

Myosin II has distinct functions in PNS and CNS myelin sheath formation

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The myelin sheath forms by the spiral wrapping of a glial membrane around the axon. The mechanisms responsible for this process are unknown but are likely to involve coordinated changes in the glial cell cytoskeleton. We have found that inhibition of myosin II, a key regulator of actin cytoskeleton dynamics, has remarkably opposite effects on myelin formation by Schwann cells (SC) and oligodendrocytes (OL). Myosin II is necessary for initial interactions between SC and axons, and its inhibition or down-regulation impairs their ability to segregate

axons and elongate along them, preventing the formation of a 1:1 relationship, which is critical for peripheral nervous system myelination. In contrast, OL branching, differentiation, and myelin formation are potentiated by inhibition of myosin II. Thus, by controlling the spatial and localized activation of actin polymerization, myosin II regulates SC polarization and OL branching, and by extension their ability to form myelin. Our data indicate that the mechanisms regulating myelination in the peripheral and central nervous systems are distinct.

Introduction

Myelin is a highly specialized membrane that wraps around axons in the peripheral (PNS) and central (CNS) nervous systems. Although the function of myelin in facilitating the efficient and rapid propagation of nerve impulses by saltatory conduction has long been known, the basic mechanisms that drive the extension and wrapping of the glial membrane around the axon remain poorly understood. During their development and differentiation into myelin-forming cells, oligodendrocytes (OL) in the CNS and Schwann cells (SC) in the PNS undergo striking morphological changes that involve the active remodeling of their cytoskeleton. Data from multiple studies have underscored the importance of the actin cytoskeleton in process extension and myelination by both SC and OL (Fernandez-Valle et al., 1997; Kim et al., 2006; Bacon et al., 2007).

SC and OL express several regulatory actin-binding proteins, which regulate actin polymerization and process forma-

tion (Bacon et al., 2007). Pharmacological inhibition of actin polymerization has a negative effect on process extension, axonal ensheathment, differentiation, and myelination by both SC and OL (Fernandez-Valle et al., 1997; Bacon et al., 2007). Similarly, mice lacking WAVE1, an actin-binding protein important for lamellipodia formation, show defective OL morphogenesis and regional hypomyelination (Kim et al., 2006). Despite the fact that myelin formation by glial cells appears to involve the assembly and progression of an inner mesaxon around one (SC) or several (OL) axons (Bunge et al., 1989), the question still remains as to whether or not this process is driven by a common actin-polymerization mechanism and whether this is regulated in a similar fashion in the CNS and the PNS. Significantly, several studies have shown that members of the small Rho-GTPase family, the main regulators of actin cytoskeleton dynamics, are also important for coordinating the formation and maintenance of the myelin sheath by SC and OL. Activation of Rac1 downstream of β 1 integrin signaling has been implicated in process extension and axonal segregation and myelination by SC (Benninger et al., 2007; Nodari et al., 2007). In contrast, in the CNS, Rac1

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Abbreviations used in this paper: ANOVA, analysis of variance; bFGF, basic FGF; CNS, central nervous system; DRG, dorsal root ganglion; MBP, myelin basic protein; MLC, myosin light chain; N-WASP, neuronal Wiskott-Aldrich syndrome protein; OL, oligodendrocytes; OPC, OL precursor cells; PNS, peripheral nervous system; ROCK, Rho-associated kinase; SC, Schwann cells; shRNA, short hairpin RNA.

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and Cdc42, although dispensable for OL differentiation and myelination, appear to be important for myelin maintenance and stability (Thurnherr et al., 2006). Although the role of Rho in myelination has not been evaluated directly, constitutive activation of Rho interferes with OL branching and maturation (Wolf et al., 2001; Liang et al., 2004), whereas its inactivation promotes plasma membrane condensation and differentiation in Olig2 cells (Kippert et al., 2007). In the PNS, inactivation of Rho-associated kinase (ROCK), a major downstream effector of Rho, does not prevent SC differentiation and myelination but results in aberrant myelin organization (Melendez-Vasquez et al., 2004). Collectively, these contrasting effects of small GTPases function in myelinating glial cells suggest that the mechanisms controlling actin dynamics in SC and OL during differentiation and myelination are regulated differently.

The spatial and temporal regulation of actin interactions with specific binding proteins and myosin motors provides a mechanism for precisely regulating actin assembly and dynamics in a variety of higher-order cellular structures (Chhabra and Higgs, 2007). In nonmuscle cells, phosphorylation of the regulatory chain of the motor protein myosin II (MLC2) by ROCK is a key regulator of actomyosin assembly (Conti and Adelstein, 2008). We have previously found that MLC2 phosphorylation is dramatically up-regulated at the onset of PNS myelination, and that inhibition of ROCK in myelinating cocultures results in a dramatic decrease of phosphorylated MLC2 levels and abnormal SC myelination (Melendez-Vasquez et al., 2004). Using a combination of pharmacological and molecular tools, we have further investigated the specific role of myosin II during myelin formation by both SC and OL. We have found in this paper that myelination in the PNS and CNS is differentially regulated by myosin II. Myosin II is necessary for SC differentiation and myelination. In contrast, inhibition of myosin II activity in OL precursors does not interfere with their differentiation or their ability to form myelin but instead potentiates myelin formation in culture. Our results indicate that the mechanisms regulating glial cell cytoskeleton and myelin formation in the PNS and CNS are distinct.

Results

Inhibition of myosin II activity reduces the number and size of SC myelin segments

To study the role of myosin II in myelination, we first examined the effects of blebbistatin, a specific inhibitor of nonmuscle myosin II (Straight et al., 2003), in SC neuronal cocultures. SC were added to established dorsal root ganglion (DRG) neuron cultures with extensive neurite outgrowth. Cocultures were then switched to media containing ascorbate to promote basal lamina formation and myelination, with or without blebbistatin (1, 5, 10, 25, and 50 μ M). As an additional control, some cultures were treated with an inactive enantiomer of blebbistatin at the same concentration (Fig. 1 A). Blebbistatin was well tolerated for the duration of the experiment, without any significant effect on SC survival as measured by a TUNEL assay (unpublished data). After an additional 2–3 wk, cultures were fixed and stained for myelin basic protein (MBP) to identify compact myelin sheaths.

The most striking and reproducible effect of blebbistatin was a reduction in the number and size of MBP⁺ segments that formed in cocultures (Fig. 1, A and C). These effects were dose-dependent, and at the highest dose used (50 μ M), myelination was completely abolished (Fig. 1 C). Because blebbistatin can interfere with cytokinesis and cell division (Straight et al., 2003), we examined the effects of the drug on SC proliferation and survival in the cocultures. There was no effect on cell survival based on the TUNEL assay but a small decrease in the index of BrdU incorporation between control and treated cultures was observed only at the highest doses of the drug (from 30% in control cultures to 20–24% in cultures treated with blebbistatin at 25 or 50 μ M; see Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200802091/DC1>). Despite reduced proliferation, myelination could not be rescued in blebbistatin-treated cultures (at 10, 25, and 50 μ M) even when an excess of SC (10^6 instead of 10^5) were added to the cultures (unpublished data). Furthermore, cocultures treated with blebbistatin for 3–7 d and then maintained for additional 1–2 wk without the drug showed normal myelination, which indicates that the effects of the drug are reversible. Thus, we conclude that the inhibition of myelination in these cultures was not caused by insufficient SC numbers or an effect on cell viability.

We also found that the addition of blebbistatin during the first 24–72 h after switching the cultures to ascorbate-containing media had a more powerful effect in inhibiting myelination (unpublished data). Adding blebbistatin to cultures 4 or 7 d after myelin induction had no apparent effect on myelin formation. These results indicate that myosin II activity is critical at early stages of myelination. Closer examination of SC morphology in blebbistatin-treated cultures revealed several abnormalities: SC nuclei were round in shape and did not elongate along the axons (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200802091/DC1>). Even among those cells that formed myelin, the nuclei appeared round and were asymmetrically localized along the internode, in contrast to the elongated and centered nuclei characteristic of myelinating SC in control cultures (Fig. 1 B). Although segments in blebbistatin-treated cultures were shorter than controls ($P < 0.0001$; Fig. 1 C), they appeared to be of normal thickness. Costaining with antibodies to laminin and sodium channels (Fig. S2) also revealed that some SC in blebbistatin-treated cultures form multiple short segments, akin to the defects previously described after ROCK inhibition (Melendez-Vasquez et al., 2004).

The reduction in myelin formation was corroborated by significantly decreased amounts of myelin proteins (MBP, P0, and myelin-associated glycoprotein) and transcription factors (Krox-20 and Oct6) based on Western blotting (Fig. 1 D). Basal lamina components such as laminin chains $\alpha 2$ and $\gamma 1/\beta 1$ were also reduced. Electron microscopy analysis of blebbistatin-treated cultures further confirmed that SC appeared to be arrested at the premyelinating stage and were unable to properly ensheath and segregate axons (Fig. 1 E). Many large-diameter naked axons were seen in these cultures, and in some cases, they were segregated but remained unmyelinated (Fig. 1 F). The basal lamina around the SC appeared thin and patchy

