Downregulation of TrkA protein expression by miRNA-92a promotes the proliferation and migration of human neuroblastoma cells

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Abstract. The aims of this study were to investigate the regulation of TrkA protein by micro (mi)RNA-92a and its effect on the proliferation and migration of human neuroblastoma cells. The BE(2)-M17 human neuroblastoma cell line was cultured and transfected with either miRNA-92a mimics or miRNA-92a inhibitors. The expression levels of miRNA-92a and TrkA mRNA were detected by quantitative polymerase chain reaction prior and subsequent to transfection. TrkA protein was quantitatively detected by flow cytometry. The proliferation and migration of neuroblastoma cells were examined *in vitro* by Cell Counting Kit-8 and Transwell assays. Transfection of BE(2)-M17 cells with miRNA-92a mimics produced significantly higher expression levels of miRNA-92a compared with those in the same cells transfected with negative controls (NCs). Increased proliferation and migration of the cells was also observed. Transfection of BE(2)-M17 cells with miRNA-92a inhibitors resulted in significantly lower expression levels of miRNA-92a when compared with those of the same cells transfected with NCs ($P<0.01$). This reduction in the miRNA-92a expression levels was accompanied by reduced proliferation and migration of the cells. The expression levels of TrkA mRNA and protein after 24 h transfection with the miRNA-92a mimics were significantly reduced when compared with the control ($P<0.01$). However, the expression levels of TrkA were significantly higher ($P<0.01$) after 48 h transfection with miRNA-92a inhibitors when compared with the control. In conclusion, miRNA-92a promoted the proliferation and migration of human neuroblastoma cells through downregulation of TrkA, which suggested that miRNA-92a may be a potential target for human neuroblastoma treatment in the future.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; miRNA, microRNA; NGF, nerve growth factor; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; Trk, tyrosine kinase

Key words: neuroblastoma, miRNA-92a, TrkA protein, proliferation

Introduction

Neuroblastoma is the most common extracranial solid tumor of childhood and exhibits various clinical manifestations and diverse prognoses (1,2). Certain tumor cells undergo spontaneous regression while others exhibit continuous progression. The prognosis of neuroblastoma is associated with the age of onset, the extent of disease and the biological characteristics of the tumor. MYCN gene amplification is an independent high risk factor for neuroblastoma. MYCN regulates various genes through multiple biochemical signaling pathways and thereby promotes the growth of malignant tumors (3). Previous studies have shown that the expression levels of the MYCN gene were associated with a variety of abnormal micro (mi)RNA regulation patterns (4-7). miRNAs are a class of endogenous non-coding RNAs ~22 nt in length, which may inhibit the translation of specific target mRNAs and induce its degradation, thereby affecting cell proliferation, differentiation, apoptosis and other biological processes (8). Various miRNA molecules have been associated with neuroblastoma; one of the MYCN gene regulating factors in neuroblastoma is the miRNA17-92 cluster (9-11).

The tyrosine kinase (Trk) receptor family includes TrkA, TrkB and TrkC. Previous studies have shown that the Trk family is associated with the prognosis of neuroblastoma (12). Neuroblastoma with TrkA expression results in an improved prognosis, as TrkA combines with its ligand, nerve growth factor (NGF), to promote spontaneous regression or differentiation of the tumor. However, neuroblastoma with TrkB expression results in a poor prognosis due to amplification of the MYCN gene. TrkB ligands from neuroblastoma, via autocrine or paracrine survival pathways, may enhance the viability, drug resistance and angiogenesis of TrkB-expressing

tumors (13). To the best of our knowledge, no studies have confirmed the association between the miRNA17-92 cluster and the TrK family. In the present study, the miRNA17-92 cluster regulated by MYCN gene was hypothesized to be associated with the TrK family and thus affect the prognosis of neuroblastoma. One of the miRNA members of miRNA17-92 cluster, miRNA-92a, was selected to investigate its effect on human neuroblastoma cells and the underlying mechanisms.

Materials and methods

Cell culture. Primary BE(2)-M17 human neuroblastoma cell lines with an amplified MYCN gene were purchased from the cell stores of the Chinese Academy of Medical Sciences (Beijing, China), and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin (Hyclone Laboratories, Inc.) at 37˚C in a 5% CO_2 atmosphere.

