Poly-L-ornithine blocks the inhibitory effects of fibronectin on oligodendrocyte differentiation and promotes myelin repair

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Abstract

The extracellular matrix surrounding oligodendrocytes plays an important role during myelination and remyelination in the brain. In many cases, the microenvironment surrounding demyelination lesions contains inhibitory molecules, which lead to repair failure. Accordingly, blocking the activity of these inhibitory factors in the extracellular matrix should lead to more successful remyelination. In the central nervous system, oligodendrocytes form the myelin sheath. We performed primary cell culture and found that a natural increase in fibronectin promoted the proliferation of oligodendrocyte progenitors during the initial stage of remyelination while inhibiting oligodendrocyte differentiation. Poly-L-ornithine blocked these inhibitory effects without compromising fibronectin's pro-proliferation function. Experiments showed that poly-L-ornithine activated the Erk1/2 signaling pathway that is necessary in the early stages of differentiation, as well as PI3K signaling pathways that are needed in the mid-late stages. When poly-L-ornithine was tested in a lysolecithin-induced animal model of focal demyelination, it enhanced myelin regeneration and promoted motor function recovery. These findings suggest that poly-L-ornithine has the potential to be a treatment option for clinical myelin sheath injury.

Key Words: differentiation; Erk1/2; extracellular matrix; fibronectin; lysolecithin-induced demyelination; oligodendrocyte; PI3K; poly-L-ornithine; proliferation; remyelination

Introduction

Ensheathment of axons by myelin is essential for rapid nerve impulse conduction as well as for preserving axonal integrity and health. In the central nervous system (CNS), oligodendrocytes (OLs) are the only cell type responsible for myelin formation. During development, OL progenitor cells (OPCs) proliferate and migrate to their final destination, where they differentiate into OLs and wrap around the axons to form dense myelin structures (Bradl and Lassmann, 2010; Stadelmann et al., 2019; Tian et al., 2019; Yang et al., 2020; Cherchi et al., 2021). OL development is regulated by a complicated interplay of intrinsic factors (transcription factors, microRNAs, epigenetic modulators) and extrinsic factors, including extracellular matrix (ECM) (Emery and Lu, 2015; Gaesser and Fyffe-Marich, 2016; Galloway and Moore, 2016; Kispert and Wegner, 2016; Liu et al., 2016; Wheeler and Fuss, 2016).

The ECM is a complex system that surrounds all cells. ECM is primarily composed of glycoproteins, collagen, and glycosaminoglycans (Paolillo and Schinelli, 2019; Rilla et al., 2019). ECM can interact with cell adhesion molecules, regulatory factors, connexins, or other proteins to trigger diverse biological activities, such as cell proliferation, migration, differentiation, and survival. In addition to maintaining tissue structure, it also participates in biological reactions essential for normal homeostasis (Birch, 2018; Manou et al., 2019). The effects of ECM components on OLs are intricate. For instance, fibronectin (FN) and decorin are positive regulators of OPC migration, whereas chondroitin sulfate proteoglycans and collagens inhibit OPC migration. Laminin, and decorin can promote OPC differentiation into mature OLs, while chondroitin sulfate proteoglycans, heparan sulfate proteoglycans, tenasin-C, thrombospondin-1, and myelin debris have been shown to impede OPC maturation (Pu et al., 2018).

FN is a glycoprotein with a high molecular weight. It is secreted as a soluble dimer with a disulfide bond by multiple cell types, including hepatocytes, neurons, and astrocytes (Pearlstein et al., 1980; Hibiits et al., 2012; Stoffels et al., 2013). FN is often referred to as the “major organizer” because it acts as a bridge between cell surface receptors (such as integrins) and extracellular molecules (such as proteoglycans, collagen, and other focal adhesion molecules). It is a ligand for several integrin receptors, including αvβ1, αvβ3, αvβ5, α5β1, αvβ6, α4β1, α4β7, α8β1, αDβ2, αMβ2, αXβ2 (Leiss et al., 2008). Although it is nearly absent in the adult CNS and only localizes to the CNS vasculature (Patten and Wang, 2021), after CNS injury, including demyelination, both neurons and astrocytes secrete FN and deposit it into the extracellular space. At the same time, FN levels increase in plasma, which can enter the demyelinated area via the damaged blood-brain barrier. FN increase within demyelinated regions suggests that FN plays a role during demyelination/remyelination. Numerous studies have shown that impaired
remyelination in multiple sclerosis (MS) is due to FN aggregation in lesions (Siskova et al., 2006, Stoffels et al., 2013; Qin et al., 2017; Wang et al., 2018b; Wertman et al., 2020). However, some studies have shown that the emergence of FN has certain positive effects (Niu et al., 2005; Stoffels et al., 2015; Tripathi et al., 2017). For instance, FN secreted by astrocytes can promote the proliferation of OPCs following CNS demyelination (Stoffels et al., 2015).

