

# MicroRNAs Are Essential for the Developmental Switch from Neurogenesis to Gliogenesis in the Developing Spinal Cord

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In the developing CNS, neurons and glia are sequentially produced from the ventricular neural progenitor cells. One fundamental question in developmental neurobiology is what signals or factors control the developmental switch from neurogenesis to gliogenesis. Here we report that microRNAs (miRNAs) play an essential role in this important developmental process. Inhibition of miRNA formation in *Olig1*<sup>Cre</sup>-mediated Dicer conditional knock-out mice disrupted both oligodendrogenesis and astroglialogenesis in the ventral neuroepithelial cells. By contrast, the early patterning and development of motor neurons were not affected in the mutant spinal cord tissue.

## Introduction

During early neural development, neurons and glial cells are sequentially generated from neural progenitor cells in the ventricular zone throughout the entire CNS. It has been well documented that different domains of neural progenitor cells produce distinct subtypes of neurons and macroglial cells (Miller, 2002; Rowitch, 2004; Richardson et al., 2006). In the ventral spinal cord, the ventricular zone is subdivided into five progenitor domains, with each domain expressing a unique combination of transcription factors and producing a distinct neuronal subtype (Briscoe et al., 2000). Motor neurons are first generated from the *Olig1/2*+ pMN domain in a *Shh*-dependent mechanism (Mizuguchi et al., 2001; Novitsch et al., 2001). Beginning at around E12.5, the *Olig1/2*+ ventral neural progenitor cells in the pMN domain cease producing motor neurons; instead, they start to produce oligodendrocyte progenitor cells (OPCs or OLPs) (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2002). Early OPCs delaminate from the ventricular zone and subsequently undergo nondirectional migration to populate all regions of the spinal cord. By contrast, astrocyte precursor cells originate from other domains of neuroepithelial cells and reach their final destinations through radial migration (Lu et al., 2002; Zhou and Anderson, 2002; Hochstim et al., 2008).

Although the spatiotemporal events of neurogenesis and gliogenesis in the developing CNS have been well described, the molecular mechanisms underlying the binary switch from neurogenesis to gliogenesis have been under intensive investigation. Recent studies have implicated microRNAs (miRNAs) in the control of the timing of many developmental programs in both

animals and plants (Banerjee and Slack, 2002; De Pietri Tonelli et al., 2008), raising the possibility that miRNAs may also play an important role in the timely generation of neurons and glia from the same pool of neural progenitor cells. miRNAs are a class of small noncoding RNA molecules (~22 base pairs) that are derived from longer RNA precursors through cleavage by the ubiquitously expressed Dicer RNase (Carthew and Sontheimer, 2009). They function as the major regulators of posttranslational gene expression (Ouellet et al., 2006) by binding to the 3'-untranslated region of specific mRNA targets, directing their degradation and/or repressing their translation (He and Hannon, 2004). In the present study, we provided the first line of genetic evidence that miRNAs are essential for the initiation of gliogenesis process in the developing spinal cord. Conditional ablation of Dicer gene in the *Olig1*+ ventral spinal neuroepithelium inhibited both oligodendrogenesis and astroglialogenesis, but had little effect on early neural patterning and neurogenesis.

## Materials and Methods

**Mouse lines and genotyping.** Mice were housed under standard laboratory conditions at the animal facility of the University of Louisville. All experimental procedures conformed to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Louisville.

*Olig1*<sup>Cre/+</sup> knock-in mice (Lu et al., 2002) were crossed with R26R mice (Soriano, 1999) to obtain *Olig1*<sup>Cre/+</sup>/R26R for LacZ histochemical analysis. *Dicer*<sup>flax/flax</sup> mice (Murchison et al., 2005) were mated to *Olig1*<sup>Cre/+</sup> to obtain the *Olig1*<sup>Cre/+</sup>*Dicer*<sup>flax/+</sup> double heterozygous mice, and conditional Dicer mutants was generated by interbreeding of double heterozygotes. For mouse genotyping, genomic DNA was extracted from embryonic tissues or mouse tails and subsequently used for genotyping by Southern analysis or by PCR. Genotyping protocols for *Olig1*<sup>Cre</sup> and *Dicer*<sup>flax</sup> mouse lines were described previously (Lu et al., 2002; Murchison et al., 2005).

