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## Astrocytic TIMP-1 Promotes Oligodendrocyte Differentiation and Enhances CNS Myelination

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### Abstract

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is an extracellular protein and endogenous regulator of matrix metalloproteinases (MMPs) secreted by astrocytes in response to CNS myelin injury. We have previously reported that adult TIMP-1KO mice exhibit poor myelin repair following demyelinating injury. This observation led us to hypothesize a role for TIMP-1 in oligodendrogenesis and CNS myelination. Herein, we demonstrate that compact myelin formation is significantly delayed in TIMP-1KO mice which coincided with dramatically reduced numbers of white matter astrocytes in the developing CNS. Analysis of differentiation in CNS progenitor cells (neurosphere) cultures from TIMP-1KO mice revealed a specific deficit of NG2+ oligodendrocyte progenitor cells. Application of rmTIMP-1 to TIMP-1KO neurosphere cultures evoked a dose-dependent increase in NG2+ cell numbers, while treatment with GM6001, a potent broad spectrum MMP inhibitor did not. Similarly, administration of recombinant murine TIMP-1 (rmTIMP-1) to A2B5+ immunopanned oligodendrocyte progenitors significantly increased the number of differentiated O1+ oligodendrocytes, while antisera to TIMP-1 reduced oligodendrocyte numbers. We also determined that A2B5+ oligodendrocyte progenitors grown in conditioned media derived from TIMP-1KO primary glial cultures resulted in reduced differentiation of mature O1+ oligodendrocytes. Finally, we report that addition of rmTIMP-1 to primary glial cultures resulted in a dose-dependent proliferative response of astrocytes. Together, these findings describe a previously uncharacterized role for TIMP-1 in the regulation of oligodendrocytes and astrocytes during development and provide a novel function for TIMP-1 on myelination in the developing CNS.

### Keywords

metalloproteinase; astrocyte; oligodendrocyte progenitor cell

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**Conflicts of Interest:** None

## Introduction

Tissue inhibitors of metalloproteinases (TIMPs) are well-recognized endogenous regulators of the matrix metalloproteinase (MMP) family of extracellular proteases (Crocker et al., 2004). Tissue inhibitor of metalloproteinase-1 (TIMP-1) is a 29kDa secreted protein first identified as a collagenase inhibiting factor in serum (Woolley et al., 1975). Expression of TIMP-1 in the CNS is significantly higher during postnatal development compared to the adult where it is very low to undetectable (Young et al., 2002a; Ulrich et al., 2005). In response to inflammatory myelin injury, TIMP-1 is robustly induced within the CNS (Pagenstecher et al., 1998; Crocker et al., 2006b; Ulrich et al., 2006), where it is predominantly expressed by astrocytes surrounding white matter lesions (Pagenstecher et al., 1998; Nygardas and Hinkkanen, 2002). These observations lend support for TIMP-1 as an endogenous factor expressed to mitigate MMP activities during neuroinflammation (Pagenstecher et al., 1998; Althoff et al., 2010). However, accumulating evidence also indicates that the biological actions of TIMPs are not solely limited to MMP inhibition (Chesler et al., 1995; Crocker et al., 2004; Stetler-Stevenson, 2008). For example, TIMP-1 has been shown to have growth factor activity (Murata et al., 1993), and anti-apoptotic potential (Jourquin et al., 2005); functions that may be mediated through ligand-receptor interactions (Jung et al., 2006; Tsagaraki et al., 2010). Hence, MMP-independent actions of TIMP-1 may also contribute to its biological effects in the developing and injured CNS.

We have previously reported that TIMP-1 deficient (TIMP-1KO) mice exhibit poor myelin recovery in experimental autoimmune encephalomyelitis (EAE), a model of T cell-mediated myelin injury (Crocker et al., 2006b). In TIMP-1KO mice, there was also a dramatic reduction in astrogliosis during EAE (Crocker et al., 2006b). These findings prompted us to reconsider the expression of TIMP-1 by astrocytes and led us to hypothesize a temporal and spatial relationship between astrocytic TIMP-1 and myelination. Herein, we report that early postnatal myelination of the CNS is markedly delayed in TIMP-1KO mice and demonstrate that TIMP-1 promotes both astrocyte proliferation and OPC differentiation in a dose-dependent and MMP-independent manner. Results from this study identify TIMP-1 as a novel astrocytic-derived factor that can directly influence CNS myelination.

## Materials and Methods

### Mice

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center (Farmington, CT). TIMP-1 knock out mice (TIMP-1KO) mice (Lee et al., 2005) were backcrossed onto a C57Bl/6 background for greater than 13 successive generations, bred as a homozygous line (Crocker et al., 2006b), and used in this study. Wild-type (WT) C57Bl/6 mice were bred and used as controls for all experiments (Jackson Laboratories; Bar Harbor, ME). All mice examined in these experiments P7 or older were male.

### Primary mixed glial and neurosphere cultures

Cells were prepared from postnatal (day 0–2) wild-type C57Bl/6 or TIMP-1KO mouse pups as previously described (Crocker et al., 2006a; Crocker et al., 2008). Mixed glial cultures were grown in DMEM containing 10% FBS [with penicillin (50U/mL) and streptomycin (50µg/mL)] and plated onto laminin-coated cover glass for immunocytochemistry in 24-well plates and fixed with 4% paraformaldehyde, as previously described (Crocker et al., 2008). Neurosphere cultures were grown in serum-free DMEM:F12 [containing penicillin (50U/mL), streptomycin (50µg/mL), B27 supplement, epidermal growth factor (EGF, 10ng/mL), fibroblast growth factor-2 (FGF-2, 20ng/mL), and heparin (2µg/mL)]. After one week in

culture, neurospheres were plated on 12mm laminin or fibronectin-coated coverslips in wells of a 24-well culture dish and differentiated into glial and neuronal populations for 7 days [DMEM containing 1% FBS and N1 supplement (Sigma, St. Louis, MO)] either with or without recombinant murine TIMP-1 (1–100ng/mL; R&D Systems, Minneapolis, MN) or a pan-MMP inhibitor GM6001 (125 $\mu$ M; Calbiochem, San Diego, CA).

