

Original Research Article

Expression of MiR-9 promotes proliferation, migration and differentiation of human neural stem cells

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Abstract

Purpose: To investigate the effect of miR-9 on the proliferation, differentiation and migration of human neural stem cells (NSCs).

Methods: The expression of miR-9 was investigated by quantitative real-time polymerase chain reaction (RT-PCR). Cell proliferation was assessed by cell counting kit-8 (CCK8) assay, while cell migration was studied by Transwell assay. The effect of miR-9 on differentiation of NSCs was investigated by western blot analysis of key differentiation marker proteins. Protein expression was determined by western blotting.

Results: Transfection and over-expression of miR-9 in NSCs significantly enhanced the proliferation of NSCs ($p < 0.05$) in a time-dependent manner, as was evident from CCK8 assay data. MiR-9 over-expression caused down-regulation of Nestin and SOX-2, and up-regulation of Tuj-1 and MAP-2. The migration of NSCs was 37 % in the cells transfected with empty vector, compared to 68 % in the cells transfected with miR-9. This effect of miR-9 on cell migration was accompanied by up-regulation of matrix metalloproteinase 9 (MMP-9) and matrix metalloproteinase 2 (MMP-2).

Conclusion: These results show that miR-9 promotes the proliferation, differentiation and migration of NSCs, and thus may be an important drug target for the generation of NSCs.

Keywords: Neural stem cells, MicroRNA, Mir-9, Migration, Differentiation, Proliferation

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INTRODUCTION

Neural stem cells (NSCs) are basically undifferentiated cells with the capacity to divide and self-renew. They also develop into neuronal and glial cells [1-3]. Although (NSCs have received considerable attention and have been thoroughly studied, the molecular mechanisms involved in their proliferation and differentiation have not been thoroughly studied. MicroRNAs are small RNA molecules that do not code for

proteins [4,5]. They have been reported to play diverse roles. It is believed that the expressions of thousands of genes in humans are regulated by microRNAs. The main processes regulated by microRNAs involve the proliferation and progression of several types of cells [6].

Recently, it was reported that miRNAs are involved in the differentiation of mouse embryonic stem cells (ES). Studies have shown that R-124 (one of the miRNAs) is highly

expressed during differentiation of ES, and that it promotes the differentiation of embryonic tumour P19 cells of mice *in vitro* [7,8]. Similar to miR-124, miR-9 has also been reported to be upregulated during the differentiation of several mouse neural stem cells [9].

In the present study, the expression of human NSCs was studied, as well as the effect of miR-9 over-expression on the proliferation, differentiation and migration of human NSCs. This was with a view to elucidating the potential of miR-9 in improving the proliferation and differentiation of NSCs.

EXPERIMENTAL

Human neural stem cells [Gibco® Human Neural Stem Cells (H9-Derived)] were obtained from Thermo Fisher. The cells were kept in DMEM F12 medium containing L-glutamine (1 mM), N₂ supplement (Gibco-BRL), 20 ng/mL each of EGF and FGF-2, and 50 ng/mL heparin. To induce cell differentiation, the cells were cultured in DMEM F12 medium with N₂ supplement containing 1 μM retinoic acid equivalent to 0.5 % retinoic acid.

Transfection

The upstream and downstream flanking sequences of miR-9 were amplified with genomic PCR. This was followed by cloning in pMSCv-pur vector. Thereafter, the vector containing miR-9 and the empty vector (negative control) were transfected into NSCs cells with help of FuGENE HD (Promega) as per the manufacturer's protocol. The transfection efficacy was checked by 5'-FAM labelled miR-9 using fluorescence microscopy.

Assessment of expression by quantitative RT-PCR

For isolation of RNA, RNeasy RNA isolation kit was used as per the instructions of the manufacturer. Thereafter, cDNA was synthesized with the help of RevertAid cDNA synthesis kit (Fermentas) following the manufacturer's protocol. In the RT-PCR assay, the cDNA was diluted 20 times and qRT-PCR was carried out in 3 replicates in ABI StepOne Real time using SYBR Green Master Mix (Fermentas). Relative quantification method ($\Delta\Delta$ -CT) was employed to determine quantitative variation in the samples. GAPDH was used as positive control.

Cell proliferation assay

The viability of the human NSCs, CCK-8 assay was used. The cells were collected at 24, 48, 72

and 96 h after transfection with miR-9 mimics or empty vector, and subjected to CCK-8 assay. Cell viability was assayed by cell numbering kit-18 (Dojindo, Japan) in accordance with the manufacturer's guidelines.

Cell migration assay

Transwell assay was used to investigate the effect of miR-9 on NSC migration. The cells were seeded in twenty-four well plates using polycarbonate membranes with 8-μm pores. The NSCs in DMEM F12 medium (100 μL) were placed at a density of 1×10^6 cells/mL in the upper chamber of the transwell assembly, while 600 μL of DMEM F12 medium supplemented with FBS (1 %) and SCF (100 ng/mL) was placed in the lower chamber. The cells were then incubated for 10 h at 37 °C. Non-migrating cells were removed by scrapping the upper surfaces of the membranes and washed in PBS. The membranes were thereafter fixed and stained with haematoxylin and eosin (H & E).

