MicroRNA-23a promotes myelination in the central nervous system

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Demyelinating disorders including leukodystrophies are devastating conditions that are still in need of better understanding, and both oligodendrocyte differentiation and myelin synthesis pathways are potential avenues for developing treatment. Overexpression of lamin B1 leads to leukodystrophy characterized by demyelination of the central nervous system, and microRNA-23 (*miR-23*) was found to suppress lamin B1 and enhance oligodendrocyte differentiation in vitro. Here, we demonstrated that *miR-23a*–overexpressing mice have increased myelin thickness, providing in vivo evidence that *miR-23a* enhances both oligodendrocyte differentiation and myelin synthesis. Using this mouse model, we explored possible *miR-23a* targets and revealed that the phosphatase and tensin homologue/phosphatidylinositol trisphosphate kinase/Akt/mammalian target of rapamycin pathway is modulated by *miR-23a*. Additionally, a long noncoding RNA, *2700046G09Rik*, was identified as a *miR-23a* target and modulates phosphatase and tensin homologue itself in a *miR-23a*–dependent manner. The data presented here imply a unique role for *miR-23a* in the coordination of proteins and noncoding RNAs in generating and maintaining healthy myelin.

MicroRNAs (miRNAs) play an important role in regulating a large number of developmental processes and diseases (1–3) through fine tuning biological networks (4, 5). Expression levels of miRNAs in oligodendroglia vary according to their differentiation stages, indicating a possible role for miRNAs in regulating developmental processes among migratory, proliferating, and myelinating oligodendrocytes (OLs) (6–9). Disruption of miRNA biogenesis by *Dicer* ablation in oligodendroglia at postdevelopmental stages results in a neurodegenerative phenotype including demyelination, inflammation, and axon loss (10), suggesting that miRNAs are also important for myelin maintenance at later developmental stages. *miR-23* is among the most abundant miRNAs in OLs (6, 7). Previously, we reported that in the presence of excess *miR-23* in vitro, a greater proportion of cells express mature markers of OLs that are paralleled by multipolar morphological appearance with increased levels of mature myelin proteins, indicating that *miR-23* can enhance oligodendrogenesis (11). In contrast, excessive lamin B1, a nuclear envelope protein and target of *miR-23*, leads to lower numbers of cells expressing mature markers with reduced levels of mature myelin proteins both in vitro and in vivo, suggesting defective differentiation of OLs. Importantly, the adverse effects of lamin B1 on OL cells can be abrogated by overexpressing *miR-23*, which functions as a negative regulator of lamin B1.

Here, we use mice in which *miR-23a* (one of the two *miR-23* isoforms: *miR-23a* and *b*) overexpression is driven by an OL-specific promoter [*2′,3′-cyclic nucleotide 3′-phophodiesterase* (*Cnp*)] to investigate the effects of *miR-23a* on OL differentiation and myelin synthesis in vivo. We demonstrated that in addition to the previously identified target, lamin B1, *miR-23a* also directly modulates the expression of two targets—phosphatase and tensin homolog on chromosome 10 (*PTEN*) and a long noncoding RNA (lncRNA), *2700046G09Rik* (RIKEN cDNA 2700046G09 gene). Through modulating expression of these molecules in myelinating glia cells, *miR-23a* fine tunes activities of the serine-threonine protein kinase Akt/mTOR (mechanistic target of rapamycin) and mitogen-associated protein kinase (MAPK) pathways that promote expression of myelin genes. Our results indicate that myelination requires tightly regulated multilayer signaling pathways partly converging downstream of PIP3 (phosphatidylinositol-3,4,5-trisphosphate) with coordinated nuclear changes such as transcription to trigger myelin gene expression, which then leads to proper membrane wrapping of axons by OLs.

Results

Generation of Transgenic Mice Overexpressing *miR-23a* in Oligodendrocytes. We have previously shown that *miR-23* overexpression enhances OL maturation in an established culture system (11). In addition, *miR-23a* and *miR-23b* are both up-regulated in OL under differentiation conditions compared with progenitor status under proliferation conditions (Fig. S1*A*). Knockdown of both *miR-23a* and *miR-23b* leads to significant reductions, whereas knockdown of either *miR-23a* or *miR-23b* individually does not cause obvious changes in expression levels of myelin genes (Fig. S1*B*), suggesting that *miR-23a* and *miR-23b* can compensate for each other in regulating OL maturation. Immunoreactivity of myelin basic protein (MBP) also demonstrated that *miR-23a* and *miR-23b* together had stronger effects on MBP expression than either one alone (Fig. S1 *C* and *D*). Given that ectopic expression of *miR-23* can promote transcription of myelin genes in cultured glia and purified OLs, we investigated the impact of overexpressing *miR-23a* on myelin formation in vivo. Northern blot analyses demonstrated that *miR-23a* displays reduced expression in *Dicer1*-ablated neurons, oligodendrocytes, and astrocytes (Fig. S2*A*), indicating ubiquitous expression of *miR-23a* in the CNS. Murine *Cnp* promoter was used to generate *miR-23a* transgenic mice (Fig. S2 *B* and *C*), as *Cnp* is highly expressed in developing OLs (12). Seven *Cnp–miR-23a* founder lines demonstrated *miR-23a* overexpression (Fig. S2*D*) and three of these lines with different expression levels were selected for further characterization. Quantitative RT-PCR

Significance

Understanding molecular mechanisms that underlie the processes for myelin synthesis and maintenance has been an intensely investigated topic. Concurrently, recent advances in noncoding RNAs (ncRNAs) have uncovered unique insights into many biological processes, and ncRNAs have become recognized as major players for epigenetic regulation. We generated a murine model overexpressing microRNA-23a (*miR-23a*) to investigate its role in myelin regulation. In addition, we used this mouse model to identify two targets of *miR-23a*: one is a protein-coding gene phosphatase and tensin homologue and the other is a long ncRNA (lncRNA) *2700046G09Rik*. Our study demonstrated a complex network comprising a protein-encoding gene, a miRNA, and a lncRNA that is central to the fine tuning and maintenance of healthy myelin.

