Fibulin 5, a human Wharton's jelly-derived mesenchymal stem cells-secreted paracrine factor, attenuates peripheral nervous system myelination defects through the Integrin-RAC1 signaling axis

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Abstract
In the peripheral nervous system (PNS), proper development of Schwann cells (SCs) contributing to axonal myelination is critical for neuronal function. Impairments of SCs or neuronal axons give rise to several myelin-related disorders, including dysmyelinating and demyelinating diseases. Pathological mechanisms, however, have been understood at the elementary level and targeted therapeutics has remained undeveloped. Here, we identify Fibulin 5 (FBLN5), an extracellular matrix (ECM) protein, as a key paracrine factor of human Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) to control the development of SCs. We show that co-culture with WJ-MSCs or treatment of recombinant FBLN5 promotes the proliferation of SCs through ERK activation, whereas FBLN5-depleted WJ-MSCs do not. We further reveal that during myelination of SCs, FBLN5 binds to Integrin and modulates actin remodeling, such as the formation of lamellipodia and filopodia, through RAC1 activity. Finally, we show that FBLN5 effectively restores the myelination defects of SCs in the zebrafish model of Charcot-Marie-Tooth (CMT) type 1, a representative demyelinating disease. Overall, our data propose human WJ-MSCs or FBLN5 protein as a potential treatment for myelin-related diseases, including CMT.

KEYWORDS
actin remodeling, Charcot-Marie-Tooth disease, Fibulin 5, mesenchymal stem cell, Schwann cell myelination

So Yeon Won and Soojin Kwon contributed equally to this study.

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1 | INTRODUCTION

The myelin sheaths of axons in the vertebrate nervous system are important for the fast and proper transmission of nerve impulses, and for the axonal homeostasis and survival.\(^1,2\) Myelin is a lipoprotein structure produced by specific types of cells: Schwann cells (SCs) in the PNS and oligodendrocytes in the central nervous system. In the PNS, myelination occurs as the SCs form myelins from multiple layers of the membrane and enwrap axons.\(^3,4\) Thus, impairment of SCs or neuronal axons results in severe myelin-related disorders, referred to as dysmyelinating and demyelinating diseases.\(^5,6\)

CMT disease is one of the most common inherited neuropathies, with a prevalence of 1:2500 worldwide.\(^7,8\) CMTs are genetically heterogeneous and mainly associated with mutations in genes that encode proteins functioning in neurons or SCs.\(^9,10\) The disease includes numerous subtypes (types 1 through 7 and X-linked forms), with common symptoms being distal weakness and muscle atrophy in the early state, foot deformities and hand atrophy in the late state.\(^11,12\) CMT type 1 is the most common type and majority of the causative genes are involved in various cellular functions such as endosomal sorting, mitochondrial dynamics, basal membrane adhesion, cytoskeleton stability, myelin formation in SCs.\(^13,14\)

Although persistent identification of CMT causative genes has accelerated understanding of the genetic basis and the cellular function underpinning disease mechanisms, no effective treatments for any type of CMTs have been developed. Thus, there has been a great need for new treatment strategies for CMTs, and cell-based therapies such as using mesenchymal stem cells (MSCs) has been considered as an alternative approach to treatment.\(^15,16\) This is because MSCs are effective in repairing damaged cells by promoting cell proliferation or preventing cell death in various types of cells.\(^17,18\) Indeed, previous clinical trials have reported that human-derived MSCs can be applied safely and efficiently to cure neurological diseases.\(^19,20\) The therapeutic effects of MSCs are mainly due to paracrine factors acting on disease-related targets or adjacent cells. Recent studies supporting this have identified significant proteins among paracrine factors by proteomic analysis, and have demonstrated that recombinant proteins have therapeutic effects comparable to MSCs in several disease model systems.\(^21,22\) From human MSCs, thus, uncovering key paracrine factors affecting SCs may be an alternative approach to developing targeted treatment of myelin-related diseases including CMTs.

In this study, we identified FBLN5 as a crucial paracrine factor from human Wharton’s jelly-derived mesenchymal stem cells (WJ-MSCs) that affect the development of SCs. The secreted FBLN5 plays as a composite of the extracellular matrix (ECM) molecules in various types of cells including neurons, SCs, endothelial cells, fibroblasts, and smooth muscle cells.\(^23\) The cell-matrix communication is important in diverse cellular processes, such as organogenesis and angiogenesis, particularly the ECM of SCs is critical for the proliferation and myelination.\(^24,25\) Previously, a mutation in FBLN5 (c.1117C>T) was identified in CMT type 1, and the effect of mutation on the conductivities of neurons and muscles was reported.\(^26,27\) However, the molecular mechanism of FBLN5 in CMT pathology has been veiled to date.

