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Original Article

Up-regulation of NT-3 expression by miR-132 via ERK signaling pathway and the potentiation of NB41A3 cell proliferation and migration

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Abstract: The proliferation and migration of neural cells play a crucial role for the repair of neural injury. However, it is difficult for neural cells to overcome scar tissues, making it of critical importance to improve migration and proliferation potency of neural cells. MicroRNA (miR) has been known to be involved in cell growth, proliferation and cell cycle modulation. This study thus investigated the role of miR-132 in neural cell migration. Synthesized miR-132 was transfected into neuroblastoma cell line NB41A3, which was tested for migration and proliferation ability using Transwell assay and clonal formation assay, respectively. Cell apoptosis after transfection was quantified by flow cytometry. The activation of ERK signaling pathway and neurotrophin-3 (NT-3) were detected by Western blotting and enzyme-linked immunosorbent assay, respectively. ERK signaling inhibitor PD98059 was then used to treat cells before transfection, followed by cell migration and NT-3 assays. MiR-132 transfection significantly increased migration and proliferation ability of NB41A3 cells, along with ERK activation and NT-3 up-regulation. The inhibition of ERK signaling pathway suppressed miR132-induced NT-3 potentiation and elevated cell migration/proliferation ability. MiR-132 can facilitate migration and proliferation of NB41A3 cells via ERK-dependent NT-3 pathway.

Keywords: MicroRNA-132, ERK signaling pathway, neurotrohpin-3, neuroblastoma, cell migration and proliferation

Introduction

The incidence of central nervous diseases has increased in recent years, leading to huge economic and social burdens [1, 2]. The repairmen of neural tissues after primary damage has now become a research hotspot for both clinicians and neurologists [3]. One major challenge for repair of neural tissues is the migration of neural cells beyond scar tissues toward distal sites, where neural progenitors initiate *de novo* regeneration [4, 5]. Such complicated process requires the elevation of migration and proliferation ability of neural cells [6]. Neurotrophin (NT) is a protein factor necessary for the growth and proliferation of neuronal cells [7]. Previous study has revealed the important role of NT-3 in stimulating the synthesis of proteins related to neuronal growth, survival, proliferation and maturation, via its specific binding onto receptors in the neural terminal and consequently retrograde transport toward cell body [8, 9]. NT-3 is thus critical for neuronal regeneration and nervous tissue repair [10]. Although the regulatory mechanism of NT-3 has been well established, the upstream pathway of NT-3, however, remains unclear.

MicroRNA (miR) is a kind of small RNA molecule containing 20~23 nucleotides. Multiple biological functions of miR have been found [9] in almost all kinds of cells from lower virus to higher animals [10]. MiR plays a vital role in various processes including cell growth [11], proliferation [12] and cell cycle regulation [13]. Its role in neural cells still needs further investigation [14]. Currently nearly 30,000 different kinds of miR have been identified regarding their unique functions on signal transduction [15], cell growth [16], proliferation [17], cell cycle mediation [18], apoptosis [19] and autophagy

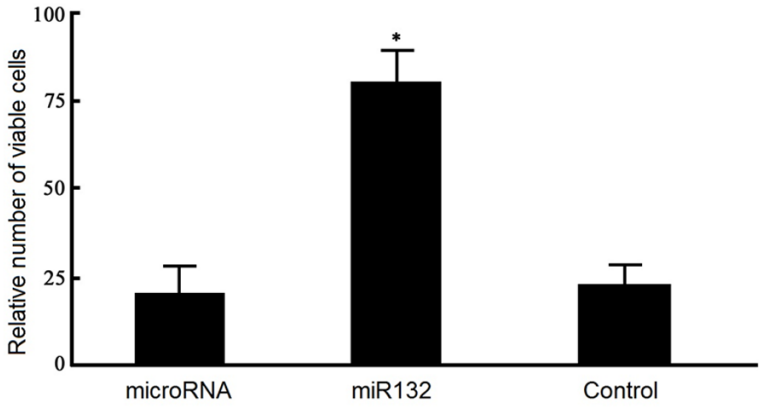

Figure 1. NB41A3 cell viability. *, P<0.05 compared to microRNA control group.