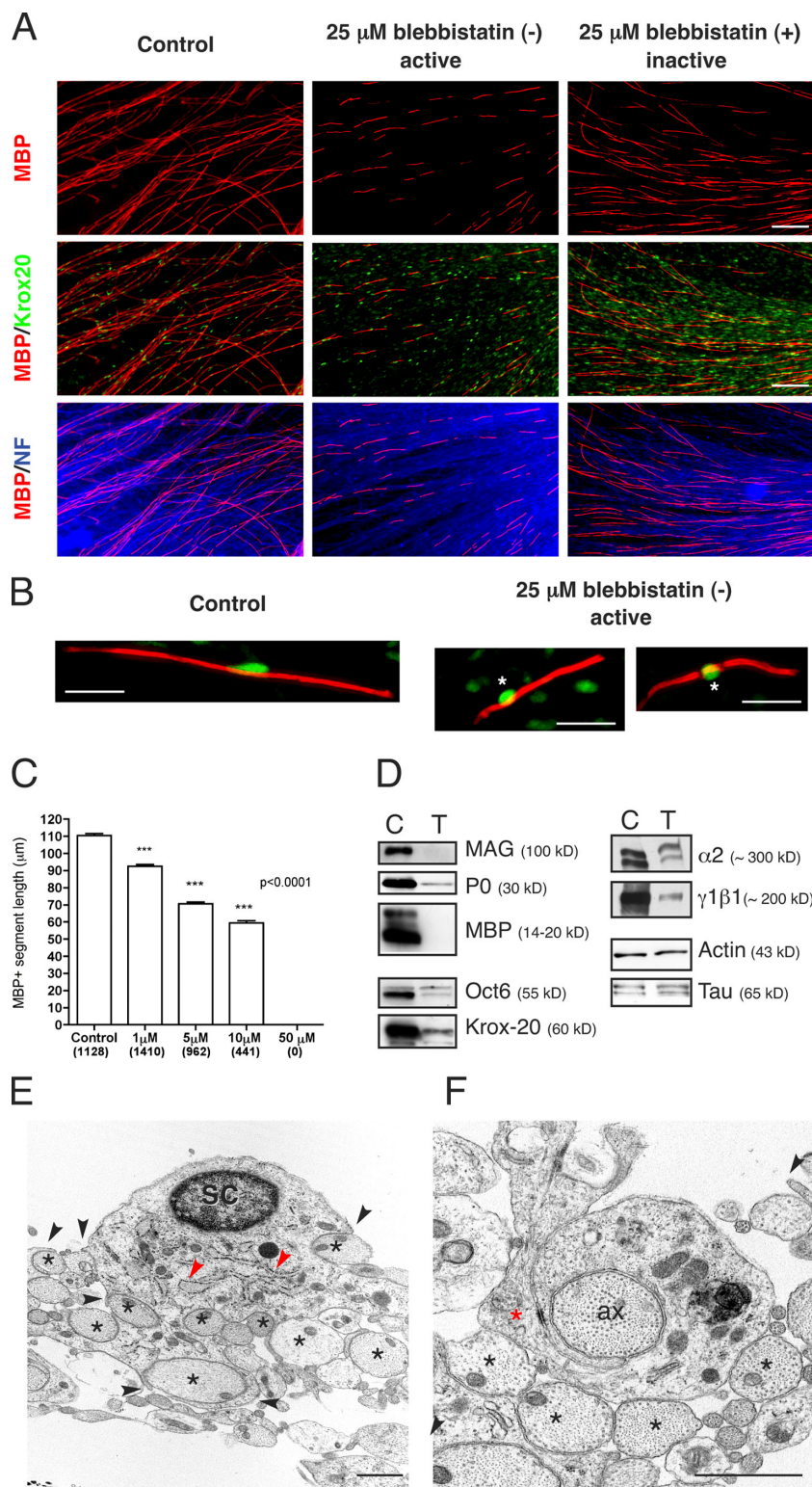


Figure 1. Inhibition of myelination in SC cocultures treated with blebbistatin. (A) 2-wk-old myelinating SC neuronal cocultures stained for MBP, Krox-20, and neurofilament. A significant reduction in myelin formation and Krox-20 expression was observed in cultures treated with the active (-) blebbistatin enantiomer compared with control cultures and cultures treated with the inactive (+) enantiomer. (B) Myelinated segments formed in blebbistatin (-)-treated cultures (right) were shorter than in control cultures (left) and frequently exhibited rounded and off-centered SC nuclei (asterisks). (C) Quantitation of internode length and number in 2-wk-old cocultures. A dose-dependent reduction in both the total number (n) and the length of the myelinated segments was consistently observed in treated cultures compared with the control. At the highest dose of blebbistatin (50 μ M), myelination was completely abolished. Data in the graph represent mean $\pm$ SEM from two independent experiments (two cultures per condition per experiment). ***, $P < 0.0001$. (D) Western blots from 2-wk-old cocultures, which confirm a significant reduction in the expression of myelin proteins, transcription factors, and laminin chains in blebbistatin-treated cultures (T). Actin and Tau levels were comparable in both treated and control cultures. (E and F) Ultrastructural analysis of 18-d-old myelinating cocultures treated with 25 μ M blebbistatin showing abnormalities in axon ensheathing and segregation. (E) An SC on top of a bundle of axons appears arrested at a premyelinating stage. Many axons (asterisks) are surrounded by very short cytoplasmic processes (black arrowheads), which fail to completely wrap or segregate them in a 1:1 association. Rough endoplasmic reticulum (red arrowheads) is very prominent in this cell. (F) A large segregated but nonmyelinated axon (ax), which is also contacted by a process (red asterisk) derived from a different SC. Many large-diameter naked axons are also observed in this field (black asterisks). The arrowhead points to a very short cytoplasmic process failing to surround an axon. Bars: (A) 100 μ m; (B) 25 μ m; (E and F) 1 μ m.

(not depicted), and they also exhibited abnormal branching and process extension. No obvious changes on the density/morphology of axons (Fig. 1 A) or in the levels of axonal-specific proteins such as Tau (Fig. 1 D) were observed between control and treated cultures. Collectively, these results suggest that myosin II inhibition greatly impairs differentiation and myelination by SC in culture.

Myosin II is required for normal SC interactions with axons and myelination

To confirm that the myelination defects observed in blebbistatin-treated cocultures were caused by inhibition of myosin II activity in SC and not by an effect of the drug on neurons, primary SC cultures were infected with lentivirus-expressing short hairpin RNA (shRNA) targeting the regulatory (light) chain of

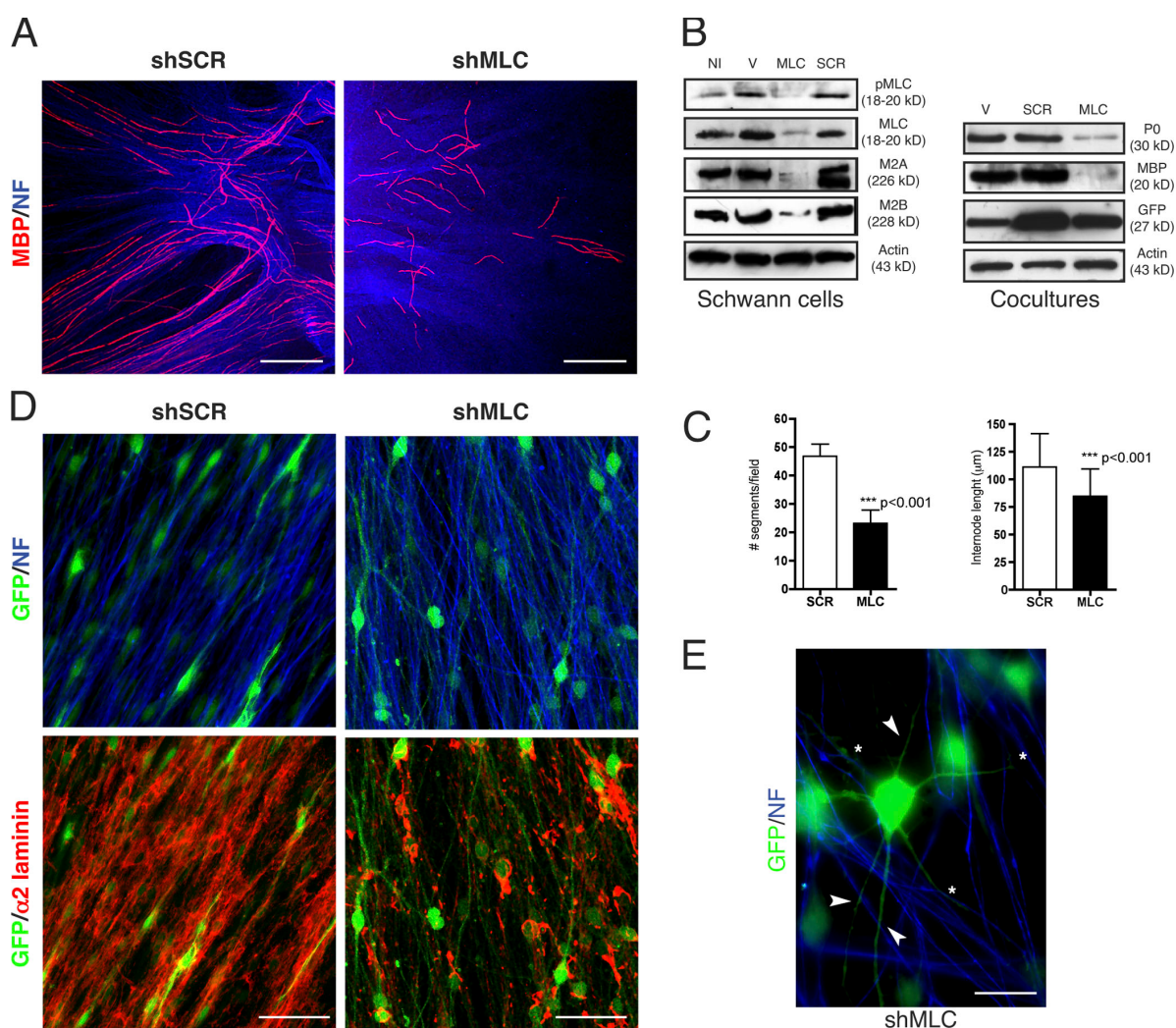


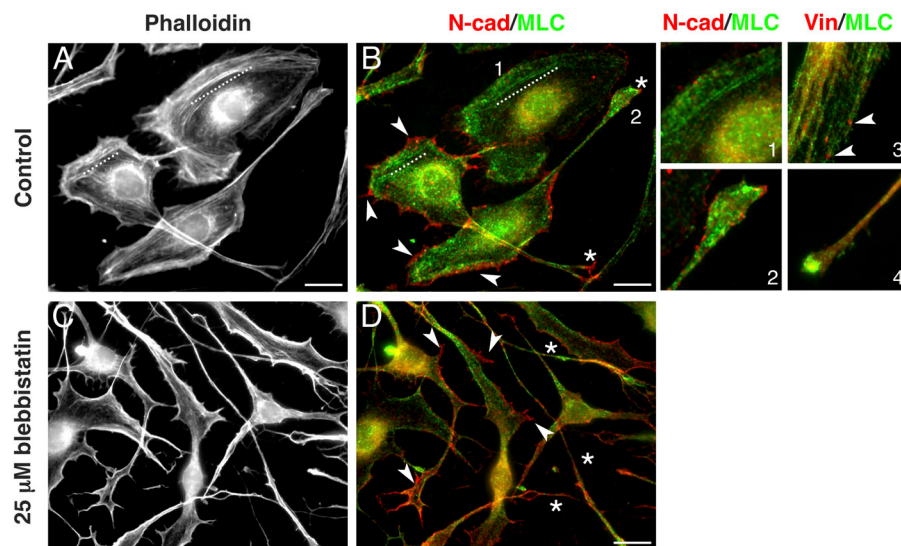
Figure 2. Knockdown of myosin II by shRNA results in abnormal SC morphology and prevents proper interactions with axons and myelination. (A) SC infected with lentivirus expressing shRNA against the regulatory chain of myosin II (shMLC) or a scrambled nontargeting sequence (shSCR) were seeded onto purified DRG neurons and allowed to myelinate. After 18 d, cocultures were stained for MBP and neurofilament. A clear reduction in the amount of myelin formed in cocultures by SC expressing shMLC compared with those expressing nontargeting (SCR) shRNA was consistently observed. (B, left) Validation of protein knockdown in SC cultures. Western blots from control noninfected SC cultures (NI) and cultures infected with either vector alone (V) or vectors expressing targeting (MLC) and nontargeting (SCR) shRNA showing a marked reduction in the levels of phosphorylated MLC, total MLC, and both isoforms of myosin II only in SC cultures infected with lentivirus shMLC. (right) Western blots of myelinating cocultures showing a marked reduction in the total amount of myelin proteins (P0 and MBP) in cocultures established with SC infected with shMLC. Control for vector expression (GFP) and protein loading (actin) are also shown. (C) Quantitation of MBP⁺ segment number and length formed in 18-d-old myelinating cocultures by SC infected with shMLC compared with nontargeting control (shSCR). Data represent mean $\pm$ SEM from two independent experiments (three cultures per condition per experiment). (D) 5-d-old cocultures stained for GFP to visualize infected SCs and also for neurofilament and laminin α 2. SC expressing shMLC shRNA have round nuclei and extend multiple long and thin processes. Knockdown cultures also showed a marked reduction in the expression of laminin α 2 chain and impaired basal lamina assembly. In control cultures (shSCR), SC exhibited the characteristic elongated morphology upon contact with the axons. (E) Detail of an SC expressing shMLC-GFP in coculture with axons (blue). The cell has multiple processes, some of which appear to contact axons (asterisks), whereas others fail to do so (arrowheads). Bars: (A) 100 μ m; (D) 100 μ m; (E) 20 μ m.

