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Tissue Inhibitor of Metalloproteinase-3 Promotes Schwann Cell Myelination

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Abstract

Tissue inhibitor of metalloproteinase-3 (TIMP-3) inhibits the activities of various metalloproteinases including matrix metalloproteinases and ADAM family proteins. In the peripheral nervous system, ADAM17, also known as TNF-α converting enzyme (TACE), cleaves the extracellular domain of Nrg1 type III, an axonal growth factor that is essential for Schwann cell myelination. The processing by ADAM17 attenuates Nrg1 signaling and inhibits Schwann cell myelination. TIMP-3 targets ADAM17, suggesting a possibility that TIMP-3 may elicit a promyelinating function in Schwann cells by relieving ADAM17-induced myelination block. To investigate this, we used a myelinating coculture system to determine the effect of TIMP-3 on Schwann cell myelination. Treatment with TIMP-3 enhanced myelin formation in cocultures, evident by an increase in the number of myelin segments and upregulated expression of Krox20 and myelin protein. The effect of TIMP-3 was accompanied by the inhibition of ADAM17 activity and an increase in Nrg1 type III signaling in cocultures. Accordingly, the N-terminus fragment of TIMP-3, which exhibits a selective inhibitory function toward ADAM17, elicited a similar myelination-promoting effect and increased Nrg1 type III activity. TIMP-3 also enhanced laminin production in cocultures, which is likely to aid Schwann cell myelination.

Keywords

ADAM17, Akt, ErbB2/3, Laminin, Nrg1 type III, N-TIMP-3

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Introduction

Myelination in the peripheral nervous system (PNS) is regulated by a close-contact signal between axons and Schwann cells. One of the axonal factors that provides a key promyelinating signal is neuregulin 1 type III (Nrg1 type III), which activates erbB2/erbB3 heterodimeric receptor complex on the Schwann cell surface (Nave & Salzer, 2006). In the absence of Nrg1 type III, axons failed to become myelinated by Schwann cells (Taveggia et al., 2005). The level of axonal Nrg1 type III determines the thickness of the myelin sheath generated by Schwann cells (Michailov et al., 2004). Furthermore, overexpression of Nrg1 type III induces early onset of myelination and myelination on small caliber axons that would normally be unmyelinated. The role of the Nrg1 type III-erbB receptor signal is mediated by the downstream PI3-kinase/Akt pathway; the activation of which is required for Schwann cell myelin formation (Maurel & Salzer, 2000; Taveggia et al., 2005).

Nrg1 type III is a membrane protein, whose expression is enriched in the PNS neurons. Unlike the other types of the Nrg1 family, type III Nrg1 acquires two transmembrane structures through the cystine-rich N-terminal domain that anchors itself into the plasma membrane (Mei & Nave, 2014). Proteolytic processing at the juxtamembrane region by various proteases generates a membrane anchored N-terminus fragment (NTF) that presents a signaling-capable epidermal growth factor (EGF) domain to the luminal space. Notably, Nrg1 type III processing by β-secretase (or BACE1) generates an NTF that activates erbB receptors and promotes Schwann cell myelination (Hu et al., 2006;

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Willem et al., 2006; Luo et al., 2011). Similarly, ADAM10, a member of the disintegrin metalloproteinase family also generates a Nrg1 NTF that activates erbB receptor signaling, albeit without an effect on Schwann cell myelination (Luo et al., 2011). In comparison, processing of Nrg1 type III by ADAM17 (or TNF-α converting enzyme, TACE) generates an NTF that attenuates erbB receptor signaling and inhibits Akt activation (La Marca et al., 2011), indicating its role as a negative regulator of the axonal Nrg1 signaling. Supporting this, knockdown of ADAM17 in the PNS enhances Nrg1 type III-associated Akt activation and increases Schwann cell myelination (La Marca et al., 2011). These findings suggest that the myelin-promoting activity of Nrg1 type III is regulated by the combined activities of proteases that modulate the signaling effectiveness of the Nrg1 NTF.

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of matrix metalloproteinases (MMPs) and ADAM family proteins. Among the TIMPs, TIMP-3 has the broadest inhibition spectrum toward ADAM proteins that include ADAM10 and ADAM17 (Nagase & Murphy, 2008). Furthermore, TIMP-3 is the only member of the TIMP family proteins that is able to effectively inhibit ADAM17, which negatively regulates Nrg1 type III signaling (Amour et al., 1998; Lee et al., 2001). The effect of TIMP-3 on Schwann cell myelination has not been investigated. Considering the role of ADAM17 as an endogenous inhibitor of axonal Nrg1 type III signaling and PNS myelination, it is possible that the inhibitory function of TIMP-3 toward ADAM17 may be effective in promoting Schwann cell myelination.

In this study, we used dorsal root ganglia (DRG) neuron-Schwann cell cocultures to investigate the effects of TIMP-3 on Schwann cell myelination. We observed that TIMP-3 promoted myelin formation by increasing both the number and the lengths of individual myelin segments generated by Schwann cells. A similar effect was observed with the N-terminal domain of TIMP-3, which exhibits a selective inhibitory function toward ADAM17 but not ADAM10. The promyelinating effect of TIMP-3 was accompanied by ADAM17 inhibition and enhanced axonal Nrg1 type III signaling in cocultures.

Materials and Methods

Media and Reagents

D10 media (Dulbecco's Modified Eagle's medium [DMEM] supplemented with 10% fetal bovine serum, 1% glutamine, 0.1 mg/ml penicillin/streptomycin); NB media (neurobasal medium with B-27 supplement, 0.08% glucose, 1% glutamine, 0.1 mg/ml penicillin/streptomycin, and 50 ng/ml nerve growth factor [NGF; #BT-5017, Harlan Laboratories, Inc.]); C10 media (Minimal Essential Medium containing 10% fetal bovine serum, 0.08% glucose, and 50 ng/ml NGF); N2 media (F12/DMEM [1:1] medium supplemented with N2 supplement and 50 ng/ml NGF). Recombinant full-length human TIMP-3 (F-TIMP-3) and N-terminus TIMP-3 (N-TIMP-3) fragment were provided by Amgen. The N-TIMP-3 is a fragment consisted of N-terminal domain (amino acid sequence 1-121) of the F-TIMP-3 (amino acid sequence 1-188). Human and rat TIMP-3 share 98% peptide sequence homology. Both recombinant proteins were expressed and purified in *Escherichia coli*.

