POST-TRANSCRIPTIONAL REGULATION OF MYELIN GENES
BY MICRORNAS

By

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<tr>
<td>3'UTR</td>
<td>3' untranslated region</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth disease</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
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<td>E</td>
<td>embryonic day</td>
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<tr>
<td>FACS</td>
<td>fluorescent automated cell sorting</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GWB</td>
<td>GW-bodies / processing bodies</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>MAG</td>
<td>myelin associated glycoprotein</td>
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<td>MBP</td>
<td>myelin basic protein</td>
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<td>MPZ</td>
<td>myelin protein zero</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendrocyte precursor cells</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
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<tr>
<td>PMP22</td>
<td>peripheral myelin protein 22</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>RSC</td>
<td>rat Schwann cell</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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By

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The conduction of electrical signals along axons of the nerves is aided by the lipid-rich membrane, myelin, which is synthesized by glial cells. Myelination in the peripheral and central nervous systems is an elaborate process that requires the precise regulation of gene expression and protein translation. Myelin genes have been shown to be regulated at both the transcriptional and post-transcriptional levels. The loss of myelin has been linked to several disease states, including multiple sclerosis and inherited peripheral neuropathies. Even the altered expression of individual myelin proteins has been linked to demyelinating diseases which reinforces the requirement for precise regulation of these genes. MicroRNAs (miRNAs) are endogenous regulatory RNA molecules that modulate gene expression at a post-transcriptional level. Regulation by miRNAs has been implicated in both developmental and disease processes. The work here details the expression profile of miRNAs during cellular differentiation in the myelinating cells of the central (Chapter 3) and peripheral nervous system (Chapter 4). In addition, we demonstrate that a disease-linked myelin gene, peripheral myelin protein 22, is subjected to miRNA regulation in the myelinating cells of both divisions of the nervous system. Finally, we reveal a critical role for Schwann cell
miRNAs during myelination whereas inhibiting their formation impairs the cells ability to differentiate and myelinate axons in vitro (Chapter 5). These experiments demonstrate a previously uncharacterized level of myelin gene regulation and provide insight into the complex process of myelination by oligodendrocytes and Schwann cells.
CHAPTER 1
INTRODUCTION

The ability for neurons to communicate rapid electrical signals, called action potentials, along their axons is an essential process for neuronal function and vertebrate life. Conduction of the action potentials is facilitated by myelin, a lipid-rich sheath that is deposited in segments, called internodes, along the axons by specialized cell types. The unmyelinated areas of the axons between the internodes are referred to as the nodes of Ravier. These nodes contain the elements, including voltage-gated sodium channels for example, that make action potential propagation possible. Myelin itself provides a high resistance and low capacitance to the axonal membrane allowing the action potential to jump from one node of Ravier to the next in a process referred to as 'saltatory conduction' (Garbay et al. 2000). This mode of conduction greatly increases the speed and efficiency at which the action potentials travel along the axons to their target. The loss of the myelin sheath is associated with slowed nerve conduction velocity and axonal damage and is a pathological feature in diseases ranging from multiple sclerosis to hereditary peripheral neuropathies (Simons and Trotter 2007).

Myelin is synthesized by different specialized cell types in each division of the nervous system, with oligodendrocytes providing the myelin to the central nervous system (CNS) and the Schwann cell myelinating the peripheral nervous system (PNS). Myelination is an elaborate process that involves the synthesis of proteins and lipids, incorporation of these constituents into the membrane, and the wrapping of the axon to form a mature internode. Although these cell types do share common functions, there are many distinct differences between oligodendrocytes and Schwann cells. In the CNS, each oligodendrocyte is able to myelinate multiple axons whereas a Schwann cell
only myelinates one axon thus defining only one internode (Simons and Trotter 2007). Myelin in the CNS and PNS serves similar functions, however there are differences in which proteins and lipids constitute the myelin in each system, as well as in the synthesis and signals governing the establishment of the myelin sheath.

**Constituents of Myelin**

Myelin is a continuation of the glial cells plasma membrane and its production requires an increase in the rate of synthesis of both lipids and proteins. Approximately 70-80% of the myelin dry weight is composed of lipids and the remaining 20-30% is proteins (Wegner 2000b). The lipids that compose the myelin include predominantly galactosphingolipids and saturated long-chain fatty acids (Mugnaini 1982). Cholesterol is an essential component of myelin being responsible for around 20-30% of total lipid content. Although there are no myelin lipids specific to either division of the nervous system, the myelin of the PNS has far more sphingomyelin than present in the CNS, ~25% vs. 5% respectively (Norton and Cammer 1984). However, the myelin synthesized by the oligodendrocytes contains more monogalactosphingolipids with cerebrosides (Gal-c) and sulfatides (SGal-C) than that made by the Schwann cells. The critical role for SGal-C in the structural integrity of myelin in the CNS has recently been demonstrated. Mice unable to synthesize Gal-C displayed progressive dysmyelination and altered paranodal structures (Fewou et al. 2009). Proper lipid syntheses is essential for both Schwann cells and oligodendrocytes to synthesis and maintain myelin. In contrast to the roughly similar lipid profiles, the proteins in the myelin differ between the two branches of the nervous system.

The myelinating cells of the PNS and CNS utilize proteins that are specific to their division of the nervous system but also use shared proteins to compose the myelin.
the PNS, approximately 20-30% of the dry mass of myelin is protein, and the majority (approximately 60%) is estimated to be glycoproteins. On the other hand, the CNS myelin only has a minor amount of glycoproteins, although the significance of the different abundances of glycoproteins has yet to be fully explained. Basic proteins are also highly abundant in the PNS myelin comprising around 30% of the total protein content. In brief, the three most abundant proteins in the PNS compact myelin are the glycoproteins myelin protein zero (MPZ) and peripheral myelin protein 22 (PMP22) and the basic protein, myelin basic protein (MBP). In the PNS, the glycoproteins MPZ and PMP22 are believed to be essential for the formation of compact myelin and reduction in the expression of either protein inhibits the myelination process (Quarles 2002). The glycoproteins, as well as MBP, interact with extracellular matrix molecules and are proposed to facilitate wrapping of the axon (Simons and Trotter 2007). There exists additional myelin specific, or myelin enriched, proteins present at lower levels, including 2’3’-cyclic AMP phosphodiesterase (CNP) and myelin associated glycoprotein (MAG). The most abundant proteins in CNS myelin are MBP, proteolipid protein (PLP), and myelin-associated oligodendrocytic basic protein (MOBP). The CNS myelin also contains CNP, yet its function remains elusive in the CNS as well as in the PNS. Besides the differences in protein content, neurotrophins can also exert contrasting effects on Schwann cells and oligodendrocytes. Nerve growth factor (NGF) for example, is a myelination stimulating agent on Schwann cells, but inhibitory on oligodendrocytes (Chan et al. 2004). In addition to its direct actions on the glial cell, it has been proposed that NGF may act on the axon affecting the diameter thus influencing myelination. The precise mechanisms by which the glial cells chose which
axons to myelinate is not completely defined, but an axonal caliber of at least 1 um appears to be the minimum, as well as specific neuregulin isoform (NGR1 type3) expression (Michailov et al. 2004; Mirsky and Jessen 1999). It is suggested that adding myelin to an axon of lesser diameter would not significantly increase the action potential conduction. Regardless, although myelin serves similar functions in the CNS and PNS and there are shared and unique constituents, abnormal myelin formation and the loss of myelin are linked to disease states.

**Myelin-Associated Diseases**

The progressive loss of myelin, referred to as demyelination, in either the CNS or PNS is associated with a number of diseases of varying etiologies. In the CNS, demyelinating diseases primarily arise from genetic abnormalities, as is the case in leukodystrophies, and inflammatory damage to the myelinating oligodendrocytes (Franklin and Ffrench-Constant 2008). However the most widely recognized CNS demyelinating disease is multiple sclerosis, which is believed to arise from an autoimmunity to several of the proteins in the myelin (Franklin and Ffrench-Constant 2008). Analyses of serum from patients with multiple sclerosis have revealed antibodies against MBP, CNP, PLP and additional myelin proteins (Grau-Lopez et al. 2009). The canonical belief is that the loss of myelin leads to slower nerve conduction through the redistribution of sodium-channels and increased axonal membrane capacitance. While these phenomenon are observed, the loss of myelin in the CNS is also associated with damage to the axons and even neuron cell loss. Interestingly, axonal damage in these diseases is primarily observed after the loss of the myelin, suggesting a protective and possibly trophic role for the myelin in the CNS (Dutta and Trapp 2007). In support of this hypothesis, there are recent studies that demonstrate
that changes in protein expression in the CNS myelin are associated with axonal loss in
the absence of myelin deficits (Garbern et al. 2002; Lappe-Siefke et al. 2003). Patients
lacking the PLP gene are associated with diagnosis of Pelizaeus-Merzbacher disease
(Garbern et al. 2002) and the loss of Cnp1 in mice are associated with axonal loss
despite no observable demyelination (Lappe-Siefke et al. 2003). These finding suggest
that loss of conduction may not be the only underlying mechanism for myelin associated
diseases but loss of trophic and mechanical support also contributing factors. Also,
abnormal wrapping and formation of the myelin sheath, referred to as dysmyelination, is
a hallmark of several disease states in the PNS and CNS. In these patients, proper
compact myelin is never formed usually due to genetic abnormalities. In the CNS,
dysmyelination is seen in mental retardation, a subset of leukodystrophies and other
developmental diseases (Karim et al. 2007; Koeppen and Robitaille 2002; Schiffmann
and van der Knaap 2004).

In the PNS, demyelinating disorders can present in several clinical
manifestations, including Charcot-Marie-Tooth (CMT) disease and autoimmune
inflammatory neuropathies (Scherer 1997). Similar to multiple sclerosis in the CNS,
autoimmunity to myelin proteins may also contribute to demyelinating neuropathies in
the PNS. In CMT1 and CMT2, antibodies to PMP22 are detected in the patients serum,
suggesting an immune component to the disease (Ritz et al. 2000). Whether this is
causative or an effect after demyelination occurs remains undefined. An autoimmune
response to MAG has also been linked to demyelination neuropathies (Gabriel et al.
1996). In addition, Guillain-Barré syndrome is associated with autoimmunity to
ganglioside in the peripheral nerve and is a rare disease (Vucic et al. 2009). However,
the majority of demyelinating diseases that are associated with PNS myelin are members of the family of inherited peripheral neuropathies. The most common is CMT, which is linked to several genes in the PNS, including PMP22, MPZ, and Egr2/Krox20 (Young and Suter 2003). These progressive neurological disorders are linked to genetic abnormalities of the Schwann cells that lead to demyelination, usually starting in the second or third decade of life (Lupski and Garcia 1992). Disorders of the PNS myelin can arise from glial gene mutation, duplication or depletion, additionally there are neuropathies that are axonal in origin. Extensive investigation has clearly demonstrated that both divisions of the nervous system are susceptible to diseases of the myelin sheath. An increased understating of how the mechanisms of myelination and the protein profile of the CNS and PNS differ may shed light on new therapeutics for these diseases.

**Myelination in the Central Nervous System**

Oligodendrocytes arise from the neural tube and migrate as progenitor cells into the developing brain. This migration is dependent on platelet-derived growth factor-α (PDGFα) and fibroblast growth factor 2 while loss of these signaling pathways impairs oligodendrocyte precursor migration (Osterhout et al. 1997). The transcription factors Sox9 and Sox10 are responsible for driving expression of the receptor for these growth factors in oligodendrocytes (Finzsch et al. 2008). Signaling through the non-receptor tyrosine kinase FYN, which is activated by PDGFα receptor, drives the formation of lamellipodia at the leading edge allowing precursor cell migration (Takenawa and Suetsugu 2007). These cells extend and contract processes as they migrate until they reach their final location and differentiate into mature myelinating cells. Since oligodendrocytes can myelinate up to 40 different axonal segments, their differentiation
requires the extension of many processes and the response to signals from the axon (Simons and Trotter 2007). For oligodendrocytes to differentiate, it is critical the RhoA activity be decreased with increased activation of Rac1 and Cdc42 promoting MBP expression. Inactivation of RhoA appears to be dependent on extracellular matrix molecules and Beta-1 integrin expression as well as factors released by neurons (Liang et al. 2004). After the extension of the cells multiple processes, mechanisms for transport of myelin assembly must be established. Although the precise steps on how the myelin components are transported remain undefined, at least two mechanisms have been suggested. First, there may be direct or indirect transport through the trans-Golgi network via an endosomal compartment. Second, it has been proposed that myelin protein may be inserted into the plasma membrane first, then internalized and transported via endosomes to the myelin in a process called transcytosis (Maier et al. 2008). Neurons have been demonstrated to release a soluble factor that signals for myelin protein incorporation into the membrane (Kippert et al. 2007). Induction of oligodendrocyte differentiation is still poorly understood, however, adenosine, insulin-like growth factor 1, and neuregulin are all positive regulators of CNS myelination (Kim et al. 2003; Stevens et al. 2002; Ye et al. 2002). Although oligodendrocytes do not synthesize a basal lamina, extracellular matrix cues are still essential for the process. Specifically, CNS myelination is modulated by laminins with the laminin receptor alpha6beta1 integrin promoting cell survival and maturation (Colognato et al. 2002). The transcription factor profile of developing oligodendrocytes is complex, with Nkx2.2, Sox10, and the bHLH proteins Olig1/2 all being required for proper maturation (Fu et al. 2002; Kuhlbrodt et al. 1998; Lu et al. 2000). Olig1 has been shown to negatively
regulate the expression of an oligodendrocyte specific g-protein coupled receptor, Grp17, which appears to involved the timing of myelination and remyelination in the CNS (Chen et al. 2009). However, it appears that only the recently characterized Myelin Gene Regulatory Factor (MRF) is a master transcriptional regulator of CNS myelination. Deletion of MRF in knock-out mice is shown to result in oligodendrocytes being halted at the premyelinating stage of differentiation. Reduced transcription and synthesis of the myelin genes are also observed, and death resulting from seizures occurs by three weeks after birth (Emery et al. 2009). Although much remains unknown regarding oligodendrocyte differentiation and myelination, the signals and mechanisms employed by Schwann cells to myelinate the PNS are more completely defined.

**Myelination in the Peripheral Nervous System**

Schwann cells, the myelin synthesizing cells of the PNS, are derived from neural crest cells. In the mature nerve, the Schwann cells can exist in either a myelinating or non-myelinating phenotype and retain the ability to transition between the two states. Axonal contact has been shown to trigger Schwann cell proliferation as well as damage to the axon. Although their primary function is to provide myelin to the axon, other essential roles have to be contributed the Schwann cells. These additional functions include but are not limited to the secretion of neurotrophic factors, synthesis of progesterone, modulation of extracellular potassium, and the transfer of molecules to the axon (Maier et al. 2002; Scherer 1997). It is becoming increasing apparent that myelinating glial cells also promote axonal survival, as evidence by axonal damage present in multiple sclerosis and peripheral demyelinating diseases. In these demyelinating disease states, axonal damage is observed after the loss of the myelin implying a protective and supportive role of the myelin on the axon.
During development, the Schwann cell precursors proliferate throughout development and form a one to one relationship with the axon through radial sorting resulting with many cells myelinating one axon. Radial sorting continues postnatally and similar to what is observed in oligodendrocytes, the sorting is dependent on Beta1-integrin recognition of extracellular matrix signals (Feltri et al. 2002). Once sorted, the Schwann cells will either initiate myelination or engulf a number of small diameter axons to form a Remak fiber (Cafferty et al. 2009). Several factors may influence Schwann fate during cell sorting, including the factors NRG1, IGFs, NT3, and BDNF (Cheng et al. 2000; Meintanis et al. 2001; Yamauchi et al. 2004). The transforming growth factor-Beta (TGF-beta) pathway and the extracellular matrix protein laminin have been shown to control the number of Schwann cells in vivo (Parkinson et al. 2001; Yang et al. 2005; Yu et al. 2005). Schwann cells express the tyrosine receptor kinases ErbB2/3 and the axonally secreted, neuregulin (NRG1 type III) promotes migration, proliferation and differentiation of the cells (Jessen and Mirsky 2005). Activation of the ErbB receptors leads to increased PI3K/AKT activation, which is associated with myelination in both the CNS and PNS. Inhibition of this pathway during development is associated with impaired myelination by oligodendrocytes which is believed to be the result of impaired differentiations (Narayanan et al. 2009). Also the NRG1/ErbB signaling pathway has been demonstrated to induce cholesterol synthesis in Schwann cells, a requirement for myelin formation (Pertusa et al. 2007). Differentiation of Schwann cells also involves the suppression of the c-Jun (N)-terminal kinase pathway (JNK) which is required developmentally for both NGR1 and TGF-Beta pathways. c-Jun is inactivated by Egr2/Krox20 during differentiation (Parkinson et al. 2004) and the prevention of this
inactivation results in loss of myelin gene expression. Notch signaling also induces Schwann cell proliferation and is reduced upon axonal contact and cellular differentiation. In addition to c-Jun, the transcription factors PAX3 and SOX2 have also been shown to be negative regulators of myelination in Schwann cell and failure to actively down-regulate their expression prevents myelin formation (Kioussi et al. 1995; Le et al. 2005). These studies help to define the profile of an immature Schwann cell, while the identification of pro-myelin transcription factors and signaling pathways such as Krox20/Egr2, octamer-binding transcription factor 6 (OCT6), brain 2 class III POU-domain protein (BRN2), and phosphatidylinositol 3-kinase (PI3K) signaling aid in defining the markers of mature Schwann cells (Le et al. 2005; Maurel and Salzer 2000; Wegner 2000b). Protein kinase A (PKA) and cyclic adenosine monophosphate (cAMP) signaling appears to be the most critical pathway for Schwann cell differentiation (Monje et al. 2009) where elevated intracellular cAMP levels are critical for myelin gene expression and myelination. Although the key players in Schwann cell differentiation are becoming more apparent, the regulation and signaling in this process is still unclear and thus poorly characterized.

The process of wrapping the axon with the myelin membrane is similar between the two divisions of the nervous system. After the cells recognize and attach to the axon, they increase their secretory pathways to allow transport of the newly synthesized membrane to the myelin sheath. The majority of the lipid components of the myelin are synthesized in the endoplasmic reticulum (ER) where some of the myelin proteins can associate with cholesterol, for example, and form a complex to be transported to the myelin sheath. After processing in the Golgi apparatus, transport vesicles are sent to
the membrane by unidentified mechanisms. However, not all myelin proteins are transported by this lipid-protein complex process. For instance, the message for MBP in Schwann cells and oligodendrocytes, is transported as RNA-protein granules for localized protein synthesis (Barbarese et al. 1999; Court et al. 2004). MOBP message has also been demonstrated to be transported for local synthesis in oligodendrocytes (Barbarese et al. 1999). The mRNA binding protein, hnRNP A2 has been shown to be involved in transport of the messages to the myelin but how the message is released and locally translated remains undefined. Regardless, it is know that these mRNA granules are actively transported along microtubules through channels in the myelin sheath, called Cajal bands, to the membrane for synthesis. In light of these finding, RNA transport and post-transcriptional gene regulation appears to be a critical factor during myelination.

Although the precise mechanism governing the induction of myelination in the PNS remains elusive, it has been established that neuregulin-ErbB signaling is essential for Schwann cell differentiation and development. Extreme examples of misregulation include the ErbB3 deficient animals which are completely void of Schwann cells (Riethmacher et al. 1997) and haploinsufficiency of the neuregulin 1 gene leads to thinner myelin (Michailov et al. 2004). Brain derived neurotrophic factor acting through its receptor p75 also increases myelin thickness (Tolwani et al. 2004). Interestingly, Schwann cells have been shown to synthesize progesterone which has a stimulatory effect of myelin gene expression and appear to have an autocrine effect (Koenig et al. 1995; Melcangi et al. 1998). As the Schwann cells sort and elongate, they secrete a basal lamina that encompasses the Schwann cell / axon which is required for the
Schwann cell to continue myelination (Bunge and Bunge 1986). As the Schwann cells differentiate, myelin gene expression increases through transcriptional mechanisms with increases in Egr2/Krox20 being associated with the induction the myelin genes MPZ and PMP22 (Topilko et al. 1994). Interestingly, PMP22 is not a direct target of Egr2/Krox20 regulation, yet MPZ, MBP and myelin associated glycoprotein (MAG) are all directly transcribed by this factor in Schwann cells (Jang et al. 2006). These studies suggest multiple levels of gene regulation during the myelination process.