myosin II (hence called MLC2). Phosphorylation of MLC2 activates the motor activity of myosin II and is required for assembly of myosin II heavy chains (Bresnick, 1999). As controls, SC cultures were infected with the empty lentiviral vector (pLL3.7) or nontargeting (scrambled) lentivirus (shSCR). Western blotting confirmed a significant and specific reduction in the amount of MLC2, phospho-MLC2, and both myosin IIA and IIB in SC infected with two different constructs expressing shMLC2 used separately (not depicted) or in combination (Fig. 2 B, left). Infected SC were then seeded onto DRG neuronal cultures and kept under myelinating conditions for 2–3 wk. In agreement

with our inhibitor data, the amount of myelin formed in cultures established with myosin-deficient SC was significantly reduced (Fig. 2 A). Western blotting also confirmed a decrease in the amount of myelin proteins (MBP and P0) expressed in knockdown cultures (Fig. 2 B, right). Quantitation of the number and length of MBP⁺ segments (Fig. 2 C) showed that in the absence of MLC2, the number of MBP⁺ segments per field was significantly reduced ($P < 0.001$) and were on average 30% shorter than controls ($P < 0.001$).

Myosin-deficient SC exhibited a morphology akin to that observed in blebbistatin-treated cultures with rounded nuclei

Schwann cells



Cocultures

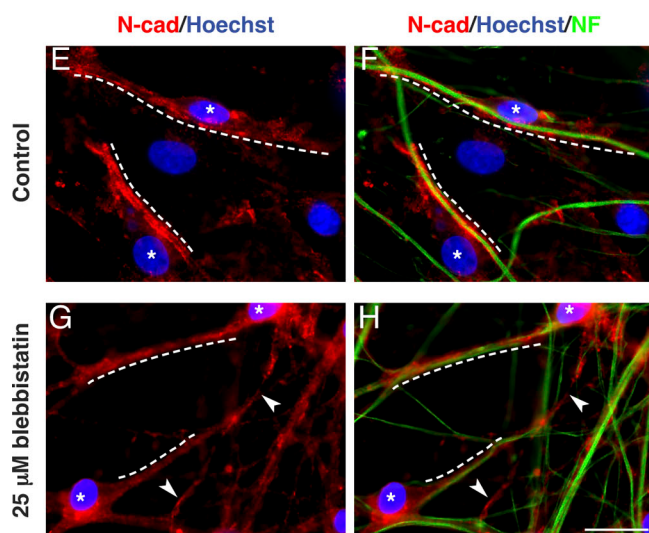


Figure 3. Myosin II regulates actin protrusive activity and is necessary to establish proper SC polarity. Subconfluent SC cultures stained with phalloidin and antibodies to N-cadherin, MLC, and vinculin. (A and B) In control cultures, SC display abundant cortical actin and stress fibers (dotted line). MLC staining is also found at stress fibers (insets 1 and 3) but not at focal adhesions (arrowheads, inset 3). (B) Two randomly polarized (front-to-back) SC showing concentration of N-cadherin staining in actin-rich protrusions at the leading edge (arrowheads) and also at the tip of the cell tail (asterisks, inset 2). Accumulation of MLC is observed at the retracting tail (inset 2), where it colocalizes with vinculin (inset 4). (C and D) SC treated with blebbistatin (25 μ M for 60 min) display striking changes in the organization of the actin cytoskeleton, including loss of stress fibers and the formation of multiple actin spikes throughout the entire cell body, where N-cadherin is highly concentrated (arrowheads). Many long and very thin cell processes (asterisks) are also observed in treated cultures. (E–H) SC-DRG cocultures maintained for 3 d in myelin-promoting media with or without blebbistatin and stained for N-cadherin, neurofilament, and Hoechst nuclear staining. In control conditions (E and F), SCs that are starting to elongate (asterisks) show a “track-like” pattern of N-cadherin staining along the axons they contact (broken line). In blebbistatin-treated cultures (G and H), multipolar SCs (asterisks) extend many N-cadherin-positive processes toward various axons. Staining in these processes at the SC–axon interface (dotted line) does not show the track-like pattern found in controls. Arrowheads indicate processes with patchy N-cadherin staining that do not align with any axon in the field. Bars, 10 μ m.

and multiple long and thin processes that failed to properly elongate along axons (Fig. 2, D and E). In addition, defects in α 2 laminin expression and basal lamina organization were also observed (Fig. 2 D). Collectively, these data suggest that knock-down of myosin II in SC impairs their ability to properly polarize, associate with axons, and myelinate.

Myosin II regulates localized actin protrusive activity and is necessary to establish proper SC polarity and contact with the axon

Several studies have demonstrated a direct role of myosin II in the control of cell polarity and localized actin-protrusive activity, which are essential for directed migration (Lo et al., 2004; Even-Ram et al., 2007). To further evaluate the effects of myosin II inhibition on SC morphology and polarity, we analyzed the changes in actin cytoskeleton and actin-associated proteins in cultures treated with blebbistatin. In control cultures, SC displayed abundant cortical actin and stress fibers (Fig. 3 A), where myosin light chain (MLC) staining was also found (Fig. 3 B, inset 1). Staining for the cell adhesion molecule N-cadherin, a protein thought to mediate the initial growth of SC processes and their alignment with axons (Wanner and Wood, 2002), was enriched at membrane protrusions (Fig. 3 B), together with ERM (not depicted). In randomly polarized (front-to-back) SC, N-cadherin was concentrated at the leading edge protrusions and also at the tip of the cell tail (Fig. 3 B, inset 2). MLC staining was also enriched at the retracting tail (Fig. 3 B, inset 2), where it colocalized with vinculin (Fig. 3, inset 4), a marker of focal adhesions (Fig. 3 B, inset 3). Treatment with blebbistatin (15–60 min) resulted in striking changes in the organization of the actin cytoskeleton (Fig. 3 C). Changes included loss of stress fibers and the formation of multiple actin spikes and ruffling lamellipodia throughout the entire cell body (Fig. 3 C and Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200802091/DC1>); N-cadherin and ERM were highly concentrated at these sites (Fig. 3 D, arrowheads).

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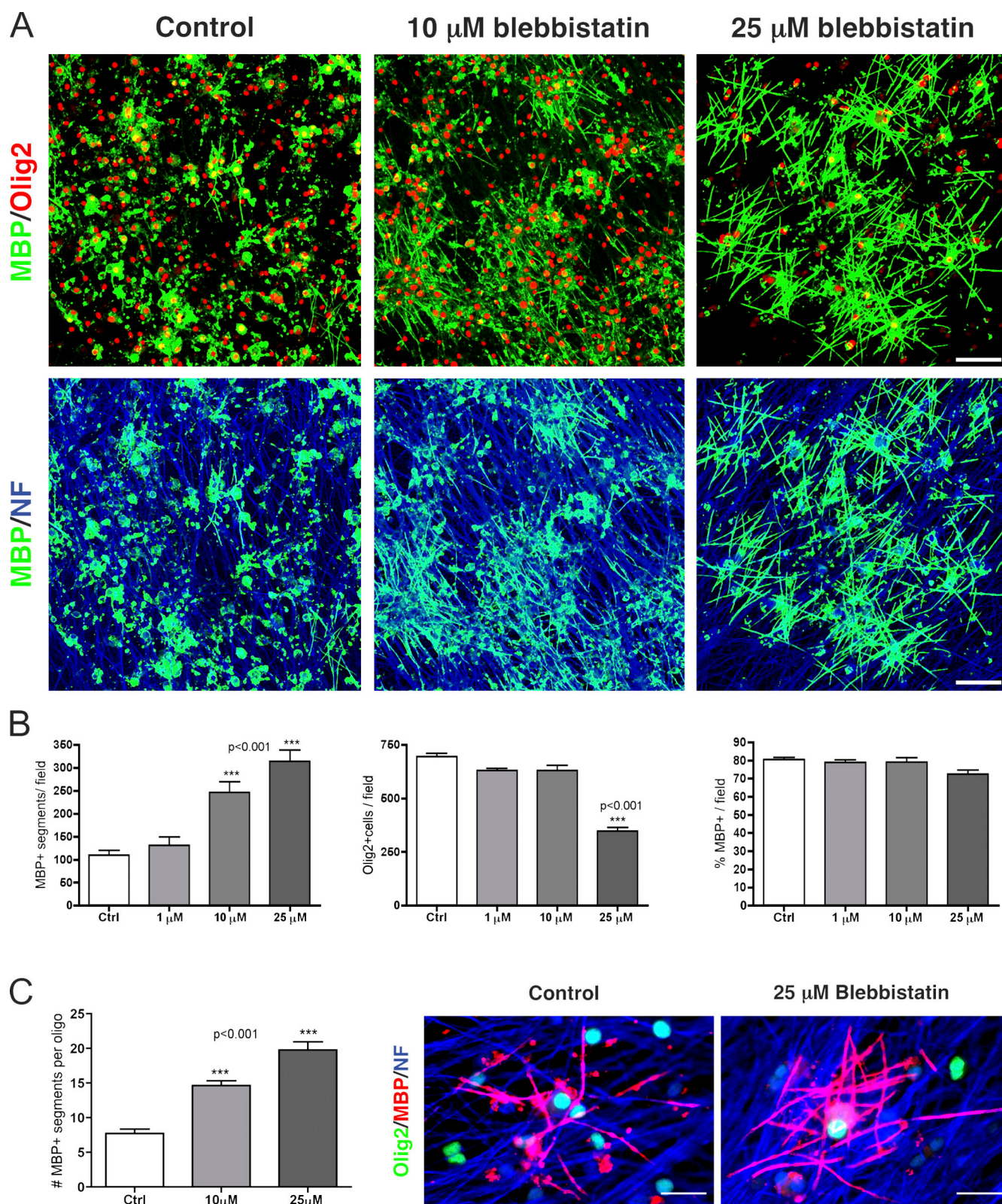


Figure 4. Myosin II inhibition in oligodendrocyte-DRG cocultures enhances myelin formation. (A) Purified OL progenitors (OPC) were seeded onto 3-wk-old DRG neuronal cultures. After 2 wk in media containing 1 μ g/ml TrkA-Fc, cultures were stained for Olig2, MBP, and neurofilament. Treatment with blebbistatin resulted in a prominent dose-dependant increase in the amount of myelin segments formed in cultures. (B) Quantitation of myelin segments (left), OL cell number (middle), and MBP expression (right) in control and blebbistatin-treated cultures. A significant dose-dependent increase in the amount of MBP+ segments was observed in cultures treated with blebbistatin ($P < 0.001$, ANOVA). The percentage of MBP+ cells is comparable in all conditions, and at the highest blebbistatin concentration (25 μ M), a significant reduction in the number of Olig2+ cells was observed. Data represent mean $\pm$ SEM from two independent experiments (two cultures per condition per experiment). (C, left) Quantitation of the number of myelin segments formed per single OL in cultures. Low-density 16-d-old myelinating cocultures were stained for Olig2, MBP, and neurofilament to identify individual OL nuclei, OL expressing MBP,

Many long and very thin dendrite-like processes were also observed in treated cultures (Fig. 3 B, asterisks).