Antibodies

The following primary antibodies were used for immunostaining or Western blot analysis: anti-mouse myelin basic protein (MBP; 1:300, #SMI-94R [Covance]), anti-rabbit Krox-20 (1:500, #PRB-236P [Covance]), anti-mouse BrdU (1:1000, #B2531 [Sigma]), anti-rabbit phospho-Akt (1:1000, #4051 [Cell Signaling Technology]), anti-rabbit phospho-Erk1/2 (1:5000, #V8031 [Promega]), anti-rabbit total-Akt (1:1000, #9272 [Cell Signaling Technology]), anti-rabbit total Erk1/2 (1:5000, #V1141 [Promega]), anti-rabbit phospho-ErbB3 (1:500, #4791 [Cell signaling]), anti-rabbit laminin (1:1000, #ab11575 [Abcam]), anti-mouse β-actin (1:5000, #A5441 [Sigma]), and anti-rabbit TIMP-3 (1:1000, #AB6000 [Millipore]).

Schwann Cell Preparation and Cultures

Schwann cells were isolated from postnatal Day 2 (P2) rat sciatic nerves. Experiments were approved by the Rutgers Institutional Animal Care and Use Committee and complied with NIH guidelines. Dissected sciatic nerves were treated with trypsin-collagenases (L15 media supplemented with 0.225% trypsin and 0.1% of collagenase) at 37°C. Thirty minutes later, cells were spun down at 50 g for 5 min and dissociated by pipetting in NB media. All dissociated cells were plated in a PLL-coated 60-mm dish. Next day, cells were treated with D10 media supplemented with 10 μM cytosine-ß-arabino furanoside hydrochloride (AraC) (#C6645, Sigma) to avoid fibroblast contamination. After, 2 to 3 days, cultures were washed with HBSS and replaced with D10 media supplemented with 10 ng/ml EGF-domain of heregulin (#396-HB-050, R&D systems) and 2.5 μM forskolin (#F6886, Sigma).

Once confluent, cells were harvested by 0.25% Trypsin-EDTA and spun at 200 g for 5 min at room temperature. Harvested cells were treated with 20 μl of Thy1.1 (#MCA04G, AbD Serotech) antibody in 1 ml of D10 media and incubated at 37°C. Thirty minutes later, cells were spun down at 200 g for 5 min at room

temperature and treated with 1 ml of rabbit complement (#S7764, Sigma), and incubated for 30 min at 37°C. After spinning cells down at 200 g for 5 min, cells were plated in PLL-coated 100 mm dish and expanded in D10 media supplemented with 10 ng/ml EGF-domain of heregulin and 2.5 μM forskolin.

DRG Neuron Cultures and DRG-Schwann Cell Cocultures

DRG neurons were dissected from E15 rat embryos and incubated with 0.25% trypsin for 30 min at 37°C. Cells were spun down at 50 g for 10 min at room temperature and resuspended in NB media. Cells were plated onto Matrigel (BD)-coated glass coverslips at the density of 0.7 to 0.8 DRG/coverslip in NB media. After 24 hr, cells were treated with NB media with 15 μM 5-fluorodeoxyuridine (FUdR) (#F0503, Sigma) for 3 days to get rid of proliferating non-neuronal cells, and then changed to NB medium without FUdR. Neuronal cells were maintained in NB medium for 8 to 10 days. Once DRG neurons reached the periphery of the coverslip fully, Schwann cells were plated onto DRG neurons at the density of 150,000 cells per coverslip in C10 media. After 5 to 6 days, cells were changed to myelinating medium by adding 50 μg/ml ascorbic acid in C10 media to initiate myelination in the absence or in the presence of the either F-TIMP-3 or N-TIMP-3.

Immunostaining for Myelin Basic Protein

Cultures were fixed in 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, samples were permeabilized in cold methanol for 20 min at −20°C and then washed with PBS. Samples were incubated with blocking solution (5% normal goat serum in PBS supplemented with 0.3% Triton X-100) for an hour at room temperature and then incubated with primary antibody for MBP in blocking solution overnight at 4°C. After washing with PBS, cultures were incubated with goat anti-mouse Alexa Fluor 488 secondary antibody (Jackson ImmunoResearch Laboratories) in PBS supplemented with 0.3% Triton X-100 for an hour at room temperature. After washing with PBS, cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Following washing with PBS, coverslips were mounted on the slides with Fluoromount-G solution (#0100-01, Southern Biotech).

In Vitro *Myelination Assay*

Myelin segments were visualized by immunostaining for MBP as described earlier. Ten images, which were randomly selected across each coverslip, were taken with the epifluorescence microscope (Nikon E800) under a 20× objective. The number of MBP-positive myelin segments was counted from the individual images by using Image J software (NIH). The total number of segments was obtained from each coverslip with three coverslips used per condition per experiment. The myelin index from four to five separate experiments was analyzed as described previously (Syed et al., 2010). Lengths of individual myelin segments were measured using Image J software (NIH). A total of 100 to 600 individual myelin segments were measured from each experiment. An average of length per condition was analyzed from four separate experiments. Student's *t* test was performed using GraphPad Prism 5.03 software.

BrdU Incorporation and Immunostaining

Cell proliferation was tested by BrdU (#B5002, Sigma) incorporation. Forty-eight hours after plating Schwann cells onto the DRG neurons, cells were incubated with 10 μM 5-bromo-2-deoxyuridine (BrdU). After 2 hr, cultures were fixed in 4% paraformaldehyde. Cultures were washed with PBS, permeabilized in cold methanol for 20 min at −20°C, and then washed in PBS. The cultures were treated with 2 N HCl for 15 min at 37°C. Cells were washed with 0.1 M borate buffer (pH 8.5) for 10 min. After washing cultures with PBS, they were incubated with blocking solution (5% normal goat serum and 0.3% Triton X-100 in PBS) for 30 min. Cultures were incubated with antibody for BrdU in blocking solution overnight at 4°C. Next day, cultures were washed with PBS and then incubated with Alexa488-conjugated goat anti-mouse Alexa Fluor 488 secondary antibody (Jackson immunoresearch laboratories) for an hour. After washing with PBS, DAPI was incubated for 10 min at room temperature to visualize nuclei. After washing with PBS, cultures were mounted on the slides using Fluoromount-G solution (#0100-01, Southern Biotech). Images were taken with the epifluorescence microscope (Nikon E800) under a 20× objective. Proliferation rate was determined by calculating the percentage of the number of BrdU-positive nuclei over DAPI-positive nuclei.

