# CHAPTER - THREE

## 3. To determine the role of ERK1/2 in the regulation of OPC migration and cytoskeletal reorganisation

### 3.1 Introduction

OPC migration is guided by many soluble and membrane bound cues(Kessaris et al., 2006; Richard Milner et al., 1997) to ensure that cells reach their final destination. Secreted molecules involved in regulation of OPCs migration are growth factors like Platelet derived growth factor (PDGF), Fibroblastic growth factor (FGF) or Hepatocyte growth factor (HGF), chemotropic molecules like netrins and secreted semaphorins, and the chemokines CXCL1.PDGF-A, a chemotactic cue generated by astrocytic glial cells in the central nervous system, serves as a major potent motogen for OPCs(Calver et al., 1998; E. E. Frost et al., 2009). Recently vascular endothelial growth factor (VEGF-A) has been demonstrated to promote OPC migration by regulating the actin reorganization and FAK-Paxillin interaction(Hayakawa et al., 2011).

Activation of PDGFRα by the growth factor PDGF-A, enhances OPC migration (RC Armstrong et al., 1990; E. E. Frost et al., 2009) and proliferation (Fruttiger et al., 1999; N. Pringle et al., 1989).Also, It is known that PDGF-A promotes OPCs migration through the non receptor tyrosine kinase Fyn which phosphorylates Cdk5. Cdk5 phosphorylates WASP (Wiskott–Aldrich syndrome protein)-family verprolin homologous protein 2 (WAVE2) at Ser-137 which further cause regulation of migration(Miyamoto et al., 2008). Another group of studies found that PDGF-A regulate the OPC migration through the ERK signalling pathway(E. E. Frost et al., 2009; Vora et al., 2011). Extracellular regulated kinase (ERK) is one of three major groups of MAPKs which has various functions in cells, and is involved in the proliferation, differentiation and survival of neurons during development(Cobb, 1999; Weber et al., 1997).Recently, PDGF-A in combination with fibronectin has been shown to augment the OPC migration in ERK dependent manner(A. Tripathi et al., 2017).

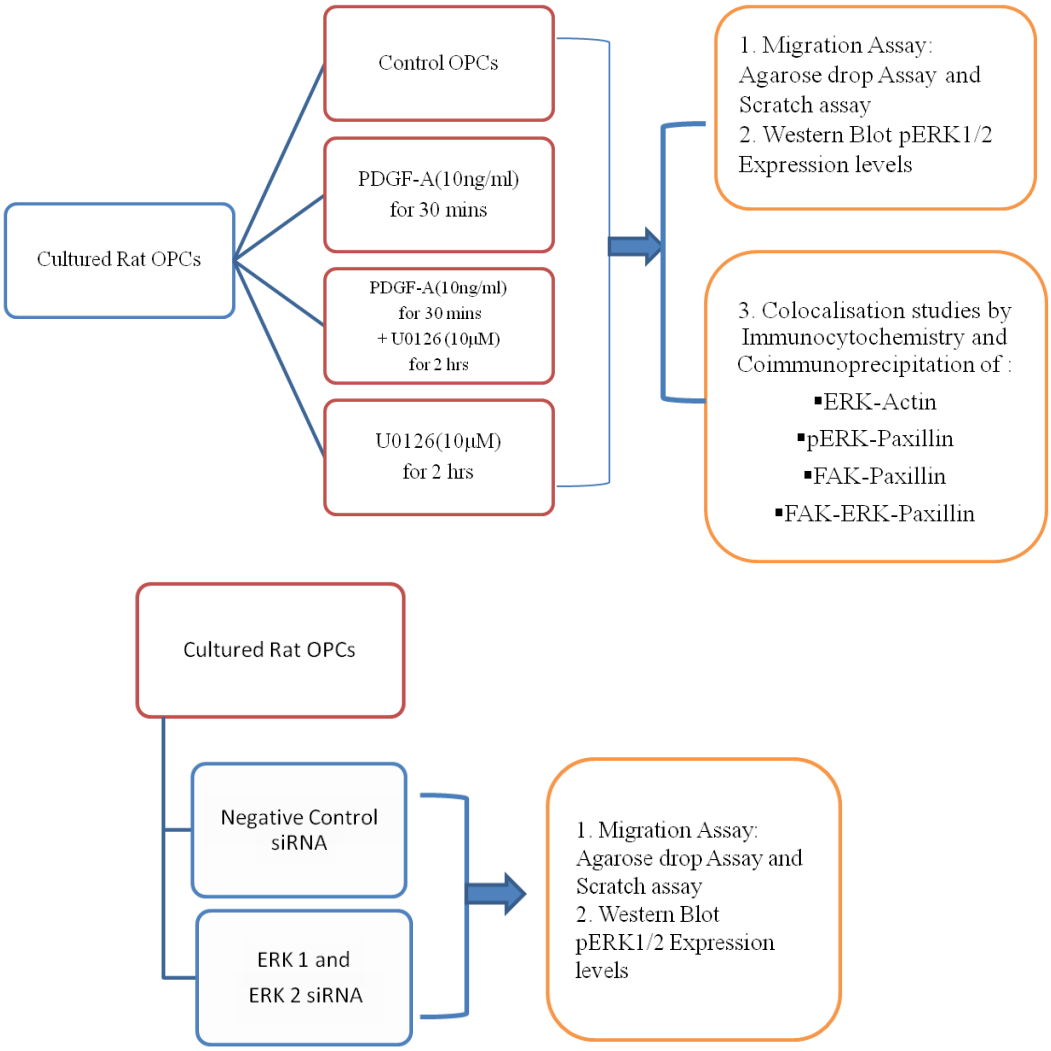
However, the downstream targets of ERK1/2 signalling which cause rearrangements of cytoskeleton and adaptor proteins leading to OPC migration largely remain unknown.Migration of oligodendrocyte involves actin cytoskeletal changes which are controlled by modification in the extracellular matrix and assembly and disassembly

of focal adhesions at the cell peripheral regions (Dennis et al., 2008; Zaidel-Bar et al., 2004). Focal adhesions (FAs) are large intracellular signalling complex assemblies which are formed due to influence of extracellular and intracellular factors. These Focal adhesion complexes consist of several adaptor and scaffold proteins such as FAK, Vinculin, Paxillin, Talin and α-Actinin(Nagano et al., 2012).The driving force that governs these activation and aggregation of these adaptor and scaffold protein still remains to be elucidated.

