MiRNA-128-3p Restrains Malignant Melanoma Cell Malignancy by Targeting NTRK3

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The functions of non-coding RNA, including microRNA (miRNA), have attracted considerable attention in the field of oncology. In this report, we examined the roles and molecular mechanisms of miR-128-3p, as related to the biological behaviors of malignant melanoma (MM). We found that miR-128-3p was expressed in low levels in these MM cells and may serve as a tumor suppressor by inhibiting proliferation, migration, and invasion, as well as inducing apoptosis in these MM cells. Moreover, neurotrophin receptor 3 (NTRK3), which serves as an oncogene that can enhance malignant behaviors of MM cells, was up-regulated in MM cells. Our current survey disclosed a complementary binding between miR-128-3p and the NTRK3 3′ untranslated regions (3′-UTR), while luciferase activities of NTRK3 3′-UTR were restrained by miR-128-3p in 293T cells. The effects of pre-miR-128-3p and sh-NTRK3 as well as anti-miR-128-3p and NTRK3(+) appeared to function synergistically in producing malignant progression. Moreover, there were possible to have counteracted effects for pre-miR-128-3p and NTRK3(+) in malignant progression. These findings established that miR-128-3p can function as a tumor suppressor by inhibiting carcinogenesis of the oncogene, NTRK3. Collectively, miR-128-3p and NTRK3 genes participate in modulating the malignant behavior of MM, and may represent new therapeutic targets for MM.

Keywords: miR-128-3p, malignant melanoma, NTRK3, proliferation, migration, invasion, apoptosis, post-transcriptional regulation

INTRODUCTION

Cutaneous malignant melanoma (MM), which originates from melanocytes in the epidermis, is a highly aggressive and the most lethal form of skin cancer worldwide (1). The incidence of MM has been increasing of late, showing one of the highest rates of cancer-related diseases (2). Treatment of MM mainly consists of surgical excision and chemotherapy, however MM is notoriously resistant to chemotherapy. Although treatment options have greatly improved in recent years, the high metastasis rate of MM, results in a 5-year survival rate of only 5–10% (3–5). Accordingly, treatments to improve the prognosis of MM represent an important issue.

Interestingly, both melanocytes and the nervous system originate from the neuroectoderm and express neurotrophins (NTs) (6). When NTs bind to neurophin receptors (NTRs), these receptors undergo dimerization and autophosphorylation, which facilitates survival and
differentiation of neuron (7). These proto-oncogene NTRs are involved in the genesis of neurogenic or neuroendocrine tumors (8, 9). A NTR of significance to this report is that of neurotrophin receptor 3 (NTRK3), which belongs to the neurotrophin receptor Trk family. NTRK3 is transmembrane receptor tyrosine kinase and binds to neurotrophin-3 (NT-3) (10). Results from in vitro experiments have revealed that human epidermal melanocytes express NTRK3 and the p75 NTR, a neurotrophin receptor known to bind all NTs with equal, but low, affinity (11). Moreover, p75NTR and NTRK3 are highly expressed in MM cells (1, 12) and P75 NTR receptor signaling contributes to promoting proliferation and migration of MM cells as show in MM cell lines (1). However, to our best of knowledge, the role of NTRK3 in human MM has not been elucidated. In this study, the regulatory mechanisms of NTRK3 on MM were assessed. MicroRNAs (miRNAs) are a class of non-coding RNAs with 18–25 nucleotides. MiRNAs can inhibit the expression of target genes at the post transcriptional level by binding to 3′ untranslated regions (3′-UTR) as identified using TargetScan 7.0 software. When collating this information, we hypothesized that miR-128-3p may play a key role in the mechanisms of miR-128-3p involved with regulating NTRK3 expression and the in vitro influence of mRNA were performed on the regulatory mechanisms of NTRK3 on MM.

