Matrix Stiffness Regulates Glial Cell Morphology and Differentiation

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MATRIX STIFFNESS REGULATES
GLIAL CELL MORPHOLOGY AND DIFFERENTIATION

By

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THE CITY UNIVERSITY OF NEW YORK
Abstract

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By

Mateusz M. Urbanski

Advisor: Carmen Melendez-Vasquez

Studies from our laboratory have shown that inhibition of non-muscle myosin II (NMII) activity has opposite effects on the formation of myelin by oligodendrocytes (OL), the myelinating glia of the central nervous system (CNS) and Schwann cells (SC), which perform the same function in the peripheral nervous system (PNS). The decrease of NMII activity in SC impairs their ability to establish polarity and myelinate, while its inhibition in OL enhances process branching and increases the amount of myelin formed in vitro an in vivo. A growing number of studies have shown that NMII also plays a role in the ability of cells to sense and respond to the stiffness of the surrounding extracellular matrix (ECM). In the PNS, the ECM consists of a dense SC-secreted basal lamina, which displays significantly higher rigidity than the more loosely organized CNS matrix.

In order to evaluate whether the opposing effects of inhibiting NMII in glial cell differentiation and myelination are partly the result of NMII-mediated sensing of ECM stiffness, we have grown cultures of primary rat OL and SC on variable rigidity polyacrylamide matrices coated with covalently bound ECM proteins. We found that stiffer matrices inhibit OL branching as well as their expression of differentiation markers, and that these effects are correlated with
increased NMII activity. SC also respond to changes in ECM stiffness, and those grown on rigid matrices adopt a more polygonal morphology with fewer actin-based protrusions than those grown on soft matrices. Interestingly, and unlike what we have observed in the OL, stimulation of SC differentiation after cAMP treatment is not affected by differences in matrix stiffness alone. However, SC differentiation is potentiated on rigid matrices at high laminin concentration, which are conditions that mimic a mature basal lamina. Taken together, our data indicate that myelinating glial cell differentiation is sensitive to changes in the mechanical properties of the ECM and that in the case of SC, these responses may be modulated by the maturity and composition of their basal lamina.
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<td>AFM</td>
<td>atomic force microscopy</td>
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<tr>
<td>BL</td>
<td>basal lamina</td>
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<td>CNPase</td>
<td>2’,3’-cyclic nucleotide 3’-phosphodiesterase</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>DRG</td>
<td>dorsal root ganglion</td>
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<td>E</td>
<td>elastic modulus</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>kPa</td>
<td>kilopascals</td>
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<td>MBP</td>
<td>myelin basic protein</td>
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<td>MLC</td>
<td>regulatory myosin light chain</td>
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<td>MLCK</td>
<td>myosin light chain kinase</td>
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<td>MS</td>
<td>multiple sclerosis</td>
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<tr>
<td>MSC</td>
<td>mesenchymal stem cells</td>
</tr>
<tr>
<td>MYPT1</td>
<td>myosin phosphatase</td>
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<tr>
<td>OL</td>
<td>oligodendrocytes</td>
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<td>OPC</td>
<td>oligodendrocyte precursor cells</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<td>ROCK</td>
<td>Rho kinase</td>
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<td>SC</td>
<td>Schwann cells</td>
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<td>T3</td>
<td>thyroid hormone</td>
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Chapter 1: Introduction

Myelination

Myelin is a specialized glial cell membrane, wrapped concentrically around axons of peripheral nervous system (PNS) neurons by Schwann cells (SC) and central nervous system (CNS) neurons by oligodendrocytes (OL). It is a concentrically laminated structure consisting of approximately 70% lipid and 30% protein, densely compacted as a result of the fusion of the inner leaflets of the plasma membrane and the extrusion of the cytoplasm.

The compact myelin membrane has a vastly higher resistance and much lower capacitance than a simple lipid bilayer. These insulator-like properties are necessary for its best known function: allowing for the saltatory propagation of action potentials, which results in a 20-100 fold increase in the velocity of nerve conduction. While this aspect of myelin function was essential for the development of complex nervous systems in higher vertebrates, myelin also provides axons with crucial neuroprotection and metabolic support (Nave, 2010) (Fruhbeis et al., 2013) (Lopez-Verrilli et al., 2013).

Central and peripheral myelin are functionally equivalent in terms of insulating capacity and structurally similar, sharing the same polarized domains. Internodes consisting of compact myelin are interrupted by 1\(\mu\)m wide nodes of Ranvier, where voltage gated \(\text{Na}^+\) channels are clustered and action potentials are regenerated during saltatory conduction. Each node is flanked by specialized internodal regions: the paranode, in which junctional complexes of caspr and contactin anchor the paranodal glial loops to the axon, and farther in the juxtaparanode containing clusters of \(\text{K}^+\) channels (Salzer, 2003). The internodal membranes are compacted and organized
through the interaction of several major myelin proteins, such as proteolipid protein (PLP) and myelin basic protein (MBP) in the CNS, and myelin protein 0 (P0) and MBP in the PNS, as well as a number of less abundant ones (Nave and Werner, 2014). However, despite the structural similarity of OL and SC myelin, the regulatory cues that control myelination and determine which axons are wrapped vary significantly between the CNS and the PNS.

In the PNS, axonal diameter and the correlated expression levels of axonal neuregulin-1 type III are major determinants of myelination, with larger axons myelinated and smaller ones ensheathed. The diameter of 1 µm is the threshold above which myelination occurs, though ectopic expression of neuregulin-1 Type III can induce the myelination of normally unmyelinated fibers (Taveggia et al., 2005). Axonally derived signals such as neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are also capable of promoting SC differentiation and myelination (Chan et al., 2001; Chan et al., 2004). In addition to axonal cues, extracellular matrix (ECM) signaling from the SC-secreted basal lamina (BL) also plays an important role in SC differentiation. GPR126, an adhesion G protein-coupled receptor (aGPCR), is among the most important mediators of this interaction. It binds to laminin ligands of the basal lamina, and is necessary for axonal sorting as well as capable of inducing a cAMP-mediated upregulation of key promyelinating SC genes such as Oct-6 and Krox20 (Mogha et al., 2013) (Petersen et al., 2015). Another target of abaxonal ECM signaling is focal adhesion kinase (FAK), which acts downstream of laminin binding by β1 integrins to promote SC spreading and radial sorting during the early stages of differentiation (Grove and Brophy, 2014).

In the CNS, on the other hand, exogenous signals which are indispensable for initiating OL myelination have proven to be somewhat elusive. In fact, CNS myelination is to a large extent regulated by inhibitory signaling molecules, such as PSA-NCAM expressed by growing axons
(Charles et al., 2000), or the LRR and Ig domain-containing, Nogo receptor-interacting protein (LINGO1) expressed by neurons in response to the neurotrophin NGF (Lee et al., 2007). In fact, in the absence of inhibitory cues, OL are capable of myelinating dead axons and plastic nanofibers (Lee et al., 2012). This certainly does not mean that there are no positive regulators of myelination, however. For example, while ectopic expression of neuregulin-1 in normally unmyelinated axons such as those of superior cervical ganglia neurons is not enough to induce OPCs to myelinate them, mice heterozygous for neuregulin-1 do exhibit some hypomyelination (Taveggia et al., 2008), which implies that while neuregulin-1 is not sufficient and essential for OL myelination, it does promote it. Similarly, while in contrast to the PNS the ECM of the adult CNS is very sparse and there is no traditional basal lamina except for that associated with the vasculature (Colognato and Tzvetanova, 2011), laminin expression has been detected in white matter tracts prior to myelination, and laminin-integrin signaling is capable of promoting oligodendrocyte precursor cell (OPC) survival (Colognato et al., 2002). CNS laminin also contributes to OL differentiation: laminin-β1 integrin signaling promotes AKT-driven extension of myelin sheets and membrane wrapping (Barros et al., 2009), while laminin-dystroglycan interactions enhance process outgrowth of differentiating OL (Eyermann et al., 2012). Other, less immediately obvious cues also contribute: neuronal activity and synaptic vesicle release are able to enhance the myelination by nearby OL (Hines et al., 2015), while spatial constraints caused by increases in the density of OPC can increase the rate of OL differentiation (Rosenberg et al., 2008).

The most striking difference between PNS and CNS myelination, however, has its basis in glial cell morphology. While in the CNS OL branch extensively and can often establish as many as 40 myelin segments in association with multiple axons, in the PNS each SC is in a one-to-one relationship with an axon, and produces only one solitary internode of myelin. The direct driving
force behind these vastly different phenotypes is the remodeling of the cytoskeleton, a process in which complexes of actin fibers and myosin motors play an essential role. Previous work from our laboratory indicates that one particular motor protein, non-muscle myosin II (NMII), is an important regulator of myelination. Inhibition of NMII promotes OL branching, differentiation and myelination (Rusielewicz et al., 2014; Wang et al., 2012; Wang et al., 2008) but impairs SC axonal sorting, differentiation and myelin formation (Leitman et al., 2011; Wang et al., 2008).

**Non-muscle myosin II**

Myosins are a family of motor proteins which generate force in an ATP-dependent manner, with the best known being the class II myosins responsible for skeletal, cardiac and smooth muscle contraction. The non-muscle class II myosins (NMII) perform a similar role, but on a much smaller scale – they interact with the actin cytoskeleton to regulate force generation and transmission at the cellular level. This makes them key players in maintaining cell shape and regulating morphology, cell migration, division, and many processes involving translating external forces into intracellular signals. Non-muscle myosins are present in all eukaryotic cells, and are hexameric molecules consisting of two 220 kD heavy chains (MHC), two 17 kD essential light chains, and two 20 kD regulatory light chains (MLC). NMII variants are differentiated by their heavy chains, and in mammals the MYH9, MYH10 and MYH14 genes encode the NMIIA, NMIIB and NMIIIC isoforms (Conti and Adelstein (2008)). The MHCs consist of a globular head domain which contains both actin and ATP binding sites, a neck region to which the essential and regulatory MLC bind, and a long alpha-helical tail domain necessary for the formation of MHC homodimers, the tails of which can further interact with other NMII molecules to form bipolar filaments capable of cross-linking the actin cytoskeleton. Force generation by these filaments relies
on the ATPase activity of the MHC heads, which in turn depends on the phosphorylation of the regulatory MLCs on Ser19, with Thr18 phosphorylation serving to further enhance NMII activity (Hirata et al., 2009; Sellers et al., 1982). The key players responsible for MLC phosphorylation status are the Ca\textsuperscript{2+}/calmodulin dependent myosin light chain kinase (MLCK) and the Rho-associated coil coil-containing kinase (ROCK), as well as the myosin phosphatase MYPT1. (Figure 1.1) MLCK and ROCK are both capable of phosphorylating the regulatory MLC on Ser19/Thr18, although ROCK has also been shown to negatively regulate MYPT1 activity (Feng et al., 1999).
Figure 1.1 Key regulators of NMII activity.