### A2B5+ Oligodendrocyte Progenitor Cells

Immunopanned OPCs were prepared from neonatal rats as previously described (Barres et al., 1992; Fok-Seang and Miller, 1992; Noll and Miller, 1994). All experiments were performed in a double-blind fashion with coded samples provided for treatment of cell cultures and decoded only following data collection. For experiments using anti-TIMP-1 (0.6 $\mu$ g/mL; R&D Systems, Minneapolis, MN), control IgG (1 $\mu$ g/mL; Sigma, St. Louis, MO), recombinant murine TIMP-1 (10ng/mL; R&D Systems, Minneapolis, MN) or vehicle (TCNB buffer), A2B5+ cultures were treated overnight, fixed, and stained for O1 by immunocytochemistry. In all experiments using glial conditioned media (GCM), A2B5+ cultures were maintained in either WT or TIMP-1KO GCM for one week. Cells were then fixed, immunostained for O1, and quantified. Conditioned media was taken at a timepoint when mixed glial cultures were approximately 75% confluent (~3–4 days) and stored at –80°C. The concentration of TIMP-1 in WT GCM was determined by ELISA (R&D Systems, Minneapolis, MN). Data are representative of results from a triplicate experiment that was repeated three times. Data are reported as proportion of control (WT) due to inter-experiment variability among independently prepared cultures and the absolute numbers of each cell type between experimental trials.

### Immunohistochemistry and Image Analysis

Spinal cord sections (6–8 $\mu$ m thick) were collected from postnatal day (P) 0, P3, P6, P10, P14 & P21 WT and TIMP-1KO mice, placed into formalin (10%) fixative and paraffin embedded. Immunohistochemistry was performed, as described previously (Crocker et al., 2006b), using the following antibodies: myelin basic protein (MBP; 1:3000, Millipore, Billerica, MA), neurofilament M (NeuroM; 1:500, Millipore, Billerica, MA), NG2 (1:500; Millipore, Billerica, MA), glial fibrillary acidic protein (GFAP; 1:1000, Dako, Denmark), A2B5 (ATCC, Manassas, VA), and O1 (Sigma, St. Louis, MO). All immunostaining was visualized using Alexa-fluorophore-conjugated secondary antisera (1:500; Invitrogen, Carlsbad, CA). All images were acquired using Northern Eclipse software (Empix Imaging, Cheektowaga, NY) on an Olympus IX71 inverted microscope. All sections used for comparative analyses of NeuroM, MBP, and GFAP used the same reagents and run in parallel at the same time. Total axon numbers were averaged by counting NeuroM+ axons on serial sections (n=3/animal) from the ventral white matter of spinal cord (n=3/group). The percentage of myelinated axons was obtained by dividing the number of concentrically myelinated axons by the total number of axons in each section. Total numbers of NG2+,  $\beta$ 3-tubulin+, and GFAP+ were counted in nine arbitrarily set fields of view (3 $\times$ 3) of a 12mm coverslip.

### Electron Microscopy

The vertebral columns from terminally anesthetized P7 WT and TIMP-1KO mice (n=4) were dissected and processed for EM as previously described (Komitova et al., 2009). Ultrathin sections (70–90nm thick) were cut and put onto 200 mesh copper grids that were visualized with a Tecnai Biotwin 12kV transmission electron microscope (FEI Corp., Eindhoven, Holland) and images were processed digitally.

## Protein Isolation, SDS-PAGE, and Western Blotting

Whole spinal cord protein lysates from P7 TIMP-1KO and WT mice were separated by SDS-PAGE and Western blotting was performed using anti-GFAP (1:3000; Dako, Denmark), proliferating cell nuclear antigen (PCNA; 1:100, Abcam, Cambridge, MA), and procaspase-3 (1:100; Millipore, Billerica, MA). Equivalent loading was verified by  $\beta$ -actin (1:20 000; Sigma, St Louis, MO). Densitometric measurements of all western blots were performed using ImageJ software (NIH, Bethesda, MD).

## BrdU Incorporation and TUNEL Assay

Mixed glial cultures were grown on laminin-coated coverslips for 48 hours and then treated with either rmTIMP-1 (10ng/mL) or GM6001 (1.25–125 $\mu$ M) and. For analysis of cell proliferation, BrdU (10 $\mu$ M) was added to each culture condition for 24hrs prior to fixation and labeling using DAB, according to manufacturer's protocol (BD Pharmingen, San Diego, CA). Parallel experiments were also performed to analyze DNA fragmentation, indicative of programmed cell death, using a TACS 2 TdT Fluorescein In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD).

## Statistical Analyses

Data are presented as mean  $\pm$  SEM. A one-way analysis of variance with Tukey's posthoc tests or unpaired Student's *t*-test was used to determine group differences. For all tests,  $p < 0.05$  was considered significant.

## Results

### Compact myelin formation is delayed in TIMP-1KO mice

We first examined the temporal expression of TIMP-1 in the spinal cord during early postnatal development by qPCR. Consistent with previous studies (Young et al., 2002b; Ulrich et al., 2005), *timp-1* mRNA was expressed at higher levels during the early postnatal period and declined with age (Supplemental Fig. 1). These data supported a temporal correlation between increased expression of TIMP-1 during the critical time of CNS myelination (Foran and Peterson, 1992). To explore the effect of TIMP-1 deficiency on developmental CNS myelination, we compared myelination in the spinal cords of WT and TIMP-1KO mice ranging from P0–P21 in postnatal age. Immunohistochemical analysis of myelin basic protein (MBP), a major protein component of compact myelin, revealed significantly fewer myelinated axons in P7 TIMP-1KO mice compared to age-matched WT C57Bl/6 mice (Fig. 1A,B). No differences in the numbers of axons (NeuroM+) or their diameters were noted between TIMP-1KO and age-matched WT mice (Supplemental Figure 2) were identified between genotypes (Fig. 1A,B), indicating that reduced myelination was not a result of delayed or impaired axonogenesis in TIMP-1KO mice. Using electron microscopy (EM), ultrastructural analysis of myelinated axons in dorsal column of the P7 spinal cord confirmed a 50% reduction in TIMP-1KO mice compared to WT mice (Fig 1B; 58.8 $\pm$ 2.4% in WT vs. 29.6 $\pm$ 8.3% in TIMP-1KO). Furthermore, these axons had 24.4% fewer myelin wraps per axon (9.9 $\pm$ 0.28 wraps in WT vs. 7.4 $\pm$ 0.35 wraps in TIMP-1KO) and there was a 28% increase in periaxonal space (Fig. 1A,B; 0.42 $\pm$ 0.01 in WT vs. 0.54 $\pm$ 0.02 in TIMP-1KO). Among the myelinated axons in the TIMP-1KO mice, myelin also appeared loosely wrapped compared to the tightly myelinated axons of WT mice. These differences were most apparent in the CNS of TIMP-1KO mice at P7. These differences also resulted in a 7.8% reduction in the G-ratio of myelinated axons in TIMP-1KO mice (Supplementary Fig. 2; 0.89 $\pm$ 0.01 in WT vs. 0.82 $\pm$ 0.01 in TIMP-1KO).