**Peripheral Myelin Protein 22**

PMP22 is a 22 kDa tetraspan glycoprotein that is predominantly expressed by the myelinating Schwann cells of the peripheral nervous system (Snipes et al. 1992). PMP22 was first identified as growth arrest-specific gene 3 (gas-3) in NIH 3T3 fibroblasts (Schneider et al. 1988) and its expression increases as cells reach density-dependant inhibition (confluency) (Manfioletti et al. 1990; Zoidl et al. 1995). The significance of the growth arrest-specific expression is still undetermined. Although PMP22 protein expression is highly restricted, PMP22 mRNA is present ubiquitously throughout the body, including the central nervous system, kidney, heart, muscle and lung (Amici et al. 2006; Baechner et al. 1995; Ohsawa et al. 2006; Parmantier et al. 1995; Suter et al. 1994). PMP22 protein is detected in Schwann cells, at epithelial and endothelial cell junctions, and in specific motor and sensory neurons (Baechner et al. 1995; Maier et al. 2003; Notterpek et al. 2001; Roux et al. 2004). In developing rat sciatic nerve, PMP22 message steadily increases and reaches maximal expression at approximately post-natal day 21 which correlates with the completion of myelination and Schwann cell differentiation state (Garbay et al. 2000). In mature sciatic nerve it represents only 2-5% of the PNS myelin proteins. In addition, PMP22 levels drop
significantly post-nerve crush injury (Snipes et al. 1992) which correlates with the de-differentiation state of the Schwann cells. These findings suggest the involvement of post-transcriptional mechanisms in controlling PMP22 expression which may be dependent upon both the cell type and differentiation state.

Although PMP22 represents only a relatively minor constituent of the PNS myelin, point mutations, duplication, and deletion of the gene are associated with demyelinating neuropathies (Lupski and Garcia 1992). Charcot-Marie-Tooth disease type 1A (CMT1A) is the most common form of inherited peripheral neuropathy with a prevalence of 1 in 2500 live births (Lupski and Garcia 1992). CMT1A has been linked with a duplication of a 1.5 Mb region on chromosome 17p11.2 (Patel et al. 1992; Roa et al. 1991) which includes the PMP22 gene. The phenotypes are proposed to be the result of altered gene dosage (Adlkofer et al. 1995; Huxley et al. 1996). The Schwann cells in neuropathic models and in patients show an impaired ability to myelinate (Nobbio et al. 2004). PMP22 levels must be tightly controlled as it is estimated that a 50% reduction in expression will result in HNPP, while a 50% increase leads to CMT1A (Maier et al. 2002). Recent data suggest that the PMP22 transcript is misregulated in a number of neurological diseases, including schizophrenia and depression (Aston et al. 2005; Dracheva et al. 2006) and also in cancer (van Dartel and Hulsebos 2004). These findings may imply leaky transcription and a requirement to regulate undesired message at a post-transcriptional level.

**PMP22 Expression and Gene Regulation**

In studies specifically examining the regulation of the PMP22 gene, the majority of the work has focused on the 5'-UTR regulatory elements. There are two characterized transcripts of the PMP22 gene that differ only in the inclusion of UTRs, primarily
untranslated exon 1. Two promoters initially characterized, P1 and P2, appear to have tissue specific functions (Maier et al. 2003; Suter et al. 1994). Transcription via promoter P1 results in the inclusion of exon 1A, is preferentially used during myelination, and is mostly Schwann cell specific (Saberan-Djoneidi et al. 2000). P1 is under the control of a CREB-dependant silencing element and a cAMP silencing element which, in the absence of cAMP, prevents PMP22 expression. This is cAMP regulatory element is common in many of the myelin protein genes. Also the PMP22 promoter region possesses many common features of mammalian promoters, including TATA and CAAT boxes (Wegner 2000a; Wegner 2000b). Promoter P2 however, appears to lack the common promoter elements found in P1 and is used ubiquitously throughout the body. The resulting transcript differs only in the inclusion of exon 1B and is is important to note that all of the transcripts encode the same protein sequence. Investigations for other regulatory regions in the PMP22 gene have revealed that both the 5'- and 3'-UTRs play critical roles in the expression and stability of the RNA transcripts (Bosse et al. 1999). The 3'UTR of PMP22 exerts a negative effect on RNA translation. In addition to the role promoter regions and UTRs have in PMP22 regulation, applicable transcription factors have also been examined. Several transcription factors that have been implicated in myelination are expressed in Schwann cells and have predicted binding regions in the PMP22 5' promoter region (Maier et al. 2003). For example, Krox20 (Egr-2) and Oct6 (SCIP) are required for Schwann cells to initiate myelination, although their precise roles are different. Oct6 appears to be primarily involved in the timing of myelination (Jaegle et al. 1996). While in Krox20/Egr2 knockouts Schwann cells, myelin fails to be synthesized altogether (Topilko et al. 1994). Although Krox20/Egr2
does directly target MPZ, MBP, and MAG in Schwann cells, PMP22 is not a direct target of this essential myelin transcription factor (Jang et al. 2006). These results suggest that there must be elaborate post-transcriptional regulation of myelin genes, including PMP22.

**MicroRNAs and Gene Regulation**

It has been established that in addition to the changes in the transcription factor profile, there are several post-transcriptional regulatory mechanisms that influence myelination (Zearfoss et al. 2008). RNA binding proteins such as Quaking have been shown to control both Schwann cell and oligodendrocyte differentiation (Chen et al. 2007; Larocque et al. 2009). In addition, the transport of MBP mRNA and local synthesis is essential for myelin formation (Barbarese et al. 1999). However another recently elucidated mechanism of post-transcriptional gene control that involves the 3'UTR is repression via microRNAs (miRNAs). MiRNAs are small, non-coding regulatory RNA molecules that bind to the 3'UTR of target genes based upon reverse complementarity and prevent their translation (Grimson et al. 2007; He et al. 2005; Valencia-Sanchez et al. 2006). MiRNAs are transcribed via RNA polymerase II, cleaved by Drosha, actively exported into the cytoplasm by Exportin 5, and then processed by the endoribonuclease Dicer to form the mature miRNA. The complex regulation of each of these steps has recently begun to be elucidated (Davis and Hata 2009; Winter et al. 2009). The binding of the miRNA to the target site on mRNA can either signal for the degradation via the RNA induced silencing complex (RISC), which contains the Argonaute proteins, or repress translation without degradation through other less defined mechanisms (Bagga et al. 2005; Pillai et al. 2005). The RISC has been localized to structures termed processing bodies (P-bodies) or GW bodies (GWB).
These cytoplasmic foci contain the RNA-binding protein GW182 and serve as the sites where miRNAs are believed to exert the majority of their function (Ding and Han 2007; Liu et al. 2005).

MiRNAs have been revealed to be involved in numerous cellular processes including cell differentiation, cell cycle, and cell death (Miska 2005). Mutations creating or deleting miRNA target sites can result in abnormal phenotypes in vivo (Clop et al. 2006). Although no direct relation has established for the role of miRNAs in the process of myelination, it has been proposed that miRNA are involved in the translational repression of myelin mRNAs during transport until local synthesis can occur (Kim et al. 2004). Loss of miRNA biogenesis by reduction of Dicer expression has been shown to affect oligodendrocyte maturation (Kawase-Koga et al. 2009). Genes that affect oligodendrocyte myelination have been shown to be subjected to miRNA mediated gene regulation (Lehotzky et al. 2009; Lin and Fu 2009). Also recently, it was reported that autoimmunity to the GW-bodies is associated with motor and sensory neuropathy in humans (Bhanji et al. 2007) although the histopathology remains undefined. Ongoing research is revealing that miRNA are likely to be involved in most cellular processes and they are likely to exert an influence on myelin gene expression in both the CNS and PNS.

The disparities between the localization of PMP22 mRNA and detectable PMP22 protein suggest that there is post-transcriptional regulation of the gene. It has been hypothesized that PMP22 mRNA may be regulated post-transcriptionally by a non-transcribed RNA molecule (Manfioletti et al. 1990). The mechanism of how the 3’-UTR of PMP22 negatively regulates expression of the message has yet to be determined.
(Bosse et al. 1999). Recently, the 3'UTR region of the PMP22 gene in medaka fish was demonstrated to possess regulatory domains again implicating this region in modulating gene expression (Itou et al. 2009). This dissertation addresses the overall hypothesis that miRNA-mediated gene regulation modulates PMP22 expression and the miRNA pathway is essential for Schwann cell myelination. In these studies we examine the miRNA expression profile (miRNAome) of oligodendrocytes and Schwann cells in response to different growth conditions or differentiation states. In addition, we show evidence that PMP22 is regulated in both the CNS and PNS by miRNAs, albeit by different miRNAs. We show that the expression of mature miRNAs is essential for proper Schwann cell myelination and differentiation. Taken together these studies demonstrate additional levels of myelin gene regulation previously uncharacterized. The elucidation of the mechanism of post-transcriptional regulation of PMP22 provides novel insight into the etiology of myelin-associated diseases and may provide new therapeutic targets in controlling myelin gene regulation.
CHAPTER 2
MATERIALS AND METHODS

Plasmids and miRNA Precursors and Inhibitors

The psicheck2 luciferase vector (Promega, Madison, WI) was used for the luciferase assays. The 3'UTR of PMP22 was inserted using the Xho1/Not1 sites. Site directed deletion of the miR-29a seed region was performed using the GenetailorTM site directed Mutagenesis System (Invitrogen, Carlsbad, CA) with specific primers designed using the PrimerX program (http://www.bioinformatics.org/primerx/). The mutagenesis primers used were 5'-ACAAGCAATCTGTGAAAATAGATTTACCAT-3' and 5'-TTTCACAGAGATGCTTGTCTCTGACGTCT-3'. The c-myc-Ago2 plasmid was a kind gift from Dr. Hannon's Laboratory (Cold Springs Harbor, NY) (Karginov et al. 2007). Pre-miRNA precursors and anti-miRNA inhibitors were obtained from Ambion (Austin, TX) and used at the indicated concentrations.

The PMP22 3'UTR fragments for the miR-9 luciferases were constructed by cloning the Xho I - Not I fragment of pGEM-T 3'UTR-PMP22 containing the full length 3'UTR of PMP22 into the psiCHECK2 reporter plasmid (Promega). Three other fragments derived from the 3'UTR of PMP22 were obtained by PCR using the following primers: For the fragment 1: 5'-GACTCGAGGGAGGAAGGAAACCAGAAAAC-3' and 5'-GAGCGGCCGCAATCCCCAATCTGAATCTGTGTCTTCTG-3'. For the fragment 2: 5'-GACTCGAGTGTCTGATTGAAGATGTATAT-3' and 5'-GAGCGGCCGCTCACTGGGTCACCCATAGTG-3'. For the fragment 3: 5'-GACTCGAGATTAGCAGGAATAATCCGC-3' and 5'-GTCGACGCGCGCCGAGTTACTCTGTATGTTTTATTTAATGACATC-3'.
Fragments of the 3'UTR of PMP22 used in assessing miR-29a binding were obtained by PCR using the rat PMP22 cDNA as template. For PMP400, the primers were 5'-AGGCCTCTCGAGGCGCCCGACGCACCATCCGTCTAGGC-3' and 5'-GAGCGGCCGCTGAGCAAAACAAAAAGATGA-3'. For PMP800, the primers were 5'-AGGCCTCTCGAGGCGCCCGACGCACCATCCGTCTAGGC-3' and 5'-GAGCGGCCGCTAAAACTGTATAATTGAGTT-3'. PCR products were digested by Xho I/Not I and cloned into the psicheck2 plasmid (Promega).

**Cell Culture and Transfection**

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) containing 10% fetal calf serum (FCS) (Hyclone, Logan, UT), 5 μM forskolin (Calbiochem, La Jolla, CA) and 10 μg/ml bovine pituitary extract (Biomedical Technologies Inc, Stoughton, MA). To analyze proliferating Schwann cells, the cell were harvested at ~75% confluency. To stimulate differentiation, the cells were subjected to 0.5% FCS/DMEM for 72 h prior to collection (Yang et al. 2004). Alternatively, the cultures were switched to a defined medium (DMEM-F12, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 100 μg/ml BSA, N2 supplement, 38 ng/ml dexamethasone, 50 ng/ml thyroxine, 50 ng/ml tri-iodothyronine) for 48 h to promote growth-arrest and differentiation (Cheng and Mudge 1996). Schwann cells cultured under these conditions have been shown to be primarily non-dividing, as determined by bromodeoxyuridine incorporation (Cheng and Mudge 1996).

The HeLa cell line was grown in DMEM (Invitrogen) containing 10% FBS and 50 μg/ml gentamycin. For transient transfection, HeLa cells were transfected using Lipofectamine 2000 (Invitrogen), 200 ng of each reporter plasmid and 10 nM of the
miRNA precursor (Ambion, Austin, TX). Luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) 48h after transfection.

Transient transfections of Schwann cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. In brief, the cells were split the day before transfection and allowed to grow overnight. The cells were then transfected with either RNA and/or DNA at the indicated concentrations. To express miR-9 in primary rat Schwann cells transient transfection using 4 µg of pcDNA6.2-GW/miR-Neg plasmid (Invitrogen) or 4 µg of pcDNA6.2-GW/EmGFP-miR-9. mir-9 was cloned according to manufacturer’s instructions using the following sequence : 5’-TGCTGTCTTTGGTTATCTAGCTGTATGAGTTTTGGCCACTGACTGACTCATACAGAGATAACCAA-3’. Schwann cells were harvested either 24h (for Northern blot) or 48h/72h (for Western blot) after transfection.

Transfection efficiency for the plasmids was approximately 30%, as judged by the expression of a plasmid encoding enhanced green fluorescent protein (EmGFP). RNA transfection efficiency was over 90% as determined using a fluorochrome-conjugated scrambled miRNA (Ambion). Cells were harvested for RNA analysis using TRIzol (Invitrogen). Protein samples were obtained by harvesting cells in gel sample buffer and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL).

Myelinating Co-cultures of Schwann Cells and Dorsal Root Ganglion Neurons

Dissociated neuronal cultures from rats were established as described (Notterpek et al. 1999b). Dorsal root ganglia (DRG) were collected from embryonic day 15 rats, digested with 0.25% trypsin (Gibco, Rockville, MD, USA), mechanically dissociated and either plated on rat tail collagen-coated (Biomedical Technologies, Inc., Stoughton, MA,
USA) 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, USA) and grown at 37°C and 5% CO₂. The following day, the cultures were treated with fluorodeoxyuridine (10 μM) for three cycles to enrich the neuronal population.

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

**Lentiviral Packaging**

A lentiviral pGIPZ shRNA vector encoding shRNA targeting Dicer and a negative, non-targeting shRNA control (Neg.) vector, both of which also encode GFP as a reporter protein, were obtained from commercial providers (Open Biosystems). Using HEK 293FT cells, the pGIPZ and Neg. shRNA lentivectors were packaged into concentrated lentiviruses using a three plasmid transfection procedure (Semple-Rowland et al. 2007). Viral titers were estimated using a Lenti-X qRT-PCR kit (Millipore, Billerica, MA) and typically averaged 2 x 10^{12} viral genomes per ml.

**Schwann Cell Transduction**

Primary rat Schwann cells were transduced with pGIPZ shRNA lentivirus as described (Bolis et al. 2009). In brief, approximately 1 x 10^5 cells were plated on a 6 cm
dish 24 h prior to transduction. Schwann cells were transduced at a multiplicity of infection (MOI) of 5. The Schwann cell cultures were incubated in 1 ml DMEM containing the virus for 4 h. After 4 h, 1 ml of 2X rat Schwann cell media was added and the cultures were then incubated for an additional 48 h. Transduction efficiency was determined to be greater than 90% by direct visualization of GFP expression. The transduced cells were selected by adding 2 µg/ml puromycin to the Schwann cell media. After 3-5 d in culture, the cells were seeded onto purified DRG neurons.

**Fluorescent Automated Cell Sorting of Oligodendrocytes**

Sprague-Dawley rats (Taconic, Hudson, NY) were handled in accordance with NIH guidelines and as approved by the NINDS ACUC Committee. P7 rat brain cells were separated on a 15–40% Percoll gradient. After staining with the A2B5 mouse monoclonal IgM antibody and GalC rabbit polyclonal antibody (Millipore, Billerica, MA), A2B5+ cells (OPCs) and GalC+ cells (OLs) were sorted using a FACS VantageSE flow cytometer (Becton Dickinson, Franklin Lakes, NJ) (Cohen et al. 2003).

**Microarrays and Bioinformatics Analysis**

Four biological replicates of A2B5+ and GalC+ cells were used for hybridization onto Rat Expression 230 Microarrays (Affymetrix, Santa Clara, CA). The microarray data were analyzed using Genespring 7.0 software (Silicon Genetics, Redwood City, CA). The data was processed using RMA and a global normalization was performed using Genespring per chip normalization (normalized to the 50th percentile) and per gene normalization (normalized to the median).

Total RNA from A2B5+ and GalC+ cells were purified using the mirVana miRNA Isolation Kit (Ambion) and used for hybridization with miRNA microarrays (LC Sciences, Houston, TX). Slides were scanned using an Axon GenePix 4000B microarray scanner.
The microarray images were background substracted using a local regression method and normalized to the statistical mean of all detectable miRNAs.

Target bias analysis was conducted using TargetScan 4.0 (www.targetscan.org) in conjunction with the Fisher's Exact Test function (fisher.test) found in the R language for statistical computing and graphics (www.r-project.org). For each miRNA and each defined window of genes ("Top", "Middle", "Bottom"), four values were determined: the number of genes in the window the miRNA targets, the number of genes in the window, the number of genes the miRNA can target regardless of window, and the number of genes assayed. The values were then used to generate a p-value for each miRNA in the three defined window of genes using a right-tailed condition ("enrichment"). Hierarchical clustering of miRNAs was done using GenePattern 2.0 (Reich et al. 2006).

**Primary Antibodies**

A previously characterized mouse monoclonal antibody (Millipore, Billerica, MA), (Notterpek et al. 1999a) was used to detect PMP22 in the immunolabeling experiments. For the PMP22 Western blots, we utilized a rabbit polyclonal antibody raised against synthetic peptide of the rat PMP22 (amino acids 117-132) (Pareek et al. 1993). We used a human anti-GWB antibody (Eystathioy et al. 2002) to detect GW Bodies in cells (kind gift from the Chan lab, Gainesville, FL) and a mouse anti-GW182 antibody (Abcam, Cambridge, MA) for the Western blot. Antibodies against c-myc (Santa Cruz, CA), phospho-histone H3 (Ser10) (Upstate, Temecula, CA), Dicer (Santa Cruz, CA) and GAPDH (Encor Technologies, Alachua, FL) were obtained from the indicated suppliers. NF-M was detected using a mouse monoclonal antibody and Myelin protein zero (MPZ) was detected using a chicken polyclonal antibody (Encor). CC1 was detected with a
polyclonal antibody (Oncogene). Egr2/Krox 20 was probed for using a rabbit polyclonal antibody (Covance, Princeton, NJ). c-Jun and Sox2 were detected using rabbit polyclonal antibodies (Santa Cruz). Oct6 was detected using a rabbit polyclonal antibody (Abcam, Cambridge, MA). MBP was probed for using rabbit polyclonal antibodies (Millipore, Billerica, MA).

