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Induction Function of miR-126 in Survival and Proliferation in Neural Stem Cells

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Data Collection B
Statistical Analysis C
Data Interpretation D
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Background: The aim of this study was to investigate the potential function of miR-126 in neural stem cells (NSCs).

Material/Methods: Expression level of miR-126 was detected by quantitative real-time PCR (qRT-PCR). MiR-126 overexpression was established by transfecting miR-126 mimics into human NSC lines (HB1.F3 and HB1.A4 cells). Its effects on cell proliferation were studied using cell-counting kit-8 (CCK8) assay, colony formation assays. Flow cytometry was performed to evaluate the effect of miR-126 on cell survival.

Results: CCK8 assay and colony formation assay showed that overexpression of miR-126 promoted cell proliferation and increased colony numbers in HB1.F3 and HB1.A4 cells. The flow cytometry confirmed the results that miR-126 inhibited cell apoptosis.

Conclusions: MiR-126 promoted the proliferation and survival of NSCs.

MeSH Keywords: Cell Proliferation • MicroRNAs • Neural Stem Cells

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Background

Neural stem cells (NSCs) are vitally important elements in the brain [1–3]. They give rise to all the other major brain cell types, which include oligodendrocytes, astrocytes, and neurons. However, molecular pathways, which are complicated and control the proliferation and differentiation of NSCs, have been characterized incompletely to date. There is multiple indirect evidence suggesting a critical role of miRNAs in NSCs [4–7]. Post-transcriptional control mediated by miRNAs was identified as a further important regulation level, especially in neural proliferation where the action of these negative modulators of gene expression is pervasive [7]. A dynamic change of miRNA expression is observed during neurulation [8]. Altered miR expression has also been detected in the maternal serum of human pregnancies [9].

MiRNAs are small single-stranded non-coding RNAs molecules which participate in the transcriptional regulation of eukaryotic genes and produce biological effects through inhibiting translation or destabilizing target mRNAs [10–12]. MiRNAs act as vital adjusters in multiple biological processes, including cell metabolism, differentiation, proliferation, and apoptosis [13,14].

miR-126 is highly enriched in endothelial cells and previous studies found that miR-126 plays critical roles in vascular integrity and can promote angiogenesis during embryonic development [15–18].

Recently, Hu et al. reported that the expression level of miR-126 was downregulated after spinal cord injury and that it can promote angiogenesis and attenuate inflammation in rats [17]. However, the effects of miR-126 in NSCs is still unclear; therefore, we intended to study its role in NSCs proliferation and survival.

Material and Methods

Cell Culture

The immortalized human NSC lines HB1.F3 and HB1.A4 were maintained with Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum (FBS).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cell lines with a mirVana™miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The expression level of miR-126 was detected by qRT-PCR according to the Taqman miRNA Assays protocol (Applied Biosystems) and normalized by U6 small nuclear RNA (RNU6B; Applied Biosystems) with the $2^{-\Delta CT}$ method.

Lentiviral transfection for stable expression clones

LV3-pGLV-H1-GFP+Puro plasmids with miR-126 mimics and negative control (LV-miR-126 and LV-miR-NC) were purchased from GenePharma (Shanghai, China). HB1.F3 and HB1.A4 stably expressing miR-126 were established by transfecting Lentivirus according to the manufacturer's instructions. The control clones were produced by the same method. Finally transfection efficiency was monitored by qRT-PCR.

Cell proliferation assay

Proliferation assays were performed using CCK8 (Dojindo, Japan). Cells were plated in 96-well plates in triplicate at approximately 1000 cells per well and cultured in the growth medium. Cells were then treated with the indicated reagent and the numbers of cells per well were measured by the absorbance (450 nm) of reduced water-soluble tetrazolium salt (WST) at the indicated time points.

Colony formation

Cells were plated into 6-well plates at a density of 1000 cells/well in 2 ml medium. The cells were incubated for 14 days. The colonies were observed using a phase-contrast microscope at a magnification of 4× (we counted the colonies containing at least 50 cells).

Flow cytometric analysis for apoptosis

Cells were transfected with miR-126 mimics or their respective controls and were harvested after transfection at 48 hours and then marked with the AnnexinV/PI double staining kit (BD Biosciences, USA) according to the manufacturer's instructions. Flow cytometry was used to assess the apoptotic cells in triplicates and all assays were repeated at least 3 times.

