Original Paper

The Role of MiR-132 in Regulating Neural Stem Cell Proliferation, Differentiation and Neuronal Maturation

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Key Words

Mir-132 • Neural stem cells • Cell proliferation • Differentiation • Neuronal maturation • Notch pathway • ERK pathway

Abstract

Background/Aims: microRNAs are of vital importance in neural development. As a brain-specific miRNA, miR-132 has been well studied in mature neurons. However, its role in neural stem cells (NSCs) remains unclear. In this study, we investigated the role of miR-132 in regulating NSCs proliferation, differentiation and neuronal maturation. **Methods:** NSCs were obtained from fetal mice spinal cord. Proliferation, cell cycle, cell apoptosis, cell motility were measured through CCK-8, BrdU, AnnexinV-FITC/PI and migration assay respectively. The expression of synaptic proteins and ERK1/2 pathway were detected by western blot. The inactivation of Notch pathway was checked using qPCR. The neurite outgrowth was recorded using Image J software and Neuron J software. Dendritic length was further analyzed through sholl analysis. Fate determination of NSCs, developmental synapse formation was assessed by immunostaining. **Results:** miR-132 negatively regulated NSCs proliferation by affecting the cell cycle and promoting apoptosis. Inactivated Notch-Hes1 pathway was observed in miR-132 overexpression cells. miR-132 was significantly increased in differentiating NSCs following activation of ERK1/2 pathway. miR-132 could impair neuronal differentiation but promote glial cell differentiation by regulating Mecp2 expression. miR-132 was implicated in neurite outgrowth but slightly inhibited postsynaptic PSD-95 expression. The differentiated neurons exhibited normal electrophysiological characteristics, and already interacted with other neurons to form synaptic-like structures. **Conclusion:** miR-132 was demonstrated as a negative regulator for NSCs self-renewal, neuronal differentiation but promoted glial cell differentiation and neurite outgrowth.

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Introduction

Neural stem cells (NSC) can proliferate and give rise to neuronal and glial lineages [1]. Theoretically, NSCs can be used in treating spinal cord injury (SCI) and other neurological disorders such as Alzheimer's disease [2], since differentiated neurons have the potential to replace damaged neurons and reconstruct neural circuits. However, the results are disappointing. NSCs transplanted into injured spinal cord hardly survive due to the ischemia-hypoxia microenvironment. Moreover, only few NSCs differentiate into neurons, the majority of them become glial cells. Ultimately, excessive glial scar can form physical barrier that hinder nerve growth [3].

Neurogenesis is a complicated process referring to neural stem cell proliferation (self-renewal), differentiation (fate specification), migration, neuronal maturation and functional integration. The whole process is regulated by the dynamic interplay between transcription factors, microRNAs (miRNA) and cell-niche signaling. Uncovering the role of molecules and signaling pathways involved in neurogenesis is vital for the development of effective therapeutic approaches in traumatic and degenerative neurological disorders [4].

MicroRNAs (miRNA) comprise a family of small, non-coding RNA molecules that are involved in a lot of biological functions, including neural development, tumor metastasis, cell proliferation, differentiation and apoptosis. Most miRNAs can negatively regulate expression of downstream target gene by directly binding to the 3'-untranslated region (3'-UTR) of a target miRNA leading to degradation of mRNA or transcription inhibition. However, miRNAs can also positively regulate gene expression by targeting gene promoter binding [5, 6]. Previous studies have demonstrated that miRNAs almost function in the whole process of neural development. For instance, miR-125b, let-7 influence the NSC self-renewal. miR-9, miR-124, miR-381 regulate neuronal differentiation. miR-219, miR-338 are required for glial cell differentiation and miR-34a is involved in neural differentiation, cell morphology and synapse formation [7-9]. Moreover, miRNAs have been reported to regulate several signaling pathways in neural development. miR-124a and miR-9 have been shown to target several components of the Notch signaling cascade [10], interaction of miR-9 with the Notch signaling pathway is also found in *Drosophila* [11]. miR-125a/b and miR-135b could target BMP/TGF β signaling cascade to promote neural induction [12, 13]. miR-135a is proved to delimits the dorsoventral extent of dopaminergic progenitors by directly interfering with Wnt signaling [14].