**Immunoblotting**

Equal amounts of protein lysates (40 µg per sample cells or 5 µg per sample nerve) were separated on sodium dodecyl sulfate gels and transferred to nitrocellulose membranes. Endoglycosidase digestion with PNGase F (New England Biolabs, Beverly, MA) was performed overnight at 37 °C prior to Western blot (Pareek et al. 1997). After transfer, the membranes were blocked in 5% nonfat milk/phosphate buffered saline (PBS) for one hour and then incubated with primary antibody in 5% nonfat milk/PBS or 5% FCS/PBS overnight at 4 °C on a shaker. The membranes were washed three times with PBS, and then incubated with secondary antibody in 5% milk/PBS for two h at room temperature. After incubation with anti-rabbit, anti-chicken or anti-mouse horseradish peroxidase (HRP) conjugated secondary antibodies (Sigma, St. Louis, MO), bound antibodies were detected with an enhanced chemiluminescent substrate (Perkin Elmer, Boston, MA). Films were digitally imaged using a GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA) and figures assembled using Adobe Photoshop 5.5. Quantification of Western blot data was performed using Scion Image (Frederick, MD). The specific band intensities were obtained and the data was normalized for GAPDH to obtain relative protein expression levels.
Immunostaining of Schwann Cells and Co-cultures

Immunofluorescence experiments were performed as described (Notterpek et al. 1999a). In brief, Schwann cells cultured on glass coverslips were fixed with 4% paraformaldehyde for 10 min and post-fixed permeabilized with cold 100% methanol for 5 min at -20°C. After blocking with 10% normal goat serum for one hour at room temperature, the samples were incubated with the indicated primary antibodies overnight at 4°C. Bound antibodies were detected using Alexa Fluor 594 (red) anti-mouse and Alexa Fluor 488 (green) anti-human antibodies (Molecular Probes, Eugene, OR; Zymed, San Francisco, CA). For PMP22 immunofluorescence in the CNS, P7 rat brain sections were incubated with mouse monoclonal CC1 (Oncogene, San Diego, CA) and rabbit anti-PMP22 antibodies. Staining was revealed using anti-mouse IgG-Alexa Fluor 488 and anti-rabbit IgG-Alexa Fluor 594. Nuclei were stained using DAPI (Molecular Probes) or Hoechst dye (10 µg/ml, Molecular Probes) was included in the secondary antibody solution to visualize nuclei where indicated. Control samples without primary antibodies were processed in parallel. Additional proteins were probed for using the previously mentioned primary antibodies. Samples were then mounted on slides using the Prolong Anti-fade kit (Molecular Probes). Images were acquired with a SPOT digital camera (Diagnostic Instrumentals, Sterling Heights, MI) attached to a Nikon Eclipse E800 microscope (Tokyo, Japan).

Immunoprecipitation

For the c-myc-Ago2 immunoprecipitation experiments, rat Schwann cells were transfected with the c-myc-Ago2 plasmid and either the negative (Neg.) scrambled miR or miR-29a (Karginov et al. 2007). Two days post-transfection, the cells were incubated in lysis buffer (10 mM Tris, pH 7.5, 10 mM KCl, 2 mM MgCl2, 5 mM DTT) supplemented
with a mixture of protease inhibitors (Complete™; Roche, Indianapolis, IN) for 15 min on ice and lysed by pipetting. Five-fold concentrated ATP depletion mix (4 units/ml RNaseIn (Promega), 100 mM glucose, 0.5 units/ml hexokinase (Sigma), 1 mg/ml yeast tRNA (Invitrogen), 450 mM KCl) was added to the cell lysates (to obtain a 1X concentration) and centrifuged at 16,000xg for 30 min at 4°C. Prior to the immunoprecipitation, anti-c-myc beads (Sigma, St. Louis, MO) were preblocked for 30 min in wash buffer (0.5% Nonidet P-40, 150 mM NaCl, 2 mM MgCl2, 2 mM CaCl2, 20 mM Tris, pH 7.5, 5 mM DTT, with EDTA-free protease inhibitors) containing 1 mg/ml yeast tRNA and 1 mg/ml BSA. Wash buffer was added to the lysates and samples were incubated and agitated with the beads for 4 h at 4°C. The beads were washed first in wash buffer and then with wash buffer containing 650 mM NaCl two times. Next, the slurry was transferred to a fresh tube and bound RNA was extracted with TRIzol (Invitrogen) and the RNA concentrations were determined.

**RNA Expression Analysis**

Real-time reverse-transcriptase PCR was performed as described (Notterpek et al. 2001). At 48 or 72 h post-transfection, RNA was isolated from Schwann cells transfected with the indicated plasmid, miRNA-precursor or inhibitor, using TRIzol (Invitrogen). QuantiTech primers specific for PMP22 RNA were obtained from Qiagen (catalog number QT00175938). To ensure equal loading of RNA, control primers for GAPDH (Qiagen QuantiTech assay catalog number QT00199633) were included. Each sample was analyzed in triplicate (0.2 μg of RNA per reaction) using the Applied Biosystems 7300 real-time PCR system. The SYBR green QuantiTech kit was obtained from Qiagen. Data was normalized to GAPDH using the $2^{-\Delta\Delta C_{T}}$ method (Livak and Schmittgen 2001).
MiRNA Expression Analysis

Expression of mature miR-29a was verified using real-time PCR and normalized to miR-125a, using the 2-\(\Delta\Delta CT\) method (Livak and Schmittgen 2001). MiR-125a is equally expressed in Schwann cells harvested from proliferating and non-proliferating cells (see data in Figure 4). Total RNA was reverse transcribed using the Taqman Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and PCR was performed in triplicate using Taqman primers specific for miR-29a and miR-125a (Applied Biosystems). To analyze RNA levels in rat and mouse sciatic nerve, the animals were sacrificed according to approved IACUC protocols and the sciatic nerves were removed and immediately frozen in liquid nitrogen. To obtain an adequate yield of total RNA, nerves from at least two animals were pooled from each treatment condition (ten crush sites were pooled for the mouse nerve analysis). The nerves were crushed under liquid nitrogen and total RNA was isolated using either the TRIzol (Invitrogen) or the mirVana miRNA Isolation kit (Ambion) according to the manufacturer’s instructions. For quantitative RT-PCR analysis, 0.1 \(\mu g\) of RNA per reaction was employed with the Quantitech SYBR Green RT-PCR kit (Qiagen) and primers specific for PMP22 (Qiagen). For nerve miRNA analysis, the NCODE miRNA first-strand cDNA synthesis and qRT-PCR kit (Invitrogen) was used with primers specific for miR-29a, miR-29b, and miR-24. Each sample was repeated in triplicate and the results were normalized using primers to GAPDH (Qiagen, for PMP22) or miR-24 (Invitrogen, for miRNA analysis). MiR-24 was used to control for equal RNA input because its expression was not affected by either Schwann cell differentiation or crush nerve injury. The relative expression of each message was determined using the 2-\(\Delta\Delta CT\) method.
MiRNA microarrays were performed by isolating total RNA from Schwann cells in proliferating and defined media (triplicate samples per condition), purified using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) and analyzed with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (5 μg) was labeled with a Cy3-conjugated RNA-linker and hybridized to Locked Nucleic Acid (LNA) based miRCURYTM arrays (Exiqon, Woburn, MA). Images were acquired using an Axon scanner (4000B) and processed with Genepix 6 (Molecular Devices, Sunnyvale, CA).

**Bioinformatics for miRNA Target Prediction**

Bioinformatic scans of the rat 3’UTR of PMP22 were conducted using three web based miRNA target prediction programs (Targetscan [http://www.targetscan.org/], miRbase [http://microrna.sanger.ac.uk/targets/v5/], and Pictar [http://pictar.bio.nyu.edu/]). MiRNAs were chosen based upon their prediction by more than one program, conservation of the binding region, and the strength of predicted interaction.

**Luciferase and Gel Shift Assays**

Luciferase assays were performed using the Dual-Luciferase assay kit (Promega). In brief, Schwann cells were co-transfected in 24-well plates with the indicated psicheck2 luciferase construct (0.4 μg/well) and miRNA precursor (10 nM). After 48 h, the cells were harvested in passive lysis buffer and luciferase activities were determined using a Bio-Tek Synergy HT luminometer (Bio-Tek Instruments Inc, Winooski, VT). The luciferase data is expressed as a ratio of Renilla Luciferase (RL) to Firefly Luciferase (FL) to normalize for transfection variability between samples. Luciferase experiments were repeated at least three independent times, in triplicate or greater, as indicated. The Hela cell line was used with the indicated psicheck2 plasmid and miRs to assess
miR-9 binding, Schwann cells were used for all additional lucifase assays where denoted.

The full length 3'UTR of PMP22 was cloned from rat sciatic nerves by PCR using the primers 5'-AGGCCTCTCGAGGCAGCGCCGACGCGTCTATGGC-3' and 5'-GTCGACGCGGCCGAGTTACTCTTGATGTTTATTTAATGATC-3'. The PCR fragment was inserted into pGEM-T easy (Promega) and used for in vitro transcription (Promega). Gel shift assays were performed with in vitro transcribed PMP22 RNA using a T7 in vitro transcription kit (Promega). The RNA was incubated for two hours with biotin-labeled miRNAs (1 pmol) at 42° C. The samples were separated on 3% agarose gels, transferred to a nylon membrane, and the complexes revealed using a nucleic acid detection kit HRP-streptavidin substrate (Pierce). Total RNA loading was monitored using SYBR Gold (Invitrogen) staining of the gel.

Sciatic Nerve Crush Injury

Experiments were performed on 8-weeks old male CD1 mice obtained from Charles River Laboratories (Wilmington, MA, USA) as described (Islamov et al. 2004). Animals were housed one per cage under standard laboratory conditions, with 12 h light/dark schedule and unlimited access to food and water. Animal protocol was approved by Animal Care and Use Committee of East Carolina University, an AAALAC-accredited Facility.

Animals were anesthetized with ketamine (18 mg/ml) –xylazine (2 mg/ml) anesthesia (0.5 ml/10 g of body wt, i.p.). An incision was made on the right thigh; the right sciatic nerve was exposed and crushed at the level of the sciatic notch for 15 sec with a fine hemostat. The wound was closed and the animals were allowed to recover for 4 or 5 days. After specified time periods animals were euthanized and sciatic nerves
were quickly dissected out, snap frozen in liquid nitrogen and stored at -80°C. The control sample (naïve nerve) was taken from the contralateral side (sciatic nerve from left leg). The excised crush sample was the injury site plus ~ 4mm up and 4mm down from the point of injury.

**Real-time PCR Analysis of Oligodendrocyte Message**

Total RNA (150 ng) from A2B5+ and GalC+ cells were treated with Turbo DNase (Ambion) for 10 min at 37°C. First strand synthesis was conducted using the Taqman Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using a Taqman Array microRNA Panel v1.0 (Applied Biosystems). PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR Systems using the Taqman 2X Universal PCR Master Mix. A geometric averaging on multiple miRNAs was performed to determine miR-203 as reference for normalization.

Real-time PCR of PMP22 was conducted using the LightCycler FastStart DNA MasterPlus SYBR Green I (Roche, Indianapolis, IN) and the following primers: 5’-TCCTCATCTGTGAGCGAATG-3’ and 5’-ACAGACCAGCAAGGATTG-3’. The beta-actin primers used for normalization were: 5’-TGTCACCAACTGGGACGATA-3’ and 5’-GGGGTTGTTGAGGTCTCAA-3’.

**Northern Blot**

miRNAs from adult tissues (Ambion) were separated by electrophoresis through a 15% TBE- urea gel (Invitrogen). After transfer to a GeneScreen Plus nylon membrane (Perkin-Elmer, Wellesley, MA), membrane was incubated overnight at 42°C with 10 pmol of biotinylated Locked Nucleic Acid (biot-LNA, IDT DNA Technologies, Coralville, IA) in ULTRAhyb Oligo Hybridization buffer (Ambion). The biot-LNA probe sequence for miR-9 was 5’- 5Biot-TCA+TAC+AGC+TAG+ATA+ACC+AAAGA-3’ (Biot = biotin and +
denotes a LNA substitution). After incubation, membrane was washed twice in NorthernMax Low Stringency Wash Solution and in NorthernMax High Stringency Wash Solution (Ambion). Detection was conducted using the Chemiluminescent DNA Detection Kit (Pierce, Rockford, IL). Blots were stripped and probed for U6 small nuclear RNA with the U6 biot-LNA 5’-5Biot-GAA+TTT+GCG+TGT+CAT+CCT+TGC+GCA-3’.

Total RNA from rat Schwann cells (10µg/lane) was separated on denaturing agarose gel and transferred overnight to a nylon membrane (Hybond). After UV-crosslink, the membranes were incubated with 32P-labelled (Random Prime kit, Amersham) PMP22 and 18S ribosomal probes. The blot was washed twice in 2XSSPE, 0.1% SDS at room temperature and once in 1XSSPE, 0.1% SDS at 65°C before exposure to film.

**Bromodeoxyuridine Assay**

To investigate the effects of Dicer shRNA on Schwann cell proliferation, equal numbers of infected cells (4 × 10⁴ 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 previously described (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 mm²) 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). As a second measure of cell division, we analyzed the cultures using a BrdU-based cell proliferation ELISA kit (Roche) according to the manufactures instructions.
Statistics

Data from multiple independent experiments were analyzed using Microsoft Excel 2007 and Graphpad Prism v5.0. For analysis of two independent groups, Student’s t-test was used with significance at p<0.05. For determination of significance between three or more groups, one-way ANOVA and post-hoc Tukey’s t-tests were utilized with significance at p<0.05. All graphs represent the means and the error bars represent the standard deviation of the mean. For correlation studies, a linear regression analysis was performed and the r2 and p-values were calculated using Graphpad Prism v5.0.
Introduction

Oligodendrocytes are glial cells of the CNS that synthesize myelin, and facilitate saltatory conduction of neuronal action potentials. In the mammalian CNS, oligodendrocyte progenitor cells (OPCs) arise in multiple ventral and dorsal locations of the forebrain through three independent proliferative waves during late embryogenesis and early postnatal periods (Kessaris et al. 2006). Oligodendrocyte differentiation has been shown to be influenced by both intracellular and extracellular cues, yet how the myelin genes are regulated and messages transported is still undefined. Elucidating the molecular mechanisms that control oligodendrocyte maturation requires examining stage-specific changes at both transcriptional and posttranscriptional levels, as oligodendrocyte lineage cells differentiate from immature OPCs into premyelinating cells (OLs).

MiRNAs are (22 nt) noncoding RNAs and are now recognized as integral components of the post-transcriptional silencing machinery. It is currently estimated that around 70% of miRNAs are processed from non-protein-coding units, whereas the less abundant intronic miRNAs are found within the introns of coding mRNAs and are usually coordinately expressed with their host genes (Saini et al., 2007). MiRNAs are initially transcribed as long primary transcripts (pri-miRNAs) and processed to mature functional molecules by two specific cleavage steps. First the enzyme Drosha cleaves the transcript, yielding the precursor miRNAs (pre-miRNAs) which are short stem-loop RNA molecules. Then, the pre-miRNAs are actively exported from the nucleus to the cytoplasm by Exportin 5 (Stefani and Slack 2008). After an additional selective
processing by the RNase III type enzyme Dicer, a small double-stranded RNA is produced. One of the strands in then incorporated into the RISC as the mature miRNA while the other stand is quickly degraded. MiRNAs act to either catalyze mRNA degradation or repress translation through base pairing within the 3' untranslated region (3'UTR) of mRNA targets (Valencia-Sanchez et al. 2006). The target genes of the miRNAs are currently being elucidated (Ambros 2004), and the search of mRNA targets mainly relies on bioinformatic analyses that are based on the phylogenetically conserved base pair complementarity between the targets and miRNAs. The characteristics of validated miRNA target sites have recently been described (Grimson et al. 2007). MiRNAs were first discovered as regulators of developmental processes in C. elegans (Lee et al. 1993) but their importance in mammalian cellular biology is now being revealed. A recent study showed that disruption of the Dicer gene in mouse Purkinje cells led to a size reduction of forebrain (Schaefer et al. 2007), in agreement with the important role of miRNAs during neuronal cell specification (Lai et al. 2005). The systematic cloning of miRNAs revealed the presence of several hundred distinct miRNAs in the rat (Miska et al. 2004), mouse, and human brain (Sempere et al. 2004). There are organ specific miRNAs, however many are also expressed ubiquitously in the body with approximately sixty percent of known miRNAs being found in the brain. Among those, few are preferentially expressed in the brain, and these include miR-9, miR-124, and miR-128.

In these experiments we identify 98 miRNAs expressed by postnatal oligodendrocyte lineage cells. We also show that 37 of these miRNAs display a mRNA target bias and that the expression level of the predicted targets of 13 miRNAs is
dynamically regulated during oligodendrocyte differentiation. Additionally, we document the miRNA mediated repression of PMP22 by miR-9 in developing and mature oligodendrocytes.

**Results**

**Characterization of MiRNAs Expressed by Oligodendrocytes**

*In vivo* miRNA expression profiles of defined neural populations have not been reported yet. To address this issue, two stage-specific populations of oligodendrocytes were obtained from postnatal rat brains: OPCs that express the A2B5 ganglioside (A2B5+ cells) and OLs that are positive for the galactocerebroside marker (GalC+ cells) (Fig. 3-1A). We performed miRNA expression profiling for these two glial populations using miRNA microarrays. The presence of 98 miRNAs was reproducibly detected by miRNA microarrays and further validated by real-time PCR (Fig. 3-1B).

The 20 miRNAs with the highest expression levels in oligodendrocytes are shown in Table 3-1. miR-9 has the highest expression level in OPCs. The class of abundantly expressed miRNAs in OPCs also includes many described brain-enriched miRNAs such as miR-26a, miR-124a, miR-125b, miR-181b and the let-7 family encompassing let-7a, let-7b, let-7c, let-7d and let-7f members. In contrast to the let-7 family whose expression is remarkably stable during differentiation, 23 miRNAs are down-regulated (with fold changes >2) and include miR-9 and miR-124a (Fig. 3-2B). Twenty miRNAs are up-regulated and some were previously identified from rat brain tissues: miR-21, miR-152, miR-142-5p and -3p, miR-338, miR-339 and miR-378 (Landgraf et al. 2007). Notably, miR-219 shows strong expression in OLs, consistent with its tight association with glial cells in the zebrafish brain (Kapsimali et al. 2007).
The fold changes from miRNA microarrays and real-time PCR are essentially similar \((r=0.99, \text{Pearson correlation})\) (data not shown). The miRNA microarray data was further validated by verifying the co-expression of intronic miRNAs and their host genes. Among the 98 validated miRNAs, 38 intronic miRNAs derive from 34 host genes. The Affymetrix microarray analysis of GalC\(^+\) cells confirmed the expression of 30 host genes (with a Normalized Expression Value (NEV) \(>0.9\)), while 4 others (MCM7, SLIT2, SMC4L1 and an uncharacterized RIKEN sequence) were not (NEV\(<0.9\)) (Table 2). Only RIMB1 was not conclusive due to the absence of RIMB1 probes. The Affymetrix microarray analysis of A2B5\(^+\) cells shows that the 4 genes not detected in GalC\(^+\) cells are indeed expressed at detectable levels in OPCs (data not shown). Overall, the comparison of expression levels of intronic miRNAs and their mRNA counterparts shows tight co-expression of 33 host genes with their intronic miRNAs during oligodendrocyte differentiation.

**Target Bias Analysis of MiRNAs in Oligodendrocytes**

To delineate miRNAs with important biological functions in oligodendrocytes, we conducted a target bias analysis (Tsang et al. 2007). In principle, if a miRNA is co-expressed with a significant number of its predicted targets, this positive correlation signature (positive target bias) would enrich for functional targets. Similarly, if one finds that a miRNA is negatively correlated with the expression of its predicted targets, this negative correlation signature (negative target bias) would also lead to enrichment of functional targets.