Here, we have investigated role of FBLN5 in the developmental processes of SCs including proliferation and myelination, and revealed that the tripeptide Arg-Gly-Asp (RGD) motif of FBLN5, highly conserved across species, is necessary for Rac Family Small GTPase 1 (RAC1) activation through binding to Integrin. In addition, we demonstrated the efficiency of FBLN5 in the recovery of defective SC myelination in a CMT type 1 zebrafish model. We hence suggest that WJ-MSCs or FBLN5 may be a potential therapeutic target for myelin-associated diseases such as CMTs.

2 | RESULTS

2.1 | Identification of FBLN5 from human WJ-MSCs affecting the proliferation of SCs

To determine the effects of WJ-MSCs on SC development, S16 cells derived from the rat sciatic nerves and utilized as an immortalized SC line\(^28,29\) were co-cultivated with human WJ-MSCs using a transwell culture system (Figure 1A). S16 cells express less myelin-related proteins including glycoprotein and galactocerebroside than the in vivo system,\(^28\) whereas the expression of myelinating SCs markers such as SOX10, S100β, peripheral myelin protein 22 (PMP22), and myelin protein zero (MPZ) is comparable to the in vivo.\(^28–32\) Thus, S16 cells may not fully reflect the physiological properties of SCs, but their fundamental features are sufficient to be utilized to examine SC development in vitro. The proliferation of co-cultured S16 cells was compared with that of S16 cells cultured in the absence of WJ-MSCs by counting the number of cells 48 hours after cultivating. The number of S16 cells was increased in the presence of WJ-MSCs (Figure 1B,C), thereby suggesting that WJ-MSCs might affect the proliferation of SCs.

Next, to identify major paracrine molecules associated with the proliferation of SCs from the WJ-MSCs, antibody microarrays were performed in the co-culture system. The antibody array was designed to detect 507 human proteins using conditioned media collected from cell culture dishes. The data analyzed by duplicated arrays showed

**Significance statement**

No effective treatments for any type of Charcot-Marie-Tooth (CMTs) have been developed to date, thus this study considered mesenchymal stem cells-based therapies. FBLN5 was identified as a key paracrine factor, important for the proliferation of Schwann cells (SCs). The RGD motif of FBLN5 was revealed to be necessary for RAC1 activation through binding to Integrin. It was further found that the FBLN5-RAC1 molecular axis modulates actin remodeling, which is essential for SCs myelination in vivo utilizing zebrafish. Finally, the results of this study demonstrated the efficiency of FBLN5 in the recovery of CMT type 1 zebrafish model.
that cytokines containing several interleukin family members are mainly secreted from WJ-MSCs under co-cultivation with S16 cells (Figure 1D). As reported, cytokines are involved in the development of SCs; we have paid attention to proteins whose role in SCs is relatively unknown. Accordingly, FBLN5, LEFTY A, and PREF 1 were selected for further validation, and all three proteins revealed elevated secretion under co-culture with S16 cells (Figure 1E and Supplementary Figure 1). In particular, we focused on FBLN5 based on the identification of mutations in FBLN5 in CMT type 1 patients. Enzyme-linked immunosorbent assay (ELISA) for FBLN5 confirmed that WJ-MSCs cultured with S16 cells secreted more FBLN5 than the cells cultured alone (Figure 1F). The concentration of FBLN5 secreted from single cultured WJ-MSCs was 12.5 ± 0.99 pg/mL, while the concentration secreted under co-culture with S16 cells increased to 18.41 ± 0.26 pg/mL (Figure 1F).

To determine if FBLN5 is a primary regulator of SC proliferation, S16 cells were treated with recombinant FBLN5 protein in a dose-dependent manner followed by cell counting kit-8 (CCK-8) analysis (Figure 2A). The results revealed that 10 ng/mL of recombinant FBLN5 was sufficient to facilitate the proliferation of S16 cells.
Next, WJ-MSCs were transfected with two kinds of verified siRNAs for FBLN5 (Supplementary Figure 2) or with control siRNAs. Subsequently, the transfected cells were then co-cultured with S16 cells to examine the effect of the presence or absence of FBLN5 on SC proliferation. The S16 cells cultivated with FBLN5-depleted MSCs showed fewer cells than S16 cells cultured with control MSCs (Figure 2B,C). In order to clarify the role of FBLN5 in SC proliferation, 5-bromo-2′-deoxyuridine (BrdU) assay was performed in S16 cells with or without recombinant FBLN5. The assay data showed that the proliferation of FBLN5-treated S16 cells was more accelerated (Figure 2D,E). Given that the FBLN family proteins increase cell proliferation through the TGF-β/ERK/MAPK pathway in 3 T3-L1 cells, we determined whether FBLN5 is also involved in the proliferation of S16 cells though ERK activity. We performed immunoblot
assays for phosphorylated ERK1/2 in the recombinant FBLN5-treated S16 cells and revealed that FBLN5 is associated with the activation of ERK in SCs (Supplementary Figure 3). Furthermore, to assess the role of FBLN5 in cell migration, characteristic of SCs at the proliferation phase, an in vitro wound-healing assay was performed. For 48 hours after wound induction by scratching, the S16 cells exhibited approximately 20% closure of the wounds, whereas the cells co-cultured with WJ-MSCs or treated with recombinant FBLN5 exhibited approximately 2.5 times more closure extent of the controls (Supplementary Figure 4). Taken together, these data suggest that WJ-MSCs, which secrete several proteins including FBLN5, play an important role in the development of SCs and that the recombinant FBLN5 protein is sufficient to control the proliferation of SCs.