### MATERIALS AND METHODS

#### Cell Lines and Cultures

The human embryonic kidney (HEK 293T) and MM (A375, M14, MeWo, and SK-MEL-5) cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, MD, USA). They were cultured with Dulbecco’s Modified Eagle Medium (high glucose) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) in a constant temperature incubator (37°, 5% CO2). Normal human epidermal melanocytes (NHEMs) were obtained from Lonza (Walkersville, MD, USA) and cultured in Melanocyte Cell Basal Medium-4 supplemented with the MGM-4 Bullet Kit (Lonza, Walkersville, MD, USA) in a constant temperature incubator (37°, 5% CO2).

#### Quantitative Real Time PCR (qRT-PCR)

The extraction and quantification of mRNA were performed according to procedures described in our previous study (13). The PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China) was used to reverse transcript mRNA into cDNA according to the instructions provided. Expressions of miR-128-3p and NTRK3 were assessed with use of SYBR® Premix Ex Taq II (TaKaRa, Dalian, Liaoning, China) according to instructions. U6 and GAPDH served as reference genes. All primers were synthesized by Lixin (Shenyang, Liaoning, China), with the sequence information contained in Table 1. The relative expressions of miR-128-3p and NTRK3 were calculated as relative quantitative values after normalizing with reference genes.

#### Cell Transfection of miRNAs

The agonior and antagonir for miR-128-3p (pre-miR-128-3p and anti-miR-128-3p) as well as their negative control (pre-miR-con and anti-miR-con) were obtained from GenePharma (Shanghai, China). Lipoctametasin 3000 (Invitrogen, Foster City, CA, USA) was applied to transfect miRNAs into A375 and M14 cells according to manufacturers’ protocols. The transfected efficiency was evaluated with use of qRT-PCR, with 48 h after transfection being the best time point for subsequent examinations.

#### Transfection and Generation of Stable Cell Lines

The expression plasmid [NTRK3(+)] (truncated isoform of NTRK3, Genbank accession number NM_001007156) and negative control plasmid (NTRK3(+)-NC) were designed and manufactured by GenScript (Nanjing, Jiangsu, China). The silenced plasmid for NTRK3 (sh-NTRK3) and its negative

### TABLE 1 | Oligonucleotides used for qRT-PCR, their Amplicon Size, GenBank accession number, and annealing temperatures.

| Gene    | Sequence                        | Amplicon size | Genbank accession number | Annealing temperature |
|---------|---------------------------------|---------------|--------------------------|----------------------|
| NTRK3   | (F) 5′TCACAGAGTGGGGAAGTTGCTCT-3′ (R) 5′-CCATGGTAAAGGCTTGA-3′ | 89            | NM_001007156              | 58°C                 |
| GAPDH   | (F) 5′-AAATGCCATCACCACCTCTCCAG-3′ (R) 5′-TGATGACCTTTTGCTGCC-3′ | 149           | NM_002046.4               | 58°C                 |
control plasmid (sh-NTRK3-NC) were designed and manufactured by GenePharma (Shanghai, China). Lipofectamine 3000 (Invitrogen, Foster City, CA, USA) was applied to transfect vectors into A375 and M14 cells according to instructions. G418 (Sigma-Aldrich, St Louis, MO, USA) was used to screen stable transfected cell lines according to procedures described in our previous study (13).

**Cell Proliferation Assay**
A375 and M14 cells (2 × 10^5 per well) were seeded into a 96-well plate. A CCK-8 dilution (Beyotime, Nantong, Jiangsu, China) consisting of 10 μl was then added to each well after 48 h. A SpectraMax M5 microplate instrument (Molecular Devices, Sunnyvale, CA, USA) was used to assess the absorbance at 450 nm, which represented cell proliferation ability.

**Cell Apoptosis Detection**
The Annexin V-PE/7-AAD staining apoptosis kit (KeyGEN, Nanjing, Jiangsu, China) was used to evaluate apoptosis of A375 and M14 cells according to procedures described in our previous study (22). The apoptosis rate was assessed using CELLQuest software (BD Biosciences, San Jose, CA, USA).