Moreover, migrating OPCs requires a continuous coordination between the FAs and actomyosin which regulate the overall dynamics of cell migration. Strong linkages of actin cytoskeleton with the Focal complex contribute a firm connection with the extracellular matrixes (ECM) but also provides tension and traction forces to alter cell morphology and a series of protrusion and retraction movements(Y.-L. Hu et al., 2014). Focal adhesion Kinase(FAK), a tyrosine protein kinase is recruited to the site of the adhesions has been reported to be the regulator of FAs assembly and disassembly. Loss of FAK results in the defect in membrane protrusion and inhibition of focal adhesion turnover (Tilghman et al., 2005).FAK in association with Paxillin has been described to play important role in FA dynamics and cell protrusion during migration(Y.-L. Hu et al., 2014). ERK signalling induce epithelial cells migration by promoting the focal adhesion formation by tyrosine phosphorylation as well as association of FAK and Paxillin(Teranishi et al., 2009). VEGF-A regulate the migration of OPCs by inducing FAK-Paxillin interaction and actin reorganization(Hayakawa et al., 2011; Hayakawa et al., 2012).

In the current study, the role of PDGF-A activated ERK signalling leading oligodendrocyte migration and cytoskeletal reorganization has been addressed.

## 3.2 Plan of Work




## 3.3 RESULTS

### 3.3.1 Reduction of ERK expression by U0126 or siRNA inhibits the PDGF-A induced OPC migration

In order to understand that PDGF-A induce the OPC migration through ERK activation, treated cells were first analyzed pERK levels in cells treated with or without PDGF-A (10 ng for 30 min) and U0126 (10µM for 2 hrs) or in combination.

The levels of pERK were found significantly reduced in cell treated with U0126 alone or with PDGF-A compared to control and PDGF-A exposed OPCs (Fig. 3.1 A & B).

To examine the effect of PDGF-A regulated ERK signalling in OPC migration, cells were transiently treated with PDGF-A, U0126 alone or in combination with PDGF-A followed by analysis of cell migration by agarose drop assay and scratch assay. It was found that PDGF-A exposure for 30 mins significantly enhance the migration of OPCs compared to control and U0126 with or without PDGF-A groups (Fig.3.2 A, B & C).

To further confirm the role of ERK signaling in OPC migration, ERK1/2 expression in OPCs was suppressed using specific siRNAs. ERK1/2 knockdown in OPCs was confirmed by western blot and found a significant reduction in the ERK1/2 protein levels in ERK1/2 siRNAs treated OPCs, compared to negative control (Fig. 3.3 C & D). Next, Migration was assessed in ERK1/2 knock down OPCs in presence or absence of PDGF-A. Significant decrease was found in ERK1/2 knock down OPCs compared to negative control or PDGF-A treated groups (Fig.3.3 A & B).

Next, since the PDGF-A is also a mitogen for OPCs, in order to prove that transient exposure enhance the migration but not proliferation, BrdU assay was performed with OPCs exposed with PDGF-A transiently (30 mins) or continuously (24 hrs). There were significant increase in number of BrdU positive cells in OPCs treated with PDGF-A for 24 hrs compared to control and PDGF-A (30 min)(Fig.3.4 A& B).There was no difference in the number of BrdU positive cells were observed between control and PDGF-A(30 min) which suggest that OPCs proliferation requires continuous exposure of PDGF-A.

### 3.3.2 PDGF-A induce actin reorganization in OPCs

OPC filopodia comprises of F-actin rich bundles which polymerizes and depolymerizes during the process of migration. Thus dynamic change in actin cytoskeleton is essential for cell migration. Therefore next the effect of PDGF-A on the actin cytoskeletal reorganization in OPCs was examined. Cells was treated with PDGF-A or U0126 followed by immunostaining for actin by phalloidin Alexa flour-488 (Figure 3.5).It was observed that PDGF-A induce the actin polymerization at the leading-edge processes in OPCs. Treatment of U0126 inhibit the PDGF-A induced actin reorganization which confirms the role of PDGF-A induced ERK signalling in

OPC migration. Similar to F-actin, PDGF-A found to induce the pERK localization at leading processes of OPCs (Figure 3.6).

### 3.3.3 PDGF-A induce pERK1/2-Paxillin-FAK complex formation in OPCs

OPCs migrate by the process of filopodia formation and protrusion from its distal ends and regular arrangement and disarrangement of focal adhesions. FAK (Focal adhesion kinase) and Paxillin are among the members of proteins associated with focal adhesion which are essential for cell spreading and migration(Furuta et al., 1995; Schaller, 2001).Interaction between this two proteins are critical for the activation of signaling cascades associated with migration(Subauste et al., 2004; Turner, 2000). Moreover, Studies have shown that HGF stimulate the association of paxillin and FAK in a MAPK dependent and inhibition of which leads to reduced cell migration and spreading(Ishibe et al., 2004; Z.-X. Liu et al., 2002).

Therefore, to understand whether PDGF-A induced ERK1/2 regulates focal adhesion formation by regulating the interaction of pERK1/2-FAK-Paxillin interactions, OPCs were treated with PDGF-A or U0126 followed by immunostaining with pERK1/2, FAK and Paxillin. PDGF-A was found to induce the interaction pERK-Paxillin (Figure 3.7) and FAK- Paxillin in OPCs (Figure 3.8). Further, FAK-Paxillin was found to be colocalized at processes and cell bodies of OPCs. Further, OPCs treatment with U0126 reduces the FAK-Paxillin interaction. Further, it was confirmed the FAK-Paxillin and pERK interaction in OPCs in response to PDGF-A. Interaction of pERK with focal adhesion proteins FAK and Paxillin has been shown to play an important role in cell migration. OPCs were exposed to PDGF-A or U0126 followed by immunoprecipitation with anti-FAK antibody and subjected to western blot analysis of with antibodies for paxillin, pERK and FAK demonstrated that PDGF-A augment the pERK-FAK-Paxillin interaction OPCs compared to control or U0126 (Figure 3.9) which suggest that PDGF-A induced ERK1/2 signaling promotes OPC migration by regulating FAK and Paxillin interaction.

