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Reduction of Dicer Impairs Schwann Cell Differentiation and Myelination

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Abstract

The process of Schwann cell myelination requires precisely coordinated gene expression. At the onset of myelination, there is an increase in the expression of differentiation-promoting transcription factors that regulate key Schwann cell genes. Further control of myelin gene expression occurs at the posttranscriptional level and, in part, is mediated by RNA binding proteins and micro-RNAs (miRNAs). miRNAs are small, endogenously derived RNA molecules that repress gene expression by specifically binding to their mRNA targets. In the experiments described here, we tested whether miRNAs were essential in controlling myelination by reducing the levels of Dicer, an essential endoribonuclease in miRNA biogenesis. We decreased the expression of Dicer by about 60% within Schwann cells using a lentiviral vector expressing an shRNA against Dicer. The reduced levels of Dicer led to a decrease in the steady-state expression of selected miRNAs and of the transcription factors Oct6 and Egr2/Krox20, both of which are critical for Schwann cells differentiation and myelination. In contrast, the levels of c-jun and Sox2 were up-regulated by the reduction in Dicer and were associated with an increase in Schwann cell proliferation. In dorsal root ganglion cocultures, Schwann cells transduced with Dicer shRNA synthesized less myelin, which was accompanied by significant reductions in the levels of myelin basic protein and protein zero. These findings support a critical role for Dicer and miRNAs in Schwann cell differentiation and myelination.

Keywords

Schwann cell; myelin; micro-RNA; Dicer; myelination

Myelination in the peripheral nervous system (PNS) by Schwann cells permits rapid saltatory conduction of action potentials along axons. Myelin defects in the PNS are associated with diseases such as inherited peripheral neuropathies, which can result from altered myelin gene dosage (Lupski and Garcia, 1992; Scherer, 1997; Vucic et al., 2009). For Schwann cells to initiate myelination, they have to exit the cell cycle and increase the expression of myelin-associated genes in a coordinated fashion, which is coupled with significant changes in their transcriptional profile (Svaren and Meijer, 2008). The transcription factors early growth response 2 (Egr2/Krox20), SRY-box containing gene 10 (Sox10), POU domain class 3 transcription factor 1 (Oct6/Scip), and POU domain class 3 transcription factor 2 (BRN2) have all been shown to be positive regulators of myelination, whereas c-Jun and SRY-box containing gene 2 (Sox2) promote a nonmyelinating Schwann

cell phenotype (Wegner, 2000; Parkinson et al., 2004, 2008; Le et al., 2005). Expression of Egr2/Krox20 is required for Schwann cell myelination, and the myelin genes *myelin basic protein* (MBP), *myelin protein zero* (MPZ), and *myelin-associated glycoprotein* (MAG) are direct targets of this critical transcription factor (Svaren and Meijer, 2008). In addition to transcriptional regulation, there is increasing evidence that posttranscriptional mechanisms involving RNA binding proteins and micro-RNAs (miRNAs) play key roles during the process of myelination in both the CNS and the PNS (Lau et al., 2008; Zearfoss et al., 2008; Verrier et al., 2009).

Posttranscriptional regulation of myelination was first demonstrated in oligodendrocytes. Analyses of the RNA-binding protein Quaking (QKI) revealed that it interacted with the MBP mRNA, and deletion of the QKI gene reduced steady-state MBP mRNA levels (Li et al., 2000). The related RNA binding proteins QKI-6 and QKI-7 have been shown to block Schwann cell proliferation and to promote myelination (Larocque et al., 2009). Recent studies indicate that miRNAs also play a role in regulating myelination (Lau et al., 2008; Kawase-Koga et al., 2009; Lin and Fu, 2009; Verrier et al., 2009) and glial cell biology (Lehotzky et al., 2009; Shin et al., 2009). The molecular mechanisms underlying the biogenesis of miRNAs in mammalian cells have been studied extensively (Valencia-Sanchez et al., 2006). In brief, mature miRNAs are derived from RNA molecules that are selectively cleaved by the ribonuclease Drosha, exported into the cytoplasm, and cleaved again by Dicer (Provost et al., 2002). Our understanding of the impact of miRNAs on cellular processes, including the development of skeletal muscle, lung, and hippocampus, has been enhanced by studies of conditional Dicer knockout mice (Harris et al., 2006; O'Rourke et al., 2007; Davis et al., 2008). Nevertheless, the roles of miRNAs in peripheral nerve development, including Schwann cell differentiation and myelination, have not been examined.