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The authors declare no conflict of interest.

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(qRT-PCR) of oligodendrocyte progenitor cells (OPCs) purified from two lines display moderate-to-high levels of *miR-23a* in proliferative medium and a further significant increase in differentiation medium (Fig. S2*E*). *Cnp–miR-23a* mice carrying one transgene allele were not overtly distinguishable from control littermates but mice carrying two transgene alleles developed a notable unilateral hindlimb paralysis as early as postnatal day 5 (P5) (Fig. 1*A*). In addition, these *Cnp–miR-23a* mice exhibited abnormal axial muscle tone (kyphosis), puffy eyes, and hindlimb ataxia from P42 (Fig. 1*A*).

Because there is no standard behavioral analysis specifically designed for myelin assessment (13), we used several tests to examine the *Cnp–miR-23a* mice for neurological dysfunction. We examined general locomotor activity of mice at 20 wk of age. *Cnp–miR-23a* transgenic mice showed motor impairment by requiring more time to traverse the balance beam (5 mm and 11 mm) (Fig. 1*B*) and a higher rate of hindlimb slips and dragging (Fig. S3 *A* and *B*). The hanging wire test was next used to examine whether the motor impairments were related to loss of muscle power, and significant differences were observed (Fig. 1*C*). Additionally, *Cnp–miR-23a* mice required more time to traverse the diagonal bar (Fig. 1*D*) and displayed a lower success rate (Fig. 1*E*) as well as reduced duration on the coat hanger (Fig. S3*C*), supporting that *Cnp–miR-23a* mice exhibited impaired motor function, which is likely a neurological consequence of *miR-23a* overexpression in OLs.

***miR-23a* Regulates Oligodendrocyte Differentiation.** To determine whether overexpressing *miR-23a* affects OL differentiation in vivo, oligodendroglia from transgenic mice under proliferation or differentiation conditions were used to analyze the expression levels of myelin genes. Interestingly, many of the myelin genes [particularly late phase genes such as myelin-associated glycoprotein (*Mag*), transferrin (*Trf*), and myelin oligodendrocyte glycoprotein (*Mog*)] are simultaneously up-regulated in *miR-23a* OLs compared with wild-type (WT) control cultures (Fig. 2*A*). Early OL gene such as proteolipid protein (*Plp*) and *Mbp* were also induced to a mild extent by *miR-23a* overexpression under proliferation conditions (Fig. S3*D*). *miR-23a* overexpression not only increased the number of OLs expressing MBP and MAG (Fig. 2*B* and Fig. S3*E*), but also increased the overall expression of PLP and MBP compared with control oligodendroglia (Fig. 2*C*), supporting *miR-23a* as a key regulator of OL differentiation.

Overexpression of *miR-23a* Leads to Enhanced Myelination in the Central Nervous System. Increased myelination in *Cnp–miR-23a* mice compared with WT control was observed in the corpus callosum stained with myelin-specific dye and CNP/MBP

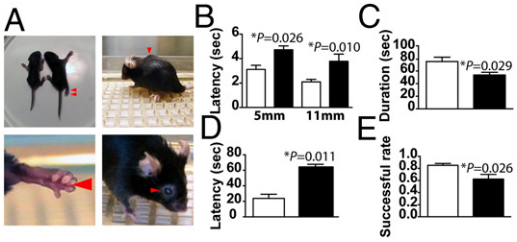

Fig. 1. *Cnp–miR-23a* mice exhibited impaired motor function. (*A*) Morphological abnormalities displayed in *Cnp–miR-23a* mice: hindlimb paralysis (*Upper Left*), loss of foot extensor tone (*Lower Left*), kyphosis (*Upper Right*) and puffy eyes (*Lower Right*). Mice at the age of P150 were analyzed with balanced beam test (*B*), horizontal wire hanging test (*C*), and coat hanger test (*D* and *E*). Measurement of time for *Cnp–miR-23a* mice to traverse balance beams (*B*) and to hang in horizontal wire (*C*) and measurement of latency to reach the diagonal bar (*D*) and successful rate to reach diagonal bar (*E*) are shown. Data are presented as means ± SEM, $n = 7$ per genotype, $^*P < 0.05$, unpaired Student *t* test. Filled bars represent *Cnp–miR-23a* and open bars denote WT mice.