Since TIMP-1KO mice exhibited a delay in CNS myelination, we next sought to determine whether this phenotype was related to differences in oligodendrocyte progenitor cell (OPC)

number. Immunohistochemistry for platelet derived growth factor alpha receptor (PDGF $\alpha$ R) was used to identify OPCs in tissues from TIMP-1KO and age matched WT mice. Throughout the CNS white matter (brain and spinal cord) of P7 TIMP-1KO mice, a 57% reduction in the number of PDGF $\alpha$ R+ OPCs cells was observed (Fig. 1C, Supplementary Fig. 3;  $178.7 \pm 14.1$  in WT vs.  $78.0 \pm 40.15$  in TIMP-1KO). These differences in OPC numbers were not observed at later developmental time points (P14 or P21) (Supplemental Fig. 3). This decrease in OPC abundance was consistent with the delayed myelination observed in the TIMP-1 KO mice at P7 and suggested that TIMP-1 might influence OPC development and/or differentiation.

### OPC differentiation is enhanced by TIMP-1

To determine whether TIMP-1 could directly influence oligodendrocyte differentiation, we used immuno-panned OPC (A2B5+) cultures from neonatal rats. Treatment of A2B5+ OPCs for 48 hrs with recombinant murine (rm)TIMP-1 resulted in a significant increase ( $34 \pm 0.45\%$ ) in the number of differentiated O1+ oligodendrocytes when compared with the A2B5+ cultures treated with vehicle ( $138 \pm 8$  in control, untreated, vs.  $185 \pm 9$  in rmTIMP-1 treated cultures;  $p < 0.001$ ; t-test vs control, Fig. 2A). Conversely, in additional sets of A2B5+ cultures treated for 48 hrs, administration of a function-blocking antibody to TIMP-1 (anti-TIMP-1), but not an IgG isotype matched control antibody (Crocker et al., 2007) resulted in an 18% decrease in number of mature O1+ oligodendrocytes ( $155 \pm 7$  in control, untreated vs.  $125 \pm 8$  in  $\alpha$ TIMP-1 treated cultures;  $p < 0.002$ ; t-test vs control Fig. 2B).

### TIMP-1 increases NG2+ OPC numbers in neurosphere cultures

While TIMP-1 significantly increased numbers of O1+ cells in a purified OPC population, we next determined whether the effects of TIMP-1 on early OPCs could be modeled in a heterogeneous cellular environment containing both glia and neurons. TIMP-1KO and WT neurospheres were grown on fibronectin or laminin, two substrates previously shown to promote growth of early OPCs (Buttery and French-Constant, 1999; Kearns et al., 2003). In this assay, OPCs were identified by immunocytochemical detection of the proteoglycan NG2 (Nishiyama et al., 2009). When cultured on either substrate, NG2+ cells were decreased by ~76% in cultures derived from TIMP-1KO mice (Fig. 3A,B). These effects were reversed by application of rmTIMP-1 (1–100ng/mL), resulting in a dose-dependent increase in the numbers of NG2+ cells (1ng/mL,  $179.3 \pm 43.1\%$  and at 10ng/mL,  $342.3 \pm 74.8\%$  increase in NG2+ cell numbers vs untreated TIMP-1KO neurosphere cultures) (Fig. 3C,D). Treatment with the pan-MMP inhibitor, GM6001, did not increase NG2+ cell numbers in TIMP-1KO cultures; suggesting that the action(s) of TIMP-1 were not a result of decreased MMP activity. We also noted that in TIMP-1KO cultures treated with rmTIMP-1 many NG2+ cells had highly branched processes reflecting a more advanced stage of differentiation (Nishiyama et al., 2009) (Fig. 3C, right panels).

### Numbers of GFAP+ astrocytes are decreased in the developing CNS of TIMP-1KO mice

We had previously reported that TIMP-1 is produced by astrocytes (Crocker et al., 2006a), and that TIMP-1KO mice exhibit poor myelin recovery following EAE (Crocker et al., 2006b). EAE pathology in TIMP-1KO mice was also accompanied by a notable decrease in the numbers of astrocytes in the injured CNS (Crocker et al., 2006b). Since astrocytes play an important role in promoting myelination (Moore et al., 2011), these findings led us to examine whether astrocyte activation differed between TIMP-1KO and WT mice during early postnatal CNS development. Immunohistochemistry for glial fibrillary acidic protein (GFAP), a marker of astrocyte activation, revealed a significant decrease in the density of reactive astrocytes populating the white matter of the spinal cord of TIMP-1KO mice (Fig. 4). Similar results were also observed throughout the CNS of TIMP-1KO mice at this same age (Supplementary Fig. 4). Western blot analysis of whole spinal cord lysates confirmed a

greater than 50% reduction in GFAP levels in P7 TIMP-1KO mice compared to age matched WT mice (Fig. 4B). To determine whether TIMP-1 could directly act as a mitogen for astrocytes, we administered rmTIMP-1 (48hrs) to primary glial cultures and counted the numbers of GFAP+ cells. Using a primary glial culture derived from TIMP-1KO mice, the addition of rmTIMP-1 (10ng/mL), but not the pan-MMP inhibitor, GM6001, doubled the number of GFAP+ cells after 48hrs (Fig. 5A,B; 108.9±15.6 in control, untreated cultures vs. 227.3±24 in rmTIMP-1 treated cultures). Similarly, application of rmTIMP-1, but not GM6001, also stimulated GFAP+ astrocyte proliferation in WT cultures (Supplementary Fig. 5; 63.3±22.3 GFAP+ cells per field in control, untreated cultures vs. 38.78±3.5 in GM6001 treated cultures vs. 195.8±57.1 in rmTIMP-1 treated cultures), although the concentration of TIMP-1 needed was higher (100ng/mL) and likely because TIMP-1 was constitutively produced in WT cultures (7.5ng/mL).

