Running Head: miR-7a in oligodendrogenesis

Specification and Maintenance of Oligodendrocyte Precursor Cells from Neural Progenitor Cells: Involvement of MicroRNA-7a

Xianghui Zhao*, †, §, Jiang Wu‡, §, Minhua Zheng‖, Fang Gao†, Gong Ju*, †

† Institute of Neuroscience, ‡ School of Stomatology, ‖ Department of Medical Genetics and Developmental Biology, the Fourth Military Medical University, 169 Changle Xi Rd. Xi’an China 710032

* Co-corresponding authors: xianghuizhao@fmmu.edu.cn; jugong@fmmu.edu.cn
Tel: +86-2984774562. Fax: +86-2983246270

§ These authors contribute equally to this article

Keywords: miRNA, oligodendrocyte, specification, proliferation, neural induction

Abstract

The generation of myelinating cells from multipotential neural stem cells in the central nervous system (CNS) requires the initiation of specific gene expression programs in oligodendrocytes (OLs). We reasoned that microRNAs (miRNAs) could play an important role in this process by regulating genes crucial for OL development. Here we identified miR-7a as one of the highly enriched miRNAs in oligodendrocyte precursor cells (OPCs), overexpression of which in either neural progenitor cells (NPCs) or embryonic mouse cortex promoted the generation of OL lineage cells. Blocking the function of miR-7a in differentiating NPCs led to a reduction in OL number and an expansion of neuronal populations simultaneously. We also found that overexpression of this miRNA in purified OPC cultures promoted cell proliferation and inhibited further maturation. In addition, miR-7a might exert above effects partially by directly repressing proneuronal differentiation factors including Pax6 and NeuroD4, or pro-OL genes involved in oligodendrocyte maturation. These results suggest that miRNA pathway is essential in determining cell fate commitment for oligodendrocyte and thus provide a new strategy for modulating this process in OL loss diseases.

Introduction

Oligodendrocytes (OLs) play a critical role in the central nervous system (CNS) by producing insulating protein membranes that ensheath axons. Significant damages to OLs result in demyelination and hinder effective communication among neurons. Correspondingly, CNS demyelinating conditions, such as spinal cord injury and multiple sclerosis will result in severe
motor, sensory and cognitive impairment. To obtain proper remyelination, the knowledge regarding the regulators of OL development has been a major focus in understanding the mechanisms promoting differentiation of oligodendrocyte precursor cells (OPCs).

Myelinating oligodendrocytes are derived from multipotential neural progenitor cells (NPCs). The process of creating a properly functional OL, including cell fate specification, OPCs migration, maturation, and myelination, is regulated by the dynamic interplay between transcription factors, epigenetic factors, microRNA (miRNA) regulators, and other cell-extrinsic signals (Emery, 2010; Yu et al., 2010). miRNAs are small, noncoding RNAs that regulate gene expression by post-transcriptional targeting RNA-induced silencing complex (RISC) to cognate messenger RNA (Bartel, 2004). Studies using Nestin-Cre line to delete the miRNA-processing enzyme Dicer in the entire CNS show that Dicer function or the maturation of miRNA is required for the proper specification and development of both neurons and oligodendrocytes during embryonic stages (Kawase-Koga et al., 2009). Moreover, disruption of Dicer function in specified OPCs and OLs by expressing Cre from the Olig1 or Olig2 promoter induces the failure of normal OL differentiation, as well as myelin formation (Dugas et al., 2010; Zhao et al., 2010; Zheng et al., 2010). Therefore, these studies indicate that mature miRNA activity is required at various stages of OL development, including the initial production of fate-specified OPCs, the differentiation of mature OLs, the generation of compact CNS myelin during development, and the maintenance of functional myelin sheaths in adult animals (Dugas and Notterpek, 2011).

Several groups have investigated the roles of individual miRNA in promoting functional CNS myelination. miR-219 and miR-338 are two of the most highly induced miRNAs in differentiating OLs and they are individually necessary and sufficient to promote normal OPC differentiation into OLs in vitro and in vivo (Dugas et al., 2010; Zhao et al., 2010); miR-23a and miR-23b have been identified as miRNAs that are induced approximately 5-fold during OL maturation, and overexpression of either one can enhance OL differentiation, probably by reducing the expression of a gene that inhibits normal OL maturation (Lin and Fu, 2009); miR-138 is also induced in differentiating OLs, and it specifically promotes the early stages of OL differentiation, while simultaneously suppressing the later stage of OL differentiation (Dugas et al., 2010; Zhao et al., 2010). In contrast to the identification of miRNAs in promoting OL differentiation, a set of OPC-enriched miRNAs has also been identified (Lau et al., 2008; Shin et al., 2009; Letzen et al., 2010), among which members of the miR-17-92 cluster were found to be
both necessary and sufficient to enhance OPC proliferation in vivo and in vitro (Budde et al., 2010). Given the critical role of miRNAs in neurogenesis (Cheng et al., 2009; Kawase-Koga et al., 2009; Shi et al., 2010; Yoo et al., 2011), we hypothesize that oligodendroglial lineage specification might be guided in part by miRNA pathways.

In Lau’s investigation, miR-7a, together with several other miRNAs showed higher level in A2B5+/GalC- OPCs than in A2B5+/GalC+ mature OLs sorted from postnatal rat brain (Lau et al., 2008). Since miR-7a shared similar temporal expression pattern with miR-17-92 cluster (Miska et al., 2004), we speculated that miR-7a might also play a role in OPC self-renewal and differentiation. In current study, through in vitro and in utero gain-of-function experiments, we investigated the effect of miR-7a in regulating the generation of OL from multipotential NPCs as well as from embryonic mouse cortex. Besides, inhibiting the function of miR-7a in NPC cultures enhanced our conclusion about miR-7a in oligodendrogenesis. Furthermore, we showed that miR-7a controlled oligodendrogenesis by directly targeting proneural genes such as Pax6 and NeuroD4, and it might impede OL differentiation by targeting regulators for this process. Our present study provides insight into the regulation of oligodendrocyte specification and proliferation by miRNAs. This work further suggests that switching off certain neuronal differentiation factors by miRNAs is a critical mechanism for the generation of oligodendrocytes.