To explore target bias in GalC\(^+\) cells, rat Affymetrix microarrays were used to establish a rank order list of mRNAs based on their Normalized Expression Values (NEV) (GEO, accession number GSE11218). This list was further examined for the
distribution of predicted targets for each of the 98 validated miRNAs. Predicted targets were compiled from TargetScan 4.0 algorithm (Grimson et al. 2007). The Fisher's Exact Test was employed to determine whether the top tenth, middle tenth, or bottom tenth percentile windows of the list of mRNAs contain more predicted targets than expected by chance. Interestingly, 30 of 98 miRNAs show target bias in either in the top tenth percentile or bottom tenth percentile (Fig. 3-2A). In total, 28 of 30 correlation signatures are negative (Fig. 3-2B). In contrast, only two miRNAs (miR-9 and miR-124a) predicted targets give a positive correlation signature. This cellular prevalence of negative correlation signatures is in line with other genome-wide studies showing that predicted targets are expressed at lower levels in tissues where the miRNA is present compared to other tissues where the miRNA is absent (Grimson et al. 2007). However, closer examination of the 30 miRNAs revealed six miRNAs among which miR-34c, miR-137, miR-146, miR-186, miR-218 and miR-449 were previously reported with positive correlation signatures in neuronal cells (Tsang et al. 2007).

To determine whether these fluctuations in correlation signatures were dependent not only on cell types (neuronal versus glial cells) but also on stages of differentiation, the set of predicted targets of these 30 miRNAs were compared to a rank order list of mRNAs expressed by A2B5\(^+\) cells (GEO, accession number GSE11218). This analysis shows an inversion of the correlation signatures for 13 of 30 miRNAs. There are two classes of inversions: the first is defined by a switch from positive to negative correlations for miR-34c, miR-146, miR-218 and miR-449 (Fig. 3-2C) and the second is defined by a reversal from negative to positive correlations for miR-9 and miR-124a (Fig. 3-2C). miRNAs in the first class are in line with studies showing before
differentiation, expression levels of predicted targets were generally higher, and some miRNAs dampen the output of the transcriptionally down-regulated mRNAs to facilitate a faster transition in gene expression (Stark et al. 2005). miRNAs in the second class may serve as buffers to silence the genetic noise of unwanted transcripts arising from “leaky” transcription (Hornstein and Shomron 2006) and such a role has been attributed to miR-9 (Li et al. 2006).

**PMP22 mRNA is not Translated into Protein in Oligodendrocytes**

To determine a functional target of miR-9, we examined the predicted targets found in the bottom tenth percentile of OPCs and in the top tenth percentile of OLs. A subsequent Gene Ontology (GO) query revealed that demyelination of sciatic nerves was ranked as a top category (P<2.0 E-5) and PMP22 was found in this category. PMP22 mRNA is transcribed in oligodendrocytes (Fig. 3-4A). Real-time PCR quantification revealed an increase of ~2 fold during the transition from A2B5+ to GalC+ cells (Fig. 3-43B). To investigate if oligodendrocytes translate the PMP22 RNA into protein, frozen sections were processed for double immunolabeling. Postnatal oligodendrocytes in the corpus callosum of rat brains were labeled with the CC1 antibody, however there was no co-staining with the PMP22 antibody (Fig. 3-4C). As positive control, the PMP22 antibody clearly stains neuroepithelial junctions in adjacent sections (Fig. 3-4D) (Roux et al. 2004). Altogether, the in vivo experiments show that PMP22 is transcribed in oligodendrocytes, however no protein is synthesized. A similar situation also exists in vitro, since PMP22 mRNA and other myelin gene messages are found in the cultured primary OPCs and O4+ cells (Fig. 3-3A), suggesting the involvement of post-transcriptional mechanisms in the control of PMP22 expression.
MiR-9 Down-Regulates PMP22 Protein Expression

A direct interaction between miR-9 and the 3'UTR of PMP22 was detected using \textit{in vitro} binding assays (Fig. 3-5). To further demonstrate a functional interaction, miR-9 was co-transfected with luciferase expression vectors containing the 3'UTR of PMP22 (r3'UTR) (Fig. 3-6A). A ~50\% repression of luciferase activity is observed with the full-length r3'UTR in the presence of miR-9 (p<0.01, Student’s \textit{t}-test, compared to co-transfection with scrambled miRNA) (Fig. 3-6B). To delineate the positions of the binding sites of miR-9 in the 3'UTR of PMP22, we employed three luciferase constructs that contain fragments of the 3'UTR (Fig. 3-6A). Only fragment 1 (positions 1-157 relative to stop codon) and fragment 2 (positions 158-498) support the down-regulation by miR-9 (p<0.01 for fragments 1 and 2, Student’s \textit{t}-test), while fragment 3 (positions 499-1127) does not. This data supports the existence of miR-9 binding sites between positions 1-498 of the 3'UTR of PMP22.

We also analyzed the down-regulation of PMP22 after transfection of miR-9 in cultured Schwann cells. Northern blot shows the absence of miR-9 in Schwann cells (Fig. 3-8A), as compared with the specific enrichment of miR-9 in the rat brain (Fig. 3-8B). Northern blot analysis of PMP22 expression reveals a reduction in PMP22 mRNA levels after transfection of miR-9 (Fig. 3-7A). Quantification from three independent experiments, after normalization with 18S ribosomal RNA, shows a ~30\% reduction in steady-state PMP22 mRNA (p<0.05, Student’s \textit{t}-test, compared to co-transfection with the empty vector). The reduction of PMP22 mRNA is accompanied by a comparable reduction at the protein level as determined by Western blot (p<0.05, Student’s \textit{t}-test) (Fig. 3-7B). The down-regulation of PMP22 was also demonstrated by immunofluorescence. Schwann cells transfected with the miR-Neg plasmid show
stronger PMP22-like pattern of immunoreactivity, as compared to cells transfected with a miR-9 expression plasmid (Fig. 3-7C). Altogether, these results show that miR-9 down-regulates PMP22 in vitro by binding to its 3’UTR.

Discussion

Profiling MiRNAs in Oligodendrocytes

Although the spatio-temporal miRNA expression pattern is proposed to be dynamically regulated during brain development (Kim et al. 2004), the miRNA expression profiles of specific neural populations (neurons, oligodendrocytes and astrocytes) have not been fully addressed. Mammalian neurons are by far the best-characterized in vitro model (Kye et al. 2007; Lee et al. 2004). We report in this study the presence of 98 miRNAs in oligodendrocytes. The expression levels of 43 miRNAs are dynamically regulated during differentiation, consistent with the actual spatio-temporal model of miRNA expression profiles in the brain. A comparison with miRNAs expressed by cortical neuronal cells shows a small overlap with 58 of 98 miRNAs (data not shown), and three miRNAs (miR-23, miR-26 and miR-29) enriched in astrocytes are also expressed by oligodendrocytes (Smirnova et al. 2005). This analysis thus suggests that neural cells may have in common a large number of miRNAs.

Modulation of the expression level of individual miRNAs may be crucial for their proper functions in the appropriate cellular context. For example, neurons and oligodendrocytes share several miRNAs such as miR-34c, miR-137, miR-146, miR-186, miR-218 and miR-449. Interestingly, these six miRNAs show negative target bias in the oligodendrocyte lineage but possess significant positive correlation signatures in neuronal cells. The contrast between neuronal and glial bias suggests that miRNAs have diverse roles that are cell-dependent. We speculate that the primary function of
these six miRNAs might be to buffer noise in gene expression, or regulate local translation in neurons. In comparison, the prevalence of negative correlation signatures in oligodendrocytes supports a modulatory role in the reinforcement of pre-existing transcriptional silencing mechanisms.

MiRNAs and the Control of Myelin Gene Expression

Aside from regulating gene expression in normal physiological conditions, miRNAs have been implicated in pathological conditions such as Alzheimer’s disease, schizophrenia and glioblastoma (Chan et al. 2005; Chen et al. 2008; Perkins et al. 2007). Of note, miR-21 was strongly expressed in glioblastoma cell lines and knock-down of miR-21 led to increased apoptosis. The anti-apoptotic effect of miR-21 was counteracted by miR-335 in a model of neural survival after ethanol exposure (Sathyan et al. 2007). During oligodendrocyte differentiation, miR-335 was down-regulated while miR-21 was strongly up-regulated, in line with the antagonistic action of miR-335 to miR-21. miR-9 is another miRNA whose expression has been well-characterized in human brain and oligodendroglioma (Nelson et al. 2006). The expression of miR-9 is very high in neuroblasts and glioblasts of fetal brain. The maturation of neuroblasts is associated with a decrease in the expression level of miR-9, and its down-regulation during the course of oligodendrocyte development is consistent with a function of miR-9 in proliferating neural cells of the brain.

What are the functions of miR-9 during oligodendrocyte maturation? In human oligodendroglioma, miR-9 expression is increased compared to normal adult brain, suggesting a potential role in neoplasia. miR-9 is a brain-enriched miRNA and is conserved during evolution, supporting important functions in neural cells. In human and rodents, there are three copies of mir-9 and only two are functional (Kim et al. 2004).
This study shows that PMP22 is a target of miR-9. PMP22 mRNA is detected in oligodendrocytes but not the protein. Of note, three studies also support the transcription of PMP22 in oligodendrocytes (Emery et al. 2009; Lau et al. 2008; Sohn et al. 2006). Interestingly, the CNP-EGFP+ mouse cells showed variable levels of expression of PMP22 mRNA between P2 and P30 (Sohn et al. 2006). Similarly, we observed an ~2-fold increase of PMP22 mRNA during the transition from A2B5+ to GalC+ cells, supporting a dynamic regulation of PMP22 during glial differentiation. Moreover, we also found by RT-PCR the presence of PMP22 mRNAs in premyelinating O4+ cells and rat Affymetrix microarray analysis confirmed an ~2-fold increase during transition from OPCs to O4+ cells (Fig. 3-3). The presence of PMP22 mRNAs in oligodendrocytes is also supported by in situ hybridization studies showing PMP22 transcripts in the CNS (Parmantier et al. 1995). The absence of PMP22 protein in oligodendrocytes is consistent with previous proteomic studies that extensively characterized proteins of CNS myelin and did not identify PMP22 (Taylor et al. 2004). More globally, the restricted expression of PMP22 protein in comparison to the broad distribution of its message further supports a post-transcriptional repression (Amici et al. 2006). Our results now point to a role for miRNAs in PMP22 regulation.

Oligodendrocytes and Schwann cells synthesize myelin in the CNS and the peripheral nervous system (PNS), respectively. Although the protein composition of their myelin sheaths is widely divergent, both cell types exert tight control over the relative abundance of the specific myelin proteins. Myelin gene dosage is primordial, as an increase of PLP in the CNS causes Pelizaeus-Merzbacher disease (PMD) (Cohen et al. 2003). Similarly, the PMP22 gene is sensitive to copy number since duplication is
found in the autosomal dominant Charcot-Marie Tooth type I disease and deletion is linked to autosomal dominant hereditary neuropathy with liability to pressure palsies (Patel et al. 1992), (Chance et al. 1993). It is thus tempting to speculate that miRNAs reinforce the control of genes sensitive to gene dosage. To support this hypothesis, the recent description of characteristics shared by dose sensitive genes includes the presence of long 3'UTRs, the target region of miRNAs (Vavouri et al. 2009). Adding an additional mechanism of regulation, might protect against protein aggregation or other consequences of increased levels of steady-state protein.

Overall, this work provides an important step toward the functional identification of miRNAs and how they interact with their targets to control oligodendrocyte identity. The significance of this work is illustrated here by attributing a role for miRNAs in the post-transcriptional regulation of PMP22. Future functional studies aimed at understanding how individual miRNAs contribute to the differences in myelin protein composition will underscore the critical importance of these small non-coding RNAs as guardians of the glial transcriptome.

Note

The work presented in this chapter was published in The Journal of Neuroscience November 5, 2008, 28(45):11720-11730. Pierre Lau, Jonathan D. Verrier, Joseph A. Nielsen and Kory R. Johnson planned and performed the experiments. Lucia Notterpek and Lynn D. Hudson aided in planning the experiments and edited the manuscript.
Figure 3-1. miRNA expression in oligodendrocytes A) FACS isolation of oligodendrocytes. Live brain cells are obtained by excluding dead cells (DAPI positive) and cell debris (low Forward Scatter Characteristics (FSC, size)). The R1 gate is used to purify oligodendrocytes. OPCs are labeled with the A2B5 antibody (Gate R2) and OLs are detected using a GalC antibody (Gate R3). B) miRNA expression in oligodendrocytes. Up-regulated miRNAs (red) are those with increased expression levels during oligodendrocyte maturation while down-regulated miRNAs (green) are clustered on the opposite side.
Figure 3-2. Target bias reversal of a subset of miRNAs during oligodendrocyte differentiation

A) Target bias analysis in OLs. Thirty of 98 miRNAs are associated with a target bias. Notably, a negative target bias is predominant (28 miRNAs in the green line above 1.3, corresponding to p<0.05, right-tailed Fisher’s Exact Test) while only 2 miRNAs are found with positive target bias (red line above the 1.3 value). As negative control, the middle window shows no target bias (blue line below 1.3 for all miRNAs). B) Targeting bias in OLs. The heatmap shows the significance values calculated for 30 miRNAs with target bias within each window (Top, Middle, Bottom). C) Targeting bias in OPCs. The 30 miRNAs with a target bias in OLs are re-analyzed using the top, middle and bottom windows obtained from the OPC rank list. This analysis reveals that 13 of 30 miRNAs are associated with target bias in OPCs (shown by arrows).
Figure 3-3. PMP22 mRNA expression in O4+ Oligodendrocytes  

A) Expression of PMP22 mRNA in OPCs and O4+ oligodendrocytes. OPCs and O4+ expressing oligodendrocytes were obtained from FACS from postnatal rat brains and RT-PCR was used to assess the presence of PMP22. Beta-actin is shown for control.

B) PMP22 mRNA level is dynamically regulated during differentiation. Microarray and RT-PCR experiments reveal MBP and FYN were highly expressed myelin genes during the O4+ stage and were used to assess the RNA quality. MPZ was also detected by microarrays and PCR. Fold differences represented the fold increase when the cells progressed from A2B5+ to O4+ stage. Error bars represent the s.e.m. n=3 for the RT-PCR and n=4 for the microarray experiments.
Figure 3-4. PMP22 is not translated in oligodendrocytes A) PMP22 mRNA in oligodendrocytes. RT-PCR shows the presence of PMP22 in OPCs and OLs. B) Quantification of PMP22 mRNA. The PMP22 PCR product from OLs (OL PMP22) appears one PCR cycle before the one obtained from OPCs (OPC PMP22). C) The CC1+ oligodendrocytes in the corpus callosum (Cc) are not immunoreactive for PMP22. Sagittal sections of postnatal rat brains are processed with a rabbit anti-PMP22 antibody (c) and the CC1 mouse antibody is used to label oligodendrocytes (b). Nuclei are visualized with DAPI (a). d, Merge picture. Scale bar, 100 µm. D) PMP22 protein is present in neuroepithelial cells. The CC1+ oligodendrocytes (f) in the cingulate cortex (Cg2 area) do not express PMP22 (g). A clear signal for PMP22 is obtained in neuroepithelial cells surrounding the ventricle. Nuclei are visualized with DAPI (e). h, Merge picture. Scale bar, 100 µm.
Figure 3-5. miR-9 interacts with the 3'UTR of PMP22 in Vitro The 3'UTR of PMP22 was transcribed in vitro and incubated with labeled miR-9. The RNA-miRNA complexes were separated from free miRNA (miRNA only) by native gel electrophoresis. A specific retardation was observed with miR-9 and not with miR-124a. The negative control corresponded to the incubation of the 3'UTR of PMP22 in the absence of any miRNA. As control, SYBR Green I gel staining before gel transfer showed the presence of 3'UTR in the three lanes (lower panel).
Figure 3-6. miR-9 binding sites within the 3'UTR of PMP22

A) Luciferase constructs containing regions of PMP22 3'UTR. Non-overlapping regions of the 3'UTR of PMP22 are used to delineate the location of miR-9 binding sites. The r3'UTR construct contains the full length 3'UTR. The remaining constructs fragment 1 (nts 1-157), fragment 2 (nts 158-498) and fragment 3 (nts 499-1127) are partitions of the full length 3'UTR. B) Determination of miR-9 effects on 3'UTR constructs. The plasmids are co-transfected with either miR-9 or Neg, a scramble miRNA. miR-9 has no effect on luciferase activity of either the empty vector or plasmid containing fragment 3 (Frag. 3). However, miR-9 does significantly reduce luciferase activity of constructs containing either the full length of PMP22 (r3'UTR), fragment 1 (Frag. 1) or fragment 2 (Frag. 2) (**p<0.01, Student's t-test, as compared to Neg).
Figure 3-7. miR-9 down-regulates PMP22 levels in Schwann cells

A) Schwann cells transiently transfected with miR-9 contain reduced steady-state levels of PMP22 mRNA, (*p<0.05, Student’s t-test, versus Neg, transfection with an empty plasmid, n=3). 18S RNA was used for normalization. Error bars represents the s.d.

B) Western blot analysis after miR-9 transfection. A down-regulation of PMP22 protein is observed after transfection of miR-9 (*p<0.05, Student’s t-test, versus Neg, n=3). N-glycosidase (PNGase) treated cell lysate is shown as a control for PMP22. PMP22 specific bands are shown by arrows. NS: Non-Specific band. Error bars represents the s.d.

C) Immunofluorescence analysis after miR-9 transfection. Transfected Schwann cells are detected by EmGFP (green, arrows). Nuclei are visualized by Hoechst staining. Scale bar, 10 µm.
Figure 3-8. miR-9 is a brain-enriched miRNA 

A) Absence of miR-9 expression in Schwann cells. Rat Schwann cells were grown in three different conditions: as growing cells, arrested by confluency or in a defined media to promote PMP22 expression. Northern blot for miR-9 showed that across all conditions, no detectable expression level of mature miR-9 can be seen. As control, human brain sample was run and revealed the presence of mature miR-9. As positive control, membranes were stripped and probed for U6 small nuclear RNA.

B) Tissue distribution of miR-9. A panel of nine tissues was obtained from P7 rats and analyzed for miR-9 expression. Membranes were stripped and probed for U6 small nuclear RNA for control.
Table 3-1. Top miRNAs expressed by oligodendrocyte lineage cells.