Western blotting analysis

Total protein from the neural stem cells was isolated in RIPA lysis buffer. The proteins were quantified by BSA protein assay kit, and equal amounts of protein extract from each group (miR-9 and empty vector transfected NSCs) were run on SDS-PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at room temperature for 1h. Thereafter, the membranes were incubated with appropriate primary antibodies overnight at 4 °C. This was followed by washing in washing buffer and incubation for 1 h with the secondary antibody. The protein bands of interest were visualized by an ECL Advanced Western Blot Detection Kit.

Statistical analysis

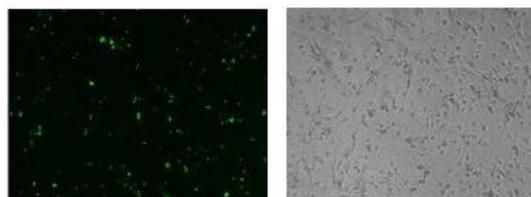
The experiments were carried out in triplicate and the results are presented as mean ± SD. Student's t-test was used for statistical analysis using GraphPad Prism software, version 7 (GraphPad Software Inc, La Jolla CA, USA). Statistical significance was assumed at $p < 0.01$.

RESULTS

Transfection of NSCs and expression of miR-9

It was observed that the expression of miR-9 was about 6.3 times higher in the miR-9 transfected cells, when compared to NSCs transfected with

empty vector (Figure 1). The transfection efficacy was further checked by fluorescence microscopy and the results showed 76 % transfection (Figure 2). Given these successful transfection results, further experiments were designed accordingly.



Effect of miR-9 on proliferation of NSCs

The effect of miR-9 transfection on proliferation of NSCs was evaluated through CCK8 assay. The results showed that transfection of NSCs with miR-9 led to significant and time-dependent enhancement of the proliferation of NSCs (Figure 3).

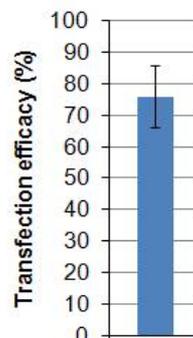


Figure 2: Transfection efficacy via fluorescence microscopy. Each experiment was carried out in triplicates, and results are expressed as mean ± SD

Effect of miR-9 on the differentiation of NSCs

The results of western blot analysis showed that miR-9 over-expression in NSCs caused significant down-regulation of Nestin and SOX-2, and significant increases in the expressions of Tuj-1 and MAP-2 (Figure 4). These results show that miR-9 promoted the differentiation of NSCs.

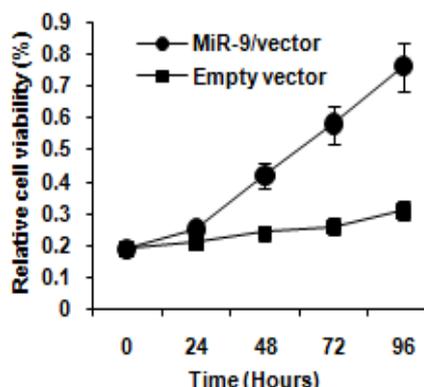


Figure 3: Relative cell viability of NSCs transfected with miR-9 or empty vector. The experiments were carried out in triplicates, and results are expressed as mean ± SD

Effect of miR-9 on the migration of NSCs

Results from transwell assay showed that over-expression of miR-9 in NSCs enhanced their capacity to migrate. There was 32 % cell migration in the NSCs transfected with empty vector, relative to 68 % cell migration in the NSCs transfected with miR-9-containing vector (Figure 5). To further validate these results, it was observed that the expressions of MMP-2 and MMP-9 were upregulated in the NSCs transfected with miR-9 (Figure 6). Taken together, these results clearly indicate that miR-9 enhanced the migration of NSCs.

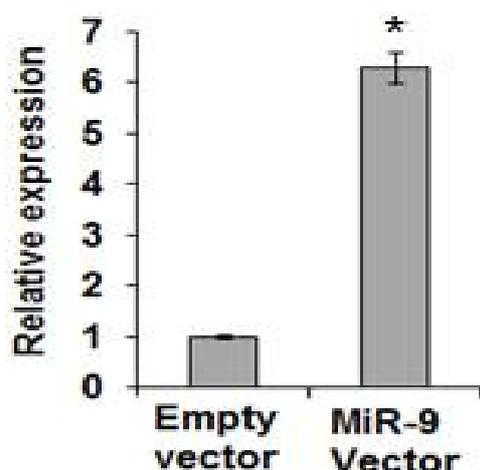


Figure 1: Relative expression of miR-9 in miR-9-transfected NSCs, and empty vector-transfected NSCs. Results are expressed as mean ± SD of three replicates; * $p < 0.05$ relative to empty vector-transfected NSCs