miR-132 is highly expressed in the central nervous system and has been well studied in differentiated neurons. Numerous studies have highlighted its role in synaptic plasticity, neurite outgrowth and neuronal morphogenesis [15-17]. However, few studies focus on its role in NSCs proliferation, migration, cell fate specification as well as neuronal maturation. In our study, we extracted NSC from fetal mouse spinal cord and investigated the effect of miR-132 on NSC self-renewal, sphere formation, differentiation, and neuronal maturation. In addition, we explored the potential target gene methyl-CpG-binding protein 2 (MECP2) of miR-132 in the process of NSC differentiation. The electrophysiological characteristics and functional relevance of new born neurons derived from fetal spinal cord NSCs were analyzed. The ERK1/2 signal pathway causing increased expression of miR-132 during differentiation, and Notch signaling maintaining stem cell proliferation, were also studied.

Materials and Methods

Cell culture and differentiation

Neural stem cells were obtained from 13.5 days of fetal mice spinal cord and cultured in growth medium with the 2% N2 (Gibco), 1%B27 (R&D), bFGF 20ng/ml (R&D), EGF 20ng/ml (R&D). The cells were growing to form neurospheres. The cell density was adjusted to 5×10^5 / ml and inoculated on 24-well plate (0.5ml per well). The medium was changed to differentiation medium containing 2% N2 and 0.5%FBS (Gibco) after the cells were basically cultured for 1-2d. The samples were collected at 1, 3,5, 7 days after differentiation.

Proliferation assays

Cell proliferation was measured by Cell Counting Kit-8 (cck-8) assay (dojindo). 10ul CCK-8 was added to each well, and then incubated in the incubator for 2 hours and further transferred to the 96-well plate for determination of the absorptivity at 450 nm. Proliferation rates were evaluated at 1, 3, 5, 7 days after treatment.

Transfection of miR-132 mimics and inhibitor

After 7 days culture, NSCs were digested using trypsin (ProSpec-Tany) and re-suspended by differentiation medium. The cells were incubated on 6-well plate, 2ml suspension was added per well. 15ul POLO3000 transfection reagent was mixed with 200ul DMEM/F12 medium, meanwhile 5ul miR-132 mimics was mixed with 200ul DMEM/F12, for 5min at room temperature. Follow that, mixed the 2 solutions and incubated for 15min, then dripped the transfection reagent mixture into cell suspension. Samples were collected 24h after transfection.

Western blot

Cell lysates were separated in 10% SDS-PAGE gels and blotted on PVDF membranes (100v, 120min). The membranes were blocked with TBST solution containing 5% skim milk. Primary Anti-bodies Mecp2, ERK, p-ERK, PSD95 and Synapsin I (abcam) were incubated overnight, after that the membranes were washed with TBST for 3 times and incubated with secondary anti-body at room temperature for 1hour. Signals were detected using ECL (Millipore).

Immunostaining

Cells were fixed with 4%PFA for 15-30min at room temperature. After washing with PBS 2-3 times, covered with 4% BSA solution containing 0.1% triton-100 at room temperature for 1 h, and then incubated overnight with 4% BSA-diluted primary antibodies Nestin, Tuj1, GFAP, MAP-2, PSD95, Synapsin I (dilution ratio 1: 50) (abcam). The samples were removed from 4 °C on the second day and reheated at room temperature for about 30 minutes, then incubated with second antibody with fluorescent label (sigma) at room temperature for 1h. Then discarded the solution and washed with PBS for 3-5 times. Nuclei were stained with DAPI (sigma).

Analysis of neurite outgrowth

Neurite number and dendritic length were determined to evaluate neuronal maturation. Neurites of labeled cells were manually traced with Image J software and the Neuron J. The averaged neurite number was recorded and dendritic length was analyzed using sholl analysis.

Statistical analysis

The relative intensities of protein bands were analyzed with the Image Lab (Bio-Rad Laboratories). Data are shown as mean±SD from three independent experiments. Results were compared using Student's Test or one-way ANOVA. Statistical analysis was performed with GraphPad Prism 7 software.

