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Pelizaeus–Merzbacher disease-associated proteolipid protein 1 inhibits oligodendrocyte precursor cell differentiation via extracellular-signal regulated kinase signaling

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ABSTRACT

Oligodendrocytes (OLs) are myelin-forming glial cells in the central nervous system (CNS) and their dysfunction causes neuropathies such as demyelinating diseases. Proteolipid protein 1 (PLP1) is an oligodendrocyte myelin-rich tetraspan membrane protein and aberration of the *plp1* gene is known to be responsible for dysmyelinating Pelizaeus–Merzbacher disease (PMD). Among previously identified gene alternations, multiplication of the *plp1* gene causes increased expression of PLP1, resulting in a phenotype with severe dysmyelination in human and also rodent models. Yet little is known about the relationship between increased PLP1 expression and oligodendrocyte precursor cell (OPC) differentiation and the intracellular molecular mechanism. Here we show that expression of PLP1 in OPCs markedly inhibits their differentiation, and that this inhibitory effect is effectively improved by inhibition of extracellular signal-regulated kinase (ERK) activity. Furthermore, in cocultures with dorsal root ganglion (DRG) neurons, ERK inhibition also improves PLP1-induced dysmyelination. Thus, ERK inhibition helps to improve defective OPC differentiation induced by PLP1 expression, suggesting that molecules belonging to ERK signaling cascade may be new PMD therapeutic targets.

1. Introduction

In development of the central nervous system (CNS), oligodendrocyte precursor cells (OPCs) differentiate into oligodendrocytes (OLs), which form myelin sheaths. The myelin sheath is essential for maximizing nerve conduction velocity. CNS myelin contains a number of specific lipids and specific membrane-associated proteins [1]. For example, myelin basic protein (MBP) is a soluble protein that binds to the cytoplasmic surface of the myelin membrane and brings the cytoplasmic faces close together, allowing a tight spiral to be formed around axons. Proteolipid protein 1 (PLP1), a tetraspan membrane protein family member, and its splice variant DM20 occupy a total of ~50% of the protein in CNS myelin [1].

Aberration of the *plp1* gene is known to cause the X-linked recessive dysmyelinating disorder called Pelizaeus–Merzbacher disease (PMD) [1–6]. Multiplication, deletion, and missense mutation of the *plp1* gene can all cause PMD. Missense mutations of this gene cause mild to severe symptoms, depending on the location of the altered amino acid. PMD of this type is characterized by dysmyelination with a thinner myelin sheath. Later in the disease course, this may lead to the virtual absence of compact myelin and, frequently, by widespread OL apoptosis [4]. The pathogenic mechanism in this case is believed to begin with the accumulation of mutation-induced, misfolded PLP1 proteins in the endoplasmic reticulum (ER) [5,6]. Patients with no *plp1* allele exhibit very mild symptoms, including slight cognitive delay and ataxia, while patients carrying multiple (mainly duplicated) alleles (50–70% of PMD cases) often display a severe dysmyelination phenotype associated with tremor of the head and neck, motor deficits, and spasticity [4]. While biochemical properties of the PLP1 protein as well as the mutant protein have been thoroughly studied in heterogenous expression systems using cell lines, it still remains unknown whether disease-associated PLP1 intracellularly transduces a signal other than an ER stress signal [5,6] and how this occurs in primary cells, especially with regard to major multiple alleles.

We have developed a retrovirus-mediated PLP1-expression system in primary oligodendrocyte precursor cells (OPCs). Using this system, we find (1) that expression of PLP1 in OPCs markedly inhibits differentiation, (2) PLP1 expression leads to dysmyelination in cocultures with dorsal root ganglion (DRG) neurons, and (3) inhibition of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling cascade

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reverses PLP1-mediated inhibition of OPC differentiation. Thus, molecules belonging to ERK signaling cascade may provide a new therapeutic target for PMD multiple alleles.