**In situ RNA hybridization and immunofluorescent staining.** Spinal cord tissues at the thoracic level were isolated from E11.5 to E18.5 mouse embryos and then fixed in 4% paraformaldehyde at 4°C overnight. Following fixation, tissues were transferred to 20% sucrose in PBS overnight, embedded in OCT media and then sectioned (16 μm thickness) on a cryostat. Adjacent sections from the control and mutant embryos were

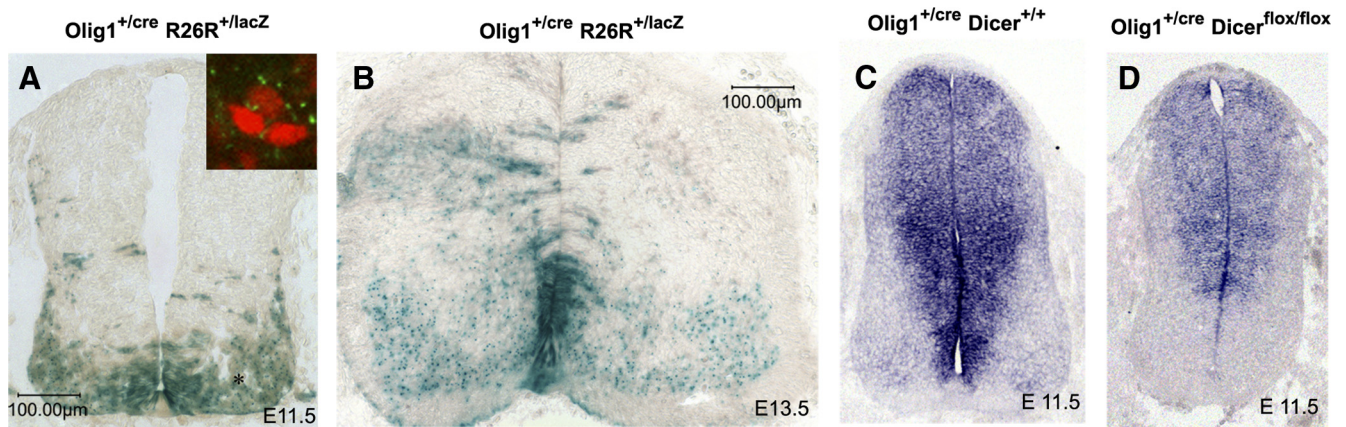
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**Figure 1.** Activation of  $\beta$ -galactosidase and Dicer activity in the ventral spinal cord by  $Olig1^{Cre}$  driver mouse line. **A, B**, Spinal cord sections from E11.5 and E13.5  $Olig1^{Cre}/R26R:LacZ$  embryos were subjected to LacZ histochemical staining. Asterisks indicate the positions of motor neurons in the ventral spinal cord. The inset in **A** represents the LacZ+/HB9+ motor neurons in the ventral horn as detected by double immunofluorescence. **C, D**, Spinal cord sections from E11.5  $Olig1^{Cre}/Dicer^{+/+}$  and  $Olig1^{Cre}/Dicer^{flox/flox}$  embryos were examined for miR-9 expression by *in situ* hybridization.

subjected to *in situ* hybridization (ISH) or immunofluorescent staining. Regular ISH was performed as described by Schaeren-Wiemers and Gerfin-Moser (1993) with minor modifications. 5'-Digoxigenin-labeled, locked nucleic acid (LNA)-modified anti-miR-9 (5'-TCATACAGCTA-GATAACCAAAGA-3') oligonucleotide probe was purchased from Exiqon Inc. and used for *in situ* hybridization as described previously (Kloosterman et al., 2006). Double immunofluorescent procedures were described previously (Qi et al., 2001). The dilution ratio of antibodies is as follows: anti-Olig2 (1:6000), anti-MAG (Millipore Bioscience Research Reagents, 1:500), anti-GFAP (Millipore Bioscience Research Reagents, 1:50), anti-Nkx2.2 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA; 1:50) (Xu et al., 2000), anti-Sox10 (1:3000) (Stolt et al., 2002), anti-PDGFR $\alpha$  (Cell Signaling Technology, 1:400) and anti-S100 $\beta$  (Millipore Bioscience Research Reagents, 1:1000).