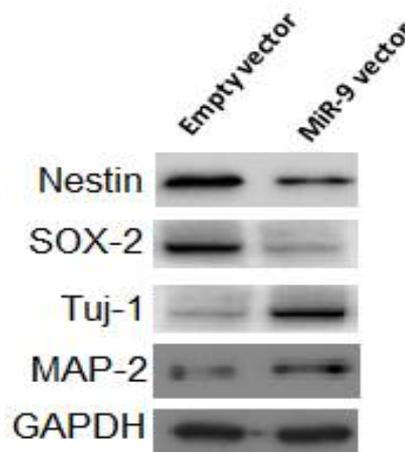


Figure 4: Western blot showing the expressions of Nestin, SOX-2, Tuj-1 and MAP-2 in empty vector- and miR-9-transfected NSCs

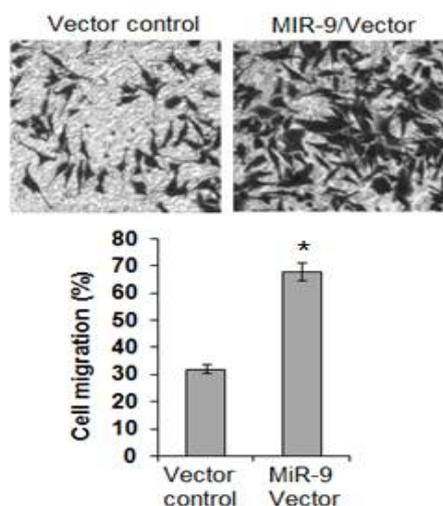


Figure 5: Cell migration capacity of NSCs transfected with empty vector or miR-9 as determined by transwell migration assay. Each experiment was carried out in triplicate, and the results are expressed as mean \pm SD; * $p < 0.05$

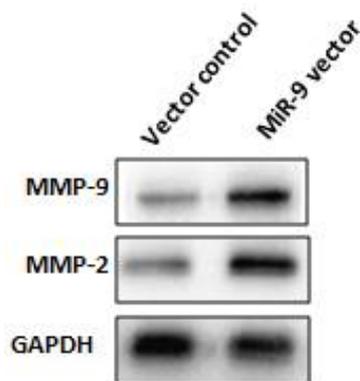


Figure 6: Western blots showing the expressions of MMP-9 and MMP-2 in empty vector- and miR-9-transfected NSCs

DISCUSSION

Human NSCs are self-renewing, multipotent cells capable of giving rise to the main phenotypes of the nervous system [10]. Several studies have been carried out on the potential clinical applications of NSCs [11]. It has been reported that NSCs could be beneficial in the treatment of neurological disorders such brain trauma and spinal cord injury [2]. Whereas the functional properties of NSCs have been studied to some extent [13], the mechanisms that govern their renewal, differentiation and migration have not been fully explored.

MicroRNAs have been reported to play vital roles in a diversity of cellular processes by regulating the expression of a number of genes. They are considered as therapeutic targets for the treatment of cancer, and they are currently

thought to be important for understanding the mechanism behind the renewal and differentiation of NSCs [14,15]. MiR-9 has been previously reported to be significantly expressed in NSCs. In the current study, the effect of miR-9 over-expression on proliferation, differentiation and migration of NSCs was investigated. The results showed that miR-9 over-expression in NSCs enhanced their proliferation in a time-dependent manner. These results are in agreement with a previous study in which miR-9 was found to regulate the proliferation of cancer cells [16]. In addition, it was observed that miR-9 over-expression in NSCs promoted their differentiation by inducing changes in the expression of several marker proteins for cell differentiation: the protein expressions of Nestin and SOX-2 were decreased, while the expressions of Tuj-1 and MAP-2 were significantly increased.

These observations suggest that miR-9 probably regulated the differentiation of NSCs by controlling the expressions of these proteins. These findings are in agreement with previous reports in which microRNAs were shown to regulate the expressions of Nestin, SOX-2, Tuj-1 and MAP-2 [17]. Results from transwell migration assay revealed that miR-9 increased the migration potential of NSCs. The increase in migration was also associated with enhanced expressions of MMP-9 and MMP-2. Taken together, these results provide new and useful insights for understanding the mechanisms that control NSC proliferation and differentiation.

CONCLUSION

The findings of this study demonstrate that miR-9 regulates the proliferation, differentiation and migration of NSCs. These results are considered helpful for unravelling some of the molecular mechanisms that regulate the proliferation and differentiation of NSCs. Thus, further studies on miR-9 will enhance knowledge on the molecular mechanisms involved in the renewal of NSCs.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The experiments in this study were done by Fei Zeng with the help of Jing Xiong, Wei Ke, Jie Pu reviewed this manuscript and gave comments for designing this study. Yanqiang Zhan drafted this manuscript and supervised the other authors.

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