2. Materials and methods

2.1. Antibodies and inhibitors

The following antibodies were purchased: anti-ERK (ERK1/2), anti-(pThr202/Tyr204)ERK (ERK1/2), anti-B-Raf, and anti-(pSer445)B-Raf from Cell Signaling Technology (Beverly, MA, USA); anti-Ras (panRas) and anti-MBP (differentiated OL marker) from Millipore (Billerica, MA, USA); anti-Olig2 (OL lineage cell marker) from Abcam (Cambridge, UK); anti-β-actin from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA); and horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary IgGs from GE Healthcare (Piscataway, NJ, USA) and Nacalai Tesque (Kyoto, Japan). The Alexa Fluor-conjugated anti-mouse and anti-rabbit IgGs were obtained from Life Technologies (Carlsbad, CA, USA) and Nacalai Tesque. U0126 and PD98059 (chemical ERK cascade inhibitors) were purchased from Biomol (San Diego, CA, USA). An annexin V apoptosis detection kit was obtained from Medical and Biological Laboratories (Nagoya, Japan).

2.2. Plasmids

PCR-amplified human PLP1 cDNA was ligated into a pRetroX-IRES-ZsGreen1, which simultaneously express ZsGreen and the inserted PLP1 construct (Takara Bio, Shiga, Japan). The PLP1 cDNA was also ligated into the retrovirus vector pMEI5 (Takara Bio). Sequencing was performed using an ABI377 automatic sequencer (Foster City, CA, USA).

2.3. Culture for primary OPCs and cocultures with primary DRG neurons

OPCs were isolated from embryonic day 15 rat cerebral cortices, as previously described [7,8]. Dissociated cells were seeded on polylysine-coated dishes. After two passages, the cells were cultured on non-coated Petri dishes (Barloworld Scientific, Staffordshire, UK). On the second day of culture, the medium was changed to DMEM-based serum-free medium with various supplements and growth factors [7,8]. The cells were cultured for an additional 2 days, then used as OPCs. To confirm cell viability under these experimental conditions, OPCs were stained with 0.4% trypan blue. Less than 5% of cells in each experiment incorporated trypan blue. To allow OPCs to differentiate, the cells were continuously cultured in a culture medium containing triiodothyronine and thyroxine without PDGF until 4 days. Maximum differentiation was reached at day 3. DRG neurons were isolated from embryonic day 15 rat spinal cords as previously described [9], then dissociated and plated onto collagen-coated coverslips. Non-neuronal cells were eliminated by cycling with medium containing 5-fluorodeoxyuridine and uridine. Myelinating cocultures were established by seeding approximately 200,000 purified OPCs onto purified DRG neuron cultures, according to the protocol of Chan et al. [9]. Cocultures were maintained for 3 weeks, with fresh medium provided every 3 days.

2.4. Retrovirus-mediated transfection

The retroviral expression vectors and the envelope expression vector were cotransfected into GP2-293 cells (Takara Bio) with a CalPhos Transfection kit (Takara Bio) as previously described [7,8]. Briefly, at 2 days after transfection, the culture supernatants were centrifuged at 10,000 rpm for 8 h to concentrate the recombinant viruses. The virus pellets were normally suspended in culture medium to adjust the multiplicity of infection (MOI) value above 1 [7,8]. OPCs infected with the recombinant retroviruses were grown in a culture medium overnight, and then cultured without PDGF for 3 days to induce differentiation or seeded on DRG neurons for cocultures, unless otherwise indicated.

2.5. Immunofluorescence

OPCs, differentiated OLs, or cocultures were fixed in 4% paraformaldehyde in phosphate-buffered saline at room temperature [7,8,10]. The fixed cultures were permeabilized with 0.05–0.1% Tween-20 and blocked with a Blocking One kit (Nacalai Tesque). Blocked cells were incubated with primary antibodies overnight at 4 °C. Unbound primary antibodies were removed by washing three times with phosphate-buffered saline containing 0.05% Tween-20. Washed cells were then incubated with secondary antibodies. After three rinses with phosphate-buffered saline containing 0.1% Tween-20, cells were mounted on a Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed under a microscope. The fluorescent images were captured using a DMI4000 microscope system (Leica Microsystems, Wetzlar, Germany) and analyzed with AF6000 software (Leica Microsystems) or captured using an Eclipse TE-300 microscope system (Nikon, Kawasaki, Japan) and analyzed with AxioVision software (Carl Zeiss, Oberkochen, Germany).