Statistics

IBM SPSS 19.0 statistical software was used to analyze the data. Student's t test or one-way ANOVA were used for analysis when appropriate. Results were considered to be statistically significant when $P<0.05$.

Results

NSCs proliferation was promoted by miR-126

To further study the potential function of miR-126 in NSCs, HB1.F3 and HB1.A4 were stably transfected with mimics or negative control (NC). The CCK8 assay was performed on the transfected cell lines. As indicated in Figure 1A, miR-126 mimics

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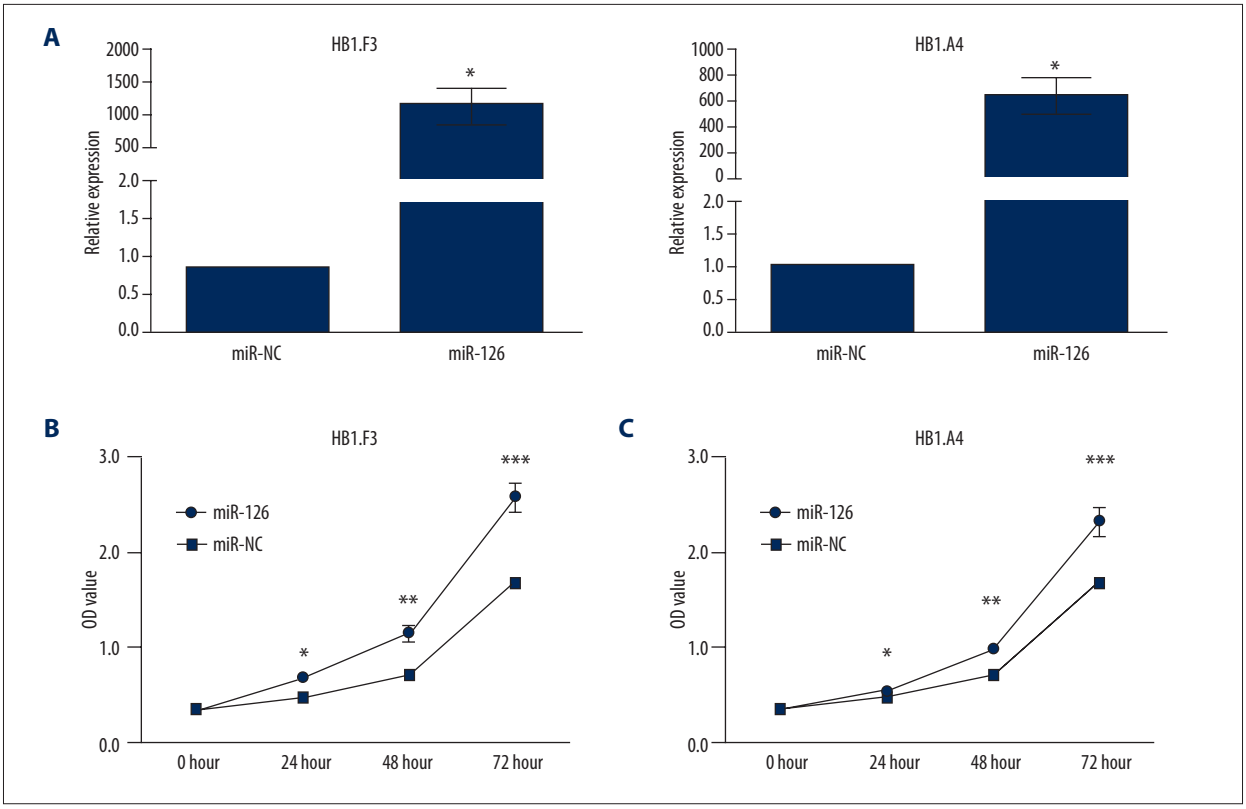

Figure 1. CCK8 assay to test the function of miR-126. Overexpression effects of miR-126 mimics in HB1.F3 and HB1.A4 cell lines (**A**). Up regulation of miR-126 promoted HB1.F3 (**B**) and HB1.A4 growth (**C**).