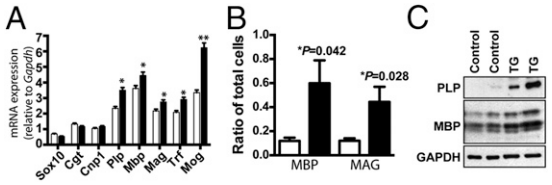

Fig. 2. Overexpressing *miR-23a* promotes OPC differentiation. (*A*) Expression of OL markers in cells purified from P7 *Cnp–miR-23a* mice was assessed using qRT-PCR. Data are presented as means ± SEM, $^*P < 0.05$, $^{**}P < 0.01$, unpaired Student *t* test. $n = 4$ per genotype. Filled bars represent *Cnp–miR-23a* OLs and open bars denote WT OLs. (*B*) Quantification of MBP- or MAG-positive cells in OPCs purified from P7 WT and *Cnp–miR-23a* mice cultured for 4 d in vitro (DIV) in differentiation medium. The total numbers of cells were determined by DAPI staining. Data are presented as ratio ± SEM from three independent experiments. $^*P < 0.05$. (*C*) Representative Western analyses showed increased expression of PLP and MBP in *Cnp–miR-23a* OLs relative to control. Protein lysates were purified from OPCs cultured at 4 DIV in differentiation media. GAPDH was used as a loading control.

antibodies (Fig. 3*A*). The size of the corpus callosum was increased in *Cnp–miR-23a* mice at P90. Expression of myelin proteins, such as CNP, MBP, and MAG was elevated (Fig. 3*B*). Electron microscopy (EM) analysis revealed an increased number of axons exhibiting focal myelin pathology such as aberrant myelin outfoldings caused by hypermyelination at P180 (Fig. 3 *C* and *D*, yellow and green arrowheads) or "invaginating" recurrent loops (Fig. 3*D*, red arrowhead), which showed similar features with the myelin sheath from which they had originated. We quantified the myelin abnormalities by comparing electron microscopy of rostral corpus callosum from *Cnp–miR23a* and controls at 6 mo of age. G ratios for the transgenic $(TG)^+/WT^-$ or TG^+/TG^+ *Cnp–miR-23a* mice are 0.6144 (±0.0043) and 0.6366 (±0.0045), respectively, which are significantly different from controls (0.7088 ± 0.0038) (Fig. 3*E*, *Upper Right*, $P < 0.01$). Increased myelin thickness was evident for axons of small calibers (Fig. 3*E*, *Upper Left*), but not all fibers were visibly hypermyelinated. The overall size distribution of callosal axons was similar in transgenic and control mice (Fig. 3*E*, *Lower*). EM demonstrated increased layers of myelin sheath wrapping in either small or large caliber axons with increased membrane wraps (Fig. 3*F*). Together, these data implicate *miR-23a* in the regulation of myelin thickness and proper myelin folding in the central nervous system (CNS).

Unbiased Search for *miR-23a* Targeted Molecules and Mechanisms. To further investigate the mechanisms of *miR-23a* in OL development and myelination, we set out to identify other relevant targets that are regulated by *miR-23a*. A total of 1,179 genes were identified to demonstrate differential expression between *Cnp–miR-23a* and WT mice [absolute fold change (FC) >1.5, multitest adjusted *P* value ≤0.05 correspondent to unadjusted *P* value ≤0.013 by cufflink] (Fig. 4*A* and Datasets S1 and S2). This included many known myelin-formation–associated genes, which were highly expressed in *Cnp–miR-23a* (Fig. 4*B*). Additionally, we found that genes specific to early stages of oligodendroglia development, such as *Pdgfra* and *Lmnb1*, displayed reduced levels in OLs purified from *Cnp–miR-23a*.

Next, we compared the differentially expressed genes with the reference lists of genes that are enriched in astrocytes, neurons, and OLs (14). The OL-enriched genes are mostly up-regulated in *Cnp–miR-23a*, whereas the neuron-enriched genes tend to be down-regulated (Fig. S4 *C* and *D*). Upon further dissection of *miR-23a* on promoting OL differentiation, we found that genes enriched in more mature OLs, including OLs and the most mature MOG^+ OLs (14), are mostly up-regulated (Fig. 4*C* and Fig. S4*E*). Collectively, the results of RNA sequencing (RNA-Seq) suggest a role for *miR-23a* in promoting the progression of less-differentiated OPCs into myelinating and mature OLs, likely by promoting the expression of mature OL-enriched genes. This