2.6. Immunoblotting

OPCs or differentiated OLs were lysed in lysis buffer (50 mM HEPES–NaOH (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 μg/ml leupeptin, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, and 0.5% NP-40) and centrifuged as previously described [7,8,10]. The proteins in the cell lysates were denatured in Laemmli buffer (0.4 M Tris–HCl (pH 6.8), 0.2 M dithiothreitol, 0.2% bromophenol blue, 4% SDS) and then separated on SDS–polyacrylamide gels. The electrophoretically separated proteins were transferred to PVDF membranes, blocked with a Blocking One kit, and immunoblotted. The bound antibodies were detected using the ECL system (GE Healthcare). At least three separate experiments were carried out, and a representative experiment is shown in each of the figures.

2.7. Recombinant proteins

Recombinant GST-tagged Ras-binding-domain (RBD) of Raf-1 was purified using *Escherichia* coli BL21 (DE3) pLysS (Takara Bio) as previously described [7,8,10]. Briefly, the transformed *E. coli* cells were treated with 0.4 μM isopropyl-1-thio-beta-D-galactopyranoside at 30 °C for 2.5 h, then harvested by means of centrifugation. The precipitates were extracted with extraction buffer (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EDTA, and 0.5% Nonidet P-40) containing 500 μg/ml lysozyme and 100 μg/ml DNaseI on ice. All purification steps were performed at 4 °C. The lysates were centrifuged, purified, and used for affinity precipitation of GTP-bound Ras.

2.8. Affinity precipitation of GTP-bound Ras

To detect active GTP-bound small GTPase from OPC or OL lysates, we performed a pull-down assay as previously described [7,8,10]. Briefly, GST-Raf1-RBD protein coupled to glutathione Sepharose 4B was added to each supernatant from an OPC or OL lysate. Samples were rotated at 4 °C for 1 h. Bound proteins were

eluted, subjected to SDS–PAGE, and immunoblotted with an anti-Ras antibody.

2.9. Statistical analysis

Values shown represent the mean ± S.D. from separate experiments. Student's *t* test was carried out for intergroup comparisons ($^{*}p < 0.01$).

3. Results

3.1. Expression of PLP1 in OPCs inhibits their morphological differentiation

We first checked for any side effects that viral infection may have had on the differentiation of rat primary OPCs. OPCs in the presence of our retrovirus extended two or fewer short and un-