Because perturbation of N-cadherin function in SC-DRG cocultures also results in multipolar SC that do not align with axons (Wanner and Wood, 2002), we next examined the distribution of N-cadherin in premyelinating SC-DRG cocultures treated with blebbistatin. As described previously (Wanner and Wood, 2002), accumulation of N-cadherin in a “track-like” staining pattern was observed along the SC–axon interface in control cultures (Fig. 3, E and F). This distribution was dramatically affected by blebbistatin treatment, as multipolar SC extended many N-cadherin–positive processes toward different axons (Fig. 3, G and H). The pattern for N-cadherin staining along these processes was different from that observed in control cultures (Fig. 3, E and F), and although some patchy accumulation was observed at points of SC–axon contact, they were not restricted to these sites but were also found in processes that did not contact axons (Fig. 3, G and H). Thus, our data suggest that myosin II in SC restricts actin protrusive activity, which in turn is important to establish SC polarity and proper contact with the axon.

Myosin II inhibition in OL-DRG cocultures enhances myelin formation

We next examined whether myosin II was also required for central myelination. To this end, cocultures of DRG neurons and purified rat cortical OL precursor cells (OPC) were grown for 2 wk in myelination-promoting conditions with or without blebbistatin. Cultures were then stained for Olig2, MBP, and neurofilament to identify individual OL nuclei, OL expressing MBP, and those actively myelinating axons. In striking contrast to SC myelination, inhibition of myosin II activity by blebbistatin resulted in a prominent increase in the amount of myelin formed in DRG-OL cocultures (Fig. 4 A). Quantitation of the number of MBP⁺ segments showed a significant dose-dependent increase ($P < 0.001$, analysis of variance [ANOVA]) in the amount of MBP⁺ segments in cultures treated with blebbistatin (Fig. 4 B). Interestingly, despite the difference in the total number of myelin segments, we found that differentiation of OL as measured by MBP expression (Fig. 4 B) was comparable between control and treated cultures (~ 70 – 80% MBP⁺ cells). Furthermore, at the highest blebbistatin concentration tested (25 μM), a significant decrease in proliferation (Table S1) and in the total number of Olig2⁺ cells was consistently observed, yet these cultures had, on average, more myelinated segments per field than control cultures ($P < 0.001$). These results strongly suggest that in blebbistatin-treated cultures, individual myelinating OL give rise to more internodes than OL in control cultures. To facilitate the quantitation of the number of internodes made by a single myelinating OL, we established cocultures with a lower number of OPC (50,000 instead of 100,000). As anticipated, the mean number of myelin segments made by a single OL in blebbistatin-treated cultures was 3–4 times more than OL in control cultures (control, 4.8 ± 3.5 ; 10 μM blebbistatin, 14.6 ± 5.5 ; 25 μM

blebbistatin, 18.5 ± 9.3 ; mean $\pm$ SEM; $P < 0.001$; Fig. 4 C). Collectively, these results indicate that myosin II activity is not required for OL differentiation and myelination, and that in the presence of blebbistatin, individual OL are more branched and make more myelin.

Knockdown of myosin II in OL-DRG cocultures by shRNA enhances myelin formation

To further investigate the contribution of OL myosin II on myelin formation, we infected OPC-DRG cocultures with shRNA lentivirus against the regulatory chain of MLC. Lentiviral infection of purified OPC cultures resulted in significant cell death (unpublished data), whereas infection of OPC in cocultures with DRG neurons was well tolerated. Incubation of cocultures with lentivirus resulted in the preferential infection of OPC rather than neurons, as indicated by GFP expression (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200802091/DC1>). As a further control, we infected established DRG cultures with the lentivirus for several days before adding OPC and inducing myelination. As shown in Fig. 5, knockdown of MLC in OPC-DRG cocultures also resulted in a significant increase ($P < 0.001$) in myelination similar to that observed in cultures treated with blebbistatin. Myelination in the presence of nontargeting lentivirus (shSCR) or vector alone was comparable to that observed in nontreated control cultures. In addition, myelination of neurons preinfected with lentivirus was comparable to controls (unpublished data), which further indicates that enhanced myelination results from the specific inhibition of myosin II in OL.

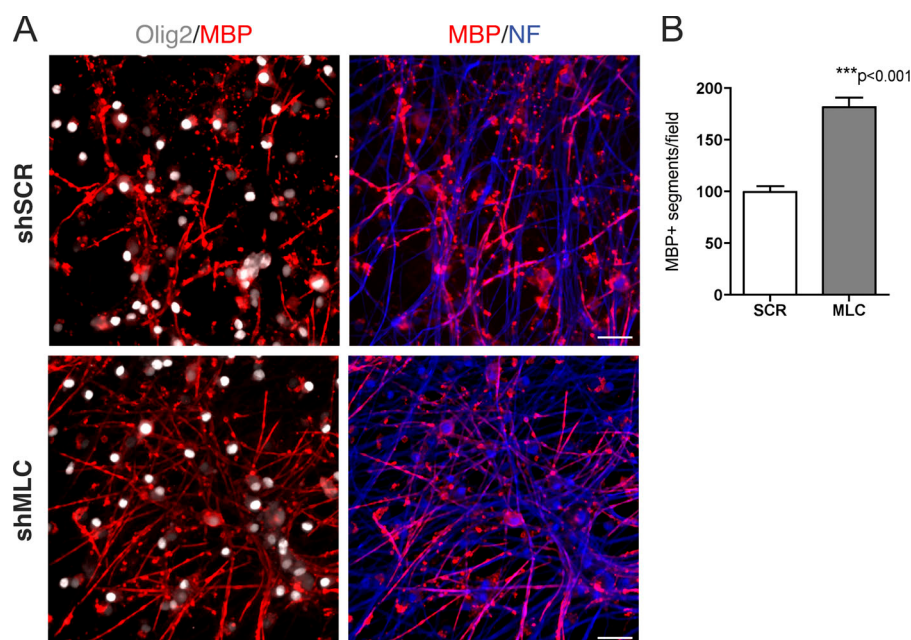
Inhibition of myosin II increases the complexity of OL branching in cultures

To further characterize the effects of myosin II inhibition on OL, we measured the effects of blebbistatin treatment on proliferation, survival, and differentiation of purified OPC cultures. In contrast to OPC in coculture with neurons, where we found a decrease in proliferation at the highest drug dose (Table S1), blebbistatin had no significant effect on the proliferation of purified OPC maintained for 3 d in media containing PDGF and basic FGF (bFGF; $35.6 \pm 1.3\%$ [control] vs. $35 \pm 1.1\%$ [25 μM blebbistatin]; mean percentage BrdU $\pm$ SEM; $P > 0.05$). Staining for caspase 3 revealed a slight increase in the percentage of apoptotic cells in blebbistatin-treated cultures; however, this difference was not statistically significant ($3.3 \pm 0.4\%$ [control] vs. $4.6 \pm 0.7\%$ [25 μM blebbistatin]; mean $\pm$ SEM, $P > 0.05$). Thus, we concluded that inhibition of myosin II did not have a significant impact on OPC proliferation and survival.

To evaluate differentiation, OPC cultures were kept for 3 d in media supplemented with T3 with or without blebbistatin and then stained for MBP expression. Unlike OPC in coculture with neurons, we found a modest but significant increase in the percentage of cells expressing MBP in blebbistatin-treated cultures compared with control ($40 \pm 1.7\%$ [control] vs. $49 \pm 2\%$

and OL actively myelinating axons. A significant increase in the mean number of myelin segments made by a single OL in treated cultures was observed ($P < 0.001$, ANOVA). Data represent mean $\pm$ SEM from two independent experiments (two cultures per condition per experiment). (right) Representative examples of myelinating OL in control and treated cultures are shown. Bars: (A) 50 μm ; (C) 20 μm .

Figure 5. Knockdown of myosin II in OL-DRG cocultures enhances myelin formation. (A) 3-wk-old myelinating OL-DRG cocultures stained for MBP, Olig2, and neurofilament. Cocultures were infected with a lentiviral vector expressing shRNA against the regulatory chain of myosin II (shMLC) or nontargeting scrambled sequence (shSCR). Knockdown of MLC in OPC-DRG cocultures resulted in a significant increase in myelination similar to that observed in cultures treated with blebbistatin. Myelination in control cultures infected with vector alone or in cultures where neurons were preinfected with lentivirus was comparable to that observed in nontreated control culture (not depicted). Bars, 50 μ m. (B) Quantitation of MBP⁺ segments per field. Data represent mean $\pm$ SEM from two independent experiments (three cultures per condition per experiment).



[treated]; mean $\pm$ SEM; $P < 0.005$; Fig. 6 A). OL in the blebbistatin-treated group also exhibit extensive membrane expansion (Fig. 6 A), a change that has been correlated with myelin sheath formation (Kachar et al., 1986; Kim et al., 2006; Sloane and Vartanian, 2007). We next examined the effects of blebbistatin treatment in OL morphological complexity and branching. We measured the fractal dimension of individual OL in control and blebbistatin-treated cultures. An increase in fractal dimension has been correlated with OL maturation in culture (Behar, 2001). We found that treatment with blebbistatin significantly increased the mean fractal dimension of OL in cultures (1.37 ± 0.12 [control] vs. 1.46 ± 0.08 [treated] mean $\pm$ SEM; $P < 0.001$; Fig. 6 B), which indicates more complex cytoskeleton branching in the absence of myosin II activity. Real-time studies also revealed a dramatic increase in protrusive activity along the OPC cell body and branches, with extensive formation of filopodia and ruffling lamellipodia. We also observed the development of new branches within 15–30 min of treatment with blebbistatin via the coalescence of some of these processes (Videos 2 and 3, available at <http://www.jcb.org/cgi/content/full/jcb.200802091/DC1>). These results suggest that myosin II inhibition influences OL morphogenesis by promoting complex branching and lamella formation, changes that have been correlated with myelin formation in vitro (Kachar et al., 1986) and in vivo (Kim et al., 2006; Sloane and Vartanian, 2007).