## Results

### $Olig1^{Cre}$ can induce reporter gene expression and Dicer deletion in the ventral spinal neuroepithelium

To determine the possible role of miRNAs in gliogenesis, we set out to disrupt miRNA formation in the ventral spinal cord using the  $Olig1^{Cre}$  knock-in mouse line (Lu et al., 2002). Previous studies showed that *Olig1* and *Olig2* are initially expressed in a broad ventral region but later confined to the pMN domain (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2000). The initial broad expression of *Olig1* in the ventral ventricular zone was confirmed by the LacZ histochemical staining in  $Olig1^{Cre/+}/R26R$  (Rosa26-lox-lacZ) double transgenic reporter embryos (Soriano, 1999). At E11.5 and E13.5 stages, LacZ staining was predominantly detected in the ventral neuroepithelial cells including the pMN and p3 domains, although few LacZ+ cells could be found in E13.5 dorsal neuroepithelium as well (Fig. 1A, B). In addition, LacZ staining was also observed in the motor neurons derived from the pMN domain (Fig. 1A, B).

We next generated the  $Olig1^{Cre}/Dicer^{flox/flox}$  conditional knock-out animals by sequential cross-mating. For unknown reasons, the  $Olig1^{Cre}/Dicer^{flox/flox}$  mutants died immediately after birth. To confirm the selective elimination of Dicer function in the ventral neuroepithelium in the conditional mutants, we compared the expression of microRNA-9 (miR-9) in the ventral spinal cord between the control and Dicer mutants. miR-9 was originally identified to be expressed in oligodendrocyte progenitor cells (Lau et al., 2008). Our recent study revealed that miR-9 was initially expressed in the ventricular zone along the entire dorsal-ventral axis (Fig. 1C). As expected, expression of miR-9 expression was nearly completely absent in the ventral ventricu-

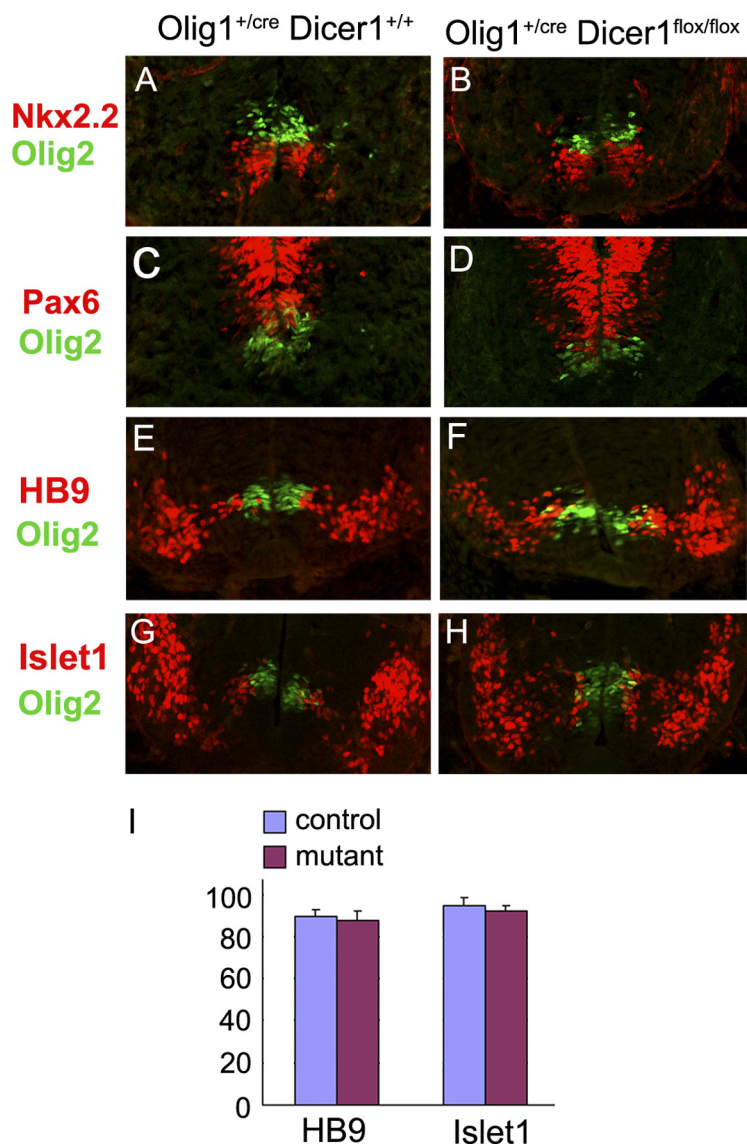
lar cells including the pMN and p3 domains in the mutants, whereas dorsal expression of miR-9 was largely spared (Fig. 1D). Therefore,  $Olig1^{Cre}$  can effectively ablate Dicer function in the ventral neuroepithelium.