Results

miR-7a expression in different brain cell types in vitro

Previous studies indicated that miR-7 was one of the top miRNAs highly expressed in FACS sorted rat A2B5+/GalC- OPCs and it was found down-regulated during OL differentiation in vivo using microRNA microarray (Lau et al., 2008). To validate the results, we examined the expression level of mature miR-7a by qRT-PCR with Taqman microRNA assay kit. Purified OPCs, differentiated mature OLs, together with primary neurosphere cultures, astrocytes and cortical neurons were first identified by immunostaining with specific markers (Fig. 1A) and the purity of individual cultures was >95%. Then total RNAs from each cell types were collected and quantitative real-time PCR showed that miR-7a expression was higher in OL lineage cells than in other neural cell types, with highest level in OPC stage (Fig. 1B). This result confirmed previous microarray data and suggested that miR-7a might be involved in the generation of oligodendrocytes.
Overexpression of miR-7a in differentiating NPCs expanded PDGFRα+ and Olig2+ OPC populations

To investigate if miR-7a is involved in the differentiation of NPCs, we induced transient overexpression of miR-7a by transfection of miRNA mimics. An increase in the intracellular miR-7a level was confirmed by qRT-PCR (Supplementary Fig. 1A).

The initial dissociated NPC culture before transfection was a ~98% Nestin+ population, with less than 5% GFAP+ cells. β-tubulin-III+ and PDGFRα+ cells were not detected at this time. Another three days’ treatment with low concentrations of bFGF and EGF, together with PDGF-AA, induced the negative control transfected NPCs differentiating into astrocytes (18.6±1.87%), neurons (17.62±2.07%), and early-stage oligodendrocytes (12±2.13%, 13.01±1.01%, 14.7±0.34%), as demonstrated by immunostaining for GFAP, β-tubulin-III, PDGFRα, NG2 and Olig2 (Fig.2A-B). We did not observe major phenotypic differences between NPCs transfected with control oligonucleotides and non-transfected cells (data not shown). Transfection of miR-7a mimics increased the percentage of OPCs up to 24~30%; about two folds to control transfection, but did not indeed change the percentage of GFAP+ astrocytes. Quantification for β-tubulin-III+ cells showed a slight decrease in miR-7a transfected group compared to control (Fig.2B).

Besides, mRNA expression level for several cell lineage specific genes revealed similar alterations after miR-7a overexpression. The basic-helix-loop-helix (bHLH) protein, Olig1 and Olig2, that have been well demonstrated the crucial role in oligodendrogenesis and myelination, together with PDGFRα, were significantly upregulated by miR-7a transfection in differentiating NPC cultures; expression for GFAP and early formed astrocyte marker S100b showed no obvious differences between two groups; and the neuron marker β-tubulin-III was decreased in miR-7a transfected NPC cultures (Fig.2C).

miR-7a induced OL specification in vivo

To determine the effect of miR-7a overexpression on OL development in mice, we electroporated a miR-7a-expressing vector into one side of the neocortical ventricular zone of developing embryos at E14.5. The cortices from electroporated embryos were collected 3 days later (E17.5). Blank vector electroporation was included as control. Overexpression of miR-7a induced a significant increase of both PDGFRα+/GFP+ and Olig2+/GFP+ OPCs in the cortex (Fig.3), which suggested that miR-7a could promote OL lineage formation in the developing mouse cortex. Moreover, there was also an increase in the number of either PDGFRα+/GFP+ or
Olig2+/GFP- cells in miR-7a treated group compared to control electroporation, which suggested a possible non-autonomous effects of miR-7a overexpression.

**Knockdown miR-7a expression repressed NPCs committing to OL lineage**

Next, we blocked the activity of miR-7a in differentiating NPCs by transfection of Hairpin microRNA inhibitors and studied the effect on cell fate commitment. qRT-PCR assay confirmed the efficiency of miRNA inhibitors in reducing the level of mature miR-7a (Supplementary Fig. 1B). In general, knocking down the level of miR-7a in differentiated NPCs resulted in an inverse differentiation pattern compared to overexpression study, as revealed by immunostaining and qRT-PCR assay: inhibiting miR-7a in differentiating NPCs significantly reduced the percentage of OPCs and the mRNA expression of several OPC markers; whereas the generation of β-tubulin-III + and Doublecortin + (DCX) neurons was greatly promoted. No obvious change in astrocytes maker expression was detected (Fig.4).

**Involvement of miR-7a in OPC proliferation and further differentiation**

According to above results, we speculated that the increase in the number of OPCs, with the decrease in the percentage of neurons at the same time, from miR-7a-overexpressing NPCs may be due to selective survival, selective stimulation of OPCs generation or proliferation, and/or a direct inhibition effect on neuron generation. As far as selective survival is concerned, no difference in cell death were observed between control NPCs and miR-7a-overexpressing or -silenced NPCs as indentified by Caspase3 immunostaining (data not shown).