Myosin II expression is down-regulated during OPC maturation in vitro and in vivo

We next examined whether the levels of myosin II protein expression change as OL mature in culture. Cell lysates were prepared from purified OPC cultures kept in proliferating media (PDGF + bFGF) for 3 d or differentiating media (T3) for 2–7 d. As shown in Fig. 6 C, levels of myosin IIB (the main isoform found in OPC and brain) are high when OPC are maintained as proliferating progenitors. When cultures are switched to differ-

entiating media and increase their MBP expression, the levels of myosin IIB are strikingly down-regulated. Staining of OPC cultures similarly showed that myosin II is expressed at high levels in OPC before their differentiation and MBP expression (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200802091/DC1>). In vivo, myosin II and phosphorylated MLC2 are also down-regulated before myelination, as indicated by Western blots of rat brains collected at different developmental stages (Fig. 6 D). Because myosin II is a known regulator of actin bundle turnover (Guha et al., 2005; Medeiros et al., 2006), we examined whether down-regulation of myosin II correlates with changes in the expression of proteins involved in promoting actin polymerization and branching. As shown in Fig. 6 D, the expression levels of WAVE and neuronal Wiskott-Aldrich syndrome protein (N-WASP) were increased at the onset of MBP expression in OPC and brain extracts, in accordance with their proposed role in myelin formation (Kim et al., 2006; Bacon et al., 2007). In contrast, the levels of total actin and Arp2/3 remained unchanged, whereas the levels of phosphorylated (inactive) cofilin were down-regulated, which is consistent with the role of the nonphosphorylated (active) form in promoting actin polymerization (Huang et al., 2006). Collectively, these results suggest that down-regulation of myosin II activity is part of the “default” differentiation program of OL in vitro and in vivo and might play a role in promoting actin polymerization at the onset of myelination.

Discussion

In this study, we have shown that myosin II differentially regulates myelin formation by SC and OL (Fig. 7). Myosin II activity is required for proper interactions between SC and axons, and its inhibition or down-regulation impairs the ability of SC to segregate axons and elongate along them, preventing the formation of a 1:1 relationship, which is critical for their differentiation

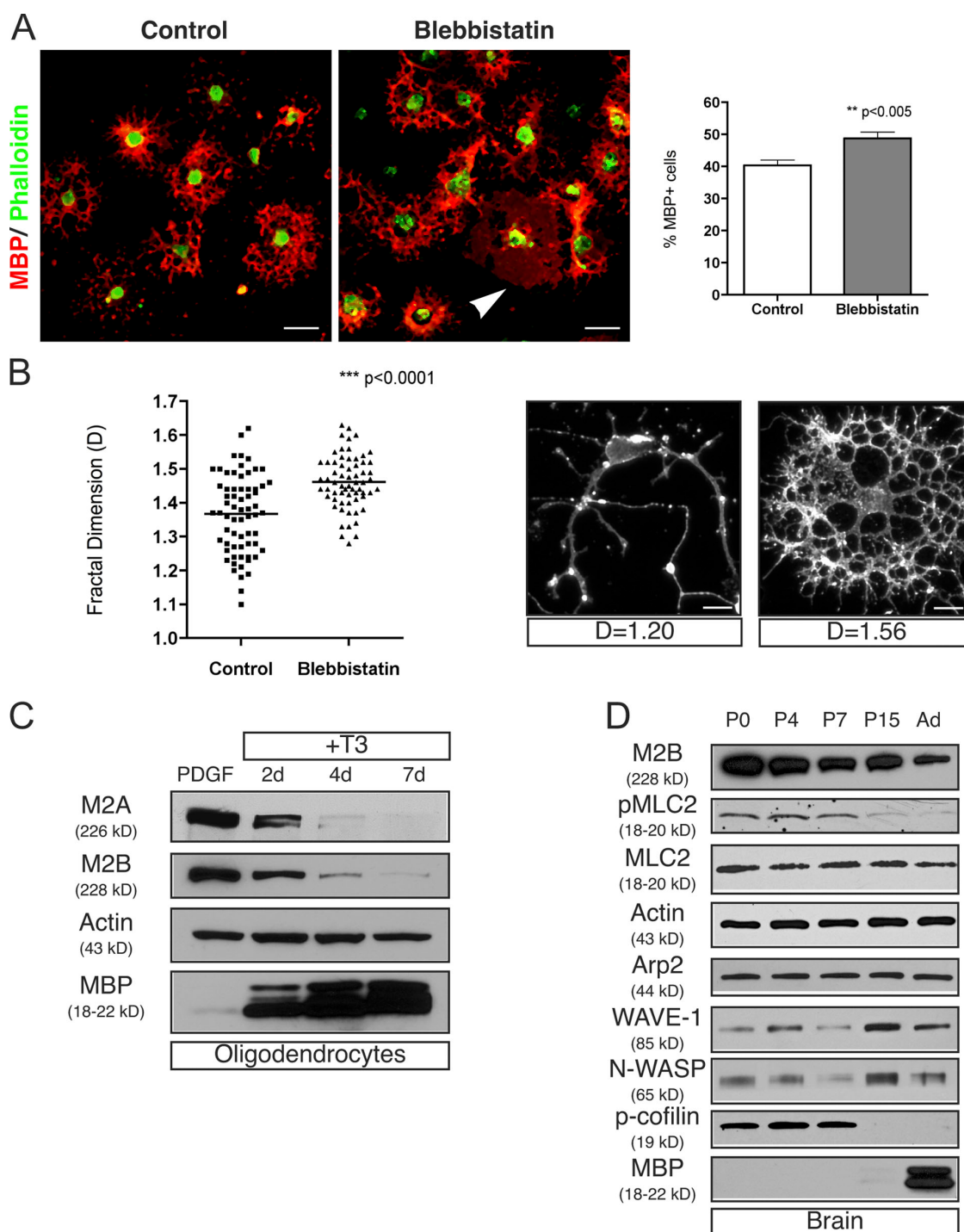


Figure 6. Blebbistatin promotes OPC differentiation in vitro and increases the complexity of OL branching in cultures. (A) Purified OPC kept for 3 d in differentiation-promoting media in the absence or presence of 10 μ M blebbistatin. Staining for MBP revealed a slight but significant increase in the percentage of MBP⁺ cells in myosin II-inhibited cultures. Examples of OL exhibiting extensive membrane expansion are indicated (arrowhead). Data in graph represent the mean $\pm$ SEM of four independent experiments (two cultures per condition per experiment). Bar, 20 μ m. (B) Fractal analysis of OPC morphological complexity was performed in cultures kept for 3 d in differentiation-promoting media in the absence or presence of 10 μ M blebbistatin. The mean fractal dimension (D) in blebbistatin-treated cultures was significantly higher than control [1.37 ± 0.12 [control] vs. 1.46 ± 0.08 [treated]; mean $\pm$ SD; $P < 0.0001$; t test], which indicates more complex cytoskeleton branching in the absence of myosin II activity. Representative examples of low- and high-complexity OPC stained with O4 are shown on the right. Bars, 10 μ m. (C) Western blot of OPC cultures kept in proliferating media (PDGF) for 3 d or differentiating media (T3) for 2–7 d. A significant reduction in the levels of myosin II (isoforms A and B) was found between cultures kept in proliferating conditions compared with T3. As OPC differentiate, the levels of myosin II are further down-regulated. (D) Developmental expression profile of myosin II, actin-binding proteins, and MBP in rat brain lysates. The levels of myosin IIB and the phosphorylated regulatory chain (pMLC2) are down-regulated as myelination progresses in rat CNS, whereas total MLC2 remain unchanged. Total actin and Arp2 also remain constant, but the levels of WAVE1 and N-WASP, proteins that are involved in actin polymerization, are up-regulated at the onset of myelination (P15). In addition, the levels of p-cofilin are down-regulated at this stage, which is consistent with the role of the nonphosphorylated form in promoting actin polymerization.

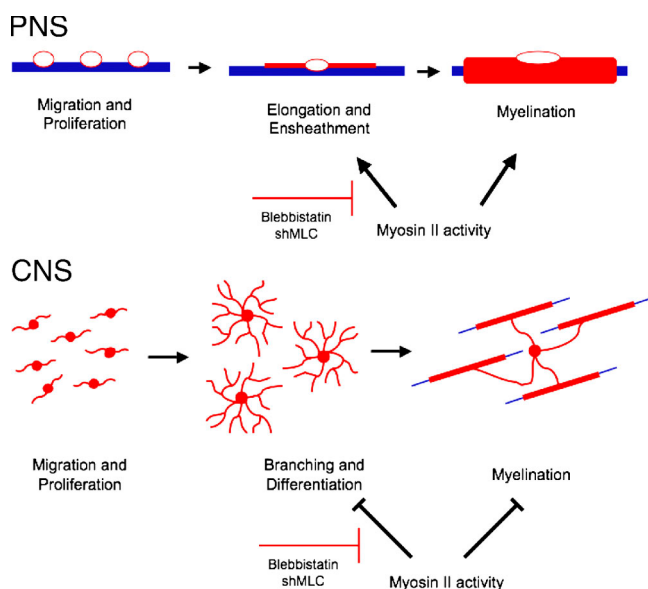


Figure 7. Summary of myosin II inhibition effects on myelination by SC and OL. Myosin II is an important regulator of SC cytoskeleton during differentiation and myelination. Myosin II appears to be necessary for the establishment of SC polarity and elongation along the axons, a crucial step in the initiation of myelin formation in the PNS. In the absence of myosin II activity, SC are unable to segregate axons in a 1:1 relationship and myelinate. In contrast, OL differentiation and myelin formation are independent of myosin II activity. Furthermore, down-regulation of myosin II promotes actin polymerization and branching, and also results in a significant increase in the amount of myelinated segments formed in culture.

and myelination. In contrast, OL branching and myelin formation are potentiated by inhibition or down-regulation of myosin II. Thus, by controlling the spatial and localized activation of actin polymerization, myosin II regulates SC polarization along the axon and OL branching, and thereby their ability to form myelin. These results indicate that the mechanisms regulating myelin formation in the PNS and CNS are distinct.