Non-muscle myosin II activity is upregulated by the phosphorylation of its regulatory light chain (MLC). MLC phosphorylation is upregulated by the Ca$^{2+}$/calmodulin dependent myosin light chain kinase (MLCK) as well as the Rho-associated coil coil-containing kinase (ROCK), and negatively regulated by myosin phosphatase MYPT1. Both MLCK and ROCK phosphorylate the regulatory MLC on Ser19/Thr18, but ROCK is also involved in the inhibition of MYPT1 activity (Sellers et al. 1982; Feng et al. 1999; Hirata et al. 2009).
Actin polymerization and NMII inhibition drive oligodendrocyte myelination

Oligodendrocytes (OL) – the myelinating glia of the CNS – are derived from the ventricular zone and cortex of the embryonic mammalian brain (Kessaris et al., 2006). In the course of their development and in myelination, OL exhibit dramatic morphological changes. While they begin as bipolar, motile and mitotically active oligodendrocyte precursor cells (OPC) which respond to platelet-derived growth factor (PDGF) (Richardson et al., 1988), maturing OL extend a large number of increasingly complex processes culminating in wraps of myelin in vivo, or elaborate lamellar structures containing myelin proteins when cultured in the absence of axons in vitro. These proteins includes such maturation markers and myelin components as 2’,3’-cyclic nucleotide 3’-phosphodiesterase (CNPase) during earlier stages of differentiation (Reynolds and Wilkin, 1988), and proteolipid protein (PLP) and myelin basic protein (MBP) as they terminally differentiate (Monge et al., 1986).

The morphological changes which occur during OL differentiation requires extensive remodeling of the actomyosin cytoskeleton, and therefore are largely driven by actin polymerization. Disrupting the mechanism of actin polymerization by the knockout of WAVE1, a key regulator of actin nucleation, results in impairment of OL process extension and branching, reduced expression of differentiation markers, and a significant reduction in myelin formation (Kim et al., 2006), indicating that morphological maturation precedes and promotes OL differentiation.

Since NMII activity promotes the retraction of actin-based processes and inhibits the formation of lamellar structures, one might predict that NMII is a negative regulator of OL differentiation, and inhibition of NMII activity should promote myelination - and that is in fact what prior work from our laboratory (Figure 1.2) established. Initial in vitro studies performed in
cultures of OL alone and in co-cultures of OL and dorsal rat ganglion (DRG) neurons verified that a decrease in Myosin II activity is an inherent part of OL maturation, and showed that inhibition of Myosin II activity through small molecule inhibitors or siRNA leads to an increase in OL morphological complexity and increases the number of myelin segments produced in co-cultures (Wang et al., 2008). These effects were further confirmed by experiments in which over-expression of myosin II or of a constitutively active form of its positive regulator RhoA led to a marked decrease in OL branching, while mouse OL in which myosin IIB had been knocked out displayed more rapid morphological maturation and higher levels of expression of one of the key myelin components, MBP (Wang et al., 2012).
Figure 1.2 Inhibition of myosin II enhances myelin formation and OL branching.

A. Myelinating OL-DRG cocultures, stained for MBP (green) and neurofilament (blue). Treatment with the myosin II inhibitor blebbistatin resulted in a prominent increase in the amount of myelin segments formed. B. Quantitation of the number of myelin segments formed per single OL in cultures. A significant increase in the average number of myelin segments made by a single OL in treated cultures was observed (p<0.001, t-test). C. Analysis of OL branching was carried out in cultures kept for 3 days in differentiation promoting media in the absence or presence of blebbistatin. The mean fractal dimension (D), which correlates with the extent of cell arborization, was significantly higher in blebbistatin treated cultures (control 1.37 ± 0.12 vs. treated 1.46 ± 0.08; mean ± SD; p<0.0001; t-test), indicating a more complex cytoskeleton branching in the absence of myosin II activity (Wang et al., 2008).
Schwann cell myelination requires NMII activity and ECM cues

Schwann cell precursors (SCP) originate within the neural crest, and migrate from it alongside developing nerves during intermediate stages of embryonic development. These SCPs give rise to immature Schwann cells, which associate with axons and either ensheath multiple small caliber axons or sort single large caliber axons for myelination (Reviewed in (Jessen and Mirsky, 2005)). The markers of Schwann cell differentiation into myelinating cells have been well characterized, and include the decrease in the levels of the negative regulator of myelination c-Jun (Parkinson et al., 2008), as well as an upregulation of positive regulatory transcription factors such as Oct-6 and Krox20 (Monuki et al., 1989; Parkinson et al., 2004).

Unlike what we have observed in the CNS, where inhibition of NMII activity is enough to produce a very significant increase in myelination, SC differentiation is actually significantly impaired by downregulation of NMII or its regulators. Prior work from our laboratory (Figure 1.3) shows that inhibition of myosin II activity in myelinating co-cultures of SC and DRG neurons by MLC shRNA treatment results in highly abnormal SC morphology (Wang et al., 2008). Rather than sort individual axons, SC extend multiple long processes that often fail to contact axons. When there is axonal contact, the expression of N-Cadherin, which promotes initial SC alignment with axons (Wanner and Wood, 2002), is patchy and aberrant. This results in a significant reduction in the number of myelin segments produced. Likewise, while inhibition of the key NMII regulator MLCK initially results in a pattern of gene expression reminiscent of SC differentiation, these SC are incapable of myelination in a SC/DRG co-culture system. Interfering with this aspect of NMII regulatory machinery apparently decouples the initiation of the differentiation program from myelin assembly (Leitman et al., 2011).
SC differentiation and myelination is also a process which depends very heavily on ECM – in this case, the abaxonal SC-secreted basal lamina – and ECM adhesion molecules. For example, genetic ablation in mice of the α2 subunit of laminin-211, which is a major basal lamina component, results in SC production of thin patchy myelin, and leads to skeletal muscle dystrophy in mice (Nakagawa et al., 2001). Similarly, disruption of laminin-411, which is also a BL component, leads to impaired radial sorting, reduced myelination, and ataxia. Comparable deficiencies can be observed in myelination by SC in which β1 integrin, a critical subunit of the laminin receptor α6β1, has been deleted (Feltri et al., 2002), demonstrating that signaling from the dense, ECM-rich BL is essential for normal SC myelination and axonal health.

Figure 1.3 Knockdown of myosin II by shRNA results in abnormal SC morphology and inhibits myelination.

A. Detail of a SC expressing shRNA targetting MLC (green) in coculture with axons (blue). The SC has multiple long processes, some of which appear to contact axons (asterisks), while others fail to do so (arrowheads). B. SC infected with a lentivirus expressing shRNA against the regulatory chain of myosin II (shMLC) or a scrambled non-targeting sequence (shSCR) were seeded onto purified dorsal root ganglion (DRG) neurons and allowed to myelinate. Cocultures were stained for MBP (red) and neurofilament (blue). A clear reduction in the amount of myelin formed in cocultures by SC expressing shMLC compared to those expressing non-targeting (SCR) shRNA was consistently observed. Right panel: Quantitation of MBP+ segments in 18 day myelinating cocultures infected with shMLC or non-targeting control (shSCR) (Wang et al., 2008).
NMII in mechanotransduction

Differentiation and development are often thought of in terms of cells responding to chemical cues by the activation of signal transduction pathways culminating in the translation of target genes. The study of mechanotransduction expands on this view by demonstrating that physical forces acting on cells are also capable of regulating both cell morphology and gene expression, guiding differentiation and development.

The most basic, and most intuitive example of mechanotransduction at work is the regulation of cell morphology by ECM elasticity. Most cells are surrounded by ECM, and connected to it by adhesive complexes tethered to the cytoskeleton. This means that any physical forces acting on the tissue and ECM are transmitted to the interior of individual cells – and in order to maintain its shape and resist deformation, a cell must regulate the contractile activity of its actomyosin cytoskeleton to match its elastic properties to those of the surrounding ECM (Reviewed in (Clark et al., 2007) and (Vicente-Manzanares et al., 2009)). The process starts when integrins, which are localized in nascent adhesive complexes, are activated by their binding to ECM ligands and by the tension of the ECM. This in turn leads to stimulation of RhoA and ROCK signaling, which promotes myosin activity by regulatory MLC phosphorylation, and also contributes to actin polymerization. Ultimately, the increase in NMII contractility and actin polymerization cause conformational changes within the adhesive complex that lead to increased integrin clustering and activation, forming a feedback loop and generating a level of tension matching the ECM (Choquet et al., 1997). This plays an important role in cell adhesion, and the resulting polarization of the actomyosin cytoskeleton is also involved in durotaxis, or cell migration from soft to rigid ECM (Wang et al., 2001) (Raab et al., 2012). This phenomenon has great physiological relevance, due to its involvement in malignant transformation and metastasis:
increased ECM stiffness upregulates focal adhesion assembly and promotes tumor invasion, and does so in a Rho GTPase dependent manner (Parri and Chiarugi, 2010).

Increased cytoskeletal tension in response to ECM engagement and rigidity also has particularly notable effects on the regulation of cell protrusion and branching and has been demonstrated repeatedly in endothelial tip cells (EC) (Fischer et al., 2009), cortical neurons (Georges et al., 2006) and mesenchymal stem cells (MSC) (Engler et al., 2006). Cells plated on soft ECM-like substrates extend larger numbers of more complex protrusions than those grown on rigid ones, and in EC and MSC the ability to respond to ECM stiffness was demonstrated to require NMII activity, with myosin contractility acting as a “brake” on the protrusion of actin-based processes and localized decreases in NMII activity often preceding the initiation of new branches (Fischer et al., 2009).

However, mechanotransduction is not limited to exerting control over morphology and migration – it can also regulate transcriptional activity and direct the differentiation of uncommitted cells. Bodily tissues vary significantly in ECM composition, and it has emerged that seeding progenitors or stem cells on substrates matching the elastic properties of specific tissues can be used to direct the cells to activate transcriptional programs corresponding to that tissue type. For example, soft ECM-like matrices (0.1-1.0 kPa) are capable of directing mesenchymal stem cells to assume an adipocyte-like profile of gene expression, while more rigid ones (25-40 kPa) direct the cells toward an osteocyte-like fate. However, NMII inhibition and disruption of cytoskeletal tension abolishes these cellular responses to ECM rigidity (Engler et al., 2006), as does the disruption of the RhoA-based NMII regulatory machinery (McBeath et al., 2004). Although the exact mechanisms by which this cytoskeletal tension is transmitted to the nucleus is
not yet completely understood, the transcriptional cofactors YAP/TAZ as well as the components of the nuclear lamina appear to play a prominent role in the process.