In the current study, we investigated the function of FN in OL development. Subsequently, we attempted to find an agent that would reverse its inhibitory effects and promote myelin repair.

Methods

Animals

Mice were housed in the Wuhan University Center for Animal Experiment/Animal Biosafety Level-III Laboratory (Wuhan, China). The rearing environment was well-lit with 12/12 hour light/dark cycles, and cages were individually ventilated. The animal experiments were carried out following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Approval for the animal experiments was granted by the institutional Animal Care and Use Committee at Wuhan University on February 20, 2014 (approved No. 20130701). Neonatal mice (n = 152, weighing 1–2 g) and specific-pathogen-free 8-week-old male C57BL/6J mice (n = 27, weighing 20–25 g) were obtained from the Animal Experiment/Animal Biosafety Laboratory in Wuhan, China (Vietcor, Colaba, CA, USA). 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin. Cells were collected at various time points for analysis (Sigma), 1% N2 supplement, 2% B27 supplement (Gibco), and penicillin-streptomycin. Medium (B104 CM), 1% N2 supplement (Gibco), 5 g/mL insulin (Sigma), and 0.5 µg/mL PLO + FN. The substrates were diluted in phosphate-buffered saline (PBS, Hyclone, Logan, UT, USA) at the following concentrations: PDL = 50 µg/mL, LN (P-L) = 20 µg/mL, PLO + FN = 10 µg/mL (FN). OPC culture was performed as described in Niu et al. (2012). Briefly, hypothermic anesthesia was performed on neonatal mice at postnatal days (P) 0–2. Mice were placed on ice with latex gloves as compartments. They became completely unconscious after 10 minutes. Then, they were quickly decapitated. Cortices were rapidly isolated, triturated, and filtered to form single-cell suspension, which was resuspended in mixed cell-medium (MCM) that comprised Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Colaba, CA, USA), 10% fetal bovine serum, and penicillin-streptomycin (Gibco, Grand Island, NY, USA). Then, the mixed glial cells in MCM were added to the coated plates and kept in culture. After 3 days, the MCM was changed in re-culture medium. When cells grew to 85–90% confluence, the MCM was replaced with modified OPC growth-medium (MOGM), which comprised DMEM/F12, 15% rat neuroblastoma conditioned medium (B104 CM), 1% N2 supplement (Gibco), 5 g/mL insulin (Sigma), and 0.5 µg/mL PLO + FN. OPCs were cultured in high-density, and purity typically reached 98% after two rounds of purification. Then, to induce OPC differentiation, the MOGM was replaced with OPC differentiation-medium (ODM), which comprised DMEM/F12, triiodo-l-thyronine (15 nM, Sigma), 1% N2 supplement, 0.2% B27 supplement (Gibco), and penicillin-streptomycin. Cells were collected at various time points for analysis (Figure 1).

Figure 1 | The flow chart.

LPC: Lysolcholein; MCM: mixed cell-medium; MOGM: modified oligodendrocyte precursor cell growth-medium; ODM: oligodendrocyte precursor cell differentiation-medium; OPC: oligodendrocyte precursor cells; PDL: poly-D-lysin; PLO: poly-l-ornithine.

Image analysis and quantification

Fractal dimension analysis

Fractal dimension (D) analysis has been used to assess the cell morphology (Behar, 2001; Fernández and Jelinek, 2001). D values of cells with simple morphology were marked as 1 while those of cells with complex morphology (highly branched or having bi-dimensional planar structure) were valued near 2. The formula for calculating the fractal dimension is as follows:

$$D = \log N / \log (1/\epsilon)$$

where N is the number of boxes of size ε that contain the object. The fractal box count was used to obtain the D value. For every independent experiment, at least 10 cells were analyzed for each group.

Quantification of fluorescence images

Images were captured with a confocal microscope with DAPI-Fluorogum (G Southern Biotech, Birmingham, AL, USA) having an aqueous final step, while manual counting was carried out for 2,3-cyclic nucleotide 3’-phosphodiesterase positive (CNP) or myelin basic protein positive (MBP) cells. Cells were classified according to morphology. MBPs were cells exhibiting interconnected with each other forming a structure similar to a ring; (3) non-interconnected for cells with branched structures; (4) ring-like structure, for cells whose branches were interconnected with each other forming a structure similar to a ring; (3) non-interconnected for cells that had membranous regions (at least one); and (5) myelin sheaths, for cells with a fully planar membrane.

For quantitative analysis, images were converted to 8-bit TIFF files. Cells were outlined and analyzed by region of interest (ROI) measurement. The fluorescence intensity of the signal area and corrected total cell fluorescence were measured according to previously described methods (Fischer et al., 2014; Lourenço et al., 2016). For each independent experiment, at least 10 cells were measured from each group.