Gene transfection. After 24 h of culture, the cells were starved in DMEM without 10% FBS and 100 units penicillin/streptomycin, and divided into four groups. The appropriate quantity of the following reagents was added to each of the four groups respectively: miRNA-92a mimics negative control (NC) sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; miRNA-92a mimics NC antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; miRNA-92a mimics sense, 5'-UAUUGCACUUGUCCCGGCCUGU-3'; miRNA-92a mimics antisense, 5'-AGGCCGGGACAAGUGCAAUAUU-3'; miRNA-92a inhibitor NC sense, 5'-CAGUACUUUUGUGUA GUACAA-3'; and miRNA-92a inhibitors antisense, 5'-ACAGGCCGGGACAAGUGCAAUA-3' (all purchased from Gene Pharma, Shanghai, China). Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) was added to the culture media of each of above groups and oligonucleotides were adjusted to a final concentration of 160 nM. All cells were incubated in DMEM containing 10% FBS at 37˚C in a humidified incubator with 5% CO_2. The miRNA-92a mimics and inhibitor were fluorescently labeled with fluorescein (Sigma-Aldrich, St. Louis, MO, USA) for verification of transfection.

Total RNA extraction. Total RNA from cultured cell lines was extracted at 0, 24, 48 and 72 h post-transfection following the manufacturer's instructions of the RNAiso Plus reagent (Takara Bio Inc., Otsu, Japan). The concentration and quality of the RNA were determined with GeneQuan Pro (Biochrom Ltd., Cambridge, UK), with the OD260nm/OD280nm between 1.8 and 2.2. The RNA was stored at -80˚C.

Quantitative polymerase chain reaction (qPCR) for miRNA and TrkA mRNA expression. qPCR analysis for miRNA-92a was performed in triplicate with the One Step Primescript® miRNA cDNA Synthesis kit (Takara Bio Inc.) and SYBR® Premix Ex TaqTMII (Perfect Real Time; Takara Bio Inc.) according to the manufacturer's instructions. U6 small nuclear RNAs served as internal controls. The mixture was incubated for 30 sec at 95˚C for 1 cycle, followed by 5 sec at 95˚C and 30 sec at 60˚C for 40 cycles using the DNA Engine Opticon2 (MJ Research Inc. Waltham, MA). The fold-change in miRNA-92a expression levels was calculated using ΔCT and $2^{-\Delta\Delta CT}$. The following primers were used respectively: miRNA-92a sense, 5'-TATTGCACTTGTCCCGGCCTG-3'; the miRNA-92a antisense strand was constructed using general primers; U6 sense, 5'-TCGCTTCGGCAGCACATA-3'; U6 antisense, 5'-TTGCGTGTCATCCTTGCG-3' (Sunbiotech Co. Ltd., Beijing, China).

TrkA mRNA expression levels were measured at 24 and 48 h post-transfection by qPCR, which was performed in triplicate using the same method as for miRNA-92a. β-actin served as the internal control. The following primers were used respectively: TrkA mRNA sense, 5'-TATTGCACTTGTCCCGGCCTG-3'; TrkA mRNA antisense, 5'-ACAAGGAGCAGCGTAGAAAGGA-3'; β-actin sense, 5'-TGACGTGGACATCCGCAAAG-3'; β-actin antisense, 5'-CTGGAAGGTGGACAGCGAGG-3' (Sunbiotech Co. Ltd.).

Cell Counting Kit-8 (CCK-8) assay. The transfected neuroblastoma cells were seeded into 96-well plates ($5.0x10^3$ cells/well) containing 100 µl DMEM medium supplemented with 10% FBS. Cell viability was detected by CCK-8 assay (Dojin Laboratories, Kumamoto, Japan) at 0, 24, 48 and 72 h post-transfection. The absorbance at 450 nm (A450) of each well was read on a Model 550 spectrophotometer (Bio-Rad, Hercules, CA, USA).