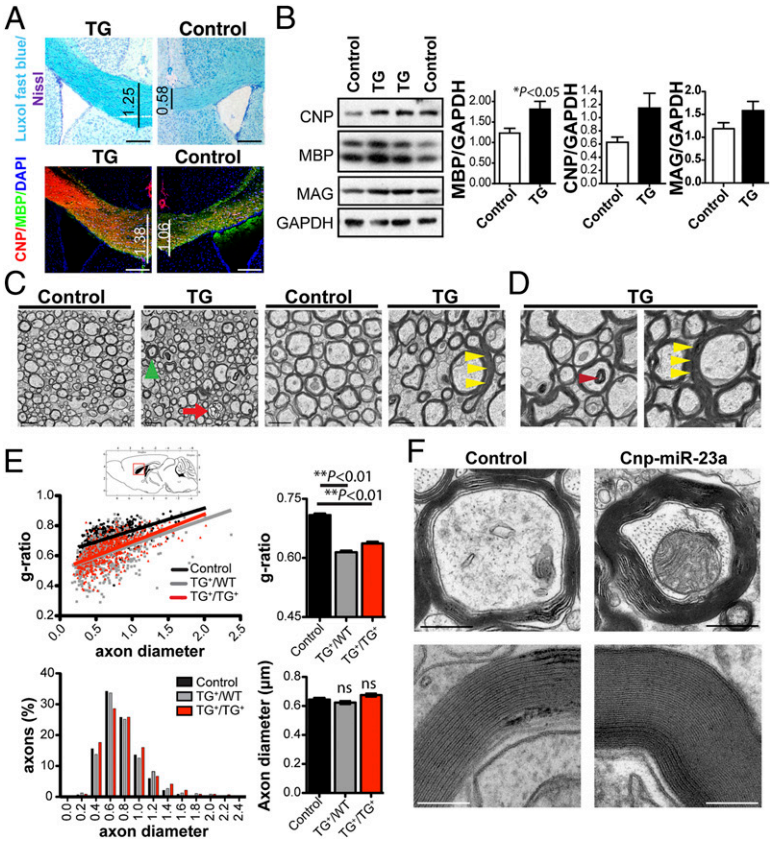

Fig. 3. Enhanced myelination in *Cnp–miR-23a* mice. (*A*) Corpus callosum in WT and *Cnp–miR-23a* mice at P90 showing enhanced myelination by Luxol Fast blue and CNP/MBP immunoreactivities. (*B*) Quantification of MBP, CNP, and MAG in corpus callosum by Western blot analysis ($n = 3$, $^*P < 0.05$, unpaired Student t test). (*C* and *D*) Myelin abnormalities in corpus callosum of *Cnp–miR-23a* mice at P180, including hypermyelination (green arrowheads), myelin debris (red arrows), aberrant outfoldings (yellow arrowheads), and invaginating recurrent loops in axons (red arrowhead). [Scale bar for *C*, 1 μm (*Left*) or 2 μm (*Right*)]. (*E*) Quantitation of myelin thickness by modified G-ratio analysis and axon size distribution for the corpus callosum (age, P180; $n = 2$ per genotype; control, WT littermates; TG^+/WT, one allele of transgene; TG^+/TG^+, two alleles of transgene). (*Upper Left*) Scatter plots comparing G ratios from TG^+/WT (gray), TG^+/TG^+ (red), and age-matched controls (black) in relation to myelin sheath inner diameter. (*Upper Right*) Myelin thickness is significantly increased in *Cnp–miR-23a* mice. (*Lower*) Axonal calibers are comparable between *Cnp–miR-23a* mice and controls. ($^{**}P < 0.01$, one-way ANOVA). (*F*) Hypermyelination in *Cnp–miR-23a* mice (*Right*) is caused by additional membrane wraps, as visualized by ultrastructure and periodicity of myelin sheaths. [Scale bar, 500 nm (*Upper*) and 100 nm (*Lower*)].

regulation may be accompanied by repression of neuron-enriched and (to a lesser extent) astrocyte-enriched genes.

***miR-23a* Target Molecules: *PTEN* and *2700046G09Rik*.** *In silico* prediction (miRANDA and TargetScan) followed by luciferase reporter assay was carried out to identify potential direct targets of *miR-23a* for regulating CNS myelination. We reasoned that true *miR-23a*–targeted genes would display positive correlation between RNA-Seq and luciferase reporter analysis. Among 35 candidates examined in this study, *PTEN* and *2700046G09Rik* (Fig. S5*A*) displayed positive correlation. In addition, their genomic locations are rather close, which raised a possibility for the lncRNA (*2700046G09Rik*) to exert *cis*-regulatory effect on the neighboring *PTEN* gene (15). Therefore, we investigated them further. The repression of *PTEN* and enhancement of *2700046G09Rik* by *miR-23a* were validated by mutagenizing their respective *miR-23a* binding elements (Fig. S5 *B–D*) *miR-23* exhibits a gradually increased expression pattern during postnatal development (11), and the true direct target genes of *miR-23a* should display expression patterns correlating with *miR-23a* expression. Indeed, the protein levels of PTEN display a gradual decrease from postnatal day 1 to 10 months of age (Fig. 5*A*), whereas the levels of *2700046G09Rik* display a gradual increase beginning at postnatal day 60 (Fig. 5*B*). We next validated the interaction of *miR-23a* and *PTEN* or *2700046G09Rik* by UV–cross-link RNA immunoprecipitation (RIP) (16). FLAG-AGO2 immunoprecipitation was conducted in

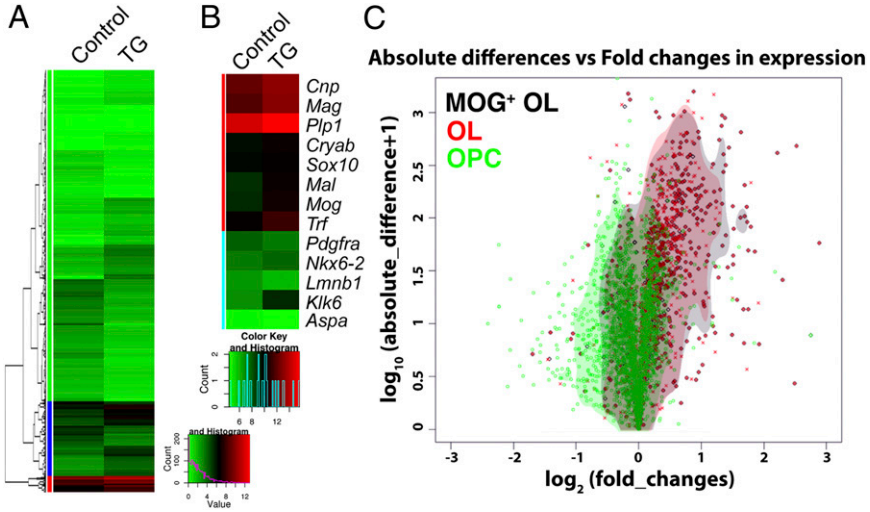

Fig. 4. Differentially expressed transcriptome in *miR-23a*–overexpressing oligodendroglia. (*A*) Hierarchical clustering and analysis of overall expressed genes in cultured OLs overexpressing *miR-23a*. (*B*) Differential expression of known myelin-associated genes plotted on a color scale (green, low expression; red, high expression). (*C*) Volcano plot and kernel density estimation demonstrate the differences in the expression patterns of genes known to be enriched at different stages of oligodendrocyte differentiation (OPC, progenitors; OL, myelinating OL; MOG^+, mature OL expressing MOG) in response to *miR-23a* overexpression.

