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ORIGINAL RESEARCH

pERK1/2 Peripheral Recruitment and Filopodia Protrusion Augment Oligodendrocyte Progenitor Cell Migration: Combined Effects of PDGF-A and Fibronectin

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Abstract Oligodendrocyte progenitor cell (OPC) migration is critical for effective myelination of the central nervous system. Not only during normal myelination but also during remyelination, the growth factors (GFs) and extracellular matrix (ECM) protein affect the OPC migration. Studies showed the altered levels of GFs and ECM in the demyelinating lesions. In our earlier studies, we have shown that the effect of platelet-derived growth factor alpha (PDGF-A) on OPC migration is dose- and time-dependent. In that we have shown that the physiological concentration (1 ng/ml) of PDGF-A was unable to induce OPC migration at transient exposure (30 min). However, the involvement of ECM in the regulation of PDGF-A mediated OPC migration was not clear. In the present study, we have used fibronectin (FN) as ECM. PDGF-A and FN have similar and overlapping intracellular signaling pathways including the extracellular regulated kinases 1 and 2 (ERK1/2). Here we demonstrate how physiological concentration of PDGF-A combines with FN to augment OPC migration in vitro. The present study is first of its kind to show the importance of the synergistic effects of PDGF-A and FN on peripheral recruitment of phosphorylated/activated ERK1/2 (pERK1/2), actin-pERK1/2 co-localization, and filopodia formation, which are essential for the enhanced OPC migration. These findings were further confirmed by ERK1/2 inhibition studies, using the pharmacological inhibitor U0126. *An understanding of these complex interactions may lead to additional strategies for transplanting genetically modified OPCs to repair widespread demyelinated lesions.*

Keywords OPC · pERK1/2 · Actin cytoskeleton · Filopodia · U0126

Introduction

Oligodendrocytes are the myelin producing cells of the CNS. The OPCs migrate extensively through the developing CNS to populate the white matter tracts of the developing brain (Levison et al. 1993; Rakic and Zecevic 2003). Abnormal cell migration can result in deferred onset or complete failure of myelination (Back et al. 2001; Volpe 2001). Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS (Noseworthy et al. 2000). Migration, proliferation, and differentiation of OPCs in the demyelinated areas are critical for remyelination (Chari 2007). Aberrant OPC migration can result from absence or failure of temporally and spatially regulated development signals (Fruttiger et al. 1999; Spassky et al. 2002; Tsai et al. 2002, 2003). Earlier studies have shown that complex alteration in the CNS ECM occurs during the MS (Sobel and Ahmed 2001; Gutowski et al. 1999; Sobel et al. 1995). Studies have supported the role of

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PDGF-A in the recruitment of OPCs into lesion area (Franklin 2002; Woodruff et al. 2004).

Our studies, along with previous others showed that PDGF-A is not only a mitogen (inducing proliferation) (Baron et al. 2000; Frost et al. 2003) but also a potent motogen (inducing migration) (Frost et al. 1996; Tsai et al. 2002; Frost et al. 2009). It has been shown that total ablation of PDGFRα is embryonically lethal (Li et al. 1996; Soriano 1997). Studies have shown that OPC migration also occurs in the absence of GFs like PDGF-A, but to do so the OPCs must be plated on a permissive substratum (Kakita and Goldman 1999; Frost et al. 1996; Fruttiger et al. 1999). Integrins, which are the receptors of ECM molecules, are differentially expressed by OPCs (Blaschuk et al. 2000; Frost et al. 1996; Milner et al. 1996; Milner and Ffrench-Constant 1994). Some of the studies using RGD peptides have demonstrated that integrin αvβ1 plays an important role in OPC migration (Milner et al. 1996). This indicates that oligodendrocytes behaviors are very complex and are regulated by a number of extracellular cues that work supportively to ensure that OPCs arrive in the correct place at the correct time in the white matter tracts.

The cytoskeleton of oligodendrocytes consists of microfilaments and microtubules but it is devoid of intermediate filaments (Song et al. 2001; Simpson and Armstrong 1999). The actin microfilaments are structured into cytoarchitectural meshworks which then generate mechanical forces which further helps a cell to migrate (Fukui and Kwang 1993; Gavin and Kwang 1997; Brandt 1998) and the same is true for oligodendrocytes (Simpson and Armstrong 1999). There are significant changes in OPC morphology during its migration, but mechanism by which they change their shape, extend their processes, and regulate their movement is still not clear.

To further gain an understanding of OPC migration and the extracellular cues regulating its actin cytoskeleton, we have checked the individual and combined effects of PDGF-A and FN on OPC migration. We show that transient exposure (30 min) of PDGF-A in the presence of FN is sufficient to elicit OPC migration. In addition, we show that transient activation of the platelet-derived growth factor receptor alpha (PDGFRα) in combination with an FN substratum leads to pERK1/2 recruitment to the cellular processes and regulating the actin organization. Further, we report for the first time that transient exposure of PDGF-A in combination with FN leads to the actin-pERK1/2 co-localization and formation of filopodia (extension of actin microfilament). Filopodia are important in sensing the environment during cell migration. The present study demonstrates how physiological concentration of PDGF-A can combine with FN to augment OPC migration in vitro.

Materials and Methods

Oligodendrocyte Progenitor Cells Isolation and Culture

OPCs were cultured by a previously described method (Chen et al. 2007; Frost et al. 2009; Vora et al. 2011; Sharma et al. 2015). Briefly, OPCs were isolated from P0–P2 day rat cortices. Dissociated cells were plated on poly-L-lysine (PLL)-coated T75 cm^2 flasks with high-glucose Dulbecco's modified Eagle's medium (DMEM-Gibco) containing 10 % fetal bovine serum (FBS-Gibco) and 1 % penicillin/streptomycin (1X-Invitrogen) then incubated at 37 °C in the presence 5 % CO_2. Cell growth medium was changed every third day for 10–12 days to obtain mixed glial cultures containing OPCs on an astrocyte monolayer. Purified OPCs were obtained by 18–20 h shaking method on an orbital shaker at 37 °C followed by differential adhesion for 1 h on the non-tissue culture plastic petri dish (Eppendorf).