### 2.2 | FBLN5 is involved in the myelination of SCs

To establish the role of FBLN5 during SC development in vivo, we performed a loss-of-function study using a zebrafish model system. The Tg(claudin K:gal4-vp16;uas:egfp)38,39 zebrafish line was used to determine the effect of FBLN5 on the myelination of SCs.

**FIGURE 3**  FBLN5 is required for myelination of Schwann cells. A, A diagram of zebrafish larvae identifying the simplified peripheral nervous system, consisting of the spinal cord tracts, lateral lines, and the spinal motor nerves. A, anterior; P, posterior. B, Lateral view images of the whole-mounted Tg(claudin K:gal4-vp16;uas:egfp) zebrafish injected with control-, fbln5-MOs, or fbln5 mRNA after DAPI staining at 4 dpf. PLLn (green), posterior lateral line; A, anterior; P, posterior. OE, overexpression. Scale bars, 20 μm. C, Quantification of cell numbers counted within the PLLn of (B) images. Statistical significance was determined using one-way ANOVA followed by Dunnett’s post hoc test (*P < .05, **P < .005). D, Lateral view images of the Tg(claudin K:gal4-vp16;uas:egfp) zebrafish injected with control-MOs or fbln5-MOs at 5 dpf. The images within the rectangles are magnified in the bottom panels. SCT, spinal cord tracts; PLLn, posterior lateral line; A, anterior; P, posterior. Scale bars, 100 μm. E, Quantification of the relative intensity of PLLn in equivalent fields of view in the images of (D). Statistical significance was determined using the unpaired Student’s t-test with Welch’s correction (***P < .001). The data are shown as the mean ± SD of three independent experiments with ≥20 embryos per condition (C and E)
monitor SC development, in which myelinating SCs were labeled with enhanced GFP. We generated fbln5-knockdown zebrafish using verified translation-blocking morpholinos (MOs) (Supplementary Figure 5). Consistent with the results from S16 cells, in the zebrafish lateral line containing SCs, the lack of fbln5 led to a decrease in cell proliferation, whereas the exogenous fbln5 increased cell proliferation (Figure 3A-C). Thus, these data support that FBLN5 is essential for the proliferation of SCs. As confirming the effect of FBLN5 on cell proliferation in physiological states, we examined the potential role of FBLN5 in the myelination of SCs. The GFP signal indicating myelinating SCs was analyzed by comparison between Tg(claudin K:gal4-vp16;uas:egfp) zebrafish injected with control MOs-injected or fbln5 MOs-injected at 5 days postfertilization (dpf). The results showed that the knockdown of fbln5 results in myelination defects in the zebrafish PNS without gross morphological defects depending on the concentration of MOs (Figure 3D,E, Supplementary Figure 5A). In demyelinating disease, reduced conductivity in the nervous system is one of the pathophysiological symptoms. Thus, we examined the

**FIGURE 4** FBLN5 regulates Integrin-mediated actin dynamics in Schwann cells. A, Images of mouse primary Schwann cells double immunostained with Sox10 and F-actin antibodies after treatment with recombinant FBLN5 alone or with an integrin inhibitor (Integrin Ihb). PBS-treated Schwann cells were used as controls. Arrowheads and arrows indicate lamellipodia and filopodia, respectively. Scale bars, 20 μm. B-D, Quantification of axial cell length in (B), lamellipodia number/cell in (C), and lamellipodia length/cell in (D) from data examined using the ImageJ software within equivalent fields of view in the images of (A). The data are shown as the mean ± SD of three independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey’s post hoc test (*P < .05, **P < .005; ns, nonsignificant)
startle response in zebrafish injected with control MOs or fbln5 MOs and revealed that control zebrafish quickly responded to stimuli, while the fbln5 knockdown zebrafish rarely responded to stimuli (Supplementary Figure 6). Together, our data suggest that FBLN5 is crucial for the myelination of SCs and its function has been evolutionally conserved.