HEK293 cells transiently coexpressing *miR-23a* and its targets. By RIP-qPCR, *PTEN* or *2700046G09Rik* coprecipitating with AGO2 was specifically enriched in *miR-23a*–transfected cells (Fig. 5*C*), indicating that *miR-23a* is facilitating AGO2 association with *PTEN* or *2700046G09Rik*. Furthermore, using a biotin-coupled *miR-23a* mimic, we observed a significant enrichment of *PTEN* or *2700046G09Rik* in *miR-23a*–captured fraction compared with control (Fig. 5*D*). Consistently, the levels of PTEN are decreased (Fig. 5*E*), whereas *2700046G09Rik* are increased (Fig. 5*F*) in spinal cord and cerebellum of *Cnp–miR-23* mice compared with WT mice. Taken together, these results strongly imply that direct interactions exist between *miR-23a* and *PTEN* or *2700046G09Rik* and that *PTEN* and *2700046G09Rik* are true *miR-23a* targets.

***2700046G09Rik* Participates in Myelin Regulation.** To understand the role of *2700046G09Rik* in oligodendroglial differentiation and myelin production, we examined its expression in various cells. RNA levels of *2700046G09Rik* were significantly higher in cultured OLs from WT mice under differentiation conditions, whereas astrocytes displayed a comparable level to cultured OPCs under proliferative conditions (Fig. S6*A*). Congruently, its level is increased in OLs from *Cnp–miR-23a* compared with WT OLs. Overexpression of *2700046G09Rik* in cultured OLs led to moderately increased expression of MAG protein (Fig. S6*B*). In addition, promoter region reporter assays revealed that two important oligodendrocyte-associated transcription factors for OPCs differentiation to OLs, YY1 and Nkx2.2 (17, 18), display positive effects on the expression of *2700046G09Rik* (Fig. 5*G*). Together, these data support a potential unique role for *2700046G09Rik* in the regulation of myelination.

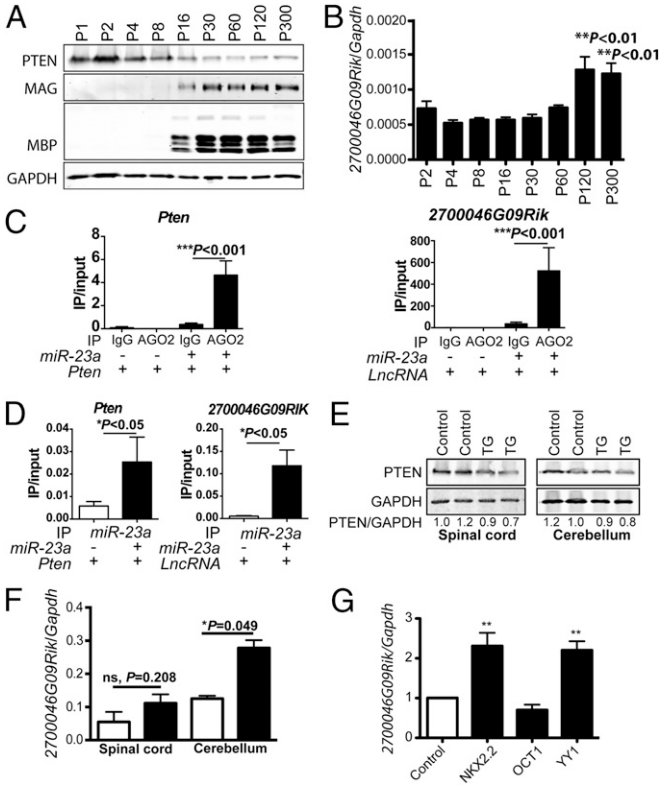

Fig. 5. *miR-23a* regulates PTEN and *2700046G09Rik*. (*A*) Western analysis of PTEN, MAG, MBP, and GAPDH from C57BL/6 brain at indicated ages. (*B*) qRT-PCR of *2700046G09Rik* from C57BL/6 brain. $^{**}P < 0.01$ compared with P0, one-way ANOVA. (*C*) Immunoprecipitation of FLAG-tagged AGO2 from HEK293 transfected with *PTEN* 3′-UTR or *2700046G09Rik* and FLAG-AGO2 plus vector or *miR-23a*. *PTEN* and *2700046G09Rik* levels were quantified by qRT-PCR, and the relative immunoprecipitate (IP)/input ratios were plotted. $n = 4$, $^{***}P < 0.001$, unpaired Student *t* test. (*D*) The 3′-end biotinylated *miR-23a* duplexes were transfected into HEK293. After streptavidin capture, input and bound fractions were evaluated by qRT-PCR. $n = 4$, $^{*}P < 0.05$. (*E*) Western analysis of PTEN in spinal cord or cerebellum from WT and *Cnp–miR-23a* mouse brain at P90. (*F*) qRT-PCR of *2700046G09Rik* transcript using spinal cord or cerebellum from control and *Cnp–miR-23a* mice. $n = 3$, $^{*}P < 0.05$. Filled bars represent *Cnp–miR-23a* and open bars denote WT mice. (*G*) Luciferase activity of firefly reporter gene fused with the 3-kb *2700046G09Rik* promoter in the presence of indicated transcription factors. $n = 4$, $^{**}P < 0.01$ compared with control, one-way ANOVA. All data are presented as ratio of means ± SEM.

