to be a direct target of miR-365, using luciferase assay and western blot. NRP1 over-expression in miR-365 expressing cells could rescue invasion and growth defects of miR-365. In addition, miR-365 expression inversely correlated with NRP1 protein levels in LC. Conclusion: Our data suggest that miR-365 functions as a tumor suppressor in LC development and progression, and holds promise as a prognostic biomarker and potential therapeutic target for LC.

Keywords: microRNA-365, NRP1, lung cancer (LC), biomarker, target therapy

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, and mortality rates continue to increase among older women with lung cancer in many countries [1]. Cancer develops as a result of an accumulation of various endogenous and exogenous causes. Gene alterations play an important role in lung cancer, and alterations in a large number of oncogenes and tumor suppressor genes have already been reported in lung cancer.

Neuropilins, including neuropilin1 (NRP1) and neuropilin2 (NRP2), are multifunctional non-tyrosine-kinase receptors that were first identified based on their critical roles in the developing nervous system [2]. NRP1 and NRP2 have 44% homology and share many structural and biological properties. NRP1 mainly exists in blood vessel endothelia, and NRP2 is mainly found in lymphatic vessels [2, 3]. Subsequent investigations identified NRP-1 as a receptor

for the vascular endothelial growth factor (VEGF)-A isoform VEGF-165 in both endothelial cells and some tumor cells [4, 5]. Research has shown that NRP1 is up-regulated in multiple tumor types and is expressed in different tumor vasculatures [3, 6], suggesting that NRP1 plays a critical role in tumor progression.

MicroRNAs (miRNAs) are small non-coding RNAs of 20-22 nucleotides. It represses gene expression through interaction with 3'untranslated regions (UTRs) of mRNAs. miRNAs are predicted to target over 50% of all human protein-coding genes, enabling them to have numerous regulatory roles in many physiological and developmental processes, including development, differentiation, apoptosis and proliferation, through imperfect pairing with target mRNAs of protein-coding genes and the transcriptional or post-transcriptional regulation of their expression [7, 8]. Thus, miRNAs may function as tumor suppressors or oncogenes, and dysregulated miRNA expression might contrib-

MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

ute to tumor cell metastasis. It remains unclear whether miRNAs can regulate the expression of NRP1 in lung cancer; therefore, we aimed to determine the target miRNAs of NRP1. Additional functions of NRP1 may be discovered by studying the target miRNAs of NRP1 and provide basis for lung cancer targeted therapy.

Material and methods

Ethics statement

Investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board.

Plasmids and plasmid construction

The expression construct pCMV-NRP1 contains the cDNA encoding the full-length human NRP1 [3, 6]. For the knockdown of endogenous NRP1, the target sequence was constructed in siRNA vector pLKO.1 as described [7]. The RNAi vector against luciferase, pLKO.1-shLuc, was used as a negative control for knockdown validation. As described previously, the DNA segments of full-length (nucleotide 1-867 from the start of 3'-UTR) and truncated (nucleotide 449 to 867) NRP1 3'-UTRs were amplified by PCR to construct reporter plasmids NRP1-3'-UTR-Luc (FL) and p NRP1-3'-UTR-Luc (449-867), respectively. For the construction of adenoviral plasmids expressing miR-365, its precursor sequences were amplified by PCR from the genomic DNA of A549 cells. The precursor DNA fragments were cloned into shuttle vector pAdTrack-CMV (Stratagene) with a GFP tracer as described.

Cell culture and transfection

Human lung adenocarcinoma A549, H1299, SK-MES-1, and BE1 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. Cells were transiently transfected by electroporation or LipofectamineTM 2000 (Invitrogen) transfection reagent. For luciferase reporter gene assay, A549 cells (5×10^5) were seeded onto 6-well plates and then transiently transfected. Twenty-four hours after transfection, the transfected cells were infected with adenoviruses expressing miRNAs or GFP. Twenty-four hours after infection, luciferase activity was measured and then normalized. Antagomir-365

and scrambled control oligonucleotides (Ambion) were transfected into cells using the LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions.

MicroRNA real-time PCR

1×10^2 - 1×10^7 cells were harvested, washed in PBS once, and stored on ice; complete cell lysate was prepared by addition of 600 μ l lysis binding buffer and vortex; 60 μ l microRNA aomogenete addictive was added to the cell lysate and mixed thoroughly by inverting several times; sample was stored on ice for 10 min, followed by addition of equal volume (600 μ l) of phenol: chloroform (1:1) solution; sample was mixed by inverting for 30-60 sec, and then centrifuged at 12000 g for 5 min; the supernatant was transferred to a new tube and the volume was estimated; 1/3 volume of 100% ethanol was added and mixed; the mixture was loaded to the column at room temperature and centrifuged at 10000 g for 15 sec; the flow-through was collected and the volume was then estimated; 2/3 volume of 00% ethanol was added and mixed; the mixture was loaded to column at room temperature and centrifuged at 10000 g for 15 sec; the flow-through was discarded; 700 μ l microRNA wash solution was added to the column, followed by centrifugation at 10000 for 10 sec; the flow-through was discarded; 500 μ l microRNA wash solution was added to the column, followed by centrifugation at 10000 for 10 sec; the flow-through was discarded; the column was transferred to a new tube and 100 μ l preheated elution solution(95 degree) was added at room temperature; RNA was collected by centrifugation at 12000 g for 30 sec.