Myosin II is a key regulator of SC cytoskeleton polarity, differentiation, and myelination

We have presented evidence that myosin II activity is an important regulator of SC cytoskeleton organization during differentiation and myelination. SC lacking myosin II activity exhibit a multipolar morphology, with round nuclei and multiple long processes that fail to elongate properly along the axons and myelinate. Multipolar morphology is also observed in myosin II-null fibroblasts (Lo et al., 2004; Even-Ram et al., 2007), which are unable to move in a directed and persistent manner. Thus, the failure to segregate and eventually myelinate the axons they contact might be partly explained by the inability of multipolar SC to orient their cytoskeleton and move in a directed manner, following cues from the axon and the ECM. EM analysis corroborated these observations, demonstrating that in myosin II-inhibited cocultures, SC associate with but fail to completely ensheath axons. Similarly, expression of myelin-specific proteins and transcription factors, and the amount of myelin formed in myosin II-inhibited cocultures, was greatly reduced. In agreement with these results, disruption of actin polymerization by

cytochalasin D inhibits SC elongation, myelin-specific mRNA expression, and myelination (Fernandez-Valle et al., 1997). Likewise, defects in SC polarity, regulation of actin-protrusive activity, and axon alignment have been shown in merlin-deficient Schwannoma cells and are thought to be involved in the process of dedifferentiation of these tumor cells (Nakai et al., 2006; Flaiz et al., 2007).

We postulate that, akin to its role in epithelial cells, myosin II plays a key role in SC polarity in regulating the formation, localization, and spreading of cell-cell contacts between SC processes and axons. Myosin II-mediated contractility controls the accumulation of adhesion complexes and the establishment of epithelial cell polarity (Ivanov et al., 2005; Miyake et al., 2006) and also helps to maintain adhesion and cadherin accumulation at the apical border of epithelial cells (Shewan et al., 2005; Ma et al., 2007).

These observations are likely to be relevant to the initial interactions between SC and axons, as during their development, SC acquire a distinct polarity with an adaxonal region contacting the axon and abaxonal surface in contact with the basal lamina (Bunge and Bunge, 1986). As for epithelial cell function, the development of this polarity is essential for SC differentiation and myelination and also plays a key role in establishing the different functional domains of myelinated axons (Salzer, 2003).

The initial contact and alignment of SC processes with the axons they myelinate is mediated by the accumulation of cell-adhesion molecules such as N-cadherin along the SC-axon interface (Wanner and Wood, 2002), where it colocalizes with the polarity protein Par-3 (Chan et al., 2006). We have found that myosin II inhibition perturbs N-cadherin accumulation at sites of SC-axon contact and results in a multipolar SC that is unable to align along the axons, which is similar to that resulting from disrupting N-cadherin function (Wanner and Wood, 2002). Thus, loss of actomyosin contractility and delocalized accumulation of cell adhesion complexes might in part explain the effects in SC morphology and differentiation caused by the inhibition or down-regulation of myosin II activity.

Down-regulation of myosin II activity promotes actin polymerization, oligodendrocyte branching, and myelin formation

In contrast to SC, terminal differentiation of OL is paradoxically promoted in the absence of myosin II activity. Thus, the percentage of OPC expressing MBP, the complexity of OL branching, and the amount of myelin segments formed in culture by individual cells are all increased. These results indicate that myosin II activity modulates key aspects of OL morphogenesis and myelination. In potential agreement, there is an inverse correlation between the levels of MBP and myosin II expression, which suggests that down-regulation of myosin II activity may be an obligate component of the differentiation program of OPC in vitro and in vivo. Interestingly, recent functional genomics studies have shown that Myh10, the heavy chain of nonmuscle myosin IIB, is enriched in acutely purified OPC and is similarly down-regulated during OL terminal differentiation (Dugas et al., 2007; Cahoy et al., 2008).

OPC in culture differentiate progressively through a sequence of stages characterized by distinct morphologies and expression of specific markers (Pfeiffer et al., 1993). As OPC mature and form lamella, the complexity and branching of their cytoskeleton increases (Behar, 2001). These cytoskeletal changes have been linked to OL differentiation and myelin formation in vitro and in vivo (Kachar et al., 1986; Kim et al., 2006; Sloane and Vartanian, 2007). The importance of the actin cytoskeleton during these events is highlighted by the myelination deficits exhibited by mice deficient for WAVE-1, an actin-associated protein important for lamellipodia formation (Kim et al., 2006). In vitro, OPC from WAVE1-null mice have fewer processes and defective lamella formation, and also exhibit regional CNS hypomyelination (Kim et al., 2006).

In the present study, we identify an inverse correlation between myosin II activity and OPC branching and myelination. Thus, in OPC cultures, down-regulation of myosin II appears to promote actin polymerization and branching, changes that translate into more myelin segments formed in culture by individual OPC. Although the direct role of myosin II in actin turnover is still not well understood, recent in vitro studies have shown that myosin II can act as an actin-depolymerizing agent (Haviv et al., 2008), and that at high myosin II concentrations, the formation and growth of actin filament bundles is inhibited. This study also found that in the presence of blebbistatin, the density of actin filaments is higher, suggesting that “inactive motors” act as passive actin cross-linkers (Haviv et al., 2008). Of note, a similar role for myosin II as a negative regulator of actin polymerization during growth cone development and consolidation has been described previously (Medeiros et al., 2006). As the distal tips of OL processes resemble growth cones in their behavior and molecular composition (Fox et al., 2006), myosin II may have a similar role in regulating the actin cytoskeleton in both structures.

Regulation of myosin II activity during myelin formation

The mechanisms that regulate myosin II activity in SC and OL during myelination are not yet known. In SC, phosphorylation of the regulatory chain of myosin II (MLC2) is up-regulated at the onset of myelination and then down-regulated (Melendez-Vasquez et al., 2004), following the same pattern of Rho activation in peripheral nerve extracts (unpublished data). Our previous studies revealed an important role for ROCK during the initial events of PNS myelination, possibly by regulating the phosphorylation status of MLC (Melendez-Vasquez et al., 2004). However, inhibition of ROCK did not interfere with the wrapping of the axon, which suggests that another ROCK-independent mechanism controls the actual motor of SC adaxonal membrane progression around the axon. Our current study extends these initial observations and provides further evidence of the important role of polarized actomyosin contraction for SC myelin formation. Similar to our previous results with a ROCK-specific pharmacological inhibitor, the effects of myosin II inactivation on SC myelination are more marked when the inhibition occurs at the early stages of myelin formation, with little effect on older cultures. This early requirement for ROCK and myosin II activity at the onset of myelination appears to be important for the sup-

pression of SC branching and the establishment of proper cell polarity (Melendez-Vasquez et al., 2004). However, unlike inhibition of ROCK-mediated MLC phosphorylation, which promotes the coordinated wrapping of the axon, inhibition of myosin II ATPase activity by blebbistatin or down-regulation of myosin II total levels by shRNA completely blocked myelin formation. These contrasting effects provide compelling evidence for the existence of different mechanisms controlling myosin II activity within the SC, as has been demonstrated in other cell types (Totsukawa et al., 2000, 2004).

In OL, constitutive activation of Rho interferes with OL branching and maturation (Wolf et al., 2001; Liang et al., 2004). Although direct proof is lacking, inactivation of Rho might affect myosin II activity in OL via down-regulation of ROCK activity and, hence, MLC phosphorylation. Our results showing that the expression levels of phosphorylated MLC by OPC are higher before MBP up-regulation both in vivo and in vitro and that down-regulation of myosin II levels and activity promotes myelination are consistent with this model. Interestingly, phosphorylation of myosin II by ROCK has been identified as a negative regulator of F-actin polymerization in developing growth cones (Loudon et al., 2006), providing a possible mechanism for the regulation of OL branching and differentiation via the Rho–ROCK–myosin II pathway.

Myosin II has three isoforms, IIA, IIB, and IIC, which are differentially localized and regulated in many cell types and that appear to have different functions during cell migration and spreading (Conti and Adelstein, 2008). We have found that SC express all three isoforms, although in cocultures, myosin IIB predominates over IIA and IIC at the initial stages of axon–SC contact, whereas IIC is up-regulated in myelinating SC (unpublished data). In SC-only cultures, myosin IIB and MLC staining are colocalized at the tip of the retracting tail, in stress fibers, and at the leading edge of polarized cells (Fig. 3 and not depicted). OL also express all three myosin II isoforms. Myosin IIB is the main isoform detected in OPC in vitro and in vivo during early development, whereas myosin IIC, which is not detected at early stages, predominates in differentiated OL (Cahoy et al., 2008) and in adult brain (Golomb et al., 2004). Myosin IIB is also the predominant isoform expressed at the leading edge of OL processes together with actin (Song et al., 2001), making it a good candidate to mediate the effects of myosin II inhibition on OL myelination. In agreement with this idea, we have found that siRNA knockdown of myosin IIB in OPC has the same effect on OL myelination as MLC2/myosin II down-regulation (unpublished data).

Collectively, these data suggest that differential spatial regulation of myosin II isoforms by various kinases or the GTPases that in turn control these enzymes might play a key role in myelination. Of note, activation of Rac-GTPase downstream of β 1-integrin has been shown to play an important role in SC process extension, axonal sorting, and myelination (Benninger et al., 2007; Nodari et al., 2007). Because delocalized activation of Rac and membrane ruffling in myosin II-null fibroblasts has been shown to cause defects in polarized cell migration (Even-Ram et al., 2007), it will be of interest to determine the activation status of myosin II in the nerves of conditional β 1-integrin or Rac knockout mice.

Myelination in the CNS and PNS is differentially regulated by myosin II

Although SC and OL have a conserved function as myelin-forming cells, they differ in developmental origin (Jessen and Mirsky, 2005; Richardson et al., 2006) and key aspects of their cell biology, including their response to axon-derived signals during terminal differentiation and myelination (Stevens et al., 1998, 2002; Chan et al., 2004; Taveggia et al., 2005, 2008) and the different patterns of association with the axons they myelinate. Thus, SC and OL initially extend processes and associate with several axons, but whereas a single OL can wrap up to 40 axons simultaneously, individual SC will ultimately segregate and myelinate only one axon. Central myelinated axons also lack the basal lamina characteristic of SC myelination. In this study, we have shown that by controlling SC polarization along the axon and promoting OL branching, myosin II differentially regulates their ability to form myelin. Our findings provide a novel conceptual framework for how the different patterns of SC and OL myelination are established and indicate that the mechanisms regulating glial cell differentiation and myelination in the PNS and CNS are fundamentally different. Further studies on the signals that regulate myosin II activity in SC and OL should help to clarify its role during myelin formation.