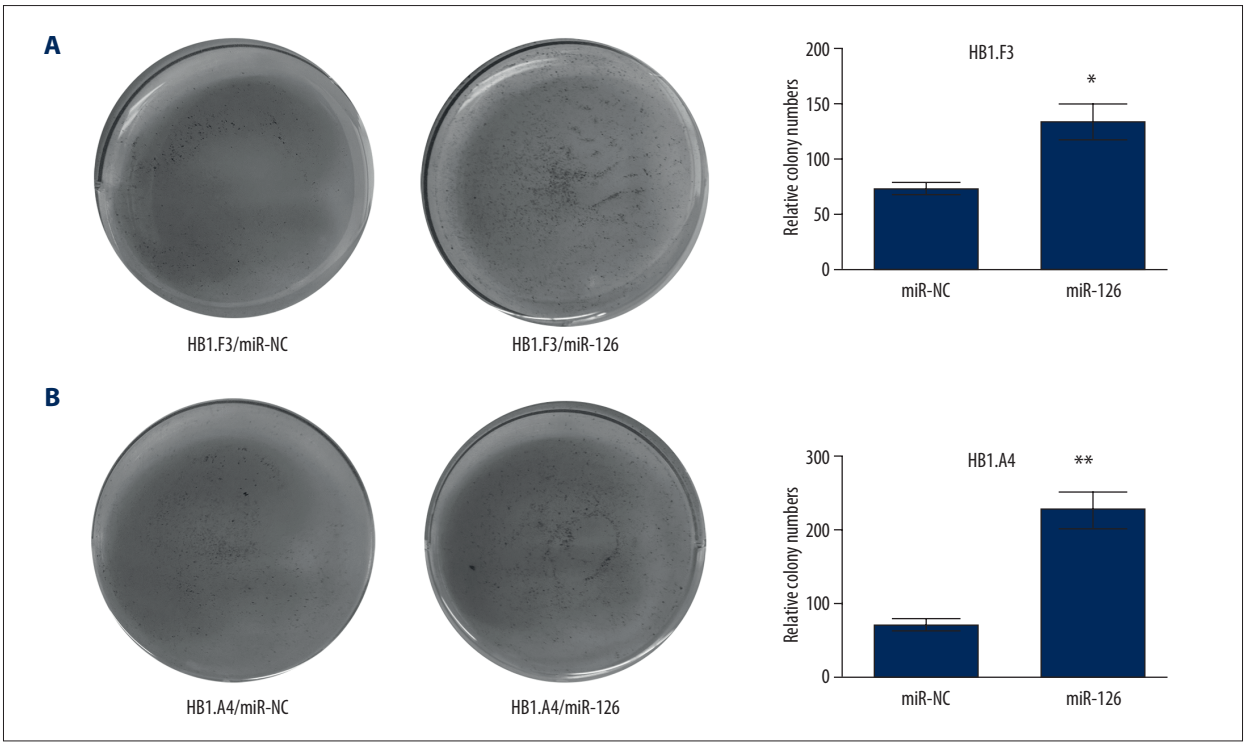

Figure 2. Colony formation assay to validate the function of miR-126. Up-regulation of miR-126 promoted HB1.F3 (**A**) and HB1.A4 growth (**B**).

