

Original Article

MicroRNA-365 inhibits growth, invasion and metastasis of malignant melanoma 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 malignant melanoma (MM) pathogenesis. Methods: We detected miR-365 expression in malignant melanoma cell lines and then investigated the effects of miR-365 on the metastasis and malignancy of melanoma cells. The correlation between miR-365 level and NRP1 (neuropilin1) was further investigated in clinical malignant melanoma specimens. Results: MiR-365 was strongly down-regulated in malignant melanoma (MM) 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 MM. We also found that ectopic expression of miR-365 inhibited MM cell proliferation and MM metastasis *in vitro* and *in vivo*. We further identified a novel mechanism of miR-365 to suppress MM 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 MM. Conclusion: Our data suggest that miR-365 functions as a tumor suppressor in MM development and progression, and holds promise as a prognostic biomarker and potential therapeutic target for MM.

Keywords: Malignant melanoma (MM), neuropilin1 (NRP1), miR-365, invasion and metastasis, pathogenesis, target therapy, cancer biomarker

Introduction

The incidence of malignant melanoma (MM) has continued to increase in the past 30 years despite aggressive education initiatives. Although most melanoma cases consist of localized disease that is curable with surgical resection, for those with regional or distant metastases, the prognosis is poor with a 10-year survival rate of only 64% and 16%, respectively [1]. Malignant melanoma develops as a result of an accumulation of various endogenous and exogenous causes. Gene alterations play an important role in malignant melanoma, and alterations in a large number of oncogenes and tumor suppressor genes have already been reported in malignant melanoma.

Neuropilins, including neuropilin1 (NRP1) and neuropilin2 (NRP2), are multifunctional nontyrosine-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 contribute to tumor cell metastasis. It remains unclear whether mi-RNAs can regulate the expression of NRP1 in malignant melanoma; 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 malignant melanoma 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 A375 cells. The precursor DNA fragments were cloned into shuttle vector pAdTrack-CMV (Stratagene) with a GFP tracer as described.

Cell culture and transfection

Human malignant melanoma A375, G361, L1-BR, and HME1 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. Cells were transiently transfected by electroporation or Lipofectamine™ 2000 (Invitrogen)

transfection reagent. For luciferase reporter gene assay, A375 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 Lipofectamine™ 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 µl lysis binding buffer and vortex; 60 µl microRNA aomogenete additive 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 µl) 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 µl microRNA wash solution was added to the column, followed by centrifugation at 10000 for 10 sec; the flow-through was discarded; 500 µl 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 µl 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 (2×10³ cells/well) directly or 24 hours after transfection 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/μL, 60-80 μL) 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 (1×10⁵) in 100 μL of serum free DMEM were added into the upper compartment of the chamber. A total of 200 μL 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× magnification). Each assay was repeated three times.

Plasmid construction and luciferase reporter assay

Wild-type 3' untranslated region (3'UTR) of PE-BP4 containing predicted miR-365 target sites were amplified by PCR from A375 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 PE-BP4 protein was amplified by PCR from A375 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 co-transfected with a control Renilla luciferase vector into A375 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 Zhengzhou University (Hangzhou, China). A375 cells were subcutaneously inoculated into nude mice (six per group, 1×10⁶ 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. 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 Zhengzhou 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-365 is down-regulated in human malignant melanoma

Using a qRT-PCR method, miR-365 levels were detected in 40 pairs of malignant melanoma tissues and their matched adjacent tissues, as well as malignant melanoma cell lines. Among