2.3 | FBLN5 regulates actin remodeling through RAC1 activation in SC myelination

Integrin plays an important role in several dynamics of SCs, such as cytoskeletal rearrangement, proliferation, and survival. Based on the structure of FBLN5 with RGD motif involved in Integrin
binding.\textsuperscript{23,41} we investigated the potential role of FBLN5 in cytoskeletal dynamics during SC myelination. First, primary SCs were isolated from the sciatic nerves of postnatal mice and myelinated in vitro. We confirmed the primary cells by immunostaining for Sox10, a molecular marker of SCs\textsuperscript{42} (Figure 4A). Consistent with the previous reports that Sox10 is a nucleocytoplasmic shuttling protein localizing in both the cytoplasm and nucleus,\textsuperscript{33,34} our data showed that Sox10 is expressed mainly in the nucleus of long-cultivated SCs (Supplementary Figure 7). Then, actin remodeling of SCs was analyzed after treatment with recombinant FBLN5 protein, because the protrusion of lamellipodia and filopodia is a prior process of myelination. The FBLN5-treated SCs gave rise to more and longer lamellipodia/filopodia protrusions than PBS-treated control SCs (Figure 4A-D). However, treatment of the Integrin inhibitor, RGD peptide, significantly repressed the enhancement of lamellipodia/filopodia generation by FBLN5 in the primary SCs (Figure 4A-D). Hence, these data suggest that FBLN5 plays a role in actin remodeling through Integrin-mediated signaling in SCs.

Next, we examined the involvement of RAC1 in an FBLN5-mediated mechanism that modulates actin remodeling of SCs, because RAC1 induces actin polymerization for extensive protrusion of cell membranes during myelination. The dorsal root ganglion (DRG) neurons were isolated from the mice spinal cord to induce myelination of the primary SCs by a co-cultivation system (Figure 5A). Then, the recombinant FBLN5 protein was treated 6 days after the co-cultivation, followed by immunostaining for the myelination marker myelin basic protein (MBP) (Figure 5A). FBLN5 promoted the myelination of primary SCs as much as that of the Laminin-treated controls SCs, whereas the Integrin inhibitor suppressed the reinforcement of FBLN5-dependent myelination (Figure 5B,C). Next, we generated the constitutive active form of mouse Rac1 (Q61L)\textsuperscript{43-47} and introduced it exogenously into the primary SCs. Notably, expression of Rac1 (Q61L) prevented the repression of SC myelination by the Integrin inhibitor (Figure 5B,C). We also produced a zebrafish fbln5 (ΔRGD) construct with a highly conserved RGD motif deleted, and assumed that its overexpression would interfere with Integrin-mediated RAC1 activation in zebrafish (Figure 5D). Remarkably, the Tg(claudin K: gag4-vp16:uas:egfp) zebrafish overexpressing the fbln5 (ΔRGD) construct showed drastically reduced myelination of SCs at 5 dpf (Figure 5E,F). Furthermore, we performed a pull-down assay to examine rac1 activation in zebrafish. We found that a lack of fbln5 decreased rac1 activation, whereas overexpression of fbln5 sufficiently activated rac1 in the fbln5-depleted zebrafish (Figure 5G). Finally, we monitored the extent of myelination in zebrafish to determine whether RAC1 activity is essential for the role of FBLN5 in SC myelination. First, we cross-sectioned the control MOs-injected or fbln5 MOs-injected zebrafish at 5 dpf and observed them using a transmission electron microscope (TEM). We examined the extent of myelination, focusing mainly on the Mauthner neuronal axons and the reticulospinal tracts, and found that the fbln5-deficient zebrafish had much less myelinated axons (Figure 5H-J and Supplementary Figure 8). Then, we produced a zebrafish rac1 (Q61L) and overexpressed the construct in the fbln5 MOs-injected zebrafish. The data revealed that expression of rac1 (Q61L) relieved the suppression of myelination in the fbln5-knockdown zebrafish PNS (Figure 5H-J). Taken together, our results suggest that activation of RAC1 through binding of FBLN5 with Integrin is essential for actin remodeling to modulate SC myelination.

\section*{2.4 | FBLN5 is effective in restoring the demyelinating peripheral neuropathy}

We next considered whether FBLN5 could be a potential treatment for demyelinating peripheral neuropathy, including CMT. It has been reported that duplication and mutation of the PMP22 gene is involved in CMT type 1.\textsuperscript{18,49} Thus, we have generated a CMT zebrafish model overexpressing zebrafish pmp22a and found that the CMT zebrafish have less myelination in the peripheral lateral line (Figure 6A,B). Then, we overexpressed fbln5 in the CMT zebrafish model and found that
the exogenous fbln5 rescues the myelination defects to normal levels (Figure 6A,B). Further TEM analysis revealed that the uncompact myelin sheaths of the Mauthner neuronal axons and reticulospinal tracts in the CMT zebrafish are significantly recovered by exogenous fbln5 (Figure 6C,D). Collectively, these results suggest that FBLN5 can be a potential target to treat demyelinating peripheral neuropathy, such as CMT.

3 | DISCUSSION

Here, we identified FBLN5 as a pivotal protein secreted from the WJ-MSCs that affected the development of SCs. We demonstrated that FBLN5 promoted SC proliferation in vitro and in vivo systems and further clarified that it was necessary for SC myelination. We found that FBLN5 bound to Integrin through the RGD motif and that the interaction was crucial for the activation of RAC1 to modulate actin remodeling (Figure 7). Notably, we revealed that recombinant FBLN5 protein effectively restored the myelination defects in the CMT zebrafish model generated by overexpressing the CMT type1 causative gene pmp22. Thus, these data imply that WJ-MSCs and the paracrine factor, FBLN5, can be applied as a targeted therapy for myelin-related disorders including CMTs.