In these experiments, we utilized an in vitro PNS myelination assay and Dicer knock-down techniques to examine the requirement for miRNAs in Schwann cell biology. We demonstrate that reduced miRNA biogenesis within Schwann cells leads to a decrease in the steady-state expression of promyelination differentiation factors and an impairment of myelination. Our findings indicate that mature miRNAs are critical for Schwann cells to switch from a proliferating, nondifferentiated state to a mature, myelin-forming phenotype.

MATERIALS AND METHODS

Primary Rat Schwann Cell Cultures

Primary Schwann cell cultures were established from newborn rat pups (Ryan et al., 2002). Schwann cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT), 5 μ M forskolin (Calbiochem, La Jolla, CA), and 10 μ g/ml bovine pituitary extract (Biomedical Technologies Inc., Stoughton, MA).

Myelinating Cocultures of Schwann Cells and Dorsal Root Ganglion Neurons

Dissociated neuronal cultures from rats were established as described elsewhere (Notterpek et al., 1999). Dorsal root ganglia (DRG) were collected from embryonic day 15 rats, digested with 0.25% trypsin (Gibco), mechanically dissociated, and plated either on rat tail collagen-coated (Biomedical Technologies, Inc.) 12-mm glass coverslips for immunolabeling or on collagen-coated tissue culture plastic for biochemical studies. Cultures were maintained in minimum essential medium (Gibco) supplemented with 10% FCS, 0.3% glucose (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES, and 100 ng/ml nerve growth factor (Harlan Bioproducts for Science, Madison, WI) and grown at 37°C and 5%

CO₂. On the following day, the cultures were treated with fluorodeoxyuridine (10 μ M; Sigma-Aldrich) for three cycles to enrich the neuronal population.

Cocultures of Schwann cells and DRG neurons were established as described previously (Einheber et al., 1993). Schwann cells were added to DRG neuron cultures 1 week after the third fluorodeoxyuridine cycle and allowed to proliferate for 10–12 days. To initiate myelination, the medium was supplemented with ascorbic acid (50 μ g/ml; Sigma-Aldrich), and the cultures were maintained for an additional 10 days. Samples were then processed for either biochemical or immunocytochemical analyses.

Lentiviral Preparation and Transduction of Primary Schwann Cells

A lentiviral pGIPZ shRNA vector encoding shRNA targeting Dicer and a negative, nontargeting shRNA control (Neg.) vector, both of which also encode GFP as a reporter protein, were obtained from a commercial provider (Open Biosystems). With HEK 293FT cells, the pGIPZ and Neg. shRNA vectors were packaged into concentrated lentiviruses by using a three plasmid transfection procedure (Sempere-Rowland et al., 2007). Viral titers were estimated with a Lenti-X qRT-PCR kit (Chemicon, Billerica, MA) and typically averaged 2×10^{12} viral genomes per milliliter. Primary rat Schwann cells were transduced with pGIPZ shRNA lenti-virus as described elsewhere (Bolis et al., 2009). In brief, approximately 1×10^5 cells were plated on a 6-cm dish 24 hr prior to transduction. Schwann cells were transduced at a multiplicity of infection (MOI) of 5. The cultures were then incubated in 1 ml DMEM containing the virus for 4 hr. After 4 hr, 1 ml of 2 $\times$ rat Schwann cell medium was added, and the cultures were incubated for an additional 48 hr. Transduction efficiency was determined to be greater than 90% by direct visualization of green fluorescent protein (GFP) expression. The transduced cells were selected by adding 2 μ g/ml puromycin to the media. After 3–5 days in culture, the cells were analyzed or seeded onto purified DRG neurons.

Quantitative Real-Time RT-PCR Analysis of miRNAs

Total RNA was isolated from puromycin-selected transduced Schwann cells using Trizol (Invitrogen) according to the manufacturer's instructions. With the miScript Reverse Transcription kit (Qiagen, Valencia, CA), equal amounts of RNA was utilized for cDNA synthesis. Then, employing the miScript SYBR Green PCR kit and the indicated miScript Primer Assays (Qiagen), real-time PCR was performed to determine relative miRNA expression levels on an Applied Biosystems 7300 real-time PCR system. miScript Primer Assays were obtained from Qiagen and were specific for the rat miRNAs let-7c, miR-129, miR-381, miR-193, miR-222, miR-145, miR-29a. The levels of the detected miRNAs were normalized to GAPDH that were run on the same reaction. Relative abundances of the detected miRNAs were determined from three independent experiments by using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Primary Antibodies

Rabbit polyclonal anti-Erg2/Krox20 (1:500 dilution; Covance, Princeton, NJ), anti-Oct6 (1:500; Abcam, Cambridge, MA), anti-Dicer (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Sox2 (1:200; Santa Cruz Biotechnology), anti-c-Jun (1:200; Santa Cruz Biotechnology), antiphospho-histone-H3 (pH-H3; 1:500; Chemicon, Billerica, MA), anti-S-100 (1:500; DakoCytomation, Denmark), and anti-MBP (1:200; Chemicon) were purchased from the indicated suppliers. Mouse monoclonal anti-NF-M (1:5,000; Encore, Alachua, FL) and chicken polyclonal anti-MPZ (1:2,500; Encore) were obtained from the indicated source. A mouse monoclonal antibody against GAPDH (1:5,000; Encore) was employed as a protein loading control.