YAP/TAZ, perhaps best known for their role in the Hippo pathway and growth regulation, have recently emerged as mechanotransduction’s master regulators, translocating between the cytoplasm and the nucleus in response to mechanical cues. In mesenchymal stem cells (MSC) grown on soft 1 kPa matrices which resemble adipose tissue, YAP/TAZ localizes to the cytoplasm, and causes the MSC to develop into adipocytes. Conversely, on 40 kPa matrices resembling cartilage, YAP/TAZ translocates to the nucleus and causes the MSC to differentiate into osteoblasts (Dupont et al., 2011). YAP/TAZ knockdown is capable of directing cells grown on a rigid matrix to develop into adipocytes, while YAP/TAZ overexpression can overcome the effects of soft ECM, and direct MSC growing on it to develop into osteocytes. Notably, YAP/TAZ only translocates in response to variations in ECM stiffness if the contractile machinery of the cells is intact – inhibition of NMII or RhoA/ROCK activity renders cells unable to respond to changes in YAP/TAZ levels and localization. YAP/TAZ localization also appears to be regulated by PKA activity, in that PKA promotes the phosphorylation and cytoplasmic retention of YAP/TAZ (Yu et al., 2013) in a process that also involves downregulation of RhoA activity (Yu et al., 2012). (See Figure 1.4 for summary of YAP/TAZ NMI-mediated regulation.) Effectively, downregulation of either NMII or YAP/TAZ causes MSC to differentiate as if they were growing on a soft matrix or developing within a soft tissue.
Figure 1.4 RhoA-dependent mechanism of YAP/TAZ localization.

Increased matrix stiffness promotes integrin engagement, RhoA/ROCK activity and actomyosin contractility, (Wada et al., 2011) leading to a nuclear localization of YAP/TAZ. YAP/TAZ direct the lineage specification of MSC, with cytoplasmic YAP/TAZ promoting adipogenesis, and nuclear YAP/TAZ osteogenesis (Dupont et al., 2011). YAP/TAZ localization is also regulated by phosphorylation via the LATS kinases, which are themselves negatively regulated by RhoA. Phosphorylated cytoplasmic YAP/TAZ is targeted for degradation in a PKA dependent process (Yu et al., 2013) that also leads to a downregulation of RhoA activity (Yu et al., 2012).
Another possible mechanism contributing to how signals are transduced to the nucleus involves the nuclear lamina. The nuclear lamina is the structural framework supporting the nuclear membrane, and its main components are the intermediate filament proteins lamin A and lamin B. Mutations in the lamin genes are associated with a wide range of pathologies, with lamin A being implicated in autosomal-dominant Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy and Hutchinson-Gilford progeria syndrome, to name a few (Dechat et al., 2008). Interestingly, while there are far fewer diseases associated with lamin B defects, one of them is adult-onset autosomal dominant leukodystrophy, in which a duplication of the lamin B gene leads to fatal loss of myelin (Padiath et al., 2006) (Lin et al., 2014). As a support structure, the nuclear lamina maintains nuclear shape, anchors the nucleus to the actin cytoskeleton, and participates in the organization and localization of chromatin (Reviewed in (Puckelwartz et al., 2011)). The breaking of this linkage, as seen for example in dilated cardiomyopathy, leads to a disruption of the cytoskeleton, abnormal nuclear shape, and aberrant heterochromatin clumping (Nikolova et al., 2004).

Recently, it was established that ECM rigidity, which translates into stiffness of the actomyosin cytoskeleton and NMII activity, is capable of regulating the expression and localization of nuclear lamins (Swift et al., 2013). These findings show that lamin A expression increases with tissue stiffness, while lamin B predominates in soft tissues such as the brain, and that changes in lamin A levels are capable of regulating MSC differentiation. Specifically, soft ECM which induces low levels of actomyosin contractility reduces the levels of lamin A and promotes MSC differentiation into adipocytes, while rigid ECM which upregulates cytoskeletal tension leads to an increase in lamin A levels and skews MSC differentiation toward osteogenesis. Furthermore, increases in lamin A levels within the nuclear envelope also appear to activate a
system of positive feedback by promoting serum response factor (SRF) activity, which in turn leads to increased actomyosin expression (Buxboim et al., 2014; Ho et al., 2013). (See Figure 1.5 for overview of lamin A response to ECM rigidity.)

In summary, ECM stiffness is capable of regulating NMII activity and the tension of the actomyosin cytoskeleton, which directly influences cell morphology and migration, and acts through secondary mediators such as YAP/TAZ and nuclear lamins to regulate the differentiation of uncommitted cells. In view of our previous work on myelination, this raises the question of whether the disparate impact of NMII activity on the morphology and differentiation of oligodendrocytes and Schwann cells is a reflection of the differences in the physical properties of the CNS and the PNS.
Increased tissue stiffness or matrix rigidity increases the nuclear levels of lamin A, a major structural component of the nuclear lamina, resulting in changes in chromatin organization and gene transcription (Swift et al., 2013). Increased lamin A expression also promotes the activity of the serum response factor (SRF). This generates a feedback loop through increased expression of actomyosin cytoskeleton components including NMII, (Buxboim et al., 2014) leading to increased stress fiber formation and greater cytoskeletal tension (Ho et al., 2013).
The mechanical properties of what are commonly called soft tissues vary widely, across multiple orders of magnitude (Reviewed in (Levental et al., 2007)), and have an acknowledged role in development (Wozniak and Chen, 2009). They have also been shown to influence various pathologies – changes in ECM composition contributes to the metastatic activity of many cancers (Pickup et al., 2014), while an increase in tissue stiffness correlates with the development of fibrosis in the liver (Georges et al., 2007) and lungs (Dolhnikoff et al., 1999).

There is ample evidence that ECM composition and elasticity of CNS tissues are also affected by disease and injury, ranging from the increased tissue stiffness observed in chronic multiple sclerosis (Cedric Raine, personal communication) (Ludwin and Raine, 2008), to fibrosis caused by spinal cord injury (Soderblom et al., 2013) or stroke (Fernandez-Klett et al., 2013). In addition, it has been shown that in active multiple sclerosis lesions there is a marked increase in the deposition of laminin, collagen and heparin sulfate proteoglycans within the brain parenchyma (van Horssen et al., 2006), while in mouse models, ethidium bromide-induced demyelination results in increased expression of tenascin, fibronectin and vitronectin (Zhao et al., 2009). This suggests that the observed increase in stiffness can be a result of increased ECM deposition. However, while implantation of elastic substrates to promote optimal wound healing and minimize scar formation has been proposed (Khaing et al., 2014), and there are ongoing efforts to generate autologous neuronal and glial progenitors for transplantation, little work has been done to evaluate the effect of ECM stiffness and mechanosensing on CNS regeneration, especially in the context of myelinating glia rather than neurons.
Similarly, while there have been many attempts to generate implantable biomaterial scaffolds or engineered tissue grafts to promote peripheral nerve repair after injury (Daly et al., 2013), and efforts to design engineered tissue grafts composed of extracellular matrix protein and SC have demonstrated that mechanical forces promote ECM reorganization conducive to nerve repair (Georgiou et al., 2013), the effect of the specific mechanical properties of these scaffolds on both axonal outgrowth and SC differentiation has not been examined.

**What role does matrix rigidity play in regulating glial cell differentiation?**

Mechanotransduction is the ability of cells to convert mechanical stimuli from their environment into biochemical signals. One common type of mechanotransduction involves the upregulation of cytoskeletal tension and NMII activity in response to increases in the rigidity of the ECM. This process has been shown to play a role in regulating the morphology of a wide range of cells, from fibroblasts to endothelial cells and neurons. It has also been implicated in controlling the morphology and lineage commitment of mesenchymal stem cells.

The differentiation of myelinating glia is also dependent on NMII activity: inhibition of NMII enhances myelination in the CNS, and impairs it in the PNS, and both OL and SC respond strongly to modulation of NMII activity. We also know that the ECM of the brain is diffuse and highly viscoelastic, while that of the PNS, where SC are surrounded by a dense basal lamina containing large amounts of laminin and collagen, is much more rigid.

We therefore hypothesize that ECM stiffness, sensed in a myosin II-dependent manner, plays a role in regulating glial cell morphology and differentiation (Figure 1.6) and propose to examine the hypothesis by testing the following:
a) Does the elasticity of the ECM affect the branching morphology of glial cells?

b) Is the branching of cells grown in a rigid ECM inhibited by myosin II activity?

c) Does variation in ECM elasticity affect the ability of glial cells to differentiate?

Figure 1.6 Proposed model of ECM regulation of glial morphology and differentiation.
Low ECM stiffness in the CNS contributes to low levels of actomyosin contractility and promotes oligodendrocyte branching and differentiation. Conversely, the rigid ECM of the PNS promotes actomyosin activity, which is required for SC differentiation and myelination.
Chapter 2: Design and validation of an in vitro model of variable ECM rigidity

In vitro models of ECM matrix stiffness allow for the study of developmentally relevant processes such as cell migration and differentiation without the many challenges and confounding factors present in an in vivo system. For our work, we elected to use an approach using synthetic polyacrylamide (PA) substrates representing ECM of specific stiffness seeded with primary rodent oligodendrocytes and Schwann cells. While it is possible to fabricate gel substrates from purified ECM proteins or protein mixtures such as collagen, fibronectin or matrigel, PA substrates offer a number of important advantages. Their elastic modulus is simple to regulate by varying the relative concentration of polyacrylamide and bis-acrylamide, the ligand-binding capacity of PA does not vary significantly with changes in its mechanical properties, nonspecific binding to the PA surface is minimal, and it is a method which has been used extensively in the mechanotransduction field. The system uses glass cover slips coated with a thin (~50 µm) layer of polyacrylamide gel that is then modified by covalent attachment of ECM proteins. The resulting “sandwich gel”, described by (Tse and Engler, 2010) and (Fischer et al., 2009) provides a uniform surface suitable for cell culture, and reliably simulates an ECM of a given rigidity. (See Figure 2.1 and the materials and methods section for details of preparation.)

