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MIR-32 AND ITS TARGET SLC45A3 REGULATE THE LIPID METABOLISM OF OLIGODENDROCYTES AND MYELIN

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INTRODUCTION

MicroRNAs (miRNAs) are short non-coding RNAs that are found in all eukaryotic cells. Seventy percentage of miRNAs are expressed in the brain (Babak et al., 2004), but understanding of the functions of these brain-specific or -enriched miRNAs is lacking. The human genome encodes over 1000 miRNAs, which may target over 60% of genes and affect most biological processes (Friedman et al., 2009). Therefore, miRNA dysregulation could trigger many diseases such as cancer, heart and neurological disorders (He et al., 2005; Thum et al., 2007; Beveridge et al., 2010) and miRNA-based therapies are also under intense investigation (Li et al., 2010).

Oligodendrocytes (OLs) are glial cells of the central nervous system (CNS) that generate large amounts of myelin as an extension of their cell membrane. This myelin is required not only for the saltatory conduction of neuronal action potentials, but also for the maintenance of axonal integrity and fast axonal transport by supplying energy and metabolites to long axonal tracts (Nave, 2010). Damaged myelin triggers many demyelinating neurological disorders such as multiple sclerosis, leukodystrophies, and demyelinating neuropathies. Therefore, understanding detailed myelination regulatory mechanisms is essential for effective treatment of these neuronal disorders.

Previously, we and others showed that miRNAs play important roles in regulating differentiation and maintenance of OLs (Shin et al., 2009; Dugas et al., 2010; Zhao et al., 2010). OL-specific postnatal ablation of Dicer, an essential endonuclease for generating miRNAs, triggers a motor coordination defect, neuronal impairment and shorter lifespan (Shin et al., 2009). Dicer mutants show demyelination, oxidative damage, inflammatory astrogliosis and microgliosis in the brain, and eventually, neuronal degeneration. Expression analysis of these Dicer mutant mice found that miR-32, miR-144, and miR-219 are expressed at significantly reduced levels in the mutant mice. Further characterization demonstrated that miR-219 modulates myelin maintenance by regulating lipid synthesis via ELOVL7 (elongation of very long chain fatty acids 7). Simultaneously, two other groups (Dugas et al., 2010; Zhao et al., 2010) used constitutional Cre that is activated from premature form to mature OLs and found miR-219 is important during OL differentiation.

Here, we report the characterization of a novel miRNA that is important for mature OLs, miR-32, and its target protein SLC45A3. As a myelin-enriched sugar transporter, SLC45A3 enhances intracellular glucose, long-chain fatty acids, and energy and metabolites to long axonal tracts. Therefore, understanding detailed myelination regulatory mechanisms is essential for effective treatment of these neuronal disorders.

Abstract—Oligodendrocytes generate large amounts of myelin by extension of their cell membranes. Though lipid is the major component of myelin, detailed lipid metabolism in the maintenance of myelin is not understood. We reported previously that miR-32 might be involved in myelin maintenance (Shin et al., 2009). Here we demonstrate a novel role for miR-32 in oligodendrocyte function and development through the regulation of SLC45A3 (solute carrier family 45, member 3) and other downstream targets such as CLDN-11. miR-32 is highly expressed in the myelin-enriched regions of the brain and mature oligodendrocytes, and it promotes myelin protein expression. We found that miR-32 directly regulates the expression of SLC45A3 by binding to the complementary sequence on the 3’UTR of cldn11 and slc45a3. As a myelin-enriched putative sugar transporter, SLC45A3 enhances intracellular glucose levels and the synthesis of long-chain fatty acids. Therefore, overexpression of SLC45A3 triggers neutral lipid accumulation. Interestingly, both overexpression and suppression of SLC45A3 reduces myelin protein expression in mature oligodendrocytes and alters oligodendrocyte morphology, indicating that tight regulation of SLC45A3 expression is necessary for the proper maintenance of myelin proteins and structure. Taken together, our data suggest that miR-32 and its downstream target SLC45A3 play important roles in myelin maintenance by modulating glucose and lipid metabolism and myelin protein expression in oligodendrocytes. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: glia, metabolism, lipids, sugar transporter, microRNA.
acids, and neutral lipid accumulation. Furthermore, we show that precise regulation of SLC45A3 expression is necessary for the proper maintenance of myelin proteins. These results demonstrate that mir-32 fine-tunes the expression of its target SLC45A3 in the myelin cells and that tight regulation of SLC45A3 levels is critical for proper myelin structure and function.