Materials and methods

Myelinating SC-DRG cocultures

DRG neurons were isolated from embryonic day 16 rat spinal cords and either trypsinized or directly plated as explants on collagen-coated coverslips (BD Biosciences). Cultures were maintained in serum-free neurobasal medium (NB medium; 2% B27 supplement, 2 mM L-glutamine, 0.4% glucose, and 50 ng/ml 2.5S NGF). Nonneuronal cells were removed by feeding the cultures with NB medium containing 5-fluorodeoxyuridine and uridine. SC were isolated from postnatal day 2 sciatic nerves and expanded for ~3 wk in D media (DME, 10% FBS, and 2 mM L-glutamine) supplemented with 4 μ M forskolin and 5 ng/ml of the EGF domain of rhNRG-1 β 1 (R&D Systems). Myelinating cocultures were established by seeding purified DRG neuron cultures with 100,000 SC in C media (MEM, 10% FBS, 2 mM L-glutamine, 0.4% glucose, and 50 ng/ml 2.5S NGF). After 3 d, cocultures were changed to media supplemented with 50 μ g/ml ascorbic acid to initiate basal lamina formation and myelination. For studies of the effects of myosin II inhibition on myelination, cocultures were switched to myelin-promoting media containing blebbistatin (EMD). Control and treated cultures were allowed to myelinate for 2–3 wk, with fresh media provided every 2–3 d. To determine the extent of myelination in SC-DRG cocultures, the total number and length of MBP⁺ segments were counted in micrographs from 10–12 random low-power fields per coverslip using ImageJ version 1.38 (total of 20–24 fields per condition per experiment; total of 2–3 experiments). Statistical tests (*t* test and ANOVA) were performed using GraphPad Prism (GraphPad Software).

Purified OPC cultures and OPC-DRG myelinating cocultures

Primary OL precursors (OPC and A2B5⁺) were purified by immunopanning from mixed glial cultures of postnatal day 1 rat cerebral cortices as described previously (Taveggia et al., 2008). Purified OPC were seeded onto poly-lysine-coated glass coverslips and maintained in Sato media (DME, 100 μ g/ml transferrin, 100 μ g/ml BSA, 20 nM progesterone, 100 μ M putrescine, 30 nM sodium selenite, 2 mM L-glutamine, 5 μ g/ml insulin, 60 μ g/ml N-acetyl cysteine, and 10 μ M forskolin) with 10 ng/ml PDGF and 10 ng/ml bFGF for proliferation or induced to differentiate in Sato containing T3 (30 ng/ml). DRG explants were isolated from rat embryonic day 16 spinal cords and plated directly onto glass coverslips coated with Matrigel (BD Biosciences). Explants were kept in NB medium and treated with antimitotic agents to eliminate nonneuronal cells. Explants were allowed to extend a dense neurite network for at least 2–3 wk before their use in coculture experiments. OPC-DRG cocultures were established as described previously (Chan et al., 2004). In brief, freshly purified OPC (50,000–100,000 cells) were

seeded directly onto DRG explants in MEM media containing 10% FBS, 2 mM L-glutamine, and 0.4% glucose. Myelination was induced the following day by addition of 1 μ g/ml TrkA-Fc (R&D Systems). For studies of the effects of myosin II inhibition on myelination, blebbistatin (EMD) was also added to the media at this point and maintained throughout the experiment. Cultures were allowed to myelinate up to 18 d, with fresh media provided every 2–3 d. Myelin segments were identified by MBP and neurofilament staining, then counted in micrographs from 8–10 random low-power fields per coverslip using ImageJ version 1.38 (total of 16–20 fields per condition per experiment; total of 2–3 experiments). Statistical tests (*t* test and ANOVA) were performed using GraphPad Prism software.

RNAi of myosin II in purified glia and myelinating cocultures

To generate shRNAs, we used the pLentiLox (pLL3.7) vector (provided by L. Van Parijs, Massachusetts Institute of Technology, Cambridge, MA; Robinson et al., 2003). Two 19-nucleotide shRNAs (MLC1, 5'-GCACGGAGCGA-AAGACAAA-3'; and MLC2, 5'-GAGGCCTCAACATGATCG-3') targeting the regulatory (light) chain of rat myosin II (MLC) were designed using Easy siRNA (ProteinLounge). The 5'-phosphorylated PAGE-purified oligonucleotides were annealed and subcloned into HpaI-XhoI sites of pLL3.7. The lentiviral vector was transfected into 293FT cells together with packaging plasmids Δ 8.9 and pCMV-VSVG (provided by J. Milbrandt, Washington University, St. Louis, MO) using Lipofectamine 2000 (Invitrogen). As control, we used the empty pLL3.7 vector or a vector encoding shRNA to a nontargeting (scrambled) sequence. Viral supernatants were collected 72 h after transfection, centrifuged at 3,000 rpm for 15 min, aliquoted for one-time use, and frozen at -80°C. Freshly plated SC were incubated for 3 d with viruses at a 2:3 dilution (vol/vol) in D media (DME, 10% FBS, and 2 mM L-glutamine) supplemented with forskolin and rhNRG-1 (EGF domain). Cells were expanded for an additional week and maintained for 3 d in D media before use for myelination experiments. Protein knockdowns were confirmed by Western blotting. For shRNA knockdown experiments in OPC-DRG cocultures, cultures were incubated with lentivirus supernatant at 1:3 dilution for 2 d. The virus was removed and cultures were allowed to myelinate for an additional 2 wk. This protocol resulted in the effective infection of OPC over neurons. As a control, we used established DRG cultures that were infected with the virus before adding the OPC and inducing myelination.

Antibodies

Antibodies used in these studies included those reactive to: MBP (SMI-94) and neurofilament (SMI-31 and SMI-32; Sternberger Monoclonals); myosin IIA and IIB (Covance), cofilin, phosphorylated cofilin, MLC2, phosphorylated MLC2, phosphorylated FAK, Arp2, and vinculin (Cell Signaling Technology); phalloidin-FITC, phalloidin-coumarin, rabbit anti-EHS laminin, anti-caspase 3, and anti-actin (Sigma-Aldrich); anti-GFP, WAVE-1, and Olig2 (Millipore); anti-N-WASP (H100; Santa Cruz Biotechnology, Inc.); anti-N-cadherin and anti-Tau (BD Biosciences). Polyclonal antibodies to Krox-20 and Oct-6 (provided by D. Meijer, Erasmus University, Rotterdam, Netherlands), P0 (M. Filbin, Hunter College, City University of New York, New York, NY), laminin α -2 chain (P. Yurchenco, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ) and myelin-associated glycoprotein were also used. Mouse A2B5 and Ran-2 hybridomas were obtained from the American Type Culture Collection. Mouse O4 was a gift from M. Chao (New York University, New York, NY). Secondary antibodies conjugated to rhodamine, fluorescein, coumarin, or cyanin 5 were obtained from Jackson ImmunoResearch Laboratories.

Immunofluorescence

Cultures (SC, OPC, and myelinating cocultures) were fixed in 4% PFA and processed for immunocytochemistry as described previously (Melendez-Vasquez et al., 2004). For surface antigen staining (A2B5 and O4), cells were blocked with 1% donkey serum and 5% BSA in PBS for 30 min, then incubated with primary antibody as described previously (Zhang et al., 2006). Cultures were examined by epifluorescence or confocal microscopy (see the following paragraph).

Image acquisition and analysis

Epifluorescence images were acquired using a microscope (DMI 4000B; Leica) with LAS 1.7.0 software equipped with a digital camera (DFC350FX; Leica) and the following objectives: N PLAN 10x/0.25 NA; N PLAN L 40x/0.55 NA; and HCX PL APO CS 63x/1.4 NA oil UV (all from Leica). Confocal images were acquired with a laser scanning microscope (LSM 510; Carl Zeiss, Inc.) using Plan-Apochromat 20x/0.75 NA or Neofluor 40x/1.3 NA oil objectives and LSM software (all from Carl Zeiss, Inc.). Image processing and quantitation (including fractal analysis) was performed

using ImageJ version 1.38 and Photoshop CS8 (Adobe). Adjustment of image brightness or contrast was performed in some cases but without misrepresenting data.

Time-lapse microscopy

Videos were acquired with a DMI 4000B microscope system (see the previous section) fitted with a stage incubator and a temperature and CO₂ digital controller (CTI Controller 37000; Pecon). Cells were plated on poly-lysine-coated glass-bottomed 35-mm tissue culture dishes (MafTek) on D media (SC) or proliferating OPC media. Phase images from SC and OPC cultures were acquired for a period of 15–30 min using an N PLAN L 40x/0.55 NA objective. Cultures were maintained at 37°C and 5% CO₂ throughout the observation period.

Proliferation and survival assays

To investigate the effect of myosin II inhibition on the proliferation and cell survival of SC and OPC, BrdU incorporation (Boehringer Mannheim GmbH) and TUNEL assays (Promega) were performed according to manufacturers' instructions as described previously (Melendez-Vasquez et al., 2004; Zhang et al., 2006).

Cell extracts and Western blotting

Lysates from rat tissues (brain and nerves), cultures of SC, OPC, and myelinating cocultures were prepared as described previously (Melendez-Vasquez et al., 2004), subjected to SDS-PAGE, and blotted onto nitrocellulose. Appropriate regions of the blots were cut and incubated with specific antibodies and developed using chemiluminescent substrate (Thermo Fisher Scientific).

Electron microscopy

Control and treated myelinating cocultures were rinsed in PBS, fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, and processed further as described previously (Einheber et al., 1995). Specimens were examined on an electron microscope (CM10; Philips).

Online supplemental material

Fig. S1 shows changes in SC morphology after blebbistatin treatment in cocultures. Fig. S2 shows an example of SC forming multiple short internodes in blebbistatin-treated cultures. Fig. S3 shows preferential infection of OPC by lentivirus in cocultures. Fig. S4 shows expression of phosphorylated MLC during OPC differentiation in vitro. Table S1 shows the quantitation of BrdU incorporation in PNS and CNS myelinating cocultures. Video 1 shows formation of ruffling lamellipodia in SC after blebbistatin treatment. Videos 2 and 3 show actin-protrusive activity of an OPC before and after treatment with blebbistatin. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200802091/DC1>.