The stiffness of ECM can be expressed in terms of elastic modulus or “E” values, which corresponds to force applied per unit area divided by the resulting change in length, measured in kilopascals (kPa). The values we selected for our substrates were ~ 1.5 kPa (“soft gels”) and ~ 30.0 kPa (“rigid gels”), and were confirmed using atomic force microscopy (AFM). (Table 2.1) The values were chosen to fall within the range of those reported for normal rodent brain tissue (0.1-1.6 kPa) and fibrotic scar tissue (30.0-40.0 kPa) (Raab et al., 2012; Tyler, 2012). For our PNS model, due to a lack of reliable published values for peripheral nerves, we elected to perform AFM
measurements of adult rodent sciatic nerves. Our data (Figure 2.2, Table 2.2) indicated a value range of 5 kPa (single teased nerves with intact basal lamina) to 50 kPa (intact nerve bundle 200 µm in diameter), with intermediate-sized bundles falling in between these two extremes, while neonatal sciatic nerves for which we have gathered preliminary AFM data have stiffness values in the 6-12 kPa range. (data not shown) This allowed us to also use the ~1.5 kPa soft gels (well below the physiological range in the PNS) and ~30.0 kPa rigid gels (the average of the measured physiological range) for the SC experiments. We also independently confirmed that the published brain elasticity values matched those obtained using our method of AFM measurement. (Table 2.2)

In order to verify that apparent effects on cell differentiation were not due to changes in cell survival or proliferation, we evaluated cell death by ethidium homodimer-1 / calcein AM labeling, and cell proliferation index by Ki-67 staining. There was no apparent change in cell viability due to matrix elasticity for either OL or SC, with overall viability > 98%. The proliferation of OL was likewise not significantly affected, but the Ki-67 index of SC grown on rigid matrices, while not high, did show a statistically significant increase over those grown on soft matrices. (Figure 2.3) This effect of ECM rigidity on proliferation had been previously reported in other cell types (Mih et al., 2012), and does not represent a significant confounding factor during the relatively short time frame of our experiments.

In summary, our in vitro model produces substrates which are highly reproducible in terms of their mechanical properties, closely models the range of elastic properties encountered in CNS and PNS tissues, and allows for culture and differentiation of primary glial cells with no adverse effects on cell survival. Chapter 3 describes our experimental results obtained through the use this system to culture and differentiate primary rodent oligodendrocytes and Schwann cells.
Figure 2.1. Preparation of ECM protein-coated polyacrylamide substrates.

Polyacrylamide coated glass cover slips are covered with a cross-linker solution (sulfo-SANPAH) and irradiated for 5 minutes with 365nm UV light. The substrates are washed, then covered with a dilute solution of ECM protein and kept overnight in a tissue culture incubator. They are then extensively rinsed with PBS, and ready to be seeded with cells (Tse and Engler, 2010).
Figure 2.2 AFM force mapping of rodent sciatic nerve.

AFM force maps of sciatic nerves embedded in elastic mounting medium. A. Large diameter non-dissociated nerves bundles composed of dozens of axons exhibit very high stiffness values. The areas of lower stiffness (20-30 kPa) visible in the lower left side of both panels represent mounting medium used to stabilize the tissue. B. Individual PNS nerve fibers surrounded by basal lamina are less stiff than intact nerves, but still several times more rigid than CNS tissue. C. Brightfield image showing the nerve segment analyzed in the top panel of section “B”, AFM probe visible on the lower left.
Figure 2.3 Proliferation of Schwann Cells (SC) and oligodendrocyte progenitors (OPC) on soft (1.5 kPa) and stiff (30.0 kPa) substrates.

There was a modest but significant increase (Mann Whitney t-test, p<0.001) in SC proliferation in stiffer matrices compared to softer ones. While OPC cultures showed the same trend, the difference in proliferation was not statistically significant. (Mann Whitney t-test, p=0.06)

Table 2.1 Measured elastic modulus of PA gels after polymerization.

<table>
<thead>
<tr>
<th></th>
<th>Acrylamide %</th>
<th>Bis-Acrylamide %</th>
<th>$E \pm SD$ (kPa)</th>
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<tr>
<td>Soft Matrix</td>
<td>7.5</td>
<td>0.05</td>
<td>1.35 ± 0.09</td>
</tr>
<tr>
<td>Rigid Matrix</td>
<td>12.0</td>
<td>0.28</td>
<td>33.15 ± 2.87</td>
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Relative concentrations of acrylamide and bis-acrylamide for the soft and rigid PA gels used in these studies and their elasticity modulus as measured by AFM. Four PA hydrogels per condition and 768 indentations per hydrogel (256 indentations in 3 separate 90x90 µm areas per gel) were assessed for these measurements.
Table 2.2 Measured elastic modulus of mouse brain and sciatic nerve.

<table>
<thead>
<tr>
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<th>E ± SD (kPa)</th>
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<tbody>
<tr>
<td><strong>Cortex &amp; White Matter</strong></td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td><strong>Teased fiber (15 μm)</strong></td>
<td>5.3 ± 0.9</td>
</tr>
<tr>
<td><strong>Intact bundle (200 μm)</strong></td>
<td>49.4 ± 19.0</td>
</tr>
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</table>

Elastic modulus values obtained from fresh adult mouse sciatic nerves (10 mm long) and fresh coronal adult mouse brain slices. After removal of the surrounding fat and connective tissue, the elasticity of exposed intact nerve bundles and tissue slices was determined using AFM nano-indentation.
Chapter 3: Myelinating glial differentiation is regulated by extracellular matrix elasticity

Rigid ECM inhibits OPC branching complexity

During oligodendrocyte differentiation in vitro OL extend increasingly complex branches in a process dependent on actin polymerization, which eventually fuse into elaborate lamellar sheets. In order to determine whether this is a mechanosensitive process which responds to ECM elasticity, we seeded rat OPCs on soft (~1.5 kPa) and rigid (~30.0 kPa) PA/matrigel substrates, chosen to correspond to the viscoelasticity of the normal brain and that of fibrous scar tissue, respectively. The cells were grown in conditions promoting proliferation (10ng/mL PDGF/bFGF) for 48 hours, followed by fixation and visualization of the actin cytoskeleton by immunofluorescence. Proliferating OPCs grown on soft 1.5 kPa matrices displayed a marked increase in branching compared to those grown on rigid 30.0 kPa ECM. (Figure 3.1 A) Quantification of the morphological differences showed that on soft ECM, the percentage of cells exhibiting complex morphology (represented as Type II and Type III cells) was significantly higher than on the rigid matrix (51%±6 vs 44%±6 of Type II; mean±SEM, p=0.003; 28% ±3 vs 22%±3 of Type III; mean±SEM, p=0.02). Conversely, the rigid 30.0 kPa matrix significantly increased the percentage of cells of low complexity (Type I) in comparison with the 1.5 kPa substrate (24%±3 vs. 14%±2; mean±SEM, p<0.001). (Figure 3.1 B) This demonstrates that soft matrices with viscoelasticity comparable to the normal brain promote OPC branching, while rigid ones significantly inhibit it. Furthermore, the inhibitory effect of stiff matrices on OPC branching was removed by treatment with the ROCK kinase inhibitor Y-27632 (Figure 3.2 A-B), which is consistent with previous reports that Rho/ROCK signaling and myosin II activity are involved in controlling OL branching (Liang et al., 2004), (Wang et al., 2012), as well as with the established
importance of Rho/ROCK activity for mechanosensing (McBeath et al., 2004). This was further confirmed by the observation that OPC purified from \textit{PlpCre/ESR1}:Rosa26-mT/mG:NMII \textit{fl/fl} and \textit{PlpCre/ESR1}:Rosa26-mT/mG:NMII \textit{B+/+} in which MIIB was conditionally ablated in oligodendrocytes (Rusielewicz et al., 2014) and grown on rigid matrices displayed increased branching complexity when compared to OPC from control animals. (Figure 3.2 C)

**Figure 3.1 Rigid ECM inhibits OPC branching complexity.**

A. OPC cultured for 48 hrs in proliferation-promoting medium display a significant decrease in branching complexity when grown on rigid substrates (right panel) compared to those grown in soft brain-like matrices (left panel). B. Quantitation of OL branching complexity. Cultures grown on the stiff matrix (30 kPa) display a large increase in the number of cells of low complexity ($p<0.001$, t-test) and a significant decrease in the number of high-complexity cells. (Type 2: $p=0.003$, t-test; Type 3: $p=0.02$, t-test). Data represent mean±SEM, approximately 1000 cells from two independent experiments were analyzed per condition. (Scale bars 25 µm.)
Figure 3.2 NMII inhibition rescues OPC branching on rigid ECM.

A

1.5 kPa

30.0 kPa

30.0 kPa + Yinh

Phalloidin/DAPI

B

1.5 kPa

30.0 kPa

30 kPa + Yinh

\% OPC

Type 0

Type 1

Type 2

Type 3

*C

**

***

C

WT

KO

\% OPC

Type 0

Type 1

Type 2

Type 3
Figure 3.2 NMII inhibition rescues OPC branching on rigid ECM.

A. In OPC cultured for 48 hrs in proliferation-promoting medium, inhibition of NMII activity with the Y-27632 ROCK inhibitor results in the rescue of OPC branching in cultures grown on rigid substrates (bottom panel). B. Quantitation of OL branching complexity. Cultures grown on the stiff matrix in the presence of Y-27632 exhibit a number of high complexity cells comparable to cultures grown on the soft, brain like matrices. Data represent mean±SEM, approximately 1000 cells from two independent experiments were analyzed per condition. C. Ablation of NMIIB rescues OPC branching in stiff matrices. OPC derived from NMIIB conditional knockout mice (KO) exhibited increased branching when grown on rigid matrix compared to cells derived from wild type (WT). (Scale bars 25 µm.)
Rigid ECM inhibits T3-induced OPC maturation

To examine whether the effects of matrix stiffness extend beyond regulation of OPC branching morphology, OPC were cultured on either soft 1.5 kPa or rigid 30.0 kPa substrates for 48 hours in the presence of 30 ng/mL thyroid hormone (T3). Similar to what we observed in proliferating cells, OPC branching complexity was significantly inhibited by rigid matrices. (Figure 3.3 A) Quantitation analysis of the T3 treated cultures indicated that on 30 kPa matrices, the percentage of low-complexity cells (Type I) increased significantly (12% ±2.6 vs. 4% ±2; mean±SEM, p<0.001) in comparison to the cultures grown on the less rigid matrices, while the percentage of high complexity cells (Type III) was decreased (55%±3.4 vs. 67%±3.5; mean±SEM, p=0.04) (Figure 3.3 B) Additionally, we also observed an effect on OPC expression of maturation markers, as cells grown on the rigid 30.0 kPa matrix exhibited a significant decrease in the levels of Rip/CNPase and MBP (Figure 3.3 A,D) when compared to those cultured on the soft 1.5 kPa matrix. (Rip: 13% ± 1.9 vs. 25% ± 1.7; MBP: 4% ± 0.4 vs. 8% ± 0.5; mean ± SEM, p<0.001) These effects on OPC differentiation were accompanied by increased expression of pMLC and therefore higher NMII activity in the 30.0 kPa condition (Figure 3.3 C), which is consistent with prior work showing that NMII inhibition has the opposite effects and promotes both branching and MBP expression (Wang et al., 2012).
Figure 3.3 Rigid ECM inhibits T3-induced maturation of OPC.
Figure 3.3 Rigid ECM inhibits T3-induced maturation of OPC.