**Experimental Procedures**

**Primary cell culture**

Oligodendrocyte progenitor cells (OPCs) were purified using the sequential immunopanning methods (Watkins et al., 2008). Fresh postnatal day 7 brains were diced and digested in papain followed by sequential immunopanning on Thy1.2 (Serotec), galactocerebrosidase (GalC, Chemicon), and then O4 antibody (Chemicon)-coated plates to select GalC+ O4+ OPCs. After rinsing nonadherent cells away, acutely purified OPCs were cultured. Cells were removed from the final panning plate with trypsin and transferred to poly-D-lysine-coated tissue culture dishes or chamber slides. All cells were cultured at 37°C, 10% CO2 in DMEM containing human transferrin, BSA, putrescine, progesterone, sodium selenite, N-acetyl-L-cysteine, B2, forskolin, bovine insulin (all from Sigma), glutamine, sodium pyruvate, penicillin–streptomycin, B-27 supplement (all from Invitrogen), Trace Elements B (Mediatech), and CNTF (10 ng/ml; PeproTech). Proliferation medium also contains OPC mitogens such as PDGF-AA and NT-3 (both from PeproTech); differentiation medium includes triiodothyronine (T3; Sigma) without OPC mitogens. The constructs in pGL3-promoter and pSV-galactosidase control vectors (Promega) were co-transfected into COS-7 cells. All cells were harvested in the reporter lysis buffer (Promega) after 48–72 h of transfection. Luciferase activities were measured as described previously (Shin et al., 2009).

**Perchod gradient mediated fractionation**

The rapid perchod gradient procedure for subcellular fractionation with frozen mouse brain tissues was conducted as previously described (Dunkley et al., 2008).

**Immunohistochemistry**

Cryosections were prepared from 4% paraformaldehyde-perfused mouse brain, permeabilized with Triton X-100, then blocked and incubated overnight with primary CC1 (Calbiochem) and SLC45A3 (Santa Cruz) antibodies. After washing with PBS, sections were incubated with secondary Cy2 anti-mouse (Amersham) and Alexa 594 anti-goat IgG (Invitrogen) antibodies. After washing (3 × 5 min) with PBS, coverslips were mounted with Vectashield (Vector Laboratories) mounting medium and DAPI.

**Glucose and free fatty acid quantification**

Intracellular glucose and free fatty acid amounts were measured using glucose assay and free fatty acid quantification kits (all from BioVision) following manufacturer’s protocols.

**Adipocyte differentiation and Oil Red-O stain**

The recombinant lentivirus particles expressing SLC45A3 or mmu-mir-32 were transduced into preadipocytes (3T3-L1) and the cells were differentiated to mature form using the Adipogenesis Assay kit (Cayman Chemical), as described previously (Shin et al., 2009). For quantification of lipid droplets, stained cells were dried completely and extracted with the dye extraction solution provided in the kit. Optical density of the extracted dye was measured at 492 nm.

**Transmission electron microscopy (EM)**

Mice were first anesthetized with 2.5% avertin and then perfused with 4% paraformaldehyde and 2.5% glutaraldehyde in PB buffer and incubated in fixative for 1 week. After being post-fixed, spinal
cords were dissected and imbedded in Epon. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate and then collected on grids. The pictures were taken with the Tecnai electron microscope at the San Francisco VA Medical Center EM Lab.

