Chapter-3

To study the combinatorial effects of PDGF-A and ECM on OPC migration

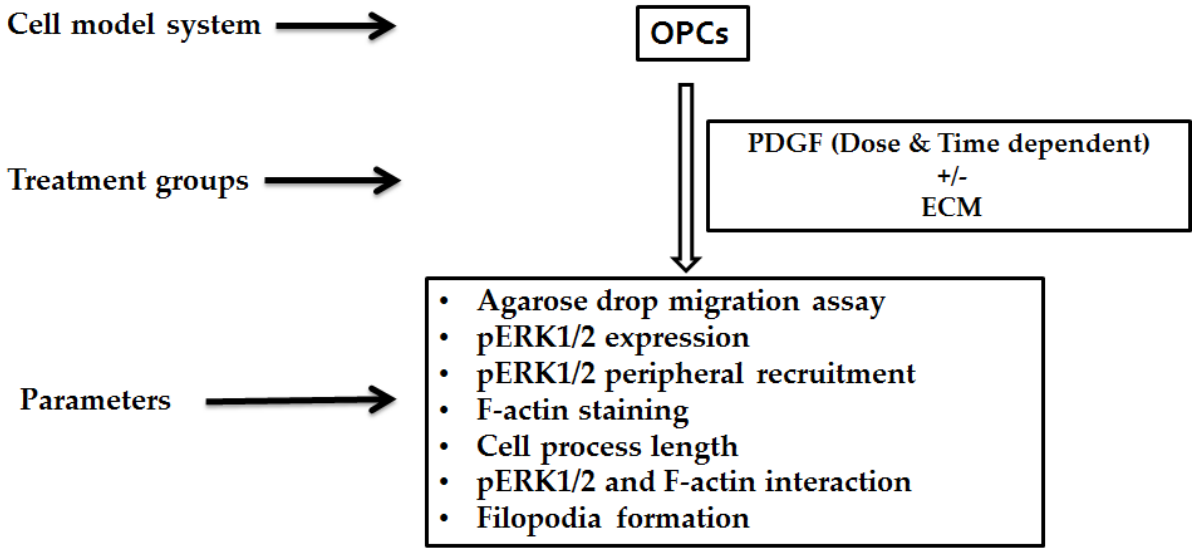
3.1 Introduction

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 embryonic lethal (Soriano 1997)and (Li et al. 1996). 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 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 mesh works which then generates mechanical forces which further helps a cell to migrate (Fukui 1993; Gavin 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. The present study demonstrate that how physiological concentration of PDGF-A can combine with FN to augment OPC migration *in-vitro*. However, higher concentration of PDGF-A doesn't require ECM engagement to enhance OPC migration.

3.2 Plan of work



3.3 Results

3.3.1 Individual effects of PDGF-A and ECM on OPC migration

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 (Fig. 3.1A). The dose dependent effect of PDGF-A on OPC migration was studied using an agarose drop migration assay (Frost et al. 2009; Frost et al. 2000; Varani et al. 1978) for 72 hrs. Two doses of PDGF-A were used, 1 ng/ml and 10ng/ml as mentioned in material and methods (Chapter 2).

Here, we show cells exposed to FN resulted in enhanced migration compared to cells exposed transiently to PDGF-A (1 ng/ml) (Fig. 3.1B, C). However, although not significant ($P>0.05$), cells migrating on an FN substratum migrate farther than cells exposed to 1ng/ml PDGF-A. This result demonstrates that OPCs migrate on FN even in the total absence of PDGF-A. This indicates the importance of ECM more than

growth factor alone at the physiological level (PDGF-A 1 ng/ml). However, OPC transiently exposed to PDGF-A at 1 ng/ml, which is closer to physiological concentrations do not migrate to the same extent as OPC transiently exposed to 10ng/ ml dose of PDGF-A (Fig. 3.1B, C). Transient exposure to PDGF-A at 10 ng/ml for 30 min resulted in significantly enhanced migration than cells exposed to PDGF-A at 1 ng/ml ($P<0.001$) (Fig. 3.1B, C).

3.3.2 Physiological dose of PDGF-A require FN engagement to augment OPC migration

Our earlier studies have shown 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. 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. 2009; Frost et al. 2000) for 72 hrs. Here, we show cells exposed to FN resulted in enhanced migration compared to cells exposed transiently to PDGF-A (1ng/ml), at (630.5 ± 38.56 μm) and (319.75 ± 35.66 μm), respectively (Fig. 3.2A, B). These results demonstrates that OPCs migrate on FN even in the total absence of PDGF-A. Overall, the data also pinpoints the importance of ECM more than GF alone at the physiological level (PDGF-A 1ng/ml). OPC migration was significantly high in [FN + PDGF-A (1ng/ml)] group compared to [PLL + PDGF-A (1ng/ml)] at (1170.41 ± 38.30 μm) and (319.75 ± 35.59 μm), respectively ($P<0.01$) (Fig. 3.2A, B). 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.

One of the study 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. 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. 3.2C, D, 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 compared to other three groups ($P<0.001$) (Fig. 3.3A, B). We have confirmed the OPC stage by double immunostaining of PDGFRα and pERK1/2 (Fig. 3.3A).

3.3.3 Physiological dose of PDGF-A require FN engagement to promote 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 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. 3.3C). 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. 3.3C (e-h)]. 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 of ‘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).

We further verified these observations by 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 10μM (Fig. 3.4 A, B, C). OPC migration was reduced by 75% in the presence of 10μM U0126. OPC migration was significantly reduced from (1133.66 ± 27.86 μm) after transient exposure to (FN + PDGF-A) to (278.58± 32.31μm) in the presence of U0126. (n = 3 with 6 replicates; P <0.001) (Fig. 3.4 D, G). Our results indicate that PDGF-A induced pERK1/2 is recruited to the OPC processes on FN but not on PLL (Fig. 3.4 E, F). 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. 3.4 E, F, H). The data from the current study demonstrates that pERK1/2 inhibition significantly decreased the (FN + PDGF-A) induced cell process formation after the 72hrs of OPC migration (Fig. 3.5 A and 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.

3.3.4 Higher concentration of PDGF-A doesn't require FN engagement to augment OPC migration

We next examined, whether higher dose of PDGF-A (10ng/ml) require FN engagement to augment OPC migration. There was no significant difference between the [PLL +PDGF-A (10ng/ml)] and [FN + PDGF-A (10ng/ml)] (Fig. 3.6A, B). Then we analyzed the pERK1/2 expression by western blot analysis. There was no significant difference between the [PLL + PDGF-A (10ng/ml)] and [FN + PDGF-A (10ng/ml)] groups (Fig. 3.7A, B, C). Moreover we have studied the F-actin staining and filopodia formation in these groups. PDGF-A (10ng/ml) alone enhanced the F-actin staining and filopodia formation (Fig. 3.8A, B, C). This shows that higher concentration of PDGF-A didn't require FN engagement to augment OPC migration.