A. OPC cultured on rigid substrates and treated with differentiation-promoting medium (T3) for 48 hours display a decrease in branching complexity and the levels of maturation markers Rip/CNPase and MBP. B. Quantitation of branching complexity. Differentiating cultures grown on the stiff matrix display a significant increase in the number of cells of low complexity (p<0.0001, t-test) and a decrease in the number of high-complexity cells. (p=0.04, t-test) Data represent mean±SEM, approximately 1000 cells from two independent experiments were analyzed per condition. C. Increased phosphorylation of the regulatory myosin light chain (pMLC2) was detected in OL grown in stiffer matrices, indicating higher NMII activity. D. Cultures grown on the stiff matrix exhibit a highly significant (p<0.001, t-test) decrease in the number Rip/CNPase+ and MBP+ cells per field. (Scale bars 25 µm.)
OL grown on rigid ECM display higher levels of nuclear Olig1

In the early stages of OL differentiation, the basic helix-loop-helix transcription factor Olig1 localizes to the nucleus and drives the expression of myelin genes, such as MBP (Li et al., 2007). However, as the OL differentiation progresses, a significant fraction of Olig1 is phosphorylated and translocates from the nucleus into the cytoplasm, (Figure 3.4 A) which is necessary for the expansion of MBP-positive membrane during OL maturation (Niu et al., 2012). Since the effects of cytoplasmic translocation of Olig1 parallel those of a reduction in NMII activity or the OL morphological response to being cultured on soft ECM, we investigated whether Olig1 localization is dependent on the mechanical properties of the ECM, as that would provide a link between mechanical forces acting on the OL and the regulation of myelin gene expression.

In order to evaluate Olig1 translocation in response to mechanical cues, we first seeded OPC onto 1.5 kPa and 30.0 kPa matrices under proliferating conditions, and found that after 48hrs in culture, the level of nuclear Olig1 as visualized by IF was significantly increased in cells grown on the 30.0 kPa matrix. (Figure 3.4 B-C) To determine whether this was also a factor during OL differentiation, we then biochemically assayed the levels of Olig1 in OPC cultured for 48hrs in a T3-containing medium. While overall expression of Olig1 did not change, nuclear levels of Olig1 were nearly 90% higher (Figure 3.4 D-E) in cells grown on the 30.0 kPa matrices than they were in cells in the 1.5 kPa condition. This was accompanied by an increase in pMLC in cells grown on the more rigid ECM. (Figure 3.4 D-E) Since the key Olig1 residue necessary for its translocation to the cytoplasm is located within a well-conserved predicted PKA phosphorylation site, we also examined the levels of phosphorylated PKA substrates, and found that PKA phosphorylation of substrates matching the molecular weight of Olig1 increased in OPC grown on soft 1.5 kPa
substrates in comparison to the 30.0 kPa ones (Figure 3.4 D-E), which is consistent with the mechanism of Olig1 translocation described by (Niu et al., 2012).
Figure 3.4 Rigid ECM promotes nuclear retention of Olig1.

A

PDGF

T3

Nuclear Olig1

B

PDGF

Olig1

DAPI

Olig1/DAPI

C

Olig1

1.5 kPa

30.0 kPa

D

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<tr>
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</tr>
<tr>
<td>Olig1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKAp(S/T)</td>
<td></td>
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</tr>
<tr>
<td>Actin</td>
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<td></td>
<td></td>
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<tr>
<td>Histone H3</td>
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</tr>
<tr>
<td>Nuclear</td>
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</table>

Relative Intensity

PDGF

Relative Intensity

PKAp(S/T)

Relative Intensity

Olig1

1.5 kPa

30.0 kPa

Relative Intensity

Nuclear Olig1

1.5 kPa

30.0 kPa
Figure 3.4 Rigid ECM promotes nuclear retention of Olig1.

A. Representative images from OPC cultures showing a decrease in nuclear Olig1 and its increased cytoplasmic localization as they progress toward a more mature phenotype. **B-C.** OPC grown on stiffer matrices under proliferative conditions for 48 hrs display a highly significant increase in nuclear Olig1 levels (p<0.0001). Data represent mean±SEM, approximately 1000 cells from two independent experiments were analyzed per condition. **D-E.** In OPC grown under differentiation-promoting conditions for 48 hrs, increased matrix stiffness promotes higher levels of phosphorylated regulatory myosin light chain (MLC2) and phosphorylated PKA substrates, as well as elevated levels of nuclear Olig1. (3 experiments) (Scale bars 25 µm.)
OL differentiating on rigid ECM exhibit elevated nuclear YAP/TAZ and nuclear lamin A

The transcriptional coactivators YAP/TAZ have been shown to play a key role in transmitting mechanical signals to the nucleus and regulating differentiation in response to matrix elasticity. Rigid ECM promotes the differentiation of MSC into osteocytes which is accompanied by nuclear YAP/TAZ localization, while soft ECM drives adipogenesis (Dupont et al., 2011) and causes YAP/TAZ to accumulate in the cytoplasm, where it can be targeted for inhibitory phosphorylation and degradation (Yu et al., 2013). Stiffness-dependent differentiation can also be modulated by the composition of the nuclear lamina - nuclear lamin A predominates in rigid tissues and organs, while lamin B levels are elevated in soft tissues (Swift et al., 2013), with the ratio of lamin A/B increasing with ECM rigidity.

We found that in OPC cultured under differentiation-promoting conditions, nuclear levels of YAP were highly elevated in response to increased matrix rigidity, while differentiating OPC cultured on stiff matrices also exhibited an increased lamin A/B ratio (Figure 3.5 A-B), demonstrating that matrix stiffness is capable of regulating OL expression of these effectors of mechanotransduction.
Figure 3.5 Rigid ECM increases nuclear levels of YAP and Lamin A.

A-B. In OPC grown in differentiation-promoting medium for 48 hrs, stiff matrix promotes increased levels of YAP and lamin type A in the nucleus, while decreasing the levels of lamin type B. (2 experiments)
Soft ECM promotes the formation and elongation of SC membrane protrusions

The next question we examined was whether SC, like OL, are capable of responding to mechanical cues. Primary rat SC were seeded onto 1.5 kPa and 30.0 kPa substrates and cultured for 48 hours with or without the presence of cAMP as an inducer of differentiation. Absent cAMP, SC on the soft substrates were more elongated, and displayed higher numbers of actin-rich processes along the membrane, while SC on the rigid substrates were more planar and had greater numbers of stress fibers. (Figure 3.6 A) Following treatment with cAMP, differences in morphology diminished with all cells assuming a more flattened morphology with fewer processes. (Figure 3.6 A)

In order to quantitate the complex changes in SC morphology, we employed measurements of aspect ratio, which corresponds to cell elongation, and solidity, which is a measurement of cell branching (Carpenter et al., 2006). For example, a stereotypical neuronal cell with many processes and one long axon would have a very high aspect ratio and low solidity, while a typical fibroblast would have a very low aspect ratio and high solidity. In the absence of cAMP, SC grown on soft matrices have a significantly higher aspect ratio and lower solidity than those grown on rigid matrices, and the difference in aspect ratio persists after the initiation of differentiation by cAMP treatment. (Figure 3.6 B-C) This demonstrates that SC are capable of responding to differences in matrix properties by regulating their morphology, and that their response to a decrease in matrix tension – increased process extension – is consistent with the effects we previously observed in SC as a result of NMII inhibition.
Figure 3.6 Soft ECM promotes Schwann cell elongation and formation of membrane protrusions.

![Figure 3.6](image-url)
Figure 3.6 Soft ECM promotes Schwann cell elongation and formation of membrane protrusions.

A. SC morphological changes in response to ECM stiffness. Undifferentiated SC grown on soft substrates showed greater elongation and process extension, while those grown on rigid substrates displayed a more polygonal morphology. While cAMP treatment induced cell spreading and process retraction, differentiating SC grown on soft matrices still displayed greater elongation. Approximately 400 cells were analyzed per condition (2 coverslips / 100 cells per coverslip / duplicate experiments).

B. Examples of aspect ratio measurement (Derived from the length and width of a best-fit ellipse based on the shape measured) and solidity (the area of the shape being measured divided by the area of the convex hull necessary to enclose it) and representative cells.

C. Quantitation of changes in SC morphology. Undifferentiated SC grown on soft matrices exhibited a significantly higher aspect ratio and lower solidity than those grown on rigid substrates, while cAMP treated cells grown on softer matrices displayed greater elongation. (p<0.001). (Scale bars 25 µm.)
Soft ECM increases SC expression of c-Jun, but does not overcome the effects of cAMP induced differentiation

Since ECM rigidity appeared able to influence SC morphology, we next investigated whether it was also capable of controlling SC differentiation. As before, SC were plated onto 1.5 kPa and 30.0 kPa matrices and cultured with or without cAMP treatment over a period of 48-72 hours. We found that in cells grown without cAMP, soft matrix produced a modest but statistically significant increase in the levels of the negative regulator of SC differentiation c-Jun (Figure 3.7 A-B), but the levels of the positive regulators Oct-6 and Krox 20 were not affected (Figure 3.7 A-D). Treatment with cAMP, which promotes SC differentiation (Morgan et al., 1991), lead to downregulation of c-Jun expression (Figure 3.7 A-B) and upregulation of Oct-6 (Figure 3.7 A-B) as well as Krox20 (Figure 3.7 C-D) levels in cells grown on both types of matrix. While the increases in Oct-6 and Krox20 were slightly greater in cells grown on the 30.0 kPa matrices, the effects on Oct-6 were slight, and the elevation of Krox20 levels was not statistically significant. Additionally, while we observed that pMLC and pFAK levels were initially elevated in undifferentiated SC grown on the rigid matrices, cAMP treatment abolished the differential. This suggests that in this culture system, forces transmitted from the ECM and actomyosin contractility do not have a strong effect on cAMP-induced SC differentiation.
Figure 3.7 Soft ECM promotes SC expression of c-Jun but cAMP-induced differentiation is not significantly affected by matrix stiffness alone.
Figure 3.7 Soft ECM promotes SC expression of c-Jun but cAMP-induced differentiation is not significantly affected by matrix stiffness alone.