To determine whether the increase in the number of OPCs after miR-7a overexpression could be ascribed to a selective miR-7a-mediated stimulation on the proliferation of newly formed OPCs, we examined their proliferative activity with the use of 5-bromodeoxyuridine (BrdU) incorporation analysis. Purified OPC cultures were transfected with miR-7a mimics and grown in differentiation medium. During transfection, to eliminate the possible effect of RNA amount rather than RNA concentration on OPC proliferation, we mixed negative control RNA with miR-7a mimics to make the equal amount of transfected RNA among different miR-7a mimic concentrations. Two day after transfection, a dose-dependent promotion of cell proliferation, as revealed by increased BrdU labeling were observed in miR-7a mimic treated cultures (Fig. 5A). The percentage of BrdU + cells was 2.03±0.24% in control group and 9.76 ± 0.56% in 50nM miR-7a treated group (p=0.014) (Fig. 5B). Meanwhile, the cultures were subjected to immunostaining for stage-specific OL markers after treatment for indicated periods. In
accompany with increased proliferation, a significant expansion of PDGFRα⁺ and NG2⁺ OPCs was observed in miR-7a overexpression group compared to control group (Fig. 5C-D). Moreover, overexpression of miR-7a resulted in a significant decrease in the number of early differentiated CNP⁺ OLs (17.52±0.91% in control group and 8.87±2.98% in miR-7a group, \( p = 0.092 \)) two days after treatment, as well as a decrease in mature MBP⁺ oligodendrocytes (30±1.87% in control group and 9±0.98% in miR-7a group, \( p = 0.017 \)) four days after treatment. These results suggest that miR-7a positively regulates OPCs proliferation but negatively controls their further differentiation.

Besides, we tested the effects of blocking miR-7a in proliferating OPC cultures by transfecting hairpin microRNA inhibitors. The immunostaining for mitosis marker Ki67 revealed a significant reduction of proliferating OPC populations in anti-miR-7a group cultured in the growth medium with mitogens (23.2±3.57% in control group and 8.67±4.5% in anti-miR-7a group, \( p = 0.029 \)) (Fig.5E-F).

We also blocked the effect of miR-7a in differentiating OPCs, but did not observe significant changes in the percentage of OL subpopulations (data not shown). We presume that since the expression of miR-7a naturally decreases with the differentiation of OL, additional repression is not as effective as in the progenitor stage (when miR-7a is highly expressed) and thus does not induce phenotypic changes.

**Target gene identification for miR-7a**

To identify the potential physiological targets of miR-7a, we analyzed the computationally predicted targets using TargetScan, PicTar, miRanda and mirBase prediction algorithms (Krek *et al.*, 2005; Grimson *et al.*, 2007; Bartel, 2009). miR-7a was predicted to target a number of genes involved in neurogenesis, astrogliogenesis and myelin gene expression (Table1).

Among these genes, Pax6 is a homeo-(HD) and paired-(PD) DNA binding domain containing transcription factor, overexpression of which has been shown to down-regulate Olig2 expression and to promote a neuronal lineage development (Jang and Goldman, 2011); NeuroD4 is a member of the bHLH family transcription factors that acts as an essential determinant for the cortical projection neuron identity. It has been shown to be a key transcriptional target and cofactor for proneural gene Ngn2, which synergizes with Ngn2 to accelerate target gene transcription in the cortex (Mattar *et al.*, 2008).
We tested miR-7a mediated repression on Pax6 and NeuroD4 by placing their 3’ UTR segments downstream to a cytomegalovirus (CMV)-driven luciferase reporter (Fig 6A) and performed reporter assays in COS-7 cells by co-transfecting with expression plasmids for miR-7a, or a control plasmid. Overexpression of miR-7a was confirmed by qRT-PCR analysis (Supplementary Fig 1C) and it significantly decreased the luciferase activity of reporters carrying Pax6 or NeuroD4 3’ UTR segments with their predicted binding sites, respectively (Fig 6B). Mutation of predicted miR-7a binding sites in the UTR segments for Pax6 and NeuroD4 (Supplementary Fig 2A) resulted in a significant recovery of their luciferase activities in response to miR-7a overexpression (Fig 6B). Thus, the mutagenesis studies suggest that there is a direct binding between miR-7a and its targets.

Next we tested whether miR-7a regulated the expression of Pax6 and NeuroD4. NPCs from rat brain were confirmed to express these genes endogenously by qRT-PCR, both of which showed none or less expression in OL lineage (Supplementary Fig 2B). Then NPC cultures were transfected with miR-7a mimics in parallel with control nucleotide. Three days after transfection, total RNAs isolated from control and miRNA-mimic-treated cells were subject to qRT-PCR measurement. The mRNA level of Pax6 was downregulated ~40% by miR-7a mimics (Fig. 6C), and protein expression was reduced to 75% as shown by Western blot analysis (Fig. 6D-E). Immunostaining with NPC maker showed a significant reduction of Pax6/Nestin double positive cells after miR-7a overexpression (Fig. 6F-G), which suggested a post-transcriptional inhibition by miR-7a. In addition, mRNA expression of NeuroD4 was drastically decreased after treatment with miR-7a mimics (Fig. 6C). In utero electroporation study, the number of Pax6+/GFP+ cells was significantly reduced in miR-7a overexpressing GFP+ cells (Supplementary Fig 3), which further corroborate the effect of miR-7a.

Conversely, knockdown of miR-7a by miRNA inhibitors resulted in an upregulation of Pax6 and NeuroD4 mRNA level in primary NPC cultures (Fig. 6H), and the number of Pax6+ cells was increased in anti-miR-7a treated group (Fig. 6I). These observations indicate that expression of neuronal genes, such as Pax6 and NeuroD4, can be negatively regulated by miR-7a during the progress of NPCs cell fate commitment, especially into oligodendrogial lineage.

Other predicted target genes of miR-7a, such as NFib and NFic (Table1), are required for astrogliogenesis and are necessary to drive the expression of GFAP (Steele-Perkins et al., 2005; Wilczynska et al., 2009). Since we did not observe alterations in the astrocyte marker expression
after miR-7a mimic treatment in differentiating NPCs under current conditions, we supposed that miR-7a did not exert the effect of promoting OL generation by inhibiting astrocyte formation. Therefore, we did not test the possibility of these two genes as functional targets for miR-7a. We also noticed that miR-7a was predicted to target a well-known myelin gene, CNPase and genes associated with OL differentiation, such as Sp1 (Table 1). Although we did not test the binding activity between miR-7a and the 3’UTR of CNPase, we had shown the downregulation of CNPase expression in miR-7a transfected OL cultures (Figure 5C-D). Additionally, miR-7a could specifically target the 3’ UTR of Sp1 (Supplementary Fig 4A-B), which is required for the myelin basic protein (MBP) gene expression (Wei et al., 2003, 2004, 2005), and repress its mRNA expression (Supplementary Fig 4C).