**Cell Migration and Invasion Assay**
MM cells (1 × 10^5 per well) were added to the medium (serum-free) in the upper compartment of the 24-well transwell, while 500 μl medium (10% FBS) per well was added to the lower compartment. The migratory and invasive ability of MM cells were examined according procedures described in our previous study (23). As viewed under a microscope, cells invading the inferior side of the membrane in 5 randomly selected fields were counted. Generally speaking, we calculated the total number of cells in the upper left, lower left, upper right, lower right, and center of the field of view, and then divided by 5 to get the average value. The average value of transmembrane A375 and M14 cells obtained by the above method represented the migration and invasion ability of the cells.

**Cell Fractionation and Western Blot Analysis**
Protein extraction and quantification were performed according to procedures described previously in our laboratory (13). The protein lysate (40 μg) was separated by polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk powder for 2 h, hybridized overnight at 4°C with the NTRK3 antibody (Upstate, New York, USA) or GAPDH (Santa Cruz Biotechnology) and incubated at room temperature for 2 h with the secondary antibody. As the antibody is directed against the entire extracellular domain of the NTRK3 receptor, corresponding to amino acid residues 1-429, the epitope of the NTRK3 antibody is against both the full length (150 kDa) and truncated isoform (50 kDa). In preliminary experiments performed in our laboratory, no significant differences in the full-length form expression were obtained in A375 and M14 cells as compared with that of NHEMs (Data not shown). Only the expression of the truncated form (50 kDa) of the NTRK3 protein was determined with use of western blot. The FluorChem M imaging analysis system (Protein Simple, San Jose, CA USA) was used to scan the protein bands and calculate relative expressions of the NTRK3 protein.

**Dual-Luciferase Reporter Assay**
The target genes of miR-128-3p were predicted with use of MiRDB and Targetscan (http://www.mirdb.org/miRDB/ and http://www.targetscan.org/). The NTRK3 3'-UTR wild-type luciferase reporter plasmid (NTRK3 wt), mutant plasmid (NTRK3 mut) and a negative control plasmid (NC, carrying non-targeting sequence) were constructed by GenePharma (Shanghai, China). The miRNAs (pre-miR-128-3p or pre-miR-con) and plasmids (NTRK3 wt and NTRK3 mut) were co-transfected into HEK 293T cells by means of Lipofectamine 3000. Luciferase activity was determined with use of the dual luciferase reporter gene assay system (Promega, Madison, WI, USA) at 48 h after transfection.

**Statistical Analysis**
Each experiment was repeated three times and the data are presented as the means ± SDs. T-tests or one-way ANOVAs were used to determine whether statistically significant differences were present between/among the groups. Bonferroni post-test was used if the ANOVA was significant. A P <0.05 was required for results to be considered as statistically significant.

**RESULTS**

**MiR-128-3p Was Down-Regulated in MM Cells**
Compared with that observed in NHEMs, miR-128-3p expression was significantly decreased in A375 and M14 cells as revealed with qRTPCR analysis (Figure 1A), but no significantly changes were observed in MeWo and SK-MEL-5 cells, thereby using A375 and M14 cells as research objects in subsequent experiments.

**MiR-128-3p Functions as a Tumor Suppressor in MM Cells**
Having identified the expression of miR-128-3p in A375 and M14 cells, we next examined the effects of miR-128-3p on the biological behaviors of MM cells. The expression of miR-128-3p displayed a nearly 10-fold up-regulation in the pre-miR-128-3p versus pre-miR-con group (Figure 1B). Following anti-miR-128-3p transfection, the expression of miR-128-3p was significantly down-regulated by nearly 0.2-fold as compared with that in the control groups (Figure 1B). Results from the CCK-8 assay showed that the proliferation of A375 and M14 cells in the pre-miR-128-3p group was decreased as compared with that observed in the control and pre-miR-con groups (Figure 1C), whereas a marked increase in cell proliferation was obtained in the anti-miR-128-3p group as compared with that of the control and anti-miR-con groups (Figure 1C). Results from the flow cytometry analysis suggested that the over-expression of miR-
128-3p markedly promoted apoptosis of A375 and M14 cells as compared with that of the control and pre-miR-con groups (Figure 1D), whereas cell apoptosis in the anti-miR-128-3p group was significantly decreased as compared with that observed in the control and anti-miR-con groups (Figure 1D). Results from the transwell assays implied that the number of migrated and invaded cells were significantly decreased in the pre-miR-128-3p versus control and pre-miR-con groups (Figure 1E), while the number of migrated and invaded MM cells was increased in the anti-miR-128-3p group as compared with that of the control and anti-miR-con groups (Figure 1E). Proliferation, apoptosis rates, and the ability for migration and invasion were not significantly different among the control, pre-miR-con and anti-miR-con groups (Figures 1C–E). These findings demonstrate that miR-128-3p can serve as a tumor suppressor in the malignant progression of MM.