Treatment Groups

Purified OPCs were plated in DMEM/F12 containing B27 supplement (Gibco), and 1 % penicillin/streptomycin (Gibco) on PLL (Sigma)-coated dishes or glass coverslips and were serum starved overnight prior to any treatment. These naïve OPCs were transiently exposed to PDGF-A (Sigma) alone and in combination with FN (Invitrogen) for 30 min (transient exposure activates signaling pathway/s that drive OPC migration behavior selectively without activating the cell proliferation) (Frost et al. 2009; Vora et al. 2011). PLL was used as control substratum which supports cell adhesion and spreading without engaging integrins. PLL is unable to activate the ERK1/2 (Chen et al. 1994; Schlaepfer et al. 1994; Miyamoto et al. 1995; Zhu and Assoian 1995). In all the experiments, the PLL and FN were used at the concentration of 10 μg/ml. For mimicking near physiological PDGF-A, 1 ng/ml concentration was used (Baron et al. 2002; Brunmark et al. 2002; Vora et al. 2011). For the inhibition studies, U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene), which has a 100-fold higher potency than PD098059 in blocking ERK signaling and acts by selectively inhibiting MEK-1 and MEK-2 phosphorylation and activation of ERKs, was used (Frost et al. 2009).

Migration Assay

OPC migration was assessed using the agarose drop migration assay (Varani et al. 1978; Frost et al. 2000, 2009). To distinguish between active migration from the

agarose drop and dividing cells in response to growth factors, the inhibitor of DNA replication i.e., aphidicolin was added to the medium (Milner et al. 1996; Ikegami et al. 1978; McKinnon et al. 1993). In all the treatment groups, cells were allowed to migrate for 72 h in the presence of aphidicolin. Hence, the emergence of cells from agarose drop was not due to proliferation but reflected entirely due to cell migration. Photomicrographs of the agarose drop assays were taken on a Nikon TS100 inverted microscope using phase optics.

Immunocytochemistry

In this study, the following primary antibodies were used: Rabbit anti-pERK1/2 was purchased from R&D Systems (diluted at 1:200); Goat anti-PDGFRα from SantaCruz (diluted at 1:50); Mouse anti-O4 from R&D Systems (diluted at 1:200); Mouse anti MBP from SantaCruz (diluted at 1:100); and Phalloidin tagged with Alexa Flour 488 (Molecular Probes). Coverslips were rinsed with phosphate-buffered saline (PBS, pH 7.4) followed by extraction with 0.5 % Triton X-100 in cytoskeleton stabilizing buffer (CSB: 1 mM EGTA, 4 % Polyethylene Glycol 8000, 0.0015 % phenol red, 100 mM Pipes, pH 6.9) for 10 min. The cultures were then fixed with 4 % paraformaldehyde in cytoskeleton stabilizing buffer (CSB) for 15 min, followed by incubation in blocking solution (3 % bovine serum albumin, 4 % goat serum). The cultures were incubated with the appropriate primary antibodies in blocking solution overnight at 4 °C, followed by incubation with secondary antibodies (1:400 in blocking solution) or Alexa-488 phalloidin in 1 ml blocking solution (Invitrogen) for 1 h. Coverslips were rinsed and mounted with Anti-fade mounting medium (Invitrogen) and sealed with nail polish. Images were visualized and captured using a Carl-Zeiss confocal microscope.

Immunoblotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose. Primary antibodies used were rabbit anti-pERK1/2 purchased from R&D Systems (diluted at 1:3000); rabbit anti-ERK1/2 from SantaCruz (diluted at 1:1000); and Mouse anti-β-actin from Pierce (diluted at 1:5000). Appropriate secondary antibodies were used. Membranes were developed using the ECL Plus chemiluminescent kit (Invitrogen).

Statistical Analysis

Confocal microscopy images were analyzed by ZEN 2012 imaging software. For statistical analysis, a minimum of 150 randomly chosen cells per condition were analyzed ($N = 3$ independent experiments with 3–4 replicates). The results of migration assay are expressed as the mean ± standard error of the mean. For filopodia counting, a minimum of 50 randomly chosen OPCs for each experimental group were analyzed, and the total number of filopodia at the growing tips per cell were counted ($n = 3$ independent experiments). Data are reported as the mean number of filopodia per cell (Eyermann et al. 2012). Differences between treatment groups were analyzed using Student's t test, or one way analysis of variance with Bonferroni's post-test where appropriate. Statistical analysis was performed with Prism 3 software (GraphPad Software Inc.). $P < 0.05$ was considered significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

Short-Term Exposure to PDGF-A Requires FN Engagement to Augment OPC Migration

Our earlier studies have shown that the effect of PDGF-A on OPC migration is dose- and time-dependent (Frost et al. 2009; Vora et al. 2011), however, the involvement of ECM in the regulation of PDGF-A mediated OPC migration was not clear. Therefore, we first investigated the individual effects of PDGF-A and FN on OPC migration in which PLL was used as control matrix. OPCs were characterized by PDGFRα staining (Supplementary Fig. S1). The dose-dependent effect of PDGF-A on OPC migration was studied using an agarose drop migration assay (Varani et al. 1978; Frost et al. 2000, 2009) for 72 h. Here, we show that cells exposed to FN resulted in enhanced migration compared to cells exposed transiently to PDGF-A (1 ng/ml), at (630.5 ± 38.56 μm) and (319.75 ± 35.66 μm), respectively (Fig. 1a, b). These results demonstrate that OPCs migrate on FN even in the total absence of PDGF-A. Overall, the data also pinpoint the importance of ECM more than GF alone at the physiological level (PDGF-A 1 ng/ml). OPC migration was significantly high in [FN + PDGF-A (1 ng/ml)] group compared to [PLL + PDGF-A (1 ng/ml)] at (1170.41 ± 38.30 μm) and (319.75 ± 35.59 μm), respectively ($P < 0.01$). Thus, OPC migration is strongly dependent on both PDGF-A as well as FN, suggesting an interaction of the GF-stimulated pathways for cell migration regulation, and those initiated upon ECM binding.