In summary, our data suggest that miR-7a may first induce OL specification by negatively regulating proneuronal factors; then maintain newly formed OPCs in their precursor stage by inhibiting pro-OL differentiation regulators as a second wave of targets for miR-7a (Fig. 6J).

**Discussion**

**miR-7a expression in oligodendrocyte lineage cells**

Consistent with earlier investigation of miRNA expression in OL lineage cells from postnatal brain (Lau et al., 2008), we found that the level of miR-7a was 4~5 folds higher in OPCs than in mature OLs identified by Taqman microRNA assay, which also revealed its relatively lower expression in neurons and astrocytes purified from postnatal cortex, as well as in neural progenitors from E15 embryonic cortex. Previous studies have suggested the possible role of miR-7a in early CNS development according to its expression patterns: during the earliest stage of rat cortical development, the level of miR-7a increased about four folds between embryonic day E11 and E13 as identified by qRT-PCR analysis (Nielsen et al., 2009); in mouse brain, the signal of Northern blot for miR-7a peaked in the fetus E12.5-E17.5 and gradually decreased after birth (Miska et al., 2004), which displayed similar temporal expression pattern to miR-9 and miR-17-92 cluster. Recently, miR-17-92 cluster showed functions in promoting OPC proliferation (Budde et al., 2010) and miR-9 was identified as one of the top miRNAs highly expressed in postnatal A2B5+/GC- OPCs in comparison to A2B5+/GC+ mature OLs (Lau et al., 2008), although its correlation with OL lineage progression has not been illustrated. Apart from these, little is known about miR-7a function in the development of vertebrate nervous system. The expression pattern of miR-7a
obtained from different neural cell subtypes indicated that it might play a role in determining the fate commitment of OL lineage, since its expression peaked in OPC stage during the development of NPCs to mature OL and it was much higher than in neurons and astrocytes.

**Oligodendrocyte generation from neural progenitor cells driven by miR-7a**

By manipulating the level of miR-7a in NPC cultures isolated from embryonic mouse brain, we found that miR-7a could promote the generation of OL lineage, but could not accelerate the formation of fully mature OLs in current culture conditions (data not shown). After overexpression of miR-7a mimics, immunostaining with OPC makers PDGFRα, NG2 and with OL lineage maker Olig2 all revealed a significant increase in the number of OPCs, which was confirmed in qRT-PCR analysis. At the same time, quantification of neuron maker β-tubulin-III revealed a slight reduction compared to control treatment, but neither the percentage of GFAP+ astrocytes nor the mRNA level of GFAP and S100b were influenced. Therefore, we speculated that miR-7a might promote oligodendrogenesis from neural progenitor cells, at least partially, by inhibiting neurogenesis. In addition, the upregulation of the mRNA expression level for Olig1 and Olig2 after miR-7a treatment is consistent with their important roles in OL development. Olig1 has been suggested a key factor for the induction of OPC formation in the brain: cortical NPC cultures infected with an Olig1–expressing adeno-virus yielded a substantial amount of OPCs (Lu et al., 2000); ectopic expression of Olig1 in embryonic mouse forebrain ventricle appears to promote OL formation in all brain regions (Lu et al., 2001); and overexpression of Olig1 initiates the differentiation of NPCs into OPCs both *in vitro* (Balasubramaniyan et al., 2004) and *in vivo* (Maire et al., 2010). Meanwhile, Olig2 is required for OPC fate specification from NPCs and subsequent OPC differentiation (Copray et al., 2006; Maire et al., 2010).

In developing mouse cortex, we found that overexpression of miR-7a was able to induce OL generation, defined by PDGFRα and Olig2 immunostaining, which further strengthened the critical function of this miRNA in oligodendrogenesis. Consistent with overexpression data, blocking the function of miR-7a during NPC differentiation lead to reduction in the expression of Olig2 as well as PDGFRα. As above presumed, the blockage of OPCs generation was accompanied by a conspicuous expansion of neuron population, as identified by early neuronal maker DCX and β-tubulin-III. Since the viability of neural subtypes was not affected by either miR-7a overexpression or knockdown treatment, the
opposite effects of miR-7a on OL specification and neuronal differentiation suggested a distinct role of miR-7a in initiating OL lineage formation while repressing neuronal development.

**A critical role of miR-7a in maintaining oligodendrocyte at precursor stage**

To investigate the further progression of OPCs in miR-7a mimic treatment, we tested its effect in purified rat oligodendrocyte cultures. BrdU incorporation assay revealed that miR-7a could promote OPC proliferation even in the differentiation condition and the formation of CNP$^+$ and MBP$^+$ oligodendrocyte was repressed by miR-7a overexpression. In opposite, blocking the endogenous activity of miR-7a in OPC cultures greatly reduced their proliferative activity even in the growth medium with mitogens. Here, Ki67 immunostaining, instead of BrdU assay, was applied to show the number of proliferating cells due to the labeling of more positive cells than in BrdU assay, as reported before (Smith et al., 1995). These results highly suggested a critical role of miR-7a in maintaining newly formed OPCs at their proliferating stage and were consistent with our observations of miR-7a expression pattern during OL differentiation. Therefore, we presume that in vertebrate brain development, miR-7a might work with other partners, such as miR-219 and miR-338 (Dugas et al., 2010; Zhao et al., 2010), to direct the formation and maturation of OL in cohort. The regulatory network for miR-7a expression remains to be explored in the further.