### Inhibition of miRNA formation in the ventral spinal cord did not affect neural patterning and motor neuron development

To assess the role of miRNAs in ventral neural patterning and neurogenesis, we first examined the expression of three progenitor identity genes *Pax6*, *Olig2* and *Nkx2.2* in the ventral spinal cord of Dicer conditional mutants. During neurogenesis stages, *Olig2* and *Nkx2.2* specifically mark the pMN domain and p3 domain, respectively; whereas *Pax6* is expressed in domains dorsal to *Nkx2.2* (Briscoe et al., 1999, 2000). Immunostaining results revealed a nearly identical pattern of *Olig2*, *Nkx2.2* and *Pax6* expression in E11.5 ventral neuroepithelium between the control and Dicer mutants (Fig. 2A–D). Consistently, a similar number of HB9+ and Islet-1+ motor neurons were generated in the ventral horn in both genotypes (Fig. 2E–I). Similarly, the expression of several ventral interneuron markers such as *Chox10*, *Evx1* and *Pax2* was not significantly altered in the conditional mutants (supplemental Fig. S1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Together, these results demonstrated that miRNAs did not play a significant role in the initial neural patterning and subsequent neurogenesis in the spinal cord.

### miRNAs are essential for oligodendrogenesis in the spinal cord

At around E12.5, neuroepithelial cells in the pMN domain cease producing motor neurons and start to give rise to migratory OPC cells. In the control embryos, *Olig2*+ cells started to migrate away from the pMN domain into the surrounding region (Fig. 3A). Expression of two other OPC markers *Sox10* (Stolt et al., 2002) and *PDGFR $\alpha$* , was also detected next to the pMN domain (Fig. 3B, C). Surprisingly, in  $Olig1^{Cre/+}/Dicer^{flox/flox}$  mutants, expression of *Olig2* was only observed in the ventricular zone, and expression of *Sox10* and *PDGFR $\alpha$*  was not detected at all (Fig. 3D–F, M). At E14.5, a large number of *Olig2*+, *Sox10*+ and *PDGFR $\alpha$* + OPCs were dispersed throughout the entire spinal cord in the control embryos (Fig. 3G–I). By contrast, in the mutant embryos, expression of *Olig2* was predominantly confined to the ventricular zone and ventrally expanded, and few *Olig2*+ cells detached from the ventral ventricular zone (Fig. 3J). The



**Figure 2.** Normal neural patterning and motor neuron development in Dicer mutant spinal cord. Spinal cord sections from E11.5 *Olig1<sup>Cre/+</sup>/Dicer<sup>+/+</sup>* and *Olig1<sup>Cre/+</sup>/Dicer<sup>flox/flox</sup>* embryos were immunostained simultaneously with anti-Olig2 and anti-Pax6 (**A, B**), anti-Nkx2.2 (**C, D**), anti-HB9 (**E, F**) or anti-Islet-1 (**G, H**). **I**, Quantification of HB9+ and Islet1+ motor neurons in the control and Dicer mutant tissues ( $n = 3$ ).

blockade of OPC generation in Dicer mutants was further confirmed by the drastic reduction of Sox10+ cells and the complete absence of PDGFR $\alpha$ + cells in the spinal cord parenchyma (Fig. 3*K, L, N*). Together, these results indicated that oligodendrogenesis in the ventral spinal cord was suppressed by the absence of miRNA formation.