Transwell migration assay. Cellular migration was measured using a modification of the method as reported previously (14), using 24-well Transwell cell culture chambers filtered with multiporous polycarbonate membranes (Corning Inc., NY, USA). The BE(2)-M17 cells were cultured for 6 h in serum-free DMEM medium without antibiotics following transfection. Each group of cells was digested to form a cell suspension and adjusted to a density of either $2x10^5$ cells per ml (miRNA-92a mimics and miRNA-92a mimics NC groups) or $3x10^5$ cells per ml (miRNA-92a inhibitor and miRNA-92a inhibitor NC groups). A volume of 100 µl cell suspension cultured in 500 µl DMEM medium with 10% FBS was extracted and plated onto a 24-well Transwell plate. The plates were placed in a humidified 5% CO_2 incubator for 36 h at 37˚C. The upper surface of the membrane was wiped with cotton swabs to remove non-migrated cells, and the remaining cells were fixed in 95% ethanol (Beijing Chemical Works, Beijing, China) for 10 min and then stained with 0.1% crystal violet (Sigma-Aldrich) for 15 min. Digital images of cells were obtained using the DMI4000B DFC500 inverted microscope (Leica Microsystems AG, Wetzlar, Germany; magnification, x200). The number of cells in each image was counted by Scion Image software (Scion Corporation, Torrance, CA, USA). Each treatment in the migration assay was performed in triplicate.

Flow cytometry assay for TrkA protein expression. A total of ~$1.0x10^6$ cells were fixed in phosphate-buffered saline (PBS) with 4% formaldehyde for 10 min at 37˚C and subsequently incubated with the primary rabbit polyclonal anti-human TrkA antibody (Abcam Plc, Cambridge, UK) at 50 µg/ml for 1 h. Following three washes with PBS, the cells were incubated with 2 µg/ml anti-rabbit IgG for 1 h (Cell Signaling Technology

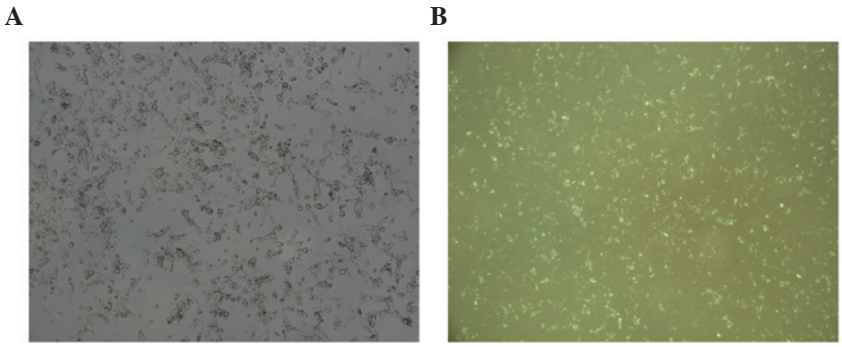

Figure 1. Cells transfected with the microRNA-92a inhibitor fluorescently labeled with fluorescein (A) under natural light and (B) under green fluorescence in the same field of vision. Magnification, x100.

Inc., Boston, MA, USA). The cells were resuspended in PBS and analyzed with the CytomicsTM FC500 flow cytometer (Beckman Coulter Inc. Fullerton, CA, USA).

Statistical analysis. All data were analyzed by SPSS software (version 13; SPSS, Inc., Chicago, IL, USA) and the significance of the difference between groups was determined by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of miRNA-92a transfection and its changes in expression. In order to analyze the effect of miRNA-92a transfection and its expression, the solution of miRNA-92a mimics, miRNA-92a mimics NC, miRNA-92a inhibitors and miRNA-92a inhibitor NC was replaced 6 h later following the transfection. Tumor cells were focused under the fluorescence microscope at a magnification of x100. The cells were observed under natural light and under fluorescence in the same field of vision (Fig. 1). Granular fluorescence was observed in a large number of the tumor cells which indicated miRNA transfection of the cells. To further verify transfection, total RNA at 24, 48 and 72 h after transfection was isolated and qPCR was employed to detect the relative expression levels of miRNA-92a at three time points. The maximum miRNA-92a expression level following miRNA-92a mimics transfection was detected at 24 h after transfection, then gradually decreased from this level (Fig. 2A). The miRNA-92a expression levels were reduced in comparison with the NC group 24 h after miRNA-92a inhibitor transfection and the inhibition reached a peak at 48 h, which lasted until 72 h post-transfection (Fig. 2B). These results demonstrate that the interference effect was significantly enhanced 24 h post-transfection with miRNA-92a mimics (P<0.01) and 48 h post-transfection with miRNA-92a inhibitors (P<0.01).