Reportedly, FBLN5 is associated with the proliferation of several types of cells, such as 3T3L1 line and smooth muscle cells. In this study, we suggest that the proliferation of SCs is also controlled by FBLN5. Given that ERK activity is dependent on the concentration of recombinant FBLN5 (Supplementary Figure 3), we have postulated that ERK activation is critical for the FBLN5-mediated development of SCs, including proliferation and myelination. However, the
involvement of ERK signaling in the myelination processes of SCs is complex. For instance, sustained activation of the MEK–ERK pathway in SCs results in hypermyelination as well as a decrease in regenerative axons and loose density of intraepithelial nerve fibers. Therefore, the investigations to uncover the mechanism by which FBLN5 regulates ERK activation, and particularly to explore the role of FBLN5 in the ERK-dependent recovery of impaired SCs will be followed.

During the myelin sheathing of axons in the PNS, Integrin-mediated signal transductions in the ECM composed of Laminin, Collagen, and Fibronectin are important in SCs. This signaling is mainly involved in the cytoskeletal dynamics of SCs during myelination, but the molecular mechanism has not yet been understood. As we have identified one of the ECM molecules, FBLN5, as an important regulator of SC myelination, we suggest a novel molecular mechanism that modulates actin dynamics by supporting the importance of ECM signaling in the process. Our data revealed that the binding of FBLN5 to the Integrin receptors was essential for RAC1 activation to promote actin protrusion, such as lamellipodia/filopodia generation. It has been previously suggested that RAC1 signaling through Integrin β-1 is essential for the myelination of SCs. RAC1 activates the Arp2/3 complex and induces lamellipodia formation through actin branching at the membrane of SCs. In addition, PAK, an effector of RAC1, is also involved in the control of SCs myelination. PAK induces the expressions of Krox-20, a transcriptional enhancer of several myelination-related genes, and Galactocerebroside, a general marker of mature SCs, through activation of cAMP. The expression of Krox-20 is also regulated by YAP/TAZ in the myelination of SCs. Previous studies have reported that FBLN5 is associated with YAP/TAZ signaling in the proliferation and migration of the airway smooth muscle cells. Based on these data, we hypothesize that FBLN5 may be important for the regulation of genes critical for myelination such as Krox-20 and YAP/TAZ, and/or for the activation of actin-associated proteins such as Arp2/3 and PAK in the myelination of SCs. Thus, the relationship between FBLN5 and these molecules will be further investigated to better understand the FBLN5-mediated mechanism.

Of note, the actin dynamics of SCs is essential throughout the processes of myelination. In the initial stages of the myelination, actin polymerization is necessary for SCs to wrap and elongate the axons. Then, SCs express MBP and widely distribute them for myelin sheathing of axons by forcing actin depolymerization. Moreover, the role of molecules involved in actin polymerization/depolymerization...
during myelination is very complex. For instance, actin depolymerization-associated factors including Cofilin-1 and Gelsolin induce the initiation of SCs myelination, where actin polymerization-related factors such as Arp 2/3 do not appear to be critical for myelin wrapping. Intriguingly, in the oligodendrocytes, MBP is highly expressed during the depolymerization of actin, and its lack leads to the accumulation of actin filaments. In the present study, we showed that the treatment of recombinant FBLN5 protein induces the expression of MBP and subsequent actin protrusions in SCs. Thus, further investigations to better understand the role of FBLN5-mediated MBP and its regulatory mechanisms in actin dynamics will be followed.

In the process of demyelination that occurs due to the failure to remove the myelin debris resulting from damages of axons or SCs, actin polymerization is required for fragmentation of myelin sheaths. Accordingly, inhibition of RAC1 prevents actin polymerization, which leads to suppression of myelin fragmentation and subsequent axonal remyelination. Here, we showed that exogenous FBLN5 effectively rescues the myelination defects in the PNS of the CMT type 1 zebrafish model. Nevertheless, this study is limited to the function of FBLN5 in the early stages of axonal myelination such as SCs proliferation, migration, and myelin formation. Thus, further investigation is needed to demonstrate the involvement of FBLN5-RAC1 molecular axis in axonal demyelination and remyelination after injury in the PNS.

Several reports have suggested that the therapies using stem cells including MSCs are potential treatments for intractable diseases. Although MSCs have limitations such as difficulty in transplantation, including postimplantation immune rejection and possibility of becoming cancer cells, it is gaining attention as an alternative treatment because of the abilities to differentiate into various types of cells and to suppress inflammation-induced immune response. Notably, it has been suggested that the application of extracellular vesicles (EVs) of MSCs instead of the entire cells may be a direct treatment by overcoming these limitations. However, the key molecules released from the EVs of MSCs and the involved mechanisms have been poorly understood in many disorders. In this study, we have investigated the effect of human WJ-MSCs on the myelination of SCs and identified paracrine factors that play a crucial role in the proliferation, cytoskeletal rearrangement, and migration of SCs. Thus our study suggests that WJ-MSCs can be a potent treatment for the myelin-related peripheral neuropathy including CMTs.