<table>
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<th>miRNA</th>
<th>Mean signal</th>
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<td>rno-miR-181b</td>
<td>21463</td>
<td>rno-miR-107</td>
<td>17520</td>
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Boldface denotes top miRNAs found in both cell populations. Mean signals were derived from three independent microarray experiments.
CHAPTER 4
PERIPHERAL MYELIN PROTEIN IS REGULATED POST-TRANSCRIPTIONALLY BY MIR-29A IN SCHWANN CELLS

Introduction

PMP22 is a 22 kDa tetraspan glycoprotein that is predominantly expressed by the myelinating Schwann cells of the peripheral nervous system (Snipes et al. 1992). PMP22 was first identified as growth arrest-specific gene 3 (gas-3) in NIH 3T3 fibroblasts (Schneider et al. 1988) and its expression increases as cells reach density-dependent inhibition (confluency) (Manfioletti et al. 1990; Zoidl et al. 1995). The significance of the growth arrest-specific expression is still undetermined. Although PMP22 protein expression is highly restricted, PMP22 mRNA is present ubiquitously throughout the body, including the central nervous system, kidney, heart, muscle and lung (Amici et al. 2006; Baechner et al. 1995; Ohsawa et al. 2006; Parmantier et al. 1995; Suter et al. 1994). PMP22 protein is detected in Schwann cells, at epithelial and endothelial cell junctions, and in specific motor and sensory neurons (Baechner et al. 1995; Maier et al. 2003; Notterpek et al. 2001; Roux et al. 2004). In developing rat sciatic nerve, PMP22 message steadily increases and reaches maximal expression at approximately post-natal day 21 which correlates with the completion of myelination and Schwann cell differentiation state (Garbay et al. 2000). In mature sciatic nerve it represents only approximately 2-5% of the PNS myelin proteins. In addition, PMP22 levels drop significantly post-nerve crush injury (Snipes et al. 1992) which correlates with the de-differentiation state of the Schwann cells. These findings suggest the involvement of post-transcriptional mechanisms in controlling PMP22 expression which may be dependant upon both the cell type and differentiation state.
PMP22 Associated Diseases

Although PMP22 represents only a relatively minor constituent of the PNS myelin, point mutations, duplication, and deletion of the gene are associated with demyelinating neuropathies (Lupski and Garcia 1992). Charcot-Marie-Tooth disease type 1A (CMT1A) is the most common form of inherited peripheral neuropathy with a prevalence of 1 in 2500 live births (Lupski and Garcia 1992). CMT1A has been linked with a duplication of a 1.5 Mb region on chromosome 17p11.2 (Patel et al. 1992; Roa et al. 1991) which includes the PMP22 gene. Since the PMP22 gene does not suffer a loss of function with the duplication, the phenotypes are likely the result of altered gene dosage (Adlkofer et al. 1995; Huxley et al. 1996). In hereditary neuropathy pressure palsies (HNPP), one copy of the PMP22 gene is deleted (Chance et al. 1993). The Schwann cells in neuropathic models and in patients show an impaired ability to myelinate (Nobbio et al. 2004). Therefore, PMP22 levels must be tightly controlled as it is estimated that a 50% reduction in expression will result in HNPP, while a 50% increase leads to CMT1A (Maier et al. 2002). Recent data suggest that the PMP22 transcript is misregulated in a number of neurological diseases, including schizophrenia and depression (Aston et al. 2005; Dracheva et al. 2006) and also in cancer (van Dartel and Hulsebos 2004). These findings may imply leaky transcription and a requirement to regulate undesired message at a post-transcriptional level.

PMP22 Expression and Gene Regulation

In studies examining the regulation of the PMP22 gene, the vast majority of the work has focused on the 5'-UTR regulatory elements. There are two characterized transcripts of the PMP22 gene that differ only in the inclusion of UTRs, primarily untranslated exon 1. Two promoters initially characterized, P1 and P2, appear to have
tissue specific functions (Maier et al. 2003; Suter et al. 1994). Transcription via promoter P1 results in the inclusion of exon 1A, is preferentially used during myelination, and is mostly Schwann cell specific (Saberan-Djoneidi et al. 2000). P1 is under the control of a CREB-dependant silencing element and a cAMP silencing element which, in the absence of cAMP, prevents PMP22 expression. This promoter possesses many common features of mammalian promoters, including TATA and CAAT boxes (Wegner 2000a; Wegner 2000b). Promoter P2, which contains exon 1B, appears to lack the common promoter elements found in P1 and is used ubiquitously throughout the body. It is important to note that all of the transcripts encode the same protein and differ only in the inclusion of UTRs. Investigations for other regulatory regions in the PMP22 gene have revealed that both the 5’- and 3’-UTRs play critical roles in the expression and stability of the RNA transcripts (Bosse et al. 1999). The 3’UTR of PMP22 exerts a negative effect on RNA translation which is observed even after the three AU-rich elements have been removed (Bosse et al. 1999). In addition to the role promoter regions and UTRs have in PMP22 regulation, applicable transcription factors have also been examined. Several transcription factors that have been implicated in myelination are expressed in Schwann cells and have predicted binding regions in the PMP22 5’ promoter region (Maier et al. 2003). For example, Krox20 (Egr-2) and Oct6 (SCIP) are required for Schwann cells to initiate myelination, although their precise roles are different. Oct6 appears to be primarily involved in the timing of myelination (Jaegle et al. 1996). While in Krox20 knockouts, myelin fails to form altogether (Topilko et al. 1994). Although Krox20 does directly target MPZ, MBP, and MAG in Schwann cells, PMP22 is not a direct target of this essential myelin transcription factor (Jang et al. 2006). When
Schwann cells establish their one-to-one relationship with an axon and begin the myelination process, the mRNAs for the myelin proteins, including PMP22, are upregulated. Once the process of wrapping has been completed, the mRNAs for the myelin proteins are reduced to approximately 20% of their peak values through undefined mechanisms (Garbay et al. 2000; Scherer and Chance 1995). These results suggest that there must be elaborate post-transcriptional regulation of myelin genes, including PMP22.

MicroRNAs and Gene Regulation

It has been established that there are several post-transcriptional processes that control myelination (Zearfoss et al. 2008). RNA binding proteins such as Quaking have been shown to control both Schwann cell and oligodendrocyte differentiation (Chen et al. 2007; Larocque et al. 2009). In addition, the transport of MBP mRNA and local synthesis is essential for myelin formation (Barbarese et al. 1999). However another recently elucidated mechanism of post-transcriptional gene control that involves the 3’UTR is repression via microRNAs (miRNAs). MiRNAs are small, non-coding regulatory RNA molecules that bind to the 3’UTR of target genes based upon reverse complementarity and prevent their translation (Grimson et al. 2007; He et al. 2005; Valencia-Sanchez et al. 2006). MiRNAs are transcribed via RNA polymerase II, cleaved by Drosha, actively exported into the cytoplasm by Exportin 5, and then processed by the endoribonuclease Dicer to form the mature miRNA. The binding of the miRNA to the target site on mRNA can either signal for the degradation via the RNA induced silencing complex (RISC), which contains the Argonaute proteins, or repress translation without degradation through other less defined mechanisms (Bagga et al. 2005; Pillai et al. 2005). The RISC has been localized to structures termed processing
bodies (P-bodies) or GW bodies (GWB). These cytoplasmic foci contain the RNA-binding protein GW182 and serve as the sites where miRNAs are believed to exert the majority of their function (Ding and Han 2007; Liu et al. 2005).

MiRNAs have been revealed to be involved in numerous cellular processes including cell differentiation, cell cycle, and cell death. Mutations creating or deleting miRNA target sites can result in abnormal phenotypes in vivo (Clop et al. 2006). Although no direct relation has established the roles of miRNAs in the process of myelination, it has been proposed that miRNA are involved in the translational repression of myelin mRNAs during transport until local synthesis can occur (Kim et al. 2004). Also recently it was reported that autoimmunity to the GW-bodies is associated with motor and sensory neuropathy in humans (Bhanji et al. 2007) although the histopathology remains undefined. Ongoing research is revealing that miRNA are likely to be involved in most cellular processes and they are likely to exert an influence on myelin gene expression in both the CNS and PNS.

The disparities between the localization of PMP22 mRNA and detectable PMP22 protein suggest that there is post-transcriptional regulation of the gene. It has been hypothesized that PMP22 mRNA may be regulated post-transcriptionally by a non-transcribed RNA molecule (Manfioletti et al. 1990). The mechanism of how the 3'-UTR of PMP22 negatively regulates expression of the message has yet to be determined (Bosse et al. 1999). Recently, the 3'UTR region of the PMP22 gene in Medaka fish was demonstrated to possess regulatory domains again implicating this region in modulating gene expression (Itou et al. 2009). In these studies we examine the miRNA expression profile (miRNAome) of oligodendrocytes and Schwann cells in response to different
growth conditions or differentiation state. In addition, we show evidence that PMP22 is regulated in both the CNS and PNS by microRNAs, albeit by different a miRNA dependent upon the cell type. In addition, we show that the expression of mature microRNAs is essential for proper Schwann cell myelination and differentiation. Taken together these studies demonstrate additional levels of myelin gene regulation previously uncharacterized. The elucidation of the mechanism of post-transcriptional regulation of PMP22 provides novel insight into the etiology of myelin-associated diseases and may identify new therapeutic targets in controlling myelin gene regulation.

Results

PMP22 Levels Inversely Correlate with GW-Body Formation and Dicer Expression

To investigate whether PMP22 is regulated by miRNAs, we first determined the formation of GW-bodies (GWBs) during different growth conditions in rat Schwann cells, where PMP22 is most expressed (Pareek et al. 1997). GWBs are cytoplasmic foci in the cell where miRNA repression is believed to occur (Rehwinkel et al. 2005) and their formation has been shown to correlate with cell cycle progression (Yang et al. 2004). Schwann cells were subjected to growth-arrest by serum starvation, and then stimulated to proliferate by the addition of 10% serum (Zoidl et al. 1995). At the indicated time points, the cells were fixed and processed for immunostaining using an anti-GWB antibody (Fig. 4-1A). Increases in both the size and abundance of GWBs occur in a time dependent manner upon release of the cells from growth-arrest. Western blot analysis on whole cell lysates demonstrates greater GW182 protein expression in actively proliferating, when compared to non-proliferating, differentiated Schwann cells (Fig. 4-1B). In addition, we investigated another miRNA associated protein, Dicer, which is required for mature miRNA biogenesis for differential expression (Valencia-
Sanchez et al. 2006). Similar to GW182, we observe the highest expression of Dicer when the cells are actively growing. The reduction in the steady-state levels of phospho-histone H3, a mitotic marker, in samples from the defined medium confirms that there are fewer cells in division (Fig. 4-1B). In comparison to the miRNA pathway associated proteins, the expression of PMP22 is low in proliferating and high in differentiating Schwann cells (Fig. 4-1C). To demonstrate that the detected heterogeneous bands at around ~22 kDa are differentially glycosylated forms of PMP22, we performed N-glycosidase reactions (Pareek et al. 1997). Upon incubation of the cell lysates with PNGase F, which completely removes the carbohydrate moiety of PMP22, all the detected bands, except the top band, shift to the core 18 kDa protein (Pareek et al. 1993). Quantification of the PMP22 protein bands in three independent experiments reveals that PMP22 expression is significantly (**p<0.01) elevated in non-proliferating, differentiated cells when compared to proliferating Schwann cells (Fig. 4-1D). This finding is consistent with previous results (Zoidl et al. 1995). In comparison, Dicer expression is high in proliferating, as compared to differentiating Schwann cells (Fig. 4-1E). Taken together, these data indicate that the expression of PMP22 inversely correlates with both Dicer and GW182, two essential proteins for miRNA biogenesis.

To further establish a functional relationship between PMP22 levels and Dicer, we inhibited Dicer expression in Schwann cells using siRNA (Fig. 4-2A). We used a negative (Neg.) scrambled siRNA to control for any non-specific effects of transfection. In the Dicer inhibited cells, we observe an increase in PMP22 protein levels at 72 h post-transfection (Fig. 4-2A), as compared to the Neg. siRNA transfected cells. Densitometric analysis of three independent experiments indicates an ~60 % increase
in PMP22 protein upon the suppression of Dicer (*p<0.05, Fig. 4-2B). The biochemical results were reinforced by immunostaining Schwann cells transfected with either the Neg. or Dicer siRNA (Fig. 4-2C). Cells with Dicer expression inhibited show less Dicer-like immunoreactivity (green), but demonstrate an increase in PMP22-like staining (red) (Notterpek et al. 1999a). Therefore, the inhibition of Dicer enhances PMP22 levels and indicates that mature miRNAs are regulating PMP22 expression in Schwann cells. MicroRNAs are predicted to target PMP22 and regulate reporter expression.

Bioinformatic scans are the “in silico” standard for assembling a list of candidate miRNAs predicted to target the 3’UTR of a given RNA. We used three programs (Targetscan, miRbase and Pictar) to generate a list of ten miRNAs with high probability to bind to the 3’UTR of PMP22. A schematic of where these miRNAs are predicted to bind is shown (Fig. 4-3A). Since RNA is known to have significant secondary structure and this can affect miRNA binding (Kertesz et al. 2007), we evaluated the binding ability of the predicted miRNAs to the 3’UTR of PMP22 using a gel shift assay (Fig. 4-3B). This assay is a qualitative measure of binding ability where biotin-labeled miRNAs were incubated with PMP22 RNA, separated on an agarose gel, and transferred to a membrane. The presence of intact RNA and relative loading in each lane is confirmed using SYBR gold staining of the gel prior to transfer. The miRNA/RNA complexes were resolved using a HRP-conjugated streptavidin and the membranes were exposed to film. MiR-29a-c, -381, and -9 all demonstrate strong binding ability, with miRs-199a, -140*, and -322/424 showing weaker binding. MiR-450 does not possess any detectable binding (Fig. 4-3B). MiR-124a is not predicted to target the 3’UTR of PMP22 and is
used as a negative control. RNA alone lane only contains the PMP22 RNA with no labeled miRNA probe.

To investigate which miRNAs may be regulating PMP22 expression in Schwann cells, we established a PMP22 3’UTR-luciferase construct with the 3’UTR of PMP22 inserted downstream and in frame with the Renilla Luciferase (RL) gene. This construct allows us to quickly and quantitatively evaluate miRNA effects on the 3’UTR of PMP22. We utilized the psicheck2 dual luciferase vector that contains a separate Firefly Luciferase (FL) gene to normalize for transfection efficiency. The 3’UTR-luciferase construct was co-transfected in Schwann cells with 10 nM miRNAs and the cells were harvested at 48 h post-transfection. MiR-29a, miR-29b, miR-29c, miR-9, and miR-381 all significantly (*p<0.05) reduce luciferase activity when compared to the Neg. scrambled miRNA, while miR-322/424 and miR-140* do not (Fig. 4-3C). As miR-381 and miR-29a are expressed endogenously in Schwann cells (see below in Fig. 4A), we examined if co-transfection of these two miRs may have an additive effect on reporter expression. As shown in Figure 4-3C, the co-transfection of miR-381 with miR-29a does not significantly enhance the inhibitory activity of miR-29a. MiR-124a serves as a non-PMP22 targeting control to ensure that activation of the miRNA pathway alone is not affecting our PMP22 3’UTR-luciferase reporter expression. These data demonstrate that specific PMP22 targeting miRNAs reduce reporter expression in Schwann cells.

**MicroRNAs are Differentially Expressed in Schwann Cells Upon Growth Condition**

To substantiate the potential functional significance of PMP22 targeting miRNAs, we next determined the miRNA expression profile (microRNAome) of actively proliferating compared to non-proliferating Schwann cells. Several miRNAs demonstrate differential expression based upon growth condition (Fig. 4-4A). In
addition, miRNAs predicted to target PMP22 are expressed by Schwann cells, including miR-29a, miR-381 and miR-140* (Fig. 4-3A). The relative expression of these particular miRNAs is consistent among the independent triplicate samples (Fig. 4-4B). MiR-9 was not detected in this microarray, nor was it found to be expressed in Schwann cells by RT-PCR or Northern blot indicating that this miRNA likely to regulate PMP22 expression in other cell types. We validated the microarray data for miR-29a, miR-381 and miR140* using RT-PCR (Fig. 4-4C). MiR-124a is included as a negative control since it was not detected by the microarray, or by RT-PCR. In agreement with the microarray data, the RT-PCR demonstrates that miR-29a is significantly (*p<0.05) down-regulated when the cells are promoted to differentiate. We observed a similar repression of miR-29a when Schwann cells are cultured in a reduced serum medium (data not shown). Based on the observed inverse correlation of miR-29a and PMP22, we decided to further characterize this specific miRNA in Schwann cells.

**MicroRNA-29a Specifically Regulates PMP22 Reporter Expression**

To demonstrate that the endogenous Schwann cell miR-29a is regulating the expression of PMP22, we employed miRNA inhibitors (anti-miRs). As shown before (Fig. 4-3C), luciferase assays were performed with the 3'UTR-luciferase construct where miR-29a reduces reporter expression (Fig. 4-5A). More importantly, the anti-miR-29a relieves the repression by the endogenous miR-29a, as compared to the Neg. control. In comparison, inhibition of other predicted PMP22 targeting miRNAs, including miR-381, miR-322/424, and miR-140*, does not relieve the repression of the reporter (Fig. 4-5B). These results indicate that although both miR-29a and miR-381 can reduce luciferase signal when their levels are elevated via transfection, only endogenous miR-29a is actively repressing PMP22 expression.
To locate binding sites for miR-29a in the 3'UTR of PMP22 and demonstrate the specificity of the interaction, two approaches were taken. First, constructs were established that contained truncations of the PMP22 3'UTR with or without the predicted binding site at 0.66 kb past the stop codon (Fig. 4-5C). Mir-29a and the Neg. miRNA were transfected with the base psicheck2 vector which did not contain the 3'UTR of PMP22. Under these conditions, miR-29a does not reduce reporter signal thus eliminating off-target effects. However, in agreement with previous studies (Bosse et al. 1999), the 3'UTR of PMP22 does instill a significant reduction (**p<0.01) on reporter activity compared to the base vector. When the PMP800 construct, which contains the predicted miR-29a site, is co-transfected with miR-29a, a further reduction in luciferase activity is seen (***p<0.001). In comparison, the PMP400 construct, in which the predicted miR-29a site is removed, demonstrates significantly greater (*p<0.05) luciferase activity than the full length 3'UTR construct (Fig. 4-5D). These data indicate that the endogenous Schwann cell miR-29a regulates PMP22 expression.

Since the 3'UTR of PMP22 contains other regulatory elements which may have been deleted in the truncations, we employed a second approach to demonstrate the specificity of miR-29a on the 3'UTR of PMP22, using site-directed mutagenesis. We deleted the 7 nt seed region in the predicted miR-29a binding site (Fig. 4-5E), which eliminated the reduction in luciferase activity (Fig. 4-5F). However, experiments with the non-mutated 3'UTR performed in parallel still retain the miR-29a associated repression of reporter signal (Fig. 4-5F). These results validate the specificity of the interaction between miR-29a and the 3'UTR of PMP22.
Endogenous Schwann Cell PMP22 is Regulated by MiR-29a

A recently developed biochemical approach to determine miRNA targets (Karginov et al. 2007) utilizes the knowledge that the Argonaute proteins of the RISC complex bind to target RNAs to exert repression (Meister et al. 2004). Thus if the Argonaute (Ago2 in these experiments) protein is immunoprecipitated 'primed' with exogenous miRNA, the precipitated protein should show an enhanced association with the PMP22 RNA. To determine if increasing miR-29a levels in Schwann cells has an influence on the association of PMP22 RNA with Ago2, we co-transfected a c-myc-Ago2 construct with miR-29a, or the scrambled Neg. miRNA. Western blot analysis with anti-c-myc antibody shows efficient and specific immunoprecipitation (IP) of the c-myc-Ago2, as compared to beads conjugated to non-specific rabbit IgG (Fig. 4-6A). The same experiments performed with non-transfected cells is shown on the right, and serves as a negative control. Next, RNA was isolated from all the IP fractions, including input, IP beads, and post-precipitation supernatant (Sup) and semi-quantitative RT-PCR was performed on equal amounts of RNA. In the input samples, co-transfection of miR-29a and c-myc-Ago2 significantly reduces (*p<0.05) the levels of steady-state PMP22 RNA when compared to the Neg. miRNA (Fig. 4-6B). In addition, cells with c-myc-Ago2 primed with miR-29a contain the majority of the PMP22 RNA associated with the Ago2 protein, pulled down in the c-myc IP fraction (***p<0.001, Fig. 4-6B). Non-specific rabbit IgG conjugated beads do not precipitate any detectable PMP22 RNA, which remained in the post-precipitation Sup fraction. These data indicate that even in the Neg. miRNA transfected cells, endogenous PMP22 RNA is associated with Ago2. Significantly, when the abundance of miR-29a is elevated, the amount of PMP22 in complex with Ago2 further increases. Therefore, the endogenous PMP22 RNA is regulated via the
RISC complex in Schwann cells and when the levels of miR-29a are increased, this interaction is enhanced.