Western blot assay

Western blot assay was used to examine the protein levels in OLs treated with different substrates. Radio immunoprecipitation assay-lysis buffer (Beyotime, Shanghai, China) was used for protein extraction. A cDNA Synthesis kit (Thermo Fisher, Waltham, MA, USA) was used for reverse transcription. The following protocol was used for real-time PCR: 95°C for 30 seconds (denaturation) and 40 cycles of 95°C for 10 seconds (elongation). GAPDH was used as the internal control. The 2^(-∆∆Ct) method was used for analysis (Schmittgen and Livak, 2008). Primers were designed on the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) and synthesized by Tsingke Bio (Beijing, China). The sequences of the primers were as follows: G-3′, reverse: 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′, forward: 5′-TAC CTG GCC ACA GCA AGT AC-3′, reverse: 5′-GTC ACA ATG TTC GTC TCT GCG CTG AT-3′, reverse: 5′-AAG TGG CAG CAA TCA TGA AGG-3′; GAPDH forward: 5′-TAC CTG GCC ACA GCA AGT AC-3′, reverse: 5′-GTC ACA ATG TTC GTC TCT GCG CTG AT-3′, reverse: 5′-AAG TGG CAG CAA TCA TGA AGG-3′.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed to examine the expression levels of myelin-related genes and integrin genes in OLs treated with different substrates. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction from cells. A cDNA Synthesis kit (Thermo Fisher, Waltham, MA, USA) was used for reverse transcription into complementary DNA. Real-time PCR (RT-PCR) was performed on a CFX 96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The following protocol was used for RT-PCR: 95°C for 30 seconds (denaturation); 55–5°C or 53°C for 30 seconds (annealing); and 72°C for 1 minute (elongation). PCR products were identified using agarose gel electrophoresis. The method was adopted for analysis (Schmittgen and Livak, 2008). Primers were designed on the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) and synthesized by Tsingke Bio (Beijing, China). The sequences of the primers were as follows: G-3′, reverse: 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′, forward: 5′-TAC CTG GCC ACA GCA AGT AC-3′, reverse: 5′-GTC ACA ATG TTC GTC TCT GCG CTG AT-3′, reverse: 5′-AAG TGG CAG CAA TCA TGA AGG-3′.
Lysoctehin-induced animal model of demyelination

Lysoctehin (LPC, Sigma) was used to induce spinal cord demyelination in 8-week-old male C57BL/6j mice. Mice were randomly designated to LPC + or saline + groups. LPC was injected into the spinal cord (one injection with 1.25% tribromoethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) (0.02 L/kg body weight) was used for anesthesia. Tissues over the thoracic (T9–T12) vertebrae were separated. The dura at level T10 was removed. Two holes were made on the metal needle (31 gauge, FL, OK), and mice were perfused with 0.1 M PBS and allowed to move freely. The effective gait was evaluated under the walkway once or twice for habituation. Mice were then tested individually and allowed to recover freely. The walkway was placed on a glass channel. The regularity index equaled the number of normal step-sequence patterns divided by the total number of paw placements. The base of support equaled the average width between the hind paws.

Results

FN promotes OL precursor cell proliferation

In the first experiment, we studied how FN affects OPC proliferation. Cells were isolated from P0–2 mice and purified to over 98% OPC purity. Our data showed that the percentage of OLGs did not depend on the coating substrate (LPC, FN, PLO, PDL) (Figure 1A). Immunofluorescence staining showed that the total number of cells was significantly greater in FN-coated plates than in PDL-coated plates (Figure 2A and B). Immunofluorescence staining showed that the total number of NPCs (NG2 cells) was significantly greater in FN-coated plates than in PDL-coated plates (Figure 2C and D). Furthermore, the percentage of proliferating OPCs (K67* NG2 cells/NG2 cells) was significantly higher in FN-coated plates than in PDL-coated plates (Figure 2E and F). These results indicated that FN promotes OPC proliferation.

FN inhibits OL differentiation

Cultured OPCs can be induced to differentiate in vitro, following a similar time course to that observed in vivo (PLO, 8 weeks; PDL, 1 week; Liquid Media, 1999; Warner et al., 2002; Emery, 2010). During this process, the cell morphology undergoes a series of changes. In the initial stage, cell shape changes from mostly bipolar to multi-process. Next, cell processes connect to form a ring-like structure, and then a double-ring structure. Then, they fuse into a multinucleate structure which change into flat myelin sheets (Brad and Lassmann, 2010; Tiane et al., 2019; Figure 3A). During this in vitro differentiation process, the cells express the same markers with the same timing as they do in vivo (Figure 3A). On day 1 in differentiation medium, the number of CNP+ (a marker for early OPC differentiation; Fressinvaux and Eyer, 2013) cells appeared fewer and cell processes were less elaborate among OPCs cultured on FN-coated plates than among those cultured on PDL-coated plates (Additional Figure 2A). The fractal dimension of the process (Figure 3B–D) was significantly higher in the FN group than in the PDL group, indicating more advanced differentiation in the FN group.