RESULTS

miR-32 is enriched in OLs and reduced in OL-specific Dicer knockout brain

Prominent demyelination is observed in the absence of Dicer in PLP-expressing OLs in vivo (Fig. 1A) (Shin et al., 2009). Since these Dicer knockouts were targeted to mature OLs, we reasoned that crucial miRNAs are involved in oligodendrocyte function. We previously found reduced production of miR-32, miR-144 and miR-219 in these OL-specific Dicer knockout (KO) mice (Shin et al., 2009). Interestingly, when these three miRNAs were examined by quantitative real time PCR in both precursor and mature OLs, miR-32 was highly expressed in the mature OLs with miR-219 (Lau et al., 2008; Shin et al., 2009), but not miR-144 (Fig. 1B). Upon further examination, we found the expression of miR-32 to be developmentally regulated, increasing more than 10-fold between postnatal day (P) 1 to P50 (Fig. 1C). As miR-32 is dramatically upregulated during a critical period for myelin development, suggesting that it may also play a vital role in oligodendrocyte development. We further confirmed miR-32 expression using in situ hybridization and verified that miR-32 is normally highly expressed in the myelin-enriched regions such as corpus callosum and white matter of cerebellum, but its levels are much lower in the OL-specific Dicer KO mice (Fig. 1D).

miR-32 promotes expression of myelin proteins

To investigate the function of miR-32 in OLs, in vitro primary cell culture and lentiviruses were used. For miR-32 overexpression and suppression, recombinant lentiviruses were transduced into OLs which were purified by the immunopanning method (Watkins et al., 2008). Interestingly, miR-32 suppression leads to reduced myelin protein expression (Fig. 1E) and overexpressing miR-32 increases expression of all myelin proteins (Fig. 1F). Overexpression of miR-32 also enhances the already well-differentiated branching structures of OLs while suppressing miR-32 reduces the branching structures (Fig. 1E, F). Together, these results imply that miR-32 promotes myelin protein expression in OLs.

SLC45A3 is a myelin-enriched miR-32 target

To identify proteins which are directly regulated by miR-32 in the OLs, in silico analysis such as Targetscan (http://www.targetscan.org) and Pictar (http://pictar.mdc-berlin.de/) were used to analyze the OL-enriched protein database (Cahoy et al., 2008). Five candidates were identified: CLDN11 (claudin 11), SLC45A3 (solute carrier family 45, member 3), RHPN2 (rhophilin, Rho GTPase binding protein 2), DOCK5 (dedicator of cytokinesis 5), and ADAM19 (A disintegrin and metalloproteinase domain 19). To verify that these genes are indeed the targets of miR-32, we used a luciferase reporter assay to determine if miR-32 binds to their 3’UTRs. Among them, we found that miR-32 suppresses both cldn11 and slc45a3 reporter activities in a dosage-dependent manner and deletions of the miR-32 binding sites in the 3’UTRs of both genes abolish these suppressions (Fig. 2A), suggesting that cldn11 and slc45a3 are directly regulated by binding of miR-32. CLDN11 has been shown to be highly expressed in oligodendrocytes and may regulate proliferation, migration, and coupling of myelin lipid bilayers (Chow et al., 2005; Tiwari-Woodruff et al., 2001; Gow et al., 1999), validating the in silico approach for identifying miRNA targets. In order to elucidate the role of other novel targets, we chose to focus on SLC45A3 as it is expressed ~32-fold higher in myelinating OLs compared to OPCs (Cahoy et al., 2008). In addition, SLC45A3 is a putative sugar transporter (Xu et al., 2001) and therefore may be linked to lipid metabolism. Interestingly, we previously found that miR-219 plays an important role in mature myelin through modulating lipid via ELOVL7 (Shin et al., 2009), indicating a possible lipid connection for miRNA and myelin. To demonstrate miR-32 regulation of SLC45A3, we reduced the level of miR-32 by transfecting shRNA lentivirus targeting miR-32 into OLs and observed up-regulation of slc45a3 (Fig. 2B). In order to validate this observation in vivo, mouse brain tissue extracts were purified by percoll gradient to separate the myelin membrane fraction. SLC45A3 was highly enriched in the myelin fraction from Dicer knockout mice when compared to the control mouse brain fractions (Fig. 2C). Furthermore, immunohistochemical analyses showed that SLC45A3 is highly induced in the OLs of Dicer knockout brains (Fig. 2D). These results attest that SLC45A3 is a myelin-enriched protein regulated by miR-32.