To discriminate whether the known biological effects of TIMP-1 to either prevent cell death (Tan et al., 2003) or stimulate cellular proliferation (Hernandez-Guillamon et al., 2009) accounted for the effects of TIMP-1 in our astrocyte cultures, we used BrdU incorporation assays and western blotting for apoptotic and proliferative markers. A significant increase in the number of BrdU+ astrocytes was observed following application of rmTIMP-1 (10ng/mL) (Fig. 5C; 58.23±1.4% in control, untreated cultures vs. 66.7±1.4% in rmTIMP-1 treated cultures). Similarly, using Western blotting techniques, an increase in proliferating cell nuclear antigen (PCNA) protein levels, a marker of cell proliferation, was observed in protein lysates from astrocytes treated with 10 ng/mL rmTIMP-1 (Supplementary Fig. 6A). Treatment with TIMP-1 did not stimulate activation of caspase-3 as levels of procaspase-3 levels remained unchanged, arguing against a significant anti-apoptotic effect of TIMP-1 in this system (Supplementary Fig. 6A).

### Glial conditioned media lacking TIMP-1 produces fewer and less-mature OPCs

To refine our understanding about whether astrocytic TIMP-1 could influence OPC growth and differentiation, we collected conditioned media from TIMP-1KO or WT primary glial cultures and used this media to culture A2B5+ immunopanned OPCs (Baracska et al., 2007). Upon examination of the numbers of O1+ oligodendrocytes one week later, A2B5+ cultures grown in WT GCM readily differentiated into O1+ oligodendrocytes with broad and expansive myelin sheets (Fig. 6A,B). In contrast, A2B5+ cultures grown in TIMP-1KO GCM exhibited less expansive myelin sheets when compared with A2B5+ OPCs grown in WT GCM (Figure 6A,B). The proportion of A2B5+ progenitors following one week of differentiation in TIMP-1KO GCM was 65% less than cultures grown in WT GCM (i.e. number of A2B5 cells in TIMP-1KO GCM was 35.5±10% of numbers grown in WT GCM; Fig. 6A,B). The number of differentiated O1+ oligodendrocytes was also reduced in these A2B5+ cultures grown in TIMP-1KO GCM when compared with the proportion of mature O1+ oligodendrocytes found A2B5+ cultures grown in WT GCM (i.e. number of O1+ cells in TIMP-1GCM was 26.1±7% of number of cells differentiated in WT GCM; Fig. 6A,B). ELISA assay for murine TIMP-1 determined that the concentration of TIMP-1 in the GCM from WT cultures was ~7.5ng/mL whereas TIMP-1 was not detected in TIMP-1KO GCM (0ng/mL). The concentration in the WT GCM was consistent with the concentration of rmTIMP-1 (10ng/mL) found to induce astrocyte proliferation, increase NG2+ cell numbers in the TIMP-1KO neurosphere assay, and increase the numbers of mature O1+ oligodendrocytes in the A2B5+ cell cultures.

## Discussion

We have identified a novel function for TIMP-1 in the regulation of OPC differentiation and astrocyte proliferation. Using purified OPC cultures, neurosphere assays, and primary glial cells, we have demonstrated that the effects of TIMP-1 can be direct, dose-dependent, and

MMP-independent. In TIMP-1KO mice, developmental myelination was markedly delayed and accompanied by a decrease in reactive astrocytes, suggesting that increased expression of TIMP-1 by astrocytes during early postnatal development may enhance the process of myelination in the CNS.

It is becoming increasingly evident that myelination is a multicellular effort requiring oligodendrocytes, neurons, and astrocytes for efficient myelin ensheathment of axons (Liedtke et al., 1996; Sorensen et al., 2008; Watkins et al., 2008). Astrocytes represent approximately 50% of the cellular number in the adult CNS and are required for maintaining neuronal homeostasis by providing trophic and metabolic support (Smit et al., 2001; Ullian et al., 2001), as well as promoting neurogenesis (Song et al., 2002). More recently, the astrocyte has been shown to also play a critical role in efficient CNS myelination (Ishibashi et al., 2006; Sorensen et al., 2008; Watkins et al., 2008). Astrocytes secrete factors that promote OPC differentiation, proliferation, and survival (Gard et al., 1995; Butzkueven et al., 2002; Moore et al., 2011), in addition to promoting oligodendrocyte sheet extension (Oh and Yong, 1996). Interestingly, astrocytes have been shown to proliferate during developmental myelination and following myelin injury (Tani et al., 1996; Pagenstecher et al., 1998), where they promote efficient myelination and remyelination, respectively (Franklin et al., 1991; Blakemore et al., 2003; Voskuhl et al., 2009). In myelinating CNS co-cultures, astrocytes enhance the rate and extent of compact myelin formation, but are not essential for the generation of myelin. Similarly, co-culture experiments lacking astrocytes reduces the numbers of myelinated axons and extent of axon myelination (Watkins et al., 2008).

Our findings suggest that astrocytic TIMP-1 is a novel regulatory molecule for myelination in the developing and injured CNS. These findings also elude to interesting parallels in the TIMP-1KO background regarding astrocytes and myelin. Specifically, we had previously reported that while there are no obvious myelin deficits in adult TIMP-1KO mice, these mice exhibited poor remyelination following EAE (Crocker et al., 2006b). Moreover, myelin injury in adult TIMP-1KO mice was also associated with reduced numbers of astrocytes (Crocker et al., 2006b). In the present study we found that recombinant TIMP-1 protein stimulated the proliferation of cultured astrocytes, while the delay in CNS myelination in TIMP-1KO mice was associated with reduced astrocytic activity in the developing CNS. This study provides new insight into a role for TIMP-1 as an autocrine and/or paracrine factor released by astrocytes that influences both OPCs and astrocytes. While TIMP-1 has been reported to be produced by several immune and CNS cell types (Toft-Hansen et al., 2004), its elevated expression by astrocytes during both development and following myelin injury provides further evidence that TIMP-1 plays a role in CNS myelination. The *in vivo* evidence that TIMP-1KO mice have delayed CNS myelination accompanied by reduced activation of white matter astrocytes is consistent with the emerging model that astrocytes play a pivotal role in CNS myelination. Since complete myelination does eventually occur in TIMP-1KO mice, our perspective is that heightened TIMP-1 expression during development, and following myelin injury in the adult, participates to enhance the overall effectiveness of CNS myelination and repair.

