Neuroscience 312 (2016) 99–107

miRNAs ARE REQUIRED FOR THE TERMINAL DIFFERENTIATION OF WHITE MATTER ASTROCYTES IN THE DEVELOPING CNS

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Abstract—While the regulation of the neurogenesis and oligodendrogenesis by microRNAs has been intensively studied, little is known about the role of microRNAs (miRNAs) in the development of astrocytes. Here, we report that microRNAs play an essential role in the differentiation and maturation of white matter astrocytes in mouse spinal cord tissues. In glial fibrillary acidic protein (GFAP)/Dicer conditional mutants, the initial generation of astrocyte progenitor cells was normal in the spinal cord. However, there was a much reduced number of GFAP+ astrocytes with shorter processes in the white matter of mutant tissues. In contrast, the expression of gray matter protoplasmic astrocyte marker was not affected. Together, our studies indicated that miRNAs are required for the differentiation and morphological maturation of white matter fibrous astrocytes in the developing spinal cord. Published by Elsevier Ltd. on behalf of IBRO.

Key words: spinal cord, glia, differentiation, Dicer, microRNAs.

INTRODUCTION

Astrocytes are the major type of non-neuronal glia found in all regions of the central nervous system (CNS). Based on their cellular morphologies and anatomical locations, astrocytes can be classified into two major categories: protoplasmic astrocytes in the gray matter and fibrous astrocytes in the white matter (Penfield, 1932). While protoplasmic astrocytes are identified with numerous, short and highly branched processes, fibrous astrocytes have long, thin and unbranched processes whose end-feet envelop nodes of Ranvier (Miller and Raff, 1984; Peters et al., 1991; Privat and Rataboul, 2007). In the developing spinal cord, fibrous astrocytes are further divided into three subtypes based on their dorsal–ventral positions and their differential expression of molecular or immunological markers (Hochstim et al., 2008).

Astrocytes exert a wide range of functions including neurotransmitter exchange, regulation of extracellular ion concentration (Scemes and Giaume, 2006), modulation of synaptic formation and efficacy, formation of blood–brain barrier, and neurotrophic support for other neural cells (Kimelberg, 2004; Kimelberg, 2007). Many CNS pathologies are associated with abnormalities in the structure and function of astrocytes (Seifert et al., 2006). When the lesioned brain and spinal cord tissues undergo the repairing process, astrocytes are reactivated and form glial scars that have a detrimental effect on neural regeneration (Sofroniew, 2009). Understanding the molecular regulation of astrocyte development could provide insights into the prevention of astrogliosis in CNS repairing. At present, little is known about the molecular regulation of astrogliogenesis both during development and in the repairing process.

Mounting evidence indicates that microRNAs (miRNAs) play important roles in the development of the CNS (Smith et al., 2010; Zheng et al., 2010; Zheng et al., 2012; Meza-Sosa et al., 2012; Meza-Sosa et al., 2014). miRNAs are small non-coding RNA molecules (~22 base pairs) processed by Dicer-mediated cleavage (Carthew and Sontheimer, 2009), and function to suppress the expression of their downstream target genes through binding to 3′ end of target mRNA sequences (He and Hannon, 2004). Recent studies demonstrated that miRNAs are involved in astrocyte proliferation and development (Kuang et al., 2012; Hong et al., 2014), and Dicer deletion in astrocytes can cause non-cell-autonomous neuronal dysfunction and degeneration (Tao et al., 2011). Meanwhile, our previous study showed that disruption of Dicer-1 gene resulted in the loss of expression of mature astrocyte markers in both the dorsal and ventral spinal cord (Zheng et al., 2010, 2012). However, it remains unclear whether the phenotypes observed in those mutants resulted from defective specification or migration of astrocyte precursor cells or from the defective differentiation and maturation of astrocytes.

In the present study, we demonstrated that disruption of the Dicer gene in $hGFAP^{cre}Dicer^{flox/flox}$ conditional mutant mice specifically inhibited the expression of

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Abbreviations: CNS, central nervous system; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; IF, immunfluorescence; ICH, immunochemistry; ISH, *in situ* hybridization; miRNAs, microRNAs.

http://dx.doi.org/10.1016/j.neuroscience.2015.11.012
0306-4522/Published by Elsevier Ltd. on behalf of IBRO.

mature markers of white matter fibrous astrocytes in the spinal cord. By contrast, expression of markers for astrocyte precursors and gray matter protoplasmic astrocytes was not compromised. Together, our studies indicated that miRNAs are selectively required for the terminal differentiation and morphological maturation of white matter astrocytes in the developing CNS.

EXPERIMENTAL PROCEDURES

Ethics statement

Animals were provided free access to food and water and housed in a 12-h light/dark cycle. All research procedures using mice were approved by the Institutional Animal Care and Use Committee at Hangzhou Normal University, Zhejiang, China. All efforts were made to minimize the number of animals used and their suffering.