## 3.4 DISCUSSION

OPCs migration is a prerequisite for the proper myelination in the CNS. OPC originates in the prenatal brain from the restricted regions of ventricular and sub ventricular zones and migrate to populate the white matter region(E. E. Frost et al., 2009; Richard Milner et al., 1996). OPCs migrate to myelinate the axons of the neurons, failure of which leads to lesions, axonal damage and demyelinating diseases such as Multiple sclerosis(N. P. Pringle & Richardson, 1993). Yet, the molecular mechanism governing the oligodendrocyte migration is complex and intricate with multiple growth factors and extracellular signals further activating several intracellular signalling pathways. OPCs are guided and controlled by different growth factors, chemotropic factors and chemokines including FGF2, PDGF-A, VEGF, CXCL1, Netrin and Semaphorins(Baumann & Pham-Dinh, 2001; Hayakawa et al., 2011; Nathalie Spassky et al., 2002; Vora et al., 2012).

Further, study by Miyamato and colleagues demonstrates Cdk5 regulates PDGF-dependent OPC migration through the direct phosphorylation of WASP (Wiskott–Aldrich syndrome protein)-family verprolin homologous protein 2 (WAVE2)) at Ser-137. Another study by Frost and colleagues (2009) has demonstrated that PDGF-A signalling regulate the OPC migration through the ERK signalling pathway in dose and time dependent manner (Vora et al., 2011).The effect of PDGF- A on long distance migration of OPCs has been has widely been discussed and understood (RC Armstrong et al., 1990; Ellison et al., 1996). Furthermore, Short term exposure to PDGF-A(10ng/ml) has been described to be sufficient to transiently induce OPC migration for up to 72 hrs. Simultaneously, continuous exposure of PDGF-A (10ng/ml) for 24hrs cause increase in the OPC proliferative capacity (Fruttiger et al., 1999; Vora et al., 2011). In present study pharmacological inhibition and siRNA mediated suppression of ERK1/2 signalling confirmed that transient exposure of PDGF-A induces the migration of OPCs via ERK signalling without promoting the proliferation. This concurs our previous lab reports (Frost et al.,2009;Vora et al.,2011) and forms the basis of our further investigation on ERK1/2 downstream targets and cytoskeletal changes.

OPC migration involves the formation of filopodia, growth cone-like structures, at the leading edges process and substrate detachment at the trailing process (Schmidt et al., 1997; Song et al., 2001). Filopodia are tightly bound actin bundles which later

polymerises and widens to form lamellipodia with the help of microfilaments branching (Bauer et al., 2009; Mattila & Lappalainen, 2008).Previously our lab has demonstrated that combined treatment of PDGF-A and fibronectin increased the pERK1/2-F-actin interaction and enhanced filopodia formation prior to OPC migration (A. Tripathi et al., 2017).In the present study, we found that PDGF-A activation alone led to the polymerisation of F-actin filaments at OPC leading edges as compared to control or U0126 treated cells. Further, pERK1/2 was also found to be localised at leading processes of OPCs similar to F-actin.

Moreover, the present study also hypothesizes that the PDGF-A activated ERK is recruited to the cell periphery where it regulates focal adhesion disassembly prior to OPC migration. Focal Adhesion (FA) comprises of tightly compact regions of the plasma membrane that are in immediate proximity to the substratum in cultured cells (Lo, 2006; Parsons, 2003; Parsons et al., 2010). FA dynamics involves an uninterrupted process of coordination between FA and actin cytoskeleton where numerous cytoskeletal proteins also come into play. Among the different focal adhesion proteins known, FAK(Focal adhesion Kinase) and Paxillin are known to be prominent cytoskeletal proteins involved in assembly and disassembly of cell adhesion and promoting directional cell movement(Y.-L. Hu et al., 2014; Schaller, 2001). ERK activates the tyrosine phosphorylation of FAK and Paxillin thereby further promoting the formation of focal adhesions in Human corneal epithelial cells(Teranishi et al., 2009). Recently, VEGF-A was shown to promote the OPCs migration by promoting the FAK-Paxillin interaction (Hayakawa et al., 2011). Current study demonstrates first time that PDGF-A induce the pERK-Paxillin-FAK interaction in OPCs, implicate a novel mechanism of PDGF-A induced OPCs migration. Alteration of FAK and Paxillin dynamics results in decreased migration by reduction in focal adhesion turnover and lamellipodia formation (Deramaudt et al., 2014).

### 3.5 CONCLUSION

In addition to earlier studies, present study further confirmed using inhibitor and siRNA that PDGF-A induce the OPCs migration via activation of ERK. Further, PDGF-A induced ERK signalling leading to formation of pERK-FAK-Paxillin complex in OPCs. The results of the study indicate the involvement of pERK-FAK-

Paxillin in focal adhesions formation and actin reorganisation prior to initiation of cell migration.