The significant decrease in PDGF $\alpha$ R+ OPCs in the white matter of P7 TIMP-1KO mice prompted us to use *ex vivo* assays to determine if exogenous TIMP-1 could directly influence OPC differentiation. We have now shown that TIMP-1 can directly promote OPC differentiation (Figure 2), thus providing compelling new evidence that in response to myelin injury, TIMP-1 can promote endogenous OPC differentiation and myelin repair independently of MMPs (Crocker et al., 2006b). Additional MMP-independent functions of TIMP family proteins have been previously reported. For example, TIMP-3 is a direct antagonist of the kinase-insert domain receptor (KDR) and disrupts VEGF signaling (Qi et

al., 2003), while TIMP-2 can bind integrin  $\alpha 3\beta 1$  and block FGF-2 and VEGF signaling during angiogenesis (Seo et al., 2003; Seo et al., 2008). To our knowledge, this report is the first demonstration that TIMP-1 can act directly to enhance OPC differentiation. The exact mechanism(s) by which TIMP-1 elicits these MMP-independent actions is presently unresolved although several possible mechanisms could be proposed. In a previous report, TIMP-1 has been shown to interact with a member of the tetraspanin family, CD63, on the surface of MCF10A breast epithelial cells (Jung et al., 2006). It is therefore plausible that similar or analogous mechanism could also mediate the actions of TIMP-1 on OPCs and astrocytes during CNS myelination. Furthermore, it is also possible that TIMP-1 inhibits other enzymes independently of MMPs that are not blocked by GM6001. Alternatively, TIMP-1 may also interact with other cellular transduction signaling pathways. For instance, TIMP-3 has been reported to sensitize cells to FasL-induced cell death (Bond et al., 2002) and also stabilize the TNF receptor (Smith et al., 1997). Thus, a possible action of TIMP-1 may be to modulate a receptor-ligand interaction that leads to proliferation and/or differentiation of OPCs. Nevertheless, though the exact mechanism through which TIMP-1 elicits its functions in the context of OPCs and myelin has yet to be characterized, and will require further study, these present findings provide a new perspective for how TIMP-1 functions in the CNS.

This study describes a previously unidentified function of TIMP-1 to directly regulate oligodendrocyte differentiation. We have also determined that TIMP-1 may enhance myelination by functioning in an autocrine, or paracrine, manner to influence astrocyte reactivity during CNS myelination. Taken together, these findings support a function for TIMP-1 to directly promote OPC differentiation while also increasing astrocyte support for OPCs and CNS myelination (Fig. 6C). In the healthy adult CNS, expression of TIMP-1 is very low, yet is highly expressed following inflammatory brain injury and in diseases such as acute demyelinating encephalomyelitis (ADEM) (Ichiyama et al., 2006), cerebral ischemia, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) [reviewed in ref. (Crocker et al., 2004)]. The revised model for a role of TIMP-1 during demyelinating diseases may be linked to promoting remyelination. For instance, in ADEM, an *acute* demyelinating disease of the CNS that spontaneously resolves, TIMP-1 levels were markedly higher than controls (Ichiyama et al., 2006). In contrast, in multiple sclerosis (MS), a *chronic* demyelinating condition characterized by minimal myelin repair, expression of TIMP-1 did not differ from healthy age-matched controls (Avolio et al., 2003; Waubant et al., 2003). We therefore hypothesize that in the diseased adult brain, increased TIMP-1 expression may contribute to remyelination and/or myelin recovery. Indeed, this idea is now supported by both our previous finding that adult TIMP-1KO mice exhibited poor myelin recovery in EAE (Crocker et al., 2006b) and a recent study by Althoff et al. (2010) which demonstrated that transgenic expression of TIMP-1 by astrocytes had a mitigating effect on the clinical development of EAE and myelin pathology. Collectively with the present study, we hypothesize that inadequate expression of TIMP-1 in MS contributes to myelin pathology by less efficient myelin repair and development of chronic myelin pathology. This emerging role of TIMP-1 in CNS myelination would also suggest that strategies used to enhance TIMP-1 expression in response to CNS injury might represent a novel therapeutic strategy to promote endogenous remyelination.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.