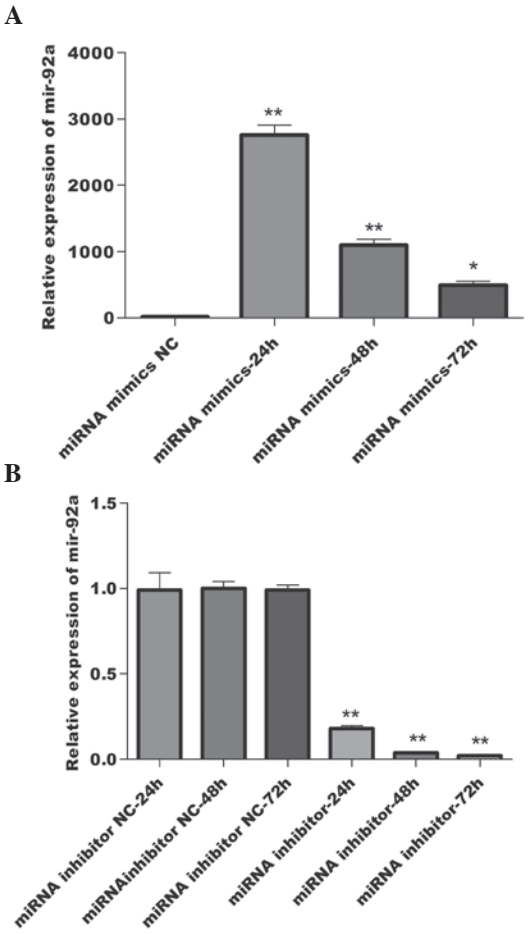

Figure 2. Quantitative polymerase chain reaction analysis of miRNA-92a at 24, 48 and 72 h after transfection. U6 served as an endogenous control. The expression levels of miRNA-92a are relative to the NC (set as 1.0). (A) The maximum expression level of miRNA-92a following transfection with the miRNA-92a mimics occurred at 24 h after transfection, but was then decreased from this level at 48 and 72 h. (B) The miRNA-92a expression level was significantly reduced 24 h after transfection with the miRNA-92a inhibitor and was further decreased after 48 h. After 72 h the expression levels remained in decline but this decline had markedly slowed down. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01, vs. the respective NC group. miRNA, microRNA; NC, negative control.

miRNA-92a promotes the proliferation of BE(2)-M17 human neuroblastoma cells. In order to investigate the functional role of miRNA-92a in neuroblastoma cells, the effect of miRNA-92a mimics and miRNA-92a inhibitors on the proliferation of BE(2)-M17 human neuroblastoma cell line was examined. The cells were transfected with either miRNA or NC for 24, 48 and 72 h. CCK-8 assay and direct cell count revealed that overexpression of miRNA-92a mimics significantly increased the proliferation of BE(2)-M17 human neuroblastoma cells and overexpression of miRNA-92a inhibitors significantly inhibited the proliferation of neuroblastoma cells (Fig. 3).

Table I. Average fluorescence intensity of Trk protein by fluorescence-activated cell sorting.

Group	miRNA-92a mimics NC	miRNA-92a mimics	miRNA-92a inhibitors NC	miRNA-92a inhibitors
Average fluorescence intensity at 24 h	31.26±0.26	29.45±0.36[a]	28.05±0.43	26.72±0.99
Average fluorescence intensity at 48 h	25.12±0.76	24.25±0.28	22.42±0.86	26.51±0.36[a]

Flow cytometry assay of TrkA protein expression levels. The average fluorescence value of the miRNA-92a mimics transfection group was significantly reduced at 24 h compared with the miRNA-92a mimics NC, while the average fluorescence value of the miRNA-92a inhibitors transfection group was significantly increased at 48 h compared with the miRNA-92a inhibitors NC. Results are presented as the mean ± standard deviation. (n=3). [a]P<0.01. Trk, tyrosine kinase; miRNA, microRNA; NC, negative control.

Effect of miRNA-92a on BE(2)-M17 human neuroblastoma cell migration. To reveal whether miRNA-92a was involved in the regulation of migration of neuroblastoma cells, a Transwell migration assay was conducted. The number of cells across the membrane in the miRNA mimics transfected group (32.4±3.7) was increased by 37.3% compared with that in the control group (23.6±2.1), which was significantly different (P<0.05) (Fig. 4A and 4B). The number of cells across the membrane in the group of cells transfected with the miRNA-92a inhibitors (21.3±2.3) was decreased by 40.8% compared with that in the control group (36.0±7.5) which was also significantly different (P<0.05, Fig. 4C and D). These data indicate that transfection with miRNA mimics was capable of enhancing the migration of cells, while transfection with miRNA inhibitors reduced the migration of cells.