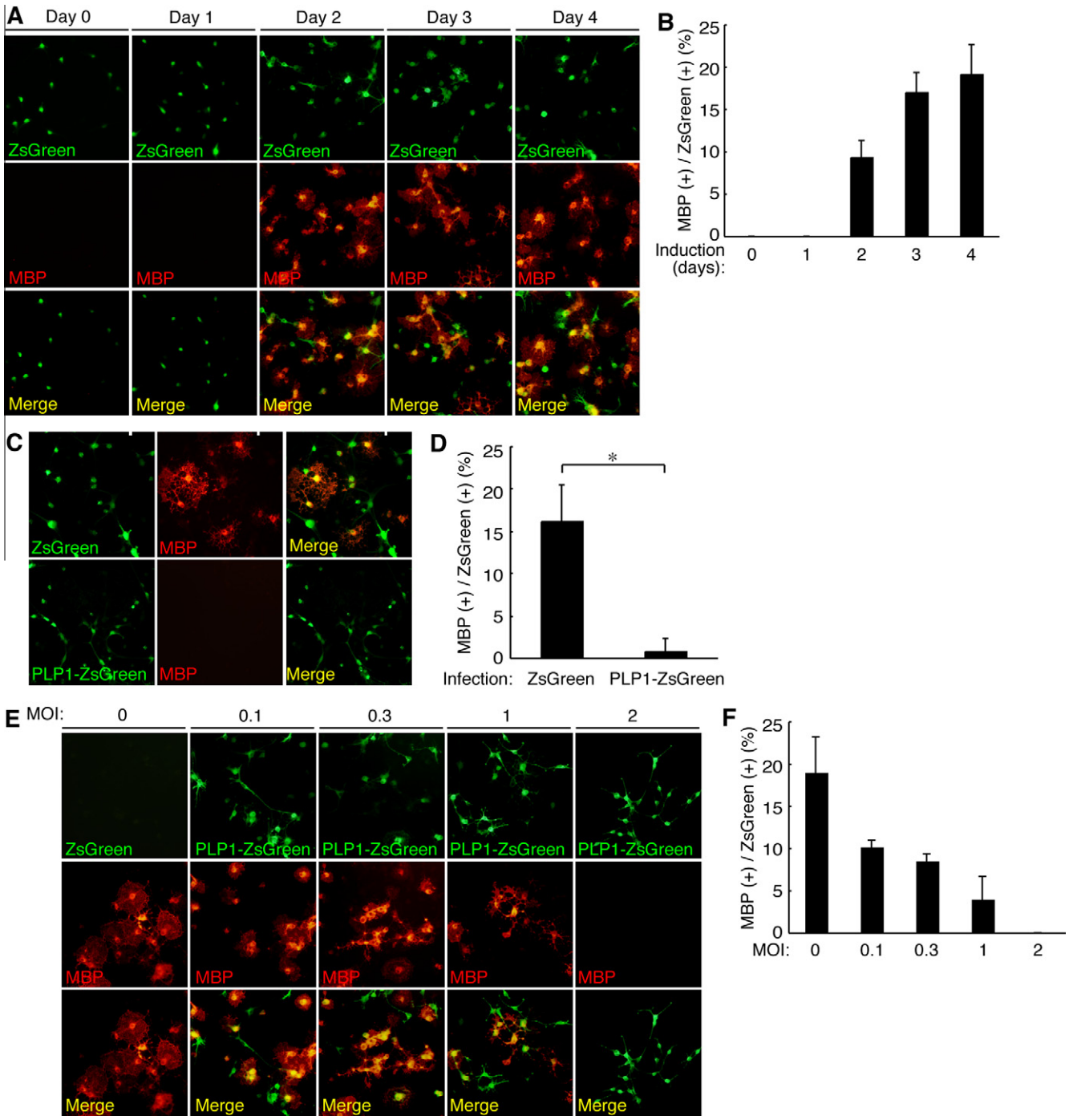

Fig. 1. PLP1 inhibits OPC differentiation. (A) OPCs were infected with a recombinant retrovirus encoding ZsGreen. After differentiation had begun (0–4 days), cells were immunostained with anti-ZsGreen (green) and MBP (red) antibodies. (C) OPCs were infected with a retrovirus encoding ZsGreen or PLP1-ZsGreen, allowed to differentiate for 3 days, and stained for ZsGreen (green) and MBP (red). (E) OPCs were infected with a recombinant retrovirus encoding ZsGreen or PLP1-ZsGreen at MOIs of 0–2, allowed to differentiate, and stained for ZsGreen (green) and MBP (red). (B, D and F) The percentages of MBP-positive cells against the total number of ZsGreen-expressing cells are shown. Data were evaluated using Student's *t* test ($n = 3$; $^{*}p < 0.01$).