Measurement of ADAM17 Activity in Cocultures

DRG neurons were pretreated with 0.3 μM of F-TIMP3 for TIMP-3 treatment conditions for 1 hr at 37°C. Schwann cells were plated onto DRG neurons at the density of 100,000 cells per coverslip in C10 media in the absence or in the presence of 0.3 μM of F-TIMP3. Twenty-four hours later, cultures were washed with PBS, and cells were collected in the assay buffer (50 mM Tris [pH 7.5], 0.1% Triton X-100, protease inhibitor [#11836170001, Roche]). Cell suspension was incubated at 4°C for 10 min and centrifuged at 2,500 g at 4°C. Supernatant was collected and used for

ADAM17 activity measurement. Totally, 30 μg of lysate from each condition was incubated with 20 μM of ADAM17 substrate (#PEPDAB064, BioZyme) (Caescu et al., 2010) for 5 min at room temperature. Fluorescence was measured using a fluorometer plate reader (Promega) every 5 min for 4 hr at room temperature.

Neuronal Induction of Akt Activation in Schwann Cells

For TIMP-3 treatment conditions, DRG neurons were pretreated with either 0.3 μM of F-TIMP-3 or 0.3 μM of N-TIMP-3 in N2 media for 1 hr at 37°C. Ten thousand Schwann cells were plated onto DRG neurons in the absence or in the presence of the either 0.3 μM of F-TIMP-3 or 0.3 μM of N-TIMP-3 in N2 media. Twenty-four hours later, cells were lysed with lysis buffer (20 mM Tris, pH7.4, 1% NP-40, 10% glycerol, 2.5 mM EGTA, 2.5 mM EDTA, 150 mM NaCl, 20 μM leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 mM sodium fluoride) to access Western blot analysis for phospho-Akt, total Atk, phospho-Erk1/2, and total Erk1/2.

Western Blot Analysis

To harvest the cell lysates, Schwann cell cultures or DRG neuron-Schwann cell cocultures were washed twice with PBS and then cells were collected in the lysis buffer (20 mM Tris, pH7.4, 1% NP-40, 10% glycerol, 2.5 mM EGTA, 2.5 mM EDTA, 150 mM NaCl, 20 μM leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 mM sodium fluoride). The same amount of total protein was loaded, separated by size in sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membrane. After blocking in 5% skim milk in tris-buffered saline (10 mM Tris and 150 mM NaCl, pH 8.0) for an hour at room temperature, the membranes were incubated with appropriate primary antibodies in tris-buffered saline containing 5% bovine serum albumin overnight at 4°C. After incubating with fluorescence-dye-conjugated secondary antibodies (LI-COR, Inc.) in 5% skim milk in tris-buffered saline containing 0.001% sodium dodecyl sulfate for an hour at room temperature, the protein bands were detected and quantified by the LI-COR Odyssey imaging system.

Reverse Transcription and q-PCR

Total RNA was extracted from SC-DRG cocultures cultured in the absence or in the presence of 0.3 μM of F-TIMP3. From 1 μg of total RNA, single-stranded cDNA was synthesized using SuperScrip III First-Strand Synthesis kit with oligo dT primer (#18080051, Invitrogen) and the final volume was adjusted up to 100 μl with water. Five microliter of the cDNA was used for quantitative polymerase chain reaction (q-PCR) with SYBR Green/ROX qPCR master mix (#K0221, ThermoFisher Scientific). Primers used were laminin-β (5′-GAGCCCTACTGTATTGTCAG-3′ and 5′-AGGAGCAAACGTTGTGACCA-3′) and Hprt (5′-TCCAACACTTCGAGAGGTCC-3′/5′-CAGACTTTG CTTTCCTTGGTCA-3′). The PCR was programmed at 95°C for 10 s, 57°C for 20 s, and 72°C for 20 s, repeated 55 times. The amount of laminin-β relative to the Hprt control gene product was calculated using LightCycler 480 software in a LightCycler 480 Real Time PCR instrument (Roche).

Statistical Analysis

For data analysis, Student's *t* test was performed using GraphPad Prism software (version 5.03). And *p* values less than .05 were considered significant.

Results

TIMP-3 Expression in Developing Sciatic Nerves

To assess endogenous TIMP-3 protein expression during PNS development, sciatic nerve lysates were prepared from postnatal rats between P0 and P30 and subjected to Western blot analysis. TIMP-3 levels were at the highest during the early postnatal period, which coincided with the onset of Schwann cell myelination prior to the appearance of the myelin protein (MBP) expression (Figure 1(a)). As the myelination progressed, TIMP-3 expression declined and was almost undetectable at P30. To determine the cellular source of TIMP-3, we assessed TIMP-3 expression in cultured Schwann cells and DRG neurons (Figure 1(b)). TIMP-3 expression was detected only in Schwann cells, not in the neurons, and the expression levels increased when Schwann cells were cocultured with DRG neurons. Our results indicate that TIMP-3 expression is limited to the early stages of PNS myelination and Schwann cells appear to be the main source of the TIMP-3 production.

TIMP-3 Promotes Schwann Cell Myelination in Cocultures

Schwann cell myelination can be assessed *in vitro* using a coculture system established using purified DRG neurons and Schwann cells. When cocultured together, Schwann cells associate with DRG neurites and proliferate in response to the contact-mediated neuronal signal. Subsequent addition of ascorbic acid to the culture initiates myelin formation by promoting Schwann cell basal lamina assembly, an event prerequisite for myelination.

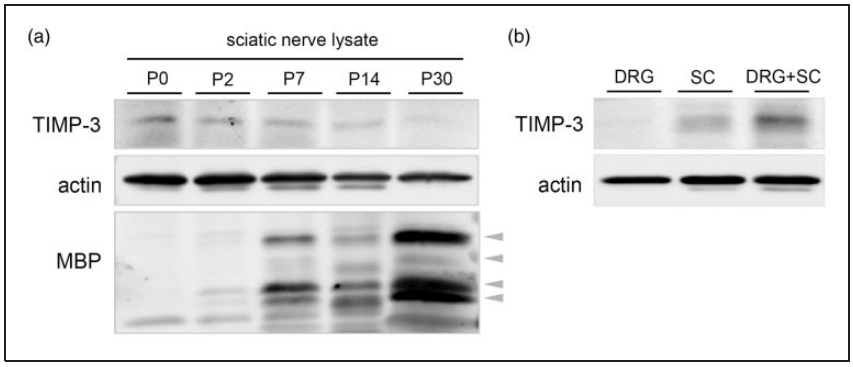

Figure 1. TIMP-3 expression in developing rat sciatic nerves and in cultured Schwann cells. (a) Sciatic nerves were collected from rats at different developmental stages ranging from postnatal Day 0 to 30. TIMP-3 expression is seen during the early postnatal period. Corresponding immunoblot for MBP shows a gradual increase in MBP expression indicating the progression of myelination. (b) TIMP-3 expression in sensory neuron and Schwann cell cultures. TIMP-3 expression is only detected in Schwann cells and further increases when cocultured with neurons. DRG = dorsal root ganglia; MBP = myelin basic protein; SC = Schwann cell; TIMP = tissue inhibitor of metalloproteinase.