Statistical analysis

No statistical methods were used to predetermine sample sizes. However, our sample sizes are similar to those reported in previous publications (Lourenço et al., 2016; Lu et al., 2018). GraphPad Prism 9.0.0 software (GraphPad Software, La Jolla, CA, USA; www.graphpad.com) was used for statistical analysis. Data are shown as mean ± standard deviation (SD). The experimental animals were either littered or similar in terms of age and weight. For in vivo experiments, at least three animals were used for each group and one additional animal was used for each group. For in vitro experiments, a higher percentage of cells with membranous structures and myelin-sheet morphology. Consistent with these differences in cell morphology, MBP staining, and lower fluorescence intensity of the signal and corrected total cell fluorescence. Statistical analysis showed that these parameters were significantly lower in the FN group than in the PDL group (Additional Figure 2C and D). These results indicated that FN suppresses OL differentiation.
structural differences between the two conditions (Figure 4G and H). These results indicate that PLO promotes OL differentiation.

Next we tested whether PLO could block the inhibitory effect of FN on OL differentiation. We co-cultured the cells with plates treated with FN and PLO (PLO + FN). On the first day, differentiation of OLs in the PLO + FN group was significantly higher than that of cells in the control group (Figure 4A and B). On the third day, analysis showed that the proportion of cells with ring-like structures had risen and was significantly greater in the PLO + FN group (PLO + FN vs. FN) (Figure 4C and D). Similarly, CNP and MBP protein levels were also significantly higher in the PLO + FN group versus the control group (PLO + FN vs. FN) (Figure 4E and F). On the fifth day, the cells in the PLO + FN group showed mature morphologies with membrane and myelin structures visible in all cells (Figure 4G). Statistical analysis showed that on this day, the proportion of cells with ring-like structures and myelin structures was significantly higher in the PLO + FN group than in the control group, while the proportion of cells with non-membranous structures was drastically lower (Figure 4H). At this point, the proportion of cells with ring-like structures and myelin structures was significantly higher in the PLO + FN group than in the control group (Figure 4I and J). These results indicated that PLO abolished the inhibitory effects of FN on OL differentiation. We also tested the influence of PLO on OPC proliferation, finding that it did not alter the amount of proliferation (PDL vs. PLO, P > 0.05). Furthermore, FN-induced increases in OPC proliferation also remained unchanged by the addition of PLO (FN vs. FN + PLO, P > 0.05) (Figure 5A and B). The levels of the differentiation marker CC1 were higher in the PLO group than in the control group (Figure 5C). These results suggest that PLO does not interfere with the FN-induced OPC proliferation.

In summary, our results suggest that PLO blocks the inhibitory effects of FN on OL differentiation without affecting its ability to facilitate OPC proliferation. PLO and FN regulate OL development via ERK and PI3K/Akt pathways. FN binds to integrin receptors in the cell membrane. Previous studies have reported that FN recognizes multiple integrin subunits, among which αvβ3 and αvβ5 are expressed on OL lineage cells (Akhtar et al., 2016; Bharadwaj et al., 2017; Suzuki et al., 2019; Wirth et al., 2020; Figure 6A). The expression levels of different integrin mRNAs during the OL development. β3 levels were high in OPCs and decreased as they differentiated into OLs. β1 rose during early differentiation (day 1) and remained in high levels during later differentiation phase (day 3). β5 levels kept increasing throughout differentiation (Figure 6B). These results indicate that OLs express different integrin subtypes during development. Thus, binding to different integrin receptors, FN can have multiple different effects during OL development.

We further investigated intracellular signaling molecules that transmit signals from membrane receptors during OL development. In the proliferative phase, the expression levels of P-Akt and P-S6K were markedly higher in OPCs of the FN and PLO + FN groups (Figure 6C and D) than in the OPCs of the PDL group, suggesting that FN promotes the proliferation of OPCs at least partially via the Akt signaling pathway. Analysis also indicated that PLO had no effect on P-Akt/P-S6K signaling during the proliferation stage. At the early stage of OL differentiation, the expression levels of P-Akt and P-S6K in OPCs were significantly lower in the FN group than in the PDL (control) group, and significantly higher in the PLO and PLO + FN groups (Figure 6E and F). At later stages, P-Erk1/2 levels did not differ among the four groups. These data suggest that FN can facilitate OL differentiation by decreasing ERK1/2 phosphorylation during early differentiation, while PLO can block this inhibition by facilitating Erk1/2 phosphorylation. At later stages of OL differentiation, levels of P-Akt (Ser473) and P-CREB were significantly lower in the FN group vs. the PDL group and significantly higher in the PLO and PLO + FN groups. Expression levels of P-ASK3 and P-Erk1/2 were not significantly different among groups at any stage of differentiation (Figure 6G and H). These results suggest that FN cannot inhibit OL differentiation by decreasing Akt and CREB phosphorylation, which can be negated by PLO-induced phosphorylation of Akt and CREB.