Interplays Among *miR-23a*, *2700046G09Rik*, and *PTEN*. The competitive endogenous RNA hypothesis (19) suggested that coding and noncoding transcripts share common miRNA binding elements and lead to altered transcriptome homeostasis. To test whether *PTEN*, *2700046G09Rik*, and *miR-23a* have interplay in regulating OL and myelin, we first investigated the possible regulatory effects of *2700046G09Rik* on *PTEN* in cell culture. The coding region plus 2 kb 3′-UTR of *PTEN* was cotransfected into HEK293 with either *miR-23a* or *2700046G09Rik* and Western blot analysis revealed that *miR-23a* and *2700046G09Rik* both exert repressive effects on *PTEN* (Fig. 6*A*). Next, luciferase reporters carrying full-length (6 kb) 3′-UTR (20) of *PTEN* was coexpressed with *miR-23a* and/or *2700046G09Rik* (Fig. 6*B*). *2700046G09Rik* did not significantly alter the level of *PTEN*, whereas *miR-23a* displayed moderate repression. Interestingly, the level of *PTEN* was reduced by *2700046G09Rik* only in the presence of the *PTEN* coding region together with 2 kb 3′-UTR (Fig. 6 *A* and *C*). This effect was abrogated by mutating the *miR-23a* binding motif of *2700046G09Rik* (Fig. 6*C*, lane 4), suggesting that an intact *miR-23a* binding motif is necessary for the full repressive effect of *2700046G09Rik* on PTEN and that there is a possible interplay between *miR-23a* and *2700046G09Rik* for regulation of PTEN. As expected, the level of PTEN was further reduced by the presence of both *miR-23a* and *2700046G09Rik* (Fig. 6*C*, lane 5).

We next investigated the possible effect of *2700046G09Rik* and *miR-23a* on each other. Inhibition of de novo transcription by actinomycin D treatment in HEK293 cells showed *miR-23a* has a longer half-life compared with U6 small nuclear RNA (snRNA) (Fig. 6*D*). Cotransfection of *2700046G09Rik* containing perfect *miR-23a* binding elements with *miR-23a* led to higher expression of *miR-23a* in HEK293 cells following 24-h treatment of actinomycin D, whereas *miR-23a* did not alter stability of *2700046G09Rik* regardless of the presence of *miR-23a* binding elements (Fig. 6*E*). These results suggested that *2700046G09Rik* enhances *miR-23a* stability. HEK293 cells expressing *miR-23a*, *2700046G09Rik*, or both were then accessed by RIP-qPCR following immunoprecipitation of DCP1 (decapping enzyme 1) for P body (processing bodies). Coexpression of *miR-23a* and *2700046G09Rik* significantly increased *miR-23a* in DCP1 immunoprecipitant (Fig. 6*F*), indicating enrichment of *miR-23a* in P bodies by *2700046G09Rik*. Altered stability and cellular localization of *miR-23a* by *2700046G09Rik* suggest a unique role for noncoding transcripts in the regulation of *PTEN* transcript. Together, these results demonstrate a network of regulatory pathway, including *miR-23a*, *2700046G09Rik*, and PTEN in the regulation of OL development and myelin gene expression.

Signaling Pathways Modulated by *miR-23a* Overexpression. Because PTEN is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K) signaling pathway (21), and accumulating evidence indicates that the PI3K/Akt/mTOR pathway regulates CNS myelination (22, 23), we investigated the possibility that *miR-23a* has a role in modulating the PI3K/Akt/mTOR signal transduction cascade. Western blot analysis revealed that the level of phosphorylated Akt was higher in *miR-23a* brain homogenate (Fig. 7*A*), indicating activation of Akt signaling. In addition, levels of PI3K signaling and MAPK activity, but not protein kinase A (PKA), were also elevated (Fig. S7 *A–C*). Ectopic expression of PTEN in cultured OLs purified from control and *Cnp–miR-23a* mice at P7 reduced expression levels of

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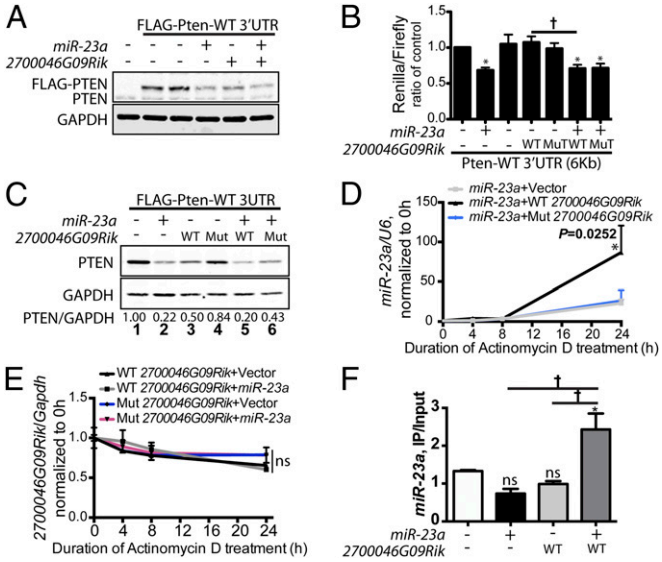