TrkA expression levels are inversely correlated with the miRNA-92a expression levels. In view of the established role of miRNA-92a as an effector for indirect MYCN-induced downregulation of protein-coding genes, it was hypothesized that this miRNA may regulate TrkA. To investigate this possibility, TrkA mRNA expression following conditional up- or downregulation of miRNA-92a was verified in BE(2)-M17 human neuroblastoma cell lines. Total RNA was extracted at 24 and 48 h post-transfection and qPCR was used to detect TrkA mRNA expression at these two time points; the TrkA protein expression levels were detected by flow cytometry. The data from the qPCR and flow cytometry measurements revealed that the TrkA mRNA expression levels (Fig. 5) and the average fluorescence value (Table I) at 24 h post-transfection were significantly reduced in the miRNA-92a mimics transfection group compared with the control group, although no statistical difference was detected at 48 h. However, when the miRNA-92a inhibitor transfection group was compared with its control, the TrkA mRNA expression levels and the average fluorescence value at 48 h post-transfection were significantly reduced, although no statistical difference was identified at 24 h. These results demonstrated that the expression levels of TrkA protein in the cells transfected with miRNA-92a mimics were significantly reduced (5.8% decrease; P<0.01) at 24 h post-transfection compared with the cells in the control group, while the expression levels of TrkA protein in the cells transfected with miRNA-92a inhibitor were significantly elevated (18.2% increase; P<0.01) at 48 h post-transfection compared with the cells in the control group. Overall, the miRNA-92a inhibitor exhibited a greater effect on TrkA protein than the miRNA-92a mimics.

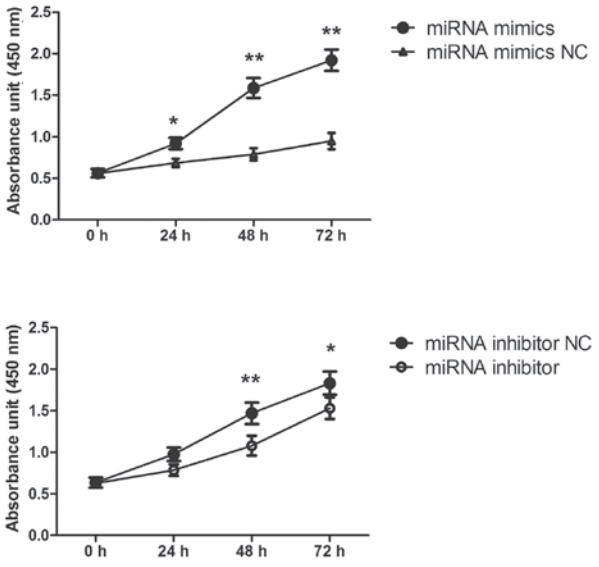

Figure 3. Cell Counting Kit-8 assay and direct cell count revealed that overexpression of miRNA-92a mimics significantly increased the proliferation of BE(2)-M17 human neuroblastoma cells, and overexpression of miRNA-92a inhibitors significantly inhibited the proliferation of the neuroblastoma cells. Data are presented as the mean ± standard deviation (n=6). *P<0.05 and **P<0.01, vs. the respective NC. miRNA, microRNA; NC, negative control.

Discussion

miRNAs are large groups of gene regulatory molecules which influence a number of gene encoding proteins. There is abundant evidence revealing >1,400 types of miRNA important in the pathogenesis of human diseases. One study has shown that miRNA inhibits the dedifferentiation and plasticity of cells in the process of tumor formation through the regulation of protein expression and the cell differentiation process. Furthermore, miRNA affects the cyclical adjustment of tumor cells, the integrity of the genomes, the stress response, apoptosis and metastasis (15).