We also noticed that miR-7 has shown its various roles in other species: it has been proved a key player in controlling photoreceptor differentiation in the *Drosophila* eye (Li and Carthew, 2005); it is expressed in certain cell subtypes in the *Drosophila* spinal cord (Aboobaker et al., 2005); and its expression in the hypothalamus neurons with sensory or neurosecretory functions has been identified in *Zebrafish* (Tessmar-Raible et al., 2007). Besides, during RA-induced differentiation of neuroblastoma cells, miR-7 expression is slightly down-regulated, and overexpression of which reduces neurite outgrowth of differentiated neuroblastoma (Chen et al., 2010). Moreover, miR-7 has been suggested as a tumor repressor and it decreases the viability and invasiveness of several cancer cell types, including glioblastoma and schwannoma (Kefas et al., 2008; Webster et al., 2009; Saydam et al., 2011). Thus miR-7a may have pleiotropic effects on different biological processes, and it will promote OL generation in a context and temporally specific manner.
**Proneural factors and myelin genes as targets of miR-7a**

The timing of OL specification, proliferation and differentiation is tightly controlled by the balance of activities between promoters and repressors. Proneural genes Pax6 and NeuroD4 could be the physiological targets for miR-7a due to the validation of direct binding between miR-7a and their 3’ UTRs, and the regulation of their expression levels by miR-7a. This conclusion is consistent with their roles in directing neuronal versus glial fate determination in the CNS: neuronal bHLH genes, NeuroD4/Mash1 double mutants showed missed two longitudinal columns of hindbrain neurons and retinal bipolar cells, and instead, those cells that normally differentiate into neurons adopted glial fate (Ohsawa et al., 2005); Pax6 is a multifunctional player regulating neural NPC proliferation and differentiation through the control of different downstream molecules in a highly context-dependent manner (Sansom et al., 2009).

In the developing ventral spinal cord, combinatorial actions of Pax6 together with other factors controlled neurogenesis and gliogenesis: Mash1 itself promoted differentiation of both neurons and OLs; Pax6, however, converted Mash1 to become neurogenic, whereas Olig2 selectively enhanced Mash1-dependent oligodendrogenesis (Sugimori et al., 2007).

After formation of OL lineage cells from NPCs, miR-7a sequentially maintained these cells at their precursor stage. The proliferation promoting effect of miR-7a in OPC cultures was similar to previous observation in other cell types, such as lung carcinoma cell (Cheng et al., 2005), although the mechanism remained unclear. But we believe that the lack of further differentiation of OPC cultures transfected with miR-7a may ascribe to its inhibitory effect on myelin gene expression directly or indirectly. CNPase is one of the well-known early myelin genes that predicated as target of miR-7a and we showed that its expression was downregulated in miR-7a transfected OL cultures. As to the function of another predicted target, Sp1 has been suggested to play an essential role in the regulation of MBP expression through binding to the GC-rich region of MBP promoter (Wei et al., 2003). It competes off the binding of Nkx2.2 to MBP promoter and then reverses the Nkx2.2-mediated repression of the MBP promoter (Wei et al., 2005). Meanwhile, through interaction with Sox10, Sp1 activates and contributes to the tissue-specific expression of MBP in the CNS (Wei et al., 2004). In current study, we demonstrated that miR-7a could bind to the 3’ UTR of Sp1 (Supplementary Fig 4B), which suggested Sp1 as a physiological target for miR-7a. Besides, several other previously reported target genes for miR-7a, such as EGFR (Kefas et al., 2008; Saydam et al., 2011) and IRS-1/2, upstream regulators of
the Akt pathway (Kefas et al., 2008), have also been identified to play roles in oligodendrocyte myelination (Aguirre et al., 2007; Flores et al., 2008). Therefore, it is conceivable that miR-7a maintains the proliferative activity of newly formed OPCs partially by inhibiting the expression of myelin genes.

Our observations suggest that an active repression of a cohort of proneuronal differentiation factors and OL differentiation activators by miR-7a can be one of the critical steps necessary for initiating OLs specification and maintaining their proliferative, progenitor state (Figure 6J). These results can therefore provide insight into the effective generation of OL lineage cell from neural progenitor cells, especially in OL loss therapies such as spinal cord injury.

**Materials and Methods**

**Primary culture of mouse cortical neural progenitor cells and rat oligodendrocyte precursor cells**

All animal experiment protocols were approved by the Animal Care and Use Committee of the Fourth Military Medical University and were conducted in accordance with the guidelines for the care and use of laboratory animals.

For mouse cortical neural progenitor cell (NPC) cultures, cortical precursors were isolated from C57/BL6 mouse embryos at E15.5 as described previously (Chen et al., 2007). Briefly, each cortex was cut into 2–3 pieces, transferred to ice-cold neurosphere growth medium (DMEM/F12 supplemented with N2 and 20 ng/ml EGF + 20 ng/ml bFGF), and dissociated by mechanical trituration with a fire-polished glass Pasteur pipette. The cell suspension was passed through a 50 µm nylon pouch and 5×10⁴ cells per ml were plated into a 100 mm dish. After 5 days, neurospheres were formed and to induce the differentiation of NPCs, dissociated secondary neurospheres were plated onto poly-D-lysine-coated dishes or coverslips in medium with 5 ng/ml EGF, 5 ng/ml bFGF and 10 ng/ml PDGF-AA as previously described (Balasubramaniyan et al., 2004).

Isolation and culture of rat OPCs were followed the protocol as previously described (Zhao et al., 2007; Zhao et al., 2010). Briefly, brains were removed from P2 Sprague Dawley rat pups and the cortices were dissected. Cortical pieces were enzymatically digested followed by mechanical dissociation. Cells were resuspended in DMEM with 10%FBS and plated onto T75 flasks. The resulting mixed glial cultures were maintained for 7-10 days. Purified OPCs were prepared by differential shaking and were seeded onto Poly-L-Ornithine-coated 35 mm dishes at the density
of $3 \times 10^4$ cells/cm$^2$ in oligodendrocyte growth medium (DMEM/F12 supplemented with 0.1% BSA, 10 nM D-biotin, 5 μg/ml insulin, 5 ng/ml sodium selenium, 50 μg/ml apo-transferrin, 10 nM hydrocortisone, 1 mM sodium pyruvate, 100 U/ml penicillin, and 10 ng/ml FGF, 10 ng/ml PDGF-AA). OPCs were amplified in growth medium for ~4 days and passaged with isolation medium (0.2% DNase I + 5 μg/ml insulin + 0.04% EDTA). To initiate differentiation, OPCs were seeded onto poly-D-lysine-coated dishes or coverslips in differentiation medium with 30 ng/ml triiodothyronine (T3), 10 ng/ml CNTF, and 5 μg/ml NAC.