Western Blot Assays

Equal amounts of protein (30 µg per sample for Schwann cell lysates or 15 µg per sample for Schwann cell/DRG cultures) were separated on sodium dodecyl sulfate gels and transferred to nitrocellulose membranes. After transfer, the membranes were blocked in 5% nonfat milk/phosphate-buffered saline (PBS) for 1 hr and then incubated with primary antibody diluted in 5% nonfat milk/PBS overnight at 4°C on a shaker. The membranes were washed three times with PBS, then incubated with the appropriate secondary antibody in 5% milk/PBS for 2 hr at room temperature. After incubation with anti-rabbit, anti-chicken, or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St. Louis, MO), the bound antibodies were detected using enhanced chemiluminescent substrate (Perkin Elmer, Boston, MA). Processed films were digitally imaged using a GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA) and quantified in Scion Image (Scion, Frederick, MD). The intensities of the specific protein bands were normalized to GAPDH to obtain relative protein expression levels. To demonstrate reproducibility of the observed effects in DRG/Schwann cultures, three independent samples from different cultures for each condition were analyzed on the same gel (see Fig. 6B).

Immunostaining of Schwann Cell-DRG Cocultures

Immunolabeling experiments were performed as described by Notterpek et al. (1999). In brief, Schwann cells cultured on glass coverslips were fixed with 4% paraformaldehyde for 10 min and then permeabilized using cold 100% methanol for 5 min at -20°C. After blocking with 10% normal goat serum for 1 hr at room temperature, the samples were incubated with the primary antibodies overnight at 4°C. Bound antibodies were detected using species-specific secondary antibodies (1:500) conjugated to either Alexa Fluor 594 or Alexa Fluor 405 (Molecular Probes, Eugene, OR). Hoechst dye (10 µg/ml; Molecular Probes) was included in the secondary antibody solution to visualize nuclei. The stained cells bound to coverslips were then mounted on slides using the Prolong Antifade kit (Molecular Probes). Schwann cell images were acquired with a Spot digital camera (Diagnostic Instrumentals, Sterling Heights, MI) attached to a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan). Single-plane images of Schwann cell/DRG cultures were obtained using a Spinning Disk Confocal Scanner mounted on an Olympus IX81 inverted fluorescent microscope.

Bromodeoxyuridine Assays

To investigate the effects of icer shRNA on Schwann cell proliferation, equal numbers of infected cells (4×10^4 viable cells per well, $n = 6$) were maintained in culture for 24 hr prior to incubation with bromodeoxyuridine (BrdU; Roche BrdU labeling and detection kit; Roche, Nutley, NJ) as described elsewhere (Johnson et al., 2005). After 8 hr of BrdU incorporation, the cells were fixed, permeabilized, and processed according to the manufacturer's instructions. BrdU-positive cells in six random fields (0.8 mm^2) were counted and divided by the total number of cells in the fields, which was determined by counting nuclei using Hoechst dye (Invitrogen, Carlsbad, CA). The number of pH-H3-positive Schwann cells was counted in same manner. As an additional measure of cell division, we analyzed the cultures using a BrdU-based cell proliferation ELISA kit (Roche).

Cellular Toxicity Assay

To determine whether transduction of either the Neg. or the Dicer shRNA lentiviral particles resulted in significant cell death, transduced cultures were evaluated by using a cellular toxicity assay (Rangaraju et al., 2008). In brief, transduced and puromycin-selected Schwann cells were seeded into 96-well plates and allowed to grow for either 24 or 48 hr prior to performing the assay. The cells were then incubated in 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (0.5 mg/ml) for 5 hr at 37°C, producing the formazan product as a result of the cleavage of the tetrazolium salt MTT by the mitochondrial enzyme succinate dehydrogenase. The amount of blue formazan product, which is detected using an automated plate reader (550 nm), is directly proportional to the number of viable cells present in each well. The effect of either shRNA on cellular viability was determined and graphed as the amount of absorbance detected in each sample. Statistical significance was determined by using Student's *t*-tests.