Results

miR-132 was highly expressed in central nervous system

The Neural stem cells (NSC) isolated from fetal mice spinal cord could proliferate and form neurospheres (Fig. 1A-B). The undifferentiated cells were identified by NSC-specific marker Nestin (Fig. 1C-D). The differentiated NSCs could express Tuj1 and GFAP which were the markers for neurons and glial cells respectively (Fig. 1E-F). In the process of differentiation, NSCs showed decreased expression of Nestin whereas Tuj1 and GFAP were increased (Fig. 1G). Finally, most cells differentiated into glial cells while only a small portion of them became neurons.

To determine the expression level of miR-132 in central nervous system, qPCR analysis was performed in various types of cells including the NSCs, neuron cells (NC) and epithelial cells (EC). miR-132 was highly expressed in NSCs and neurons compared to epithelial cells

(Fig. 1H). Moreover, miR-132 was significantly increased during embryonic development, and there was no obvious difference in miR-132 expression between spinal cord, cerebral cortex and hippocampus (Fig. 1I). During induced differentiation *in vitro*, miR-132 was gradually increased and reached the highest level at 7th day of differentiation (Fig. 1J).

miR-132 abrogated NSCs proliferation and migration

To explore the role of miR-132 in NSC proliferation and migration, negative control RNAs, miR-132 mimic and miR-132 inhibitor were transfected into NSCs respectively (Fig. 2A-B). Results analysis pointed to the conclusion that miR-132 can inhibit cell proliferation by affecting the cell cycle and promoting apoptosis (Fig. 2C-F). Moreover, miR-132 abrogated cell motility (Fig. 2G).

miR-132 impaired neural differentiation but promoted glial cell differentiation

We transfected the miR-132 mimics into NSCs and induced differentiation, the Tuj1 positive and GFAP positive cells were assessed separately by immunostaining. As shown in Fig. 3A-E, miR-132 overexpression led to enlarged GFAP positive cells and reduced Tuj1 positive cells. Further, we predicted the miR-132 target genes at miRTarBase and TargetScan. Among all the candidates, Mecp2 aroused our interest. In addition, Mecp2 showed the most obvious reduction in protein expression level after miR-132 mimics transfection (Fig. 3F). Interestingly, miR-132 could bind to 3'UTR of Mecp2 with complementary sequences and was verified as a miR-132 target gene (Fig. 3G). Moreover, we also found decreased Mecp2 expression in NSCs transfected with miR-132 mimics (Fig. 3H) and this was further confirmed by immunostaining (Fig. 3I).

miR-132 directly target Mecp2 during NSC differentiation

To further confirm whether miR-132 directly suppressed the Mecp2 expression in the process of NSC differentiation, Mecp2 Knock-Down Lentivirus was constructed (Fig. 4A-C) and transfected into NSCs (Fig. 4D). As shown in Fig. 4E, fluorescence intensity was dramatically reduced in Mecp2-KD cells compared to NC group. Reduced tuj1 positive cells and increased GFAP positive cells were found in Mecp2-KD group (Fig. 4F). This was consistent with the result obtained in miR-132 overexpression NSCs. In addition, miR-132 overexpression led to decreased Mecp2 expression. Furthermore, Mecp2 was a target gene of miR-132 with complementary sequences of 3'UTR. These combined evidence confirmed that Mecp2 expression was directly regulated by miR-132.

miR-132 promoted neurite outgrowth and differentiated neurons formed synaptic-like structures

Differentiated neurons were gradually maturing with dendritic spine and synapse formation. We investigated whether miR-132 involved in neuronal maturation. miR-132

Fig. 1. miR-132 was increasing during differentiation. NSCs extracted from fetal mice spinal cord could self-renewal and form neurospheres (A-B). Cell identification by immunostaining (C-F). Cell specific markers changed during differentiation (G). Relative miR-132 expressions in different cell types were measured by qRT-PCR (H). Relative miR-132 expressions during embryonic development (I). Relative miR-132 expressions during differentiation (J). *, P<0.05; **, P<0.01; ***, P<0.001 compared with control group.