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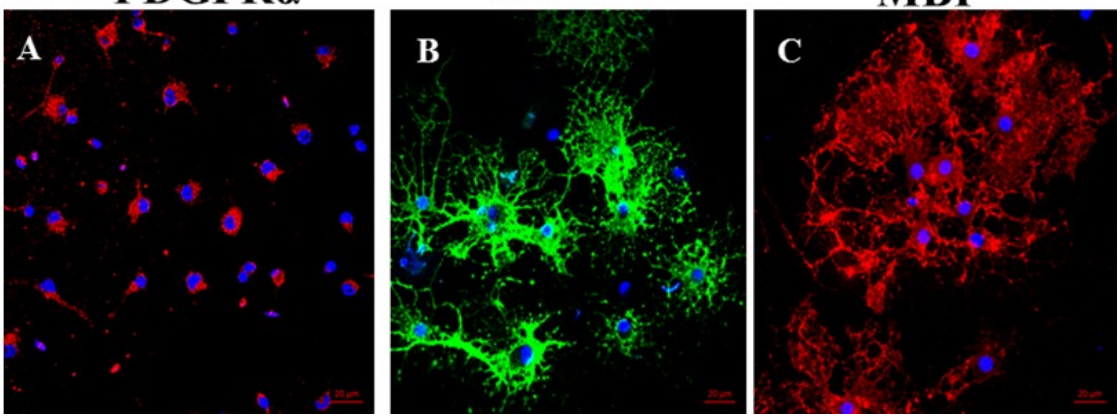
3.1(A)



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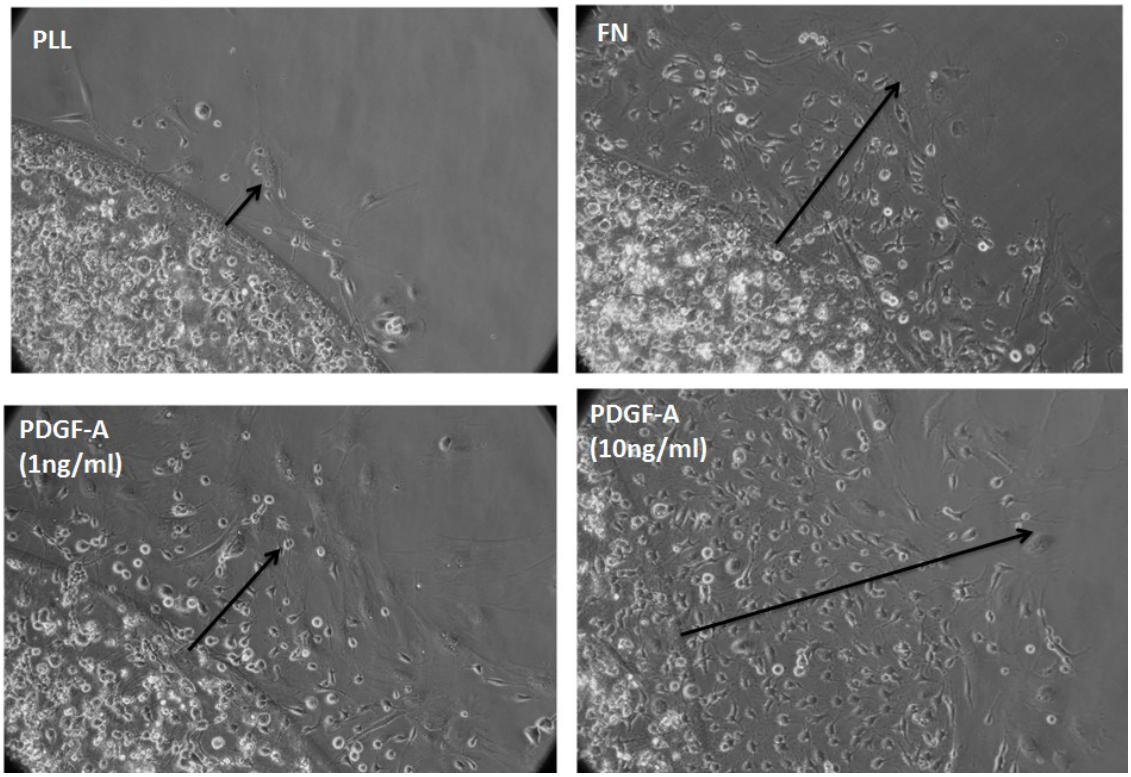


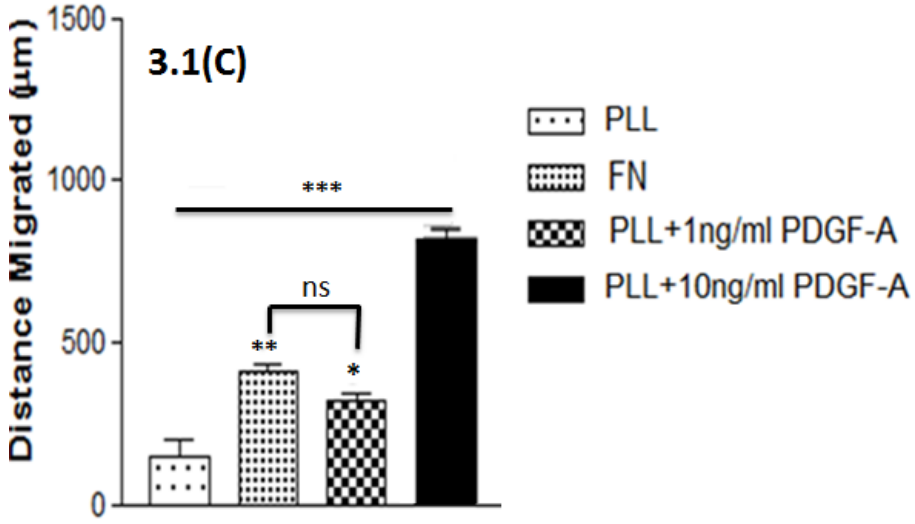


Fig. 3.1- Characterization of oligodendrocyte with cell specific markers.

(A) *After overnight shaking, isolated oligodendrocytes were fixed at 12-hours, 4th day and 10th day, followed by immunostaining. Representative Immunostaining images showing a-12-hour OPCs with anti-PDGFRα (red), b-4th day immature oligodendrocytes with anti-O4 (green), c- 10th day mature oligodendrocytes with anti-MBP (red). The nucleus was stained with DAPI (blue). Scale bar=20µm* ***(B)-****Agarose drop migration assay (Frost et al., 2000 and Vora et al., 2011) in response to PDGF-A and FN treatment. Phase contrast photomicrograph showing PLL, FN, PDGF-A (1ng/ml) and PDGF-A (10ng/ml). OPCs migrate away from the edge of the agarose drop. Migration of OPCs in suspension (40,000 cells per well). These are 20X phase contrast images. Migration was measured 72 h after the addition of growth factor.* ***(C)*** *Graph showing the effects of PLL, FN, PDGF-A (1ng/ml) and PDGF-A (10ng/ml) 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).*