SLC45A3 regulates glucose and lipid metabolism of OLs

The proteins classified in the SLC45 family have been predicted to be putative sugar transporters. However, the substrates of these transporters are not known except for SLC45A1, which transports glucose and galactose (Amler et al., 2000; Shimokawa et al., 2002). Sugar is very important not only as an energy source but also as a substrate for the synthesis of fatty acids (Tamura et al., 2009) and therefore myelin lipids. To investigate the function of SLC45A3 in OLs, we measured the intracellular glucose levels in OLs either overexpressing or suppressing SLC45A3. Interestingly, serial increases of slc45a3 shRNAs reduced intracellular glucose levels, and conversely, sequential increment of slc45a3 cDNA lentiviral particles increased glucose levels (Fig. 3A), suggesting that SLC45A3 regulates intracellular glucose concentrations in OLs. We next tested whether SLC45A3 affects long-chain fatty acids, which are used for the synthesis of very long chain of fatty acids (VLCFAs) in cells. Palmitic acid is a representative fatty acid with 16 carbons and can be measured by enzymatic assays. For the measurements of the fatty acids, we began by using OLs, but the results were inconsistent, likely due to the complexity and saturation of fatty acids in these
uniquely lipid-rich myelin-forming cells. We hence performed these measurements in 3T3-L1 adipocytes which have proven useful for the analysis of free fatty acids and lipids metabolism (Jefcoat et al., 2008). Palmitic acid levels were upregulated in the differentiated 3T3-L1 adipocyte cells by SLC45A3 in a dosage-dependent manner.

Fig. 1. miR-32 promotes myelin protein expression in OLs and is dysregulated in the OL-specific Dicer KO. (A) Ultrastructure of adult spinal cord indicates that loss of Dicer results in thinner myelin and an absence of myelin in some axons. (B) Quantitative miRNA real time PCR. miR-32 expression is increased in mature OLs compared to premature OLs (OPCs), but miR-144 does not show a difference. (C) Developmental expression of miR-32. Total RNAs from postnatal mouse brains (1, 7, 21, 30, and 50 day-old) were used for the syntheses of cDNAs and then analyzed by individual miRNA-specific real time PCRs. Data were normalized by the result of U6 control. (D) In situ hybridization against mmu-miR-32 on mouse brain sagittal sections. Suppression of miR-32 reduces myelin protein expression (E) and ectopic overexpression of miR-32 increases myelin protein expression (F). Whole cell extracts were analyzed by Western Blot with antibodies against myelin-specific proteins. OPCs were differentiated into mature forms with tri-iodothyronine in vitro for 2 days, transduced with the lentivirus harboring miR-32 shRNA (E) or transcript (F), differentiated with tri-iodothyronine for 5 more days (total 7 days in vitro differentiation), and then stained with MBP antibody (red). miR-32 suppression downregulates OL maturation, and conversely its overexpression upregulates OL differentiation with more myelin membrane-like sheets in culture. DIV stands for ‘days in vitro differentiation’. Both GAPDH and GFP were used for the normalization controls.
(Fig. 3B), indicating SLC45A3 increases fatty acid synthesis. We then examined whether SLC45A3 affects lipid synthesis in mature adipocyte cells. SLC45A3 overexpression was accomplished by transducing recombinant lentivirus harboring its coding sequence into the 3T3-L1 preadipocyte cells. These cells were allowed to differentiate into mature adipocytes and lipid accumulation was measured by Oil Red O stain. SLC45A3 overexpression causes a 20% increase in lipid droplets (Fig. 3C, D) for these cells. Taken together, these results suggest that SLC45A3 may play a novel role in glucose regulation and/or fatty acid synthesis in oligodendrocytes. Furthermore, as both processes are crucial for oligodendrocyte development and function, this also suggests that SLC45A3 may be one of the main downstream targets of miR-32.