Mouse lines and genotyping

hGFAP^{cre} (FVB-Tg(GFAP-cre)25Mes/J) (stock # 004600) and Dicer$^{flox/flox}$ (STOCK Dicer1^{tm1Bd}h/J) (stock # 006001) mouse lines were purchased from JAX. Dicer$^{flox/flox}$ mice were mated to hGFAP^{cre} to obtain the hGFAP^{cre} Dicer$^{flox/+}$ double heterozygous mice, and conditional Dicer mutants were generated by interbreeding of double heterozygotes. For tissue preparation, animals received an overdose of Nembutal (200 mg/kg body weight) before perfusion. For genotyping, genomic DNA extracted from mouse tails was used as templates for PCR reactions. Primers for genotyping glial fibrillary acidic protein (GFAP) transgene are oIMR1900: 5′ACTC CTTCATAAAGCCCT3′, oIMR1901: 5′ATCACTCGTTGC ATCGACCG3′. Transgene appeared as a 190-bp band. Primers for Dicer genotyping: oIMR6305: 5′CCTGACAG TGACGGTCCAAAG3′, oIMR6559: 5′CATGACTCTTCA ACTCAAACT3′. Mutant allele is 420 bp, whereas wide-type allele is 351 bp.

In situ RNA hybridization, immunofluorescent and immunochemistric staining

Spinal cord tissues isolated from E12.5 to P15 mice were fixed in 4% paraformaldehyde at 4 °C overnight. Following fixation, tissues were transferred to 30% sucrose in PBS overnight, embedded in OCT media and then sectioned on a cryostat at the thickness of 18 μm for *in situ* hybridization (ISH) or 14um for immunfluorescence (IF) and immunochemistry (ICH). Adjacent sections from the control and mutant tissues were used for ISH, IF and ICH staining. Regular ISH and the combined immunohistochemistry and ISH were performed as described by Schaeren-Wiemers and Gerfin-Moser (1993) with minor modifications.

Double immunofluorescent procedures were described previously (Qi et al., 2001). The dilution ratio of primaryantibodies is as follows: anti-cre (Millipore, 1:500, Boston, Massachusetts, USA; Novagen, 1:2000), anti-GFAP (Millipore, 1:2000, Boston, Massachusetts, USA), anti-GS (Chemicon, 1:200), anti-Sox10 (1:2000), anti-MBP (Abcam, 1:500). The dilution ratio of secondary antibodies is as follows: Goat anti mouse IgG1(γ1) [594] (Invitrogen, 1:3000), Goat anti mouse IgG1(γ1) [488] (Invitrogen, 1:3000), Goat anti Rabbit IgG(H + L) [594] (Invitrogen, 1:3000), Goat anti Rabbit IgG(H + L)[488] (Invitrogen, 1:3000), Goat anti Rat IgG(H + L) [594] (Invitrogen, 1:3000), Goat anti Rat IgG(H + L) [488] (Invitrogen, 1:3000), Goat anti mouse IgG(H + L) (Vectorlab 1:500).

Western immunoblotting

Spinal cord tissues from P15 control and conditional mutant pups were isolated and lysed in tissue lysis buffer (Sigma, St. Louis, Missouri, USA) in the presence of protease inhibitor cocktail (Sigma, St. Louis, Missouri, USA). 25 μg protein from control and mutant tissues was loaded for SDS–PAGE electrophoresis and subsequently detected with anti-GFAP (Millipore, 1:5000, Boston, Massachusetts, USA), mouse anti-GAPDH (Sigma, 1:5000, St. Louis, Missouri, USA) antibodies according to the standard protocol. Second antibodies are as follows: Goat anti mouse IgG(H + L) HRP (Promega 1:2000, Madison, Wisconsin, USA).

RESULTS

hGFAP promoter drives CRE expression in neuroepithelial cells and astrocytes in the spinal cord

To decipher the function of miRNAs in astrocytic development, we utilized the hGFAP^{cre} transgenics to disrupt Dicer recombinase for inhibition of miRNA biosynthesis in spinal astrocytes. Previous studies showed that hGFAP-driven CRE expression in radial glia and astrocytes in the brain (Zhuo et al., 2001; Casper and McCarthy, 2006). However, CRE expression under hGFAP promoter in the developing spinal cord has not been characterized in detail.

Immunofluorescent staining with CRE antibody revealed that the hGFAP-induced CRE was not detected in the ventricular zone of spinal cord until E13.5 (Fig. 1A, B), when neurons are no longer produced and NSCs are committed to glial lineage (Gabay et al., 2003; Ogawa et al., 2005; Deneen et al., 2006; Mukouyama et al., 2006). From E13.5 to E16.5, CRE expression was observed in the parenchyma in a ventral-to-dorsal sequence (Fig. 1B, C). Double labeling experiments revealed that CRE+ cells did not co-express neuron marker NeuN and oligodendrocyte marker Sox10 (Fig. 1D, E). In contrast, many CRE+ cells in the white matter co-expressed the astrocytic marker GFAP (Fig. 1F, H, J). The expression of GFAP is increased gradually in white matter astrocytes from P0 to P7. In the gray matter, nearly all CRE+ cells were co-labeled with glutamine synthetase (GS), a marker for protoplasmic astrocytes, form P0 to P7 (Stanimirovic et al., 1999) (Fig. 1G, I, K). Thus, CRE in hGFAP transgenic spinal tissue is initially expressed by ventricular progenitor cells but later restricted to cells of astrocyte lineage after they are detached from the VZ zone.

To further verify that the CRE expression is restricted to the glial lineage, we examined the expression of Mir-124, a well-known neuron-specific miRNA species (Makeyev et al., 2007; Maiorano and Mallamaci, 2010), in hGFAP–Dicer mutants. No apparent difference was detected in its neuronal expression between the

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