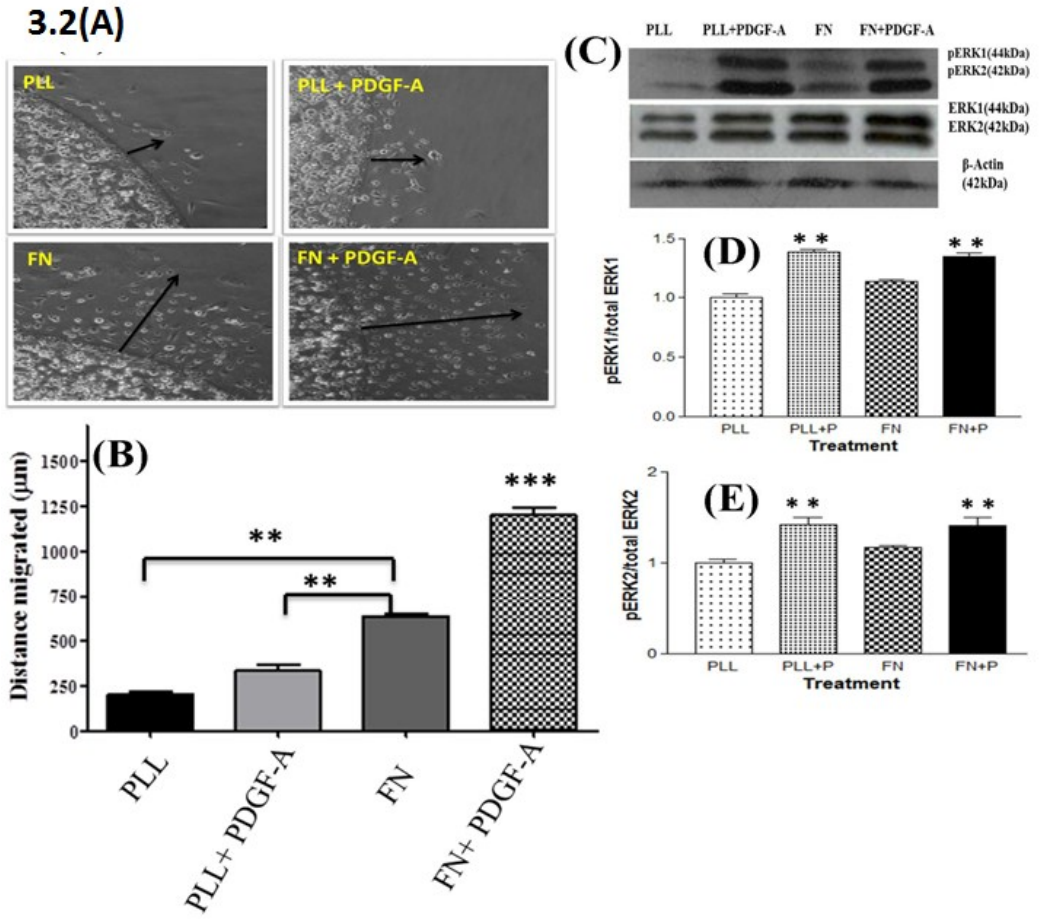
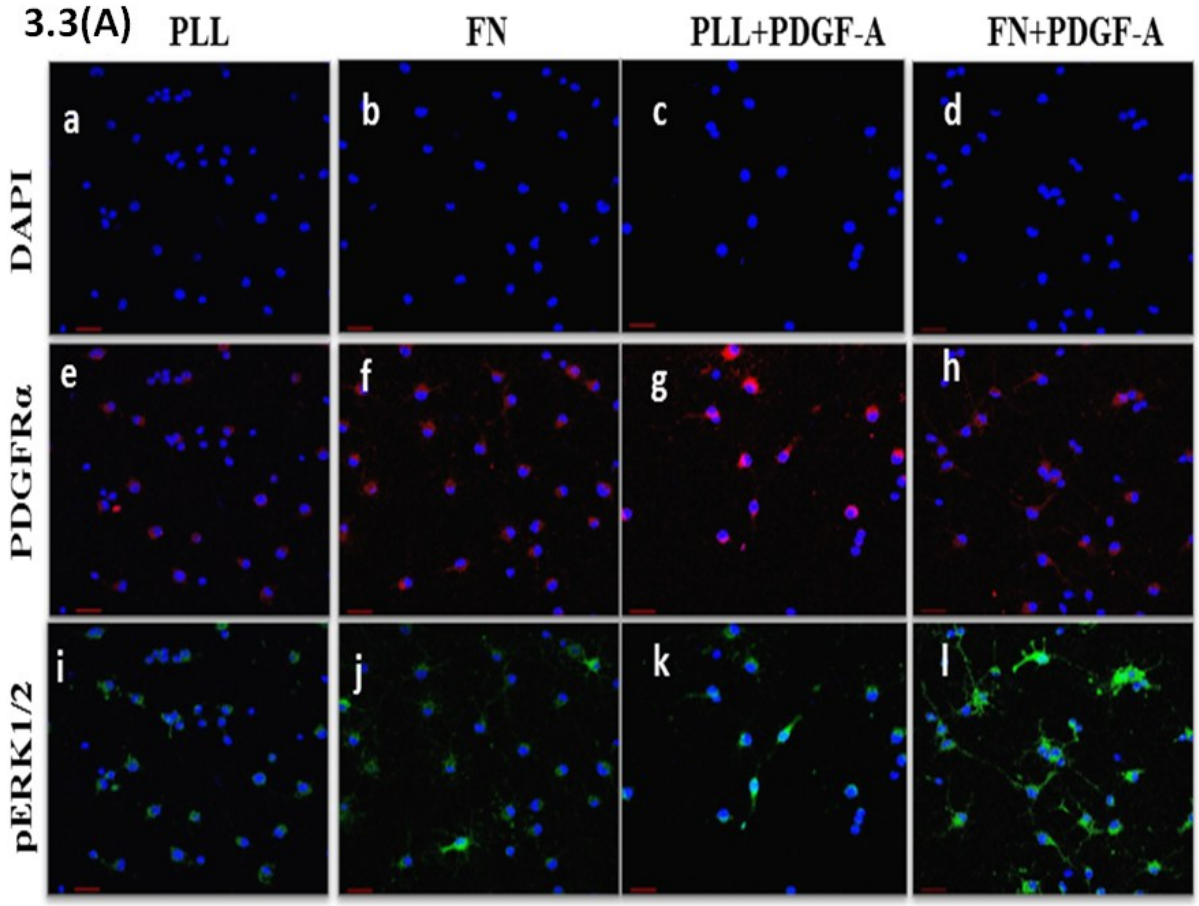


Fig. 3.2 – Physiological dose of PDGF-A (1ng/ml) require FN to stimulate OPC migration and ERK1/2 phosphorylation.