**NTRK3 Was Up-Regulated in MM Cells**

As based on an online bioinformatic analysis, it was predicted that NTRK3 might be a target gene of miR-128-3p. Therefore,
qRT-PCR and western blotting were used, with the results that the expression of NTRK3 was significantly elevated in A375 and M14 cells as compared with that of NHEMs (Figures 2A, B). Conversely, no significantly changes in NTRK3 expression were obtained in MeWo and SK-MEL-5 cells. Therefore, Subsequent experiments were only performed in A375 and M14 cells.

MiR-128-3p Negatively Regulates NTRK3 Expression

NTRK3 mRNA and protein expressions were down-regulated in the pre-miR-128-3p as compared to that of the control and pre-miR-con groups (Figures 2C, D). In contrast, transfection with anti-miR-128-3p increased the expression of NTRK3 mRNA and protein as compared with that observed in its control groups (Figures 2C, D). No statistically significant differences were obtained among the control, pre-miR-con and anti-miR-con groups (Figures 2C, D). These finding provided further evidence that miR-128-3p is regulating the expression of NTRK3.

NTRK3 Is a Direct Target Gene of MiR-128-3p

With use of TargetScan software 7.0 it was forecasted that there is a binding site of miR-128-3p at 209-215 bp of NTRK3 3'-UTR (Figure 2E.a). The seed for miR-128-3p to NTRK3 3'-UTR is shown in Figure 2 E.a. NTRK3 was a direct target gene of miR-128-3p.
128-3p as revealed from results of the luciferase assay. In accord with these expectations, a co-transfection with NTRK3 wt and pre-miR-128-3p, but not NTRK3 wt and pre-miR-con, significantly reduced the relative luciferase activity as compared with that of control (Figure 2E.b). To further verify whether miR-128-3p directly targeted NTRK3 through the predicted binding site, a luciferase reporter vector containing the Mut-type 3′-UTR of NTRK3 (NTRK3-3′UTR-Mut) was constructed. Co-transfection of NTRK3 mut and pre-miR-128-3p did not affect the relative luciferase activity. Taken together, these results demonstrate that miR-128-3p can bind NTRK3 3′-UTR and NTRK3 was a target gene of miR-128-3p.

NTRK3 Is an Oncogene in MM Cells

After verifying that NTRK3 was up-regulated in MM cells and was direct target of miR-128-3p, we next attempted to elucidate some of the effects of NTRK3 on the biological behaviors of MM cells. CCK-8 assay results indicated that the proliferation of A375 and M14 cells in the sh-NTRK3 group was significantly decreased compared with that of the control and sh-NTRK3-NC groups (Figure 3A), while proliferation in the NTRK3(+) group was significantly increased compared with the control and NTRK3(+-)NC groups (Figure 3A). A down-regulated expression of NTRK3 (sh-NTRK3) resulted in an increased apoptotic ratio of A375 and M14 cells as compared with that in the control and sh-NTRK3-NC groups (Figure 3B), whereas an up-regulation of NTRK3 [NTRK3(+)] significantly suppressed apoptosis in A375 and M14 cells as compared with the control and NTRK3(+-)NC groups (Figure 3B). Transwell assays were conducted to assess the effects of NTRK3 on the migratory and invasive ability of A375 and M14 cells. As predicted, the migratory and invasive ability in the sh-NTRK3 group was reduced compared with that of the control and sh-NTRK3-NC groups (Figure 3C), whereas increased migratory and invasive capacities were observed in the NTRK3(+) versus the control and NTRK3(+-)NC groups (Figure 3C). No statistically significant differences were obtained among the control, sh-NTRK3-NC and NTRK3(+-)NC groups (Figure 3A–C). Collectively, these results showed that NTRK3 can be considered as an oncogene in A375 and M14 cells.