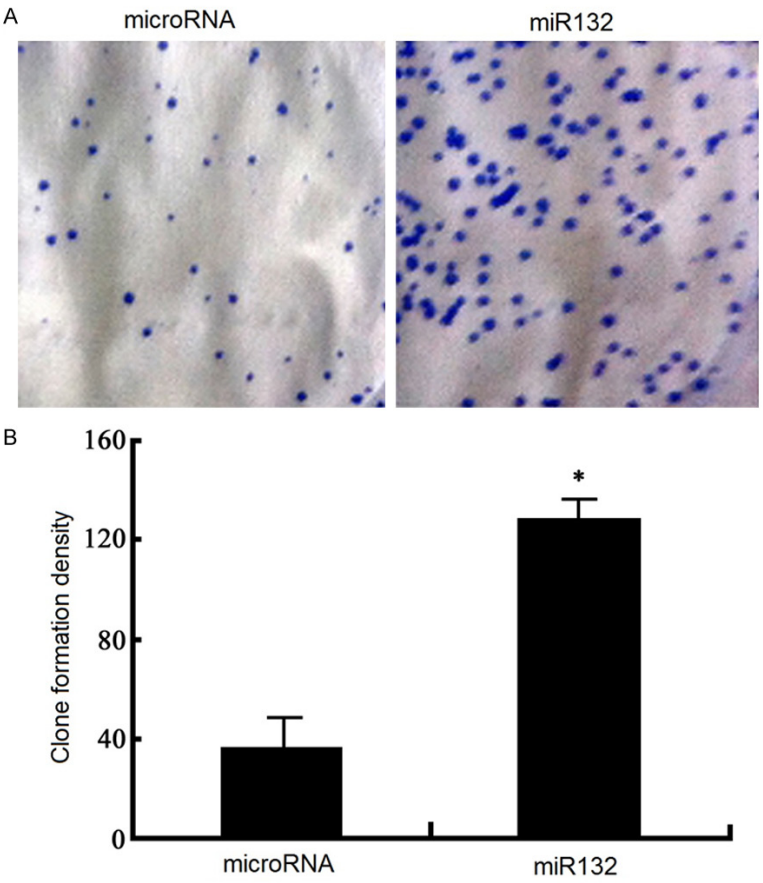

Figure 2. Clone formation ability of NB41A3 cell. A. Representative images showing clone formation of controlled (left) and miR-132 transfected (right) cells. B. Quantitative results of clone formation density. *, P<0.05 compared to microRNA control group.

[20]. MiR-132 has been implicated in the repair of neural tissue injuries [21], but leaving its role in proliferation and maturation of neural cells largely unknown.

This study thus investigated the molecular mechanism of miR-132 on neuroblastoma cell line NB41A3 model, in order to provide more evidences for clinical treatment of neural diseases.

Materials and methods

Cell culture and transfection

NB41A3 cells (ATCC, US) were resuscitated in 37°C waterbath until completely thawing. Cell preservation buffer was removed by centrifugation. Cells were re-suspended into DMEM medium containing 10% FBS (Gibco, US), and were cultured in a 37°C humidified chamber perfused with 5% CO_2.

Based on previous reports of miR-132 in other tumor cell lines [1] and preliminary study. 20 μM miR-132 (Forward, 5'-CGGCT GTAAA ACGAC AGT-3'; Reverse, 5'- GCTAC AGGAA ACATG ACC-3') and controlled nonsense miR were used to transfect NB41A3 cells. In brief, one day before transfection, cells were seeded into 96-well plate at 10^3 cells each to reaching 85% density. MiR-132 was mixed with Lipo200 reagent (Invitrogen, US) diluted in DMEM medium. After 10-min incubation, transfection mixture was added into each well, followed by continuous incubation at 37°C.

MTT assay

Proliferation of NB41A3 cells was quantified by MTT assay kit (Dingguo, China) following the manual instruction. In brief, cells were seeded into 96-well plate in triplicates. 20 μL MTT reagents (20 mg/mL) were added to each well for 37°C incubation for 6 hours. The reaction was quenched by PBS