**Overexpression and suppression of SLC45A3 reduces the number of matured oligodendrocytes in vitro**

To further reveal the role of SLC45A3 in myelination, we examined overexpression and suppression of SLC45A3 in OLs achieved by recombinant lentivirus in the primary OL cultures. Intriguingly, both overexpression and suppression of SLC45A3 cause reduction in myelin protein expression levels (Fig. 4A, C). Furthermore, immunocytochemistry of these primary OLs with antibody against MBP demonstrated that changing the expression level of SLC45A3 can trigger alteration in OL morphology (less branches) (Fig. 4B, D), suggesting modulated SLC45A3 expression is necessary for proper OL differentiation or maintenance.
The lipid characteristics of myelin and the presence of myelin-specific proteins are known to be critical for proper myelin structure and function. Expression of genes encoding structural myelin proteins and enzymes involved in myelin lipid biosynthesis are regulated in a highly coordinated fashion, implying tight control of a balanced and local production of these two key components of myelin assembly (Nagarajan et al., 2002; Verheijen et al., 2003). Though little is known about myelin lipid turnover in vivo, renewal of myelin lipids in adult life has been shown to be necessary (Ando et al., 2003). In addition to synchronized expression of transcripts encoding structural myelin proteins and enzymes involved in myelin lipid biosynthesis, recent data suggest that glial lipid levels regulate myelin protein trafficking and thus also affect myelin assembly (Simons et al., 2000; Saher et al., 2009). Here, we present data demonstrating that a target protein of miR-32, SLC45A3, is critical for the proper maintenance of OLs by regulating its glucose concentration and/or lipid metabolism.

One of the major characteristics that separate myelin from other membranes is its high lipid-to-protein ratio with lipids accounting for at least 70% of the dry weight of myelin membranes. The specific myelin lipid composition likely to be important for the appropriate packing of the myelin membrane and changes in lipid composition can affect lipid–protein interactions causing an altered packing of proteins in the membrane (Coetzee et al., 1996; Lee, 2003). Many of the lipids that are enriched in myelin play roles in its axon-insulating properties. Thus, lipids control insulation of the axon and proper structure of the myelin membrane (Chrast et al., 2011), suggesting that lipid metabolism plays a critical role in the integrity of the myelin sheath. SLC45A3, also known as prostate cancer-associated protein 6 or Prostein, is highly expressed in the prostate (Xu et al., 2001) and in mature OLs (Cahoy et al., 2008), yet the function of SLC45A3 is not known before this study. According to the function of another SLC45 isoform (SLC45A1), SLC45 family proteins might be involved in the transport of sugars such as glucose and galactose. As sugar comes into the cell, acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to

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**DISCUSSION**

The lipid characteristics of myelin and the presence of myelin-specific proteins are known to be critical for proper myelin structure and function. Expression of genes encoding structural myelin proteins and enzymes involved in myelin lipid biosynthesis are regulated in a highly coordinated fashion, implying tight control of a balanced and local production of these two key components of myelin assembly (Nagarajan et al., 2002; Verheijen et al., 2003). Though little is known about myelin lipid turnover in vivo, renewal of myelin lipids in adult life has been shown to be necessary (Ando et al., 2003). In addition to synchronized expression of transcripts encoding structural myelin proteins and enzymes involved in myelin lipid biosynthesis, recent data suggest that glial lipid levels regulate myelin protein trafficking and thus also affect myelin assembly (Simons et al., 2000; Saher et al., 2009). Here, we present data demonstrating that a target protein of miR-32, SLC45A3, is critical for the proper maintenance of OLs by regulating its glucose concentration and/or lipid metabolism.