MiR-128-3p Exerts Tumor Suppression by Inhibiting Carcinogenesis of the Oncogenic NTRK3

Having established that miR-128-3p and NTRK3 functioned as a tumor suppressor and an oncogene, respectively, and that NTRK3 was a target of miR-128-3p, the next issue to address was whether miR-128-3p exerted a tumor inhibitor effect by
hindering carcinogenesis of the oncogenic NTRK3. To accomplish this goal, pre-miR-128-3p or anti-miR-128-3p were transfected into stable NTRK3(+) or sh-NTRK3 MM cells prior to assessing their malignant biological behavior. Results from the CCK8 assay indicated that proliferation of A375 and M14 cells was remarkably reduced in the pre-miR-128-3p + sh-NTRK3 group as compared with all other groups (Figure 4A). Similarly, with the exception of the pre-miR-128-3p + sh-NTRK3 group, A375 and M14 cell proliferation in the anti-miR-128-3p + sh-NTRK3 group was dramatically reduced compared to all other groups, while the proliferation of A375 and M14 cells in the anti-miR-128-3p + NTRK3(+) group was remarkably elevated in comparison with all other groups (Figure 4A). No statistically significant differences were observed among the control, the pre-miR-con + sh-NTRK3-NC, pre-miR-con + NTRK3(+)-NC, pre-miR-128-3p + NTRK3(+), anti-miR-con + sh-NTRK3-NC, and anti-miR-con + NTRK3(+)-NC groups (Figure 4A). Moreover, A375 and M14 cells treated with pre-miR-128-3p + sh-NTRK3 exhibited increased apoptotic ratios as compared with that obtained in all other groups (Figure 4B). With the exception of the pre-miR-128-3p + sh-NTRK3 group, higher rates of apoptosis were observed in A375 and M14 cells within the anti-miR-128-3p + sh-NTRK3 group as compared with all other groups, whereas apoptosis was dramatically suppressed in NTRK3(+) cells with anti-miR-128-3p transfection versus that observed in all other groups (Figure 4B). No statistically significant differences were detected among the control, the pre-miR-con + sh-NTRK3-NC, pre-miR-con + NTRK3(+)-NC, pre-miR-128-3p + NTRK3(+), anti-miR-con + sh-NTRK3-NC, and anti-miR-con + NTRK3(+)-NC groups (Figure 4B). In addition, the migration and invasion of MM cells demonstrate the same tendency as their proliferation, and the number of A375 and M14 cells showing migration and invasion were remarkably reduced in the pre-miR-128-3p + sh-NTRK3 group as compared with all other groups (Figure 4C). As predicted, with the exception of the pre-miR-128-3p + sh-NTRK3 group, the migratory and invasive ability within the anti-miR-128-3p + sh-NTRK3 group was attenuated versus that of all the other groups, while the number of A375 and M14 cells showing migration and invasion were dramatically increased in the anti-miR-128-3p + NTRK3(+) group as compared with all other groups (Figure 4C). There were no statistically significant differences among the control, the pre-miR-con + sh-NTRK3-NC, pre-miR-con + NTRK3(+)-NC, pre-miR-128-3p + NTRK3(+), anti-miR-con + sh-NTRK3-NC, and anti-miR-con + NTRK3(+)-NC groups (Figure 4C).
MiRNAs leads to regulatory abnormalities and dysfunctions that evidence has indicated that aberrant expression of endogenous miRNAs can modulate many biological processes (32). Accumulating evidence has indicated that aberrant expression of endogenous miRNAs leads to regulatory abnormalities and dysfunctions that can contribute to the pathogenesis of many types of tumors (19, 33). The miRNA under investigation in this report, miR-128-3p, is a brain-rich miRNA encoded by two individual genes, miR-128a and miR-128b, which encode the same mature sequence (34). Results from previous studies have shown that miR-128-3p was down-regulated in several cancers including bladder cancer, glioma, esophageal squamous-cell cancer, colorectal carcinoma, and hepatocellular carcinoma (17–19, 35, 36). Consistent with these findings, our current data also demonstrated a decrease in the expression of miR-128-3p in MM. However, it should be noted that there exist reports indicating that miR-128-3p expression is increased in gastric and breast cancers (33, 37), which is contrary to our current results. Such seemingly inconsistent findings may be the result of different roles exerted by miR-128-3p within specific types of tumors. Overall, when combining the results of these previous reports with our current findings, it appears that changes in miR-128-3p expression might be essential for the occurrence and malignant progression of MM.