Seven to 10 days after the initiation, myelin segments can be visualized by immunostaining for MBP. To determine the effect of TIMP-3 on Schwann cell myelination, cocultures were treated with increasing doses of recombinant full-length TIMP-3 (F-TIMP-3) at the time of initiating myelin formation. The cultures were then maintained in the continuous presence of F-TIMP-3 for 9 days, after which the cultures were fixed and immunostained for MBP. Control cultures were maintained in the absence of F-TIMP-3. Under myelinating condition (+ascorbic acid), F-TIMP-3 treatment enhanced myelin formation in a dose-dependent manner, shown by an increase in the number of myelin segments generated in the cocultures (Figure 2(a)). Representative images of the MBP-positive myelin segments in the cultures are shown in Figure 2(b). TIMP-3 (0.3 μM) also increased internodal length of individual myelin segments (Table 1). Full-length TIMP-3 had no effect on neuronal contact-mediated Schwann cell proliferation (Figure 2(d)), indicating that the increase in the number of myelin segment was not due to an increase in Schwann cell number. TIMP-3-mediated myelination was accompanied by an increase in Krox 20 expression, a promyelinating transcription factor that is necessary for Schwann cell commitment to myelination (Figure 2(e)).

TIMP-3 N-Terminus Domain Is Sufficient to Increase Schwann Cell Myelination

TIMP-3 targets ADAM10 and ADAM17 (Rapti et al., 2008), which have been shown to cleave Nrg1 type III and modulate its signaling activity. Processing by ADAM10 generates Nrg1 N-terminus that enhances erbB receptor activation (Luo et al., 2011) whereas ADAM17-mediated processing attenuates the signaling function of Nrg1 and inhibits Schwann cell myelination (La Marca et al., 2011). Considering that axonal Nrg1 type III is a key regulator of Schwann cell myelination, it is possible that the promyelinating function of TIMP-3 may be due to its inhibition of ADAM17 that in turn enhances Nrg1 signaling in Schwann cells. Although full-length TIMP-3 inhibits both ADAM10 and ADAM17, N-terminal TIMP-3 domain (N-TIMP-3) exhibits selective inhibitory effects toward ADAM17 and does not inhibit ADAM10 (Lee et al., 2001; Rapti et al., 2008). Therefore, F-TIMP-3 and N-TIMP-3 are useful agents for discriminating between the activities of ADAM10 and ADAM17. We observed that treatment with N-TIMP-3 had similar myelination-promoting effects in cocultures as seen with F-TIMP-3: Both the number of myelin segments (Figure 2(c)) and the myelin internodal length (Table 1) increased to equivalent levels observed with F-TIMP-3 treatment. N-terminus TIMP-3 also increased Krox 20 and MBP expression cocultures (Figure 2(e)). These results suggest an N-terminus-specific function of TIMP-3 in promoting Schwann cell myelination, which possibly involves ADAM17 inhibition.

TIMP-3 Inhibits ADAM17 Activity in Cocultures and Enhances Nrg1 Type III Signaling in the Schwann Cells

To determine whether TIMP-3 inhibits ADAM17 in cocultures, we employed a fluorimetric assay utilizing a ADAM17-specific fluorescence resonance energy transfer (FRET) substrate. In this assay, ADAM17 activity can be monitored by detecting an increase in the fluorescence, which is generated when ADAM17 cleaves the FRET substrate in two fragments (Caescu et al., 2010). Cell lysates prepared from control- or TIMP-3-treated cocultures were incubated with the ADAM17 substrate, and the fluorescence was monitored over time. In control lysates (-TIMP-3), the substrate fluorescence intensity steadily increased, indicating the presence of endogenous