#### miRNAs are required for astroglial development in the ventral spinal cord

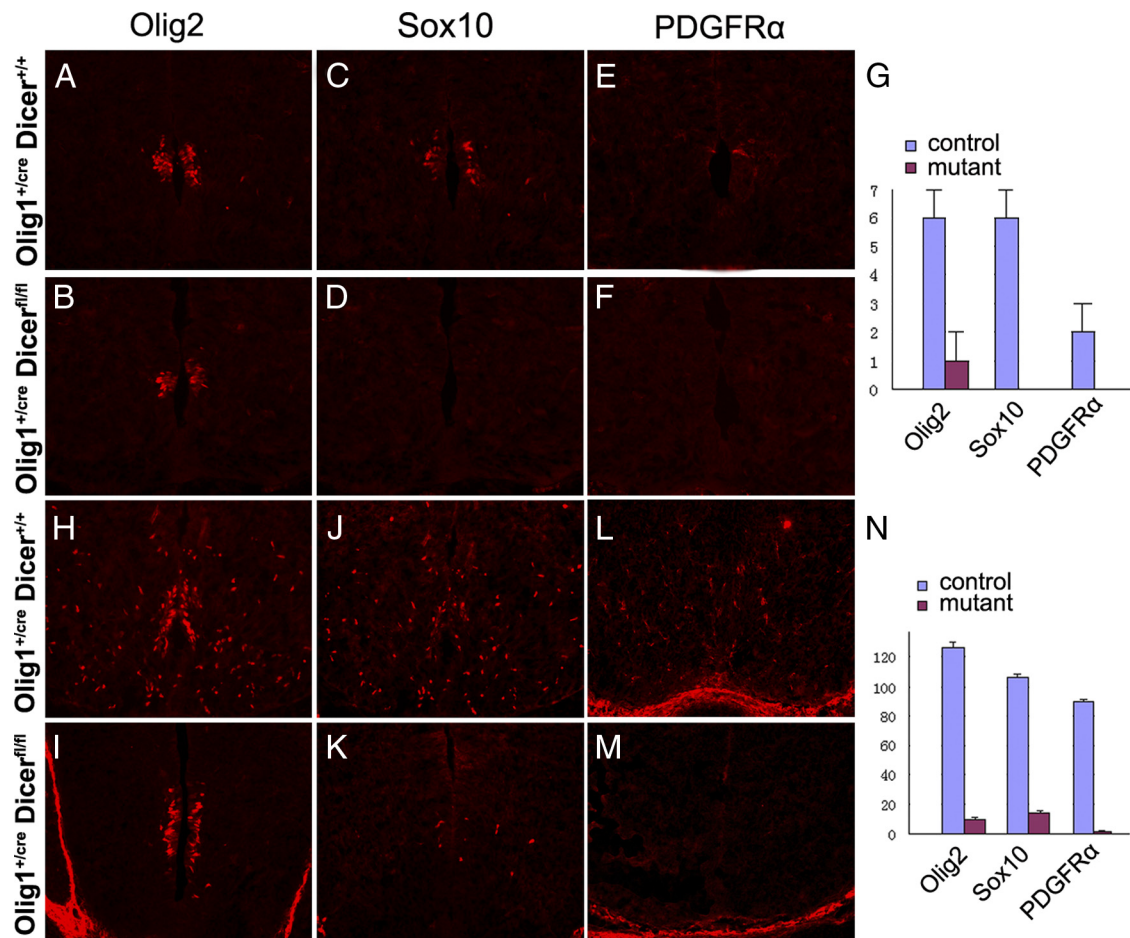
To address the role of miRNA function in astrocyte development, we examined the expression of the well defined mature astrocyte marker GFAP in the Dicer mutant spinal cord. In E18.5 control pups, GFAP immunofluorescent staining was observed in the entire white matter region of the spinal cord. Strikingly, GFAP immunostaining in *Olig1<sup>Cre/+</sup>/Dicer<sup>flox/flox</sup>* animals was completely absent in a triangular region immediately flanking the floor plate (Fig. 4*A, B*). Similarly, expression of another two astrocyte markers ID3 and S100 $\beta$  (Langley et al., 1984) was also

absent in the same region (Fig. 4*C–F*). These results indicated that the conditional ablation of Dicer disrupted the development of a subset of astrocytes derived from the ventral *Olig1*+ neuroepithelium, probably from the ventralmost p3 progenitor domain. In support of this idea, the expression of Slit 1, a specific marker for the ventralmost white matter astrocytes derived from the p3 domain (Hochstim et al., 2008), was completely inhibited in the Dicer mutants (supplemental Fig. S2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).