**Duplex miRNA mimics and Hairpin miRNA inhibitor transfection**

For *in vitro* transfection study, dissociated NPCs or purified OPCs were transfected with 50 nM of miRIDIAN miRNA mimics (Dharmacon, C-310591-07) or Hairpin inhibitors (Dharmacon, IH-310591-08) using Lipofectamine 2000 (Invitrogen) one day after plating. For both transfections, either miRIDIAN miRNA mimic negative control (Cat#: CN-001000-01) or Hairpin inhibitor negative control (Cat#: IN-001005-01) transfection were included, respectively. The transfection efficiency was evaluated by qRT-PCR with Taqman MicroRNA assay (Applied Biosystems). Three or five days posttransfection as indicated, cultures were harvested for immunocytochemistry and qRT-PCR assay.

**RNA Extraction and qRT-PCR**

Total RNAs were purified from tissue or cell cultures using TRizol reagent according to the manufacturer’s instruction (Invitrogen). RNA was transcribed to cDNA with PrimeScript II 1st Strand cDNA Synthesis Kit (Takara). Quantitative real-time PCR was performed using the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc), and the relative gene expression was normalized to internal control Gapdh. Primer sequences for SybrGreen probes of target genes are as follows. Rat and mouse Gapdh: “acaagatgtgtaaggtcgtcgctgtga” and “agcttcccatctctcagcctgtgact”; mouse Pdgfra: “ggagactcaag taaccttgac” and “tcagtctgcgttctgtcataa”; mouse Olig2: “gggaggtcgctcagttcgc” and “ctcc agcagttttgttga”; mouse Olig1: “ggagctctagatctgcttc” and “cagatggtggatgctgactc”; mouse Gfap: “tctcgatgtagcctatgga” and “aagctgctcgcttgagcat”; mouse β-tubulin-III: “cccaggggcaactatgg” and “ccagacccgaactgtcga”; rat and mouse Pax6: “aagagtgcgggtcaggtcgggtt” and “tttatcataaagtgtctgatg” and “agagctcagacaggtcga” and “agagctcagacaggtcga”; rat and mouse Sp1: “agagctcagaggtcga” and “aagctgctcgcttgagcat”.

13
For mature miRNA expression analysis, cDNA was synthesized using Taqman MicroRNA Reverse Transcription kit (Applied Biosystems) and 10 ng total RNA along with miR-7a specific stem loop RT primer supplied in miR-7a Taqman MicroRNA Assay (Applied Biosystems, product ID 000268). Quantitative real-time PCR was performed with Taqman PCR master mix according to manufacturer’s instruction and U6 snRNA Taqman probe (Applied Biosystems, product ID 001973) was used as endogenous control.

**Cell proliferation assay**

A BrdU incorporation assay was used to assess cell proliferation. OPC cultures were exposed to 20 μM BrdU for 12 hours and fixed in 4% formaldehyde. Cells were subjected to DNA denaturation with 2 M hydrochloric acid for 30 minutes and then neutralized with 0.1 M sodium borate at pH 8.5 for 10 minutes. After permeabilization with 0.03% TritonX-100 in 5% normal donkey serum for 15 minutes, cultures were incubated with primary antibody against BrdU (Sigma, 1:200) for 1 hour at room temperature followed by donkey anti-mouse secondary antibody conjugated to Alexa 594 (Molecular Probe, 1:1000) for another hour. Hoechst was used to label nuclei. BrdU incorporation was evaluated by examining five random fields per coverslip and the percentage of cells positive for BrdU was compared to that of the control condition. Experiments were replicated using cells from three different primary cultures.

**In utero electroporation**

For *in utero* electroporation, DNA solution (1μl) in PBS containing 0.01% fast green was injected into the lateral ventricle of the C57 mice embryos at E14.5. After injection, electroporation (five 50 ms square pulses of 35 V with 990 ms intervals) was carried out. Plasmid DNAs (1 mg/ml) used for electroporation were pCIG-miR-7a and control pCIG. Embryos were harvested 72 hr after electroporation and processed for immunohistology. At least five embryos from three to four female mice with the expression of each vector were analyzed and characterized. To compare the effect of miR-7a and control vector treatment on OL generation, the number of PDGFRα+/GFP+, PDGFRα+/GFP− and Olig2+/GFP+, Olig2+/GFP− were counted in defined areas (0.25mm²).

**Immunostaining and western blot assay**

Immunostaining was performed on tissue or cell cultures using above standard protocols along with fluorescent secondary antibodies (Millipore). Hoechst was used to stain nuclei and determine the percentage of immunopositive cells. The primary antibodies were: mouse anti-
CNPase (1:300, Sigma), rabbit anti-NG2 (1:300, Millipore), mouse anti-MBP (1:1000, Abcam), rabbit anti-PDGFRα (1:500, Abcam), rabbit anti-Olig2 (1:200, Abcam), rabbit anti-Pax6 (1:400, Proteintech), mouse anti-Nestin (1:100, Chemicon), mouse anti-β-tubulin-III (1:500, R&D), mouse anti-GFAP (1:4000, Sigma), rabbit anti-Ki67 (1:1000, Abcam).