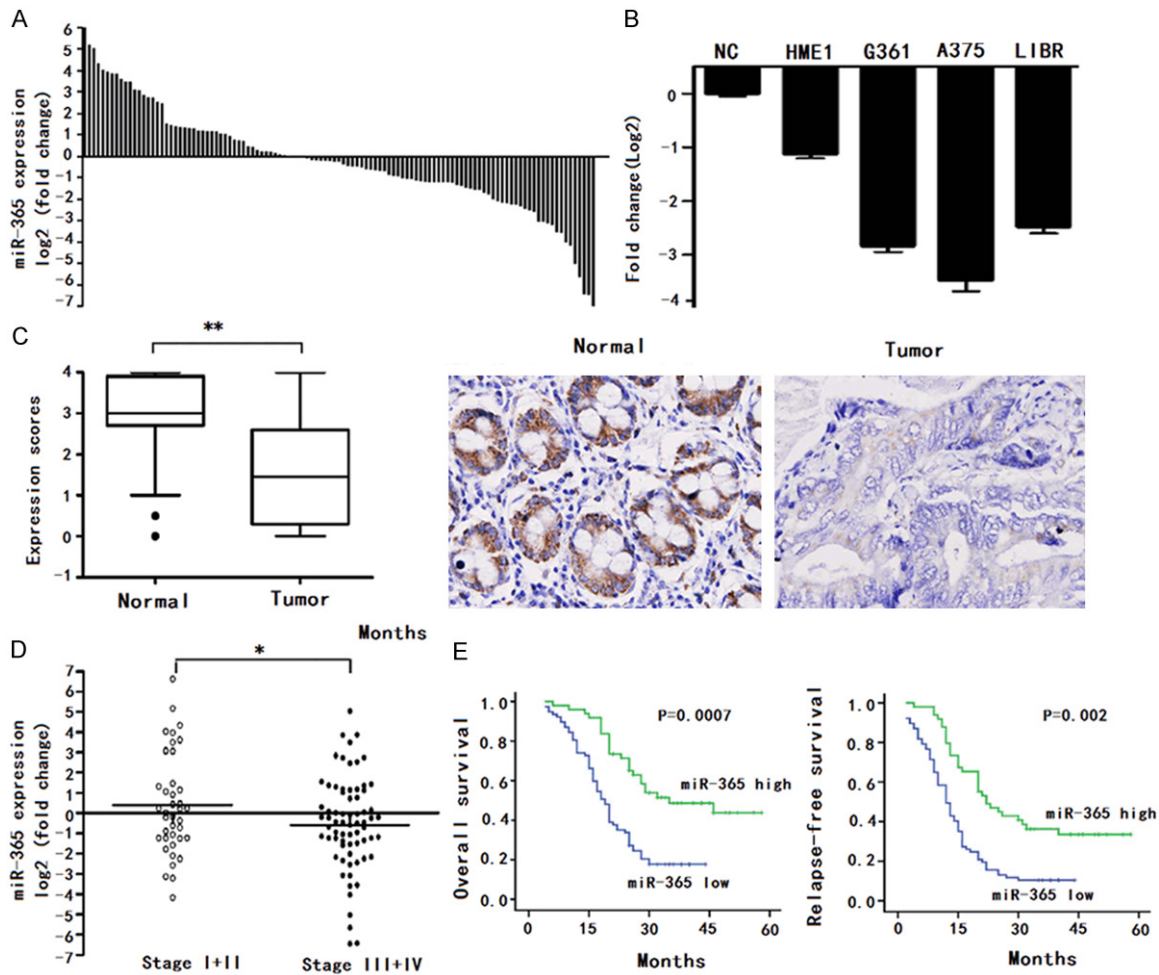


Figure 1. miR-365 is frequently downregulated in human malignant melanoma (MM) and correlates with malignant melanoma prognosis. **A.** miR-365 was detected in 40 malignant melanoma patients by qRT-PCR. Data are shown as log2 of the fold change in malignant melanoma tissues (tumor) relative to adjacent normal tissues (normal). **B.** Relative expression of miR-365 in 4 cell lines derived from malignant melanoma and one nonmalignant control cell line was determined by qRT-PCR. Data are shown as log2 of the fold change in MM cell lines relative to NC. **C.** miR-365 expression was analyzed in adjacent normal tissues (Normal) and MMs (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: $\times 200$. **D.** Expression levels (as log 2 of fold change of MM 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 MM. OS and RFS curves for 126 MM patients with high or low miR-365 expression were constructed using the Kaplan-Meier method and evaluated using the log-rank test.