ERK1/2 Activation and Peripheral Recruitment are Essential for the OPC Migration

One of the studies showed that ERK1/2 is critical for the transition of OPCs from pre-progenitor to the late progenitor stage, but it is not essential for the transition of immature oligodendrocytes to the mature oligodendrocytes

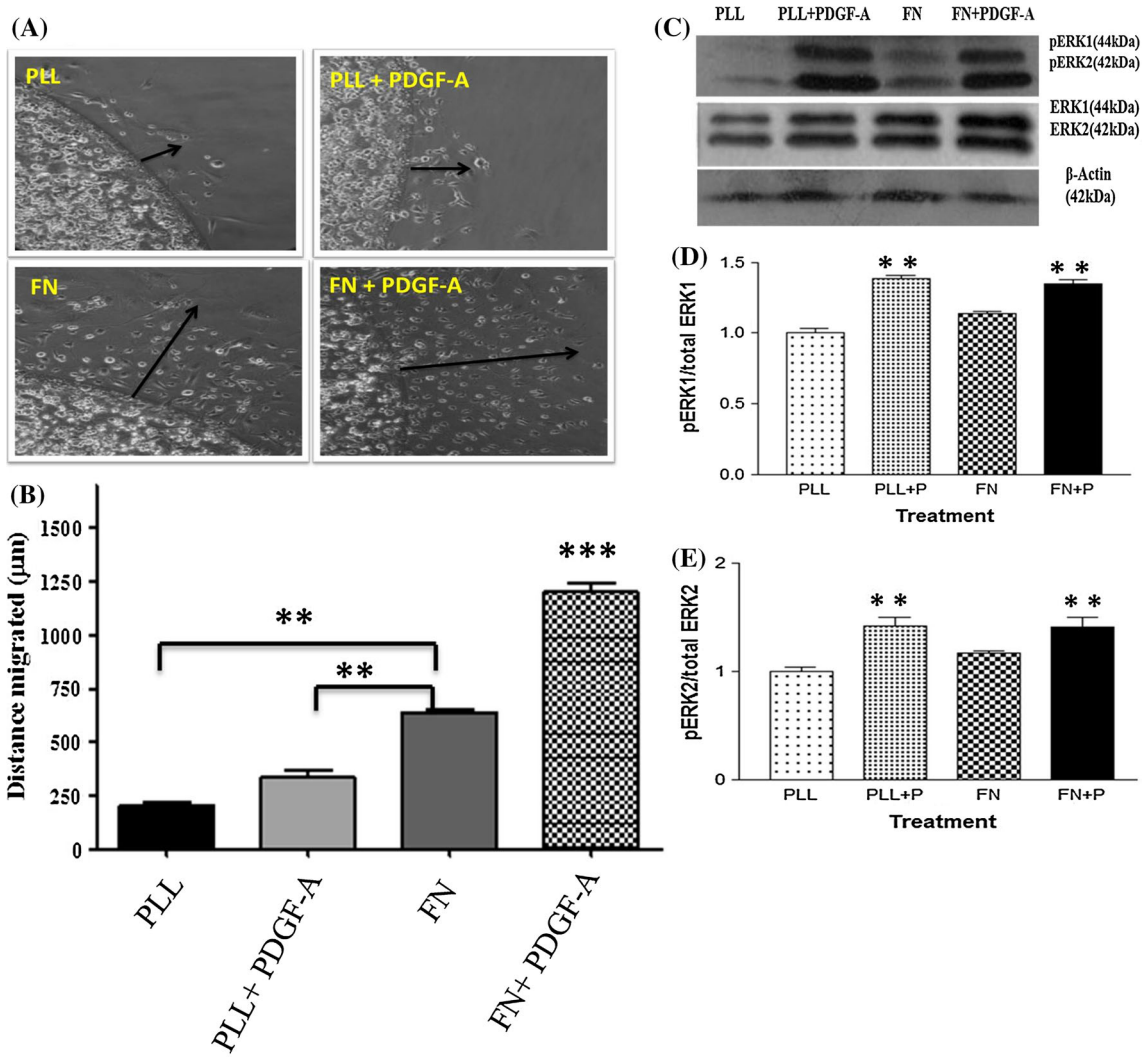

Fig. 1 Combination of PDGF-A and FN stimulates OPC migration and ERK1/2 phosphorylation. **a** Phase contrast photomicrograph showing OPCs migrate away from the edge of the agarose drop after PDGF-A and FN exposure. These are 20X phase contrast images. Migration was measured 72 h after the addition of growth factor. **b** *Graph* showing the effects of PDGF-A, with or without FN on OPC migration. Each point represents the mean ± SEM of three separate experiments. $N = 3$ with 3 replicates of each N. Data were evaluated using One way ANOVA-parameter ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). **c** Representative Western blot for phosphorylated and total ERK1/2 after treatment of OPCs with PDGF-A alone and in combination with FN. **d** and **e** After densitometric analysis, pERK1/2 data were normalized to total ERK1/2. Three different cell preparations were analyzed three times $N = 3$. Error bars represent ± SEM. Data were evaluated using One way ANOVA—parameter ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

(Guardiola-Diaz et al. 2012). This shows the importance of ERK1/2 in early development of oligodendrocytes, during the migratory stage. We used Western blot analysis to assess changes in the expression levels of pERK1/2 in the OPCs transiently exposed to PDGF-A and FN. There was no significant difference in pERK1/2 levels in the OPCs exposed to PDGF-A on PLL or on FN ($P > 0.05$) (Fig. 1c–e). However, confocal imaging revealed that pERK1/2 was differentially localized in the cell processes. When OPCs were treated with (FN + PDGF-A), pERK1/2 was significantly recruited to the cell process in comparison to other three groups ($P < 0.001$) (Fig. 2a, b). We have confirmed the OPC stage by double immunostaining of PDGFRα and pERK1/2 (Supplementary Fig. S2).