Quantification and Statistical Analyses

When quantification is presented, experiments were repeated at least three independent times, and data were analyzed in Microsoft Excel 2007. Comparisons of two independent groups were carried out using Student's *t*-tests with the significance set at $P < 0.05$. Means $\pm$ standard deviations were calculated in Graph-Pad Prism 5.0 and were plotted on all graphs.

RESULTS

Inhibition of Dicer Leads to Reduced miRNA Levels in Schwann Cells

To determine whether Dicer expression is required for Schwann cell differentiation and myelination, we transduced primary rat Schwann cells with Dicer shRNA carrying lentivirus. We chose this approach because lentiviral shRNA-mediated knock-down of gene expression is efficient in Schwann cells (Hu et al., 2005) and reducing the steady-state levels of Dicer hinders the formation of mature miRNAs (Asada et al., 2008). Cells were transduced at an MOI of 5 and then cultured in puromycin selection media for 3–5 days. Efficient selection and establishment of pure, transduced Schwann cell populations were confirmed by direct visualization of GFP (Fig. 1A). The steady-state expression of Dicer was reduced in the Dicer shRNA Schwann cells compared with Neg. shRNA-transduced samples, as evaluated by Western blot analyses (Fig. 1B). Quantification of independent transductions revealed that the steady-state levels of Dicer were reduced by ~60% in the Schwann cells expressing Dicer shRNA, relative to control (Fig. 1C; $n = 4$, $**P < 0.01$). To reinforce the biochemical results and to confirm uniform levels of Dicer knock-down in transduced cells, we carried out immunolabeling experiments. By using a previously employed polyclonal antibody (Verrier et al., 2009), we found that the Dicer-like immunoreactivity is visibly reduced in puromycin-selected Dicer shRNA-transduced cells compared with the Neg. shRNA (Fig. 1D). In addition, the decrease in Dicer-like immunoreactivity is uniform among the cells.

Dicer is critical for the biogenesis of miRNAs (Kanellopoulou et al., 2005), and, although the steady-state levels of Dicer decrease during glial differentiation, the protein continues to be expressed at readily detectable levels (Potenza et al., 2009; Verrier et al., 2009). To determine whether inhibition of Dicer in primary Schwann cells affects miRNA biogenesis, we performed semiquantitative RT-PCR on RNA isolated from cultures transduced with either the Neg. or Dicer shRNA lentiviral particles. We chose to test a set of specific miRNAs that are readily detectable in rat Schwann cells, as previously determined from microarray profiling (Verrier et al., 2009). Inhibition of Dicer resulted in a significant decrease in the expression of miR-129, miR-145, miR-193, miR-222, and miR-29a (Fig. 2; $n = 3$, $**P < 0.01$, $***P < 0.001$). In comparison, we did not detect a statistically significant change in miR-381 or let-7c, indicating that not all miRNAs were affected by an ~60% reduction in Dicer. These data indicate that the majority of the tested Schwann cell prevalent miRNAs were significantly reduced upon inhibition of Dicer.

Dicer Knock-Down Impairs Schwann Cell Differentiation

The loss of Dicer expression has been shown to affect cellular division and differentiation as well as cell death (Harris et al., 2006; Carleton et al., 2007; Bu et al., 2009). Before testing the viability and the mitotic potential of Dicer-deficient cells, we immunolabeled the cultures with the Schwann cell-specific marker S100 beta (Chan et al., 2000). As shown in the micrograph (Fig. 3A, red), every cell in the field of view displays S100-like immunoreactivity, indicating pure cultures of Schwann cells. To assess whether a reduction of steady-state Dicer levels affects Schwann cell viability, we performed MTT assays on Neg. and Dicer shRNA-transduced cultures. Schwann cells transduced with the Dicer shRNA show increased signal compared with the Neg. shRNA control at both 24 hr and 48 hr postplating (Fig. 3B; $***P < 0.001$), indicating that they are viable.