the 40 patients with malignant melanoma, 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 MM (**Figure 1A**). Moreover, miR-365 expression was reduced in all malignant melanoma cell lines (A375, HME1, LIBR and G361) compared with the nonmalignant normal control (**Figure 1B**). To further verify the results concerning the biological role of miR-365 in

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

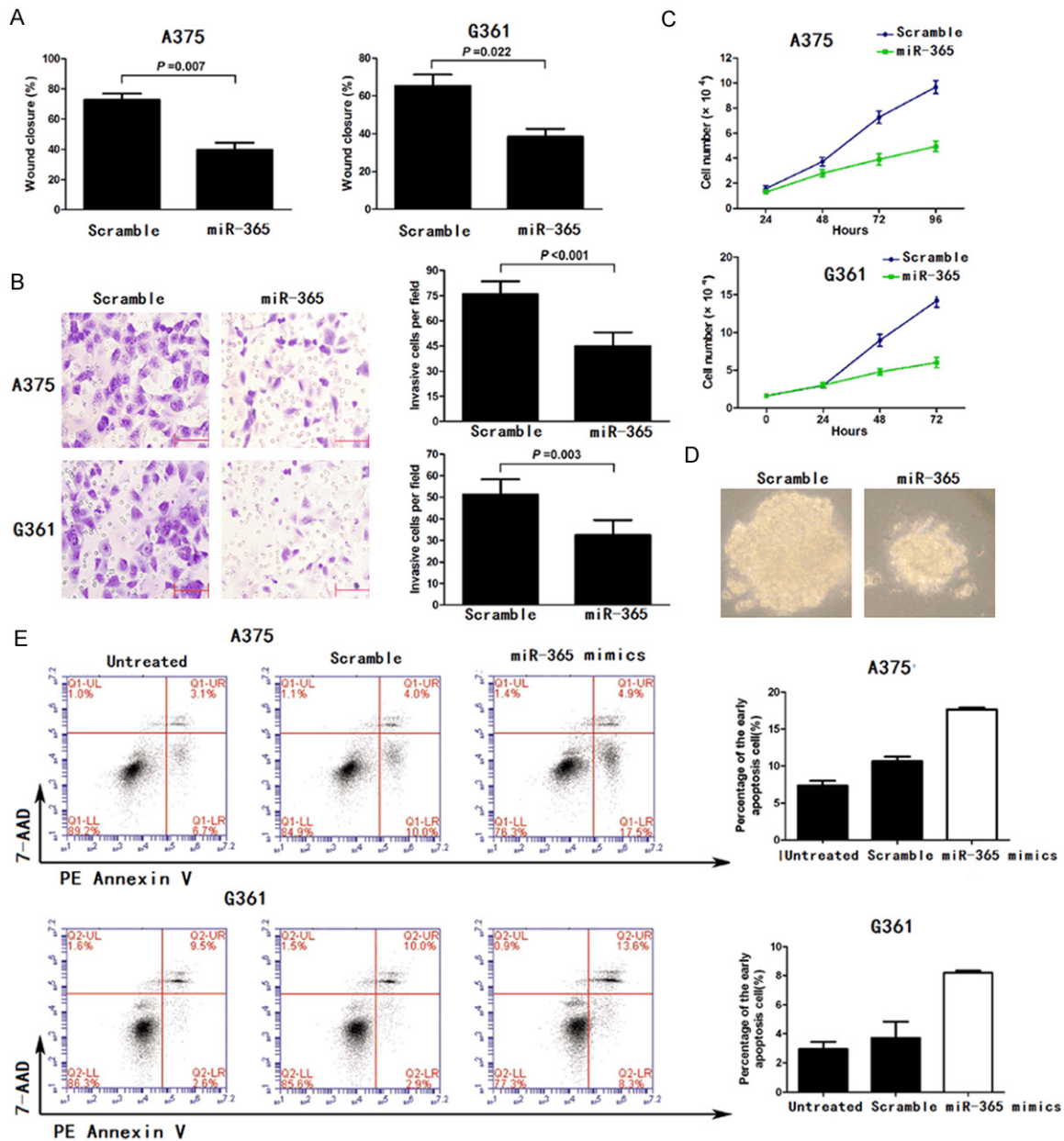


Figure 2. miR-365 inhibits cell migration, invasion, growth and soft agar colony formation, and induces cell apoptosis in MM. (A, B) The wound healing assay (A) and invasion assay (B) of A375 and G361 cells infected with miR-365 or scramble lentivirus. The invasion assay was measured by way of Transwell assays with Matrigel. (C) The growth of A375 and G361 cells infected with miR-365 or scramble lentivirus was assayed. (D) Colony growth assays in soft agar were performed on A375 with