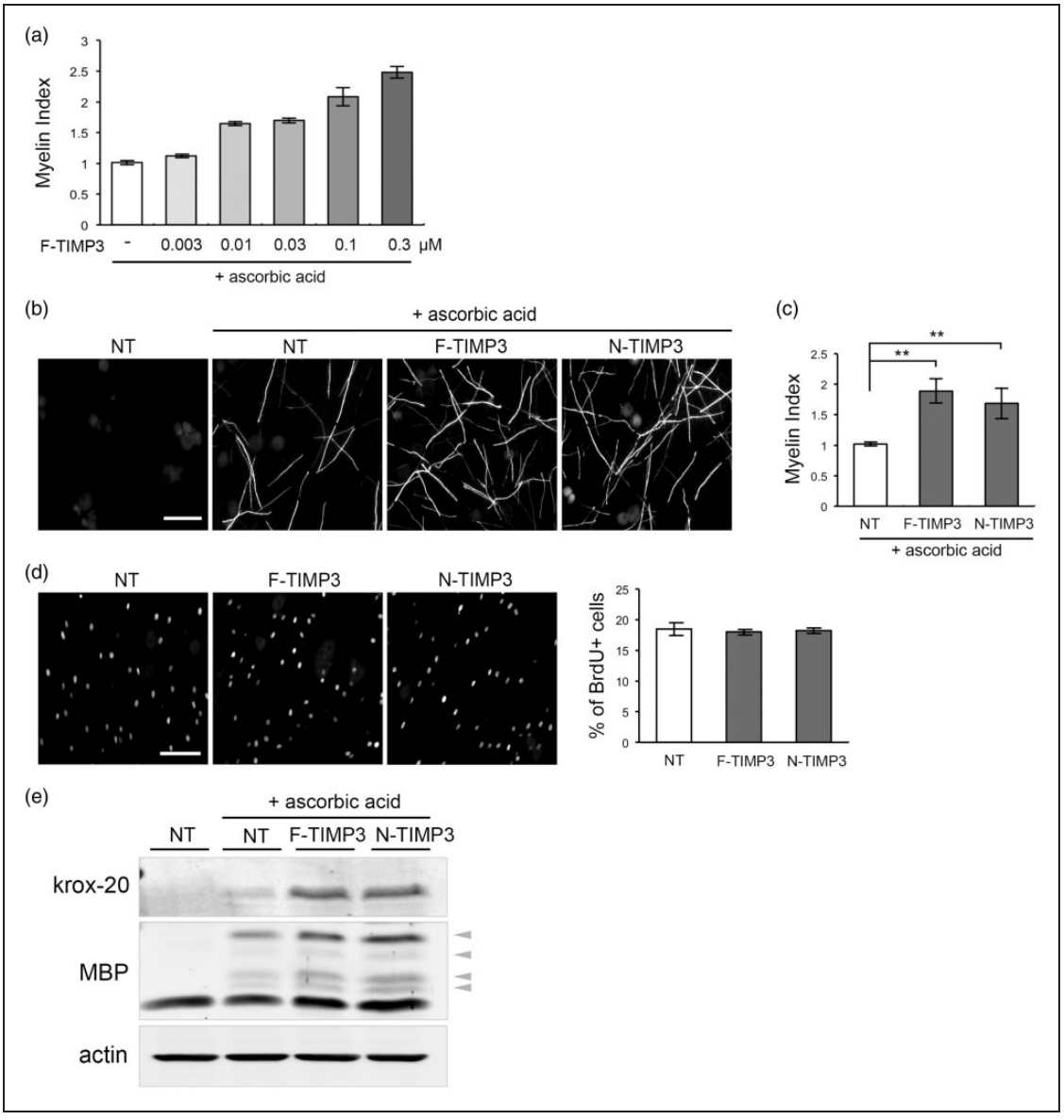

Figure 2. TIMP-3 promotes Schwann cell myelination. (a) Dose-dependent effect of TIMP-3 on Schwann cell myelination. Schwann cell-DRG neuron cocultures were treated with different concentrations (0.003, 0.01, 0.03, 0.1, and 0.3 μM) of F-TIMP-3 at the time of initiating myelination (+ascorbic acid). Nine days later, cultures were analyzed by immunostaining for MBP. The means ± *SEM* were determined from two coverslips/condition from two independent experiments. (b) Representative images of MBP + myelin segments generated in myelinating cocultures treated with F-TIMP-3 (0.3 μM) or N-TIMP-3 (0.3 μM). Scale bar: 100 μm. (c) N-terminus domain of TIMP-3 (N-TIMP-3, 0.3 μM) elicits a similar myelination-promoting effect as F-TIMP-3. $^{**}p < .01$. The means ± *SEM* were determined from three coverslips/condition from four to five independent experiments. (d) TIMP-3 does not affect neuronal contact-mediated Schwann cell proliferation. Schwann cell proliferation in cocultures was assessed by BrdU incorporation. The result is presented as percentage BrdU+ cells detected in the cultures. Scale bar: 100 μm. The means ± *SEM* were determined from three coverslips/condition from four independent experiments. (e) TIMP-3 increases the expression of myelin-associated protein. Krox 20 and MBP expression were assessed in cocultures maintained under myelinating condition for 9 days in the presence or absence of TIMP-3. Both F-TIMP-3 and N-TIMP-3 increased the expression levels of Krox 20 and MBP. The protein expression is absent in cultures kept under nonmyelinating condition (−ascorbic acid). MBP = myelin basic protein; NT = no treatment; TIMP = tissue inhibitor of metalloproteinase.

ADAM17 activity in cocultures. In TIMP-3-treated cultures, there was a significant decrease in the rate of the fluorescence increase over time, indicating an attenuated ADAM17 activity. Overall, there was a 26.3% decrease in the endogenous ADAM17 activity in TIMP-3-treated cultures (Figure 3(b)).

ADAM17 inhibits Nrg1 type III activity toward erbB receptors and inhibits Schwann cell myelin formation (La

Table 1. Average Length of Myelin Segments in Control- and TIMP-3-Treated Cocultures.

	Ascorbic acid	Ascorbic acid + 0.3 μM F-TIMP-3	Ascorbic acid + 0.3 μM N-TIMP-3
Mean segment length(μm)	158.57	170.51***	171.66**
SEM	3.82	5.02	3.54
Number of myelin segments analyzed	1,105	1,389	1,459
Percentage increase over control	–	7.5	8.2

Note. Schwann cell-DRG cocultures were treated with F-TIMP-3 or N-TIMP-3 at the time of initiating myelination and maintained for 9 days, at which time the cultures were fixed and immunostained for MBP. Lengths of individual myelin segments were measured from three coverslips/condition from four to five independent experiments. MBP: myelin basic protein; TIMP: tissue inhibitor of metalloproteinase.
**$p < .01$, ***$p < .001$.

Marca et al., 2011). Therefore, TIMP-3-mediated ADAM17 inhibition is expected to relieve the block on Nrg1 activity and enhances erbB receptor signaling in Schwann cells. To investigate this, we monitored the effect of TIMP-3 on Nrg1 type III activity in cocultures. A previous study has shown that in Schwann cell-DRG neuron cocultures, the neuronal Nrg1 type III is the sole activator of the PI3-kinase/Akt pathway in the Schwann cells (Taveggia et al., 2005), thus we used Akt phosphorylation as a readout for the endogenous Nrg1 type III activity. Schwann cells were placed on DRG neurons to initiate contact-mediated Nrg1 signaling. Twenty-four hours later, the levels of Akt phosphorylation were assessed by Western blot analysis. Neuron- or Schwann cell-only mono cultures served as controls. Although Akt phosphorylation was minimal in the mono cultures, placing Schwann cells on DRG neurons induced Akt phosphorylation, which is expected of the Nrg1 type III-mediated PI3-kinase pathway activation in the Schwann cells (Figure 3(c)). In the presence of F-TIMP-3, the Akt phosphorylation increased significantly, indicating an increase in the Nrg1 type III activity. N-TIMP-3 elicited a similar effect as F-TIMP-3 (Figure 3(d)). Erk activation, which occurs independent of Nrg1 type III activity in coculture (Taveggia et al., 2005), was not affected by the TIMP-3 treatment. Altogether, these results suggest that TIMP-3 inhibits ADAM17 activity in cocultures and, in turn, enhances Nrg1 type III signaling in the Schwann cells.