Western blot

Cells were harvested, washed twice in PBS, and lysed in lysis buffer (protease inhibitors were added immediately before use) for 30 min on ice. Lysate was centrifuged at 10000 rpm and the supernatants were collected and stored at -70 in aliquots. All procedures were carried out on ice. Protein concentration was determined using BCA assay kit (Tianlai Biotech).

Cell viability assay

Cells were seeded into 96-well plates (2×10^3 cells/well) directly or 24 hours after transfec-

MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

tion and allowed to attach overnight. Freshly prepared cisplatin was then added with different final concentrations. Forty-eight hours later, cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as described previously [8].

Transwell invasion assay

Transwell filters were coated with matrigel (3.9 mg/ μ L, 60-80 μ L) on the upper surface of a polycarbonic membrane (diameter 6.5 mm, pore size 8 mm). After incubating at 37°C for 30 minutes, the matrigel solidified and served as the extracellular matrix for analysis of tumor cell invasion. Harvested cells (1×10^5) in 100 μ L of serum free DMEM were added into the upper compartment of the chamber. A total of 200 μ L conditioned medium derived from NIH3T3 cells was used as a source of chemo-attractant, and was placed in the bottom compartment of the chamber. After 24 h incubation at 37°C with 5% CO₂, the medium was removed from the upper chamber. The non-invaded cells on the upper side of the chamber were scraped off with a cotton swab. The cells that had migrated from the matrigel into the pores of the inserted filter were fixed with 100% methanol, stained with hematoxylin, and mounted and dried at 80°C for 30 minutes. The number of cells invading through the matrigel was counted in three randomly selected visual fields from the central and peripheral portion of the filter using an inverted microscope (200 \times magnification). Each assay was repeated three times.

Plasmid construction and luciferase reporter assay

Wild-type 3'untranslated region (3'UTR) of PEBP4 containing predicted miR-365 target sites were amplified by PCR from A549 cell genomic DNA. Primers used: Forward: GAT CTG CAG GGG TTA GCT TGG GGA CCT GAA C; Reverse: GAT CAT ATG AGA GTG ACA TAC TGA TGC CTA C. Mutant 3'UTRs were generated by overlap-extension PCR method. Both wild-type and mutant 3'UTR fragments were subcloned into the pGL3-control vector (Promega, Madison, WI) immediately downstream of the stop codon of the luciferase gene. DNA fragment coding PEBP4 protein was amplified by PCR from A549 cell cDNA, and cloned into pCMV-Myc expression vector (Clontech, Mountain View, CA). Primers used: Forward:

GCT GAA TTC ATG CCG GTG GAC CTC AGC AAG T; Reverse: CTG CTC GAG CTA CTT CCC AGA CAG CTG CTC G. For luciferase assay, the reporter plasmid was cotransfected with a control Renilla luciferase vector into A549 cells in the presence of either miR-15b or NC. After 48 h, cells were harvested, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Animal studies

Five-week-old female BALB/c nude mice were purchased from the Animal Center of Zhejiang University (Hangzhou, China). For *in vivo* chemosensitivity and metastasis assays, A549 cells (infected with either the miR-15b-overexpressing lenti-virus or the mock lentivirus) and A549/CDDP cells (infected with either the miR-15b-knockdown lenti-virus-mediated antagomir or the antagomir-NC) were subcutaneously inoculated into nude mice (six per group, 1×10^6 cells for each mouse). Tumor growth was examined every other day, and tumor volumes were calculated using the equation $V = A \times B^2/2$ (mm³), where A is the largest diameter and B is the perpendicular diameter. When the average tumor size reached ≈ 50 mm³, cisplatin was administered via intraperitoneal injection at a dose of 5 mg/kg, 1 dose every other day, with 3 doses in total. After 2 weeks, all mice were sacrificed. Transplanted tumors were excised, and tumor tissues were used to perform hematoxylin & eosin (H&E) staining. All research involving animal complied with protocols approved by the Zhejiang medical experimental animal care commission.

Data analysis

Image data were processed using SpotData Pro software (Capitalbio). Differentially expressed genes were identified using SAM package (Significance Analysis of Microarrays, version 2.1).

Results

MiR-26a is down-regulated in human lung cancer

Using a qRT-PCR method, miR-365 levels were detected in 40 pairs of lung cancer tissues and their matched adjacent tissues, as well as lung

MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

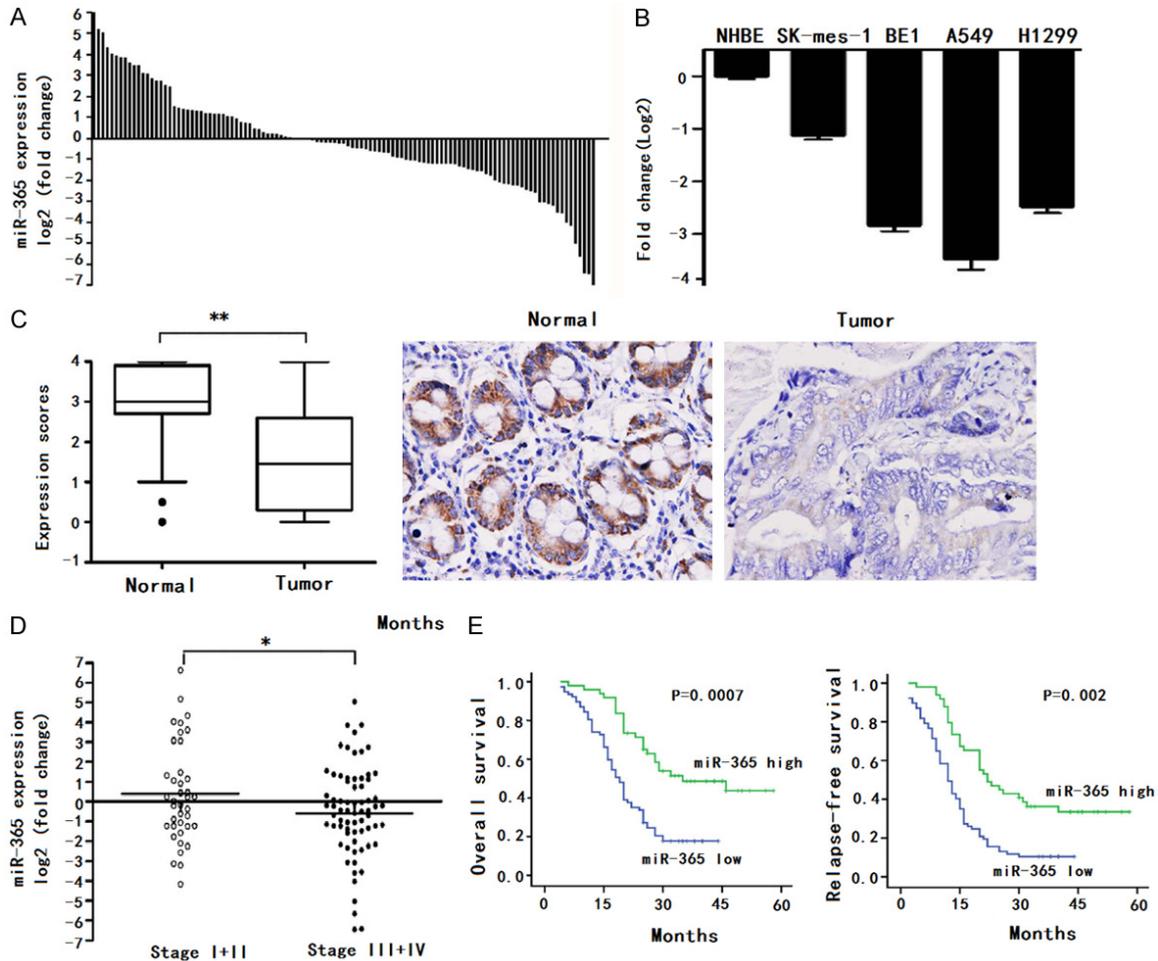
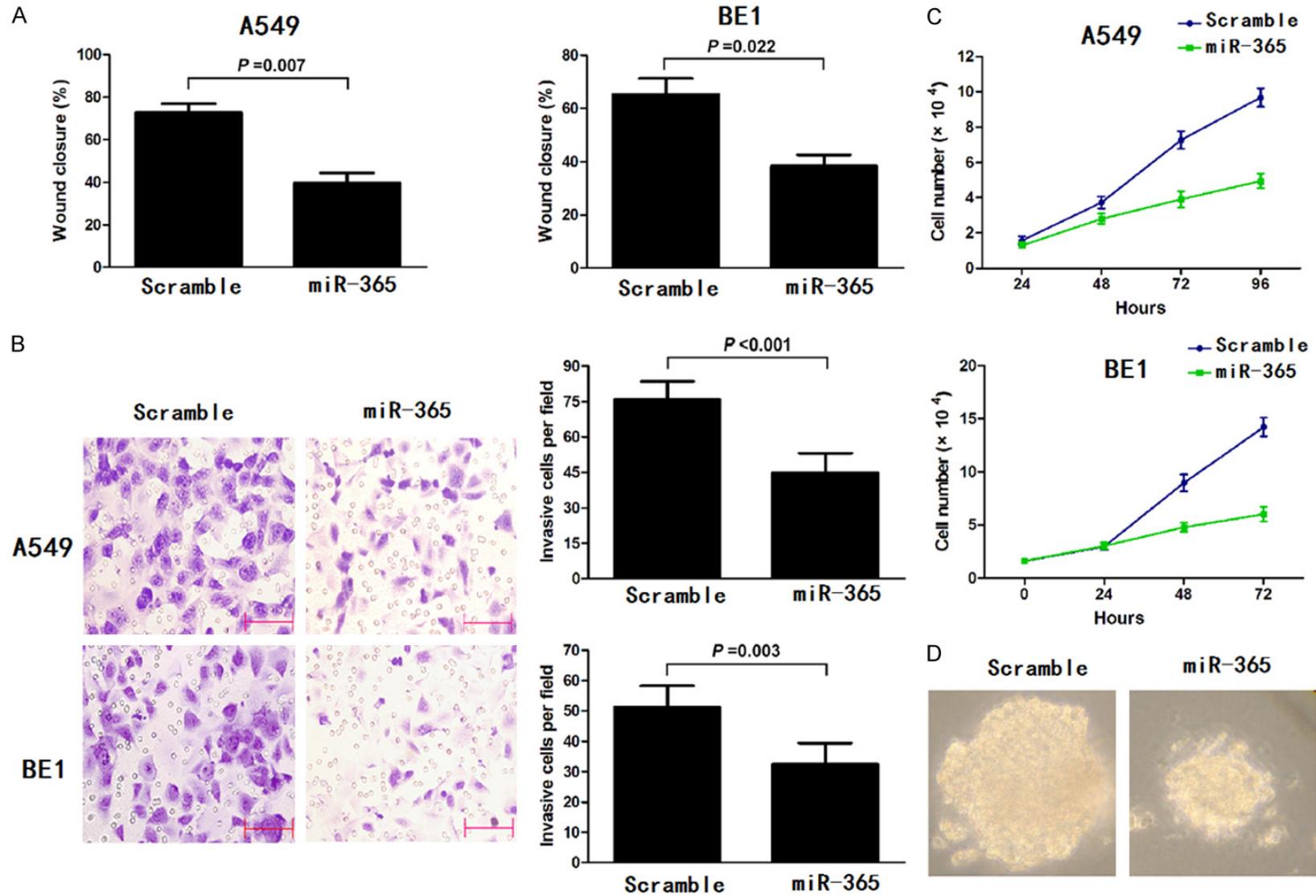