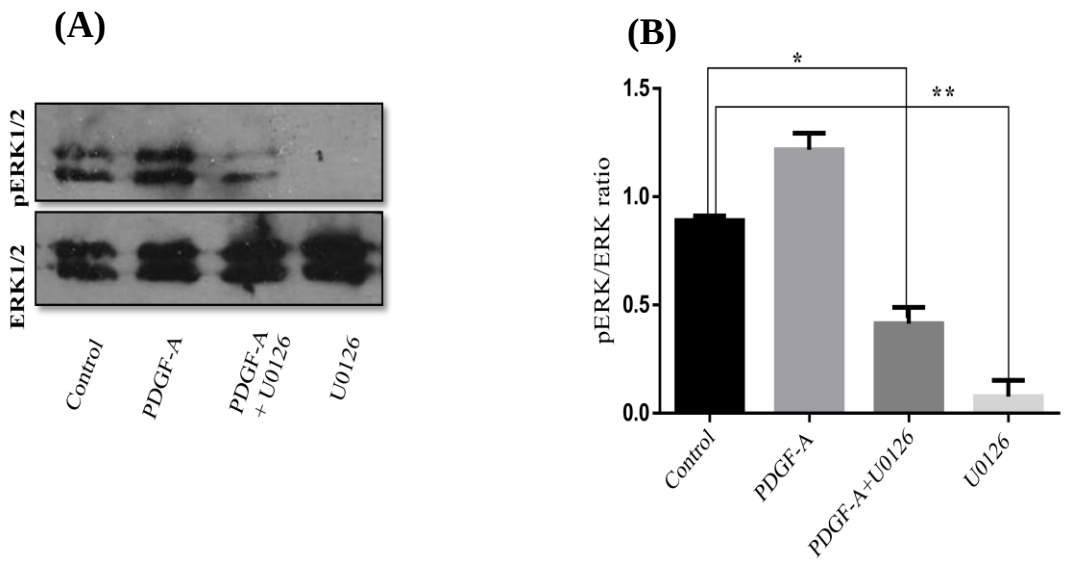
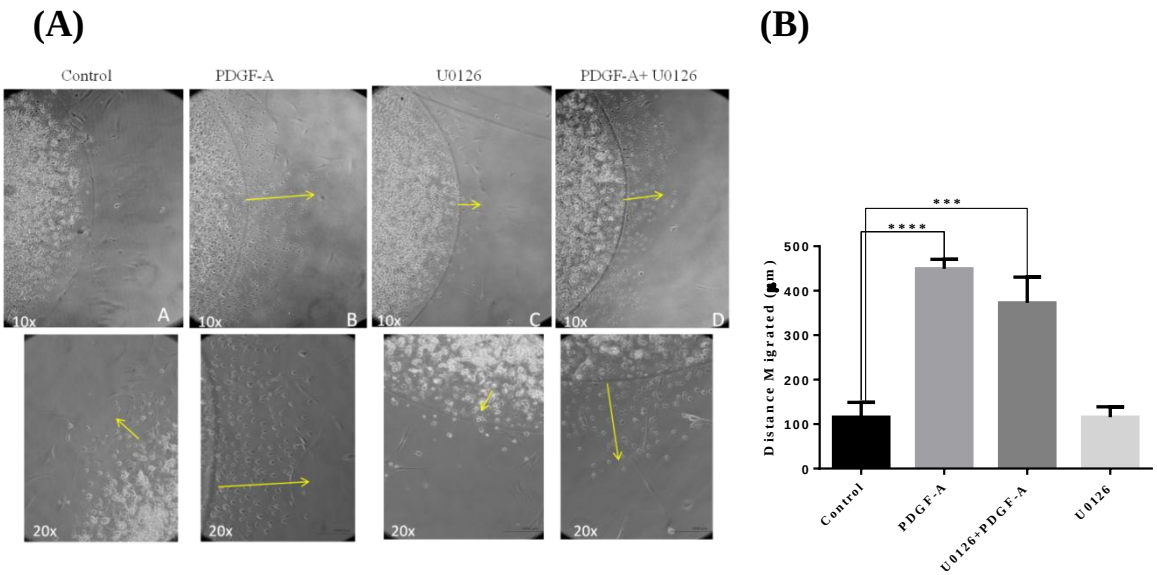



**Figure 3.1 : PDGF-A activates ERK signalling in OPC** (A) Western Blot analysis of ERK phosphorylation in OPC cells Lane1: Control(No PDGF) Lane 2: OP cells harvested immediately after 30 mins exposure to 10 ng/ml PDGF-A, Lane 3: OP cell harvested immediately 2hrs exposure to 10µM U0126 and then 30 mins exposure to 10ng/ml PDGF-A, Lane 4: OP cells harvested after 2 hours exposure to U0126 (B) Relative density bands for pERK1/2 protein band obtained. Values were normalized to the control. (N=3) *P<0.05;**P<0.01; ***P<0.001,****P<0.0001 compared to control , one way ANOVA followed by dunnett test for multiple comparison