#### Discussion

In the developing CNS, different progenitor domains as defined by their unique combinatorial expression of progenitor transcription factors give rise to distinct subtypes of neurons and glia at different time windows. The molecular events underlying the sequential generation of neurons and glia remain one of the most important questions in developmental neurobiology. In the present study, we provided the genetic evidence that miRNAs play an essential role in the transition from neurogenesis to gliogenesis. In the *Olig1<sup>Cre</sup>/Dicer<sup>flox/flox</sup>* conditional knock-out mice, miRNA biosynthesis is largely inhibited in the ventral neuroepithelium due to the Cre-mediated ablation of Dicer gene, as confirmed by the absence of miR-9 expression in the ventral ventricular zone of the Dicer conditional mutants (Fig. 1). In association with the absence of miRNA biogenesis, the production of oligodendrocyte progenitor cells was nearly completely suppressed. In Dicer mutants, very few Olig2+ neural progenitor cells were able to delaminate from the ventral ventricular zone during gliogenesis stages to form migratory OPC cells. As a result, expression of oligodendrocyte progenitor markers Sox10 and PDGFR $\alpha$  was almost completely absent in the conditional mutants (Fig. 3). Interestingly, at E14.5, Olig2 expression in the ventricular zone was ventrally expanded in the mutant embryos (Fig. 3*H, K*). The increased number of Olig2+ neural progenitor cells was probably due to the lack of OPC emigration from this zone, as we failed to detect significant differences in the proliferation or cell death of Olig2+ neural progenitor cells between the control and conditional mutants (our unpublished observations).

Similarly, development of astrocytes was also disrupted in the ventral spinal cord of the Dicer mutants, as evidenced by the complete lack of expression of two astrocyte markers, GFAP and S100 $\beta$ , in the ventral region surrounding the floor plate (Fig. 4*A–D*). In addition, we found that ID3 transcription factor has specific expression in spinal cord astrocyte cells, and its expression was also absent in the ventral triangular region as observed in GFAP expression (Fig. 4*E, F*). In the conditional mutants, Cre activity was observed in both the pMN domain and the p3 do-



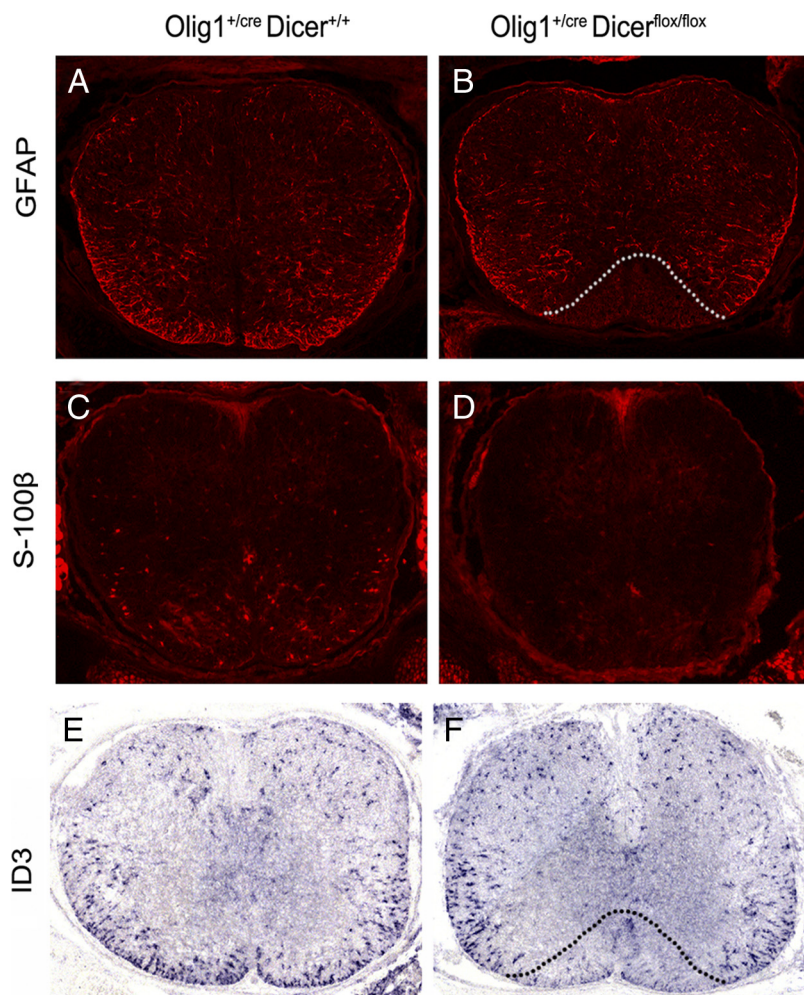
**Figure 3.** Inhibition of oligodendrogenesis in *Olig1<sup>cre</sup>/Dicer<sup>fl/fl</sup>* mutant spinal cord. Transverse spinal cord sections from E12.5 (**A–F**) and E14.5 (**G–L**) *Olig1<sup>Cre/+</sup>/Dicer<sup>+/+</sup>* and *Olig1<sup>Cre/+</sup>/Dicer<sup>fl/fl</sup>* embryos were immunostained with anti-Olig2 (**A, D, G, J**), anti-Sox10 (**B, E, H, K**) and anti-PDGFRα (**C, F, I, L**). **M, N**, Statistical analysis of Olig2+, PDGFRα+, and Sox10+ OPCs in E12.5 (**M**) and E14.5 (**N**) control and Dicer mutant tissues ( $n = 3$ ).