Although the role of miRNAs in cell cycle regulation appears to be cell type specific, recent studies indicate that the levels of Dicer are altered in a number of cancer cell lines (Kumar et al., 2009). To evaluate the effect of Dicer suppression on Schwann cell proliferation, we performed immunolabeling experiments with an antibody for the mitotic marker phospho-histone H3 (pH-H3). Cultures transduced with Dicer shRNA lentiviral particles show a number of cells with mitotic profiles, which prominently react with the pH-H3 antibody (Fig. 3C, red). In agreement, quantification of pH-H3-positive cells reveals a significant increase in dividing cells resulting from Dicer shRNA transduction (Fig. 3D; $***P < 0.001$). In addition to pH-H3 staining, we performed BrdU incorporation assays on cells transduced with either Neg. shRNA or Dicer shRNA. After 8 hr of incubation, BrdU incorporation was evaluated by using immunostaining and colorimetric measurements. As shown in the graph (Fig. 3E; $n = 6$, $*P < 0.05$), an ~ 60% loss in Dicer (Fig. 1B,C) promotes Schwann cell proliferation, as determined from direct counting of BrdU⁺ cells. Analyses of similarly treated cultures using an ELISA-based colorimetric assay confirmed the increased uptake of BrdU by Dicer shRNA cells compared with Neg. control (Fig. 3F; $n = 6$, $**P < 0.01$). Therefore, the inhibition of Dicer is associated not with Schwann cell loss but rather an increase in cell proliferation.

Because proliferating, undifferentiated Schwann cells are a nonmyelinating phenotype (Woodhoo et al., 2009), we examined the expression of known Schwann cell transcription and differentiation factors in Dicer shRNA cells. Two weeks post-transduction and selection in puromycin, whole-cell lysates were analyzed for the expression of a panel of Schwann cell proteins (Fig. 4A). As shown in Figure 1B, Dicer levels remained reduced compared with Neg. shRNA control at 2 weeks post-transduction, indicating that the expression and functionality of the shRNA were maintained. The inhibition of Dicer reduced the steady-state levels of the promyelination transcription factors Oct6 and Egr2/Krox20 and of the myelin protein MPZ. Quantification of four independent transductions revealed a significant decrease in steady-state Oct6 (Fig. 4B; $n = 4$, $**P < 0.01$) and Egr2 (Fig. 4C; $n = 4$, $**P < 0.01$) proteins in Dicer shRNA cells. Dicer shRNA transduction also resulted in elevated c-Jun expression compared with Neg. control (Fig. 4D; $n = 4$, $*P < 0.05$). In addition, we detected a significant decrease in MPZ levels (Fig. 4E; $n = 4$, $*P < 0.05$), indicating that the inhibition of Dicer is associated with an impairment in myelin-related gene expression in cultured Schwann cells.

Inhibition of miRNA Biogenesis Impairs Schwann Cell Myelination

To examine further the effects of reduced Dicer protein levels on myelin gene expression, we examined myelin formation in cocultures of Schwann cells and DRG neurons. Pure populations of Schwann cells expressing the Dicer shRNA were obtained using puromycin selection (see Fig. 1) and were seeded onto DRG neuron cultures. Schwann cells were allowed to proliferate through radial sorting along the axons. After 2 weeks of culture in

myelination-promoting medium, the cells were either fixed for immunolabeling or lysed for Western blot analyses. The expression of Neg. shRNA vector alone did not affect the ability of the cells to form mature myelin (Fig. 5A). Schwann cells expressing MBP and GFP were readily detected in myelinating cocultures (arrows). On occasion, we found nontransduced Schwann cells (GFP negative) that were also positive for MBP (arrowheads), but these cells represented a minor fraction of the myelin segments. Western blot analyses of Schwann cell-DRG cocultures grown under myelinating conditions seeded with cells expressing Dicer shRNA revealed a dramatic reduction in the steady-state expression of MPZ and MBP, whereas the levels of NF-M remained constant (Fig. 5B). Each Western blot lane represents an independent coculture to demonstrate reproducibility of the observed prominent phenotype. GAPDH expression confirmed equal protein loading in each lane. Myelination formation was also evaluated by immunostaining the samples for MBP (Fig. 5C, red). Cultures seeded with Dicer shRNA Schwann cells contained much fewer MBP-positive myelin segments compared with the Neg. controls. We confirmed the presence of axons in the field of view by probing with an antibody for neurofilament-M (blue).

To determine whether the down-regulation of Dicer affects the expression of negative regulators of myelination, we analyzed myelinating cocultures for Sox2 (Wegner, 2000). Although Sox2 would normally decrease upon the initiation of myelination, it remained elevated when Dicer was suppressed (Fig. 6A). Quantification of Western blots from three independent experiments reveals a significant increase in the steady-state levels of Sox2 upon Dicer shRNA transduction compared with control (Fig. 6B; $n = 3$, $*P < 0.05$). The increase in steady-state Sox2 levels is in agreement with the reduction of miR-145 in response to Dicer shRNA transduction (Fig. 2), insofar as miR-145 is known to regulate Sox2 expression in human stem cells (Xu et al., 2009). These data support the hypothesis that loss of miRNA biogenesis impairs the ability of Schwann cells to differentiate and myelinate axons.