TIMP-3 Increases Laminin Contents During Schwann Cell Myelination

In addition to targeting ADAM proteins, TIMP-3 inhibits a wide range of MMPs that degrade components of extracellular matrix (ECM; Apte et al., 1995; Brew & Nagase, 2010). This presents a possibility that the pro-myelinating function of TIMP-3 may involve modulation of the ECM contents during myelin formation. One of the components is laminin, which is essential for Schwann cell differentiation and myelin ensheathment (Baron-Van Evercooren et al., 1986; Eldridge et al., 1989; Court et al., 2006). Schwann cells also increase laminin production at the onset of myelination (Fragoso et al., 2003). To this end, we monitored laminin levels in cocultures treated with TIMP-3 maintained under non-myelinating (−ascorbic acid) and myelinating (+ascorbic acid) condition. We focused on the first 5 days of initiating myelination, a period that is mostly impacted by laminin. Antibodies against pan-laminin were used to detect the total laminin contents using Western blot analysis. In control cultures without TIMP-3, laminin levels increased with the onset of myelination (NT, −ascorbic acid vs. +ascorbic acid) (Figure 4(a)). Treatment with TIMP-3 further increased the total laminin contents, more profoundly under myelinating condition (F-TIMP-3, +ascorbic acid). To determine whether the increase was due to a decrease in laminin proteolysis, we monitored the levels of the degradation products at lower molecular weights (Figure 4(b)). There was no obvious difference in the degraded laminin contents between control- and TIMP-3-treated cultures, indicating that laminin processing was not affected by TIMP-3. TIMP-3, however, drastically increased the laminin mRNA levels in cocultures (Figure 4(c)). This result indicates that TIMP-3 enhances laminin production at the transcription levels during Schwann cell myelination. The increase in the total laminin contents is likely to aid in the myelination process.

Discussion

Myelination in the PNS is dependent on contact-mediated signaling between Schwann cells and the associated axons. One of the axonal signals that is essential for myelination is Nrg1 type III, which binds and activates the erbB2/3 receptor complex on the Schwann cell surface (Michailov et al., 2004; Taveggia et al., 2005). Subsequent activation of the PI3-kinase pathway in the Schwann cells is then required for initiating myelin formation (Maurel & Salzer, 2000).

The signaling function of Nrg1 type III is regulated by proteolytic processing that modulates the receptor activating EGF domain within the N-terminus (Hu et al., 2006; Willem et al., 2006; La Marca et al., 2011; Luo et al., 2011; Fleck et al., 2013). A variety of metalloproteinases have been implicated in Nrg1 type III cleavage, including β-secretase and ADAM17. Previous studies have shown that Nrg1 cleavage by these proteases has opposing functional effects on Schwann cell myelination. Processing by β-secretase activates Nrg1 and is

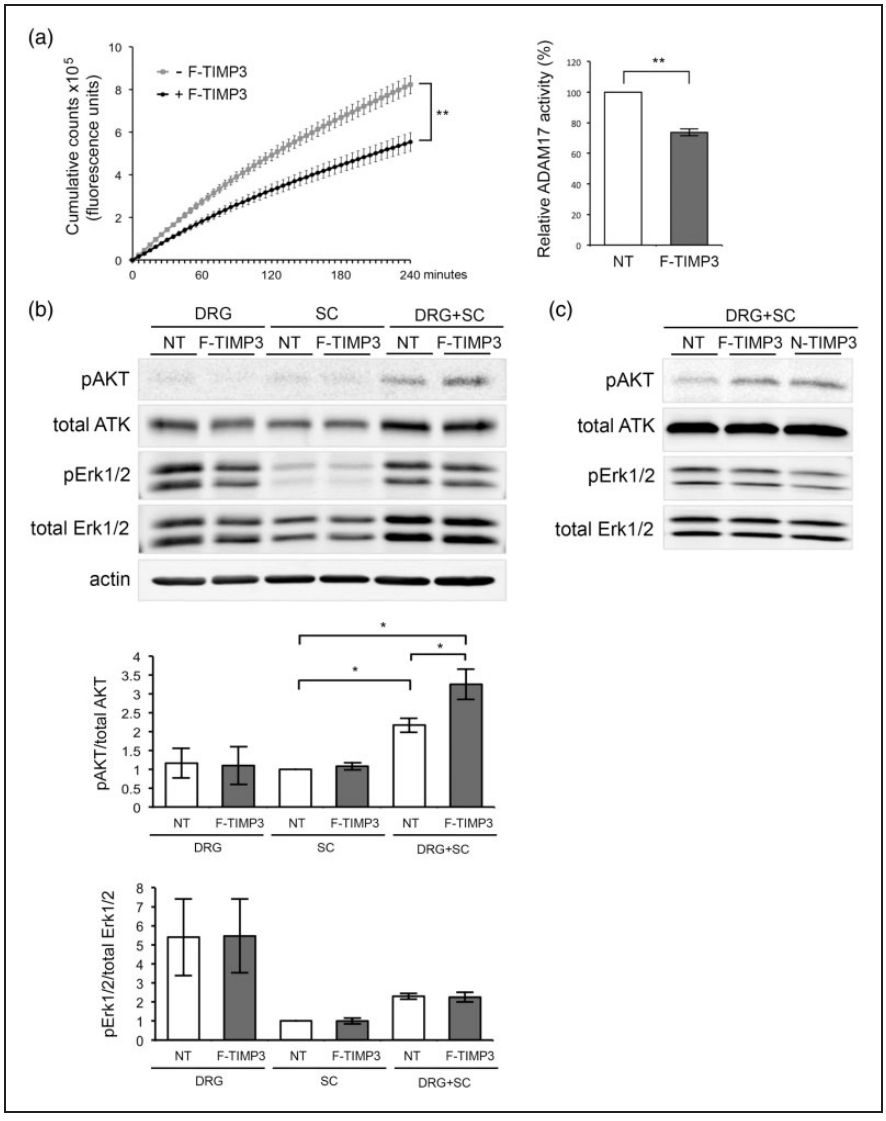

Figure 3. TIMP-3 inhibits endogenous ADAM17 activity and enhances Nrg1 type III signaling in cocultures. (a) Schwann cell-DRG neuron cocultures were treated with F-TIMP3 (0.3 μM). Twenty-four hours later, lysates were prepared and incubated with ADAM17 substrate for 5 min. Fluorescence was measured every 5 min for 4 hr. Treatment with F-TIMP-3 decreases endogenous ADAM17 activity in cocultures. Quantification of the relative ADAM17 activity at 4 hr time point is shown on the right. $^{**}p < .01$. The means ± *SEM* were determined from three independent experiments. (b) Sensory neurons were pretreated with F-TIMP-3 for 1 hr, after which Schwann cells were plated on the neurons to initiate contact-mediated Nrg1 signaling. Twenty-four hours later, lysates were prepared, and the levels of phospho-Akt, phospho-Erk as well as total Akt and Erk were assessed by immunoblotting. Control lysates were prepared from neuron-only or Schwann cell-only cultures treated with F-TIMP-3. Quantitative results of the immunoblots are shown at the bottom $^{*}p < .05$. The means ± *SEM* were determined from three independent experiments. (c) Akt activation in cocultures treated with F-TIMP-3 or N-TIMP-3. Treatment with N-TIMP-3 increased Akt activation as seen in F-TIMP-3-treated cultures. DRG = dorsal root ganglia; NT = no treatment; SC = Schwann cell; TIMP = tissue inhibitor of metalloproteinase.