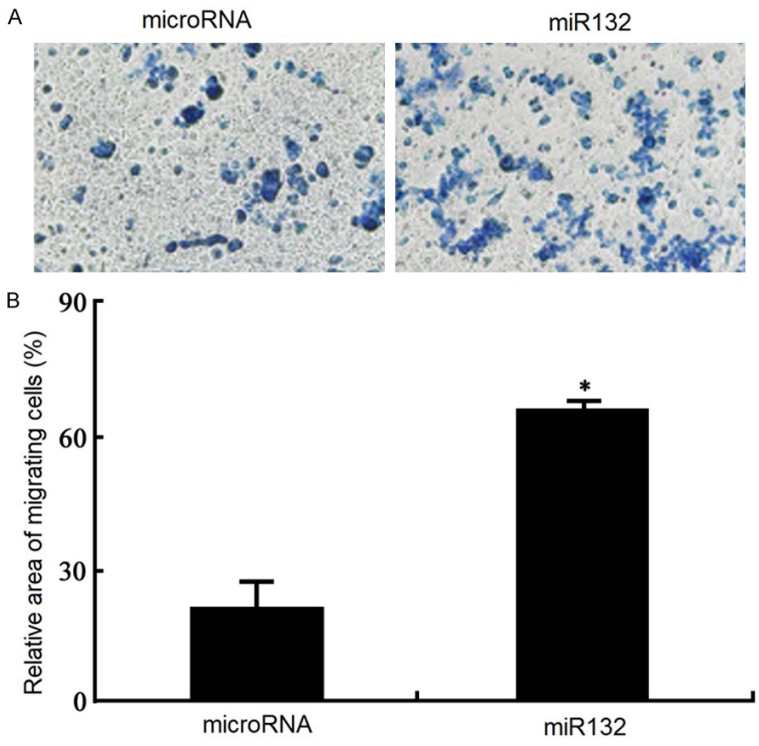

Figure 3. NB41A3 cell migrations. A. Representative images of migrated cells in Transwell chamber; B. Relative area occupying by migrating cells. *, P<0.05 compared to microRNA control group.

washing for 6 times. 0.2 mL DMSO was then added to teach well for 20-min development. A microplate reader was then used to quantify absorbance value at 492 nm.

Cell apoptosis assay

NB41A3 cells after transfection were examined for cell apoptosis using phosphatidyl serine method. In brief, cells were re-suspended (10^5 per mL) and mixed with Annexin V and Annexin V-EGFP (Beyotime, China) solution. After 20-min dark incubation, cells were loaded for flow cytometry assay to detect the percentage of phosphatidyl serine flipping cells.

Enzyme-linked immunosorbent assay (ELISA)

Transfected cells were collected for supernatants of culture medium. NT-3 expression level was detected by ELISA kit (BD, US) following the manual instruction.

Western blotting

Total proteins were extracted from transfected NB41A3 cells. Western blotting was deployed to quantify ERK and p-ERK levels as previous reported [21].

Clone formation assay

NB41A3 cells were examined under clone formation assay according to previous documentation [22]. In brief, cells in all groups were firstly counted and diluted serially. Cells were then inoculated into agarose gel and cultured in 37°C chamber for 2 weeks. Culture medium was discarded until clone formation. Cells were fixed in method at room temperature for 15 min, and were stained in Giemsa dye for 30 min. The number of clones was counted under an inverted microscope.

Transwell assay

Cells from all groups were tested for the migration ability using Transwell assay as previously documented [22]. In brief, the upper surface of Transwell plate was pre-coated with solid medium. The bottom of plate was filled with culture medium containing chemotactic factors. NB41A3 cells after transfection were seeded on the top layer. After 48-hour incubation, the number of cells in the lower phase was counted.

Statistical analysis

SPSS 18.0 software package was used to process all collected data, which were presented as mean ± standard deviation (SD). The comparison across groups was performed by analysis of variance (ANOVA). A statistical significance was defined when P<0.05.

Results

MiR-132 facilitated NB41A3 cell growth

MTT assay showed the facilitation of cell growth after miR-132 transfection when compared to controlled cells (P<0.05, **Figure 1**). As no difference existed between blank control cells and those cells with nonsense microRNA trans-