Figure 1. miR-365 is frequently downregulated in human lung cancer (LC) and correlates with lung cancer prognosis. A. miR-365 was detected in 40 lung cancer patients by qRT-PCR. Data are shown as log₂ of the fold change in lung cancer tissues (tumor) relative to adjacent normal tissues (normal). B. Relative expression of miR-365 in 4 cell lines derived from lung cancer and one nonmalignant lung cell line (NHBE: normal human bronchial epithelial cells) was determined by qRT-PCR. Data are shown as log₂ of the fold change in LC cell lines relative to NHBE. C. miR-365 expression was analyzed in adjacent normal tissues (Normal) and LCs (Tumor) samples on the tissue microarrays by in situ hybridisation. Expression scores are shown as box plots. Representative images of miR-365 expression by in situ hybridization are shown. Original magnification: ×200. D. Expression levels (as log₂ of fold change of LC tissues relative to non-tumor adjacent tissues) of miR-365 in I-II stages (n=39) versus III-IV stages (n=71) of the cancer patients. E. Survival analysis of LC. OS and RFS curves for 126 LC patients with high or low miR-365 expression were constructed using the Kaplan-Meier method and evaluated using the log-rank test.

cell lines. Among the 40 patients with lung cancer, approximately 70% (28 of 40 patients) of tumors revealed a more than two-fold reduction in miR-365 levels, with a 5.76-fold reduction relative to adjacent normal tissues, suggesting that reduction of miR-365 was a frequent event in human LC (**Figure 1A**). Moreover, miR-365 expression was reduced in all lung cancer cell lines (A549, H1299, Sk-mes-1 and BE1) compared with the nonmalignant lung cell line NHBE (**Figure 1B**). To further verify the results concerning the biological role of miR-

365 in human lung carcinogenesis, we employed in situ hybridisation to evaluate miR-365 expression in 126 LCs and 41 non-tumor tissues on tissue microarrays (TMAs). The results revealed that the expression scores of miR-365 were significantly decreased in LCs compared with normal tissues (**Figure 1C**). Moreover, we found low expression of miR-365 in 37% and 77% of lung tumors classified as stage I/II and stage III/IV, respectively. Therefore, miR-365 expression inversely correlates with lymph node metastasis and clinical

MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression



MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

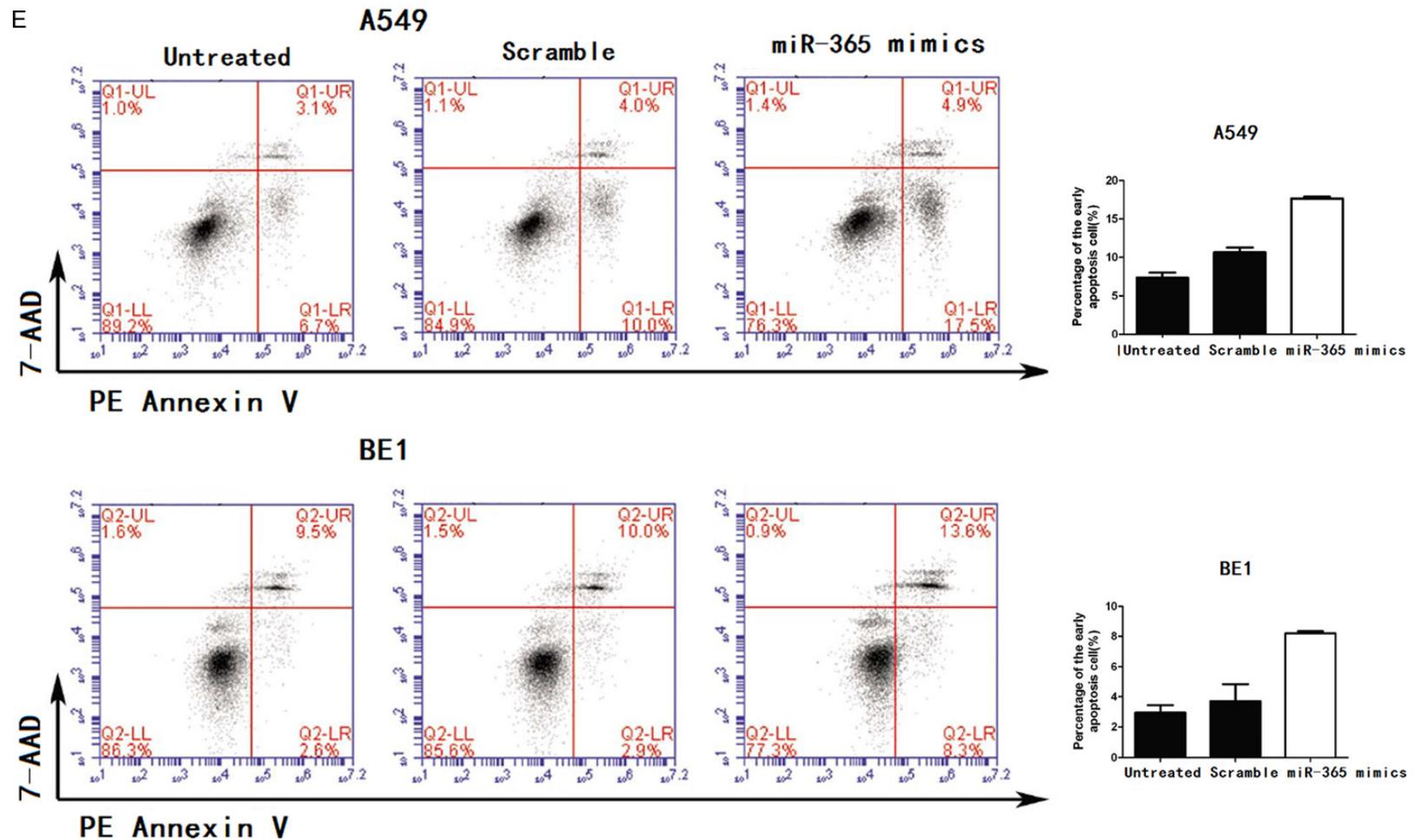


Figure 2. miR-365 inhibits cell migration, invasion, growth and soft agar colony formation, and induces cell apoptosis in LC. (A, B) The wound healing assay (A) and invasion assay (B) of A549 and BE1 cells infected with miR-365 or scramble lentivirus. The invasion assay was measured by way of Transwell assays with Matrigel. (C) The growth of A549 and BE1 cells infected with miR-365 or scramble lentivirus was assayed. (D) Colony growth assays in soft agar were performed on A549 with overexpression of miR-365 or scramble. Representative images of the assays are shown (left panel). Original magnification: $\times 200$. (E) A549 and BE1 cells were stained with PE Annexin V and 7-AAD 72 h after treatment with miR-365 mimics or scramble. Early apoptotic cells are shown in the right quadrant. *, $P < 0.05$; **, $P < 0.001$.

MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

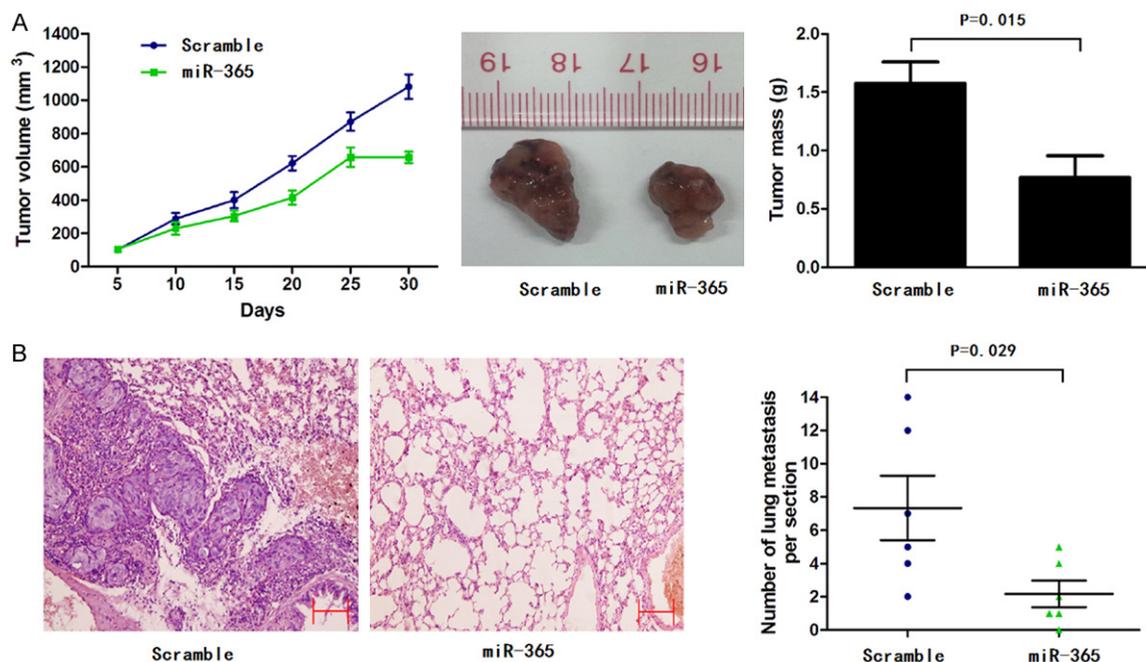


Figure 3. miR-365 inhibits LC cell growth and metastasis *in vivo*. A. Tumor growth in mouse xenograft models. A549 cells infected with miR-365 or scramble lentivirus were injected subcutaneously into nude mice. Tumor size was measured every 5 days. After 30 days, the mice were killed, necropsies were performed, and tumors were weighed. B. Tumor metastasis in mouse xenograft models. A549 cells with overexpression of miR-365 or scramble were injected into the tail vein of nude mice. After 45 days, the mice were killed. Micro-metastases in liver per HE-stained section in individual mice were calculated. Each group had six mice. Original magnification, $\times 100$; scale bar: 50 mm. All data are shown as mean \pm s.e.m.

stage ($P=0.019$ and $P<0.001$, respectively). However, miR-365 did not correlate with age, gender, cell differentiation, or invasion depth (T stage). These results suggest that the miR-365 might play a critical role in the LC metastasis and progression (Figure 1D). To further analyze the significance of miR-365 in terms of clinical prognosis, Kaplan-Meier survival analysis was performed using patient overall survival and relapse-free survival. The results demonstrated that patients with low miR-365 expression had shorter median OS and RFS than did patients with high miR-365 expression (21.6 months vs. 39.0 months, $P<0.001$ for OS; 15.2 months vs. 31.6 months, $P=0.002$ for RFS; Figure 1E).

MiR-365 suppresses GC growth and metastasis

Noting the inverse correlation between miR-365 levels and metastasis, we investigated the effect of miR-365 re-expression on the migration and invasion abilities of LC cell lines. Two LC cell lines (SLC-7901 and AGS) with relatively low basal expression of miR-365 (Figure 1B)

were infected with either miR-365 or control lentivirus and selected with 5 mg/l puromycin for two weeks. Next, wound healing assay and transwell assay were performed. As expected, overexpression of miR-365 significantly suppressed cell migration and invasion abilities (Figure 2A, 2B). To demonstrate the effect of miR-365 on LC growth, we performed LC cell proliferation assay. The proliferation assay showed that ectopic expression of miR-365 in A549 and BE1 attenuated cell proliferation compared with control cells (Figure 2C). Moreover, ectopic miR-365 expression inhibited colony formation ability in soft agar (Figure 2D). To further address whether up-regulation of miR-365 would induce LC cell apoptosis and cell death, the number of early apoptotic A549 and BE1 cells following treatment with miR-365 mimics was examined. As expected, few early apoptotic cells (10% in A549 or 2.9% in BE1) were detected in the scramble-treated cells, whereas miR-365 mimics treatment increased the percentage of early apoptotic cells (17.5% in A549 or 8.3 in BE1) as judged by Annexin V staining (Figure 2E). Therefore, we concluded that miR-365 could affect cell survival in LC cells.

MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

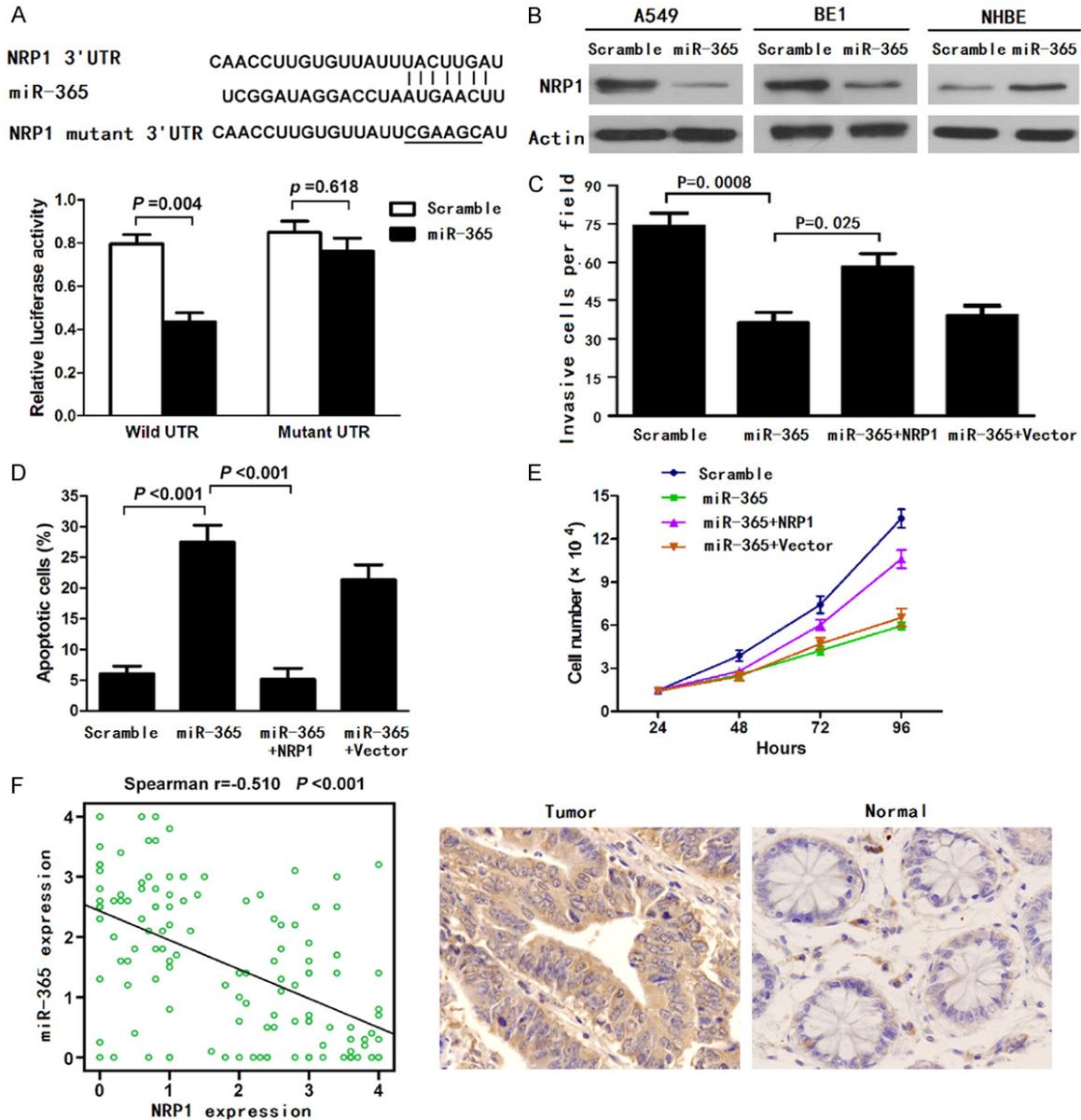


Figure 4. miR-365 directly targets NRP1. (A) The 3'-UTR element of NRP1 messenger RNA is partially complementary to miR-365. miR-365 or scramble control and luciferase reporter containing either a wildtype or a mutant 3'-UTR were co-transfected into HEK-293T cells. And a Renilla luciferase expressing construct exerts as internal control. (B) Western blot analysis of NRP1 expression in A549 and BE1 cells infected with miR-365, and NHBE transfected with miR-365 inhibitors (Anti-miR-365). (C-E) NRP1 abrogates the suppressive roles of miR-365 in LC cell invasion and growth. A549 cells stably expressing miR-365 or scramble were transfected with or without NRP1 plasmids. Invasion assays (C), Apoptosis analysis (D), and Cell proliferation analysis (E) were performed with the above cells as described in Materials and Methods. Data are presented as mean \pm s.e.m from at least three independent experiments. (F) Spearman's correlation scatter plot of the levels of miR-365 (determined by in situ hybridization) and NRP1 protein (determined by immunohistochemistry) in 126 LC specimens. Representative images of NRP1 expression by immunohistochemistry are shown (right panel). Original magnification: $\times 200$.

miR-365 inhibits LC cell growth and metastasis in vivo

Next, we tested whether miR-365 could play a role in tumorigenesis by using nude mouse xenograft models. We found that overexpres-

sion of miR-365 in A549 cells significantly suppressed tumor growth in nude mice (Figure 3A). Then, A549 cells infected with either miR-365 or control lentivirus were injected into the tail vein of nude mice to examine lung metastasis. As shown Figure 3B, a significantly lower

MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

number of macroscopic lung metastases could be observed in miR-365 overexpressing cells than control cells. These results indicate that miR-365 may repress LC growth and metastasis.

miR-365 directly targets and inhibits NRP1

To understand how miR-365 suppresses LC growth and metastasis, we used three algorithms (Targetscan, Pictar and Miranda) to help identify miR-365 targets in human lung cancers. Of these target genes that were predicted by all three algorithms, NRP1 attracted our attention immediately as it has been implicated in tumorigenesis or metastasis [9]. We cloned the full-length NRP1 3'-UTR into a luciferase reporter vector. Luciferase assay revealed that miR-365 directly bound to NRP1 3'-UTR, and by which it remarkably reduced luciferase activities (**Figure 4A**). However, mutation of the putative miR-365 sites in the 3'-UTR of NRP1 abrogated luciferase responsiveness to miR-365 (**Figure 4A**). To directly assess the effect of miR-365 on NRP1 expression, we performed western blot analysis. As seen in **Figure 4B**, lentiviral induced ectopic miR-365 dramatically suppressed the NRP1 protein levels in A549 and BE1 cells. Furthermore, knockdown of miR-365, through transfection of anti-miR-365, in NHBE cells increased NRP1 protein levels (**Figure 4B**). Taken together, these results indicate that NRP1 is a direct downstream target for miR-365 in LC cells. The above results prompted us to examine whether miR-365 suppresses LC growth and metastasis through repressing NRP1 expression. For this purpose, NRP1 was re-expressed in miR-365 transfected A549 cells. In miR-365-expressing cells, re-expression of NRP1 rescued the invasion and growth defects of miR-365 (**Figure 4C-4E**). Finally, we tested if miR-365 expression correlated with NRP1 protein levels in LC. There was an inverse correlation between the NRP1 protein levels, indicated by immunohistochemistry staining, and miR-365 expression assessed by in situ hybridization in 126 LC tissues on TMAs as used above (**Figure 4F**). Our findings demonstrate that miR-365 has properties consistent with tumor suppressor function. The ability to modulate NRP1 levels might explain, at least in part, why miR-365 can inhibit LC growth and metastasis.