overexpression of miR-365 or scramble. Representative images of the assays are shown (left panel). Original magnification: $\times 200$. (E) A375 and G361 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. * $P<0.05$; ** $P<0.001$.

clinical 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 MM metastasis and progression (Figure 1D). To fur-

ther 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

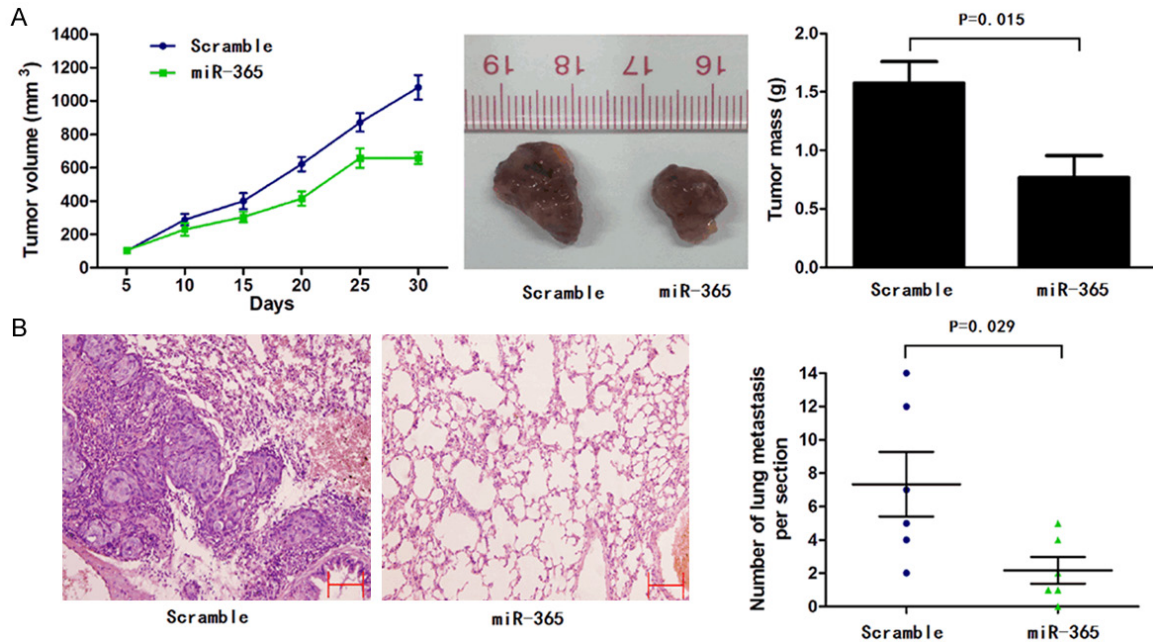


Figure 3. miR-365 inhibits MM cell growth and metastasis *in vivo*. A. Tumor growth in mouse xenograft models. A375 cells infected with miR-365 or scramble lentivirus were injected subcutaneously into nude mice. Tumor size was measured every 5 days. After 30 days, the mice were killed, necropsies were performed, and tumors were weighed. B. Tumor metastasis in mouse xenograft models. A375 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 μ m. All data are shown as mean \pm S.E.M.