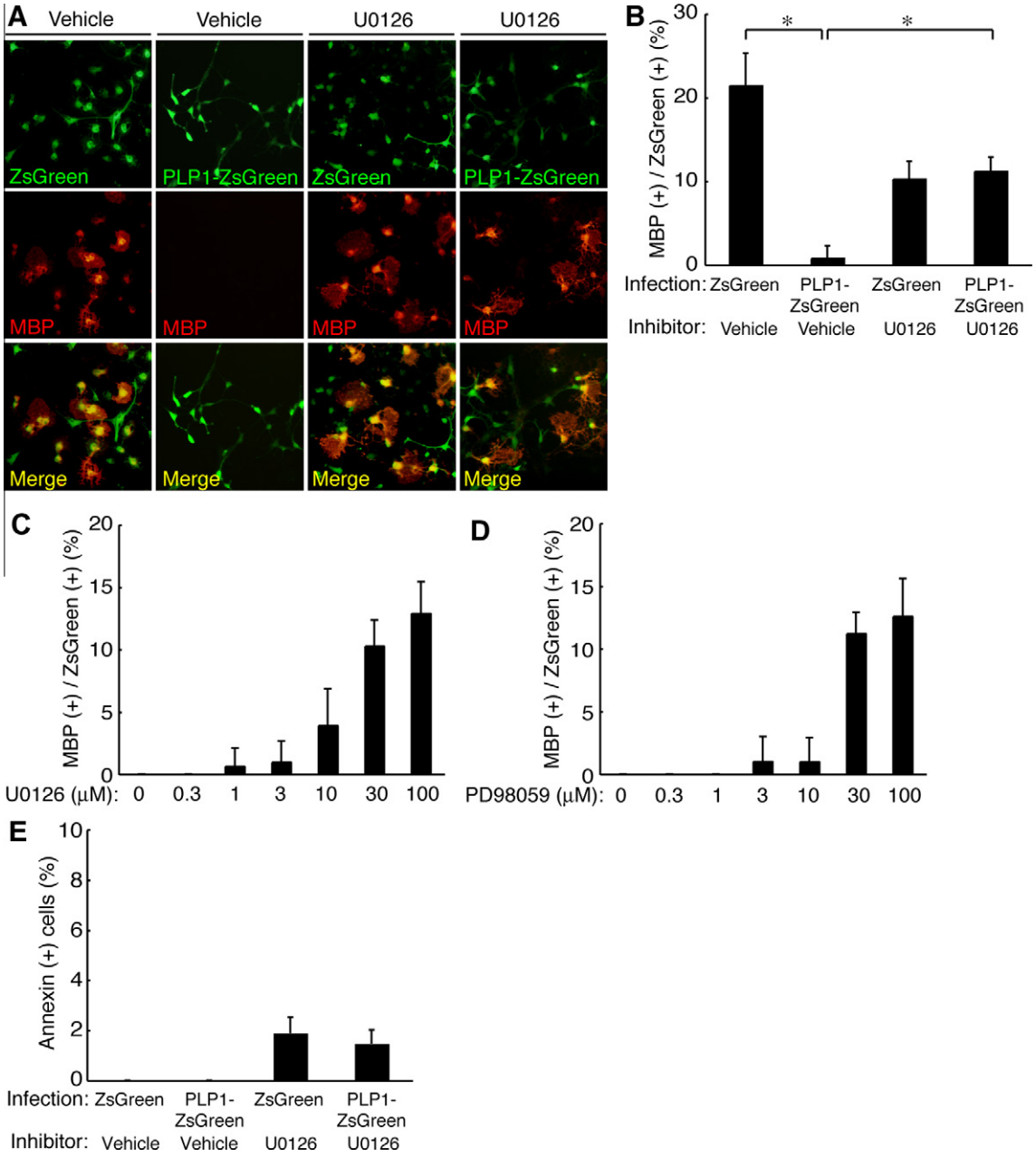

Fig. 2. PLP1 inhibits OPC differentiation through the ERK cascade. (A and B) OPCs were infected with a retrovirus encoding ZsGreen or PLP1-ZsGreen with or without U0126 (30 μM), allowed to differentiate for 3 days, and stained for ZsGreen (green) and MBP (red). (C and D) PLP1-ZsGreen-infected OPCs were treated with U0126 or PD98059 at various concentrations and allowed to differentiate for 3 days. (E) OPCs were infected with a ZsGreen or PLP1-ZsGreen retrovirus. The percentages of annexin V-positive, apoptotic cells relative to the total number of DAPI-positive cells were calculated. Data were evaluated using Student's *t* test ($n = 3$; $^*p < 0.01$).

branched processes and did not express any MBP, the specific marker of mature differentiated OLs (Fig. 1A and B). At 3–4 days after the induction of differentiation, ZsGreen-expressing cells extended processes with high branches to form MBP-positive myelin web-like structures (Fig. 1A and B). This time course of OPC morphological differentiation was the same as that followed by non-infected OPCs [7]. Accordingly, we allowed OPCs to differentiate for 3 days and used the resulting assortment of cells in the experiments described below.

We infected OPCs with a retrovirus coding PLP1-IRES-ZsGreen or ZsGreen as the control. PLP1-expressing cells displayed markedly suppressed myelin web-like structures with low-branched processes (0.79 ± 1.5% in PLP1-expressing cells in comparison with 16 ± 4.3% in control cells, Fig. 1C and D). We next examined whether the expression level of PLP1 protein influences the differentiation efficiency. OPCs were infected with retroviruses at MOIs 0–2. PLP1 inhibited differentiation in a manner dependent on the MOI value (Fig. 1E and F). At an MOI of 2, the highest value, no MBP-positive web-like structure was observed. These results seen in primary OPCs support the *in vivo* data indicating that the severity of dysmyelination is proportional to the expression level of PLP1 [11]. Collectively, expression of PLP1 in primary OPCs inhibits morphological differentiation in a PLP1-dose-dependent manner.