**(C)**

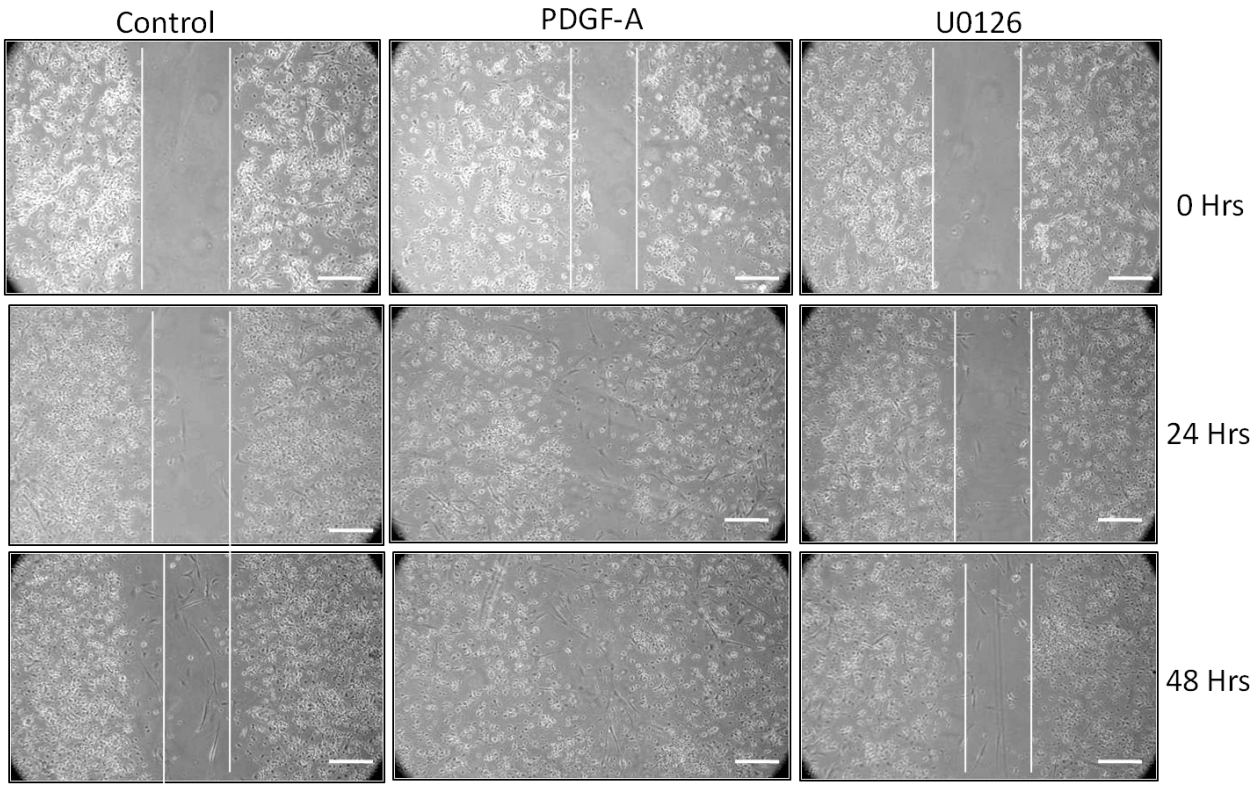



**Figure 3.2 PDGF-A activated ERK signaling regulates OPC migration**. (A) Migration was analyzed by Agarose Drop assay in Control, PDGF-A(10ng/ml) for 30mins,U0126(10µM) for 2 hrs with PDGF-A (10ng/ml) for 30 mins and U0126 (10µM) alone for 2 hours (N=3). Micro pictographs were taken after 72 hrs. Scale Bar =200µM(10X),400µM(20X)(B) Quantitative analysis of distance migrated by OPCs from the corona of agarose drop(µm) ***P<0.001,****P<0.0001 compared to control , one way ANOVA followed by Dunnett test for multiple comparison **(C)** Migration was analyzed byScratch Assay in Control, PDGF-A(10ng/ml) for 30mins and U0126 (10µM) alone for 2 hours (N=3). Micro pictographs were taken after 0 hr, 24 hrs and 48 hrs after the making the scratch. Scale Bar =200µM (10X).