DISCUSSION

Posttranscriptional regulation of gene expression by miRNAs is critical in a wide variety of cellular processes (Ambros, 2004). Here we investigated the effect of reducing the miRNA biogenesis protein Dicer on Schwann cell myelination and differentiation. The inhibition of Dicer in primary Schwann cells led to reduced steady-state miRNA levels and increased cellular proliferation. In addition, the cells maintained low levels of known Schwann cell differentiation markers and failed to produce myelin (Figs. 4, 5). These experiments support an essential role for miRNAs during Schwann cell differentiation and myelination.

The expression of Dicer is critical for embryonic development, insofar as Dicer-deficient animals fail to survive past E7.5 (Bernstein et al., 2003). Postnatally, the loss of Dicer function alters the differentiation of T cells, embryonic stem cells, and pancreatic islet cells; oocyte maturation; and cardiac muscle function (Muljo et al., 2005; Kanellopoulou et al., 2005; Murchison et al., 2007; Lynn et al., 2007; Chen et al., 2008). Within the CNS, conditional deletion of Dicer in forebrain excitatory neurons leads to reduced dendritic branching and microcephaly (Davis et al., 2008). Recently it was shown that Dicer and Argonaute protein expression is maintained during cellular differentiation of neurons and Schwann cells, suggesting a role for miRNA function in peripheral nerve development (Potenza et al., 2009; Verrier et al., 2009). In support of this idea, patients with autoimmunity to the RNA-induced silencing complex-associated protein GW182 often present with motor and sensory neuropathies (Eystathiou et al., 2002). In agreement, we have shown recently that transient reduction of Dicer in Schwann cells leads to altered expression of a neuropathy-linked gene, namely, PMP22 (Verrier et al., 2009). These data indicate a critical role for miRNAs in peripheral nerve development and disease processes.

MiRNAs have been shown to be involved in cellular differentiation in both in vitro and in vivo models (Miska, 2005; Harris et al., 2006). Specifically, miR-23 has been shown to influence lamin B1 expression in oligodendrocytes and to modulate oligodendrocyte development and myelin gene expression (Lin and Fu, 2009). The tubulin polymerization-promoting protein, which is required for oligodendrocyte myelination, is also known to be regulated by an miRNA (Lehotzky et al., 2009). Here we reduced Dicer levels by shRNA specifically in Schwann cells, allowing us to address the role of glial miRNAs in myelination and differentiation (Fig. 1). Inhibition of Dicer expression led to reduced steady-state levels of several Schwann cell miRNAs (Fig. 2), including miR-29a, which specifically regulates PMP22 (Verrier et al., 2009). Interestingly, not all tested miRNAs were reduced, suggesting either high basal expression levels or long half-lives. With regard to Schwann cell differentiation, we observed a decrease in *Egr2/Krox20*, which is in agreement with the apparent failure of the cells to up-regulate myelin genes when cocultured with DRG neurons (Fig. 5). We also demonstrated that known negative regulators of myelination, c-Jun and Sox2, remain elevated in Dicer shRNA cells. These data imply that the loss of mature miRNAs interferes with Schwann cell differentiation, a phenotype observed in other cell types (Kapinas et al., 2009; Kawase-Koga et al., 2009). Significantly, recent studies with the developing CNS have revealed that the deletion of Dicer drastically reduces oligodendrocyte maturation (Kawase-Koga et al., 2009). Together, these findings indicate a shared requirement for miRNA regulation during the differentiation of myelinating glial cells in the CNS and the PNS.

We previously demonstrated that PMP22 expression is subject to miRNA regulation in the CNS as well as the PNS (Lau et al., 2008; Verrier et al., 2009). In comparison, MPZ is apparently not a direct target of miRNA-mediated repression in Schwann cells, at least under the in vitro conditions employed (Fig. 4). The reduction of steady-state MPZ upon transduction of Dicer shRNA is consistent with the observed effect on *Egr2/Krox20* levels, which transcriptionally control MPZ (Jang et al., 2006; Jang and Svaren, 2009). It is interesting to note that PMP22 has not been shown to be a direct target of *Egr2/Krox20*, suggesting a distinct regulatory mechanism for this myelin gene. In addition to myelin gene expression, exit from the cell cycle is also a marker of Schwann cell differentiation (Scherer, 1997; Berger et al., 2006). Here we demonstrate that the loss of Dicer leads to increased cellular division, a characteristic phenotype of undifferentiated Schwann cells (Fig. 3). Similarly, gliomas increase their division upon loss of specific miRNAs (Kumar et al., 2007), and studies in cancer cell lines demonstrate a reduction of cellular proliferation with miRNA depletion (Zhang et al., 2009). These findings, together with our observation of impaired myelin formation in Schwann cells expressing Dicer shRNA, support the hypothesis that mature miRNAs are required for Schwann cells to switch from a dividing, nonmyelinating cell to a differentiated, myelinating phenotype.