4 | MATERIALS AND METHODS

4.1 | Cell culture

S16 cells (ATCC CRL-2941, American Type Culture Collection, Rockville, Maryland), the rat SC line, were cultured in Dulbecco’s Modified Eagle’s medium (Biowest S.A.S, Nuaille, France) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, California), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) in 5% CO2 at 37°C. Human WJ-MSCs were isolated according to the previously specified procedure and cultured in Alpha Minimum Essential Medium (Gibco) supplemented with 10% FBS and 50 μg/mL gentamicin (Gibco) in 5% CO2 at 37°C. For co-cultivation, the WJ-MSCs (1.5 × 10^5 cells) were seeded into the upper chamber of transwell inserts (pore size of 1 μm, BD Biosciences, Franklin Lakes, New Jersey) when the S16 cells reached a confluence of almost 70%. These cells were co-cultured for 24 hours in a condition of serum-free media in 5% CO2 at 37°C. Human cell subject research was reviewed and approved by the Institutional Review Board of Samsung Medical Center, and informed consent was obtained from pregnant mothers (IRB#2016-07-102).

4.2 | Characterization of WJ-MSCs

According to the MSC criteria of the International Society for Cell Therapy (ISCT), immunophenotypic analysis of WJ-MSCs was performed by flow cytometry to determine the expression of the following markers: CD44, CD73, CD90, CD105, CD11b, HLA-DR (MHC-II), CD34, CD45, and CD19 (BD Biosciences). At least 10 000 events were acquired on a BD FACSVerse (BD Biosciences, New Jersey), and the results were analyzed with BD FACSuite software version 10 (BD Biosciences). The differentiation of WJ-MSCs was tested according to the procedure outlined in a previous report.

4.3 | Antibody array

The RayBio Biotin Label-based Human Antibody Array (#AAH-BLG-1-4), which is available to detect a total of 507 of human proteins, was applied for the analysis of secreted proteins from the conditioned media for the co-cultivation of MSCs and S16 cells. All array data obtained using the Axon GenePix 4000B microarray scanner was analyzed with GenePix Pro 6.0 software. The average score of protein expression was normalized with that of internal biotinylated controls in duplicated arrays. The clustering analysis of hits was performed using the MeV software (Dana-Farber Cancer Institute, Boston, Massachusetts). The heatmap was generated by candidates with log 2 (fold change) >1.5.

4.4 | Enzyme-linked immunosorbent assay

The ELISA kit (LSBio, LS-F6448) was used for the analysis of the expression of proteins selected from the antibody array. The protein concentration was calculated from the intensity value measured at a wavelength of 450 nm.

4.5 | CCK-8 assay

The CCK-8 (CCK-8, Dojindo, Tokyo, Japan) was applied for analysis of the proliferation of S16 cells. For the assay, S16 cells (1 × 10^4 cells/well) were seeded into 96-well cell plates, including 100 μL of
conditioned culture medium, and incubated overnight. The CCK-8 reagent of 10 μL was added to each well and incubated for 1 hour at 37°C. Then a microplate reader (xMark Microplate Absorbance Spectrophotometer) was used to analyze the cellular absorbance at a wavelength of 450 nm.

4.6 | BrdU assay

S16 cells, cultured on round glass coverslips in 12-well plates, were pretreated with BrdU (10 μM) for 1 hour, and then treated with 10 ng/mL of recombinant human FBLN5 protein in a serum-free state. The cells were fixed in 3% formaldehyde and treated with 2 N HCl for 30 minutes at 37°C for antigen retrieval followed by neutralization with boric acid (pH 8.4). After blocking for 1 hour at room temperature, the cells were stained with anti-BrdU antibody (#ab6326, Abcam), secondary (#112-165-143, Jackson ImmunoResearch Laboratories), and Hoechst 33342 (#H1399, Life Technologies Corporation) antibodies. The immunostained images were acquired using an LSM 700 confocal microscope (Carl Zeiss AG, Jena, Germany). The images were analyzed with ImageJ software.

4.7 | Transfection

The verified siRNAs for FBLN5 (Supplementary Table 1) were transfected to WJ-MSCs using Lipofectamine 3000 (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. The non-targeting control siRNAs (Bioneer, SN-1001, Korea), which have at least four mismatch nucleotides to the human, mouse, and rat Fibulin genes, were transfected as a control. The transfected cells were applied for the next experiments after 24 hours of incubation in a complete medium.

4.8 | In vitro wound healing assay

S16 cells reached to 90% confluence in 6-well plates were treated with mitomycin (10 μg/mL) for 1 hour to inhibit cell proliferation. Confluent monolayers of the cells then were scratched using a 200 μL pipette tip to generate the wound. The cells were washed with PBS and cultured with nothing, WJ-MSCs, or recombinant FBLN5 for 48 hours. To determine the extent of wound closure, photographs of the wound were taken 0, 24, and 48 hours later using a phase contrast microscope (Olympus, CKX41, Japan). Wound closure was measured proximate cells length using ImageJ software (NIH).