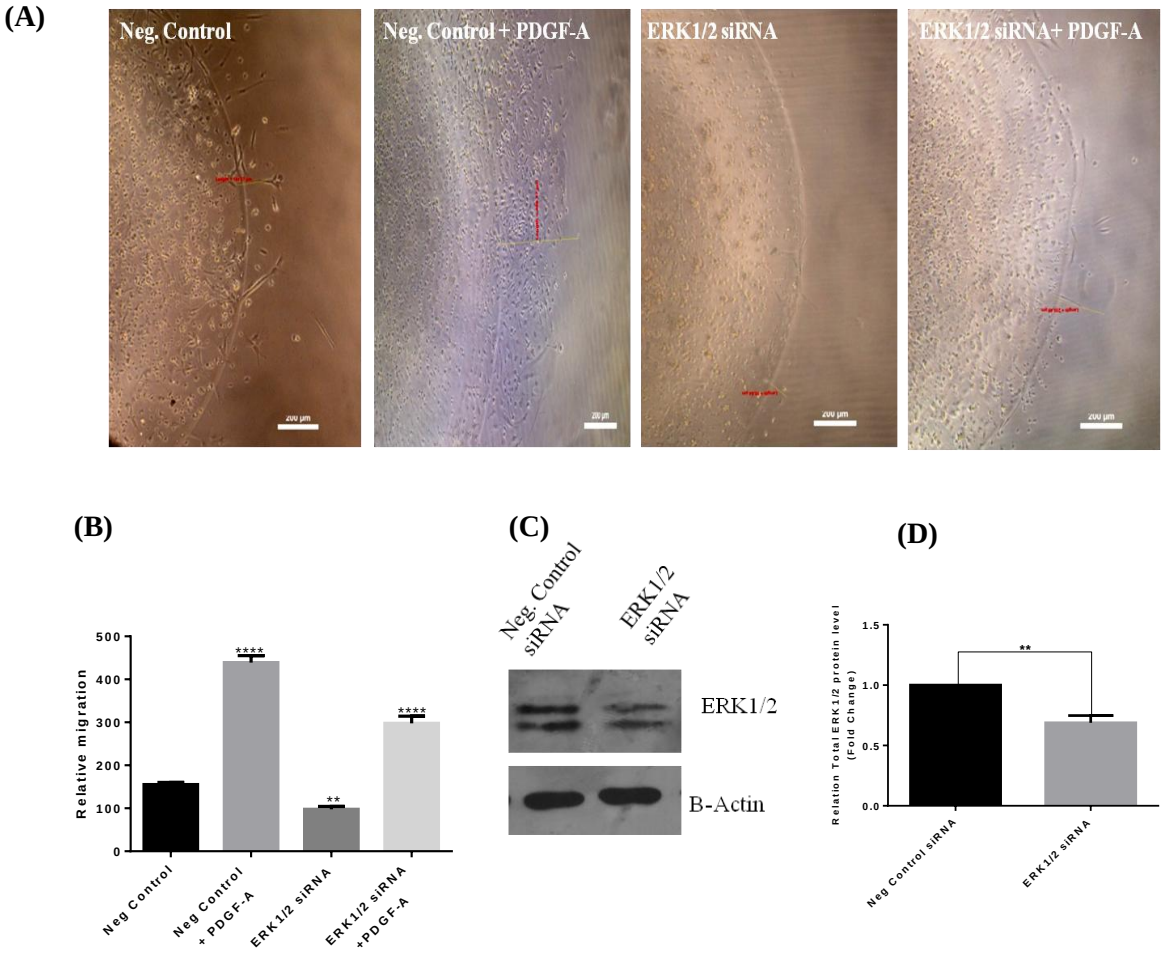



**Figure 3.3 : Knockdown of ERK in Oligodendrocyte progenitor cells (OPCs) : (A)** OPC Migration was analyzed by agarose drop assay in Negative Control siRNA , Negative Control siRNA with PDGF-A(10gn/ml) for 30 mins , ERK1/2 siRNA (10nmol)and ERK1/2 siRNA with PDGF-A (10ng/ml) for 30 mins.(Scale Bar: 200µm).**(B)** Quantitative analysis of distance migrated by OPCs from the corona of agarose drop(µm) **(C)** Representative western blot of Negative control and ERK 1/2 siRNA knockdown .**(D)** Relative change in the ERK1/2 protein expression. Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001,****P<0.0001 compared to control .

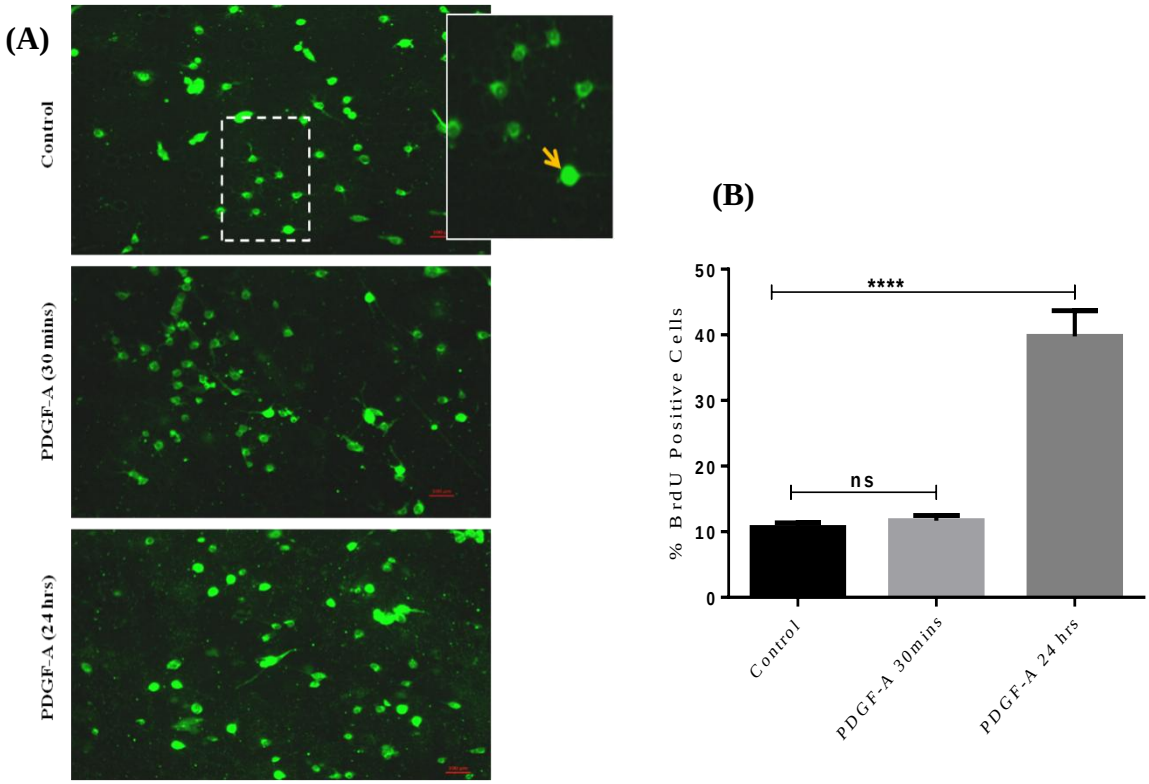



**Figure 3.4: Continuous exposure PDGF-A for 24hrs leads Oligodendrocyte progenitor cells proliferation.(A)** Representative fluorescence Images of BrdU-positive cells in oligodendrocytes progenitors cells pre-treated for 30 mins and 24hrs with PDGF-A (10ng/ml ) respectively. Scale bar = 50 µm, **(B)** The statistical data showed percentage BrdU-positive cells calculated from panel A. Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001,****P<0.0001 compared to control , one way ANOVA followed by dunnett test for multiple comparison

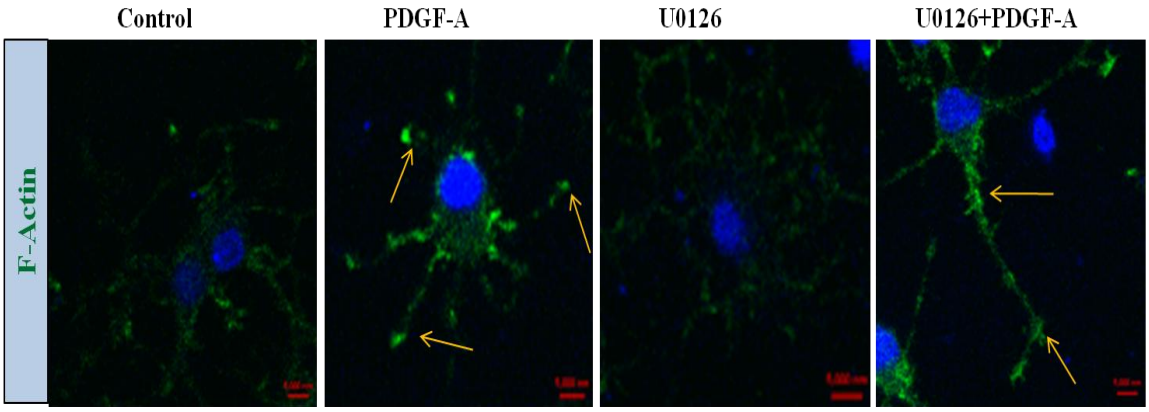



**Figure 3.5: PDGF-A reorganized actin cytoskeleton in OPC cultures**.
Cells were fixed and stained with phalloidin Alexa Flour- 488 (green) to visualize F-actin. And DAPI (blue) to stain nuclei. Compared with control OPC group, PDGF-A-

treated cells showed polymerized F-actin fibres. (Scale bar-. 1000 nm) The experiment was performed three times with similar results.