The role of miR-128-3p in malignant progression of MM was further investigated. As predicted, our data showed that an up-regulation of miR-128-3p restrained the proliferation, migration, and invasion of MM cells, as well as increased the rate of apoptosis of MM cells, while silencing of miR-128-3p showed the opposite effects. We also found that M14 and A375 cells demonstrated similar migratory and invasive rates as revealed from results of the transwell assay. Consistent with these findings were the results of Wen et al. reporting similar migratory abilities of M14 and A375 during the process of Rho GTPases influence on cytoskeletal and migratory activity (38). In this way, miR-128-3p serves as a tumor inhibitor in MM. Similarly, anti-tumor effects of miR-128-3p have been observed in several other types of tumors (17–19, 34, 36, 39–46). For example, Liu et al. reported that an over-expression of miR-128-3p, miR-101, and miR-27b inhibited VEGF-C secretion of gastric cancer cells, thereby blocking HUVEC migration, proliferation, and tubulation (44). Conversely, there are reports demonstrating that miR-128-3p facilitated the occurrence and development of some cancers. Notably, MiR-128-3p has been shown to be a new, robust oncogene candidate in T cell acute lymphoblastic leukemia (20), a result which differs from our current findings. One possible explanation may be due to the potential for tumors with different origins to exert diverse mechanisms involved with tumorgenesis. Taken together, it seems that miR-128-3p might function as a tumor suppressor in MM, which suggests a novel potential treatment protocol against MM.

Recently, increasing evidence has accrued showing that miRNAs, as modulators of cancer, could affect the expression of critical genes connected with tumourigenesis and malignant behaviors (33, 45). MiRNAs restrain the expression of target genes with the aid of guiding a RNA induced silencing complex (RISC) to bind the 3′-UTR of miRNAs and seed sequence of miRNAs. This combination of events leads to an inhibition of translation or mRNA degradation (20). Our present data also reveal that the expression of NTRK3 was elevated, an effect which was modulated by miR-128-3p in MM cells. Others have
In conclusion, our data suggest that miR-128-3p in MM cells could serve as a tumor inhibitor by negatively modulating malignant biological behaviors of MM cells. The findings that NTRK3 promoted malignant biological behaviors of MM cells, indicates that NTRK3 serves as an oncogene in MM cells. Combining our findings obtained with miR-128-3p and NTRK3, it appears that miR-128-3p exerts its tumor suppression by inhibiting the carcinogenesis of the oncogene, NTRK3. These results provided a novel and more comprehensive understanding of miR-128-3p and NTRK3 functions as related to MM. Accordingly, they can serve as a foundation for the development of new genetic diagnostic markers and targeted therapies involving miR-128-3p and NTRK3 for MM treatment.

DATA AVAILABILITY STATEMENT
All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS
XZ and TM conceived and designed the experiments. XZ, JH, and QW performed the experiments. QW and JH analyzed the data. XZ and TM drafted the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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