Fig. 2. miR-132 impaired NSCs proliferation by affecting the cell cycle and promoting apoptosis. Relative miR-132 expression after mimics and inhibitor transfection (A-B). CCK-8 was performed to assess cell proliferation (C). Cell cycle assay (BrdU) (D-E). Analysis of cell apoptosis (AnnexinV-FITC/PI) (F). Migration assay to assess cell motility (G).

Fig. 3. miR-132 impaired neuronal differentiation but promoted glial cell differentiation. Immunostaining was performed to separately detect Tuj1 positive and GFAP positive cells after miR-132 mimic transfection (A-E). Relative expression of predicted target genes after miR-132 mimics and inhibitor transfection (F). miR-132 could bind to 3'UTR of Mecp2 with complementary sequences (G). Mecp2 was decreased after Mir-132 mimics transfection confirmed by fluorescence intensity and WB (H-I).

mimics was transfected 3 days after differentiation. We found that miR-132 promoted neurite outgrowth with increased neurite number and dendritic length (Fig. 5A-C). At 14th day of differentiation, the neurons were stained with MAP-2, these differentiated neurons connected together (Fig. 5D), and formed synaptic-like structures (Fig. 5E). The evoked AP was recorded by whole-cell current clamp recordings (Fig. 5F), but the action potential duration-APD₅₀ was much longer than neurons derived from stem cells in the subventricular Zone. Neurotransmitters such as ChAT and Glu that highly expressed in spinal cord were confirmed by immunostaining (Fig. 5G). However, miR-132 slightly inhibited postsynaptic protein PSD-95 expression while it did not change the presynaptic protein Synapsin I expression (Fig. 5H).

Activated ERK1/2 signaling led to increased miR-132 expression during NSCs differentiation

Previous studies have demonstrated that ERK1/2 signaling pathway is crucial for cell differentiation. Since miR-132 was increased in differentiating NSCs, we wondered whether ERK1/2 caused the up-regulation of miR-132. The expression of ERK1/2 and p-ERK1/2 were detected at different time points of differentiation, according to WB results, p-ERK1/2 was increased and maintained the high level of expression throughout the whole process (Fig. 6A-C). Similar results were obtained in expression of miR-132. Next, we investigated the relationship between miR-132 and ERK1/2 pathway. No obvious change was found in NSCs transfected with miR-132 mimics or miR-132 inhibitor (Fig. 6D). On the other hand, when spontaneous ERK1/2 activity was suppressed by SCH772984 (an inhibitor of MAPK/ERK kinase), miR-132 was dramatically decreased (Fig. 6E-F). Interestingly, blocking ERK1/2 increased PSD-95 expression (Fig. 6G). Further, immunostaining revealed that inactivated ERK1/2 pathway caused impaired neuronal differentiation (Fig. 6H-I). These results suggested that miR-132 expression was regulated by ERK1/2 pathway, but with opposite effects on fate determination of NSCs. It reminded us that the activation of ERK1/2 pathway leading to increased miR-132 expression might be a trigger to initiate differentiation, then miR-132 might switch to interact with other pathways.

miR-132 did not affect neurosphere formation of NSCs but inhibited cell proliferation through Notch-Hes1 pathway

Neurosphere formation was one of most important stem cell characteristics. According to our results, miR-132 and Mecp2 appeared to have no effect on sphere formation (Fig. 7A). Mecp2-KD promoted cell proliferation (Fig. 7B), in contradiction with the result we

Fig. 4. miR-132 directly target Mecp2 during NSC differentiation. Construction of Mecp2-KD Lentivirus (A-C). The lentivirus transfected into NSCs (D). Mecp2 expression was significantly decreased after transfection (E). Decreased Tuj1 positive cells and increased GFAP positive cells were found in Mecp2-KD group (F).

Fig. 5. miR-132 promoted neurite outgrowth and differentiated neurons formed synaptic-like structures. miR-132 mimics treatment caused increased neurite number. Image J and Neuron J were used to assess neuronal maturation (neurite number and dendritic length) (A-C). Differentiated neurons connected together and formed synaptic-like structures (D-E). The evoked AP was recorded by whole-cell current clamp recordings (F). Neurotransmitters (ChAT and Glu) that highly expressed in neurons in spinal cord were confirmed by immunostaining (G). miR-132 slightly inhibited postsynaptic protein PSD-95 expression while it did not change the presynaptic protein Synapsin I expression (H).