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 MM 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 MM cell lines. 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 MM growth, we performed MM cell proliferation assay. The proliferation assay showed that ectopic expression of miR-365 in A375 and G361 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 MM cell apoptosis and cell death,

the number of early apoptotic A375 and G361 cells following treatment with miR-365 mimics was examined. As expected, few early apoptotic cells (10% in A375 or 2.9% in G361) were detected in the scramble-treated cells, whereas miR-365 mimics treatment increased the percentage of early apoptotic cells (17.5% in A375 or 8.3% in G361) as judged by Annexin V staining (**Figure 2E**). Therefore, we concluded that miR-365 could affect cell survival in MM cells.

miR-365 inhibits MM 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 overexpression of miR-365 in A375 cells significantly suppressed tumor growth in nude mice (**Figure 3A**). Then, A375 cells infected with either miR-365 or control lentivirus were injected into the tail vein of nude mice to examine melanoma metastasis. As shown **Figure 3B**, a significantly lower number of macroscopic melanoma metastases could be observed in miR-365 overexpressing cells than control cells. These results

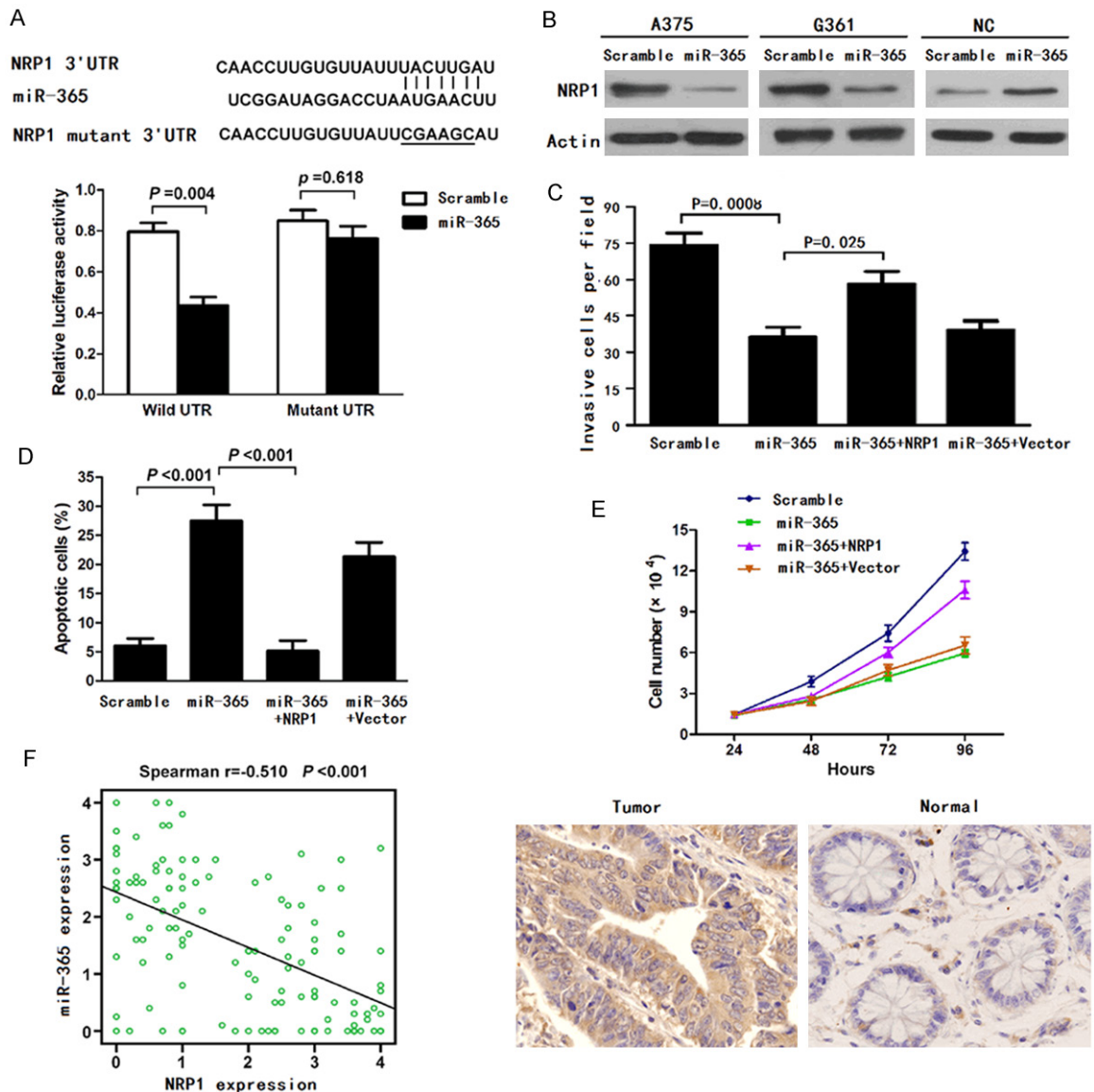


Figure 4. miR-365 directly targets NRP1. (A) The 3'-UTR element of NRP1 messenger RNA is partially complementary to miR-365. miR-365 or scramble control and luciferase reporter containing either a wild type or a mutant 3'-UTR were co-transfected into HEK-293T cells. And a Renilla luciferase expressing construct exerts as internal control. (B) Western blot analysis of NRP1 expression in A375 and G361 cells infected with miR-365, and NC transfected with miR-365 inhibitors (Anti-miR-365). (C-E) NRP1 abrogates the suppressive roles of miR-365 in MM cell invasion and growth. A375 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 MM specimens. Representative images of NRP1 expression by immunohistochemistry are shown (right panel). Original magnification: $\times 200$.

indicate that miR-365 may repress MM growth and metastasis.

miR-365 directly targets and inhibits NRP1

To understand how miR-365 suppresses MM growth and metastasis, we used three algo-