To further show that miR-29a regulates endogenous PMP22 in Schwann cells, we transfected the cells with miR-29a, anti-miR-29a, and Neg. miR, followed by protein analysis 72 h later (Fig. 4-7A). Transfection of miR-29a reduces steady-state PMP22 protein levels in Schwann cells when compared to the Neg. miRNA control. However, when endogenous miR-29a is inhibited via transfection of anti-miR-29a, the steady-state levels of PMP22 protein are greater than in Neg. control miRNA cells (Fig. 4-7A). Quantification of four independent experiments reveals that miR-29a reduces PMP22 protein levels by approximately 45%, when compared to Neg. controls (*p<0.05).

Furthermore, inhibition of endogenous miR-29a results in a significant increase in steady-state PMP22 protein levels (**p<0.01, n=4) (Fig. 4-7B). Since miRNA-mediated gene regulation can ultimately reduce the steady-state levels of the target RNA (Wu and Belasco 2008), we used real-time RT-PCR to determine the effect of miR-29a on PMP22 RNA. In cells with elevated miR-29a, the levels of PMP22 message are reduced, as compared to control (Fig. 4-7C). In comparison, when endogenous miR-29a is inhibited, the steady-state levels of PMP22 RNA are significantly (*p<0.05) elevated. Taken together, these results demonstrate that miR-29a actively modulates PMP22 expression within Schwann cells, which can be detected both at the protein and RNA levels.

**PMP22 and MiR-29 Expression are Inversely Correlated In Vivo**

PMP22, both RNA and protein, expression increases as the nerve develops and as the Schwann cells differentiate and synthesis myelin (Garbay et al. 2000; Snipes et al. 1992). To investigate an in vivo correlation between PMP22 and miR-29a levels in
rat sciatic nerve, animals were sacrificed at post-natal day 2, 4, 8, 16, and 21 and their sciatic nerves were collected. In rats, the process of myelination occurs post-natally and is believed to be completed by P21 (Garbay et al. 2000), justifying the time-points for these experiments. To obtain adequate RNA for these experiments at least two animals were pooled per sample and three independent samples were analyzed for each time-point. Total RNA, containing small RNA molecules including miRNAs, was isolated from the nerves. Quantitative RT-PCR reveals that PMP22 RNA expression in sciatic nerve steadily increases with age reaching a maximal level at P16 (Fig. 4-8A). The same samples were analyzed for miR-29a expression using RT-PCR and it is shown that miR-29a levels are the highest at P2, but by P4 its expression has been significantly reduced and remains low at the later time points (**p<0.001, Fig. 4-8B).

To determine a correlational relationship between PMP22 and miR-29a levels, a linear regression analysis was performed (Fig. 4-8C). There is a significant correlation between the two expression levels with increased miR-29a levels being associated with decreased PMP22 expression (r²=0.78, p<0.05). These data reveal an inverse relationship between PMP22 and miR-29a levels in vivo supporting that Schwann cell differentiation state affects the miRNA profile expression during sciatic nerve development.

To further investigate a functional relationship between PMP22 and miR-29, RNA and protein from mice subjected to sciatic nerve crush injury were analyzed. It is establish that post-crush injury, Schwann cells de-differentiate and proliferate allowing the axon to heal (Jessen and Mirsky 2008). The myelin genes, including PMP22, are down regulated rapidly post-injury (Bosse et al. 2006; Snipes et al. 1992). MiRNA
microarray analysis of control and crush injured nerves revealed that miR-29b to be the
predominate form of miR-29 expressed in response to nerve injury. MiR-29 levels were
low in mature myelinated nerve and there was an approximately 2-fold increase in miR-
29b expression at 4 d post-injury, as detected by microarray (data not shown). MiR-29a
and miR-29b have identical binding regions and are located in the same miRNA cluster
in the mouse, rat, and human genome. MiR-29b has the same predicted binding site in
the 3’UTR of PMP22 as miR-29a and it is possible that mice preferentially use the miR-
29b form over the miR-29a that is used in rat. In addition as shown in Fig. 4-3C, both
miR-29a and miR-29b had similar effects on the PMP22 3’UTR-luciferase reporter
expression. We show here that PMP22 RNA (Fig. 4-9A) and protein (Fig 4-9B) are
reduced post-injury when compared to control, in agreement with the current literature
(Bosse et al. 2006; Snipes et al. 1992). To validate the microarray results, miR-29b
levels were determined using quantitative RT-PCR on RNA isolated from crush and
control nerves. In agreement with the microarray data, miR-29b levels are elevated in
nerve subjected to crush injury when compared to control (*p<0.05, Fig. 4-9C). These
data provide additional support to the hypothesis of a functional relationship between
miR-29 and PMP22 in sciatic nerve as well as implicate the miRNA pathway in
peripheral nerve repair.

Discussion

Myelin gene expression is regulated by transcriptional and post-transcriptional
events (Svaren and Meijer 2008; Wegner 2000b), and here we show that functional
RNA molecules within glia are involved in this process. Specifically, we found
differential miRNA expression profiles based upon Schwann cell phenotype, which also
correlates with an induction of Dicer and GWB formation. The expression of PMP22
inversely correlates with Dicer, and steady-state PMP22 levels can be increased by the inhibition of Dicer. In addition, we demonstrate that several miRNAs present in Schwann cells bind to the 3'UTR of PMP22 and are able to reduce the expression of a luciferase reporter. Although a number of miRNAs had negative effects on reporter expression, only the inhibition of endogenous miR-29a relieved the miRNA-mediated repression, supporting a functional relationship between miR-29a and PMP22. In agreement, miR-29a specifically interacts with the 3'UTR of PMP22 regulating the expression of the endogenous PMP22 protein and RNA and the expression of miR-29 and PMP22 are inversely correlated in vivo.

GWBS are the main sites of miRNA-mediated gene regulation in the cell (Ding and Han 2007; Liu et al. 2005) and the formation of these structures appears to be regulated with the cell cycle (Lian et al. 2006; Yang et al. 2004). We detected GWBS in Schwann cells, and in agreement with previous reports (Yang et al. 2004), their formation is increased when cellular division is stimulated (Fig. 4-1). These data indicate that although GWBS are present in non-proliferating Schwann cells, they may be more important during cellular division. It will be of interest to determine if Schwann cells require GWBS and/or miRNA regulation to retain their mitotic ability in the mature peripheral nerve, which is necessary for repair upon axonal injury (Clemence et al. 1989). An impairment of miRNA processing may reduce the ability of the cells to divide and remyelinate post-injury. Dicer, an essential miRNA biogenesis protein, is required for developmental processes and has been implicated in disease states, including retinal degeneration, abnormal neuronal spine length, heart failure, and skeletal muscle development (Chen et al. 2008; Damiani et al. 2008; Davis et al. 2008; O'Rourke et al.
The data presented in this report indicate that Dicer expression in Schwann cells is differentially regulated depending on the growth condition. These findings are in accord with the observations that miRNA regulation appears to be important in cell cycle control (Carleton et al. 2007; Vasudevan et al. 2008). In agreement with the growth regulatory activity of PMP22 (Zoidl et al. 1995), Dicer and PMP22 protein levels have an inverse relationship in Schwann cells, which can be utilized to modify PMP22 expression (Figs. 4-1 & 4-2). Our data suggest that loss of Dicer expression or function could be detrimental to Schwann cell biology by leading to alteration in PMP22 levels.

Using biochemical and molecular approaches, we have identified miR-29a as a regulator of PMP22 in Schwann cells. We employed current prediction programs to assemble a list of potential PMP22-targeting miRNAs (Fig. 4-3) and compared their relative expression to PMP22 levels using microarrays (Fig. 4-4). MiR-29a expression inversely correlates with the levels of PMP22, which may signal a functional relationship. Although three miRNAs are able to down regulate the 3'UTR-luciferase reporter (Fig. 4-3C), miR-29a is the only tested endogenous miRNA that when inhibited in rat Schwann cells, leads to increased luciferase activity (Fig. 4-5B). While miR-381 is also expressed in Schwann cells and can repress reporter expression when transfected (Figs. 4-3 and 4-4), inhibition of endogenous miR-381 does not relieve the repression of the reporter (Fig. 4-5B). Therefore, mere binding ability alone does not necessarily dictate functionality in vitro. The de-repression observed with miR-29a anti-miRNA and the maximal effect on the luciferase assays identifies endogenous miR-29a as a critical miRNA governing PMP22 expression in Schwann cells. In addition, we demonstrate that miR-29a represses the luciferase reporter by binding at one specific region on the
3'UTR of PMP22 (Fig. 4-5F). The deletion of the predicted binding site, which is conserved between rat, mouse, human, and chicken (http://www.targetscan.org/), abolishes the miR-29a dependent repression. However, since the miR-29a seed deletion construct did not have greater luciferase signal then the intact 3'UTR in presence of the Neg. miR (Fig. 4-5F), we cannot eliminate the contribution of other regulatory domains in the 3'UTR of PMP22. AU-rich elements have been implicated in miRNA-mediated repression (Jing et al. 2005) and the 3'UTR of PMP22 contains three such regions (Bosse et al. 1999), one in close proximity to the predicted miR-29a seed target site. In support of a functional role of miR-29a in regulating endogenous PMP22, we co-immunoprecipitated Argonaute 2 (Ago2), the catalytic component of the RISC, 'primed' with exogenous miR-29a, and PMP22 RNA (Fig. 4-6). This result is a powerful qualitative measure to confirm PMP22 as a target RNA for miR-29a. Future studies will examine if other myelin gene RNAs are associated with this complex in Schwann cells.

Schwann cells exist in at least two different phenotypes in the adult nervous system, myelinating and non-myelinating, and can transition between the two states upon injury (Garbay et al. 2000; Jessen and Mirsky 2008). Both of these processes, namely myelination and cellular division, require a rapid and extensive change in gene expression (Scherer and Chance 1995). Here we characterize the miRNAome of actively-dividing Schwann cells and compare it to non-proliferating cells cultured in defined media (Cheng and Mudge 1996). There is unique expression of miRNAs based upon growth condition (Fig. 4-4), suggesting there are different subsets of genes that are post-transcriptionally regulated depending on the cells phenotype. It is also possible that certain genes are preferentially regulated by miRNAs in one of these
growth conditions. Our data suggest that PMP22 is regulated by miRNAs primarily in proliferating cells where the expression of miR-29a is highest (Fig. 4-4). Other PMP22 binding miRNAs that did not change their relative expression levels might be implicated in conferring cellular identity and controlling leaky transcription (Mattick and Makunin 2005). Such mechanism would support the detection of PMP22 RNA throughout the body and the restricted distribution of the protein (Amici et al. 2006; Baechner et al. 1995). Therefore, PMP22 is likely regulated by miR-29, and additional miRNAs in non-neural tissues, as well. Our examination of miR-29 expression during rat sciatic nerve development (Fig. 4-8) supports our hypothesis that the miRNA expression profile of Schwann cells is dependent on the differentiation state. The inverse relationship between miR-29a and PMP22 levels not only supports a function role for miR-29 expression in vivo but also implicates the miRNA pathway in nerve development. It will be of interest to determine the target messages of the additional differentially regulated miRNAs in Schwann cells and search for their possible roles in neuropathic states or developmental abnormalities as well as determine the axonal verses Schwann cell contribution to miRNA expression during development.

MiRNAs have been implicated in several disease phenotypes, including Alzheimer’s disease, cancer and heart disease (Blenkiron and Miska 2007; Hebert et al. 2008; van Rooij and Olson 2007). Not only has misexpression of miRNAs been associated with diseases, but novel therapeutic approaches utilizing artificial miRNAs for treatment have recently been described (Hammond 2006; McBride et al. 2008). Although no reports have directly associated miRNA regulation with peripheral nerve health, recent observation have shown that PNS axons in vivo and in vitro contain
functional effector complexes (Murashov et al. 2007) as transfection with siRNAs into distal axons selectively downregulated the target and abolished Sema3A-dependent growth cone collapse (Hengst et al. 2006). Since siRNA and miRNA pathways share common effector proteins, these observations indicate that peripheral nerve function may be regulated by the miRNA biosynthetic pathway. In addition, the recent identification of patients with auto-immunity to GWBs suggests such a role for miRNA process in peripheral nerve health (Bhanji et al. 2007). Clinical studies indicate that these patients most often present with motor and sensory peripheral neuropathy. Although it is not known whether the resulting neuropathy is axonal or glial in origin, it is tempting to hypothesize that any impairment in the miRNA machinery in Schwann cells may alter myelin gene expression and lead to neuropathy. Since certain myelin genes, including PMP22, are dose-sensitive (Berger et al. 2006), loss of a required regulatory process could result in abnormal gene dosages, a mechanism known to lead to disease (Roa et al. 1991). The elevated levels of miR-29 in response to sciatic nerve crush injury and the inverse relationship to PMP22 (Fig. 4-9) suggest that the miRNA pathway responds to nerve injury and may be involved in the regulation of the demyelination process as well as the developmental regulation of myelin genes. Future studies will further elucidate and characterize the miRNA response to nerve crush injury and determine the axonal and Schwann cell contributions.

Recent studies detected miR-29a repressed in lung cancer (Fabbri et al. 2007), and to target Mcl-1 expression (Mott et al. 2007), as well as Tcl1 levels (Pekarsky et al. 2006). In the central nervous system, the expression of MiR-29a is high in astrocytes (Smirnova et al. 2005), although the functional significance of this finding is
undetermined. The linkage of miR-29a to these different paradigms support the hypothesis that each miRNA can regulate many genes (Valencia-Sanchez et al. 2006). MiRNAs have been shown to exert their function by either signaling for mRNA degradation via siRNA-like mechanisms, or inhibiting translation (Bagga et al. 2005; Pillai et al. 2005). We provide evidence that miR-29a ultimately does lead to reduced steady-state PMP22 RNA levels (Fig. 4-7C). It is currently unknown if other myelin genes, such as myelin basic protein or myelin protein zero, are co-regulated by the same miRNAs. Myelin basic protein message has been shown to be translationally repressed and transported for local synthesis in oligodendrocytes (Gould et al. 2000), a mechanism that may involve miRNAs. Nevertheless, it appears that the miRNA regulation of PMP22 may be cell-type specific. Related studies in oligodendrocytes indicate that additional brain-enriched miRNAs target PMP22 in the central nervous system (Lau et al. 2008). Together, these data show that the individual miRNAome of the cell may contribute to refining the genetic profile, whereas more than one miRNA may target a specific RNA dependent on cell type.

In conclusion, here we demonstrate that a disease-associated peripheral myelin gene is regulated by miRNAs. Elucidating this pathway will provide novel insights to the understanding of the molecular signals and mechanisms required for myelination. In addition, miRNAs may be therapeutic tools for diseases associated with altered gene dose in glial cells, such as in demyelinating neuropathies. Future studies will seek to further characterize the role of miRNAs in the myelination process, sciatic nerve development and in peripheral nerve injury.

**Note**
The work presented in this chapter was published in *Glia* 2009 Sep;57(12):1265-79. Jonathan D. Verrier and Pierre Lau planned and performed all experiments. Lynn D Hudson, Alaxander Murashov, Rolf Renne and Lucia Notterpek aided in planning the experiments, provided critical reagents and edited the manuscript.
Figure 4-1. GW body formation and Dicer expression are enhanced in actively-proliferating Schwann cells. A) Rat Schwann cells were subjected to growth arrest and then induced to divide. Cells were fixed and labeled with a human anti-GWB antibody. Hoechst dye is used to visualize nuclei. The inset in the upper right corner represents a no primary antibody control. Scale bar, 10 μm. B) Western blot analysis on total lysates of proliferating and non-proliferating (non-prolif.) cells are shown using the indicated antibodies. Phospho-Histone H3 (pH-H3) serves as a mitotic marker. C) Upon incubation of the cell lysates with PNGase F (N), the indicated ~22 kDa PMP22 bands shift to the core 18 kDa core protein (*). C: no enzyme control. (B, C) GAPDH serves as a loading control. D) Quantification reveals increased PMP22 expression in non-proliferating cells (n=3,**p<0.01). E) Quantification demonstrates that Dicer expression is upregulated in proliferating cells (n=3,*p<0.05). (D, E)
Figure 4-2. Suppression of Dicer increases PMP22 levels. A) In whole cell lysates the suppression of Dicer by siRNA is confirmed using an anti-Dicer antibody. Increased PMP22 protein is observed in cells transfected with Dicer siRNA. B) Quantification reveals that inhibition of Dicer expression results in an increase in PMP22 protein levels, as compared cells transfected with Neg. siRNA (n=3, *p<0.05) C) Dicer suppression is confirmed by immunolabeling using an anti-Dicer antibody (green). Increased PMP22 protein in cells treated with Dicer siRNA is detected using an anti-PMP22 antibody (red). Hoechst dye is used to visualize nuclei. Scale bar, 10 µm.
Figure 4-3. The binding and regulatory ability of predicted PMP22 targeting miRNAs. A) MiRNAs that are predicted to bind to the 3'UTR of PMP22 and the location of the binding sites are shown. B) Binding of candidate miRNAs are detected as miRNA/PMP22 RNA complexes by a gel shift assay. C) Luciferase assays were performed after co-transfection of the PMP22 3'UTR luciferase reporter construct and the indicated miRNA-precursors. All three isoforms of miR-29 (a, b and c), miR-381 and miR-9 show repression of luciferase signal compared to the Neg. miR (*p<0.05, n=3)
Figure 4-4. Growth conditions alter the miRNA expression profile of Schwann cells. A) The miRNA expression profiles of Schwann cells grown in serum-containing (proliferating) or defined medium (non-proliferating) were determined using an Exiqon microarray (n=3 for each condition, color scale bar indicates fold-change). B) Three miRNAs predicted to target the 3'UTR of PMP22 are expressed in Schwann cells and show unique profiles upon growth condition. C) The expression of predicted PMP22 regulating miRNAs was validated using RT-PCR. MiR-29a and miR-381, but not miR-140*, are reduced in proliferating, as compared to non-proliferating Schwann cells (*p<0.05, n=3). MiR-124a serves as a negative control.
Figure 4-5. miR-29a regulates PMP22 3'UTR-luciferase reporter expression through one specific binding site. A) Co-transfection with the reporter construct and miR-29a, or anti-miR-29a, leads to a reduction or increase in luciferase activity, respectively (**p<0.01, n=4). B) Inhibition of miR-322/424, miR-381, and miR-140* does not significantly increase PMP22 3'UTR reporter expression when compared to Neg. control (n=3, p>0.05). C) Schematic representation of PMP22 3'UTR truncation constructs. The predicted miR-29a binding site is indicated at 0.66 kb after the stop codon (grey line). D) MiR-29a has no effect on the empty psicheck2 vector, but significant (**p<0.01) repression is observed with the PMP22 3'UTR construct. The PMP400 fragment demonstrates greater luciferase activity (*p<0.05), whereas the PMP800 construct has less compared to the 3'UTR + Neg. control (**p<0.001, n=4). E) A schematic depicting the deletion of the predicted 7 nt seed region for the miR-29a binding site. F) Luciferase assays were performed after transfecting either the full length or the 29a seed Del PMP22 3'UTR construct in the presence of miR-29a. The intact construct demonstrates a reduction in luciferase activity in the presence of miR-29a (*p<0.05), which is abolished in the deletion mutant (n=3).
Figure 4-6. Endogenous PMP22 RNA associates with Ago2 and the interaction is enhanced by miR-29a over-expression. (A) C-myc-Ago2 transfected and non-transfected Schwann cells were processed for immunoprecipitation (IP) using either a non-specific rabbit IgG (Rbt IgG) or rabbit anti-c-myc conjugated (c-myc IP) agarose beads. Western blot using an anti-c-myc antibody shows enrichment of c-myc-Ago2 in the IP fraction compared to the input, with no enrichment in the control Rbt IgG IP sample. (B) RNA was isolated from total cell lysates (input), IP fractions (IP) and post-immunoprecipitation supernatants (Sup), from the samples analyzed in panel A. Semi-quantitative RT-PCR was performed using primers specific for PMP22 RNA. The cells transfected with miR-29a show less total PMP22 RNA compared to Neg. control (n=4, *p<0.05). There is an approximately 4-fold increase in the PMP22 bound to Ago2 when the cells are co-transfected with miR-29a compared to the Neg. miRNA (n=4, ***p<0.001).
Figure 4-7. miR-29a regulates endogenous PMP22 levels in Schwann cells. A) Schwann cells were transfected with Neg. miR, miR-29a, or anti-miR-29a and harvested for protein analysis. The increase of miR-29a reduces PMP22 protein, while the anti-miR-29a results in elevated steady-state PMP22 levels. B) Quantification reveals that transfection of miR-29a reduces steady-state PMP22 levels (n=4,*p<0.05), while inhibition of endogenous miR-29a relieves the miRNA-mediated repression (**p<0.01). PMP22 levels were normalized to GAPDH. C) Quantification of real-time RT-PCR experiments on RNA from Schwann cells transfected with Neg. miR, miR-29a, or anti-miR-29a (n=4). PMP22 RNA levels in cell transfected with miR-29a are significantly reduced as compared to Neg. control (**p<0.001). Inhibition of miR-29a increases the steady-state levels of PMP22 RNA (*p<0.05).
Figure 4-8. PMP22 and miR-29 expression are inversely correlated in developing rat sciatic nerve. A) Total RNA was isolated from rat sciatic nerves at the indicated time points (in days). Relative quantitative RT-PCR reveals the expression of PMP22 RNA increases as the animals’ age increases. GAPDH was used to normalize for equal loading of RNA and relative change in expression (n=3, * p<0.05, *** p<0.001). B) RT-PCR on the same RNA samples determines the expression of miR-29a in sciatic nerve during development (Fig. 4A) (n=3, * p<0.05, *** p<0.001). C) The relative expression levels of PMP22 (x-axis) and miR-29a (y-axis) as determined in panels A and B are plotted together. Linear regression analysis reveals that expression of PMP22 and miR-29a in developing rat nerve is highly correlated (p<0.05, r^2 = 0.078).
Figure 4-9. Nerve crush injury reduces PMP22 expression and elevates miR-29 levels.  
A) Total RNA was isolated from both control or crush injured mouse sciatic nerve. Relative expression of PMP22 was determined using quantitative RT-PCR analysis. Sciatic nerve crush injury results in decreased PMP22 RNA expression at 4 d post-injury when compared to control (each sample was run in triplicate and is a pool of 10 crush or control nerves, *** p<0.001). B) Western blot analysis of total nerve lysates reveals that PMP22 protein levels are reduced at 5 d post-crush injury to the nerve. C) Relative quantitative RT-PCR reveals that miR-29b levels are elevated at 4 d post-injury in mouse nerves (each sample was run in triplicate and is a pool of 10 crush or control nerves, * p<0.05).
CHAPTER 5
REDUCTION OF DICER IMPAIRS SCHWANN CELL DIFFERENTIATION AND MYELINATION