Fig. 6. *miR-23a* and *2700046G09Rik* repress PTEN. (*A*) Representative Western analysis of PTEN from HEK293 coexpressing *PTEN* coding sequences fused to 2-kb *PTEN* 3′-UTR with either *miR-23a*, *2700046G09Rik*, or both. (*B*) Luciferase activity of firefly reporter gene fused with the 6-kb full-length *PTEN* 3′-UTR (without coding region) in the presence of indicated RNA. WT, *2700046G09Rik* with normal *miR-23a* binding elements. Mut, *2700046G09Rik* with mutated *miR-23a* binding elements (M123, Fig. S5). Data presented as ratio of means ± SEM $*P < 0.05$ compared with control; $^{\dagger}P < 0.05$ compared with WT *2700046G09Rik*; one-way ANOVA. (*C*) Representative Western analysis of PTEN from HEK293 coexpressing *PTEN* coding sequences fused to 2-kb 3′-UTR (containing WT *miR-23a* binding elements) together with *miR-23a* and/or *2700046G09Rik*. (*D*) qRT-PCR analysis of *miR-23a* in HEK293 transfected with *miR-23a* plus vector (gray), *2700046G09Rik* carrying WT (black), or Mut (blue) *miR-23a* binding elements followed by actinomycin D treatment. $n = 6$, $*P < 0.05$ compared with vector control. One-way ANOVA analysis with Newman–Keuls test at 24 h. (*E*) qRT-PCR analysis of *2700046G09Rik* transcript in HEK293 transfected with *miR-23a* plus *2700046G09Rik* carrying WT (black) or Mut (blue) *miR-23a* binding elements followed by actinomycin D treatment. $n = 6$, NS, nonsignificant. (*F*) Immunoprecipitation of DCP1 from HEK293 cells transfected with vector, *miR-23a*, *2700046G09Rik*, or *miR-23a* plus *2700046G09Rik*. *miR-23a* levels were quantified by qRT-PCR, and the relative immunoprecipitate (IP)/input ratios were plotted. $n = 4$, NS, nonsignificant, $*P < 0.05$, $^{\dagger}P < 0.05$.

MAG compared with vector control (Fig. 7*B*). Overexpressing dominant-negative Akt (AKT-DN) dramatically reduced expression of myelin proteins in *Cnp–miR-23a* (Fig. 7*C*), consistent with the hypothesis that Akt acts downstream of *miR-23a* to mediate myelin formation. Rapamycin, the mTOR inhibitor, treatment reduced expression levels of several myelin proteins (PLP, MOG, and MAG) in cultured OLs isolated from *Cnp–miR-23a* mice (Fig. 7*D*). Collectively, these results confirm that PTEN/PI3K/Akt/mTOR is part of the cascade in *miR-23a*–mediated regulation and that mTOR acts downstream of *miR-23a* to mediate myelin production.

Discussion

The deposition of a precise amount of myelin around axons is necessary for proper impulse transmission, whereas too much or too little myelin surrounding axons causes nerve dysfunction in various neurological diseases. We have generated a mouse model overexpressing *miR-23a* that produces excessive myelin protein and myelin formation in the CNS. These mice display severe motor deficits beginning in postnatal life. Because *Cnp* expresses in both central and peripheral nervous systems (PNS), we cannot exclude the possibility that the motor deficits observed in *Cnp–miR-23a* mice were caused, at least in part, by peripheral myelin abnormality. Using this mouse model, we also produced a profile for differentially expressed genes that are associated with *miR-23a* overexpression. This *miR23a*-myelin transcriptome offers a useful resource for future investigation in understanding the signaling networks and factors that are required for the regulation of OL development/myelination and other *miR-23a*–regulated biological functions.

Our demonstration of *miR-23a* overexpression in OLs resulting in hypermyelination of mouse brain establishes a regulatory role for *miR-23a* in myelin production. Overexpression of *miR-23a* leads to up-regulation of genes in OL clusters, but substantial down-regulation of genes in neuronal clusters. This suggests that *miR-23a* not only functions to enhance OL lineage progression and promote myelin proteins but also safeguards against expression of genes for other cell lineages that might interfere with the progression of OL maturation and myelin sheath formation. This is consistent with previous reports that miRNAs function as guardians to enhance lineage-related protein identity and to repress other nonlineage protein expression (6, 9).

Elevated PIP3 signaling or loss of PTEN in myelinating glia has been shown to cause hypermyelination in the CNS (21, 24) and PNS (25). PTEN antagonizes PI3K signaling and negatively regulates the ERK1/2-MAPK pathway (26). Akt and mTOR, downstream effectors of PI3K signaling, promotes OL differentiation and myelin generation (22, 27, 28). Transgenic mice overexpressing constitutively active Akt kinase in OLs enhance myelin formation in the CNS but not PNS (22), and this is mediated through downstream mTOR signaling (23). The present study revealed that elevated level of *miR-23a* in OLs is sufficient to promote formation of myelin that can last until older age (at least 1-y old) partly through the Akt/mTOR and MAPK signaling pathways by targeting *PTEN*. A recent study reported that the cAMP-response element-binding protein (CREB) promotes glioma formation by up-regulating *miR-23a*, leading to down-regulation of its direct target, *PTEN* (29). Thus far, we have not observed obviously increased incidence of glioma formation in murine brains overexpressing *miR-23a* in myelinating glia.