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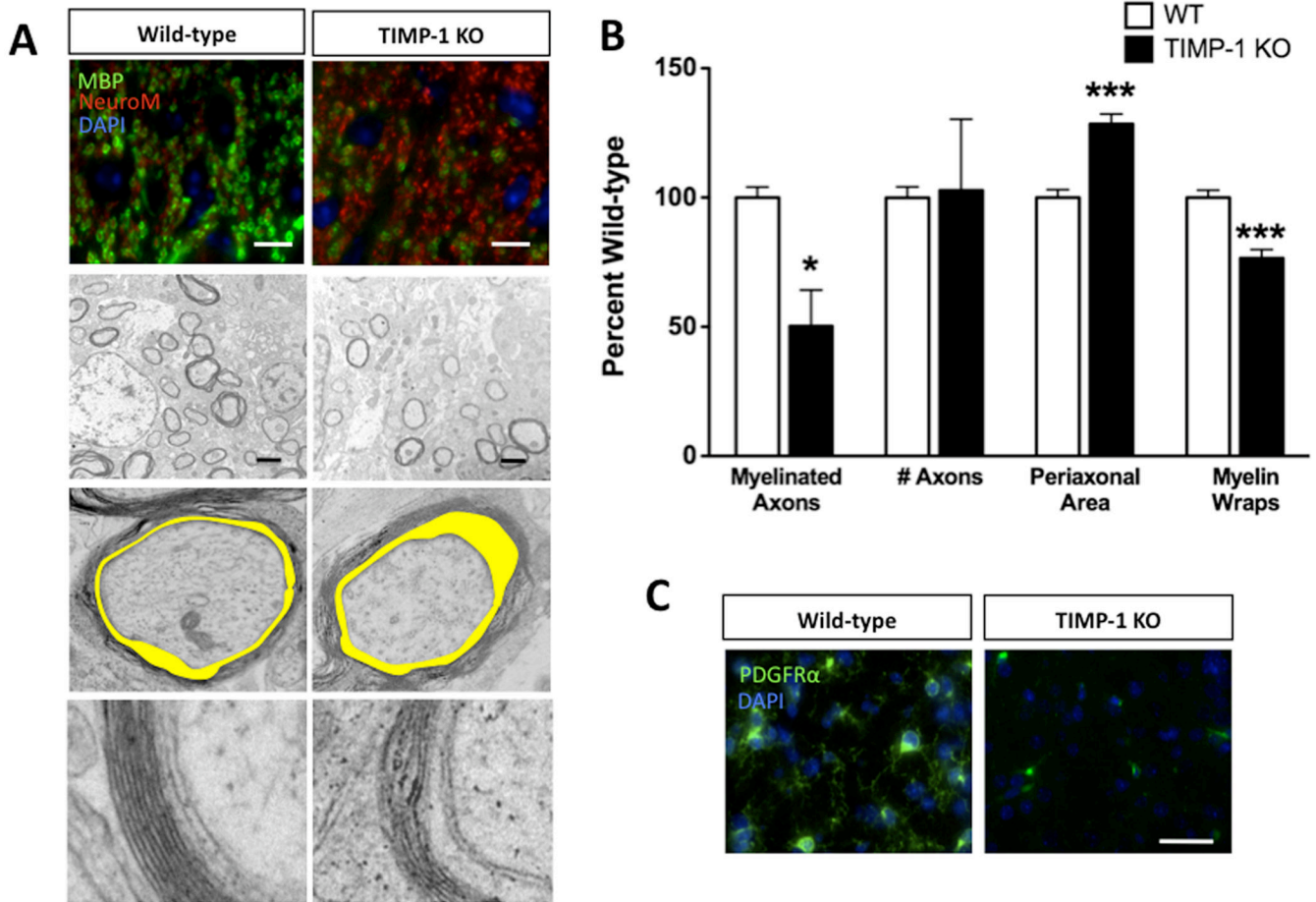
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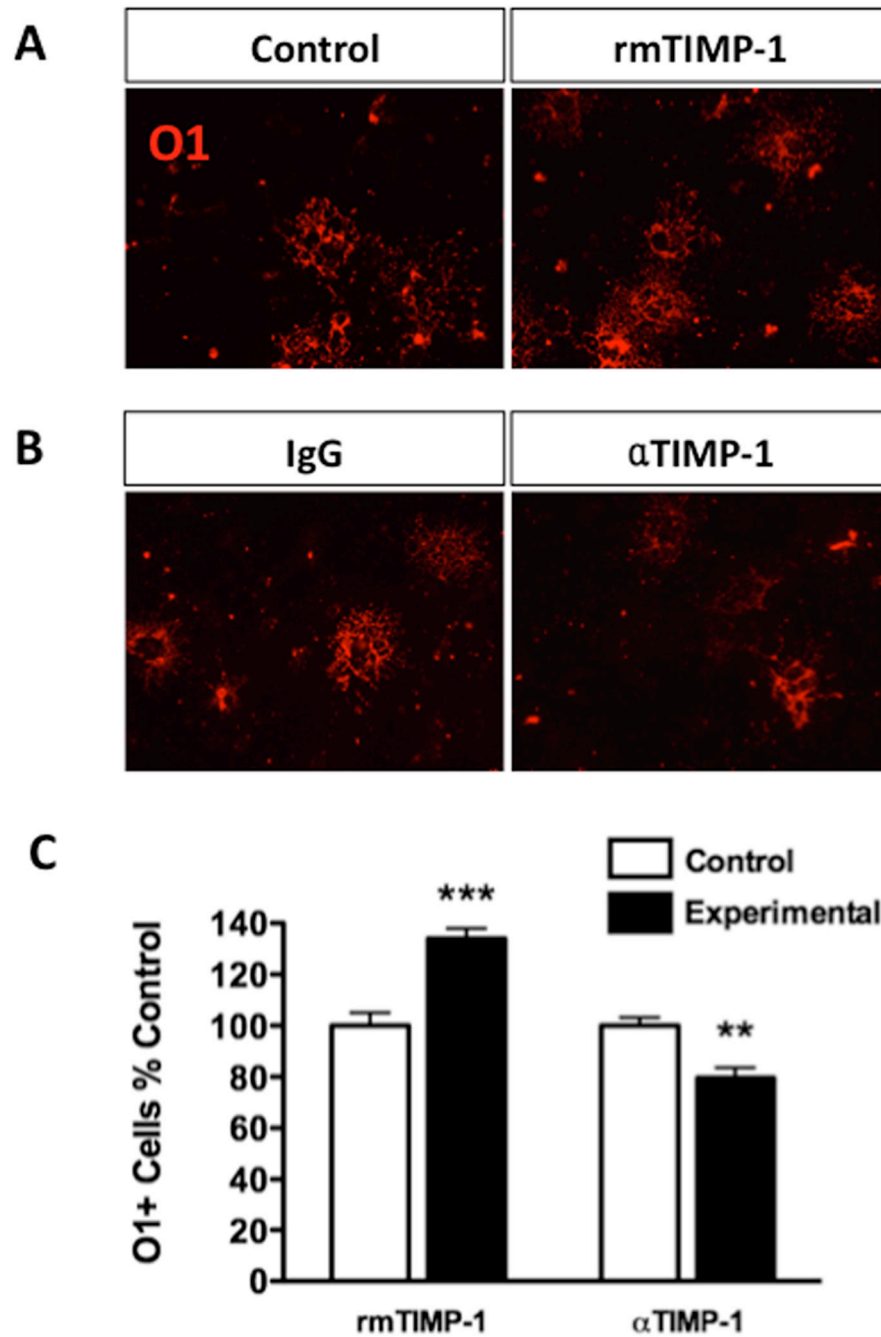
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**Figure 1.**

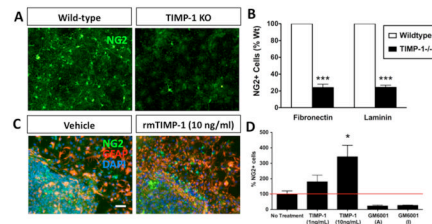
Analysis of myelination in the spinal cord of TIMP-1KO mice. **(A&B)** Fluorescent immunohistochemistry for myelin basic protein (green) and axons, labeled by NeuroM (red), in the dorsal column of WT and TIMP-1KO P7 spinal cords identified a 30% reduction in the proportion of myelinated axons in TIMP-1KO compared to WT. No differences were observed in axon number. Electron microscopy performed on P7 spinal cords from WT and TIMP-1KO mice confirmed the decrease in fully myelinated axons, while revealing other morphological differences: an enlarged periaxonal space (shaded yellow) and fewer myelin wraps in TIMP-1KO mice. Comparison of the periaxonal space was calculated by dividing the total area of the myelinated axon by the axonal area (ImageJ software; NIH). Analysis was performed on  $n=3$  animals/genotype; data represent 44 and 53 axons counted for WT and TIMP-1KO, respectively. **(C)** A significant decrease in the number of PDGFR $\alpha$ + cells was observed in the white matter of spinal cords from P7 TIMP-1KO mice compared to WT. Scale bars = 20 $\mu$ m and 2 $\mu$ m in upper and lower panels respectively in panel A. Scale bars = 20  $\mu$ m in Panel C. \* $p<0.05$ ; \*\*\* $p<0.001$ . Error bars represent the mean ( $n=3$ /genotype)  $\pm$  SEM.