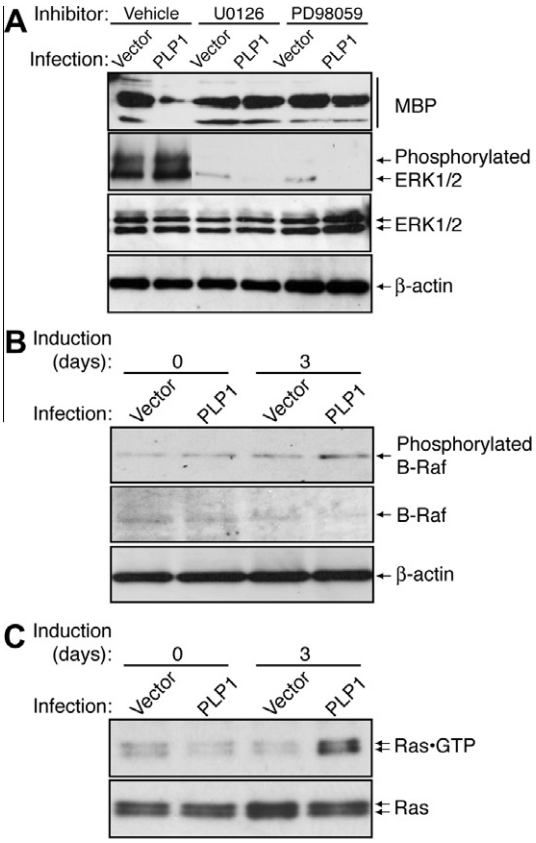

Fig. 3. Effect of PLP1 expression on ERK signaling. (A) Infected cells were allowed to differentiate for 3 days with or without U0126 (30 μM) or PD98059 (30 μM), lysed, and immunoblotted with an antibody specific for MBP, phosphorylated ERK1/2, ERK1/2, or β-actin. (B) OPCs were infected with a retrovirus encoding ZsGreen or PLP1-ZsGreen and either used immediately or allowed to differentiate for 3 days. The cell lysates were immunoblotted with an anti-phosphorylated B-Raf, B-Raf, or β-actin. (C) The cell lysates were affinity-precipitated with GST-Raf1-RBD and immunoblotted with an anti-Ras antibody.

3.2. Inhibition of the ERK cascade helps PLP1-expressing cells to differentiate

Of several kinase inhibitors, we found that U0126, the specific ERK cascade inhibitor (ERK upstream kinase inhibitor) [12], improves defective formation of myelin web-like structures by PLP1-infected OPCs (11 ± 1.7% in U0126-treated PLP1-expressing cells in comparison with 0.79 ± 3.9% in vehicle-treated PLP1-expressing cells, Fig. 2A and B). This effect was observed in a U0126-dose-dependent manner (Fig. 2C), although U0126 did not cause apoptosis (Fig. 2E) according to experiments using the surface phosphatidylserine-binding protein annexin V [13]. PD98059, another ERK cascade inhibitor structurally different from U0126, also had a similar protective and dose-dependent effect (Fig. 2D). These results confirm that inhibition of the ERK cascade improves defective morphological differentiation by PLP1. The ERK cascade is also known to participate in many aspects of normal myelination processes *in vivo* [14,15]. It is conceivable that proper regulation of ERK activity in cells may allow OPCs to differentiate.

Next, we used an anti-(pThr202/Tyr204)ERK antibody, which recognizes the active state, and tested whether expression of PLP1 in OPCs mediates ERK signaling. As expected, expression of PLP1 promotes ERK phosphorylation over the basal level (Fig. 3A, lanes 1 and 2 of second row), but the presence of either U0126 or PD98059 inhibits ERK phosphorylation and promotes MBP expression in PLP1-expressing cells (Fig. 3A, lanes 4 and 6 of first and second rows); this observation is consistent with immunofluorescence results indicating that ERK inhibitors improve formation of myelin web-like structures (Fig. 2A). We further examined whether PLP1 expression activates brain-rich Raf kinase (B-Raf) and small GTPase Ras proteins, which act upstream of ERK in neuronal lineage cells [16]. PLP1 expression increased B-Raf phosphorylation (Fig. 3B), which was detected by an antibody recognizing active, phosphorylated B-Raf [17]. Similarly, an affinity-precipitation assay using a Ras-binding domain [18] revealed that PLP1 increased active, GTP-bound Ras (Fig. 3C).