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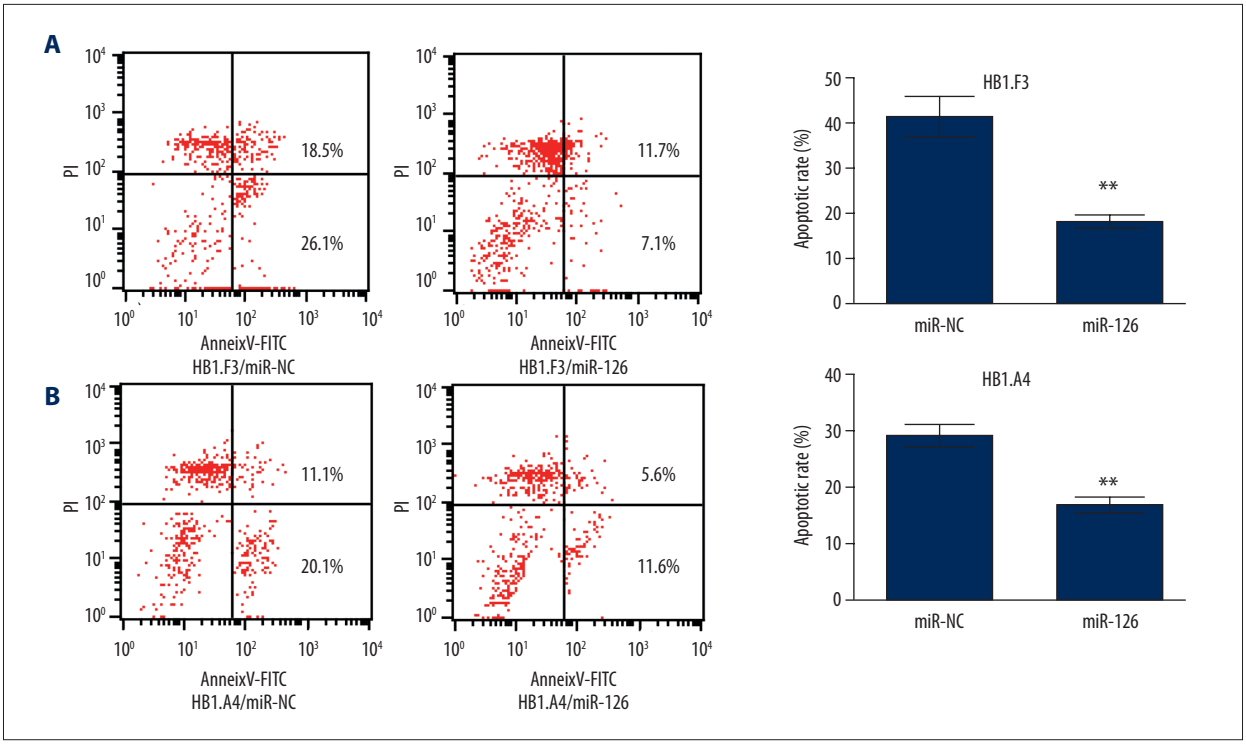

Figure 3. Flow cytometric analysis to test the role of miR-126 on cell apoptosis. miR-126 inhibited HB1.F3 (**A**) and HB1.A4 growth (**B**) apoptosis.

showed good overexpression effects both in HB1.F3 and HB1.A4 and both cell lines overexpressing miR-126 showed marked cell growth increase (Figure 1B, 1C).

To further confirm the function of miR-126 on cell proliferation and growth, soft agar colony formation assay was performed. As shown in Figure 2A, 2B, the colony formation was significantly increased by miR-126 compared with the negative control group. Results demonstrated that the promotion effect of miR-126 was significant.

miR-126 inhibited NSCs apoptosis

We next investigated the function of miR-126 in the regulation of cell apoptosis by flow cytometry. Cells overexpressing miR-126 displayed a significant decrease in the apoptotic rate when compared with the control group (Figure 3A, 3B). These results suggest that miR-126 may inhibit apoptosis in NSCs, which may contribute to the growth induction features of miR-126.

Discussion

MiRNAs have a vital role in regulating cellular activities, including proliferation and differentiation [8,12,19,20]. They can profoundly regulate the expression of massive target genes that encode proteins, which may finally lead to the change of biological function. miR-126, which is located in intron 7 of the EGF-like domain 7 (EGFl7) gene [21], is a microRNA that is highly enriched in endothelial cells [22] and regulated by the transcription factors Ets-1 and Ets-2 in endothelial cells [23]. Previous studies found that miR-126 promoted angiogenesis during embryonic development and after injury by targeting SPRED1 and PIK3R2 [17]. Also, knockdown of miR-126 resulted in delayed angiogenic sprouting, collapsed blood vessels, widespread hemorrhages, and partial embryonic lethality during zebrafish and mouse embryogenesis [24,25]. It is also involved in cell growth regulation in several organs, such as colorectal cancer, gastric cancer, and liver carcinoma, by regulating multiple target genes, including insulin receptor substrate, p85, PI3K, akt, and Crk [26–29]. Hu et al. reported that miR-126 plays an important role in angiogenesis and inflammation after contusion spinal cord injury in rats [17].

Conclusions

In the current study, we verified that when the expression level of miR-126 was overexpressed by mimics transfection, the proliferation and survival of NSCs were both significantly promoted. CCK8 assay and colony formation assay both demonstrated that miR-126 can induce the proliferation of NSCs. Apoptosis assay showed that miR-126 has inhibits apoptosis in NSCs. Although the mechanism is not yet completely understood, our study provides further evidence in this area.

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