**Figure 2.**

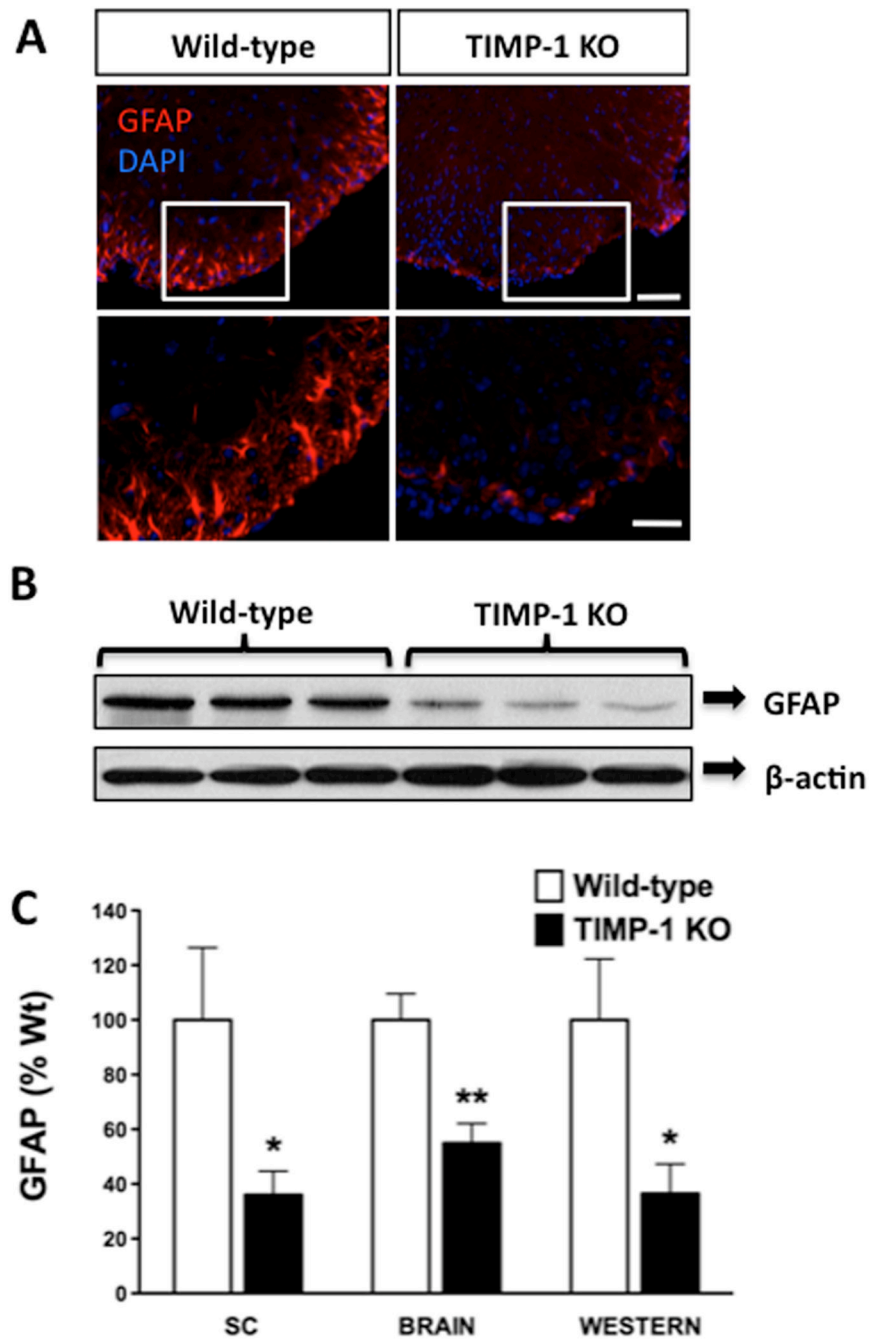
Differentiation of primary (A2B5+) OPC cultures into mature O1+ oligodendrocytes can be increased or decreased by adding or blocking TIMP-1 respectively. (A) Following a 48 hr treatment with rmTIMP-1 (10ng/mL) to purified immunopanned A2B5+ rat OPC cultures, increased numbers of mature (O1+) oligodendrocytes were observed. Control cultures were also treated for 48hrs in similar volumes, but lacking TIMP-1 (\*\* $p < 0.001$ , t-test). (B) Administration of a blocking anti-TIMP-1 antibody (0.6 $\mu$ g/mL) to immunopanned A2B5+ cultures for 48 hrs decreased O1+ cell numbers compared to control IgG (1 $\mu$ g/mL) (\*\* $p < 0.0002$ , t-test). All data using A2B5+ cultures are representative of three

independent experiments performed in triplicate using coded samples in a double-blinded fashion. Error bars represent the mean  $\pm$  SEM.

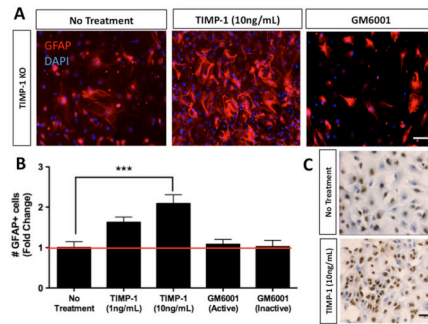
**Figure 3.**

Recombinant TIMP-1 restores the NG2+ cell deficit observed in neural stem cells derived from TIMP-1KO mice. **(A)** Representative immunocytochemical labeling of oligodendrocyte progenitors (NG2+) derived from WT or TIMP-1KO neurosphere cultures following one week of differentiation. **(B)** A significant decrease in the number of neural stem cell derived NG2+ cells was observed in the TIMP-1KO cultures when plated on either fibronectin or laminin substrates. **(C&D)** Administration of recombinant murine TIMP-1 (1 & 10ng/mL), but not the pan-MMP inhibitor GM6001 (active/inactive; A/I), dose dependently reversed the deficiency (>3-fold increase) of NG2+ progenitor cells in TIMP-1KO neurosphere cultures. Scale bars = 200 $\mu$ m. \*\*\* $p$ <0.0001; \* $p$ <0.05.  $n$ =3/group. Error bars represent the mean  $\pm$  SEM.