Sox2 and c-Jun are negative regulators of myelination and markers of immature nonmyelinating Schwann cells (Jessen and Mirsky, 2008; Parkinson et al., 2008). We demonstrate that, when Dicer is suppressed via shRNA, the steady-state levels of Sox2 are increased (Fig. 6). Previous investigations into miRNA repression of differentiation-related transcription factors have revealed Sox2 to be a direct miRNA target in several cell types (Tay et al., 2008; Xu et al., 2009). In agreement with these reports, we observed a significant reduction in miR-145, an miRNA known to target Sox2, upon Dicer shRNA transduction (Fig. 2). In addition to being a target for miRNAs, Sox2 has been shown to transcribe miR-302 in human embryonic stem cells, which in turn regulates the expression of several proteins involved in cell cycle regulation (Card et al., 2008). Sox2 transcription of miR-302 was shown to increase the proportion of cells in S phase, and loss of Sox2 abolished the effect. It remains unclear whether Sox2 regulates the transcription of miR-302 or cellular proliferation-inducing miRNAs in Schwann cells. In addition to Sox2, we also detected

elevated levels of c-Jun in response to inhibition of Dicer (Fig. 4). c-Jun was previously demonstrated to regulate specific miR-NAs in keratinocytes affecting their differentiation (Sonkoly et al., 2009). Whether c-Jun is directly controlling the expression of certain miRNAs in Schwann cells has yet to be determined. Nonetheless, in addition to Sox2 and c-Jun, there are likely to be other genes that contribute to the cell's failure to differentiate and myelinate.

The prediction of functional miRNA gene targets currently relies heavily on bioinformatics programs. The inhibition of Dicer leads to a global decline of miRNAs (Kanellopoulou et al., 2005), and here we demonstrate that even a 60% reduction in steady-state Dicer results in alterations in several Schwann cell miRNAs (Fig. 2). The specific miRNAs evaluated were chosen based on previously published miRNA microarrays, which identified these miRNAs as of potential interest in glial differentiation (Murchison et al., 2007; Verrier et al., 2009). Bioinformatics scans for predicted target genes implicated in Schwann cell biology and differentiation by these miRNAs include but are not limited to PMP22; collagens I, IV, and V, which are targets for miR-29a; Sox2 and integrin beta 8, targets of miR-145; and p27(Kip), a predicted target gene of miR-222 (predictions performed with targets.org search engine). In addition, miR-222 has been shown to control cell cycle progression (Zhang et al., 2009) and regulate cellular differentiation (Cardinali et al., 2009; Qian et al., 2009). Although recent studies have shed light on many of the precise requirements for miRNA-mediated gene regulation, functional validation for each of these predictions within Schwann cells will be necessary to verify their biological significance. Future studies will determine how overexpression and/or inhibition of these specific miRNAs may impact Schwann cell myelination and differentiation.

The data presented here suggest that inhibition of the miRNA pathway in Schwann cells may lead to demyelination or dysmyelination (Figs. 4, 5). It is tempting to hypothesize that this might be a contributing mechanism for the etiology of the clinical manifestations observed with autoimmunity to RISC proteins (Eystathiou et al., 2002; Bhanji et al., 2007). However, why the peripheral nerve is preferentially targeted or particularly susceptible to these phenomena still warrants further investigation. The data presented here support additional investigations into the signaling mechanisms that regulate specific miRNA expression. Insofar as targeting gene therapy to the Schwann cells presents only technical hurdles, elucidating which signaling pathways regulate individual miRNAs may be a more feasible approach. The ability to control a single miRNA is a critical step for utilizing miRNAs for therapeutic purposes. Further experiments will investigate whether axonal signals influence the expression of miRNAs in Schwann cells and ultimately affect their myelination.

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Fig. 1. Lentiviral transduction of Dicer shRNA reduces steady-state Dicer expression. **A:** Schwann cell cultures were transduced with either control or Dicer shRNA lentiviral particles. GFP fluorescence (left) and phase-contrast (right) images of live Schwann cell cultures are shown. **B:** Western blot analyses of whole Schwann cell lysates after transduction with Dicer shRNA or Neg. shRNA. Molecular mass in kDa. **C:** Quantification of Dicer in protein lysates from four independent cultures after normalization to GAPDH ($n = 4$, $**P < 0.01$). **D:** Schwann cells transduced with the Dicer shRNA (right) or Neg. shRNA (left) were immunostained with an anti-Dicer antibody. Hoechst dye is shown to visualize nuclei (blue). Scale bars = 10 μm .