4.9 | Cultivation of DRG and primary SCs

The DRG and sciatic nerves were isolated from c57/BL6NJ mice at postnatal day 15 (P15) and transferred to the conical tube containing trypsin of 0.25% (25-053-CI, Corning, New York) and 1 mg/mL of collagenase (C2139, Sigma-Aldrich, Saint Louis, Missouri). The primary tissues were incubated for 30 minutes at 37°C for digestion followed by centrifugation. The cell pellets were suspended in DMEM (10-013-CV, Corning) containing fetal bovine serum of 15% (35-016-CV, Corning) and penicillin-streptomycin of 1% (30-002-CI, Corning) after being washed with DPBS (21-030-CVR, Corning). The primary cells were cultivated on the coverslips in 35-mm dishes or 6-well plates coated with poly-o-lysine hydrobromide (P6407, Sigma-Aldrich) in 5% CO₂ at 37°C.

4.10 | Zebrafish housing and manipulations

Adult Tg(claudin K:gal4-vp16:uas:egfp) zebrafish were maintained with a cycle of 13 hours of light and 11 hours of dark in an automatic system (Genomic-Design, Korea) at 28.5°C and pH 7.0-7.9. The zebrafish embryos were collected by natural breeding and incubated in clean petri dishes containing E3 medium (297.7 mM NaCl, 10.7 mM KCl, 26.1 mM CaCl₂, and 24.1 mM MgCl₂) with 1% methylene blue (M2662, Samchun Chemicals, Korea) at 28.5°C. To inhibit the formation of melanin, which interferes with immunostaining, the zebrafish larvae were raised in E3 medium containing 0.2 mM N-phenylthiourea (P7629, Sigma Aldrich). Animal subject research was reviewed and approved by the Institutional Animal Care and Use Committee at the Samsung Biomedical Research Institute and the Sungkyunkwan University (IACUC#20191204002).

4.11 | DNA plasmid construction

The zebrafish fbln5, pmp22α, and rac1 were amplified with cDNAs obtained from zebrafish larvae at 5 days postfertilization (dpf). The coding sequences of fbln5, pmp22α, and rac1 were subcloned into pCS2+ vectors for mRNA expression in zebrafish. The cloned rac1 and fbln5 plasmids were mutated using a QuickChange II Site-Directed Mutagenesis Kit (#200524, Aglient) to generate rac1 (Q61L) and fbln5 (ΔRGD) constructs, respectively. The mouse Rac1 coding sequences were subcloned into a pCMV vector and mutagenized to generate Rac1 (Q61L). The designed oligos for the above subclonings are presented in Supplementary Table 2.

4.12 | Microinjection into zebrafish

To block the expression of zebrafish fbln5, translation blocking antisense morpholino oligonucleotides (MOs) (Supplementary Table 3) were designed and synthesized by GeneTools (Philomath, Oregon). The MOs were dissolved in nuclease-free water and microinjected into zebrafish embryos using a gas-powered microinjection system (PV83 Pneumatic PicoPump, SYS-PV830, World Precision Instruments, Sarasota, Florida). The capped mRNAs of zebrafish genes (fbln5, pmp22, and rac1) were synthesized using a mMESSAGE mACHIN T3 kit (AM1340, Ambion). The mRNAs synthesized
in vitro (600-800 ng/μL) were injected into zebrafish embryos with or without fbln5 MOs.

4.13 Immunoblot assay

The cell extracts were prepared by ultrasonication (Branson Ultrasonic, Danbury, CT) in a buffer (9.8 mol/L of UREA, 4% of CHAPS, 130 mmol/L of dithiothreitol, 40 mmol/L of Tris-HCl, 0.1% of sodium dodecyl sulfate, 1 mmol/L of EDT, and a protease/phosphatase inhibitor cocktail). The zebrafish extracts were prepared at 5 dpf. The yolks of zebrafish larvae anesthetized with 0.02% of Tricaine (AS040, Sigma) were removed by gentle pipetting, and homogenized with a 1-mL syringe in T-PER buffer (78 310, Thermo Fisher Scientific, Massachusetts). Zebrafish or cellular protein extracts (20-40 μg) were separated by SDS-PAGE, and the resolved proteins were transferred to a 0.45-μm PVDF membrane (IPVH00010, Millipore). After blocking in 5% skim milk (Cat.232100, BD Biosciences) for 30 to 60 minutes at room temperature (RT), each membrane was blotted with a primary antibody (mouse anti-Rac1 [ARC03], Cytoskeleton, Colorado), rabbit anti-MBP (sc-271 524, Santa Cruz, 1:200) antibodies at 4°C overnight, or mouse body (mouse anti-Rac1 [ARC03], Cytoskeleton, Colorado), rabbit anti-Fibulin5 [A9961], ABclonal, Massachusetts, 1:2000), or mouse body (mouse anti-Rac1 [ARC03], Cytoskeleton, Colorado), rabbit anti-MBP (sc-271 524, Santa Cruz, 1:200) antibodies at 4°C overnight