A-B. Undifferentiated SC (-cAMP) growing on soft substrates showed a modest but significant increase in c-Jun expression (p<0.05). Addition of cAMP (+cAMP) abolished the differences in cell morphology and down-regulated c-Jun expression to comparable levels in both soft and stiff matrices. Upregulation of Oct6 expression after cAMP treatment was also observed in both conditions, although it was slightly higher in the stiff matrices. Approximately 400 cells were analyzed per condition (2 coverslips / 100 cells per coverslip / duplicate experiments) C-D. Increased phosphorylation of the regulatory myosin light chain (pMLC2) and FAK (pFAK) were detected in undifferentiated SC plated on stiffer matrices, indicating NMII activity and cell tension. These differences were abolished after addition of cAMP. Increased Krox-20 expression was observed in both matrices after cAMP treatment. (Scale bars 30 µm.)
Laminin potentiates the effects of stiff ECM on cAMP-induced SC differentiation

While ECM mechanical properties alone are not sufficient to regulate SC differentiation, it is well established that ECM components such as laminins are necessary for normal SC radial sorting and myelination (Nakagawa et al., 2001; Wallquist et al., 2005). One receptor recently identified as essential for SC radial sorting and myelination is GPR126, an adhesion G-protein coupled receptor that interacts with laminin-211 (Petersen et al., 2015), and which may be responsive to the mechanical properties of the ECM (Liebscher et al., 2014) (Petersen et al., 2015).

In order to examine whether the developmental status of the basal lamina may modulate the SC response to ECM rigidity and cAMP upregulation, we cultured SC on 1.5 kPa and 30.0 kPa matrices that were treated with collagen I and either 5 µg/mL or 20 µg/mL of mouse laminin, simulating the composition of either the immature or fully developed basal lamina (Grove and Brophy, 2014). Our data indicate that upon cAMP treatment the expression of Krox20 by cells grown on laminin-coated substrates was significantly increased in the 30.0 kPa condition, and that it was further enhanced by increased levels of laminin. (Figure 3.8 A-B) This indicates that while SC differentiation can be upregulated by increasing ECM rigidity, this response is laminin-dependent and may be influenced by the maturity of the basal lamina.
Figure 3.8 Increased laminin concentration potentiates SC differentiation on stiff ECM.

A. Representative images from cultures of differentiating SC showing the effect of matrix stiffness and laminin concentration on the expression of Krox20. B. Quantitation of Krox20 nuclear fluorescence. Krox20 expression and percentage of Krox20 positive cells were upregulated in SC grown on stiff laminin-coated matrices, with highest response observed at the highest laminin concentration. (p<0.001, 2 experiments) (Scale bars 20 µm.)
Chapter 4: Discussion

ECM dysfunction in CNS pathologies

Pathological changes have been shown to influence the composition and mechanical properties of the ECM in various tissues. For example, development of rat liver fibrosis correlates with an increase in tissue stiffness (Georges et al., 2007) and similar changes are observed in the fibrotic lung (Dolhnikoff et al., 1999). In the CNS, the effect of injury and disease on mechanical tissue properties has not been studied as extensively, since most work has focused on chemical cues produced in response to injury (Galtrey and Fawcett, 2007), the effects of which on regrowth and remyelination are by now well established (Levine et al., 2001; Silver and Miller, 2004; Yiu and He, 2006).

However, there does exist a growing body of work indicating that ECM mechanical properties do play a role in CNS regeneration and remyelination. One of the key responses to CNS injury is astrocyte activation and the formation of the astrocytic scar, which contains the damage but may present a physical barrier to nerve repair and remyelination (Georges et al., 2006). The effects of this can be seen in multiple sclerosis (MS), where in acute lesions, myelination is more widespread before scar formation (Ludwin and Raine, 2008), while in chronic MS lesions that are notably more rigid than the healthy brain (Cedric Raine, personal communication), myelin repair is limited to a narrow area at the lesion margin that is in contact with healthy tissue (Prineas and Connell, 1978). Moreover, there is evidence that astrocytic activation itself is enhanced by increases in ECM rigidity (Janmey and Miller, 2011). The effects of these changes in ECM composition on OL regulatory pathways are largely unexamined, but recent work (Furusho et al., 2015) demonstrates that FGF signaling is dispensable for remyelination in acute cuprizone-
induced lesions but necessary in chronic ones, adding support to the idea that changes in the physical microenvironment can substantially regulate OL remyelination.

CNS scarring that increases ECM density is also present in other pathologies: fibrosis occurs as a consequence of proliferation of stromal cells following stroke (Fernandez-Klett et al., 2013), as well as due to perivascular fibroblast infiltration in spinal cord injuries (Soderblom et al., 2013), leading to the formation of a dense, ECM protein-rich core surrounded by reactive glial cells. Given this range of pathologies which dysregulate the ECM of the CNS, and the generally poor prospects for regeneration and repair following CNS injury, it is important to consider all possible factors that may influence recovery, including the impact of mechanical forces on the differentiation of and myelination by CNS glia.

**ECM mechanical properties regulate OL differentiation**

Process extension by OL is necessary for their morphological maturation as they establish contact with multiple axons, and strongly correlates with their differentiation and expression of myelin proteins. Previous work from our lab has shown that these processes are regulated by non-muscle myosin II, and that higher levels of NMII activity decrease OL branching, expression of myelin proteins, and the number of myelin segments produced *in vitro* (Wang et al., 2012; Wang et al., 2008). Conversely, in a mouse model in which demyelinating lesions of the corpus callosum were induced with lysolecithin, inhibition of NMIIB activity in the oligodendrocyte lineage significantly accelerated the rate of lesion repair and remyelination (Rusielewicz et al., 2014), demonstrating that NMII is a negative regulator of CNS myelination.
Mechanotransduction is also NMII dependent. Since NMII activity increases in proportion to ECM rigidity, and a number of CNS pathologies result in both glial and fibrotic scarring as well as increased ECM deposition, we investigated whether OL maturation and differentiation are affected by changes in the mechanical properties of the ECM. We found that increases in ECM stiffness result in a significant reduction of OL branching, and that this was accompanied by elevated levels of the phosphorylated NMII light chain, leading to an increase in NMII activity. This was consistent with the findings of (Wang et al., 2008) as well as with similar observations performed on endothelial tip cells by (Fischer et al., 2009). These effects of rigid (30.0 kPa) ECM were abrogated by treatment with Y-27632, an inhibitor of ROCK, or by the genetic ablation of NMII, providing evidence that OL mechanotransduction is a process dependent on NMII activity. In addition to its impact on OL morphological maturation, we also found that an increase in ECM rigidity has an inhibitory effect on key OL maturation markers, such as CNPase/Rip and MBP, demonstrating that ECM properties are also capable of regulating OL differentiation. In light of the findings from our laboratory that a reduction in cytoskeletal tension resulting from NMII ablation can significantly enhance the recovery from an acute demyelinated lesion (Rusielewicz et al., 2014), this suggests that NMII inhibition could also be used to promote myelin repair in chronic or fibrotic lesions featuring increased ECM stiffness.

Interestingly, recently published studies show that while the ability of OL to extend processes and ensheath axons depends on actin polymerization and Arp 2/3 activity, myelin wrapping itself appears to require actin disassembly, with movement of the inner tongue of the OL myelin membrane driven by alternating cycles of actin polymerization and depolarization, dependent on gelsolin and coflin activity (Nawaz et al., 2015; Zuchero et al., 2015). While these findings are consistent with our data, since increased NMII activity opposes actin protrusion, they
suggest that the inhibitory effect of ECM stiffness on OL maturation and differentiation would primarily be a factor during the early stages of OL differentiation, when OL branch and axonal contact is established. The effect of ECM stiffness on the movement of the inner myelin tongue would likely be minimal, because of the lack of direct contact between the ECM and the inner myelin membranes, as well as the fact that the movement of the inner tongue appears to be adhesion-independent (Nawaz et al., 2015).

**Mechanisms of OL mechanotransduction**

In an attempt to elucidate the mechanisms responsible for the effects we observed in OL, we have examined a number of transcription factors and regulatory pathways which play key roles in OL differentiation or NMII-dependent mechanotransduction. One of these was Olig1, a transcription factor essential for OL development and maturation which is present in the OL nucleus early during OL development, but must be phosphorylated and undergo a transition to the cytoplasm during differentiation (Niu et al., 2012), as nuclear Olig1 overexpression greatly reduces the spread of MBP-positive OL membrane. We found that in OL cultured on rigid matrices, Olig1 is retained in the nucleus at nearly twice the level found in OL grown on compliant substrates. This suggests that the reduced OL complexity and significantly lower numbers of OL with MBP positive processes we observed in cultures grown on stiffer matrices are a result of mislocalization of Olig1 caused by an increase in ECM stiffness.

YAP/TAZ are transcriptional cofactors in the Hippo regulatory pathway, which have been shown to play an essential role in matrix elasticity dependent mechanosensing that requires RhoA and NMII activity (Dupont et al., 2011). Soft ECM causes YAP/TAZ to accumulate in the
cytoplasm where it can be targeted for degradation, while rigid ECM promotes nuclear localization and activation of target genes. Consistent with this, nuclear levels of YAP were increased in OL cultured on rigid substrates, and reduced on compliant ones, demonstrating that an essential, NMII-dependent mechanosensing pathway is engaged in the process of ECM-regulated OPC differentiation.

In addition to YAP/TAZ, nuclear lamins have also recently been implicated as mechanosensitive molecules involved in transcriptional regulation. As integral components of the “nucleoskeleton”, they regulate changes in nuclear shape and are involved in maintaining chromatin organization (Puckelwartz et al., 2011) (Dechat et al., 2008). Increased nuclear lamin A levels are observed in cells grown on rigid matrices, while lamin B is more predominant on soft substrates or in soft tissues, and have been shown to direct the fate specification of mesenchymal stem cells (Swift et al., 2013). We found that in OL cultured on stiff matrices, the ratio of lamin A to lamin B was increased, as expected in cells grown on a rigid matrix, suggesting that nuclear lamins may be involved in the ECM-mediated regulation of OL morphology and differentiation. This is an especially interesting finding in light of the fact that lamin B overexpression resulting from gene duplication is the cause of adult-onset autosomal dominant leukodystrophy (Padiath et al., 2006) (Lin et al., 2014). Lamin B overexpression was also experimentally shown to cause defects in lipid and cholesterol synthesis (Rolyan et al., 2015), which is of potential interest because of cholesterol’s importance in maintaining the fluidity and elasticity of the cell membrane.