3.3. ERK inhibition increases myelin formation in cocultures

Finally, we sought to determine whether ERK inhibition contributes to myelin formation in PLP1-infected OPCs, using the coculture system with DRG neurons [9]. Cocultures were costained against Olig2 and MBP to identify individual OL lineage cell nuclei and mature OLs expressing MBP, respectively. Treatment with U0126 dramatically increased the number of MBP-expressing, Olig2-positive cells (13 ± 1.2% in U0126-treated, PLP1-expressing cells in comparison with 1.3 ± 1.7% in vehicle-treated, PLP1-expressing cells, Fig. 4A and B). U0126-treated cells also exhibited mature myelin structures (*right*, *bottom* panel of Fig. 4A). Additionally, although U0126 weakly or moderately increased cell numbers, as revealed by the number of Olig2 staining-positive cells (22 ± 1.2% in U0126-treated, mock-expressing cells in comparison with 16 ± 1.4% in vehicle-treated, mock-expressing cells), these findings support our findings that PLP1 inhibits OPC differentiation through the ERK cascade, possibly leading to dysmyelination.

4. Discussion

We herein demonstrate that PLP1 itself inhibits OPC differentiation and that its effect is mediated through the ERK signaling cascade. Importantly, ERK inhibition effectively promotes myelination by OLs overexpressing PLP1 in cocultures. The question of how PLP1 inhibits differentiation through ERK remains unanswered even after the current study. Harrisingh et al. report that the sustained activation of the Ras/Raf/ERK cascade induces de-differentiation of Schwann cells, the peripheral myelin-forming glial cells, reversing cells to the developmental stage that occurs just before the initiation of differentiation [19]. They show that induction of Raf-1 activation as well as infection of constitutively activated, oncogenic Ras in Schwann cells results in an increase in phosphorylated ERK and the upregulation of cell cycle regulator $p21^{Cip1}$ (also known as $p21^{Waf1}$). The proper regulation of the cell cycle, and especially of the transition from G1 to S phase, is necessary for cells to proliferate; altering the cell cycle, on the other hand, can arrest growth. These phenomena are likely to apply to OLs as well. Cyclin-dependent kinase (Cdk) inhibitors of the Cip/Kip family known as $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ block the functional Cdk2 complex in OLs [20]. $p21^{Cip1}$ is required for differentiation; but, this role is independent of its ability to control exit from the cell cycle [21]. $p27^{Kip1}$ inhibits OPC proliferation, thereby inducing differentiation [22]. The level of $p57^{Kip2}$ protein regulates how many times an OPC divides before differentiation begins [23]. We find in the present study that PLP1 inhibits OL differentiation through the signaling cascade to ERK. It will be interesting to examine whether $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ act as the ERK pathway's downstream targets.

Charcot–Marie–Tooth (CMT) disease is the most common hereditary peripheral neuropathy; it affects either the Schwann cells (generally called CMT disease type 1) or the neurons (generally called CMT disease type 2). CMT disease type 1A (CMT1A) is caused by alteration of the *peripheral myelin protein* (*pmp*) *22* gene. Multiplication, deletion, and point mutation of the *pmp22* gene are