Fig. 6. miR-132 was regulated by ERK1/2 pathway. WB was performed to determine phosphorylated ERK1/2 expression during differentiation (A-C). Phosphorylated ERK1/2 expression after mimics and inhibitor transfection (D). SCH772984 could effectively suppress ERK1/2 pathway (E). Relative miR-132 expression after SCH772984 treatment (F). Relative PSD-95 expression after SCH772984 treatment (G). Immunostaining was performed to assess the role of ERK1/2 pathway in NSCs fate determination (H-I).

obtained in overexpression of miR-132 (Fig. 2C). Notch signal pathway has been proved to play a role in maintaining NSCs in undifferentiated state. It is known that Notch pathway affect cell proliferation by regulating downstream targeted genes, such as Hes1. Although not being a predicted target of miR-132, down-regulation of miR-132 led to increased Hes1 expression (Fig. 7C). Increased Hes1 expression caused by miR-132 inhibitor was partly rescued by utilizing RO4929097 (Notch signaling inhibitor) (Fig. 7D).

Discussion

Many studies have demonstrated the important role of miR-132 in nervous system. miR-132 has been reported to be involved in neuronal morphogenesis, dendritic growth, synapse formation, physiological rhythm and drug addiction [18-22]. Reduced miR-132 expression is also implicated in several neurodegenerative disease such as Alzheimer's disease (AD) and Parkinson's disease (PD) [23, 24]. Besides its numerous roles in neuronal development and related diseases, it has been shown that miR-132 also mediates inflammatory processes [25]. Therefore, miR-132 is recently regarded as a 'NeurimmiR' that regulates both neuronal and immune functions, plays a role of cross-talk between two systems [17]. However, most research have focused on miR-132 functions in cultured neurons. Yet, few studies have investigated its functions in NSCs proliferation, migration, and differentiation. In this study, we showed that miR-132 was highly expressed in NSCs and overexpression of miR-132 could block Notch-Hes1 pathway to inhibit cell proliferation. It also affected cell cycle, promoted apoptosis and abrogated cell motility but with no effect on sphere formation. More than that, increased miR-132 following ERK signaling activation inhibited neuronal differentiation by regulating Mesp2 expression. miR-132 could promote neurite outgrowth of differentiated neurons, and these neurons formed synaptic-like structures at 14th day of differentiation.

Consistent with previously studies, we observed highly expressed miR-132 in NSCs and neurons. It is well documented that miR-132 play crucial roles in the regulation of cell proliferation and migration. Park JK et al. have demonstrated that miR-132 could promote the proliferation of pancreatic cancer cells [26]. Conversely, miR-132 has been proved to inhibit proliferation and migration of ovarian cancer cells [27]. Luo J et al. have showed that miR-132 negatively regulate the proliferation of lung cancer cells [28]. Clovis YM et al. have

Fig. 7. miR-132 did not affect neurosphere formation of NSCs but inhibited cell proliferation through Notch-Hes1 pathway. No significant difference in sphere formation was observed (A). Mesp2-KD could promote cell proliferation (B). miR-132 negatively regulated Hes1 expression (C). Increased Hes1 expression caused by miR-132 inhibitor was partly rescued by utilizing RO4929097 (D).

reported that miR-132 impair radial migration of neurons by targeting Foxp2[29]. These results are well aligned with our findings.

Notch signaling pathway is associated with delayed neurogenesis, increased gliogenesis and is crucial for maintaining neural stem cells in an undifferentiated state [30]. Salta E et al. have noticed that miR-132 dampens radial glial (embryonic neural stem cells) proliferation but promotes oligodendrocyte differentiation by fine tuning Notch signaling [31]. Decreased expression of miR-132 promotes neuronal differentiation of embryonic stem cells (ESC)[32], that was in line with the result we obtained in NSCs. Overexpression of miR-132 showed impaired neuronal differentiation and enhanced glial differentiation. Meanwhile, miR-132 overexpression resulted in decreased Mesp2 in this process. In addition, Mesp2-KD also led to decreased number of neurons but increased glial cells. Furthermore, miR-132 could bind to 3'UTR of Mesp2 with complementary sequences [33]. Those combined results demonstrated that miR-132 inhibited neuronal differentiation by regulating Mesp2 expression in NSCs.