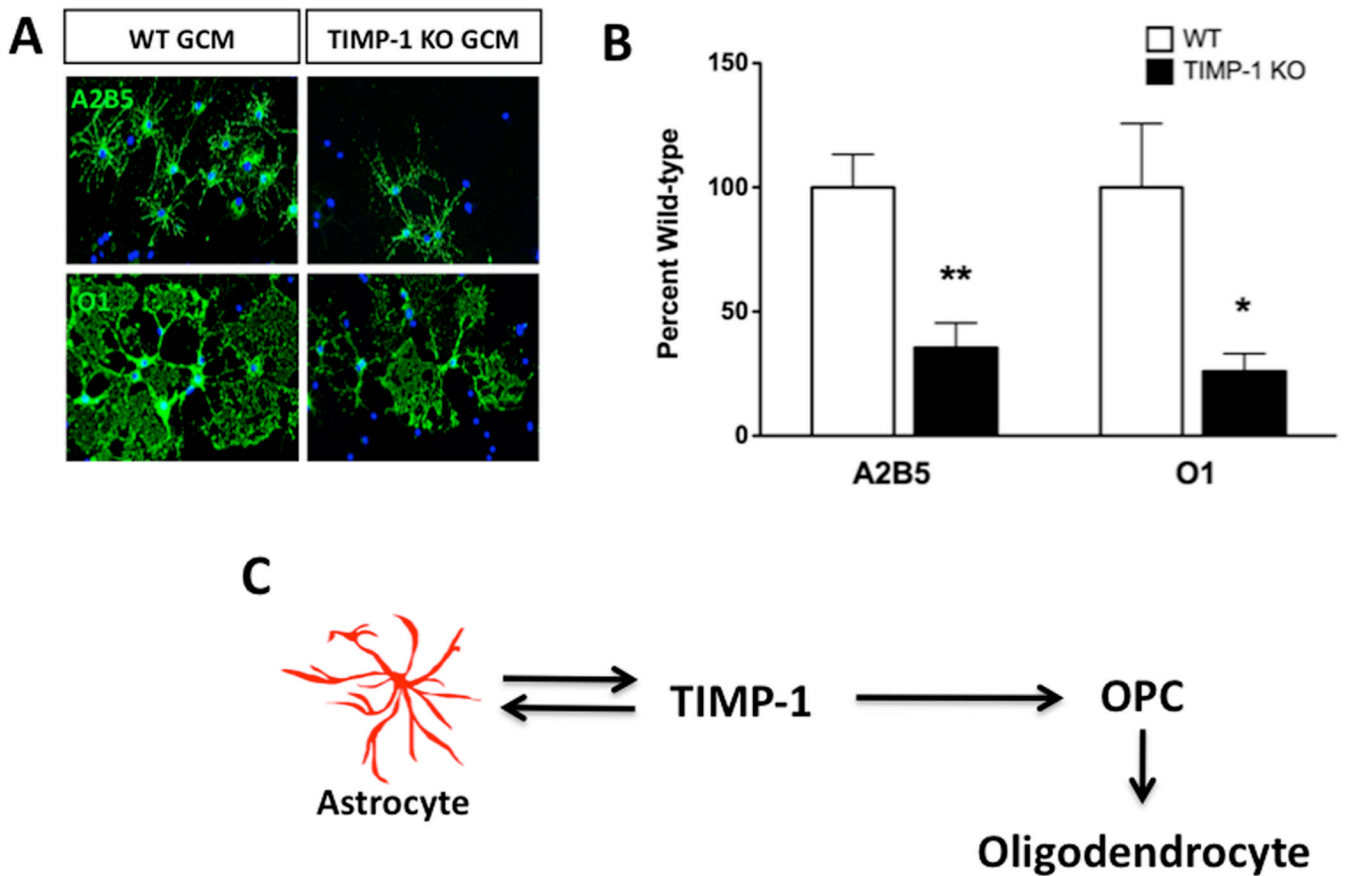




**Figure 4.** Decreased reactive astrocyte numbers in spinal cords of P7 TIMP-1 KO mice. **(A)** P7 spinal cord sections immunostained for GFAP revealed a dramatic decrease in the numbers of reactive astrocytes between WT and TIMP-1KO mice. **(B)** Representative Western blot analysis of P7 spinal cord lysates confirmed reduced GFAP expression levels in the spinal cords of TIMP-1KO mice (n=5/group). **(C)** Integrated pixel density of GFAP IHC in both the spinal cords (SC) and brains was performed in addition to the densitometry on the Western blot relative to the loading control  $\beta$  relative to the loading control  $\beta$ -actin. Scale bar = 200 $\mu$ m, \*p<0.05. Error bars represent the mean  $\pm$  SEM.



**Figure 5.** Recombinant TIMP-1 increases proliferation of astrocytes. **(A&B)** Addition of rmTIMP-1 to primary glial cultures from TIMP-1KO mice resulted in a dose-dependent increase in astrocyte numbers while treatment with GM6001 (active/inactive; A/I) did not alter astrocyte numbers (n=9/group). **(C)** Astrocytes incubated with BrdU followed by immunohistochemistry for BrdU show a significant increase in the numbers of BrdU+ labeled cells. Scale bars = 20 $\mu$ m; \*\*\*p<0.001. Error bars represent the mean  $\pm$  SEM.

**Figure 6.**

Glial conditioned media (GCM) derived from TIMP-1KO mixed glial (astrocytes and microglia) cultures poorly supports primary oligodendrocyte growth and differentiation. **(A)** Primary oligodendrocyte precursor cells (A2B5+) were split and grown in either conditioned media collected from either WT C57Bl/6 or TIMP-1KO primary glial cultures. Immunocytochemistry for A2B5 and O1 following 5 days of culture revealed fewer A2B5+ cells, while O1+ cells exhibited a stunted morphology with smaller membranous fans of prospective myelin. **(B)** Quantification of A2B5+ and O1+ cells confirmed a 65% and 74% decrease, respectively, in the numbers of cells grown in GCM derived from TIMP-1KO mice. Error bars represent the mean  $\pm$  SEM. **(C)** Hypothesized model of TIMP-1 in the regulation of astrocyte functions during oligodendrocyte differentiation and CNS myelination. Our data indicate that astrocyte production of TIMP-1 functions directly to promote OPC differentiation. Coincidentally, TIMP-1 also signals as an autocrine or paracrine factor on astrocytes that may too support OPC differentiation. An important effect of astrocytic TIMP-1 expression therefore is to enhance CNS myelination.