Combined Effects of FN and PDGF-A Promoted F-actin and Filopodia Formation

Disruption or changes in the cytoskeleton and the aggregation of abnormal cytoskeletal proteins are the usual signs of neurodegenerative diseases (Goedert 2001; Forman et al. 2004). We studied the effects of PDGF-A and FN to further understand the distribution and organization of microfilaments prior to migration in OPCs. When cells were exposed

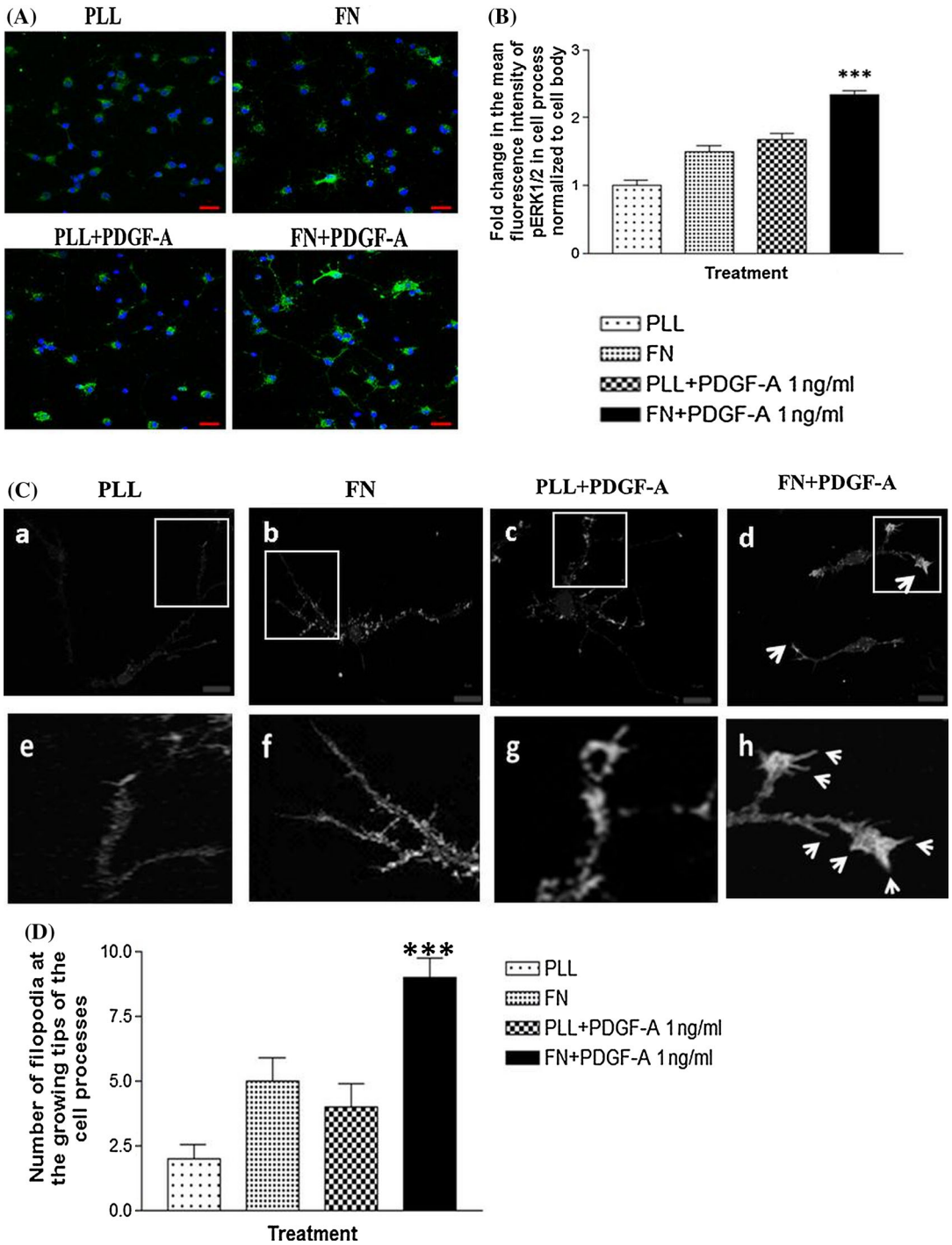

Fig. 2 pERK1/2 peripheral recruitment and filopodia formation. **a** OPCs were exposed to PDGF-A alone and in combination with FN for 30 min, Nucleus was stained with DAPI (4′,6-diamidino-2-phenylindole) (*blue*) and pERK1/2 (anti-pERK1/2 *green*). *Scale bar* 20 μm. **b** Mean fluorescence intensity of pERK1/2 in the cell process was normalized to the intensities in the cell body within each individual cell. *Values* represent mean ± SEM. Statistical differences between the stimulated and control samples were determined by one way ANOVA-parameter (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) **c** OPCs were exposed to PDGF-A alone & in combination with FN. The cells were stained with phalloidin Alexa 488-*green* to study F-actin organization [**c**(*a–d*)]. Images are shown in *gray scale*. *Scale bar* 10 μm. **c**(*e–h*) are cropped images of **c**(*a–d*), to show the microfilament extension (filopodia) from the OPC's growing tips. Filopodia was found increased in the FN + PDGF-A group [**c**(*h*), shown by *white arrows*] **d** graph shows increased number of filopodia at the growing tips of the cell process in (PDGF-A + FN)-treated OPCs. *Values* represent mean ± SEM. Statistical differences between the stimulated and control samples were determined by one way ANOVA-parameter (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (Color figure online)

to both PDGF-A and FN, the F-actin staining was significantly increased at the growing tips of the cell processes, compared to the individual treatments (Fig. 2c). Structural polarization is the initial criteria for cell migration (Lauffenburger and Horwitz 1996). Combination of PDGF-A and FN stimulated the development of filopodia (extension of actin microfilaments) from the processes of OPCs (Fig. 2d). These observations indicate that OPCs organize actin in the cell processes prior to migration when transiently exposed to PDGF-A and FN in combination. Following that filopodia was seen to form along the length and near the leading distal edge of the processes. Actin reorganization is one of the early events prior to cell migration and for a cell to sense the environmental cues present in the surrounding, filopodia plays a very essential and primary role as 'antennae' (Mattila and Lappalainen 2008). Microtubules follow microfilaments at the leading edges of the process and new branching sites during the branch formation and process extension in oligodendrocytes (Song et al. 2001).