PLO promotes remyelination and motor function recovery after LPC-induced demyelinating injury

Thus far, we have shown that in culture, PLO can block FN-regulated inhibition of OL differentiation while sparing OPC proliferation. To evaluate the practical potential of PLO as a therapeutic agent for diseases of demyelination, we used an experimental model of lipid-induced demyelinating disease. This groups were used in the culture studies. Local injection of LPC into the spinal cord causes selective and focal myelin loss in the ventrolateral white matter (Torrence et al., 2020; Figure 7A). Fourteen days after injection, which is during active remyelination and OPC proliferation, we observed that LPC-induced demyelination was significantly higher in the PLO-treated group than in controls (LPC and LPC + PDL). These results were confirmed by immunostaining with CC1 and MBP antibodies, showing a higher density of CC1+ mature OLs and a significantly enlarged area of MBP protein expression following LPC treatment (Figure 7D and E). We also used eriochrome cyanine staining for myelin, which revealed that the area of demyelination was significantly lower following PLO treatment than after the control treatments (Figure 7F and G). We examined astrocyte and microglial response in LPC-treated demyelinated regions (injury and found no significant differences between groups in the numbers of iba1+ microglia/macrophages or GFAP+ astrocytes (Figure 7H and I).

We next investigated the functional recovery of the modal mice using the Catwalk system. Demyelination of the spinal cord in the lower thoracic region affects hindlimb function, as the descending and ascending fibers play critical roles in proprioception and coordination. In our study, we observed significant improvements in motor function in LPC-treated mice relative to the control group 14 days after surgery (Figure 7J). We also assessed recovery using the regularity index, a fractional measure of inter-paw coordination (Garrick et al., 2021). This index was significantly higher in PLO-treated mice than in control mice (Figure 7K), indicating better coordination. Furthermore, our results showed that PLO-treated animals exhibited expedited functional recovery, suggesting that PLO promotes functional recovery of LPC-induced demyelinating injury.

We next explored how dosage affects PLO-facilitated myelin repair. At a low concentration (20 µg/mL), PLO helped to reduce the size of the demyelinated area (Figure 7A–C). At a median dose (500 µg/mL), PLO exhibited an effect on promoting myelin repair. Compared with controls, at this dosage, the area of myelin damage was greatly reduced and the number of CC1+ OLs was significantly higher in the damaged area (Figure 8A–D). However, at the high PLO concentration of 500 µg/mL, the effect was the opposite; the size of the damaged area was larger and CC1+ cell density was lower (Figure 8A–D) compared with the control. Thus, our data suggests that high levels of PLO will worsen myelin repair and wound recovery.

Discussion

In this study, we found that FN plays dual roles during OL development: it promotes OPC proliferation and inhibits OL differentiation. This is consistent with the finding that FN increases during the early stage of remyelination and is downregulated soon afterward (Zhao et al., 2009; Steffels et al., 2013). During remyelination, FN is believed to be secreted by the OPCs of the OPC pool (i.e., the number of potential OLs). However, FN becomes detrimental as time goes on because it suppresses OL differentiation and thus the formation of myelin sheets. For successful remyelination to be successful, it must be removed. Studies in mouse and other lab models have shown that FN mediates the proliferation of OPCs in vitro (Steffels et al., 2015), and hinders morphological differentiation of OLs (Sisková et al., 2006, 2009; Qin et al., 2017). In addition, injection of astrocyte-derived FN into demyelinating lesions results in reduced OL differentiation and remyelination (Steffels et al., 2013).

Many of the biological functions of FN rely on integrins, which have multiple family members. Different ECM proteins, including FN, trigger intracellular signaling cascades by binding to integrins (van der Flier and Sonnenberg, 2001; Labrat-Boyer, 2012). Among FN’s integrin receptors, only the αvβ1/β3/5 subunits are expressed in OLs (Sisková et al., 2016; Barros et al., 2009; Friedland et al., 2009). Our data showed that these integrins have different temporal expression patterns during OL development. The β3 subunit was highly expressed in OPCs and was downregulated during differentiation. The β1/β5 subunits were more highly expressed during differentiation than proliferation, with β1 levels being highest in the early stages of differentiation and β5 levels being highest in the late stages. The temporal expression pattern of the β1/β5 integrins was quite similar to that of integrins affect OL survival (Mazaheri et al., 2018), migration (Zhu et al., 2016; Suzuki et al., 2019), and differentiation (Quiñela-López et al., 2019). FN has been reported to bind to integrin β1 on the cell membrane of OLs. This is thought to be that FN activates the integrin signaling pathway (van der Flier and Sonnenberg, 2001). In later stages of differentiation, β3 levels were high in OLs and decreased as they differentiated into OLs, which would also inhibit differentiation.