required for myelination in the PNS (Hu et al., 2006; Willem et al., 2006). In contrast, ADAM17 processing attenuates Nrg1 signaling. *In vivo* inhibition of ADAM17 results in increased Nrg1 activity and Schwann cell hypermyelination in the PNS (La Marca et al., 2011). Other members of the ADAM family, such as ADAM10, have also been implicated in regulating Nrg1 signaling (Luo et al., 2011). Therefore, PNS myelination is regulated in part by modulation of the proteases activities that alter the signaling function of Nrg1.

In this study, we investigated the effect of TIMP-3 on Schwann cell myelination. Proteins of the TIMP family are endogenous inhibitors of MMP and ADAM proteins. Among the TIMPs, TIMP-3 exhibits a strong inhibitory activity toward ADAM17, whose function has been shown to negatively regulate Nrg1 and Schwann cell

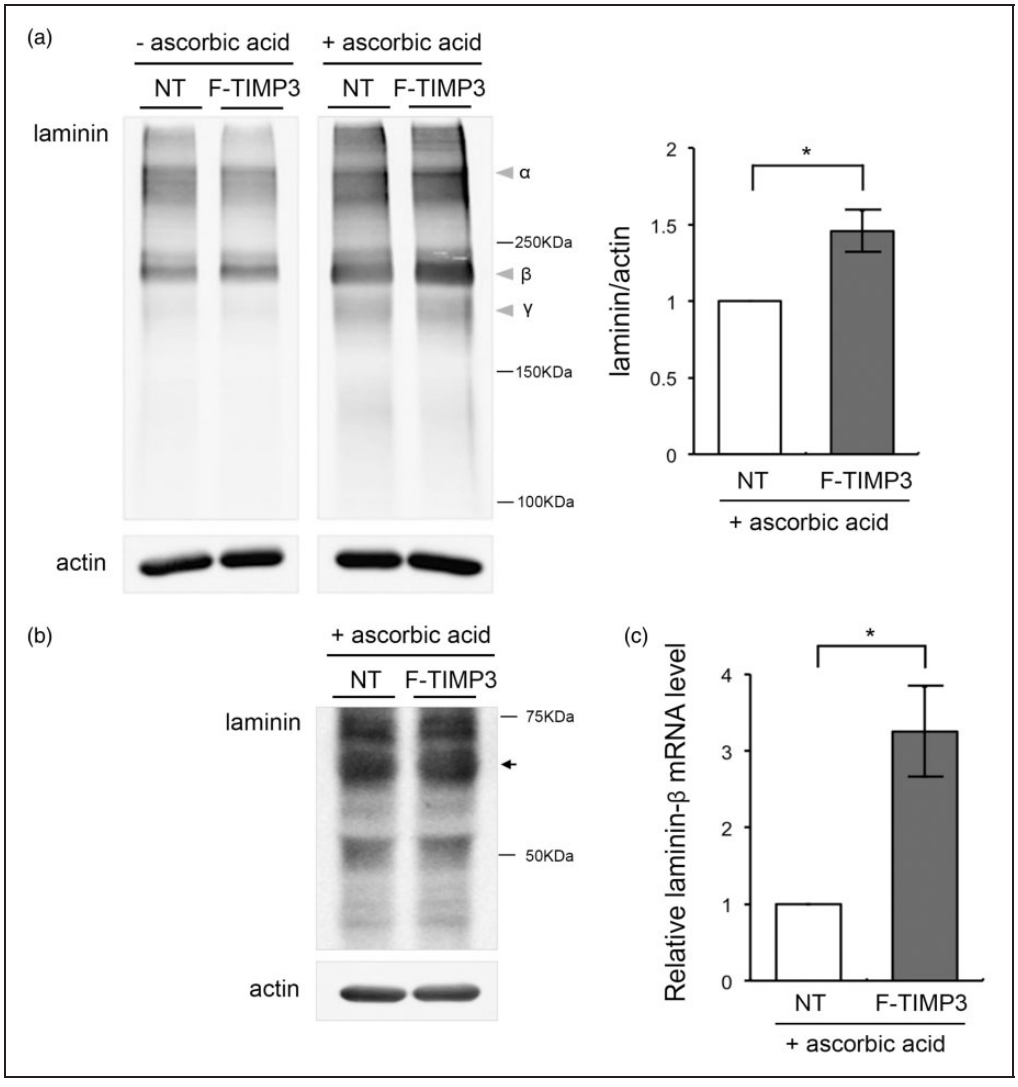

Figure 4. TIMP-3 treatment increases laminin contents during Schwann cell myelination. (a) Schwann cell-DRG cocultures were treated with F-TIMP-3 and placed under nonmyelinating (−ascorbic acid) or myelinating condition (+ascorbic acid) in the continuous presence of F-TIMP-3. Control cultures were maintained in the absence of TIMP-3 (NT). Five days later, total laminin contents were determined using antibodies against pan-laminin. Overall laminin contents increased in cultures under myelinating condition. Treatment with TIMP-3 further increased the levels of laminin. Arrows indicate positions of bands corresponding to laminin α, β, and γ chains. Quantitative result of the immunoblot is shown on the right. *$p < .05$. The mean ± *SEM* was determined from three independent experiments. (b) Lysates used in (a) were ran on a 10% gel to visualize bands at lower molecular weights, which likely represent the laminin degradation products. The arrow indicates a 60-kDa cleaved product expected from laminin-β1 cleavage by MMP-2 (Horejs et al., 2014). There was no difference in the amount of the lower molecular weight products between control- (NT) and F-TIMP-3-treated cultures. (c) TIMP-3 increases laminin production. Expression levels of laminin-β mRNA were determined by qRT-PCR in control- and TIMP-3-treated cocultures 5 days into myelination. *$p < .05$. The mean ± *SEM* was determined from three independent experiments. NT = no treatment; TIMP = tissue inhibitor of metalloproteinase.

myelination (Amour et al., 1998; La Marca et al., 2011). TIMP-3 also inhibits ADAM10, which cleavages and activates Nrg1, but without a significant consequence on myelin formation (Rapti et al., 2008; Luo et al., 2011). Our data show that TIMP-3 increases neuronal contact-mediated Akt activation in Schwann cell-DRG neuron coculture, indicating an increase in neuronal Nrg1 type III activity. This is a result expected of TIMP-3 targeting negative regulators of Nrg1 signaling, such as ADAM17. Accordingly, we show that TIMP-3 inhibits ADAM17 activity in cocultures. We also show that TIMP-3 treatment enhances myelin formation by Schwann cells, which is likely to occur if TIMP-3 relieves the myelination block imposed by ADAM17. Altogether, the positive impact of TIMP-3 on Nrg1 signaling and myelination implicates its role in targeting ADAM17.