pERK1/2-Dependent OPC Migration and Actin Organization

We further verified these observations using the pharmacological inhibitor U0126, which selectively inhibits ERK1/2 without affecting cell survival. U0126 significantly inhibited (FN + PDGF-A) induced ERK1/2 phosphorylation at a concentration of 10 μM [Supplementary Fig. S3]. OPC migration was reduced by 75 % in the presence of 10 μM U0126. OPC migration was significantly reduced from (1133.66 ± 27.86 μm) after transient exposure to (FN + PDGF-A) to (278.58 ± 32.31 μm) in the presence of U0126. ($n = 3$ with 6 replicates; $P < 0.001$) (Fig. 3a, d). Our results indicate that PDGF-A induced pERK1/2 is recruited in the OPC processes on FN but not on PLL (Fig. 2a, b). Moreover, following confocal microscopy studies, we found that inhibition of pERK1/2 with U0126 adversely affected actin cytoskeleton organization, actin-pERK1/2 co-localization, and filopodia formation in the (FN + PDGF-A) set (Fig. 3b, c and e). The data from the current study demonstrate that pERK1/2 inhibition significantly decreased the (FN + PDGF-A) induced cell process formation after the 72 h of OPC migration (Fig. 4a, b). Further pERK1/2 was also found to be in close proximity to the actin cytoskeleton, thus is likely to interact with actin stress fibers to mediate cytoskeletal organization during process outgrowth prior to OPC migration.

Discussion

PDGF plays a substantial role in OPC migration (Armstrong et al. 1991; Frost et al. 1996, 2009; Tsai et al. 2002). PDGF is crucial for normal myelination of the CNS (Calver et al. 1998; Tsai et al. 2002). Our studies along with the others' have confirmed that OPC migration does not require continuous exposure to PDGF and it is regulated by the ERK-signaling pathway (Frost et al. 1996, 2009; Tsai et al. 2002). Our earlier study (Vora et al. 2011), showed that transient exposure to PDGF-A at low concentration (1 ng/ml) did not show any significant migration compared to the control cells. Even so the transient exposure at 10 ng/ml showed comparable OPC migration to that of continuously exposed cells (Vora et al. 2011). In the present study, we checked the effect of 1 ng/ml (physiological concentration) of PDGF-A on a FN substratum. PLL was used as a control substratum. PLL is a biologically inert coating that allows cells to adhere to the culture surface, it enhances electrostatic interaction between negatively charged ions of the cell membrane and positively charged ions on the plastic. In order to investigate the role of integrin activation on OPC migration, we used FN as ECM or substratum. Integrin αvβ1, αvβ3, αvβ5, and αvβ8 have been identified as FN receptors on the oligodendrocyte surface (Milner et al. 1997; Milner and Ffrench-Constant 1994). αvβ1 plays significant role in OPC migration (Milner et al. 1996). Our results show that when combined, the effects of PDGF-A and FN synergise. OPC on a FN substratum when exposed to 1 ng/ml PDGF-A migrates significantly further than either treatment alone.

Activation of PDGFRα by PDGF-A induces a series of intracellular signaling cascade events including phospholipase C-γ, PI3K, the Src family of tyrosine kinases, and MAPKs including ERK cascades (Heldin and Westermark 1999). There are a number of studies which proves that integrin outside signaling is similar to and overlaps with GFR signaling pathways, which independently signal to trigger the same signaling molecules. One of the overlapping signaling molecules between PDGFRα and integrins is ERK1/2 (Giancotti and Tarone 2003; Schwartz and Ginsberg 2002; Cabodi et al. 2004). It has also been shown that threshold levels of ERK phosphorylation are required to activate different downstream behaviors (Avrov and Kazlauskas 2003; Stork 2002; Frost et al. 2009). ERK1/2 is thought to be important in the regulation of cell adhesion and cytoskeleton network formation which ultimately leads to cell migration process (Fincham et al. 2000). Our previous study showed that a 30-min pulse of PDGF-A is sufficient to activate the ERK signaling pathway and that ERK regulates PDGF-A-induced OPC migration (Frost et al. 2009). Our earlier study showed that ERK1 levels were not affected by growth factor treatment, whereas ERK2 levels were significantly elevated at both high and low concentrations of PDGF-A, compared to untreated controls (Vora et al. 2011). The importance of ERK2 is well evident from the ERK2 deficient mice, which die early in development. (Hatano et al. 2003; Yao et al. 2003;