miR-132 is also implicated in neural plasticity. In miR-132-knockout mice, miR-132 could influence synaptic transmission and plasticity [34]. More than that, miR-132 could regulate axonal growth by targeting Rasa1 [35]. Yoshimura et al. have pointed out that miR-132 negatively regulated synapse maturation by indirectly down-regulating PSD-95 [36]. Besides, we also found increased neurite number and longer dendritic length in newborn neurons with highly expressed miR-132. Dendritic and axon growth, neuronal plasticity as well as synapse formation are all involved in neuron maturation. It is interesting that miR-132 promoted neurite outgrowth while suppressing synapse protein expression.

miRNAs could simultaneously regulate multiple targets [37]. Several studies have confirmed that MeCP2, Sox4, PTEN, PIK3R3, p250GAP are targets of miR-132 [33, 38-41]. However, in different cell types, targets of miRNAs might change and even the same miRNA could have the opposite effect on cell behaviors. For example, positive effect of miR-132 on dendritic growth and arborization of neurons by repressing p250GAP expression [42], is in contradiction with the observation that MeCP2 also facilitates neuronal maturation, since Mesp2 and p250GAP are both reported target genes of miR-132 [43]. Accumulating evidence suggest that miR-132 regulates several targets involved in promoting or blocking neurite outgrowth and arborization.

Contradictory results are obtained with regard to the role of Mesp2 in neuronal development. Kish N et al. have proposed that MeCP2 mutation did not affect neural precursor proliferation and differentiation, suggesting it is mainly involved in neuronal maturation rather than cell fate determination [44]. However, several studies have demonstrated that ectopic expression of MeCP2 represses glial differentiation but promotes neuronal differentiation in mice [45, 46]. Similar results are found in zebrafish that mesp2 knockdown increases neural precursors and gliogenesis, but impairs neurogenesis [47]. On the other hand, there are studies reporting that Mesp2 binds to astrocyte-specific marker GFAP and S100B to subsequently suppress their expression, and Mesp2 knockdown accelerates glial cell differentiation with elevated expression of GFAP [48, 49]. In our study, the loss of Mesp2 promoted NSCs proliferation and glial cell differentiation. Interestingly, GFAP is also a downstream target of Notch signaling and Mesp2 could interact with Notch signal pathway to regulate cell proliferation and gliogenesis [47].

ERK1/2 signaling is crucial for neuronal proliferation, differentiation as well as neurite outgrowth [50]. It has been demonstrated that miR-132 up-regulation is related to ERK1/2 pathway activated by BDNF in differentiated neurons [51]. In our study, we found dramatically increased expression of miR-132 paralleling the activation of p-ERK1/2 during NSCs differentiation. When ERK1/2 was suppressed by SCH7729 84, we observed a clearly decrease in miR-132 expression and neuronal differentiation which was contrary to the effect of miR-132 in this process.

Stem cell behaviors like self-renewal and differentiation were quite complicated processes that involved activation of multiple signal pathways and their interaction with key molecules as well. In fact, this study also had yielded confused but interesting results. For instance, why inactivation of ERK1/2 pathway leading to decreased miR-132

expression impaired neuronal differentiation, but similar result was obtained in miR-132 overexpression? As one of the potential target genes of miR-132, why Mecp2 knocking down accelerated NSC proliferation while miR-132 mimics did the reverse? However, we couldn't exclude that MeCP2 may exert an inhibitory effect on miR-132 transcription in this process, since a feedback loop of miR-132, BDNF and Mecp2 had been proposed already [33], suggesting a homeostatic mechanism for maintaining MeCP2 at a certain level.

Conclusion

miR-132 was demonstrated as a negative regulator for NSCs self-renewal, neuronal differentiation but promoted glial cell differentiation and neurite outgrowth.

Disclosure Statement

The authors declare that they have no competing interests.

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