For clinical remyelination, the best-case scenario is a treatment that keeps the OPC proliferative property of FN while at the same time blocks its ability to inhibit OL differentiation. We found that PLO would be an ideal candidate for this treatment. PLO is a positively charged synthetic amino acid polymer that is widely used as a coating substrate (Sato et al., 2018; Hosseini Farahabadi et al., 2020). It has been used successfully for the attachment and study of neural cell differentiation and outgrowth. For example, PLO enhances the migration of neuronal stem/progenitor cells by promoting the binding of α-actin 4 to actin filaments (Ge et al., 2016). Furthermore, PLO promotes the preferential differentiation of neuronal stem/progenitor cells into neurons and astrocytes (Ge et al., 2016). In vitro, PLO expression is expressed in laminin coating substrates (Hosseini Farahabadi et al., 2020; Setien et al., 2020). For example, the combination of PLO and laminin coating promoted neuronal arborization and maturation (Setien et al., 2020), and PLO with FN has also been used for neuronal stem cells (Lewicka et al., 2012).

In our in vitro study, we combined PLO with FN and observed enhanced OPC proliferation (which results in increased OL differentiation) and in a study that was performed in an LPC-induced demyelination/remyelination mouse model, PLO promoted myelin repair and motor function recovery.
**Research Article**

PLO blocks the negative effects of FN on oligodendrocyte differentiation and promotes oligodendrocyte maturation.

**Figure 1** FN promotes the proliferation of NG2+ cells.
(A) Representative immunofluorescence images of OPCs plated on glass coverslips coated with FN or PDL and cultured for 2 days in proliferation medium. The number of cells was significantly higher in the FN group than in the PDL group. Scale bar: 100 µm. (B) Quantitative analysis of A. Data were from three independent experiments. (C) Representative immunofluorescence images of OPCs stained for NG2 (an OPC marker, red, stained with Alexa Fluor555), Ki67 (a proliferating-cell marker, green, stained with Alexa Fluor488), DAPI (blue, nucleus staining), cultured for 2 days in proliferation medium. Compared with the PDL group, the number of NG2+ cells and the proportion of Ki67+ cells in NG2+ cells were significantly higher in the FN group. Scale bar: 50 µm. (D) Quantitative analysis of NG2+ cells in C. (E) Quantitative analysis of proliferating OPCs (Ki67+ NG2+ cells/NG2+ cells) in the different treatment groups. Data are expressed as mean ± SD from at least three independent experiments. **P < 0.01, ***P < 0.001 (Student's t-test). DAPI: 4′,6-diamidino-2-phenylindole; FN: fibronectin; NG2: nerve-glia antigen 2; OPC: oligodendrocyte precursor cell; PDL: poly-D-lysine; PM: proliferation medium.

**Figure 2** FN suppresses the differentiation of oligodendrocytes.
(A) The diagram illustrates changes in cell morphology revealed by typical markers during oligodendrocyte differentiation in vitro. OPCs cultured for 2 days in proliferation medium (2 d PM) were stained with NG2 antibody (red, stained with Alexa Fluor555). CNP antibody was used after 1 and 3 days in the differentiation medium (1/3 d DM). MBP antibody was used to mark OLs cultured for 5 days in the differentiation medium (5 d DM). (B) Representative immunofluorescence images of OLs stained for Olig2 (green, stained with Alexa Fluor488) and CNP (red, stained with Alexa Fluor555), cultured for 3 days in differentiation medium. Arrow point to cells with ring-like structure. The proportion of OLs with ring-like structure was significantly lower in the FN group than in the PDL group. (C) Quantification of the percentage of cells in B having ring-like structure or non-ring-like structure. (D) Quantification of mRNA expression levels (normalized by the PDL group) of MBP and CNP from OPCs cultured for 3 days in differentiation medium. (E) Quantification of the protein expression levels (normalized by the PDL group) of MBP and CNP from OPCs cultured for 3 days in differentiation medium. Data were collected from three independent experiments. **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by Tukey’s multiple comparison test). CNP: 2′,3′-Cyclic nucleotide 3′-phosphodiesterase; DM: differentiation medium; FN: fibronectin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MBP: myelin basic protein; NG2: nerve-glia antigen 2; Olig2: oligodendrocyte transcription factor 2; OPC: oligodendrocyte precursor cell; PDL: poly-D-lysine; PM: proliferation medium.