The miRNA genes are located in introns or non-coding regions of the chromosome and are first transcribed as primary transcripts of 500-3,000 nt termed pri-miRNA. These molecules are then cut to ~70-bp miRNA precursors and then

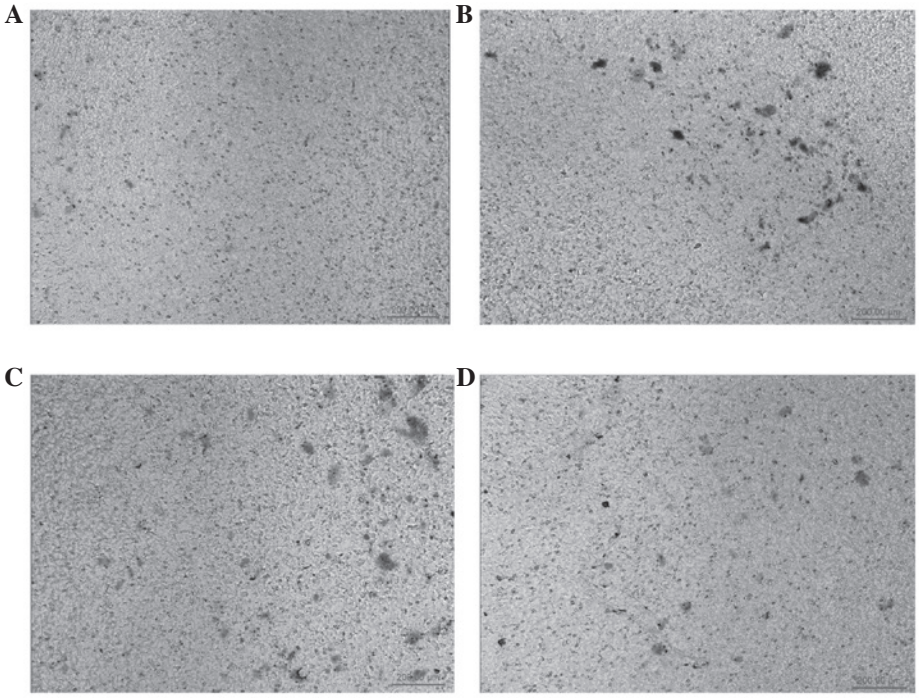

Figure 4. Transwell migration assay. Compared with the miRNA-92a mimics negative control group (A), the miRNA-92a mimics-transfected group (B) exhibited a significantly increased number of cells located across the membrane (P<0.05). Compared with the miRNA-92a inhibitors control group (C) the miRNA-92a inhibitors transfected group (D) exhibited a significantly reduced number of cells across the membrane (P<0.05). Magnification, x200. miRNA, microRNA.

to ~22-bp mature miRNA assembled into RNA-induced silencing complexes to perform RNA interference gene silencing. When the miRNA molecule and the 3'UTR nucleotide of the target mRNA are complementary, the complex may inhibit the translation of the mRNA to protein and may induce the degradation of target mRNA (8,16).

In the present study, double-stranded miRNA mimics, which have greater stability than single stranded ones, were selected to increase miRNA-92a expression levels. The miRNA-92a expression levels significantly increased (P<0.01) with miRNA mimics compared to an NC, achieving a peak 24 h after transfection and then gradually decreasing at 48 and 72 h after transfection. The miRNA-92a expression levels were found to progressively decrease compared with the NC following transfection with miRNA inhibitors. The effective regulation of miRNA-92a expression by transfection indicated that the research was reliable.

Studies have revealed that miRNA may affect a multitude of target genes and that one target gene may be regulated by multiple miRNA molecules (17-19). The miR-17-92 cluster has been considered to be the most effective carcinogenic miRNA, and hundreds of target genes of this cluster have been reported (20-23). One study observed a clear reduction in the expression levels of endogenous p21 mRNA and protein in SK-N-AS 17-5p cluster cells, as well as in SK-N-AS cells transiently transfected with miR-17-5p, although not in those transfected with miRNA-92 (21). Another study demonstrated that miRNA-17-92 is a potent inhibitor of transforming growth factor (TGF)-β signaling. By functioning upstream and downstream of pSMAD2, miRNA-17-92 activation triggers downregulation of multiple key effectors along the TGF-β signaling cascade as well as direct inhibition of the

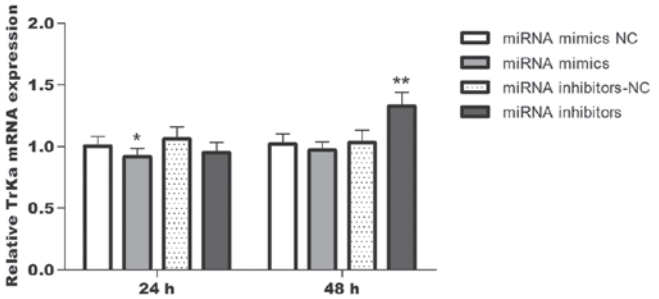

Figure 5. Quantitative polymerase chain reaction analysis of TrkA mRNA at 24 and 48 h after transfection. β-actin served as an endogenous control. Expression levels of TrkA mRNA are relative to the NC (set as 1.0). The TrkA mRNA of the miRNA-92a mimic transfection group was significantly reduced at 24 h, while the TrkA mRNA of the miRNA-92a inhibitor transfection group was significantly increased at 48 h. Results are expressed as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01, vs. the respective NC. miRNA, microRNA; NC, negative control.