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References

- Bacon, C., V. Lakics, L. Machesky, and M. Rumsby. 2007. N-WASP regulates extension of filopodia and processes by oligodendrocyte progenitors, oligodendrocytes, and Schwann cells—implications for axon ensheathment at myelination. *Glia*. 55:844–858.
- Behar, T.N. 2001. Analysis of fractal dimension of O2A glial cells differentiating in vitro. *Methods*. 24:331–339.
- Benninger, Y., T. Thurnherr, J.A. Pereira, S. Krause, X. Wu, A. Chrostek-Grashoff, D. Herzog, K.A. Nave, R.J. Franklin, D. Meijer, et al. 2007. Essential and distinct roles for cdc42 and rac1 in the regulation of Schwann cell biology during peripheral nervous system development. *J. Cell Biol.* 177:1051–1061.
- Bresnick, A.R. 1999. Molecular mechanisms of nonmuscle myosin-II regulation. *Curr. Opin. Cell Biol.* 11:26–33.
- Bunge, M.B., and R.P. Bunge. 1986. Linkage between Schwann cell extracellular matrix production and ensheathment function. *Ann. N. Y. Acad. Sci.* 486:241–247.
- Bunge, R.P., M.B. Bunge, and M. Bates. 1989. Movements of the Schwann cell nucleus implicate progression of the inner (axon-related) Schwann cell process during myelination. *J. Cell Biol.* 109:273–284.
- Cahoy, J.D., B. Emery, A. Kaushal, L.C. Foo, J.L. Zamanian, K.S. Christopherson, Y. Xing, J.L. Lubischer, P.A. Krieg, S.A. Krupenko, et al. 2008. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* 28:264–278.
- Chan, J.R., T.A. Watkins, J.M. Cosgaya, C. Zhang, L. Chen, L.F. Reichardt, E.M. Shooter, and B.A. Barres. 2004. NGF controls axonal receptivity to myelination by Schwann cells or oligodendrocytes. *Neuron*. 43:183–191.
- Chan, J.R., C. Jolicoeur, J. Yamauchi, J. Elliott, J.P. Fawcett, B.K. Ng, and M. Cayouette. 2006. The polarity protein Par-3 directly interacts with p75NTR to regulate myelination. *Science*. 314:832–836.
- Chhabra, E.S., and H.N. Higgs. 2007. The many faces of actin: matching assembly factors with cellular structures. *Nat. Cell Biol.* 9:1110–1121.
- Conti, M.A., and R.S. Adelstein. 2008. Nonmuscle myosin II moves in new directions. *J. Cell Sci.* 121:11–18.
- Dugas, J.C., A. Ibrahim, and B.A. Barres. 2007. A crucial role for p57(Kip2) in the intracellular timer that controls oligodendrocyte differentiation. *J. Neurosci.* 27:6185–6196.
- Einheber, S., M.J. Hannocks, C.N. Metz, D.B. Rifkin, and J.L. Salzer. 1995. Transforming growth factor-beta 1 regulates axon/Schwann cell interactions. *J. Cell Biol.* 129:443–458.
- Even-Ram, S., A.D. Doyle, M.A. Conti, K. Matsumoto, R.S. Adelstein, and K.M. Yamada. 2007. Myosin IIA regulates cell motility and actomyosin-microtubule crosstalk. *Nat. Cell Biol.* 9:299–309.
- Fernandez-Valle, C., D. Gorman, A.M. Gomez, and M.B. Bunge. 1997. Actin plays a role in both changes in cell shape and gene-expression associated with Schwann cell myelination. *J. Neurosci.* 17:241–250.
- Flaiz, C., K. Kaempchen, C. Matthies, and C.O. Hanemann. 2007. Actin-rich protrusions and nonlocalized GTPase activation in Merlin-deficient schwannomas. *J. Neuropathol. Exp. Neurol.* 66:608–616.
- Fox, M.A., F.S. Afshari, J.K. Alexander, R.J. Colello, and B. Fuss. 2006. Growth cone-like sensorimotor structures are characteristic features of postmigratory, premyelinating oligodendrocytes. *Glia*. 53:563–566.
- Golomb, E., X. Ma, S.S. Jana, Y.A. Preston, S. Kawamoto, N.G. Shoham, E. Goldin, M.A. Conti, J.R. Sellers, and R.S. Adelstein. 2004. Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *J. Biol. Chem.* 279:2800–2808.
- Guha, M., M. Zhou, and Y.L. Wang. 2005. Cortical actin turnover during cytokinesis requires myosin II. *Curr. Biol.* 15:732–736.
- Haviv, L., D. Gillo, F. Backouche, and A. Bernheim-Groswasser. 2008. A cytoskeletal demolition worker: myosin II acts as an actin depolymerization agent. *J. Mol. Biol.* 375:325–330.
- Huang, T.Y., C. DerMardirossian, and G.M. Bokoch. 2006. Cofilin phosphatases and regulation of actin dynamics. *Curr. Opin. Cell Biol.* 18:26–31.
- Ivanov, A.I., D. Hunt, M. Utech, A. Nusrat, and C.A. Parkos. 2005. Differential roles for actin polymerization and a myosin II motor in assembly of the epithelial apical junctional complex. *Mol. Biol. Cell.* 16:2636–2650.
- Jessen, K.R., and R. Mirsky. 2005. The origin and development of glial cells in peripheral nerves. *Nat. Rev. Neurosci.* 6:671–682.
- Kachar, B., T. Behar, and M. Dubois-Dalq. 1986. Cell shape and motility of oligodendrocytes cultured without neurons. *Cell Tissue Res.* 244:27–38.
- Kim, H.J., A.B. DiBernardo, J.A. Sloane, M.N. Rasband, D. Solomon, B. Kosaras, S.P. Kwak, and T.K. Vartanian. 2006. WAVE1 is required for oligodendrocyte morphogenesis and normal CNS myelination. *J. Neurosci.* 26:5849–5859.
- Kippert, A., K. Trajkovic, L. Rajendran, J. Ries, and M. Simons. 2007. Rho regulates membrane transport in the endocytic pathway to control plasma membrane specialization in oligodendroglial cells. *J. Neurosci.* 27:3560–3570.
- Liang, X., N.A. Draghi, and M.D. Resh. 2004. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. *J. Neurosci.* 24:7140–7149.
- Lo, C.M., D.B. Buxton, G.C. Chua, M. Dembo, R.S. Adelstein, and Y.L. Wang. 2004. Nonmuscle myosin IIb is involved in the guidance of fibroblast migration. *Mol. Biol. Cell.* 15:982–989.
- Loudon, R.P., L.D. Silver, H.F. Yee Jr., and G. Gallo. 2006. RhoA-kinase and myosin II are required for the maintenance of growth cone polarity and guidance by nerve growth factor. *J. Neurobiol.* 66:847–867.
- Ma, X., J. Bao, and R.S. Adelstein. 2007. Loss of cell adhesion causes hydrocephalus in nonmuscle myosin II-B-ablated and mutated mice. *Mol. Biol. Cell.* 18:2305–2312.
- Medeiros, N.A., D.T. Burnette, and P. Forscher. 2006. Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nat. Cell Biol.* 8:215–226.

- Melendez-Vasquez, C.V., S. Einheber, and J.L. Salzer. 2004. Rho kinase regulates schwann cell myelination and formation of associated axonal domains. *J. Neurosci.* 24:3953–3963.
- Miyake, Y., N. Inoue, K. Nishimura, N. Kinoshita, H. Hosoya, and S. Yonemura. 2006. Actomyosin tension is required for correct recruitment of adherens junction components and zonula occludens formation. *Exp. Cell Res.* 312:1637–1650.
- Nakai, Y., Y. Zheng, M. MacCollin, and N. Ratner. 2006. Temporal control of Rac in Schwann cell-axon interaction is disrupted in NF2-mutant schwannoma cells. *J. Neurosci.* 26:3390–3395.
- Nodari, A., D. Zambroni, A. Quattrini, F.A. Court, A. D'Urso, A. Recchia, V.L. Tybulewicz, L. Wrabetz, and M.L. Feltri. 2007. β 1 integrin activates Rac1 in Schwann cells to generate radial lamellae during axonal sorting and myelination. *J. Cell Biol.* 177:1063–1075.
- Pfeiffer, S.E., A.E. Warrington, and R. Bansal. 1993. The oligodendrocyte and its many cellular processes. *Trends Cell Biol.* 3:191–197.
- Richardson, W.D., N. Kessaris, and N. Pringle. 2006. Oligodendrocyte wars. *Nat. Rev. Neurosci.* 7:11–18.
- Rubinson, D.A., C.P. Dillon, A.V. Kwiatkowski, C. Sievers, L. Yang, J. Kopinja, D.L. Rooney, M. Zhang, M.M. Ihrig, M.T. McManus, et al. 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* 33:401–406.
- Salzer, J.L. 2003. Polarized domains of myelinated axons. *Neuron.* 40:297–318.
- Shewan, A.M., M. Maddugoda, A. Kraemer, S.J. Stehbens, S. Verma, E.M. Kovacs, and A.S. Yap. 2005. Myosin 2 is a key Rho kinase target necessary for the local concentration of E-cadherin at cell-cell contacts. *Mol. Biol. Cell.* 16:4531–4542.
- Sloane, J.A., and T.K. Vartanian. 2007. Myosin Va controls oligodendrocyte morphogenesis and myelination. *J. Neurosci.* 27:11366–11375.
- Song, J., B.D. Goetz, P.W. Baas, and I.D. Duncan. 2001. Cytoskeletal reorganization during the formation of oligodendrocyte processes and branches. *Mol. Cell. Neurosci.* 17:624–636.
- Stevens, B., S. Tanner, and R.D. Fields. 1998. Control of myelination by specific patterns of neural impulses. *J. Neurosci.* 18:9303–9311.
- Stevens, B., S. Porta, L.L. Haak, V. Gallo, and R.D. Fields. 2002. Adenosine: a neuron-glial transmitter promoting myelination in the CNS in response to action potentials. *Neuron.* 36:855–868.
- Straight, A.F., A. Cheung, J. Limouze, I. Chen, N.J. Westwood, J.R. Sellers, and T.J. Mitchison. 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. *Science.* 299:1743–1747.
- Tavecchia, C., G. Zanazzi, A. Petrylak, H. Yano, J. Rosenbluth, S. Einheber, X. Xu, R.M. Esper, J.A. Loeb, P. Shrager, et al. 2005. Neuregulin-1 type III determines the ensheathment fate of axons. *Neuron.* 47:681–694.
- Tavecchia, C., P. Thaker, A. Petrylak, G.L. Caporaso, A. Toews, D.L. Falls, S. Einheber, and J.L. Salzer. 2008. Type III neuregulin-1 promotes oligodendrocyte myelination. *Glia.* 56:284–293.
- Thurnherr, T., Y. Benninger, X. Wu, A. Chrostek, S.M. Krause, K.A. Nave, R.J. Franklin, C. Brakebusch, U. Suter, and J.B. Relvas. 2006. Cdc42 and Rac1 signaling are both required for and act synergistically in the correct formation of myelin sheaths in the CNS. *J. Neurosci.* 26:10110–10119.
- Totsukawa, G., Y. Yamakita, S. Yamashiro, D.J. Hartshorne, Y. Sasaki, and F. Matsumura. 2000. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* 150:797–806.
- Totsukawa, G., Y. Wu, Y. Sasaki, D.J. Hartshorne, Y. Yamakita, S. Yamashiro, and F. Matsumura. 2004. Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts. *J. Cell Biol.* 164:427–439.
- Wanner, I.B., and P.M. Wood. 2002. N-cadherin mediates axon-aligned process growth and cell-cell interaction in rat Schwann cells. *J. Neurosci.* 22:4066–4079.
- Wolf, R.M., J.J. Wilkes, M.V. Chao, and M.D. Resh. 2001. Tyrosine phosphorylation of p190 RhoGAP by Fyn regulates oligodendrocyte differentiation. *J. Neurobiol.* 49:62–78.
- Zhang, Y., C. Tavecchia, C. Melendez-Vasquez, S. Einheber, C.S. Raine, J.L. Salzer, C.F. Brosnan, and G.R. John. 2006. Interleukin-11 potentiates oligodendrocyte survival and maturation, and myelin formation. *J. Neurosci.* 26:12174–12185.