For western blot analysis, protein lysates were resolved by SDS-PAGE and blotted using standard procedures. Antibodies used were: rabbit anti-Pax6 (Proteintech), mouse anti-β-actin (Sigma) and HRP conjugated secondary antibodies (Jackson Immuno Research). Signals were revealed by chemiluminescence with the ECL kit (Pierce) according to the manufacturer’s instruction.

**miRNA Expression Vectors and Luciferase Reporter Assays**

Mouse miR-7a locus on chromosome 13 with its ~500 bp flanking sequences was PCR amplified from genomic DNA and inserted into pCMV6 or pCIG expression vector (Zhao et al., 2010). The expression of mature miRNA was verified by qRT-PCR with Taqman MicroRNA assay. Segments carrying the putative miR-7a binding sites in 3’ UTR of Pax6, NeuroD4 and Sp1 were cloned into pMir-REPORT vector (Ambion, Inc). Primer sequences for cloning target genes are:

- mouse miR-7a, “ccatgggttccgacttgc” and “tagacagtagtagcaggtg”;
- mouse Pax6-3’UTR, “tcatgcatgtgtgact” and “tacaa ggtggctgtg”;
- mouse NeuroD4-3’UTR, “tagcctcaagttctactggga” and “tctctctctatgccaacgt”;
- mouse Sp1-3’UTR-segment1, “atgggatgtgacctacca” and “tccatggtgtgctc”;
- mouse Sp1-3’UTR-segment2, “atcactgctgctctccact” and “agggcatgtgcaacactcgaa”.

Using site-directed mutagenesis based on overlap extension PCR (Ho et al., 1989), the predicted miR-7a binding sites in pMir-reporter-Pax6-UTR were changed from “TTTTTC C” to “ATAAGG” and from “TCTTCC” to “TGAAGG” respectively; the predicted binding sites in pMir-reporter-NeuroD4-UTR were changed from “GTCTTC” to “CAG AAG” and from “TTTTCC” to “CTAAGG” respectively; the three predicted binding sites in pMir-reporter-Sp1-segment1 were changed from “GTCTTT” to “CAGAA”, from “TCTTTC” to “AGCATG” and from “CTTCC” to “GAAGG”.

For luciferase reporter assays, 10 ng luciferase reporter construct DNA was cotransfected with 200ng vector expressing miR-7a into COS-7 cells by Lipofectamine 2000. The pRSV-renilla luciferase plasmid was included to control the transfection efficiencies. Luciferase activity was...
assayed 48 hr after transfection using the dual-luciferase reporter assay system (Promega). Three transfection assays were performed to obtain statistically significant data.

**Statistic Analysis**

Quantifications were performed from at least three independent experiments and data were presented as mean ± SEM in the graphs. Student’s t test was used to compare two sets of data and one-way analysis of variance analysis was applied in multiple comparisons. p < 0.05 was considered statistically significant.

**Acknowledgement**

We thank Haifeng Zhang for help with Confocal laser scanning microscopy, Ms. Lingling Fei and Jianyong Qiu for technical support. We would also like to thank Pro. Jian Wang and Dr. Yazhou Wang for discussion the results. This work was supported by the grants from National Natural Science Foundation of China (30800325), the Scientific Research Foundation for the Returned Overseas Chinese Scholars from State Education Ministry and Outstanding Young Researcher Foundation of Fourth Military Medical University.

**References**


Table 1. Predicted mRNA targets of miR-7a

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax6</td>
<td>paired box 6</td>
<td>Neurogenesis</td>
</tr>
<tr>
<td>NeuroD4</td>
<td>neurogenic differentiation 4</td>
<td>Neurogenesis</td>
</tr>
<tr>
<td>Gsk3β</td>
<td>glycogen synthase kinase 3 beta</td>
<td>Neurogenesis, neuronal migration, neuronal polarization and axon growth/guidance</td>
</tr>
<tr>
<td>MeCP2</td>
<td>methyl CpG binding protein 2</td>
<td>Neuronal maturation and synaptogenesis</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like factor 4</td>
<td>Indispensable for pluripotency</td>
</tr>
<tr>
<td>Sp1</td>
<td>Sp1 transcription factor</td>
<td>Required for myelin gene expression</td>
</tr>
<tr>
<td>CNP</td>
<td>2',3'-cyclic nucleotide 3' phosphodiesterase</td>
<td>Myelin gene</td>
</tr>
<tr>
<td>NFib</td>
<td>nuclear factor I/B</td>
<td>Astrogliogenesis, required for the expression of astrocyte marker GFAP</td>
</tr>
<tr>
<td>NFic</td>
<td>nuclear factor I/C</td>
<td>Astrogliogenesis, required for the expression of astrocyte marker GFAP</td>
</tr>
<tr>
<td>SNCA</td>
<td>alpha-synuclein</td>
<td>Modulator of oxidative damage</td>
</tr>
<tr>
<td>NAIF1</td>
<td>nuclear apoptosis inducing factor 1</td>
<td>Involved in cell death</td>
</tr>
<tr>
<td>Parp1</td>
<td>poly(ADP-ribose) polymerase-1</td>
<td>Mediator of cell death</td>
</tr>
</tbody>
</table>
Figure Legend

Fig 1. miR-7a expression in different brain cell types

(A) Representative images of purified neurospheres (Nestin⁺), neurons (β-tubulin-III⁺), astrocytes (GFAP⁺), oligodendrocyte precursor cells (PDGFRα⁺), mature oligodendrocytes (MBP⁺) from embryonic or neonatal rat cortex used for studying the expression level of miR-7a. Scale bars, 50 µm.

(B) Taqman MicroRNA Assay was applied to analyze the relative expression level of miR-7a from different cell cultures as indicated. U6 RNA was used as internal control. Data were from three independent experiments and represented mean±SEM. *p<0.05 (Student’s t test).
Fig 2. Overexpression of miR-7a promoted oligodendrocyte generation in vitro

(A) Mouse NPCs were transfected with miR-7a mimics and scrambled miRNA negative control as indicated. The transfected cells were cultured for another 3 days and subjected to immunostaining with antibodies against NG2, PDGFRα, Olig2, GFAP and β-tubulin-III. Scale bars, 50 μm.