While we have yet to identify a direct mechanistic link between ECM properties and OL differentiation, the data thus far suggest avenues for future work. Olig1 nuclear-cytoplasmic translocation is regulated by phosphorylation on serine 138, which is located within a well conserved protein kinase A (PKA) phosphorylation motif (Niu et al., 2012). These proposed PKA-
mediated effects on Olig1 localization are consistent with the fact that OPC differentiation is
promoted by cAMP and that a number of studies have shown the involvement of cAMP, PKA and
cyclic AMP-response element binding protein (CREB) in promoting OPC differentiation and OL
myelination (Baron et al., 1998) (Afshari et al., 2001) (Syed et al., 2013). Interestingly, PKA has
also been shown to inhibit RhoA, which in turn indirectly promotes the inhibitory phosphorylation
of YAP/TAZ, leading to their translocation from the nucleus to the cytoplasm (Yu et al., 2013).
(Figure 1.4)

This suggests that PKA activity can potentially drive increased Olig1 cytoplasmic
localization, cytoplasmic translocation and degradation of YAP/TAZ, as well as inhibition of
RhoA and downregulation of NMII activity, accounting for the majority of the effects we have
observed as a consequence of culturing OL on soft matrices and promoting OL differentiation.
However, while mechanical forces have been shown to, for example, activate PKA/CREB
signaling in cardiac fibroblasts (Husse and Isenberg, 2010) or induce chondrocyte differentiation
in a PKA dependent manner (Juhasz et al., 2014), there is currently no evidence that PKA activity
is regulated by mechanical cues in oligodendroglia, leaving it open to investigation.

Another possible modulator of YAP/TAZ localization is GPCR activity. A number of G
protein subunits have been shown to regulate YAP/TAZ phosphorylation, with G\textsubscript{12}/G\textsubscript{13} \(\alpha\) subunits
strongly inhibiting LATS kinases and therefor reducing YAP/TAZ phosphorylation, and G\textsubscript{as}
exerting the opposite effect (Yu et al., 2012). Moreover, the PKA-dependent regulation of
YAP/TAZ described above occurs downstream of cAMP signaling that is driven by G\textsubscript{as} activity,
and opposes G\textsubscript{12}/G\textsubscript{13} mediated RhoA activation (Yu et al., 2013). These findings are particularly
interesting in light of that fact that an adhesion GPCR, GPR56, has been shown to regulate
myelination by promoting OPC proliferation, and may do so in a G12/G13 and RhoA dependent manner (Ackerman et al., 2015).

Mechanical forces alone fail to regulate SC differentiation

NMII contractility plays an important role in SC myelination – its downregulation by the knockdown of MLC causes SC to aberrantly extend multiple processes, and prevents them from efficiently myelinating axons in DRG/SC co-cultures (Wang et al., 2008). This dependence on NMII activity suggested that SC were likely to be sensitive to mechanical cues, most likely originating from the dense ECM they inhabit. However, while this proved to be the case, the overall impact of ECM rigidity on SC morphology was initially much less notable compared to what we observed previously in OL.

SC which were not treated with cAMP and grown on soft collagen-coated matrices did exhibit a higher degree of elongation, increased numbers of actin-based protrusions and lower levels of MLC phosphorylation compared to those cultured on rigid matrices, in a manner consistent with the established effects of soft ECM on cell morphology and consistent with the effects we have previously observed as a result of NMII inhibition in SC. However, when differentiation was induced with cAMP treatment, most morphological differences were greatly diminished.

Similarly, while matrix stiffness proved to influence the expression of key regulators of SC differentiation, the differences were not strongly pronounced. While SC cultured on soft matrices without cAMP exhibited lower levels of phosphorylated MLC and phosphorylated focal adhesion kinase (FAK), as well as increased levels of the negative regulator of myelination c-Jun, the
elevation of c-Jun on soft ECM was slight, and the differences were completely abolished following the addition of cAMP. Likewise, while the expression of positive regulators of myelination Oct-6 and Krox20 was somewhat increased in differentiating SC grown on stiff matrices, the difference in Oct-6 was very slight, and the raise in Krox20 level was not statistically significant. This suggests that while matrix stiffness alone does affect SC morphology and transcription factor expression, the effect is very modest when compared to those brought about by cAMP signaling (Morgan et al., 1991) (Monje et al., 2010).

**SC mechanotransduction requires ECM laminin**

Schwann cells are radially polarized, in that they have an inner, adaxonal membrane which interacts with the axolemma, and an outer abaxonal surface which contacts the basal lamina (Salzer, 2003). SC begin to secrete the basal lamina after axonal envelopment (Clark and Bunge, 1989). It is their only direct source of ECM contact and the proteins which comprise it, laminin-211 and 411 in particular, play an important role in normal myelination (Nakagawa et al., 2001; Wallquist et al., 2005). The basal lamina secreted by immature SC is thin and incomplete, but becomes increasingly dense as myelination progresses (Cornbrooks et al., 1983), and Focal Adhesion Kinase (FAK) activity, which promotes cell adhesion and spreading, is necessary to compensate for the limited integrin engagement offered by the immature lamina (Grove and Brophy, 2014).

Since our initial experiments used collagen-coated substrates, their results reflected the effects of changes in ECM rigidity, but not the changes in composition of the basal lamina occurring during SC differentiation and myelination. To increase the physiological relevance of
our model system, we incorporated varying levels of laminin into our ECM-like substrates. We found that SC differentiation in response to cAMP, as measured by Krox20 expression, was significantly enhanced in cells growing on the rigid matrices at the high laminin concentration representing the mature basal lamina.

This finding was particularly interesting in view of the recently described laminin-211 dependence of GPR126, an adhesion G-protein coupled receptor required for mammalian PNS myelination (Monk et al., 2011). GPR126 consists of two primary domains: a transmembrane C-terminal fragment (CTF) which promotes SC differentiation and wrapping of axons by regulating cAMP levels through \( G_{\alpha S} \) and \( G_{\alpha i} \) signaling (Mogha et al., 2013), and an autoproteolytically cleaved extracellular N-terminal fragment (NTF) which interacts with laminin-211 and is necessary for the radial sorting of axons (Petersen et al., 2015). The NTF also associates with the CTF at the membrane, inhibiting CTF activity. This inhibitory interaction, which leads to a reduction in cAMP levels, can be stabilized by low levels of laminin, such as are present in an immature basal lamina. However, as laminin concentration and polymerization increase, the forces transmitted through the laminin fibers dissociate the NTF from the CTF, relieving the inhibitory effect. This mechanism is fully consistent with the effects we observed, in that Krox20 levels are at their highest in cells which are exposed to both high concentrations of laminin and high matrix rigidity.

Intriguingly, laminin-211 has also been implicated as a negative regulator of myelination. The work of (Heller et al., 2014) demonstrates the convergence of two opposing regulatory pathways on to PI 3-kinase, which is known to be a critical factor in the initiation of myelination (Maurel and Salzer, 2000). The first, which is mediated by axonal neuregulin-1 type III is pro-myelinating, while the second, involving \( \alpha 6\beta 4 \) integrin and laminin-211 is inhibitory and prevents
hypermyelination. However, these apparently paradoxical effects of laminin-211 are not contradictory, since there is data showing that laminin-211 / GPR126 / cAMP signaling, while necessary for the initiation of myelination in conjunction with neuregulin-1 type III signaling, is not required for long-term myelin maintenance (Glenn and Talbot, 2013).

This increasing evidence showing the importance of integration of various converging adaxonal and abaxonal signals also helps to place in context our laboratory’s earlier findings (Leitman et al., 2011) that while NMII inhibition via MLCK knockdown in SC in vitro promotes the expression of Krox20 and myelin proteins, the cells are unable to wrap and produce compact myelin when exposed to axons. This led us to propose a biphasic model of SC dependence on NMII activity, in which NMII inhibition caused by axonal signaling promotes differentiation, but this is not sufficient for myelination in the absence of NMII activity-promoting interactions between integrins and the abaxonal basal lamina.

**ECM-dependent approaches to PNS regeneration**

The role of ECM-derived signaling in SC differentiation can be particularly important given current research into surgical PNS repair, in which nerve engraftment is a common technique. Due to limited availability of donor tissue, a great deal of research has been directed at developing engineered alternatives, using a staggering array of materials, ranging from natural ones based on collagen, fibrin or chitosan, to synthetic ones fabricated from silicone, poly(lactic-co-glycolic acid) (PLGA) or poly(caprolactone fumarate) (PCLF) (Daly et al., 2013; Gerth et al., 2015). There have also been numerous attempts to improve their performance, ranging from relatively simple micro-patterning of the grafts to encourage growth and migration, to highly
elaborate ones like seeding them with Schwann cells engineered to over-express neurotrophic factors (Meyer et al., 2015).

However, to our knowledge, there have been no attempts to examine how the mechanical properties of either biomaterial grafts or of the healthy PNS ECM affect the morphology and differentiation of Schwann cells. Since there is evidence that in engineered tissues mechanical forces play a role in promoting cell and ECM organization conducive to nerve repair (Georgiou et al., 2013), and that mechanical stimulation has been shown to promote nerve repair and remyelination (Kim et al., 2013), examining how SC differentiation is affected by mechanical forces can have important implications for developing strategies for peripheral nerve regeneration.

**Concluding remarks**

The developmental niches inhabited by oligodendrocytes (OL) and Schwann cells (SC) vary significantly in their mechanical properties. In the central nervous system (CNS), the extracellular matrix (ECM) is diffuse and highly elastic compared to that of the peripheral nervous system (PNS), which consists of much denser nerve bundles in which SC secrete their own basal lamina. ECM plasticity is capable of regulating cell morphology and gene expression in a process known as mechanotransduction, which depends on the regulation of cytoskeletal tension by NMII activity, and our work shows that both OL and SC are capable of responding to changes in ECM properties. While low matrix stiffness promotes OL morphological maturation and expression of markers of myelination, SC differentiation is upregulated by increased matrix rigidity in a process that requires the presence of ECM laminin. (Figure 4.1) These findings enhance the understanding of the basic cellular mechanisms of myelination. Additionally, since ECM mechanical properties...
in both the PNS and CNS can vary following disease and injury, studying how these mechanical factors regulate OL and SC development may be important for optimizing therapies designed to promote nerve regeneration and remyelination.