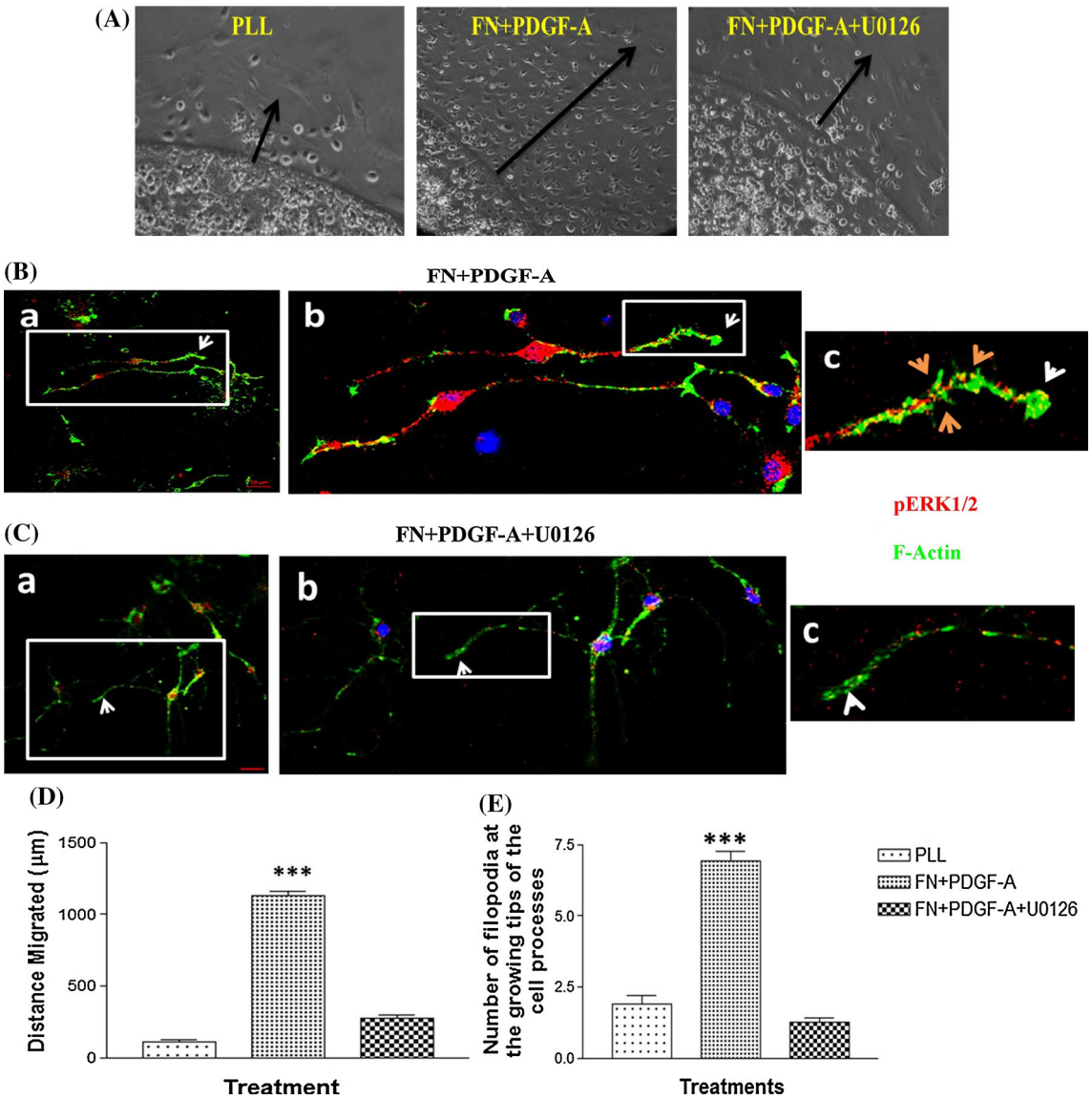

Fig. 3 pERK1/2 inhibition by U0126 significantly decreased the OPC migration and filopodia formation. **a** Phase contrast photomicrograph showing OPC migration in (FN + PDGF-A) with or without U0126. OPCs migrate away from the edge of the agarose drop. Migration was measured 72 h after the addition of growth factor. **b** and **c** Immunocytochemistry showing co-localization of F-actin and pERK1/2. OPCs were exposed to (FN + PDGF-A) with or without U0126 for 30 min. The cells were studied for the formation of microfilament extensions (filopodia). Cells were stained for (phalloidin 488-*green*) and pERK1/2 (*red*). Co-localization of F-actin and pERK1/2 shown in the (FN + PDGF-A) without U0126 (**b** *white arrows*) and the microfilament extensions from the cell process [**b**(*c*) *orange arrows*]. *Scale bar* **b**(*a*) 20 µm; **b**(*b*, *c*) are cropped images. But the Co-localization of F-actin and pERK1/2 was not observed in the (FN + PDGF-A) with U0126 (**c** *white arrows*). *Scale bar* **c**(*a*) = 20 µm; and **c**(*b*, *c*) are cropped images. **d** Graph showing the effect of (FN + PDGF-A) with or without U0126 on OPC migration **e** graph showing the effect of (FN + PDGF-A) with or without U0126 on the number of filopodia at the growing tips of the cell process. *Values* represent mean ± SEM. Statistical differences between the stimulated and control samples were determined by one way ANOVA-parameter (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (Color figure online)

Fincham et al. 2000; Saba-El-Leil et al. 2003). One of the studies showed that ERK1/2 is critical for the transition of OPCs from pre-progenitor to the late progenitor stage, but it is not essential for the transition of immature oligodendrocytes to the mature oligodendrocytes (Guardiola-Diaz et al. 2012). This shows the importance of ERK1/2 in early development of oligodendrocytes, during the migratory stage.