(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).*



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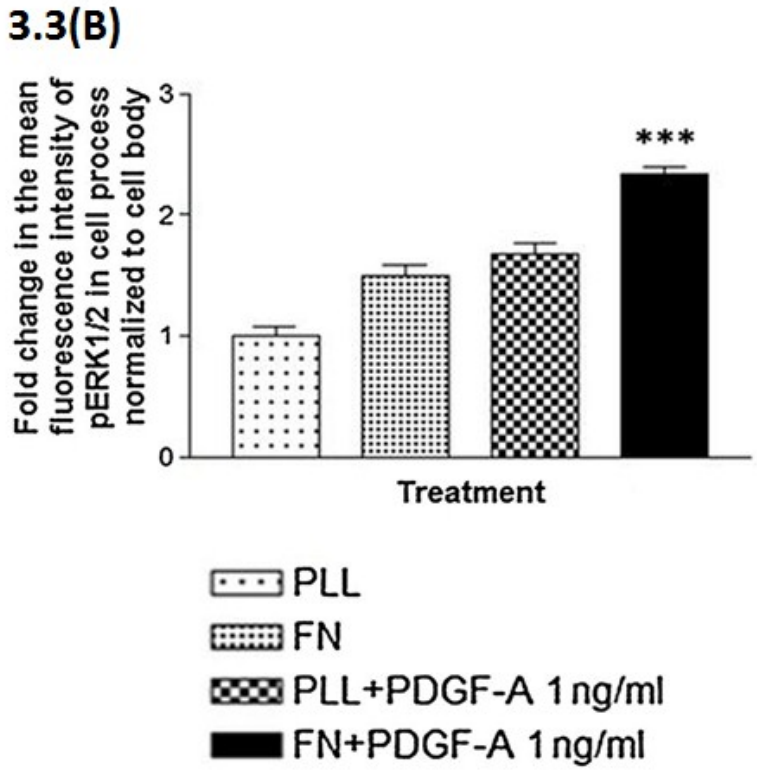
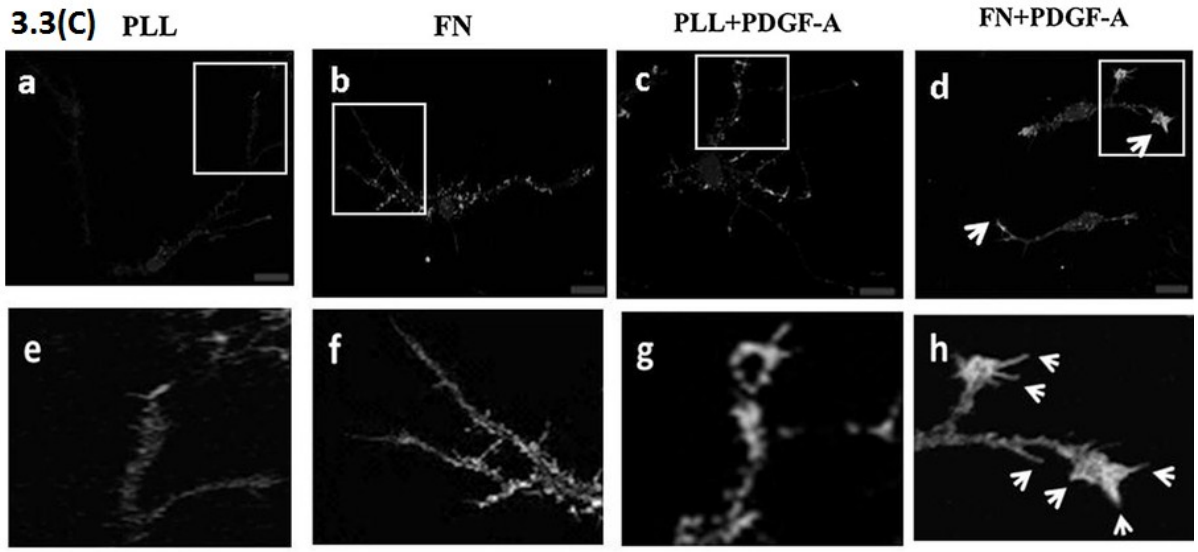
To study the combinatorial effects of PDGF-A and ECM on OPC migration





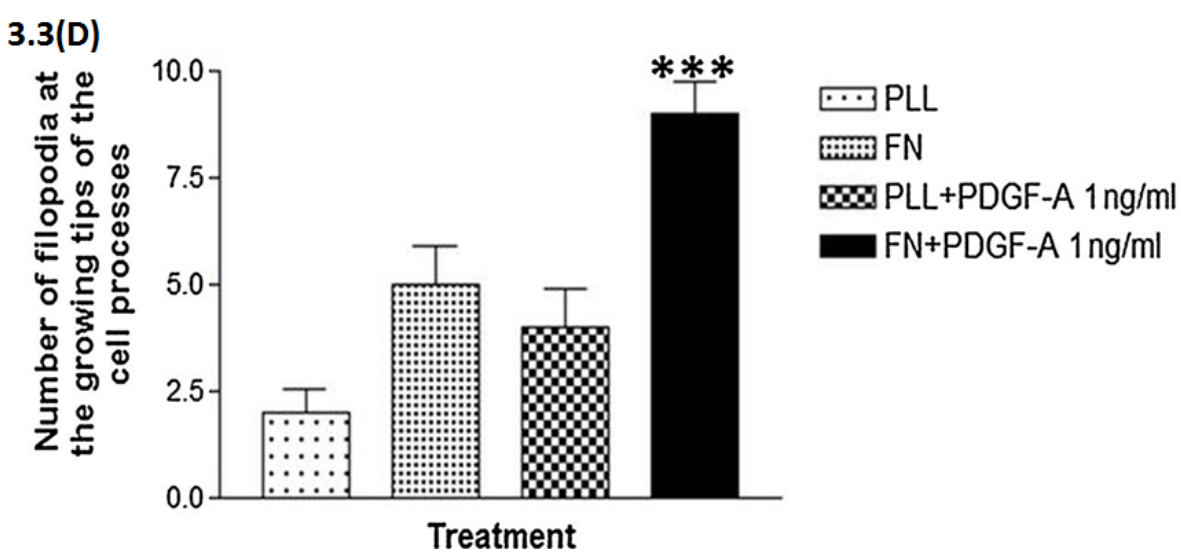


Fig. 3.3- pERK1/2 peripheral recruitment and filopodia formation.

(A) *OPCs were exposed to FN, PDGF-A & in combination for 30 min. Nucleus was stained with DAPI (blue; a-d), PDGFRα (anti- PDGFRα red; e-h) & pERK1/2 (anti-pERK1/2 green; i-l). Scale bar=20µm. We have stained the OPCs for PDGFRα in the same cell with pERK1/2 stained, to confirm that the OPCs were in progenitor stage.* ***(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 phalloidinAlexa 488-green to study F-actin organization [C(a-d)]. Images are shown in grey scale. Scale bar =10µm. C(e-h) are cropped images of C(a-d), to show the microfilament extention (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)*