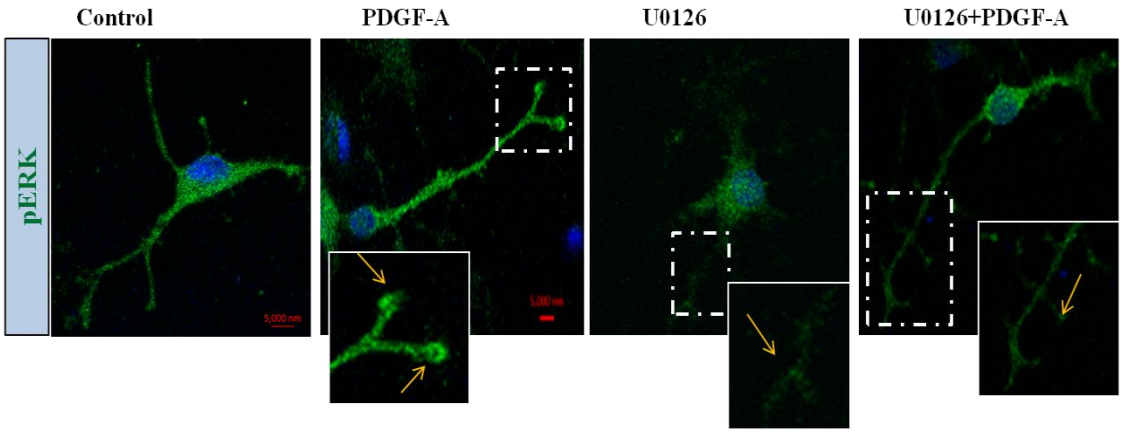



**Figure 3.6 PDGF-A activated ERK1/2 localization in cytoskeleton of OPC cultures**. Cells were fixed and immunostained with anti-ERK1/2 antibody (FITC-green) and DAPI (blue) to stain nuclei. Compared with control OPC group, PDGF-A-treated cells showed ERK1/2 localised in the leading processes. (Scale bar-. 1000 nm) The experiment was performed three times with similar results.

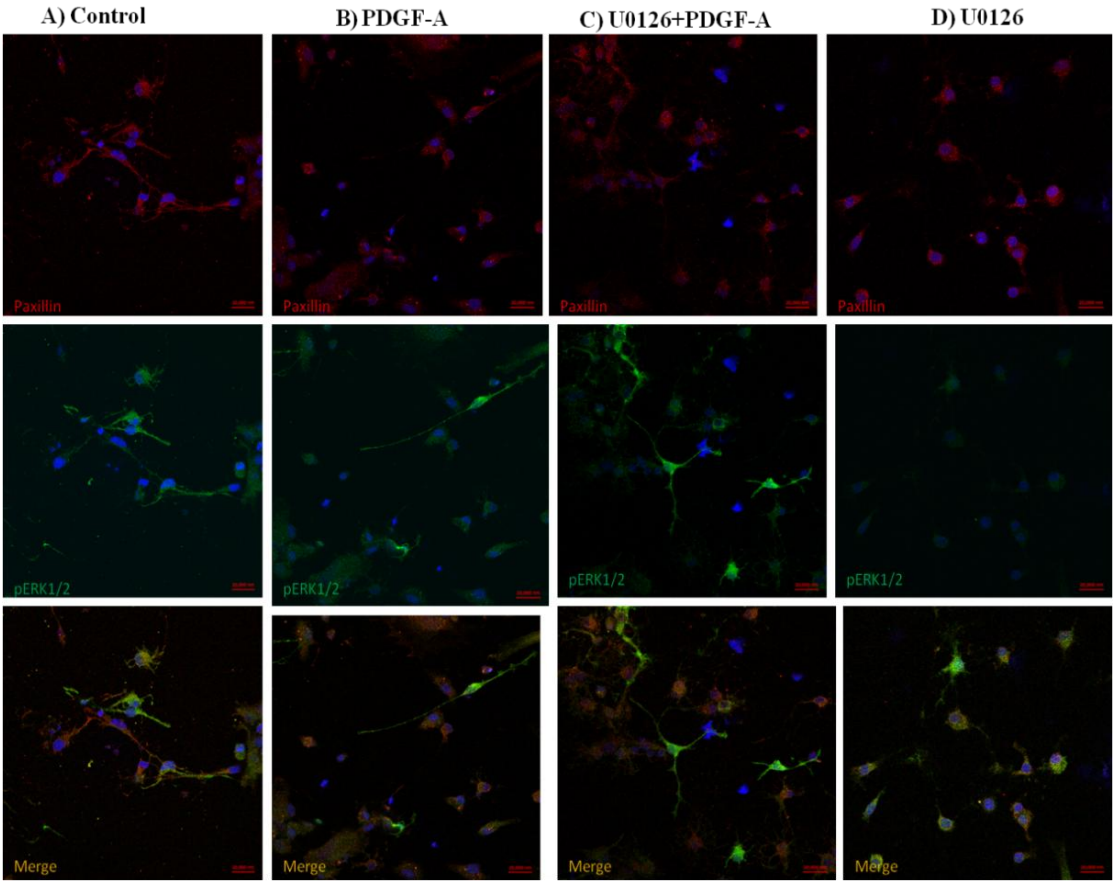


**Figure 3.7 Interaction of pERK1/2 and Paxillin :** Association of pERK1/2 with paxillin was visualized by Immunostaining with pERK1/2 (*green)* and anti-Paxillin (*red*) antibodies in Control, PDGF-A (10ng/ml), U0126 (10uM) for 2hr prior to 30 min exposure PDGF-A and U0126 (10uM ) alone for 2hr treated OPCs. PDGF-A induces the pERK1/2-paxillin complex formation in OPCs.(Scale bar-. 1000 nm) The experiment was performed three times with similar results.