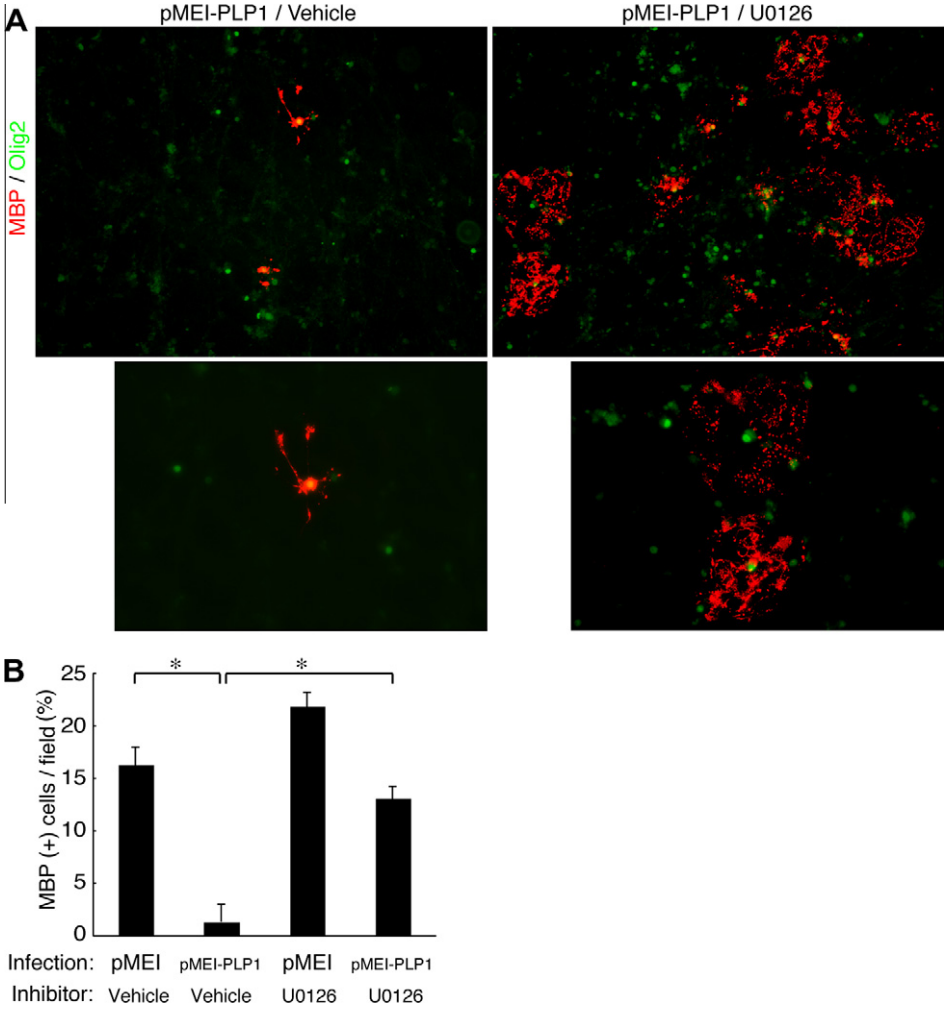

Fig. 4. ERK inhibitor increases myelin formation in cocultures. (A and B) OPCs were infected with a retrovirus encoding pMEI-PLP1 and cocultured with DRG neurons in the presence or absence of U0126. Cells were stained with antibodies against Olig2 (for identification of OL lineage cells, green) and MBP (red). The small panels in the second row represent magnifications of differentiated OLs that appear in the large panels in the top row. The percentages of MBP-positive cells against the total number of Olig2-expressing cells are shown. Data were evaluated using Student's *t* test ($n = 3$; $^{*}p < 0.01$).

responsible for the pathology of this disease [24]. The properties of the PMP22 protein are very similar to those of the PLP1 protein in many ways – some biochemical and other pertaining to cell biology. Topologically, PMP22 is predicted to belong to the tetraspan membrane protein family. A newly-biosynthesized PMP22 protein is normally transported to the Schwann cell surface where it participates in maintenance of the myelin membrane structure, making up 2–5% of all myelin membrane proteins. Significantly, Schwann cells derived from animal models of CMT diseases show enhanced proliferation, defective differentiation, and demyelination *in vitro* and *in vivo* [24–26]. Certain aspects of this cellular phenotype are similar to those of PLP1-expressing OPCs. Additionally, the ERK signaling pathway plays a key role in myelination by Schwann cells [27,28]. Further studies may allow us to clarify whether modification of the ERK signaling pathway has a protective effect against CMT1A as a peripheral dysmyelination disorder. If it can be shown that this is indeed the case, then the effects of excessive PMP22 on Schwann cell differentiation and myelination might be similar to that of PLP1 on OPC differentiation.

In this study, we identify the ERK cascade as one of the signals leading to defective OPC differentiation and dysmyelination caused by excessive PLP1. Further studies on ERK signaling and its precise target will be necessary to increase our understanding not only of how excessive PLP1 activates ERK to inhibit OPC differentiation but also of whether the molecular mechanism presented here is conserved *in vivo*. Furthermore, the application of our culture models may be invaluable in the elucidation of diseases such as PMD and for developing drug-target-specific medicines.

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