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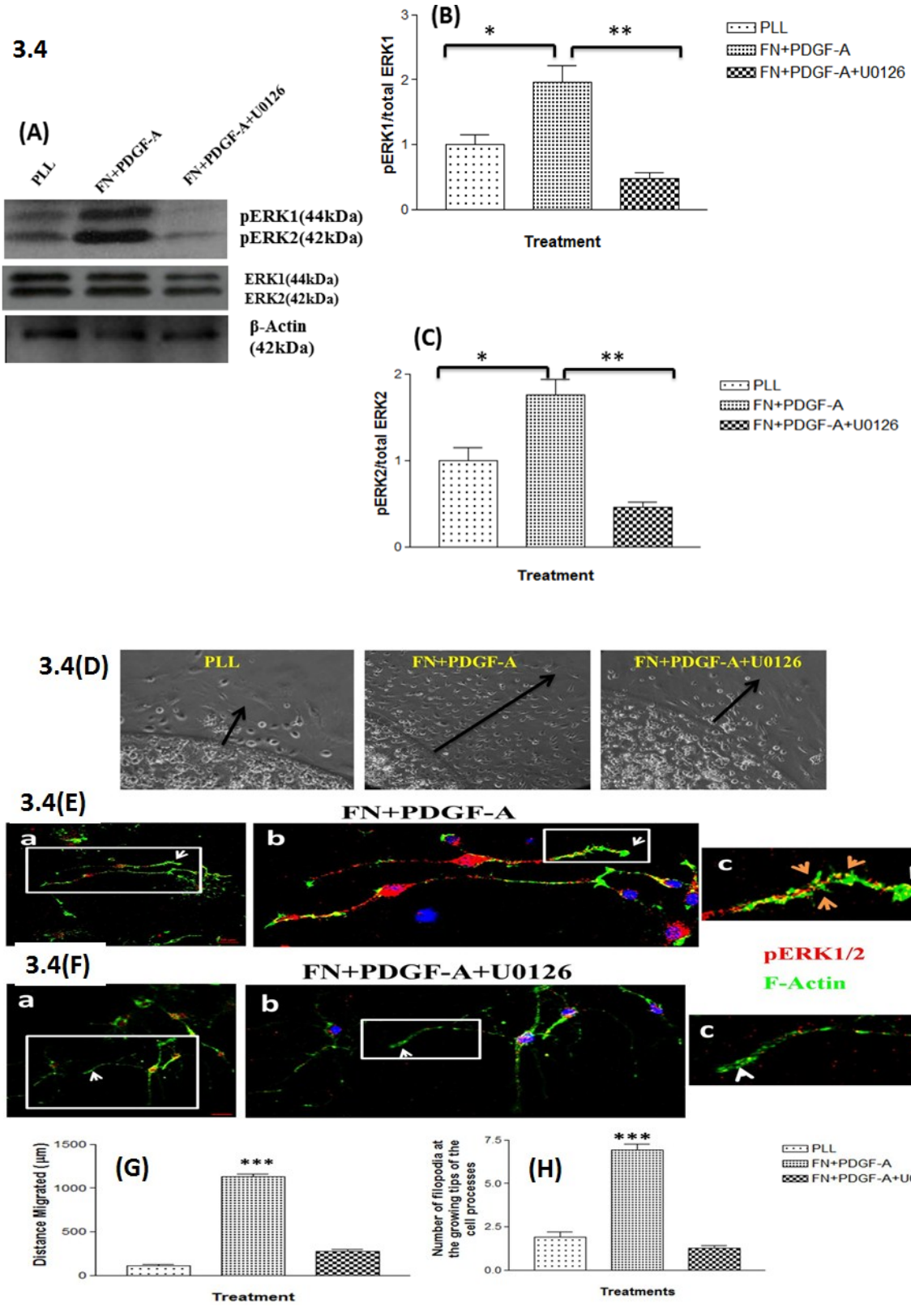


3.4(I)

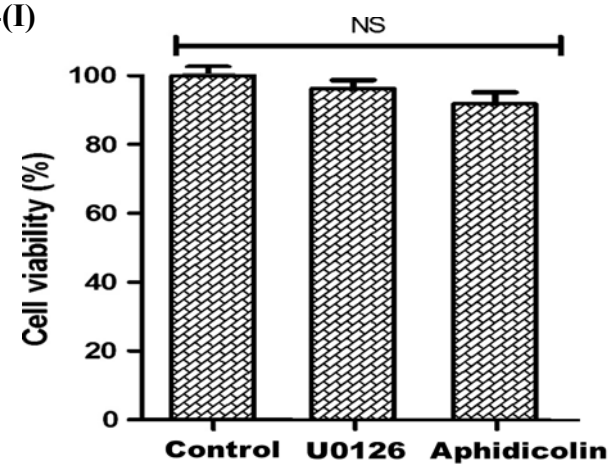


Fig. 3.4- pERK1/2 inhibition by U0126 significantly decreased the OPC migration and filopodia formation.

(A) *Representative Western blot for phosphorylated and total ERK of OPCs treated with PDGF-A + FN with or without U0126.* ***(B-C)*** *After densitometric analysis, pERK1/2 data were normalized to total ERK1/2 and represented in graphical form. 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).* ***(D)*** *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.* ***(E)*** *and* ***(F)*** *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 F-Actin (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 E(a)=20µm; E(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 F(a)=20µm; and F(b,c) are cropped images. ***(G)*** *Graph showing the effect of (FN + PDGF-A) with or without U0126 on OPC migration* ***(H)*** *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)* **(I)** Effects of inhibitors U0126 and aphidicolin were checked on the OPC survival. Data shows that no significant difference between control and inhibitors treated OPCs.