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form malonyl-CoA and fuels malonyl-CoA for de novo synthesis of long-chain fatty acids, which leads to subsequent chain elongation by fatty acid synthase and further elongation by ELOVL (elongation of very long chain fatty acids) protein. These products are incorporated into neutral lipids such as cholesterol esters and phospholipids, and used for steroidogenesis and energy production (Cahoy et al., 2008; Shin et al., 2009; Tamura et al., 2009). Hence, sugar overload can cause over production of VLCFAs. Extra VLCFAs might be incorporated into cell
membranes of white matter tracts and disrupt the stability of the axonal or myelin membranes by occupying the lateral chains of proteolipid proteins, gangliosides, and phospholipids (Ho et al., 1995). In addition, lipid accumulation in cell membranes can result in elevated oxygen consumption, which can trigger oxidative damage (Airt et al., 2002). Therefore, appropriately regulated sugar levels are required for proper OL and myelin maintenance. Our results indicate that SLC45A3 is a myelin-enriched putative sugar transporter that regulates myelination through its role in lipid metabolism. Interestingly, a recent report showed that myelination and OLs are gradually reduced as the glucose concentration is lowered, indicating survival of OLs and myelination is dependent on having adequate glucose levels (Rinholm et al., 2011).

Here, we found that both suppression and overexpression of SLC45A3 lead to lowered levels of myelin proteins and altered OL morphology, indicating the expression level of SLC45A3 itself is crucial for myelin protein maintenance. Consistent with these results, suppressing miR-32 (which would increase SLC45A3) also leads to decreased myelin protein levels and altered OL morphology. However, contrary to the results from suppressing SLC45A3, overexpressing miR-32 increased myelin protein expression and enhanced the branching morphology of OLs (Fig. 1F), indicating the presence of other target proteins in the mature OLs regulated by miR-32 and that the results we observed in suppressing miR-32 represent the overall consequences of the effects by miR-32 on all targets. Indeed, CLDN11 is also regulated by miR-32 (Fig. 2A). CLDN11 is a tight junction-associated protein found in CNS myelin, and may determine the permeability between layers of myelin sheaths via focal adhesion (Chow et al., 2005; Tiwari-Woodruff et al., 2001). In addition, it was shown to be involved oligodendrocyte proliferation and migration (Tiwari-Woodruff et al., 2001). Interestingly, homozygous CLDN11 null mice exhibit tremors, impaired coordination, hindlimb weakness, abnormal myelination of the cranial nerves, increased auditory thresholds, and abnormal stria vascularis (Gow et al., 1999). Since CLDN11 is a candidate auto-antigen in the development of autoimmune demyelinating disorders (Kauhsansky et al., 2007), the regulation of CLDN11 by miR-32 could also affect the overall phenotype shown in the miR-32 overexpression and suppression (Figs. 1E, F and 4E) and needs to be elucidated in the further study.

CONCLUSION

We characterized mature OL-specific functions of miR-32 and one of its target proteins, SLC45A3. Our results suggest that miR-32 participates in the optimization of sugar levels by regulating expression of SLC45A3, thus modulating intracellular glucose and long-chain fatty acid levels. Our data also imply that stringent regulation of SLC45A3 expression (by miR-32 and other factors) is essential for the maintenance of myelin proteins and structure. When the levels of intracellular glucose and long-chain fatty acids are perturbed, they could lead to excessive lipid accumulation or defective OL differentiation (Fig. 4E). Hence, miR-32 and its downstream target SLC45A3 play important roles in myelin maintenance by modulating glucose and lipid metabolism in OLs. Future experiments using SLC45A3 and miR-32 oligodendrocyte-specific conditional knockout animals would be necessary to elucidate and verify their function in vivo. The results presented here, together with our recent findings with miR-219 (Shin et al., 2009), imply that miRNAs are integral components of the network that ensures proper formation of compact myelin and support of axonal integrity by regulating lipid homeostasis for mature OLs.

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