rithms (Targetscan, Pictar and Miranda) to help identify miR-365 targets in human malignant melanomas. 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 A375 and G361 cells. Furthermore, knockdown of miR-365, through transfection of anti-miR-365, in control cells increased NRP1 protein levels (**Figure 4B**). Taken together, these results indicate that NRP1 is a direct downstream target for miR-365 in MM cells. The above results prompted us to examine whether miR-365 suppresses MM growth and metastasis through repressing NRP1 expression. For this purpose, NRP1 was re-expressed in miR-365 transfected A375 cells. In miR-365-expressing cells, re-expression of NRP1 rescued the invasion and growth defects of miR-365 (**Figure 4C-E**). Finally, we tested if miR-365 expression correlated with NRP1 protein levels in MM. 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 MM 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 MM 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 malignant melanoma. We thereby in this study aimed to investigate the role of miR-365 in the pathogenesis of malignant melanoma.

In our study, the expression levels of miR-365 were first measured in human malignant melanoma samples and samples of adjacent normal mucosa tissues. We then evaluated the expression of miR-365 in 4 malignant melanoma cell lines and in normal skin 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 overexpressed miR-365 could inhibit malignant melanoma cell migration, invasion and proliferation, and promote apoptosis. Meanwhile, those tumorigenic 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 malignant melanoma, 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 malignant melanoma 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 malignant melanoma, whereas the forced expression of miR-365 inhibited EMT. miR-365 may therefore regulate malignant melanoma 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 overexpression. 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]. 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 overexpressed NRP1. These data suggest that miR-365 can attenuate tumor D-MVA via NRP1. But why reduced NRP1 in malignant melanoma 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 malignant melanoma. MiR-365 can decrease migratory, invasive 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 malignant melanoma.

Disclosure of conflict of interest

None.

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