TGF-β-responsive gene, but miRNA-92a did not affect the luciferase signals of SMAD2 (9). This suggests that every member of the miRNA-17-92 cluster exhibits its own characteristics, and the signaling pathway and mechanism of miRNA-92a may be different from the miRNA-17-92 cluster, although this requires further investigation.

Neuroblastoma cells have been observed to undergo differentiation and dissipation when TrkA is highly expressed, while cells with low TrkA expression levels exhibit the opposite behavior and become more invasive (3). In the present study, the proliferation and migration of neuroblastoma cells were found to be enhanced when TrkA expression was reduced, while the proliferation and migration

capacity decreased when the TrkA expression was increased in the neuroblastoma cells.

Numerous studies have shown that high expression of TrkA in neuroblastoma tissue is associated with improved clinical characteristics in patients and lower MYCN gene amplification, while patients in the advanced stages of neuroblastoma usually exhibit lower expression levels of TrkA and the tumors cannot be treated with NGF to inhibit differentiation (24-26). The NGF/TrkA signaling pathway is important in promoting neuroblastoma differentiation and natural regression (13,27,28). High expression of TrkA protein in neuroblastoma cells is associated with differentiation and regression (28-30), while lower TrkA expression is associated with increased invasiveness. One study suggested that activated Ras in the NGF/TrkA signaling pathway stimulates nuclear translocation of p53 and induces growth arrest by induction of p21WAF1 in PC12 cells (31). Activation of TrkA induces the phosphorylation and activation of SHC, phoshoinositide 3-kinase and phospholipase Cγ1, which are the primary effectors of Trk activity in NGF-treated PC12 cells (32). Ras/MAPK and AKT are activated downstream of these signaling pathways. Ras sequentially activates a series of kinases, including RAF1, mitogen-activated protein kinase kinase, mitogen-activated protein kinase (MAPK) and ribosomal S6 kinase (RSK) (13). MAPK and RSK translocate to the nucleus to initiate the activation of transcription factors that regulate NGF-inducible genes, leading to survival and neuronal differentiation. Other signaling proteins important for normal biological responses to ligand binding include SH2B/APS, fibroblast growth factor receptor substrate 2 and AKT (32). Further studies may be conducted to investigate whether miRNA-92a regulates the Ras/MAPK signaling pathway.

In the present study, transfection with miRNA-92a inhibitors was found to exert a more marked impact on TrkA protein expression levels than transfection with miRNA-92a mimics. Since the BE(2)-M17 neuroblastoma cell line exhibits MYCN gene amplification, the cell line was hypothesized to have high expression levels of miRNA-92a. Therefore, reducing the expression of miRNA-92a is more efficient than increasing the expression. Furthermore, the expression levels of the target protein were found to be reduced at 24 h after transfection with the miRNA-92a mimics, although these were restored to the levels of the NC after 48 h. This may be considered to be an intracellular adjustment mechanism to correct target protein expression back to normal levels.

Although an association between the expression levels of miRNA-92a and TrkA has been observed, the mechanism and signaling pathway of TrkA expression regulated by miRNA-92a remains unclear. The present study has limitations in that there is not a great quantity of data and the findings have not been replicated in other neuroblastoma cell lines. If a particular solution were found to inhibit or restore the TrkA signaling pathway when the study is repeated, the results may be more convincing.

In conclusion, the present study demonstrated that the biological behavior of neuroblastoma cells was markedly altered when the expression levels of miRNA-92a were elevated or reduced. The proliferation and migration capacity of neuroblastoma cells exhibited a positive correlation with the expression levels of miRNA-92a in tumor cells, and a negative correlation with TrkA protein expression levels. miRNA-92a may affect neuroblastoma cell proliferation and migration capacity by regulating TrkA protein expression levels. This may provide an experimental basis for the treatment of neuroblastoma with miRNA-92a.

Acknowledgements

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