Introduction

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 including 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 need to exit the cell cycle and increase the expression of myelin-associated genes in a coordinated fashion, which is associated with significant changes in the transcriptional profile of these cells (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, while c-Jun and SRY-box containing gene 2 (Sox2) promote a non-myelinating Schwann cell phenotype (Le et al. 2005; Parkinson et al. 2008; Parkinson et al. 2004; Wegner 2000b). Expression of Egr2/Krox20 is required for Schwann cells to myelinate 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 post-transcriptional mechanisms involving RNA binding proteins and miRNAs play key roles during the process of myelination in both the CNS and PNS (Lau et al. 2008; Verrier et al. 2009; Zearfoss et al. 2008).

Post-transcriptional 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 the 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 show that miRNAs also play a role in regulating myelination (Kawase-Koga et al. 2009; Lau et al. 2008; Lin and Fu 2009; Verrier et al. 2009). The molecular mechanisms underlying the biogenesis of miRNA in mammalian cells has been described 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, hippocampus and lung have been enhanced by studies of conditional Dicer knock-out mice (Davis et al. 2008; Harris et al. 2006; O'Rourke et al. 2007). However, the roles that miRNAs have in peripheral nerve development and on Schwann cell differentiation and myelination have not been examined.

In these experiments, we utilized an in vitro model of Schwann cell myelination and Dicer knock-down techniques to examine the effects of miRNAs on myelination. We demonstrate that loss of miRNA biogenesis leads to decreased steady-state levels of pro-myelination differentiation factors in Schwann cells and an impairment of myelination. Our findings suggest that mature miRNAs are required for Schwann cells to switch from a proliferating, non-differentiated state to a mature myelin forming cell.
Results

Inhibition of Dicer Levels in Schwann Cells Using Dicer shRNA

To determine if Dicer expression is required for Schwann cell differentiation and myelination we transduced primary rat Schwann cells with lentivirus carrying shRNA targeting Dicer. We chose this approach because lentiviral shRNA-mediated knock-down of gene expression is efficient in Schwann cells (Hu et al. 2005), and reducing steady-state Dicer levels can efficiently inhibit 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 three to five days. Efficient selection and establishment of pure, transduced Schwann cell populations was confirmed by direct visualization of GFP (Fig. 5-1A). The steady-state levels of Dicer protein were reduced in the Dicer shRNA Schwann cells when compared to Neg. shRNA transduced cells, as evaluated by Western blot analyses (Fig. 5-1B). 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 cells (Fig. 5-1C, n=4, ** p<0.01). These data show that it is possible to efficiently transduce primary rat Schwann cells with lentivirus and to reduce expression of Dicer protein using the shRNA approach.

Dicer Knock-Down Impairs Schwann Cell Differentiation

The loss of Dicer expression has been shown to affect cellular division and differentiation (Bu et al. 2009; Carleton et al. 2007). To evaluate the effect of Dicer suppression on Schwann cell proliferation, we analyzed primary rat Schwann cells transduced with either Neg. shRNA or Dicer shRNA using a bromodeoxyuridine (BrdU) incorporation assay. After 8 hours of incubation, BrdU incorporation was evaluated using immunostaining and colorimetric measurements. As shown on the graph,
suppression of Dicer levels by shRNA lead to increased Schwann cell proliferation as determined from direct cell counting (Fig. 5-2A, n=6, *p<0.05). Analyses of similarly treated cultures using an ELISA-based colorimetric assay confirmed the increased uptake of BrdU by Dicer shRNA cells (Fig. 5-2B, n=6, **p<0.01). Since proliferating, undifferentiated Schwann cells are a non-myelinating phenotype (Woodhoo et al. 2009), these data suggest that under normal conditions, Dicer and likely mature miRNAs are necessary for Schwann cell differentiation.

To examine the effects of Dicer shRNA on the expression levels of known Schwann cell transcription and differentiation factors, we analyzed total protein lysates of cultures that had been transduced with Dicer shRNA virus (Fig. 5-2C). Dicer levels remained reduced when compared to Neg. shRNA control cells at two weeks post-transduction and selection in puromycin, indicating that the expression and functionality of the shRNA was maintained. The inhibition of Dicer expression reduced steady-state levels of the pro-myelination transcription factors, Oct6 and Egr2/Krox20, and the myelin protein MPZ. Dicer shRNA transduction also resulted in increased c-Jun expression as compared to Neg. control shRNA. Quantification of independent transductions revealed a significant decrease in Egr2 protein levels in the Dicer shRNA cells (Fig. 5-2D, n=4, **p<0.01). These data suggest that reduction of Dicer levels is associated with impairment in pro-myelin transcription factor and MPZ expression in cultured Schwann cells.

Inhibition of miRNA Biogenesis Impairs Schwann Cell Myelination

To further examine the effects of reduced Dicer protein levels on myelin gene expression, we examined myelin formation in Schwann cell/DRG co-cultures. Pure populations of Schwann cells expressing the Dicer shRNA transgene were obtained
using puromycin selection (see Fig. 5-1) and were seeded onto DRG neuron cultures. Schwann cells were allowed to proliferate through radial sorting along the axons. After two weeks of culture in myelination promoting medium, the cells were either fixed for immunofluorescence or harvested for Western blot analysis. Prior to harvesting the cells, we confirmed that the Dicer shRNA transgene was being expressed by monitoring direct GFP fluorescence in live cells (Fig. 5-3A). The myelination capacity of the Dicer shRNA Schwann cells was evaluated by examining MPZ protein levels in Schwann cell/DRG co-cultures that had been maintained in myelinating conditions for 2 weeks (Fig. 5-3). MPZ-like immunoreactivity was reduced in DRG cultures seeded with Schwann cells expressing Dicer shRNA compared to those expressing the Neg. shRNA (Fig. 5-3A). Western blot analyses of the myelinating Schwann cell/DRG co-cultures confirmed that expression of Dicer shRNA severely reduced MPZ protein levels (Fig. 5-3B). Each Western blot lane represents an independent co-culture to demonstrate reproducibility of the observed phenotype. In comparison, the level of neurofilament medium chain (NF-M) was similar in the samples. These observations support our hypothesis that Dicer and miRNAs plays a role in triggering Schwann cell differentiation and myelination.

We also examined the effects of reduced Dicer expression on Schwann cell myelination by monitoring the levels of MBP, a component of compact myelin (Fig. 5-4). The expression of Neg. shRNA vector alone did not affect the ability of Schwann cell to form mature myelin (Fig. 5-4A). Schwann cells expressing MBP and GFP were readily detected in myelinating co-cultures (arrows). Non-transduced Schwann cells (GFP negative) in the cultures were also positive for MBP (arrowheads) and represented only
a small fraction of the total myelin observed. Western blot analyses of Schwann cell/DRG co-cultures grown under myelinating conditions seeded with cells expressing Dicer shRNA revealed a reduction in the steady-state expression of MBP, while the levels of NF-M remained constant (Fig. 5-4B). GAPDH expression confirmed equal protein loading. Myelination formation was also evaluated by immunostaining the cultures for MBP (red, Fig. 5-4C). Dicer shRNA cells exhibit less MBP-like immunoreactivity when compared to the Neg. control shRNA samples. To control for the presence of axons in the field of view, we also probed from NF-M as a neuronal marker (blue). Similar results were obtained when Schwann cells transduced with shRNA targeting GW182, a protein required for miRNA function, were seeded onto DRGs (Fig. A-1).

To determine if reducing Dicer levels affects the expression of negative regulators of myelination, we analyzed myelinating co-cultures for Sox2 (Wegner 2000b). While Sox2 normally would be down-regulated upon the initiation of myelination, it remained elevated when Dicer was suppressed (Fig. 5-5A). Quantification of these experiments reveals a significant increase in the steady-state levels of Sox2 upon Dicer shRNA transduction when compared to control (Fig. 5-5B, n=3, p<0.05). These data support the hypothesis that loss of miRNA biogenesis impairs the ability of Schwann cells to differentiate and myelinate axons.

**Discussion**

Post-transcriptional regulation of gene expression by miRNAs is important in a wide variety of cellular process (Ambros 2004). Here we investigate the effect of reducing the miRNA biogenesis protein, Dicer, on Schwann cell myelination and differentiation. We demonstrate that inhibition of Dicer expression results in an
increased proliferation rate in primary rat Schwann cells. In addition, the cells fail to produce myelin and maintain low levels of known Schwann cell differentiation markers (Figs. 5-2, 5-4). These experiments reveal an essential role for miRNA regulation during Schwann cell differentiation and myelination.

The correct level of Dicer expression is critical for cellular and organismal function. Dicer protein is essential for embryonic development since Dicer null animals fail to develop past E7.5 (Bernstein et al. 2003). Loss of Dicer function alters the differentiation of T-cells, embryonic stem cells, 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). Conditional deletion of Dicer in forebrain excitatory neurons leads to reduced dendritic branching and microcephaly (Davis et al. 2008). Patients with autoimmunity to the RNA-induced silencing complex associated protein GW182 often present with motor and sensory neuropathies indicating a role in the PNS (Eystathioy et al. 2003). 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 Schwann cell development (Potenza et al. 2009). Also we have shown that transient reduction of Dicer in Schwann cells leads to altered PMP22 expression (Verrier et al. 2009). These data implicate miRNAs in a wide variety of developmental and disease processes, including glial differentiation.

MiRNAs have been shown to be involved in cellular differentiation at both the in vitro and in vivo (Harris et al. 2006; Miska 2005). Specifically, in studies of myelinating glial cells, miR-23 has been shown to influence lamin B1 expression in oligodendrocytes and to modulate their development and myelin gene expression (Lin and Fu 2009).
Also tubulin polymerization-promoting protein, which is required for oligodendrocyte myelination, has been shown to be regulated by a miRNA (Lehotzky et al. 2009). Here we reduce Dicer levels by shRNA specifically in Schwann cells allowing us to address the role of glial miRNAs in myelination and differentiation (Fig 5-1). We observed decrease in Egr2/Krox20, which is in agreement with the reduction or apparent failure to up-regulate myelin genes when cultured with DRG neurons (Figs. 5-3 and 5-4). We also demonstrated that the known negative regulator of myelination, c-Jun and Sox2, remain elevated in the Dicer shRNA cells. These data imply that loss of mature miRNAs inhibits Schwann cells differentiation, a phenotype observed in other cell types (Kapinas et al. 2009; Kawase-Koga et al. 2009). Recent studies in the developing CNS have revealed Dicer deletion to drastically reduce oligodendrocyte maturation (Kawase-Koga et al. 2009). These finding, in conjunction with our results, suggest a shared requirement for miRNA regulation for differentiation of the myelinating glial cells in both the CNS and PNS.

We previously demonstrated that PMP22 expression is subject to miRNA regulation in the PNS and CNS (Lau et al. 2008; Verrier et al. 2009). In comparison, MPZ is apparently not a direct target of miRNA repression in Schwann cells, at least under the employed in vitro conditions (Fig. 5-2). The reduction of steady-state MPZ upon transduction of Dicer shRNA is consistent with the observed effect on Egr2/Krox20 levels, which transcriptionally controls MPZ (Jang et al. 2006; Jang and Svaren 2009). Preliminary data indicate that Egr2/Krox20 itself is subjected to miRNA-mediated repression in Schwann cells (Fig. A-2). 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 cell cycle is also a marker of
Schwann cell differentiation (Berger et al. 2006; Scherer 1997). Here we demonstrate
that the loss of Dicer leads to increased cellular division, a characteristic of an
undifferentiated Schwann cell (Fig. 2). Loss of Dicer has been associated with altered
proliferation and appears to be cell type dependant. For example, cancer cell lines
demonstrate reduction of cellular proliferation with miRNA depletion (Zhang et al. 2009),
while others such as gliomas increase their division upon loss of specific miRNAs
(Kumar et al. 2007). 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, non-
myelinating cell to a differentiated, myelinating phenotype.

We demonstrate that when Dicer is suppressed via shRNA, the steady-state levels
of the transcription factor Sox2 is increased (Fig. 5-5). Sox2, along with c-Jun, are
negative regulators of myelination and markers of immature non-myelinating Schwann
cells (Jessen and Mirsky 2008; Parkinson et al. 2008). The increase of Sox2 observed
in Schwann cells with Dicer shRNA is in agreement with the current literature examining
miRNAs in regulating differentiation (Card et al. 2008; Xu et al. 2009). Previous
investigations into miRNA repression of differentiation related transcription factors has
revealed Sox2 to be a direct miRNA target in several cell types (Tay et al. 2008; Xu et
al. 2009). In addition to being target by miRNAs, in human embryonic stem cells, Sox2
has been shown to transcribe miR-302 which in turn regulates the expression of several
proteins that are 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 if Sox regulates transcription of miR-302 or cellular proliferation-inducing miRNAs in Schwann cells. In addition to Sox2, we also demonstrated elevated levels of c-Jun in response to Dicer shRNA (Fig. 5-2). c-Jun was previously demonstrated to regulate specific miRNA expression 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. However, 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 data presented here suggest that inhibition of the miRNA pathway in Schwann cells may lead to demyelination or dysmyelination (Fig. 5-2, 5-3, 5-4). It is a tempting hypothesis that this may be a contributing mechanism for the etiology of the clinical manifestations observed with autoimmunity to RISC proteins (Bhanji et al. 2007; Eystathioy et al. 2002). However, why the peripheral nerve is preferentially targeted or particularly susceptible to these phenomenon still warrants further investigation. The data presented here support additional investigations into the signaling mechanisms that regulate specific miRNA expression. Currently, targeting gene therapy specifically to the Schwann cells presents technical hurdles, including cell specific delivery, elucidating which signaling pathways regulate specific miRNA expression may be a more feasible approach. The ability to control an individual miRNA is a critical step to utilize specific miRNAs for therapeutic purposes. It has been shown that both Schwann cells and oligodendrocytes display dynamic miRNA profiles during differentiation (Lau et
Further experiments will investigate if axonal signals may govern the expression of miRNAs in Schwann cells and ultimately affect their myelination.

The elucidation of post-transcriptional myelin gene regulation contributes to the understanding the underlying mechanisms of myelination. The experiments discussed here suggest that mature miRNAs are essential for Schwann cell differentiation and the initiation of myelination. Further dissection of the observed phenotype and molecular profile of miRNA depleted Schwann cells will provide novel insight into which essential differentiation factors/pathways are under miRNA mediated regulation. These results will be important to understanding both the development and disease related biology of Schwann cells and how we can potentially modulate their miRNA profile for therapeutic potential.
Figure 5-1. Lentiviral transduction of Dicer shRNA reduces steady-state Dicer levels. (A) Schwann cell cultures were transduced with either control or Dicer shRNA lentiviral particles. GFP fluorescence (left panel) and phase contrast (right panel) images of live cultures are shown. Scale bar, 10 µm. (B) Western blot analyses of whole cell lysates of Schwann cells transduced with Dicer shRNA or Neg. shRNA. Molecular mass, kDa. (C) Quantification of Dicer protein levels after normalizing to GAPDH, reveals that expression of Dicer shRNA leads to a significant reduction in the steady-state levels of Dicer protein (n=4, ** p<0.01).
Figure 5-2. Suppression of Dicer levels results in increased proliferation and reduced expression of Schwann cell differentiation markers. (A) Quantification of BrdU incorporation reveals an increase in the number of labeled cells in cultures treated with Dicer shRNA, as compared to those treated with Neg. shRNA (n=6 field of view per condition, *p<0.05). (B) An ELISA based colorimetric BrdU incorporation assay shows a significant increase in BrdU uptake in Schwann cells treated with Dicer shRNA cells, as compared to those treated with Neg. control shRNA (n=6, **p<0.01). (C) 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, kDa. (D) Quantification of independent experiments reveals that expression of Dicer shRNA leads to a significant reduction in the steady-state levels of Egr2/Krox20 protein (n=4, **p<0.01).
Figure 5-3. MPZ protein levels are reduced in myelinating co-cultures containing Schwann cells expressing Dicer shRNA. (A) Transduced Schwann cells were analyzed for MPZ expression using an anti-MPZ antibody (red). NF-M is detected (blue) to identify axons. GFP (green) indicates transduced cells. Scale bar, 20 µm. (B) Western blot analyses of NF-M and MPZ expression in Schwann cell/DRG co-cultures after two weeks in myelinating conditions. GAPDH is shown as a protein loading control and each lane represents an independent co-culture. Molecular mass, kDa.
Figure 5-4: Dicer shRNA transduced Schwann cells show impaired myelination in Schwann cell / DRG co-cultures. (A) Lentiviral Neg. shRNA transduced Schwann cells retain myelination capacity (arrows indicate transduced myelinating cells, arrowhead denote residual non-transduced cells). Mature myelin is detected using an anti-MBP antibody (red). GFP expression denotes transduced cells (green) and Hoechst dye is used to visualize nuclei (blue). Scale bar, 10 µm. (B) Western blot analysis of Schwann cell/DRG co-cultures grown under myelinating conditions for 2 weeks reveals that Dicer shRNA impairs the production of myelin. GAPDH is shown as a loading control. Molecular mass, 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 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 bar, 20 µm.
Figure 5-5. Reduction in Schwann cell Dicer levels results in increased Sox2 expression. (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 is shown as a loading control. Molecular mass, kDa. (B) Quantification of independent experiments reveals a significant increase in the levels of Sox2 in the Schwann cells transduced with the Dicer shRNA, as compared to the Neg. shRNA controls (n=3, * p<0.05).
CHAPTER 6
CONCLUSIONS AND SIGNIFICANCE

Overview of Findings

Elegant studies have detailed the molecular and transcriptional machinery required for myelination (Svaren and Meijer 2008). Only recently has the contribution of post-transcriptional mechanisms governing myelin gene regulation been appreciated (Verrier et al. 2009; Zearfoss et al. 2008). The data presented in this dissertation reveal an essential role for miRNA-mediated gene regulation during the process of Schwann cell myelination. We demonstrate that not only is an individual myelin gene, PMP22, regulated in both the CNS and PNS by specific miRNAs (Chapter 2 and 3), we show inhibition of miRNA biogenesis by Dicer reduction severely impairs Schwann cell myelination (Chapter 5). These data reveal a critical function for miRNAs in the biology of the peripheral nervous system and contributes to the understanding of the intricate process of myelination.