Our earlier studies confirmed that short-term exposure to PDGF-A activates only the migratory activity of OPCs (Frost et al. 2009; Vora et al. 2011). The long-term or continuous exposure is required for the proliferation by activating other intracellular pathways, such as PI3 K (Tsai et al. 2002; McKinnon et al. 2005). As discussed above, transient exposure to PDGF-A at a higher concentration

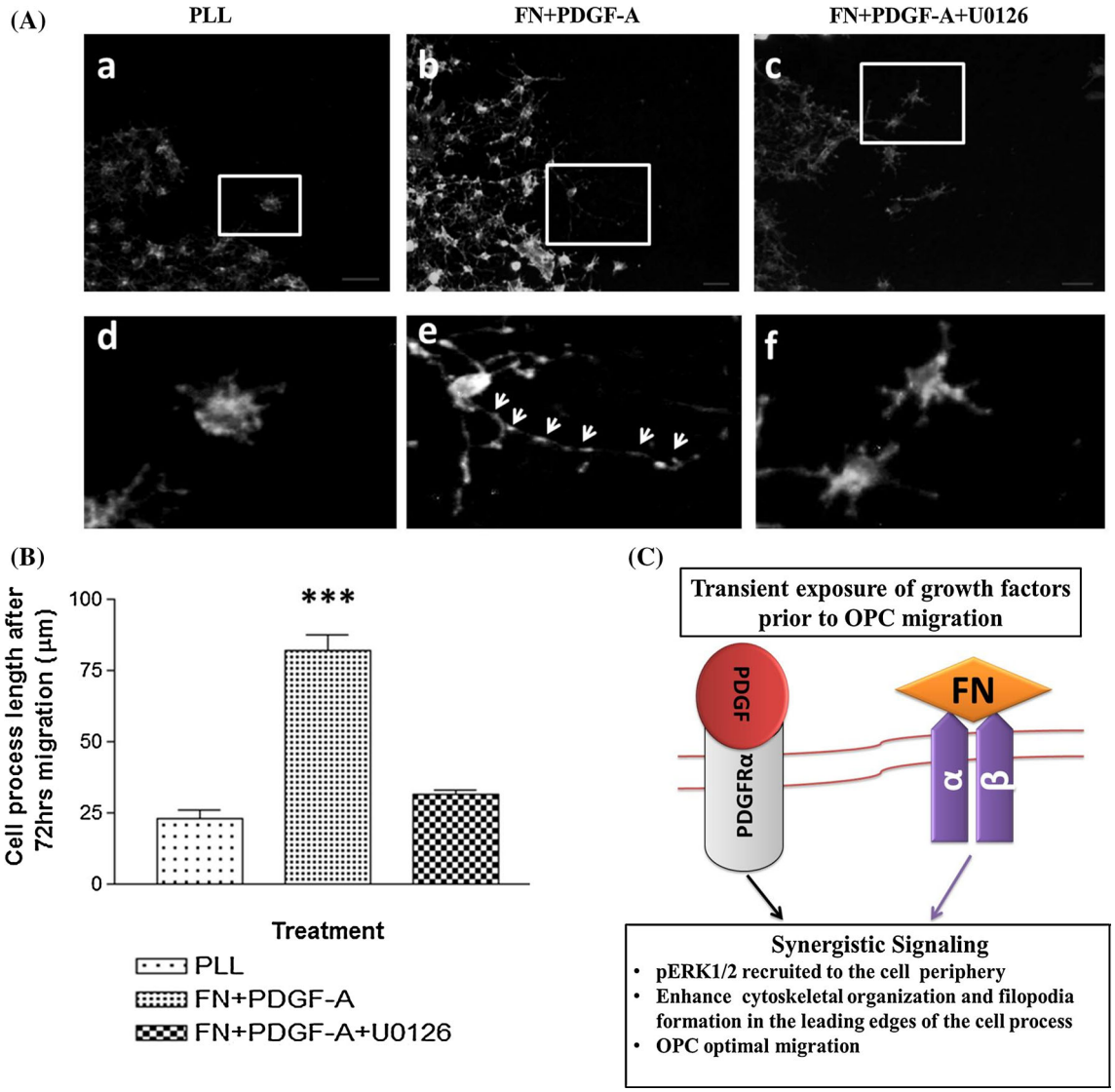

Fig. 4 pERK1/2 inhibition on FN + PDGF-A affected the cell process formation. **a** After 72 h of cell migration assay, the cells were stained for F-actin (phalloidin 488-*green*), here shown in *gray scale* and studied for the length of cell processes. *Scale bar* **a**(*a–c*) = 20 µm, **a**(*d–f*) are cropped images. Process length was significantly more in (FN + PDGF-A) compared to PLL and in presence of U0126 [*white arrows* (*e*)] **b** graph showing the effect of (FN + PDGF-A) with or without U0126 on length of cell processes. Each point represents the mean ± SEM of three separate experiments. $N = 3$ with 3 replicates. Data were evaluated using one way ANOVA parameter ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). **c** A schematic diagram showing PDGF-A and FN synergistic signaling model of OPC migration. FN alone initiates OPC migration and PDGF-A enhances and provides directionality. FN and PDGF-A combined stimulation results in pERK1/2 peripheral recruitment, cytoskeletal organization, and filopodia formation prior to OPC migration

(10 ng/ml) showed comparable OPC migration to that of continuously exposed groups. However, transient exposure to PDGF-A at a low concentration (1 ng/ml) which is near to physiological concentration (Baron et al. 2002; Brunmark et al. 2002; Vora et al. 2011), did not demonstrate any significant migration compared to control (untreated) cells (Vora et al. 2011; Frost et al. 2009). One possible explanation is receptor activation threshold levels and according to this, the sustained exposure of low concentration of PDGF-A is required to achieve threshold levels of receptor activation, resulting in OPC migration (Vora et al. 2011). Moreover, previous studies suggest that OPC migration induced by PDGFRα activation is regulated by cell-surface receptors and not by the endocytosed receptors which are responsible for OPC proliferation (Sorkin and Von Zastrow 2002; Heldin and Westermark 1999; Heldin and Ericsson 2001). In addition, we have shown that the short-term exposure to PDGF-A results in the activation of a self-regulating positive feedback loop of the ERK signaling pathway (Bhalla et al. 2002; Frost et al. 2009; Vora et al.

2011) which is involved in the sustained migration of OPCs. This signaling mechanism may allow PDGF to initiate OPC migration, which is then directed by a complex interaction of chemoattractive and chemorepulsive signals.