4.14 RAC1 activity assay

Four hundred micrograms of cell lysates were incubated with 10 μg of P21-associated kinase (PAK)-PBD beads (BK035-S, Cytoskeleton, Colorado) on the rotator for 1 hour at 4°C. The beads were then washed in a buffer (25 mM Tris pH 7.5, 30 mM MgCl2, and 40 mM NaCl). Finally, the bead-bound proteins were denaturized by boiling in a 5x sample buffer for 2 minutes and separated by 8% SDS-polyacrylamide gel electrophoresis.

4.15 Immunocytochemistry

Cells fixed in 4% PFA at RT for 30 minutes were permeabilized with 0.1% triton X-100 in PBS for 5 minutes after washing with 1x PBS. Then, the cells were washed 2 to 3 times and blocked in PBS with 1% BSA at RT for 1 hour. The cells were incubated with primary antibodies: mouse anti-Sox10 (sc-365 692, Santa Cruz, 1:200), mouse anti-MBP (sc-271 524, Santa Cruz, 1:200) antibodies at 4°C overnight in blocking solution. After washing with 1x PBS, the cells were incubated with Alexa Flour 594-conjugated secondary antibodies (mouse A11005, Invitrogen, 1:500) or Phalloidin-iFluor 488 reagent (ab176753, Abcam) at RT for 1 h. For generating imaging data, randomly selected fields (≥3) were photographed using a confocal microscope (Carl Zeiss, LSM 700, Germany) and analyzed using ImageJ software (NIH) in all of imaging data.

4.16 Zebrafish motility analysis

Using Daniovision (Noldus, Wageningen, The Netherlands), the velocities of the zebrafish were analyzed by a tap test. Individual zebrafish larvae injected with MOs were transferred into 24-well plates, with each well with 1 mL of E3 media. After the zebrafish acclimatized in the chamber for 30 minutes, double tapping stimuli (first at level 4 and second at level 8) were applied for the zebrafish every 10 seconds. The movements of the zebrafish were recorded and analyzed using Ethovision XT software (Noldus, Wageningen, The Netherlands).

4.17 Transmission electron microscopy

Zebrafish larvae anesthetized with 0.02% tricaine (AS040, Sigma) were fixed in a buffer (2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4) for 24 hours. After being washed with 0.1 M phosphate buffer, the larva were postfixed in a buffer (1% osmium tetroxide [02236, Polysciences] in 0.1 M phosphate buffer) for 2 hours and then gradually dehydrated in ethanol (50%-100%) and infiltrated with propylene oxide. The fixed zebrafish were embedded in a Poly/Bed 812 kit (#80791, Polysciences) and polymerized in an electron microscope oven (TD-700, DOSAKA, Japan) for 24 hours at 65°C. The embedded zebrafish blocks were sectioned with an ultramicrotome (Leica EM UC-7, Leica Microsystems, Austria) with a diamond knife (Diatome) at a thickness of 200 nm. The sectioned samples were stained with 1% toluidine blue (T3260, Sigma) and then observed by bright-field microscopy. The regions for TEM were selected and re-sectioned at a thickness of 70 nm. The ultrathin sectioned samples were transferred onto the copper/nickel grids for double staining with uranyl acetate and lead citrate. The stained samples were observed using TEM (JEM-1011, JEOL, Japan).

4.18 Statistics

All statistical analyses were performed using the GraphPad Prism version 5 (GraphPad Software, La Jolla, California). Values were presented as the mean ± S.D. or fold changes relative to the mean controls. Differences between two groups were evaluated using unpaired Student's t-tests with Welch's correction. Datasets having more than two groups or conditions were subjected to ANOVA followed by Tukey’s, Dunnett’s, or Bonferroni’s post hoc test. A P-value less than .05 was considered significant for any statistical test used.
5  |  CONCLUSION

To seek novel therapeutic targets for demyelinating diseases, we screened the paracrine factors secreted from MSCs co-cultured with SCs and identified FBLN5 using antibody array analysis. We showed that after recombinant FBLN5 treatment, SCs proliferated more through activation of ERK. We further demonstrated that the RGD motif of FBLN5 is essential for binding to integrin, and this interaction is involved in actin remodeling via RAC1 activity during myelination of SCs. Finally, we revealed that FBLN5 effectively restores myelination defects in the zebrafish model of demyelinating disease, suggesting that FBLN5 may be a potential target for myelin-related disorders.

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CONFLICT OF INTEREST
The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
S.Y.W., S.K., H.S.J.: conducted experiments and analyzed data; K.W.C., B.-O.C., J.E.L.; and J.W.C.: designed experimental designs and commented on the manuscript; J.E.L.: designed the overall experiments and wrote the manuscript with substantial contribution from J.W.C., S.Y.W., and S.J.K.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

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