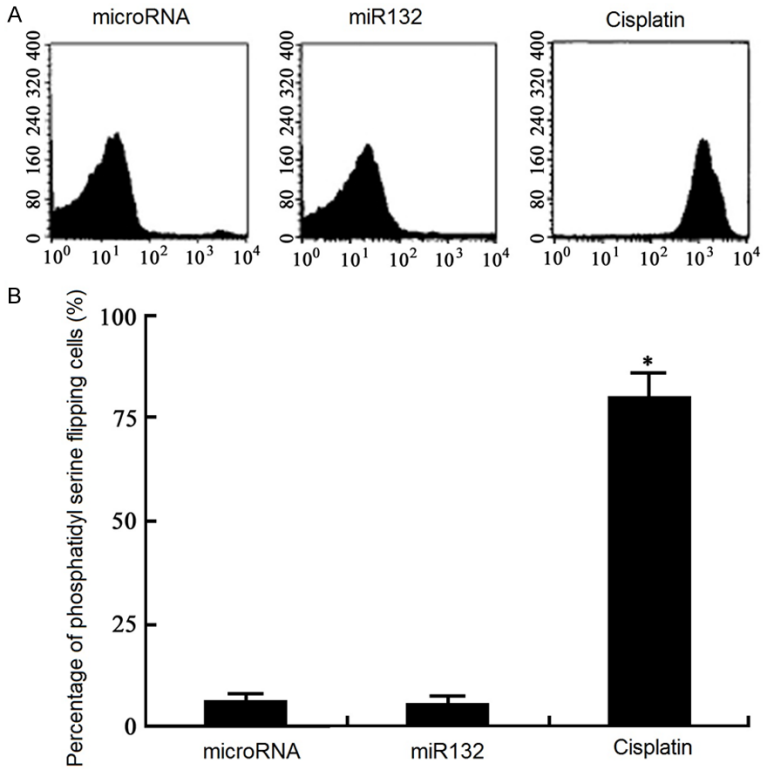

Figure 4. NB41A3 cell apoptosis. A. Flow cytometry data; B. Percentage of cells with phosphatidyl serine flipping. *, P<0.05 compared to microRNA control group.

fection, blank control group was omitted in the following study.

Elevated NB41A3 cell proliferation after miR-132 transfection

As shown in **Figure 2**, NB41A3 cells transfected with miR-132 had relatively stronger ability in clone formation, when compared to microRNA controlled group (P<0.05, **Figure 2**).

MiR-132 potentiated migration ability of NB41A3 cells

Transwell assay showed significantly facilitated migration ability of NB41A3 cells after miR-132 transfection, as compared to microRNA control group (P<0.05, **Figure 3**).

MiR-132 did not induce NB41A3 cell apoptosis

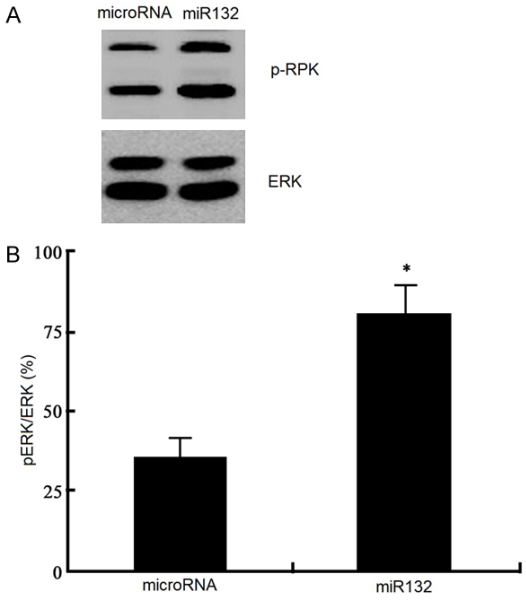

Figure 5. ERK signaling pathway. A. Western blotting bands showing p-ERK and ERK protein levels; B. p-ERK/ERK ratio. *, P<0.05 compared to microRNA control group.

As shown by flow cytometry, miR-132 transfection did not cause the flipping of phosphatidyl serine, in contrast to those cell transfected with cisplatin, which worked as the positive control (P<0.05, **Figure 4**).

MiR-132 activated ERK signaling pathway in NB41A3 cells

Western blotting results showed significantly elevated ERK phosphorylation level, in contrast to those in control group (P<0.05, **Figure 5**), suggesting the activation of ERK signaling pathway by miR-132.

MiR-132 increased NT-3 expression in NB41A3 cells

ELISA results showed elevated NT-3 level in the culture supernatants from miR-132 transfected NB41A3 cells (P<0.05, **Figure 6**), suggesting the potentiation of NT-3 expression by miR-132.