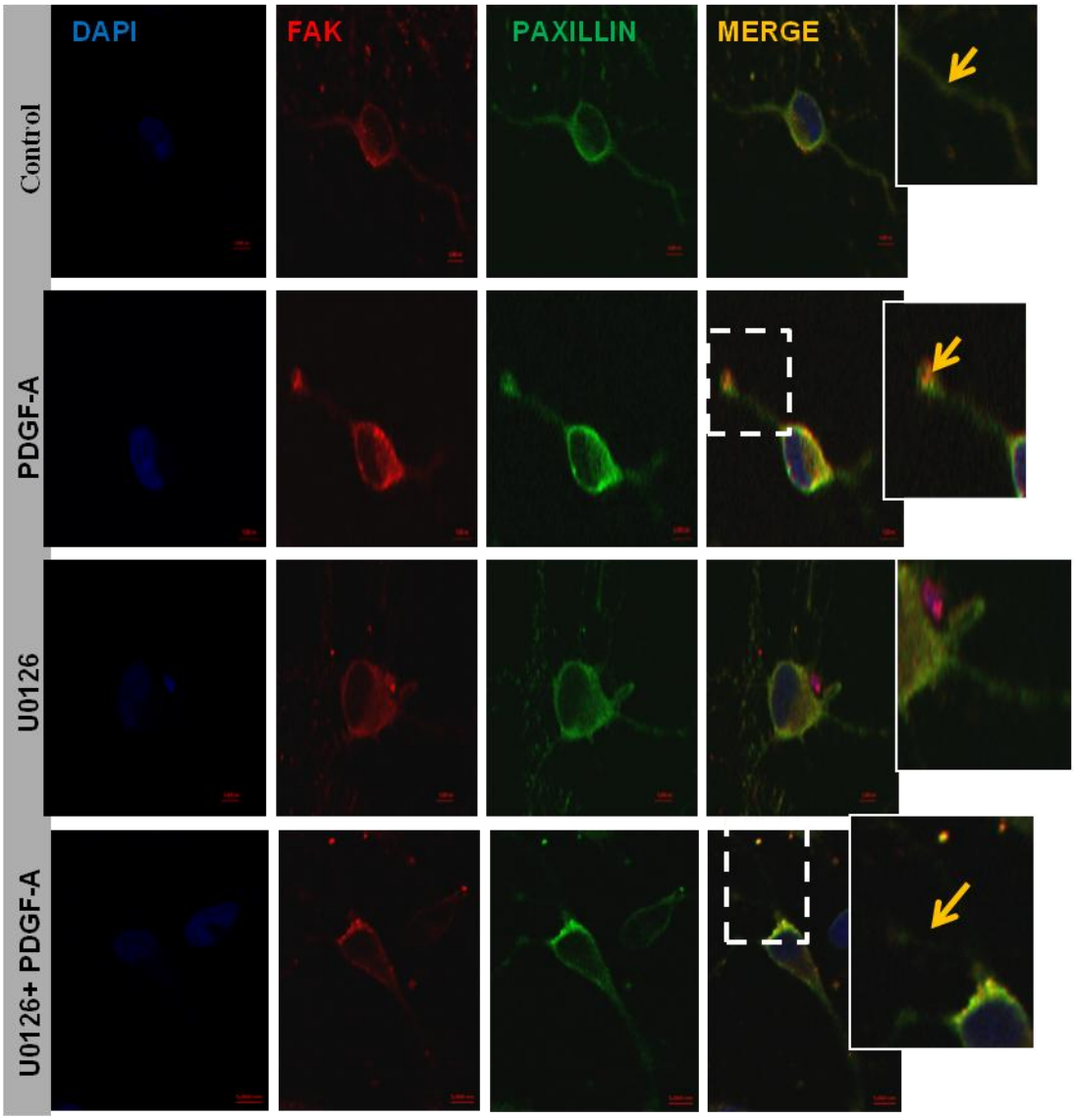



**Figure 3.8: PDGF-A induced FAK-Paxillin colocalization :** Association of FAK with Paxillin OPCs was visualised by Immunostaining with anti- FAK (TRITC-Red) and anti- Paxillin (FITC-Green) antibodies in Control, PDGF-A (10ng/ml) ,U0126 (10µM) with and without PDGF-A treated OPCs . (Scale bar-. 1000 nm) The experiment was performed three times with similar results.

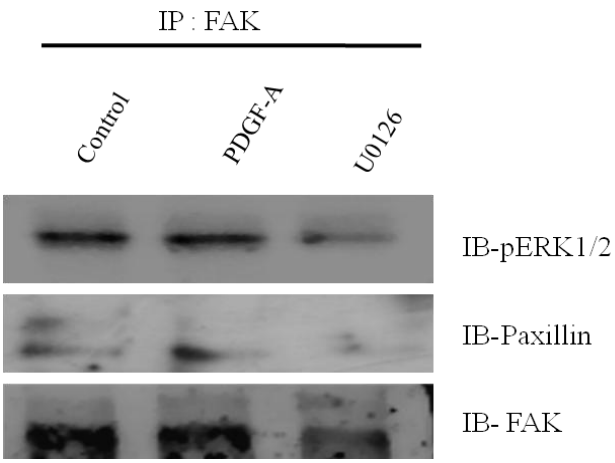



**Figure 3.9: PDGF-A induced pERK1/2-FAK-Paxillin interactions.** Cells were exposed to PDGF-A(10ng/ml) and U0126(10µM) for 30 mins and 2 hrs respectively. Cell lysates (1 mg protein) were subjected to immunoprecipitation (IP) with anti-FAK antibody conjugated to Dynabeads, and immunoprecipitates were subjected to SDS-PAGE and Western blotting with anti-FAK, anti-pERK1/2 and anti -paxillin antibodies. Shown is a representative blot from 3 experiments.