main (Fig. 1*A, B*). Since pMN domain only produces only oligodendrocytes (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002), the most likely explanation for the lack of expression of astrocyte markers in the ventralmost white matter area is that the ventral neuroepithelial cells from the p3 domain failed to produce astrocyte cells in the Dicer conditional mutants. Consistent with this idea, expression of VA3 astrocyte marker Slit1 (Hochstim et al., 2008) was inhibited in the mutants (supplemental Fig. S2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Thus, in the absence of miRNA formation, the pMN domains failed to generate OPC cells, and the p3 domain failed to produce astrocytes.

Two most recent papers (Dugas et al., 2010; Zhao et al., 2010) reported that miRNAs are essential for oligodendrocyte maturation, but not for OPC generation and astrocyte development in the brain. The apparent differences between our data and their findings could be attributed to the regional differences in Olig-Cre gene expression and the different developmental stages examined. Since Olig1-Cre and Olig2-Cre are not expressed in the ventricular zone of the cerebral cortex, it may not be surprising to see normal OPC generation and astroglialogenesis in this region, given that OPC cells and astrocytes can arise from the local sub-ventricular zone of the embryonic cortex (Kessaris et al., 2006). In addition, any defect in early OPC generation in the cortex could be compensated at later postnatal stages by increased cell proliferation (Zhao et al., 2010).

Intriguingly, miRNA function did not appear to act in early neural patterning and the development of motor neurons, as suggested by the more or less normal expression of three ventral patterning genes (*Pax6*, *Olig2* and *Nkx2.2*) and motor neuron-specific markers (*HB9* and *Islet1*) in the conditional mutant spinal cord (Fig. 2). Despite the fact that gliogenesis is greatly suppressed in Dicer mutants, there was no prolonged or ectopic motor neuron formation, suggesting that neurogenesis and gliogenesis can be mechanistically separated. In addition, the lack of ectopic expression of *Olig2* in postmitotic motor neurons (Fig. 2*B*) also indicated that miRNA-mediated silencing of gene expression did not account for the rapid downregulation of *Olig2* mRNA or protein expression in these neurons. It is likely that gene regulation at the transcriptional level rather than the post-transcriptional level is responsible for the suppression of many progenitor genes in postmitotic neurons.

Although the present study clearly demonstrated that miRNAs are required for gliogenesis in the spinal cord, it has also raised several outstanding questions regarding the regulation of gliogenesis by miRNAs. First, which miRNA species are involved in this gliogenesis process? It would be interesting and important to know whether the same miRNA molecules control both oligodendrogenesis and astroglialogenesis. Second, what are the miRNA downstream target genes that are involved in gliogenesis? Considering that miRNAs are negative regulators of gene expression, it is plausible that miRNAs function to repress the expression of



**Figure 4.** Abnormal development of astrocytes in *Olig1<sup>cre</sup>/Dicer<sup>flox/flox</sup>* mutant spinal cord. Transverse spinal cord sections at the thoracic level from E18.5 wild-type and mutant embryos were immunostained with anti-GFAP (**A, B**) and anti-S100β (**C, D**) antibodies, or subjected to ID3 *in situ* RNA hybridization. (**E, F**). The region devoid of GFAP or ID3 signal in the mutants is outlined by dashed lines.

some inhibitory factors that normally suppress gliogenesis at early neurogenesis stages. The derepression mechanism appears to be a common phenomenon for transcriptional regulation of cell fate specification and differentiation (Muhr et al., 2001). Third, given the conserved mechanism for gliogenesis throughout the CNS, are miRNAs also essential for gliogenesis in the rostral brain regions. Answers to these questions in the future will provide important new insights into the mechanistic control of glial development and regeneration in the CNS.

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