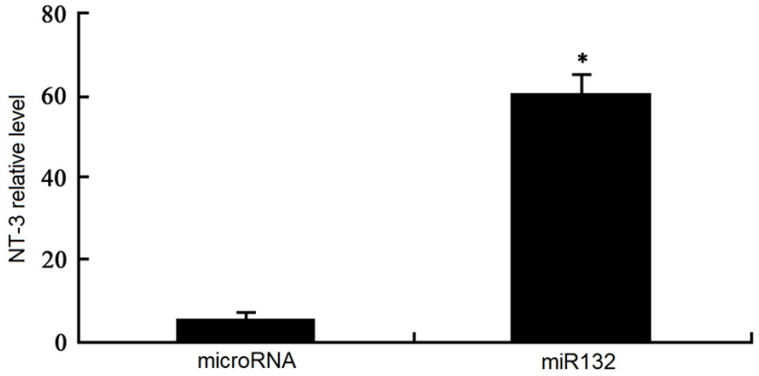

Figure 6. NT-3 expression levels. *, P<0.05 compared to microRNA control group.

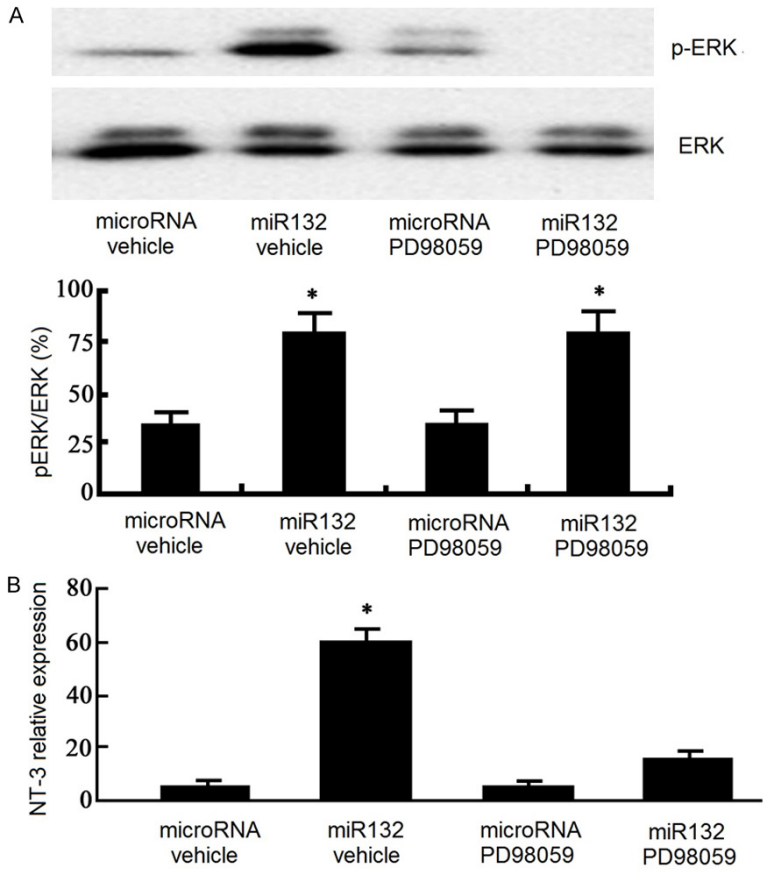

Figure 7. NT-3 expression and ERK signaling pathway modulated by miR-132. A. Western blotting bands showing p-ERK level in cells after applying ERK inhibitor (upper panel), along with quantitative data (lower panel). B. NT-3 relative level. *, P<0.05 compared to control group.

ERK inhibition suppressed miR-132-induced NT-3 expression

As shown in **Figure 7**, ERK signaling pathway inhibitor PD98059 significantly suppressed miR-132-induced ERK activation (P<0.05, **Figure 7A**). Moreover, miR-132- induced NT-3 potentiation was also significantly decreased by PD98059 (P<0.05, **Figure 7B**). These results supported the ERK-dependent NT-3 up-regulation by miR-132.

ERK signal blocking inhibited cell migration and proliferation

The application of PD98059 significantly suppressed cell proliferation and migration that were induced by miR-132 transfection (P<0.05, **Figure 8**). All these results clearly demonstrated that miR-132 can induce cell proliferation and migration via ERK signaling pathway.

Discussion

Various neural injuries severely affect patients' life quality and brings huge economic for the society [17]. Therefore the systematic study of neural regeneration is of critical importance for both neural development and clinical treatment [18].