Discussion

In recent years, accumulated evidence has led oncologists to speculate that unrevealed

molecular factors, particularly noncoding RNAs previously classified as "junk", play important roles in tumorigenesis and tumor progression. Depending on their mRNA targets, miRNAs can function as tumor suppressors or promoters of oncogenesis. However, the mechanisms that dysregulated miRNAs have not been widely studied, including aberrant miRNA biogenesis and transcription [10, 11], epigenetic alteration [12, 13], and amplification or loss of genomic regions that encode miRNAs [14].

Using gene chips and bioinformatics, we predicted that the target miRNA of NRP1 was miR-365. Research has shown that the expression of miR-365 varies in different tumors. Some reports showed that miR-365-3p suppressed liver cancer cell invasion by targeting smoothened [15]. Moreover, in melanomas, miR-193a, miR-365, and miR-565 were shown to be under expressed in patients with a BRAF mutation [16]. From these data, we inferred that miR-365 might act as a tumor suppressor. There is no published literature regarding whether miR-365 is under expressed in lung cancer. We thereby in this study aimed to investigate the role of miR-365 in the pathogenesis of lung cancer.

In our study, the expression levels of miR-365 were first measured in human lung cancer samples and samples of adjacent normal mucosa tissues. We then evaluated the expression of miR-365 in 4 lung cancer cell lines and in normal lung mucosa cell lines. We found that the expression of miR-365 was down-regulated in both the tumor tissues and the cancer cell lines. Moreover, the over-expressed miR-365 could inhibit lung cancer cell migration, invasion and proliferation, and promote apoptosis. Meanwhile, those tumourigenic qualities can be completely restored by NRP1 over-expression.

In addition, the luciferase reporter assays suggested that miR-365 targets NRP1 directly. Thus, we conclude that miR-365 acts as a potential tumor suppressor in lung cancer, a function that is accomplished by curbing the expression of NRP1. The phenotypic transition from an epithelial to a mesenchymal like cell state represents an important mechanism of epithelial plasticity and cancer metastasis. MicroRNAs have recently emerged as potent regulators of EMT due to their ability to target multiple components involved in epithelial

integrity or mesenchymal traits [17, 18]. The miR-200 family has been shown to directly target EMT transcription factor families [19]. In human mammary epithelial cells, miR-9 directly targets E-cadherin, thus promoting the mesenchymal phenotype, including increased cell migration and invasion [20-23]. MiR-27 promotes human lung cancer cell metastasis by inducing the epithelial-to-mesenchymal transition [23, 24]. NRP1 enhances signaling in three major pathways that have been linked to EMT, i.e., TGF- β , Hh and HGF/cMet [25-30]. In the previous study, over-expressed NRP1 promoted EMT and si-NRP1 restrained EMT was found in lung cancer, whereas the forced expression of miR-365 inhibited EMT. miR-365 may therefore regulate lung cancer cell EMT via NRP1.

In vivo, we found that tumor growth was significantly inhibited by the forced expression of miR-365 but was restored by NRP1 over-expression. Thus, we can infer that the reduction in tumor growth may have been due to the decrease in NRP1, which was curbed by miR-365. Reports indicated that silenced NRP1 on oral cancer cells can regulate xenografted tumor angiogenesis [31, 32]. Meanwhile, some experiments found that reduced NRP-2 expression on pancreatic tumor cells can attenuate the tumor D-MVA *in vivo* [33, 34]. These findings indicate that reduced NRP expression in tumor cells can inhibit xenografted tumor angiogenesis. In our study, NRP1 expression was reduced by miR-365 in tumor cells and reduced NRP1 expression attenuate the xenografted tumor DMVA. Moreover, the reduced tumor D-MVA could be restored in the tumors that over-expressed NRP1. These data suggest that miR-365 can attenuate tumor D-MVA via NRP1. But why reduced NRP1 in lung cancer cells can curb the D-MVA of xenografted tumor? We guess that the effects on development of the tumor vasculature may be due to altered angiogenic mediator expression in the tumor cells themselves, and we will detect protein levels of several known angiogenic mediators such as VEGF-A, VEGF-C and so on in our next study. In general, NRP1 may mediate the effects of miR-365 on tumor progression indirectly by affecting angiogenesis, or directly, through its effects on tumor cells.

In conclusion, our study is the first to document the tumor suppressor role of miR-365 in lung cancer. MiR-365 can decrease migratory, inva-

sive and proliferative behaviors by attenuating the expression of NRP1. Our findings indicate that the restoration of the tumor suppressor miR-365 might be useful in the treatment of lung cancer.

Disclosure of conflict of interest

None.

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MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

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MiR-365 inhibits growth, invasion and metastasis of LC by targeting NRP1 expression

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