Fig. 2.

Reduction of Dicer protein alters miRNA levels within Schwann cells. Semiquantitative real-time RT-PCR was performed on RNA isolated from shRNA transduced Schwann cells. The levels of miR-129, miR-145, miR-193, miR-222, and miR-29a are significantly decreased in Dicer shRNA-transduced cells compared with Neg. shRNA controls ($n = 3$, $**P < 0.01$, $***P < 0.001$). The steady-state levels of let-7c and miR-381 are not significantly affected ($n = 3$, $P > 0.05$). The levels of the miRNAs are normalized to GAPDH RNA, and the relative RNA abundance was determined by using the $2^{-\Delta\Delta C_t}$ method.

Fig. 3.

Suppression of Dicer affects the differentiation state of Schwann cells. **A:** The purity of the transduced cultures is shown using the Schwann cell marker S100 (red). Hoechst dye is used to visualize nuclei (blue). **B:** The influence of Dicer shRNA transduction on cellular viability was determined at 24 and 48 hr postplating using an MTT assay ($n = 8$, $P < 0.001$). **C:** Representative immunostaining of Dicer shRNA-transduced Schwann cells with the mitotic marker pH-H3 reveals ongoing cell division. Hoechst dye (blue) is shown to visualize total cell number. **D:** Quantification of pH-H3-positive cells reveals a significant increase in mitotic cells in response to Dicer shRNA transduction when compared with controls ($n = 8$ fields of view, $***P < 0.001$). **E:** Quantification of BrdU incorporation in cultures treated with Dicer shRNA compared with Neg. shRNA ($n = 6$ field of view per condition, $*P < 0.05$). **F:** An ELISA-based colorimetric BrdU incorporation assay shows a significant increase in BrdU uptake in Schwann cells treated with Dicer shRNA compared with Neg. control ($n = 6$, $**P < 0.01$). Scale bars = 10 μm .

Fig. 4.

Protein expression profile of Dicer-inhibited Schwann cells. **A:** Western blot analyses of Schwann cells expressing Dicer shRNA (30 µg/lane). The steady-state levels of Dicer, Oct6, Egr2/Krox20, c-Jun, and MPZ are shown. GAPDH is shown as a loading control. Molecular mass in kDa. Quantification of four independent experiments reveals that transduction of Dicer shRNA leads to a decrease in the steady-state expression of Oct6 ($n = 4$, $**P < 0.01$; **B**) and Egr2/Krox20 ($n = 4$, $**P < 0.01$; **C**). **D:** Quantification from independent experiments reveals that Dicer shRNA leads to elevated c-Jun protein levels ($n = 4$, $*P < 0.05$). **E:** Quantification of steady-state MPZ levels in response to Dicer shRNA transduction compared with Neg. shRNA controls ($n = 4$, $P < 0.05$).

Fig. 5.

Dicer shRNA-transduced Schwann cells show impaired myelination. **A:** Lentiviral Neg. shRNA-transduced Schwann cells (green) retain myelination capacity. Mature myelin is detected by using an anti-MBP antibody (red). Arrows indicate transduced myelinating cells, whereas the arrowheads denote residual nontransduced cells. Hoechst dye is used to visualize nuclei (blue). **B:** Schwann cell/DRG cocultures grown under myelinating conditions for 2 weeks were analyzed by Western blots for NF-M, MPZ, and MBP. GAPDH is shown as a protein loading control, and each lane represents an independent coculture. Molecular mass in kDa. **C:** The myelination capacity of the Schwann cells was determined using an antibody for MBP (red, left panels). The Dicer shRNA Schwann cells (bottom panels) produced fewer MBP-positive myelin tracts than the Neg. shRNA-transduced cells (top panels). The axonal marker NF-M is shown to demonstrate the presence of neurons (blue, right panels). Scale bars = 10 μ m in A; 20 μ m in C.

Fig. 6.

Sox2 expression is elevated by the down-regulation of Dicer in Schwann cells. **A:** Western blot analysis of myelinating Schwann cell/DRG cocultures is shown using an anti-Sox2 antibody. NF-M is shown as a marker for neuronal content. GAPDH serves as a protein loading control. Molecular mass in kDa. **B:** Quantification of independent experiments reveals a significant increase in the levels of Sox2 in Schwann cells transduced with the Dicer shRNA compared with controls ($n = 3$, $*P < 0.05$).