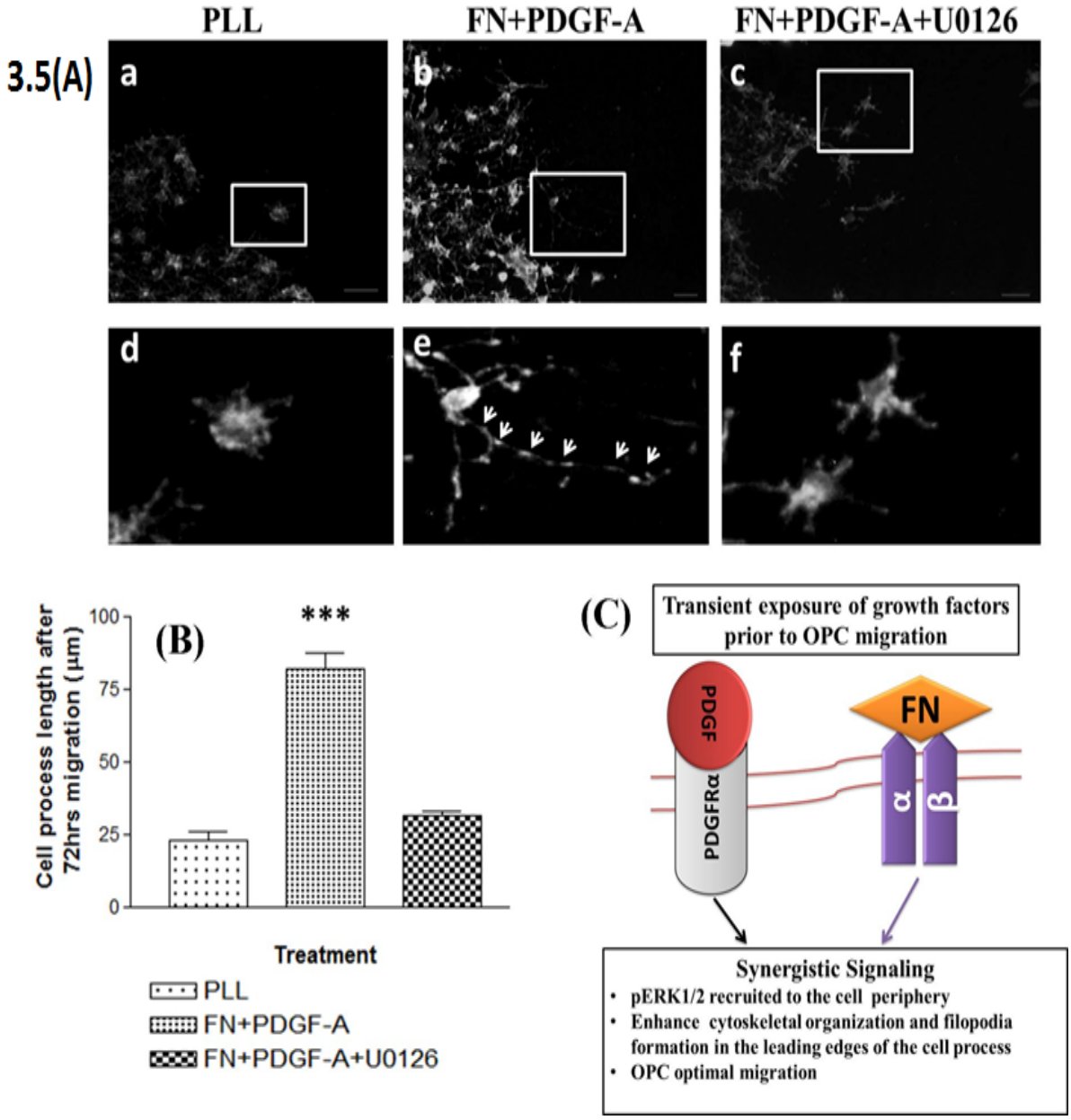
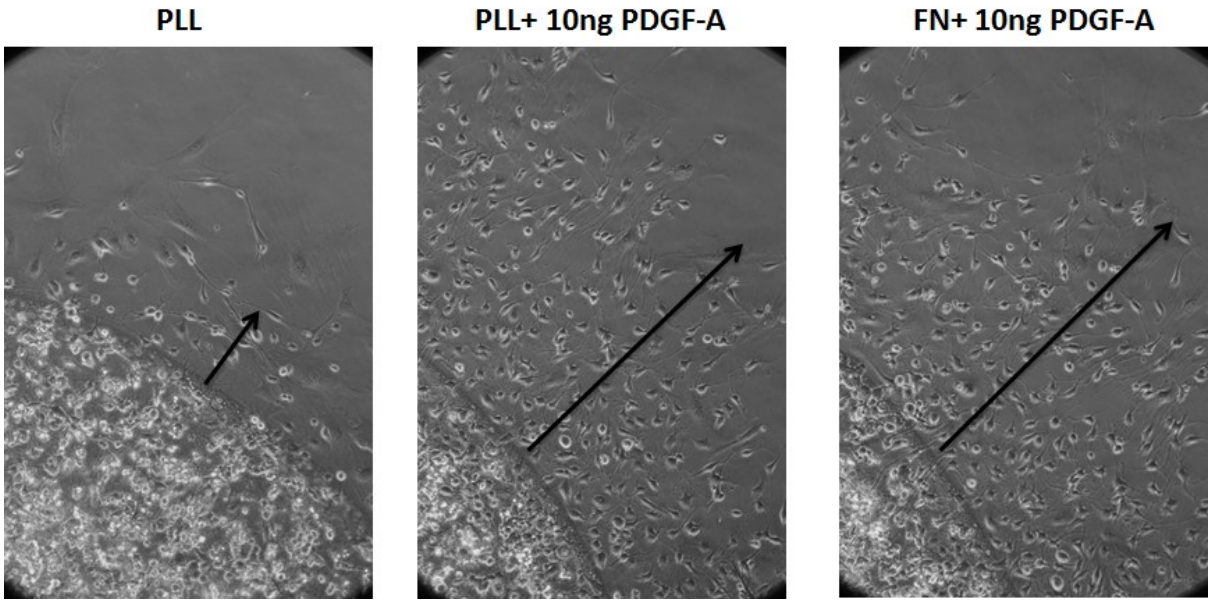


Fig. 3.5- pERK1/2 inhibition on FN + PDGF-A affected the cell process formation. ***(A)*** *After 72hrs of cell migration assay the cells were stained for F-Actin (phalloidin 488-green), here shown in grey scale and studied for the length of cell processes. Scale bar A(a-c) =20µm , A(d-f) are cropped images. Process length was significantly more in (FN+PDGF-A) than 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.*

3.6(A)



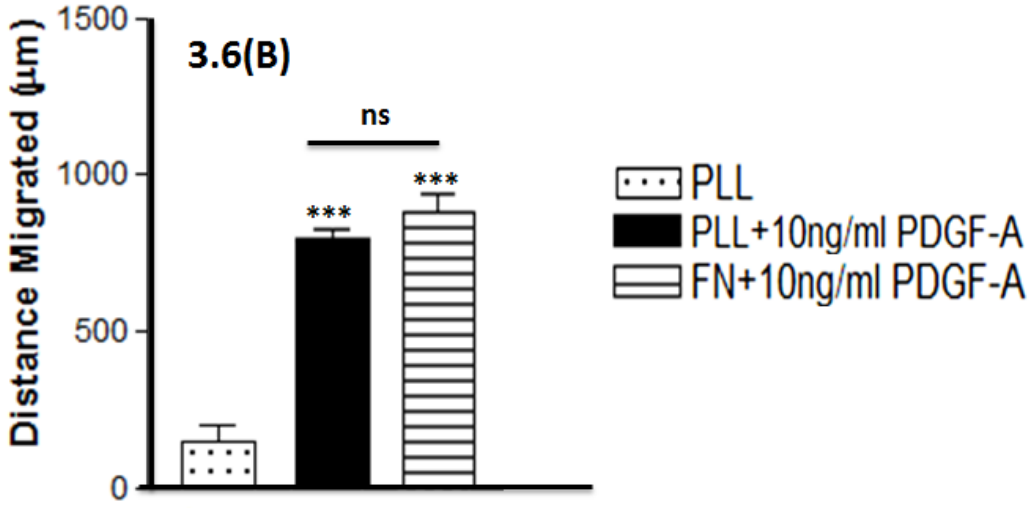


Fig.3.6 Individual and combined effects of PDGF-A (10ng/ml) and FN on OPC migration

(A) *Phase contrast photomicrograph showing OPCs migrate away from the edge of the agarose drop after PDGF-A (10ng/ml) with or without FN. 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).*