**Figure 4.1 Revised model of ECM regulation of glial cell differentiation.**

The mechanical properties of the ECM are capable of regulating the morphology of both oligodendrocytes (OL) and Schwann cells (SC). In OL, increased ECM stiffness leads to an increase in myosin activity, inhibiting both OL branching and expression of differentiation markers. Conversely, increases in ECM rigidity can promote SC differentiation, but this effect is greatly potentiated by increased levels of ECM laminin.
Chapter 5: Materials and Methods

Preparation of glial cultures on ECM protein substrates of varying elasticity

Thin polyacrylamide substrates (PA) of different viscoelastic moduli were prepared as previously described (Tse and Engler, 2010) (Fischer et al., 2009). Briefly; NaOH-cleaned 12 mm glass coverslips were coated with 0.5% 3-aminoproyltrimethoxysilane in diH₂O for 10 minutes at RT, rinsed extensively, incubated with 0.5% glutaraldehyde in PBS for 30 min at RT, rinsed for 30 min under running diH₂O, and allowed to air-dry in a laminar flow hood. Glass slides were coated with a thin layer of Rain-X (ITW Global Brands), allowed to dry, wiped with 100% ethanol, rinsed under running water for ~10 min and allowed to dry as above. PA solutions ranging from 7.5 acrylamide/0.05% bis (Young’s modulus $E \sim 1.5$ kPa) to 12% acrylamide/0.28% bis (Young’s modulus $E \sim 30$ kPa) prepared in PBS and activated with 10% ammonium persulfate and TEMED (3µl of 10% APS and 2 µl TEMED per 500µl of PA solution). The activated solutions were immediately pipetted on to the Rain-X treated slides, covered with the treated 12mm cover slips (6 µl of PA solution per 12mm cover slip) and allowed to polymerize for 10 min at RT. After polymerization, the slides and cover slips were placed in PBS solution, and a razor was used to gently dislodge the PA-coated 12mm cover slips from the Rain-X treated slides. The substrates were placed in 24-well plates in PBS, rinsed, allowed to sit at 4°C overnight with gentle shaking to allow any unpolymerized PA to diffuse out, then washed 3 times with PBS. To make a sandwich gel, PA gels were activated for crosslinking with 365 nm UV light using Sulfo-SANPAH (Pierce) and incubated with ECM protein (0.1mg/ml Collagen I or 0.1 mg/ml Collagen I plus 5-20 µg/ml mouse laminin-111 for PNS cultures; 20µg/ml matrigel for CNS cultures) overnight at 37°C. After
extensive washing, primary glial cells (3-5x10^4 for IF or 1x10^5 for WB) were plated on top of the cross-linked substrates and allowed to adhere overnight.

**Atomic force microscopy (AFM)**

The elastic modulus of soft and stiff PA gels was measured by AFM nano-indentation and confirmed to match the predicted values (Table 2.1). Briefly, an Asylum Research MFP-3D-BIO Atomic Force Microscope was used to collect force curves and force maps of the two groups of gels. A CP CONT-PS C (NanoAndMore.com) probe with a 6.1 \( \mu \)m polystyrene bead was used for the spherical Hertz model indentations, and an Olympus TR400PB Long Cantilever (AsylumResearch.com) was used for the pyramidal Hertz model indentations. The Asylum Research GetReal calibration method was utilized for calibration of the spring constant. We have independently verified that the GetReal calibration gives adequate calibrations when compare directly to the thermal tune method for these probes. The same probe was used for all measurements amongst and between groups, and the groups were rotated between measurements. Force maps were conducted in PBS. Curves were collected to a trigger point of 100 nN for spherical indenters and 25 nN for pyramidal indenters. A 1 second dwell was incorporated into the force curve to elucidate viscous behavior. A 50 \( \mu \)m/s indentation velocity was employed because we found the gels to have a high rate of creep, with slower velocities causing the gel to yield to the bead and preventing clean force curves from being collected. The curves were fit with the Hertz model as utilized in the Asylum Research software. Each force map samples a grid across a 90 \( \mu \)m x 90 \( \mu \)m area. We sampled 4 gels from each group with the spherical indenter, and each gel was sampled 3 times, producing a total of 12 force maps consisting of 256 curves per
sample, or a total of 3072 measurements for each type of gel. For tissue measurements, fresh brain and nerves (teased vs. unteased fibers) were compared, as well as spherical vs. pyramidal indenters.

**Glial cell purification**

A2B5+ oligodendrocyte precursors (OPC) were purified by immunopanning (negative Ran2 panning, positive A2B5 panning) from mixed glial cultures of postnatal day 2 rat cerebral cortices as previously described (Wang et al., 2008). Purified OPC were seeded on to PA/ECM elastic substrates in 24-well plates and maintained in either proliferation media with PDGF (10 ng/ml) and bFGF (10 ng/ml), or induced to differentiate in media containing T3 (30 ng/ml). For inhibition studies, Y267632 (Calbiochem) was added to the media (10 µm) and maintained throughout the experiment (1-3 days).

Mouse OL were prepared as previously described (O'Meara et al., 2011). Briefly, mixed glial cultures were prepared from postnatal day 2 mouse cerebral cortices of PlpCre/ESR1:Rosa26-mT/mG;NMIIfl/fl and PlpCre/ESR1:Rosa26-mT/mG;NMIIIB+/+ animals (Rusielewicz et al., 2014), and used to generate OL-enriched glial cultures by separating the OL from the astrocyte monolayer by orbital shaking, followed by purification by differential adhesion to plastic. Purified OPC were seeded on to PA/ECM elastic substrates. Recombination was induced with 1 µM tamoxifen and the cells were allowed to differentiate for 48-72 hours in medium containing T3 (30 ng/ml).

Schwann cells (SC) were isolated from postnatal day 2 rat sciatic nerves and expanded for 3 weeks in M media (MEM, 10% FBS and 2 mM L-glutamine), supplemented with 4 µM forskolin (Sigma), and 5 ng/ml of the EGF domain of rhNRG-1-β1 (R&D Systems), hereafter called M+ media. For studies on the effects of cAMP analogues on SC differentiation, cultures maintained in
M+ media were switched to media without growth factors for 3 d (M media). SC were either starved overnight in serum-free M media (MEM and 2 mM L-glutamine), or placed in defined media DM (DMEM/F12, 5 µg/ml insulin, 5 µg/ml transferrin), prior to treatment with 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP) (Calbiochem), for 24h, 48h and 72h.

**Immunofluorescence**

Glial cell cultures on PA/ECM substrates were fixed in 4% PFA for 10 minutes and processed for immunocytochemistry as previously described (Wang et al., 2008). Briefly, cells were permeabilized for 5 minutes with 0.5% Triton X-100 and blocked with 5% BSA, 2% donkey serum and 0.2% Triton X-100 for 1 hour at room temperature. This was followed by an overnight incubation with primary antibodies in blocking solution at 4°C, and a 1 hour room temperature incubation with secondary antibodies in blocking solution. The stained cultures were mounted using ProLong Gold anti-fade reagent, and imaged by epifluorescence using a Leica DMI4000 microscope with LAS 1.7.0 software equipped with a digital camera (DFC350FX; Leica) and the following objectives: N PLAN 10 × /0.25 NA; N PLAN L40 × /0.55 NA; and HCX PL APO CS63 × /1.4 NA oil UV (all from Leica). Image analysis (see below) was performed using ImageJ.

**Image analysis**

OPC were counted in micrographs from 10-12 random 20X fields/coverslip, using ImageJ (total of 20-30 fields per condition per experiment; total of 2-3 experiments). For the evaluation of morphology, OPC were counted and classified according to their branching complexity as follows: (0): no branching; (1) low complexity: cells with at least one or two branches directly from cell
body; (2) medium complexity: cells with secondary processes extending from primary branches
and (3) high complexity: cells with tertiary processes extending from secondary branches.

The shape of Schwann cells was quantified by manually tracing individual cells using the
ImageJ polygon selection tool, and using the ImageJ measure tool to determine their aspect ratio
and solidity (Analyze > Set Measurements > Shape Descriptors). Aspect ratio is a measure of the
extent of cellular polarization and is defined as the ratio of length (major axis) to width (minor
axis) of a best-fit ellipse derived from the cell. Solidity corresponds to cell convexity, and is
derived by dividing the traced area of the cell by the area of a convex hull applied to that cell
(Carpenter et al., 2006). While aspect ratio corresponds to cell polarization, solidity is reflective
of cell branching. Cells with a large number of processes would have low solidity while more
polygonal cells would have higher solidity.

For quantitation of nuclear fluorescence intensity, random 20X fields were captured from
cultures co-stained with DAPI and phalloidin as well as immunostained for either c-Jun, Oct6,
Krox-20 or Olig1. The DAPI channel was used to establish areas of interest corresponding to the
nuclei by using the ImageJ nucleus counter tool with manual corrections. The ROIs established by
the nucleus counter were added to the ROI manager, and then used, along with the ImageJ measure
tool, to record the mean gray value of c-Jun, Oct6, Krox-20 or Olig1 staining within the nuclear
regions. Background fluorescence was measured in the areas outside the nuclear ROIs, averaged,
and subtracted from the mean nuclear gray value. Statistical tests (t-test and ANOVA) were
performed using Graph Pad Prism software.
Cell extracts and Western blotting

To prepare lysates, seeded PA/ECM culture substrates were transferred to new 24-well plates, washed gently with PBS, and treated with lysis buffer (95 mM sodium chloride, 25 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid, 2% sodium dodecylsulfate, pH 7.5, protease and phosphatase inhibitor cocktail). Protein concentration was assayed by BCA, equal protein amounts (5-10 μg) per condition were loaded and separated by SDS-PAGE and blotted onto nitrocellulose. Appropriate regions of the blots were cut and incubated with specific antibodies and developed using chemiluminescent substrate (Pierce). Nuclear and cytoplasmic fractions were prepared using the Active Motif Nuclear Extract Kit (AM 40010). Briefly, the cells were lysed using detergent treatment under hypotonic conditions and the nuclei separated by centrifugation at 14,000g, followed by SDS-PAGE, as above. In order to quantify protein levels, films from 2-3 independent experiments were scanned, and the absolute intensity of the protein bands of interest as well as the corresponding loading controls was measured using ImageJ (Analyze > Gels). The relative intensity of the protein bands was then calculated by dividing the absolute intensity value of the protein band by the absolute intensity of the loading control. Actin and tubulin were used as controls for whole cell lysates, and histone H3 as the control for the nuclear fractions.

Antibodies

Antibodies used in these studies included those reactive to: MBP (Covance SMI-94R), anti-Rip antigen supernatant (DS Hybridoma Bank), Olig1 (Millipore AB5540), M2B (Covance PRB-44P), MLC2 (Cell Signaling 3672S), phosphorylated MLC2 (Cell Signaling 3674S), P-(S/T) PKA substrate (Cell Signaling 2261S), YAP (Santa Cruz SC-271134), lamin A/C (Cell Signaling
4777S), Lamin B1 (Santa Cruz SC-20682), histone H3 (Cell Signaling 4499), c-Jun (BD Technologies 610326), Oct6 (GenWay 18-003-42328), Krox-20 (Covance PRB-236P), FAK (Cell Signaling 3285), pFAK (Cell Signaling 3281s) actin (Sigma A2066), tubulin (Epitomics 2871-1), Ki-67 (Millipore). Staining was also performed with phalloidin-FITC (Thermo A12379) Acti-Stain 555 (Cytoskeleton PHDH1) and phalloidin Acti-Stain 670 (Cytoskeleton PHDN1). Secondary antibodies conjugated to rhodamine, fluorescein, coumarin, or cyanin 5 were obtained from Jackson Laboratories.
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