**Figure 3** PLO blocks the negative effects of FN on oligodendrocyte differentiation and promotes oligodendrocyte maturation.
(A) Representative immunofluorescence images of OLs (stained with Alexa Fluor555) cultured for 1 day in differentiation medium (1 d DM) under different coating conditions. Compared with the PDL group, OLs in the FN group exhibited less ring-like structure, while those in the PLO + FN group exhibited more ring-like structure. (B) Quantification of the ratios of cells in A with/without ring-like structure. Data were collected from three independent experiments. (C) Representative western blots of MBP and CNP proteins from OPCs cultured for 3 days in differentiation medium. (D) Quantification of the protein expression levels (normalized by the PDL group) of MBP and CNP from E. (E) Representative immunofluorescence images of OLs stained for Olig2 (green, stained with Alexa Fluor488) and MBP (red, stained with Alexa Fluor555), cultured for 5 days in differentiation medium. The OLs in the FN group exhibited less membranous and myelin-sheet structures than did those in the PDL group. Scale bars: 50 µm (A), 100 µm (B, G). (H) Quantification of G, showing the percentages of cells bearing non-membranous, membranous, or myelin sheet structures. Data are expressed as mean ± SD from three independent experiments. **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by Tukey’s multiple comparison test). CNP: 2′,3′-Cyclic nucleotide 3′-phosphodiesterase; DM: differentiation medium; FN: fibronectin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MBP: myelin basic protein; NG2: nerve-glia antigen 2; Olig2: oligodendrocyte transcription factor 2; OPC: oligodendrocyte precursor cell; PDL: poly-D-lysine; PM: proliferation medium.

**Figure 4** PLO blocks the negative effects of FN on oligodendrocyte differentiation and promotes oligodendrocyte maturation.
(A) Representative immunofluorescence images of OLs (stained with Alexa Fluor555) cultured for 1 day in differentiation medium (1 d DM) under different coating conditions. After treatment with the PLO and FN combination, OLs showed more complex morphology than those in the PDL or FN groups. (B) Quantitative analysis of D values on cells in A. (C) Representative immunofluorescence images of OLs stained for Olig2 (green, stained with Alexa Fluor488), and CNP (red, stained with Alexa Fluor555) after being cultured for 3 days in differentiation medium (3 d DM) under different coating conditions. Compared with the PDL group, OLs in the FN group exhibited less ring-like structure, while those in the PLO + FN group exhibited more ring-like structure. (D) Quantification of the ratios of cells in A with/without ring-like structure. Data were collected from three independent experiments. (E) Representative western blots of MBP and CNP proteins from oligodendrocytes cultured for 3 days in differentiation medium under different coating conditions. (F) Quantification of G, normalizing the PDL group data from at least three independent experiments. (G) Representative images of OLs stained for Olig2 (green, stained with Alexa Fluor488) and MBP (red, stained with Alexa Fluor555), cultured for 5 days in differentiation medium (5 d DM) under different coating conditions. Arrow 1: cells with non-membranous structure; arrow 2: cells with membranous structure; arrow 3: cells with myelin sheets. Compared with the PDL group, OLs in the FN group exhibited less membranous structure and no myelin sheets, while those in the PLO + FN group exhibited more membranous structure and myelin sheets. Scale bars: 50 µm in A, 100 µm in C and G. (H) Quantification of the percentages of cells having non-membranous/membranous/myelin sheet structures, as revealed by MBP immunostaining in E. Data are expressed as mean ± SD of at least 10 cells from each of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by Tukey’s multiple comparison test). CNP: 2′,3′-Cyclic nucleotide 3′-phosphodiesterase; DAPI: 4′,6-diamidino-2-phenylindole; DM: differentiation medium; FN: fibronectin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MBP: myelin basic protein; NG2: nerve-glia antigen 2; OL: oligodendrocyte; Olig2: oligodendrocyte transcription factor 2; PDL: poly-D-lysine; PL0: poly-L-ornithine.

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PLO has no significant effects on OPC proliferation and does not interfere with the proliferative effect of FN.

Erk1/2 and FN function through ERK and PI3K/Akt signaling pathways.

We speculate that PLO might work through lipid rafts on the cell membrane.

PLO might function by altering the downstream intracellular signaling pathways mediated by FN. Studies have shown a close correlation between integrin signals and lipid rafts in the membrane (Decker et al., 2004). Lipid rafts and integrin activation has been reported to regulate OL survival through the PI3K/Akt signaling pathway (Baron et al., 2003; Decker and ffrench-Constant, 2004). Lipid rafts are rich in cholesterol, sphingolipids and related proteins, which are generally considered as micro-domains on the cell membrane (Lingwood and Simons, 2010; Levental et al., 2020).