In the present study, we hypothesized that 1 ng/ml PDGF-A (physiological concentration) requires ECM (FN) synergy for the activation of ERK signaling pathway which is involved in the sustained migration of OP cells. Our data from the present study show that there was no significant difference in the protein levels of pERK1/2 in (PLL + PDGF-A) and (FN + PDGF-A) groups (Fig. 2). However, confocal imaging demonstrates that pERK1/2 was differentially localized in the cell process in both (PLL + PDGF-A) and (FN + PDGF-A) groups, respectively. OPCS exposed to both PDGF-A and FN showed the recruitment of pERK1/2 to the cell process in comparison to the PDGF-A alone treated and untreated control cells (Fig. 3). pERK1/2 is recruited to these peripheral sites of signal initiation prior to OPC migration. Our data demonstrate that upon the interaction of integrin's with FN, pERK1/2 is targeted to the cell periphery which also suggests the importance of FN on which the PDGF-A acts as a fuel for the pERK1/2 distribution. Our data confirm that the physiological concentration of PDGF-A (1 ng/ml) requires ECM synergy for sustained OPC migration.

Oligodendrocytes have a complex architecture, but are devoid of an intermediate filament system (Wilson and Brophy 1989; Barry et al. 1996; Norton et al. 1984). The orientation of the microfilaments within the cell appears to be random. They are situated immediately underneath the plasma membrane. They are thought to generate the mechanical forces necessary for diverse functions such as cell migration and development (Fukui and Kwang 1993; Gavin and Kwang 1997; Brandt 1998; Simpson and Armstrong 1999). Disruption or changes in the cytoskeleton and the aggregation of abnormal cytoskeletal proteins are the usual signs of neurodegenerative diseases (Goedert 2001; Forman et al. 2004). Polymerization of G-actin into F-actin and its subcellular localization plays important roles in the generation of the force required for cell movement (Lauffenburger and Horwitz 1996; Mitchison and Cramer 1996). During oligodendrocyte development, signals pertinent to process formation must be transduced into proper modifications in cytoskeletal organization. The oligodendrocyte lineage begins as a pre-progenitor cell, which then become migratory bipolar OPCs, ultimately differentiated into non-migratory multipolar oligodendrocytes (Armstrong 1998). Structural polarization is the initial criteria for cell migration (Lauffenburger and Horwitz 1996). Oligodendrocyte cells possess cone-like growth structures similar to neuronal growth cones, which are used to explore their environment (Schmidt et al. 1997). In the present study, we have examined the effects of PDGF-A and FN to further interpret the distribution and organization of microfilaments in OPCs prior to migration. Combined treatment (PDGF-A + FN) resulted in the enhanced microfilaments in the cell process (Fig. 2c). A previous study reported the direct or indirect interactions of microfilaments and microtubules (Novak and Titus 1997). Microtubules follow microfilaments at the leading edges of the process and new branching sites during the branch formation and process extension in oligodendrocytes (Song et al. 2001). Studies suggest that during neuronal growth cone turning, intact microfilaments are essential for microtubule reorientation (Challacombe et al. 1996). Hence, the adverse effect on microfilaments directly affects the microtubules function.

Cells use filopodia to sense and probe the environment or surroundings. These are thin, actin-rich plasma-membranes which protrude from the cell membrane (Mattila and Lappalainen 2008). Filopodia play a significant role in cell migration, neurite outgrowth, and as precursors for dendritic spines in neurons. Neurite outgrowth in the case of cortical neurons, is dependent on filopodia formation (Dent et al. 2007; Jang et al. 2010). Process outgrowth by oligodendrocytes is more or less same as neurite outgrowth. Accurate polymerization and crosslinking of actin filaments followed by convergence are required for filopodia formation. In general, filopodia are formed first and are then stretched out and transformed into lamellipodia by microfilament branching. Hence the leading edge of the oligodendrocyte process is pushed out (Bauer et al. 2009). There are studies which confirm the importance of filopodia in OPCs. Time-lapse imaging revealed that OPCs in the cortex are highly dynamic. With the motile filopodia, OPCs survey their local environment, extend growth cones, and continuously migrate (Hughes et al. 2013). In many chronically demyelinated white matter lesions, the impaired OPC homeostasis may lead to insufficient local interactions, which are required to stimulate oligodendrogenesis along with detection of demyelinated axons (Franklin et al. 1997; Chang et al. 2000; Hughes et al. 2013). In the present study, we have demonstrated that PDGF-A and FN collectively enhanced filopodia formation prior to OPC migration. In OPCs, F-actin was found in filopodia extending from the distal point of the leading edge process. The better understanding of the various actin-associated proteins during the initiation and elongation of filopodia will provide an insight into the mechanisms of filopodia formation in oligodendrocytes. The focal restoration of filopodia bearing OPC numbers would enhance the OPC migration potential in chronic lesions that may be therapeutically beneficial in MS (Hughes et al. 2013). Finally, the present study also demonstrates a critical role for pERK1/2 in OPC migration and cytoskeleton organization, which was confirmed using U0126 inhibitor studies (Fig. 3).

In summary, the data from the present in vitro study support our hypothesis that optimal OPC migration is controlled by the dual signaling of FN and PDGF-A, which binds to two different families of cell-surface receptors, i.e., integrins and PDGFRα (Fig. 4c). Data from the current study suggest that the parallel signaling pathways of both FN and PDGF-A are required to reconcile the extent of the signals and are also strictly coordinated in terms of their strength/biological activity resulting in enhanced cell migratory behavior. Hence, the present study proposes that one of the driving factors for significant migration observed in the combination group (FN + PDGF-A) may be PDGFRα-integrin interactions that promote the formation of focal adhesions, providing the translocation machinery for pERK1/2, which is relocated to the filopodia of the migrating cell. By understanding the above interactions, regulation of the ECM secretion and the controlled release of GFs at the sites of demyelinated lesion may serve as a driving force for the directional OPC migration in the demyelinating diseases.

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Compliance with Ethical Standards

Conflict of Interest The authors report no conflicts of interest.

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