This notion is further corroborated by the observation that N-TIMP-3, which has selective inhibitory activity toward ADAM17, elicits a similar myelination-promoting effect on Schwann cells.

The promyelinating function of TIMP-3 was associated with an increase in Krox20 expression, indicating its role during the very early stages of Schwann cell myelination. In fact, we show that in developing sciatic nerves, TIMP-3 expression is at the highest during the early postnatal period, implicating its role during the early stages of Schwann cell myelination. As a promyelinating transcription factor, Krox20 induction allows Schwann cells in one-on-one contact with individual axons to proceed past the premyelinating stage and initiate radial membrane wrapping of the axons (Topilko et al., 1994). Our data show that TIMP-3 increases the number of myelin segments generated in cocultures, indicating an increase in the number of Schwann cells committed to form myelin. TIMP-3 treatment also increased internodal length of individual myelin segments (Table 1) indicating enhanced growth of myelin sheath. Similar effects have been observed in cocultures with enhanced Nrg1 signaling. For example, ectopically increasing Nrg1 activity in culture increases Krox 20 expression, number of myelin segments, and internodal length (Syed et al., 2010). *In vivo*, expression of Nrg1 type III in PNS neurons increases Krox 20 expression and myelin thickness (Michailov et al., 2004; Taveggia et al., 2005). Therefore, the myelination-promoting effect of TIMP-3 mimics the effects of enhanced Nrg1 type III signaling on Schwann cell myelination. This is in agreement with our observation that TIMP-3 treatment enhances Nrg1 type III signaling in cocultures.

In addition to targeting ADAM proteins, TIMP-3 inhibits a wide range of MMPs that cleave and modify components of ECM (Apte et al., 1995; Brew & Nagase, 2010). Schwann cells and DRG neurons express MMP-2 (Muir, 1995; Lehmann et al., 2009; Ali et al., 2014) which degrades ECM components (Horejs et al., 2014). Schwann cells also express MMP-9, whose function has been implicated in ECM remodeling following PNS injury (La Fleur et al., 1996; Ferguson & Muir, 2000; Kim et al., 2012). Both MMP-2 and MMP-9 are targeted by TIMP-3. In this regard, MMP-2 and MMP-9 produced by Schwann cells have been shown to modulate cytoplasmic compartmentalization in myelinating Schwann cells. Furthermore, inhibition of the MMPs rescues internodal length defect in dystrophic mouse model (Court et al., 2009). Therefore, lengthening of myelin segments seen in TIMP-3-treated cultures could be a consequence of the TIMP-3 function that inhibits MMP-2 and MMP-9 activities, independent of the enhanced Nrg1 signaling.

Laminin is a major component of Schwann cell basal lamina that is important for myelin formation (Court et al., 2006). In PNS, expression of laminin is upregulated during the first week of birth in rats, when basal lamina assembly and myelin formation by Schwann cells commence (Masaki et al., 2002). In cocultures, laminin expression increases with the onset of myelination when ascorbic acid is added (Fragoso et al., 2003). Furthermore, addition of purified laminin is sufficient to initiate and increase Schwann cell myelination in coculture (Eldridge et al., 1989; McKee et al., 2012). Our data show that TIMP-3 treatment increases total laminin content produced during the early stages of myelin formation (Figure 3). Since TIMP-3 inhibits various MMPs, one can speculate that TIMP-3-mediated inhibition of MMPs that degrade laminin may contribute to the increased laminin contents. However, we did not detect a change in the amount of laminin degradation products using Western blot analysis, suggesting that TIMP-3 did not alter the laminin proteolysis in cocultures. Interestingly, however, TIMP-3 increased contents of the laminin transcripts, indicating a function that enhances laminin production. The mechanism by which TIMP-3 promotes laminin synthesis is unclear. A previous study has shown that the PI3-kinase/Akt pathway promotes laminin synthesis at the transcription level (Li et al., 2001). Since TIMP-3 treatment increases Akt activation in coculture, it is possible that the laminin production was mediated by the TIMP-3 function that increases Nrg1 type III-Akt signaling. Regardless of the mechanism, considering the promyelinating function of laminin in Schwann cells, accumulation of laminin in TIMP-3-treated culture is likely to contribute in part, to enhanced myelin formation.

In summary, we show that TIMP-3 treatment increases Schwann cell myelination in cultures. The finding suggests that TIMP-3 functions as a positive regulator of the process that enhances axonal Nrg1 type III signaling necessary for myelin formation. TIMP-3 also increases internodal length, which may indicate its function in attenuating MMP activities that modulate cytoplasmic compartmentalization in myelinating Schwann cells. Furthermore, we show that TIMP-3 promotes laminin production. Activities of MMPs, including MMP-2 and MMP-9 increase following PNS nerve injury or inflammatory neuropathy, which may disrupt ECM assembly necessary for proper myelin repair (La Fleur et al., 1996; Ferguson & Muir, 2000; Chattopadhyay et al., 2007; Zhao et al., 2010; Kim et al., 2012). In fact, myelin segments formed after remyelination in injured PNS are shorter and thinner than normal (Hiscoe, 1947). The promyelinating effect of recombinant TIMP-3 along with the positive effect on ECM production presented in this study may provide a potential therapeutic strategy for improving myelin repair in the PNS under demyelinating pathologic conditions.

Summary

We show that TIMP-3 treatment enhances Schwann cell myelination in coculture. The promyelinating effect of TIMP-3 is accompanied by an increase in neuronal Nrg1 type III signaling and accumulation of laminin during the early stages of myelin formation.

Declaration of Conflicting Interests

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