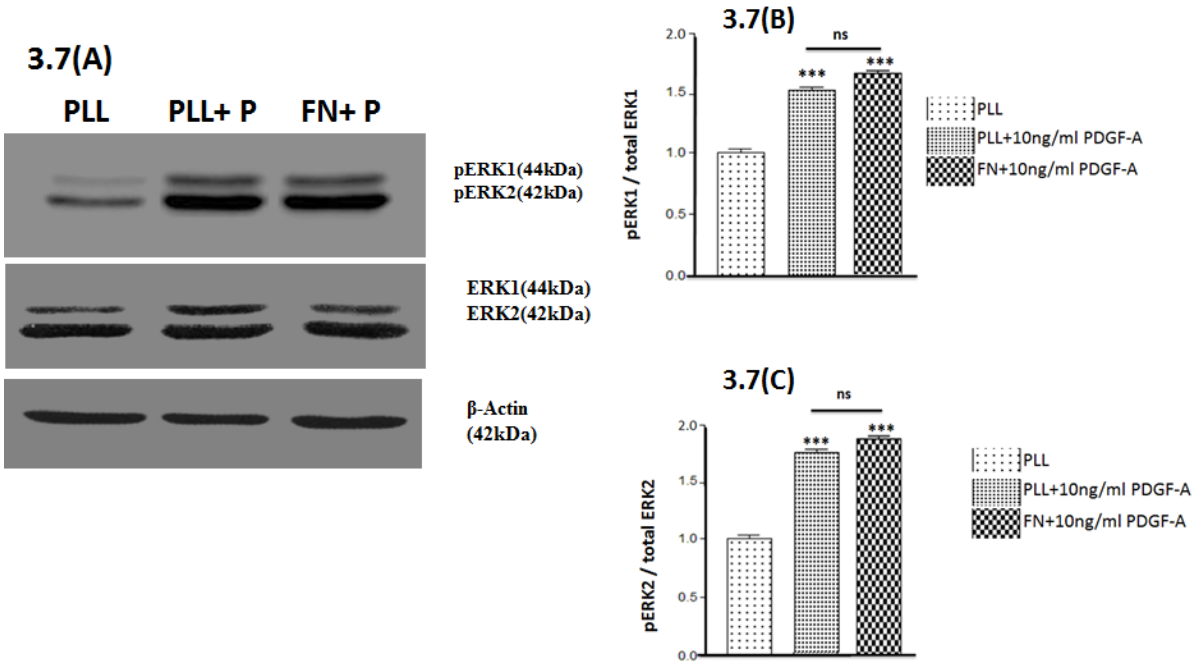
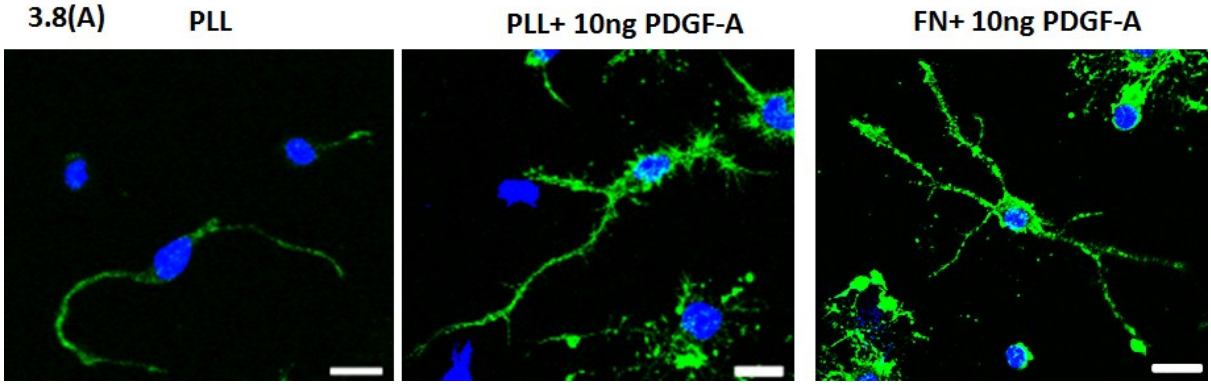


Fig.3.7 Individual and combined effects of PDGF-A (10ng/ml) with FN on ERK1/2 phosphorylation.

(A)- *Representative Western blot for phosphorylated and total ERK1/2 after treatment of OPCs with PDGF-A alone and in combination with FN.* ***(B) and (C)*** *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).*



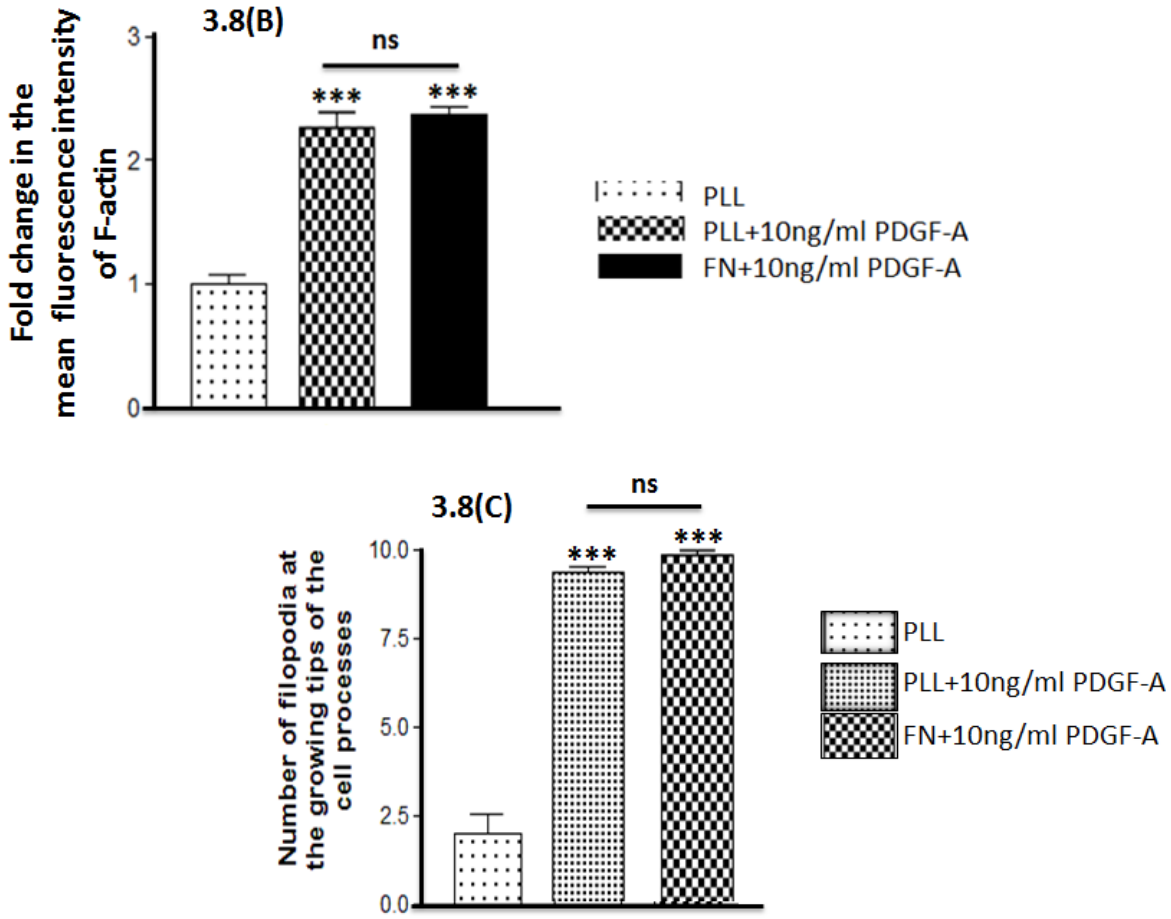


Fig. 3.8 ***Individual and combined effects of PDGF-A (10ng/ml) with FN on F-actin staining and filopodia formation***