As an important miR molecule, the molecule mechanism of miR-132 in mediating neural cells still requires comprehensive illustration [19]. This study thus investigated the modulatory mechanism of miR-132 in neural cells using neuroblastoma cell line NB41A3 as the model. Our results found potentiated cell migration and proliferation abilities after miR-132 transfection but without cell apoptosis. MiR-132 also elevated NT-3 expression levels of neural cells. Such potentiation effect is probably dependent on ERK signaling pathway as its specific inhibitor can depress NT-3 secretion, as well as NB41A3 cell migration

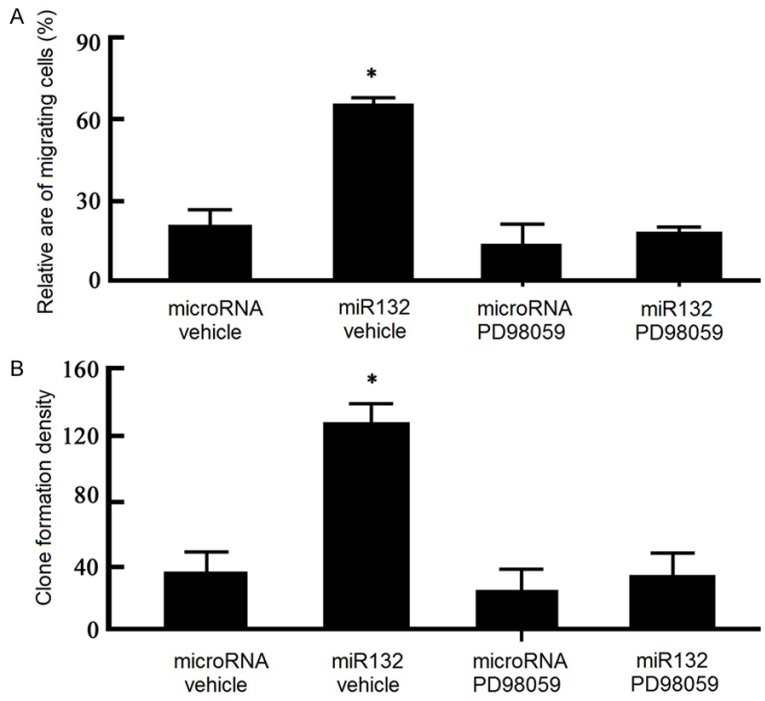

Figure 8. Cell migration (A) and clone formation (B) abilities after ERK inhibitor. *P<0.05 compared to the control group.

and proliferation. These results were consistent with previous studies [20].

Our study for the first time reported the enhanced neural cell migration and proliferation but not cell apoptosis, in addition to ERK activation and NT-3 secretion after miR-132 transfection. The inhibition of ERK signaling pathway depressed miR-132-induced NT-3 expression and cell migration/proliferation. These results collectively suggested the potency of miR-132 as one novel molecular marker and drug target for treating neural injuries. The modulation of miR-132 level in neural cells thus may improve proliferation and migration potency of neural cells.

Our results had certain differences compared to previous studies in which over-expression of miR actually facilitated cell apoptosis [21]. No apoptosis, however, has been discovered in our study. In contrast, miR-132 actually facilitated cell growth and proliferation. We also for the first time reported the enhancement of neural cell migration induced by miR-132. Such inconsistency may be attributed to the difference of cell types and miR molecules [22]. As certain miR, such as miR-126 and miR-143 exert pro-apoptotic roles [23] while others (such as miR-107 and miR-192) have anti-apoptotic functions [24]. Moreover, differential expressional level of miR across different tissues [25] may also cause the various biological functions of miR.

Certain limitations also existed in this study, as only cell model was used to demonstrate the biological function of miR-132 in regulating neural cell growth, proliferation and migration, but without clinical samples from neural injured patients as supporting evidence to show the relationship between neural cell growth and miR-132 [26, 27]. In future, animal models with neural injury [28] can be used to substantiate the molecular model suggested by our research. Furthermore, the correlation between miR-132 expression level and severity of neural injuries can be examined in patients, along with small interference RNA (siRNA) approach to further elucidate the molecular mechanism of miR-132 in neural cells.

In summary, this study showed the potentiation of NB41A3 cell migration and proliferation by miR-132 via ERK-dependent NT-3 secretion. This result provided evidences and suggested the potency of miR-132 as the molecular marker for neural injury and regeneration.

Acknowledgements

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Disclosure of conflict of interest

None.

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