The animal model of demyelination revealed the potential therapeutic value of PLO. After treatment with PLO, the mice with LPC-induced demyelination showed a much better recovery. Histological changes were obvious and indicated quicker and better remyelination. Functional changes were less obvious but still significant. We also found that the effect of PLO on remyelination was dosage-dependent. While beneficial at low and medium dosages, the high dosage was detrimental. Excessive PLO might disrupt the integrin signals (Mezu-Ndubuisi and Maheshwari, 2021) or other components of the ECM, such as fibulin-2 (a glycoprotein in the ECM; Schaeffer et al., 2018), which can participate in the remodeling of the ECM within the glial scar. PLO is clinically safe and commonly used to make alginic microcapsule systems for drug delivery and cell transplantation (Wang et al., 2018a; Vong et al., 2019; Somor et al., 2020; Xu et al., 2022). The current study only tested the effect of PLO in one animal model of demyelination. In the future, we would like to test it in other animal models to better evaluate its clinical potential.
R.LPC+PDL

**LPC**

**LPC**

**LPC+PDL**

**LPC+PLO**

**LPC+20 μg/mL**

**LPC+PLO**

**LPC**

**PLO**

**LPC+PLO**

**LPC+PLO**

**LPC**

**PLO**

**LPC+PLO**

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**LPC+PDL**

**LPC+PLO**

**LPC+PLO**

**LPC**

PLO promotes functional myelin repair after 14 days of lysolecithin-induced demyelination injury. (B) RNA in situ hybridization for MBP and PLP1 on spinal cords with different treatments. The white dashed lines mark off the lesion area. PLP + “MBP” Ols were significantly greater in the PLO-treated group. Scale bar: 50 μm. (C) Quantification of PLP + “MBP” Ols in the lesion area with different treatments as shown in B (n = 3). (D) Representative MBP (red, stained with Alexa Fluor555) and CC1 (red, stained with Alexa Fluor555) immunofluorescence staining. Compared with control groups, MBP-stained areas and the number of CC1 positive cells were significantly greater in the PLO-treated group. The white dashed lines mark off areas of increased cell density (as shown by DAPI staining) representing the location of PLO demyelination. Scale bar: 50 μm. (E) Quantification of CC1 + “MBP” Ols in C (n = 3). (F) EC staining showing myelin in the spinal cord after different treatments. The area of demyelination was significantly lower in the PLO-treated group than in the control groups. The white dashed lines mark off the demyelinated area. Scale bar: 100 μm. (G) Quantification of the size of the demyelinated region in F. Three animals were used for each group (n = 3). (H) Representative immunostaining for astrocytes [GFAP + “Iba1”] and microglia (“Iba1” cell, green, stained with Alexa Fluor488) in lesion areas 14 days after injury. The number of astrocytes or microglia in the PLO-treated group was similar to that in the control groups. The white dashed lines mark off the lesion area. Scale bar: 50 μm. (I) Quantification of GFAP + “Iba1” cells in H. Three animals were used for each group (n = 3). (J) Scores for “base of support” were compared among different groups one day before surgery and 14 days after surgery (n = 5). (K) Regularity index values for the different groups (n = 4). All data are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 (Student’s t-test was used to compare two groups, and one-way analysis of variance followed by Tukey’s multiple comparison test was performed for comparisons of three or more groups). CC1: Adenomatous polyposis coli; DAPI: 4′,6-diamidino-2-phenylindole; dpl: days post-lesion; EC: eriochrome cyanine; GFAP: glial fibrillary acidic protein; Iba-1: ionized calcium binding adaptor molecule 1; LPC: lysolecithin; MBP: myelin basic protein; PDL: poly-D-lysine; PLO: poly-L-ornithine; PLP: proteolipid protein.

In conclusion, our study confirmed that FN both promotes OPC proliferation and inhibits OL differentiation. Pathologically accumulated FN as observed in multiple sclerosis could thus contribute to remyelination failure. Our studies further showed that PLO could block the negative effect of FN on OL differentiation, while maintaining the benefits of OPC proliferation. It will be highly worthwhile to extend the current study to test the medical potential of PLO.

**Author contributions:** Study design: HF, JP; demyelination animal model establishment: PYH, ZH; other experiment implementation: YIX, SHS; data analysis: SHS; manuscript draft: HF, JP, YIX. All authors approved the final version of the manuscript.

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**Availability of data and materials:** All data generated or analyzed during this study are included in this published article and its supplementary information files.

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**Additional files:**

**Additional Figure 2:** Different coating substrates do not affect the percentage of OPCs in culture.

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