(A) *OPCs were exposed to PDGF-A (10ng/ml) alone & in combination with FN. PLL was taken as control. The cells were stained with phalloidinAlexa 488-green to study F-actin organization [A(a-c)]. Scale bar =10µm.* ***(B)*** *Graph shows the fold change in the mean fluorescence intensity of F-actin in OPCs exposed to PDGF-A (10ng/ml) with or without FN.* ***(C)*** *Graph shows the number of filopodia at the growing tips of the cell process in OPCs treated with PDGF-A (10ng/ml) with or without FN. 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)*

3.4 Discussion

PDGF-A plays a substantial role in OPC migration (Armstrong et al. 1991; Frost et al. 1996; Frost et al. 2009; Tsai et al. 2002). PDGF-A is crucial for normal myelination of the CNS (Calver et al. 1998; Tsai et al. 2002). Studies have confirmed that OPC migration doesn't require continuous exposure to PDGF-A and it is regulated by the ERK signaling pathway (Frost et al. 2009; Frost et al. 2000; Tsai et al. 2002). Earlier study (Vora et al. 2011), showed that transient exposure to PDGF-A at low concentration (1ng/ml) didn't show any significant migration compared to the control cells. Even so the transient exposure at 10ng/ml showed comparable OPC migration to that of continuously exposed cells (Vora et al. 2011). However, the involvement of ECM in the regulation of PDGF-A mediated OPC migration was not extensively explored. Integrin-linked kinase play key role in OPC migration (O'Meara et al. 2016). In the present study, we have checked the individual and combined effects of PDGF-A and FN. There were two concentrations of PDGF-A used; one was 1ng/ml (physiological concentration) and 10ng/ml (higher concentration), while, FN was used as an ECM in the current study. Earlier studies have shown that αvβ1 integrin plays significant role in OPC migration on fibronectin. Fibronectin is a recognized ligand for αvβ1 whereas, laminin, is a ligand for integrin α6β1 (Milner et al. 1996). α6β1-integrin is responsible for laminin mediated enhancement of growth factor survival signalling. Another study showed that when OPCs were grown on vitronectin in the presence of PDGF-A, it was found that αvβ3-integrin physically interact with PDGFR-α, which is responsible for the proliferation of OPCs (Baron et al. 2002). Hence, the best ECM candidate to study the OPC migration was fibronectin, whereas laminin is involved in the PDGF-A dependent survival and vitronectin in the PDGF-A dependent proliferation signalling in OPCs. PLL was used as a control substratum. PLL is a biologically inert coating that allows cells to adhere to the culture surface. PLL enhances electrostatic interaction between negatively-charged ions of the cell membrane and positively-charged ions on the plastic. As discussed above, in order to investigate the role of integrin activation on OPC migration, we used FN as ECM or substratum. Integrins 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 synergize. OPC on a FN substratum, exposed to 1ng/ml PDGF-A migrate significantly further than either treatment alone. However, higher concentration of PDGF-A (10ng/ml) doesn't require prior activation of integrin via FN engagement to enhance the OPC migration.

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-in 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). 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). 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; Fincham et al. 2000; Saba-El-Leil et al. 2003). One of the study 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.

Chapter-3

To study the combinatorial effects of PDGF-A and ECM on OPC migration

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 PI3K (Tsai et al. 2002) (McKinnon et al. 2005). As discussed above that transient exposure to PDGF-A at a higher concentration (10ng/ml) showed comparable OPC migration to that of continuously exposed groups. However, transient exposure to PDGF-A at a low concentration (1 ng/ml) simulates nearly the physiological concentration (Baron et al. 2002; Brunmark et al. 2002; Vora et al. 2011), did not demonstrate any significant migration compared to the 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 signalling pathway (Bhalla et al. 2002; Frost et al. 2009; Vora et al. 2011) which is involved in the sustained migration of OPCs. This signalling mechanism may allow PDGF-A to initiate OPC migration, which is then directed by a complex interaction of chemoattractive and chemorepulsive signals.

In the present study, we hypothesized that 1ng/ml PDGF-A (physiological concentration) require ECM (FN) synergy for the activation of ERK signalling pathway which is involved in the sustained migration of OP cells. Data from the present study show that there was no significant difference in the protein levels of pERK1/2 in [PLL + PDGF-A (1ng/ml)] and [FN + PDGF-A(1ng/ml)] groups (Fig. 3.2C, D, E). However, confocal imaging demonstrates that pERK1/2 was differentially localized in the cell process in both [PLL + PDGF-A(1ng/ml)] and [FN + PDGF-A(1ng/ml)] 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.3.3). pERK1/2 is recruited to these peripheral sites of signal initiation prior to OPC migration. Our data demonstrates 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. Finally, results confirm that the physiological concentration of PDGF-A (1ng/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 1993; Gavin 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 play 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 pre-progenitor cells, 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 growth cone-like 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 [FN + PDGF-A (1ng/ml)] resulted in the enhanced microfilaments in the cell process (Fig. 3.3C). However, PDGF-A (10ng/ml) was alone sufficient to increase the

microfilament staining in the cell process (Fig. 3.8 A, B). 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 (Jang et al. 2010)(Dent et al. 2007). Process outgrowth by oligodendrocytes is more or less same as neurite outgrowth. Accurate polymerization and crosslinking of actin filaments followed by convergence is 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 oligodendroctes. 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). Recently it has been shown that electric signals regulate directional OPC migration by β1 integrin associated with actin cytoskeleton (Zhu et al. 2016). Finally, the present study also demonstrates a critical role for pERK1/2 in OPC migration and cytoskeleton organization, which was confirmed by using U0126 inhibitor studies (Fig. 3.4). However, the higher concentration of PDGF-A (10ng/ml) didn't require FN engagement to enhance the OPC migration (Fig. 3.6). Higher concentration of PDGF-A alone was able to increase the pERK1/2 levels (Fig. 3.7). Moreover, F-actin staining and filopodia formation by the [FN+PDGF-A (10ng/ml)] was not significantly higher than [PLL+PDGF-A(10ng/ml)] group(Fig. 3.8).

Recent study has shown that overexpression of PDGF-A in OPCs can repair spinal cord injury (Yao et al. 2017). It would be very interesting to see how the higher concentration of PDGF-A (10ng/ml) can modulate the OPC migration machinery without involvement of ECM. For that we have hypothesized that "PDGFRα activation causes integrin switching, lipid raft microenvironment formation and cytoskeletal rearrangement", which is discussed in detailed in the chapters 4 and 5.