Duplications of *LMNB1* (which encodes lamin B1) have been identified to cause adult-onset autosomal dominant leukodystrophy (30). Excessive lamin B1 expression reduces occupancy of Yin Yang 1 (YY1) transcription factor on the promoter region of PLP, thus leading to down-regulation of PLP abundance, conferring myelin loss in the mouse brain (31). Reduced levels of both PTEN and LMNB1 by *miR-23a* are likely to participate in hypermyelination observed in the present study.

To date, long noncoding RNAs have been shown to function as regulators of gene expression transcriptionally and posttranscriptionally through working at the DNA level (32), modulation of chromatin modifications, or transcriptional interference by antisense transcription (1). miRNAs bind to the coding sequences or 3′-UTRs of target transcripts, thus leading to impaired translation or increased degradation of transcripts (33). Interestingly,

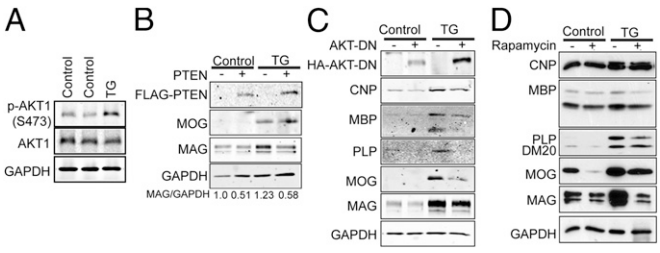

Fig. 7. *miR-23a* overexpression leads to activation of the Akt/mTOR pathway. (*A*) Western analysis of phosphor-AKT (S473) expression in corpus callosum of P90 WT or *Cnp–miR-23a* mice. (*B–D*) Representative Western analyses of myelin proteins in mouse OPCs from WT or *Cnp–miR-23a* mice cultured for 4 DIV in differentiation media. Purified OPCs were overexpressing PTEN (*B*) or dominant-negative AKT1 (AKT-DN) (*C*). (*D*) Purified OPCs were treated with 15 nM rapamycin.

2700046G09Rik, one of the *miR-23a* targets identified in this study, is a lncRNA *in cis* with the neighboring *PTEN* gene. *miR-23a* up-regulates the *2700046G09Rik* transcription, and *2700046G09Rik* in turn lengthens the half-life of *miR-23a*, thus potentiating its repressive effects. *2700046G09Rik* also can lead to a reduced level of *PTEN* expression. This down-regulation is independent of *miR-23a*–responsive elements (MREs) on *PTEN*, but requires the *miR-23a* MREs on *2700046G09Rik*. Therefore, repressive effects on PTEN can either occur with *miR-23a* alone or in coordination with *2700046G09Rik*. It is possible that *2700046G09Rik* targets the *PTEN* with the assistance of *miR-23a.* In addition, *2700046G09Rik* may aid in the cellular recompartmentation of *miR-23a* into P bodies, which could also contribute to the regulation of PTEN level. Interplay of *miR-23a* and *2700046G09Rik* in this study infers additional molecular processes in regulating mRNA decay (Fig. S8). lncRNAs are tightly controlled by environmental cues and inducible functions (34). Intriguingly, we discovered that promoter of *2700046G09Rik* can be activated by two important transcription factors (YY1 and Nkx2.2) (17, 18, 35) in OL development. Our results are consistent with a previous report that dynamic changes of lncRNA transcriptome are important for glia differentiation (36). We propose that the presence of *2700046G09Rik* in oligodendroglia potentiates and signals the activation of the *miR-23a*/PTEN/Akt/mTOR and MAPK cascades in the correct developmental stage, thus regulating the expression of myelin genes in OLs.

Noncoding RNAs (ncRNAs) have emerged as a major component in epigenetic regulation, specifically in orchestrating neural gene expression and function and gene–environment interactions (2, 37). Elevated levels of *miR-23a* are identified in severe demyelinated regions of brains derived from the murine model of autosomal dominant adult-onset leukodystrophy (Fig. S9) and active multiple sclerosis lesions in human brains (38, 39). Interestingly, increased frequency of *miR-23a* rs(refSNP) 3745453 C allele has been identified as a potential risk factor of multiple sclerosis (39). Therefore, *miR-23a* may participate in not only modulating genes in normal myelin regulation but also in myelin repair. Through identification of *miR-23a* targets, we revealed that *miR-23a* and some lncRNAs (*2700046G09Rik*) interact in a complex regulatory network to modulate genes that are important for myelin formation/maintenance. Understanding the layers of complexity in the molecular mechanisms that underlie the elaboration of myelin synthesis and maintenance has undergone intensive investigation and recent advances have uncovered unique insights into these processes. Identifying targets of *miR-23a* and developing future demyelinating experiments using this murine model will provide the opportunity to better understand RNA regulatory networks and offer future therapeutic approaches against demyelinating diseases.

Materials and Methods

Details for generation of *Cnp–miR-23a* mice oligodendrocyte culture, RNA-Seq analysis, immunoblotting, immunostaining, and qRT-PCR are provided in *SI Materials and Methods*. *Cnp-miR-23a* mice were generated and maintained in C57BL/6 background. All animal experiments were performed under approval by the Animal Care and Use Committee at the University of California, San Francisco.

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