Abnormal expression of myelin genes is associated with neuropathic phenotypes in humans (Lupski and Garcia 1992). Specifically, the misexpression of PMP22 has been extensively studied and linked to a family of hereditary peripheral neuropathies (Young and Suter 2003). However, the precise mechanisms governing the levels of PMP22 expression have remained elusive. The regulation of this dose-sensitive, disease-linked gene by miRNAs provides novel insight into how PMP22 levels are controlled. Recent descriptions of the shared characteristics of dose-sensitive genes include a short half-life, complex 3’UTR and a propensity to aggregate (Vavouri et al. 2009). PMP22 possesses each of these characteristics. For example, abnormal expression or mutations in the gene are associated with the formation of intracellular
protein aggregates (Ryan et al. 2002). In addition, the majority of PMP22 normally presents with a half-life of less than an hour (Pareek et al. 1997). To address each of these issues, approaches utilizing the Schwann cells’ endogenous protein homeostatic mechanisms have been employed (Fortun et al. 2003; Rangaraju et al. 2008). However, the experiments described in this dissertation address the functional role the 3'UTR in regulating PMP22 expression. The results presented here demonstrate post-transcriptional regulation of PMP22 by miRNAs targeting the 3'UTR in both the CNS and PNS (Chapters 3 and 4). These data provide an additional approach to modulate PMP22 expression, a desirable effect in peripheral neuropathies associated with altered PMP22 levels.

PMP22 message has been shown to be regulated at the post-transcriptional level. While the 3'UTR of PMP22 has been demonstrated to contribute a negative influence on gene expression (Bosse et al. 1999) and the message was shown to be regulated post-transcriptionally (Manfioletti et al. 1990), the underlying mechanism remained undefined. Also PMP22 message has been detected rather ubiquitously in the body, yet the protein is restricted in its expression (Amici et al. 2006). In the CNS, PMP22 message is detectable in most cell types yet the protein is only expressed at the neuroepithelial junctions (Lau et al. 2008; Parmantier et al. 1995; Taylor et al. 2004). This dissertation reveals that PMP22 translation in the CNS is repressed by miR-9 in developing and mature oligodendrocytes (Chapter 3). The presence of PMP22 message in oligodendrocytes is an interesting observation since the protein is not utilized in the myelin made by the cell (Ishii et al. 2009). The precise functions of PMP22, particularly in the CNS, still remain undefined, although its roles in myelin and
junction formation has been demonstrated (Notterpek et al. 2001; Pareek et al. 1997). PMP22’s association with the cell cycle and cellular growth lacks mechanism and remain correlational (Zoidl et al. 1995). Experiments in this dissertation confirm PMP22 message expression in the CNS, specifically in oligodendrocytes, with the lack of detectable protein, however for the first time we also show that miRNA mediated repression at least contributes to this phenomenon.

PMP22 protein is most highly expressed in the myelinating Schwann cells of the PNS (Snipes et al. 1992). The molecular mechanisms dictating its expression in Schwann cells only partially explain the observed message and protein levels. The two transcripts found differ only in untranslated regions (Suter et al. 1994). However, their expression has not been linked to the known myelination inducing transcription factors and only correlates with cAMP levels, cell cycle and initiation of myelination (Jang et al. 2006; Wegner 2000b). The data presented here contributes to the understanding of how PMP22 expression is regulated in Schwann cells and oligodendrocytes. We demonstrate that the Schwann cell’s miRNA profile dynamically changes with the growth/differentiation state of the cell (Fig. 4-4). These data imply that a different subset of miRNAs is required during proliferation than is needed during differentiation. A subpopulation of these miRNAs are upregulated in differentiated Schwann cells, a phenomenon that we propose to be critical for myelination to occur (Chapter 5). It is shown here, and in recent published studies, that Schwann cells retain Dicer expression throughout differentiation (Chapter 3 and 5) (Potenza et al. 2009). It is tempting to hypothesize that some miRNAs involved in Schwann cell differentiation and myelination are directly regulated by cAMP levels, since this second messenger molecule is
required for myelination. To support this hypothesis, miR-142-3p has been shown to regulate cAMP levels in T cells by targeting adenyl cyclase 9 (Huang et al. 2009) and miR-132 has been shown to be directly transcribed by CREB (Vo et al. 2005). Since Schwann cell differentiation is dependent on elevated cAMP levels, it would be of interest to investigate which miRNAs in Schwann cells are implicated in the cAMP pathway and which genes they regulate. In addition, it would be useful to determine which miRNAs themselves are elevated by increased cAMP levels in Schwann cells and if their target genes may be involved in the cells differentiation and myelination processes. Here we show that miR-29a actively modulated PMP22 mRNA and protein levels in Schwann cells (Chapter 4). In agreement with the literature on PMP22 levels and cell cycle, miR-29a is highest in growing cells and lower in growth arrested/differentiated cells. The expression levels are inversely correlated with PMP22 and may help define the mechanism by which the message and protein are elevated upon growth arrest (Schneider et al. 1988). It is interesting to note that mature miR-9, the miRNA demonstrated to regulate PMP22 in the CNS, is not expressed in Schwann cells (Fig. 3-8). Recent work has detailed the molecular machinery involved in the processing of the pre-miRNA into the mature, active form (Davis and Hata 2009). Characterization of the precise mechanisms governing which miRNAs are expressed in Schwann cells aids in utilizing this pathway for therapeutic potential.

The miRNA expression profiles of both Schwann cells and oligodendrocytes are dynamic during their differentiation and maturation. This dissertation extensively characterizes the miRNA expression profiles of developing oligodendrocytes and Schwann cells (Chapters 3 and 4). The miRNA pathway has been show to be essential
for both cellular differentiation and embryogenesis (Bernstein et al. 2003). Here we demonstrate that the miRNAome of these glial cells is plastic where there are significant changes throughout differentiation. Interestingly in oligodendrocytes, miR-9’s expression levels only change slightly during differentiation but its target bias is greatly refined indicating an increase repression of target RNAs in mature cells (Fig. 3-2). These data imply that mere expression of the miRNA is not the only contributing factor in denoting functionality. In Schwann cells, we have identified several subpopulations of miRNAs whose expression is dependent on the growth and differentiation state of the cell. The identification of these populations of miRNAs provides insight into which specific miRNAs are essential for normal Schwann cell biology.

The loss of miRNA function is essential for life and specific organ targeted deletion of miRNAs has demonstrated developmental roles in many tissues (Bernstein et al. 2003; Muljo et al. 2005; Murchison et al. 2007). We provide data here suggesting that the miRNA pathway may be essential for Schwann cells to differentiate into myelinating cells (Chapter 5). It is establish that miRNAs are involved in the differentiation process of many cell types (Ambros 2004; Miska 2005; Stefani and Slack 2008) yet there has been limited investigation into what genes and processes miRNAs modulate in the myelinating glial cells (Lau et al. 2008; Lin and Fu 2009; Verrier et al. 2009). Recently it was shown that deletion of Dicer in oligodendrocytes greatly impairs the maturation of OPCs and ultimately affects myelination (Kawase-Koga et al. 2009). Here we inhibited Schwann cell Dicer expression, thus reducing miRNA levels in these cells and proceeded to induce differentiation and myelination. The Dicer shRNA cells displayed a transcription factor profile of an immature, undifferentiated Schwann cells, with reduced
Egr2/Krox20 and Oct6 expression and enhanced c-Jun levels (Fig. 6-2). These results are strikingly similar to what has been reported when Schwann cells are forced to remain undifferentiated (Parkinson et al. 2008). Myelination was greatly impaired in the Dicer shRNA cells when compared to control, indicating a role for mature miRNAs in differentiation and myelination of Schwann cells. These findings are translationally relevant whereas patients with autoimmunity to a protein required for miRNA function present with peripheral neuropathies, albeit of unknown origin or etiology (Bhanji et al. 2007). Additionally, an antibody isolated from plasma from a human patient with neuropathy recognized the miRNA processing bodies of Schwann cells. Data provided in this dissertation indicate that the loss of miRNA function in Schwann cells results in impaired myelination.

The studies here describe the modulation of myelin gene expression and myelination by miRNAs. The further elucidation of which particular miRNAs are implicated in Schwann cell differentiation and myelination will identify new therapeutic avenues for myelin-associated diseases. The expanded understanding of miRNAs and the elucidation of their targets will provide new ways to modulate gene expression. The miRNA pathway is an attractive avenue because the modulation of endogenous pathways is a more feasible option for gene regulation than the introduction of exogenous material to the genome. The introduction of artificial shRNAs in vivo has been associated with cellular toxicity and may limit their therapeutic potential (McBride et al. 2008). The observed toxicity is proposed to be due to interference with, even possible saturation, of the endogenous miRNA pathway (Grimm et al. 2006). These
issues are being addressed by limiting transgene expression (John et al. 2007) and further clinical trials utilizing gene therapy are currently underway.

Recent reviews have highlighted the potential ways to utilize the miRNA pathway for treatment of disease (Brown and Naldini 2009). As miRNA focused research expands, the precise signaling mechanisms governing individual miRNA expression is beginning to be revealed. For example, recently it was shown in osteoblasts that miR-29 is regulated through canonical WNT signaling (Kapinas et al. 2009). MiR-29 has been shown to regulate osteoblast differentiation and extracellular matrix molecule secretion (Li et al. 2009b). The combined knowledge of these experiments presents a logical hypothesis that modulation of WNT signaling will affect miR-29 levels and thus osteoblast differentiation. Estrogen and progesterone have been shown to have opposing influences on miRNA expression in smooth muscle cells (Pan et al. 2008) suggesting a previously unappreciated consequence of hormone replacement therapy. Also Src tyrosine kinase has been shown to influence miRNA expression in cancer cells (Li et al. 2009a), again targeting this activity of this kinase will have additional global effects by altering miRNA expression. These data suggest that the miRNA pathway may be influenced by currently used therapeutics in several diseases and may contribute to side effects. Continuing research defining the signaling mechanisms governing individual miRNA expression will allow targeted expression/repression of desired miRNAs and enhance their therapeutic potential.

It is predicted that 60% of human genes contain miRNA target sites in their 3’UTR (Davis and Hata 2009). A large percentage of these sites are highly conserved throughout different species. The evolutionary retention of these regulatory regions
implies a significant, essential role for miRNAs in many cellular process, including the maintenance of vertebrate life (Bernstein et al. 2003). The data presented here implicate miRNA-mediated gene regulation in glial biology and differentiation.

**Unresolved Issues and Future Studies**

The experiments present in this dissertation provide novel and exciting insight into miRNA mediated myelin gene regulation. However, the results presented here highlight some unresolved issues that can be addressed in future studies. The most obvious questions that arise from our findings is to determine what other genes are regulated by miRNAs in both developing and myelinating Schwann cells and oligodendrocytes. Also the elucidation of which miRNAs are the most critical to the process of myelination is an important issue raised by the data presented in the dissertation. Finally, the characterization of the precise mechanism by which the inhibition of Dicer leads to such a severe phenotype in Schwann cells is another major unanswered question.

In these experiments, we provide microarray profiles of Schwann cells and oligodendrocytes (Chapter 3 and 4). These data sets provide the foundation for further investigation into the genes in both cell types regulated by miRNAs. Recently these data were used to demonstrate that a gene required for oligodendrocyte differentiation is under regulation by a miRNA (Lehotzky et al. 2009). To elucidate additional miRNA targets, gene expression arrays can be performed on cell populations and co-cultures grown in the same conditions as the samples using in the described experiments (Chapters 4 and 5). The two data sets can then be employed for correlational target bias analysis bioinformatics. These experiments would aid in narrowing the search for miRNA target genes in these cell types. Further delineation of miRNA regulated genes in myelinating glial cells would require further 3'UTR luciferase assays followed by point
mutagenesis to ensure specificity (as performed in Chapters 3 and 4). Finally, overexpression and inhibition of the miRNA in the cell type of interest would demonstrate endogenous regulatory functionality. As our knowledge base increases on what factors determine a functional miRNA target (Grimson et al. 2007), our prediction and validation of miRNA targeted gene relationships will accelerate. The continued demonstration of functional miRNA targets will contribute to the understanding of glial biology and may shed light on diseases of unknown etiology.

Although we obtained a drastic phenotype due to the inhibition of Dicer in Schwann cells, and it is logical to assume that it was due to the alteration of miRNA biogenesis, the precise mechanism remains undefined. Reduction of Dicer was associated with impaired myelination by Schwann cells (Chapter 5) and we propose that this may be due to impaired differentiation of the cells. Recently it was shown that Dicer and Argonaute levels are maintained during Schwann cell differentiation implicating miRNA regulation in glial cell development (Potenza et al. 2009). These data imply a requirement for maintained miRNA mediated regulation is all phases of Schwann cell development. Also it was shown that timed deletion of Dicer in OPCs leads to the inhibition of oligodendrocyte maturation, again suggesting miRNAs are required for glial cell differentiation (Kawase-Koga et al. 2009). To support this hypothesis in the PNS, we demonstrate that the Dicer shRNA Schwann cells have increased proliferation, impaired myelination and elevated levels of markers of immature cells (Chapter 5). In addition to further suggest that loss of miRNAs is affecting Schwann cell differentiation, a wide variety of differentiation markers were evaluated. As expected the Schwann cells had reduced Egr2/Krox20 levels upon Dicer knock-
down (Fig. 5-7). Although these data support our hypothesis, these experiments need to be performed in myelinating Schwann cell/DRG co-cultures and the precise mechanism behind these observations remain undefined. For example, is the lack of myelin formation a result from the retained expression of negative regulators of myelination or from the inability to respond to promyelinating cues from the axons? After this extended characterization of the effect of Dicer reduction in Schwann cells, experiments to match the reduced miRNAs to the factors controlling differentiation would need to be performed. As described earlier, these experiments would rely heavily on bioinformatics and laborious transfection and mutational analyses. Since not all of the signals and factors governing Schwann cell myelination are known, one may need to characterize additional regulators of myelination prior to search for the effect of miRNAs on the pathway. For example, only recently has an orphan g-protein coupled receptor been shown to regulate the intracellular levels of cAMP in Schwann cells (Monk et al. 2009). It has been demonstrated that activation of this receptor is required for Schwann cell myelination. As the precise mechanisms controlling Schwann cell differentiation and myelination become better defined, they will provide new molecular targets for miRNA related investigation.

One lingering hypothesis derived from this dissertation, as well as years of previous studies examining PMP22 message in different tissues, is that PMP22 message itself encodes a functional RNA. PMP22 is conserved across the animal kingdom and particular regions of the message are shown to be very highly conserved (Itou et al. 2009). Bioinformatical analysis (www.esembl.org) predicts a non-coding RNA gene, snoU13, to be located in the first intron of the PMP22 gene and a region that
is highly conserved (Itou et al. 2009). SnoRNAs are small function molecules that are not translated to protein and help guide modification to RNA transcripts (Kawaji and Hayashizaki 2008). Only recently has it been appreciated that snoRNA can be processed into functional mature miRNAs (Ender et al. 2008; Scott et al. 2009; Taft et al. 2009). In support of this hypothesis, recent estimates are that approximately 40% of known miRNAs are derived from the introns of coding genes (Winter et al. 2009). The hypothesis that the PMP22 gene does encode for a small functional RNA molecule is tempting as it would help to explain the apparent disconnect between PMP22 message and protein expression. To obtain the snoRNA/miRNA from the PMP22 gene, only transcription and not translation would be needed. It is further tempting to speculate that in CMT1A, the increased gene dosage of hypothesized RNA molecule may contribute to the observed cellular phenotypes. Finally, since PMP22 expression is ubiquitously detected in tissues yet the protein expression is restricted, could other miRNAs be involved in repressing PMP22 message in these cell types? Experiments similar to those describe in this dissertation (Chapters 3 and 4) could be utilized to determine which miRNAs silence PMP22 expression in tissues such as cartilage, lung and gut. These speculations and hypotheses will need to be experimentally tested and verified.

In summary, the work described here details the regulation of PMP22 by miRNAs in the myelinating glial cells of both divisions of the nervous system. Additionally, the process of myelination in the PNS is severely impaired by selective inhibition of miRNA biogenesis in Schwann cells, indicating an essential role for the miRNA pathway in myelination. Therefore, this dissertation provides the groundwork for future studies of
the functions that individual miRNAs play in governing the onset and maintenance of myelination. The miRNA pathway, specifically PMP22 targeting miRNAs, could provide new therapeutic avenues for PMP22-associated diseases and help determine the cause of peripheral neuropathies of unknown etiology. Ultimately, these findings expand the knowledge of glial biology, regulation of PMP22 and miRNAs in the control of PNS myelination and differentiation.
Figure A-1. Schwann cells transduced with GW182 shRNA show reduced myelin formation in vitro. Schwann cell transduced with either Neg. shRNA or GW182 shRNA were seeded onto pure DRG neurons and allowed to myelinate for 2 weeks. Myelin formation is shown using an anti-MBP antibody (red) and less MBP-like immunoreactivity is seen in the GW182 shRNA cultures when compared to the Neg. shRNA. Equal neuronal content is shown using an anti-NF-M antibody (blue) and GFP is shown as a marker for transduced cells (green).
Figure A-2. Egr2/Krox20 reporter expression may be modulated by a dynamically regulated miRNA in Schwann cells. A) Microarray experiments performed on primary SCs subjected to either proliferation or differentiation (non-prolif) media for 48 h prior to harvest reveals miR-140 and miR-140* expression to be dynamically regulated. B) Primary rat Schwann cells were co-transfected with an Egr2-Luciferase reporter construct and either a Neg. (non-coding) miR, miR-140 or anti-miR-140 for 48 h and then harvested for luciferase assay. MiR-140 reduced luciferase signal while anti-miR-140 resulted in increased signal when compared to control.
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BIOGRAPHICAL SKETCH

Jonathan Verrier was born in Newburyport, Massachusetts, in 1980 to William and Leslie Verrier and raised in the nearby town of Byfield. After graduating from Triton Regional High School in 1998, he attended The University of Vermont and obtained a Bachelor of Science in biology, with a concentration in neurobiology, in 2002. After graduation he moved to Boston, Massachusetts, and worked as a laboratory technician for a year under the supervision of Dr. Jo-Ellen Murphy at the Harvard Institute of Medicine at Brigham and Women’s Hospital. While residing in Boston, he next obtained a Master of Science in pharmacology from the Massachusetts College of Pharmacy while under the supervision of Dr. Timothy Maher in 2005. He then moved to Gainesville, Florida to begin his studies in the Interdisciplinary Program for Biomedical Research at the University of Florida. He joined the laboratory of Dr. Lucia Notterpek in 2006.