(B) Histogram depicts the percentage of NG2+, PDGFRα+, Olig2+, GFAP+, and β-tubulin-III+ cells, which represent three cell types as indicated in differentiated NPCs. Data were from three independent experiments.

(C) Relative expression level of neural lineage specific genes was analyzed by qRT-PCR from miR-7a transfected differentiating NPC cultures. GAPDH was used as internal control. Data represent mean±SEM. *p< 0.05, **p< 0.01 (one-way ANOVA).
Fig 3. In utero electroporation of miR-7a promoted oligodendrocyte specification in embryonic mouse cortex (A-B) Mouse embryos at E14.5 were electroporated with expression vector for miR-7a and harvested at E17.5. The sections of electroporated cortices were analyzed by immunostaining with antibodies to PDGFRα and Olig2, respectively. pCIG plasmid electroporation was included as control. Arrows indicate electroporated GFP+ cells co-labeling with PDGFRα or Olig2. Dashed line shows the edge of lateral ventricle (LV). Scale bar, 100μm. (C-D) Quantification of PDGFRα+ or Olig2+ cells in a defined cortical area (0.25mm²). Y axis indicated the ratio of the number of PDGFRα+/GFP+, PDGFRα+/GFP− and Olig2+/GFP+, Olig2+/GFP− cells in miR-7a-overexpressing cortices to that of the control. Data represent mean ± SEM. **p<0.01, *p<0.05 (one-way ANOVA).
Fig 4. Inhibition of miR-7a repressed oligodendrogenesis and induced neurogenesis in vitro

(A) Mouse NPC cultures were transfected with miR-7a Hairpin inhibitor and negative control. Three days after transfection, cells were subjected to immunostaining with antibodies against PDGFRα, Olig2, GFAP, β-tubulin-III and DCX. Scale bars, 50 μm.

(B) Histogram depicts the percentage of PDGFRα+, Olig2+, GFAP+, β-tubulin-III+ and DCX+ cells, which represent three cell types as indicated in differentiated NPCs. Data were from three independent experiments.

(C) Relative expression level of several neural lineage specific genes was analyzed by qRT-PCR from miR-7a Hairpin inhibitor transfected differentiating NPC cultures. GAPDH was used as internal control.

Data represent mean±SEM. * p < 0.05, **p < 0.01 (one-way ANOVA).
Fig 5. Manipulating the expression level of miR-7a in purified oligodendrocyte cultures controlled the proliferation and differentiation

(A-B) Purified rat OPCs were transfected with miR-7a mimics at different concentrations as indicated, and cultured in OL differentiation medium. Two days after transfection, cultures were subjected to BrdU incorporation assay. Representative images of BrdU immunostaining (A) and quantification of the percentage of BrdU+ cells (B) showed that overexpression of miR-7a induced an increase in the number of proliferating OPCs in a dose depended manner.

(C-D) Stage specific makers immunostaining indicated that overexpression of miR-7a increased the number of PDGFRα+ and NG2+ OPCs, whereas repressed their further differentiation as shown by CNPase and MBP immunostaining.

(E-F) Blocking the activity of miR-7a by inducing hairpin miRNA inhibitors into OPC cultures significantly reduced the number of proliferating cells as indicated by Ki67 staining.

Scale bars, 50 μm; data represent mean±SEM. * p < 0.05 (one-way ANOVA in B; Student’s t test in D and F).
Fig 6. miR-7a targeted proneural genes

(A) Sequence analysis for 3’UTR of mouse Pax6 and NeuroD4 transcripts. The rectangle represented the 3’UTR of each gene, in which the recognition sites of miR-7a were indicated by red bars. Black lines underneath depicted the regions of 3’UTRs that were cloned into pMir-reporter: Pax6 3’UTR, 1056bp; NeuroD4 3’UTR, 700bp.

(B) Luciferase reporter assays for the effects of miR-7a expression on activities of reporters carrying the 3’UTR segments of Pax6 or NeuroD4, respectively. Their mutant forms with corresponding “seed” sequence mutations were included as negative control. The histogram showed the ratio of the luciferase activity normalized to control expression vector.
(C) qRT-PCR analysis of Pax6 and NeuroD4 expression using RNAs isolated from NPCs 3 days after transfection with miR-7a mimics. Scrambled miR transfection was included as control.

(D-E) Western blot analysis showed that Pax6 protein level was downregulated in NPC cultures by miR-7a overexpression compared with either control transfection or no transfection (indicated as “Blank”). β-actin was included as an internal control.

(F-G) Expression of Pax6 in NPC cultures was examined by immunostaining and quantified 3 days after transfection with miR-7a mimics. Nestin staining was used to quantify the percentage of Pax6⁺ cells. Scale bars, 50 μm.

(H) qRT-PCR analysis of Pax6 and NeuroD4 expression using RNAs isolated from differentiating NPC cultures after transfection with anti-miR-7a as well as the scrambled oligonucleotide control as indicated.

(I) Quantification the number of Pax6⁺ cells among Nestin⁺ cell in differentiating NPCs transfected with anti-miR-7a revealed by immunostaining.

(J) Schematic diagram for the function of miR-7a in regulating oligodendrocyte generation. During the NPC to OPC transition, miR-7a suppresses the expression of proneuronal differentiation genes (such as Pax6 and NeuroD4) to initiate the generation of OL lineage, and then suppresses the expression of pro-OL factors (such as Sp1 and CNPase) to maintain the cells at proliferating precursor stage. Inhibition of expression/stage transition is shown by black lines with bars; promotion of stage transition is shown by red dashed lines with arrow heads. Astrogliogenesis is not involved in current model due to the lack of confirmed relevance to miR-7a.

Data were from three independent experiments. * p<0.05 (one-way ANOVA)