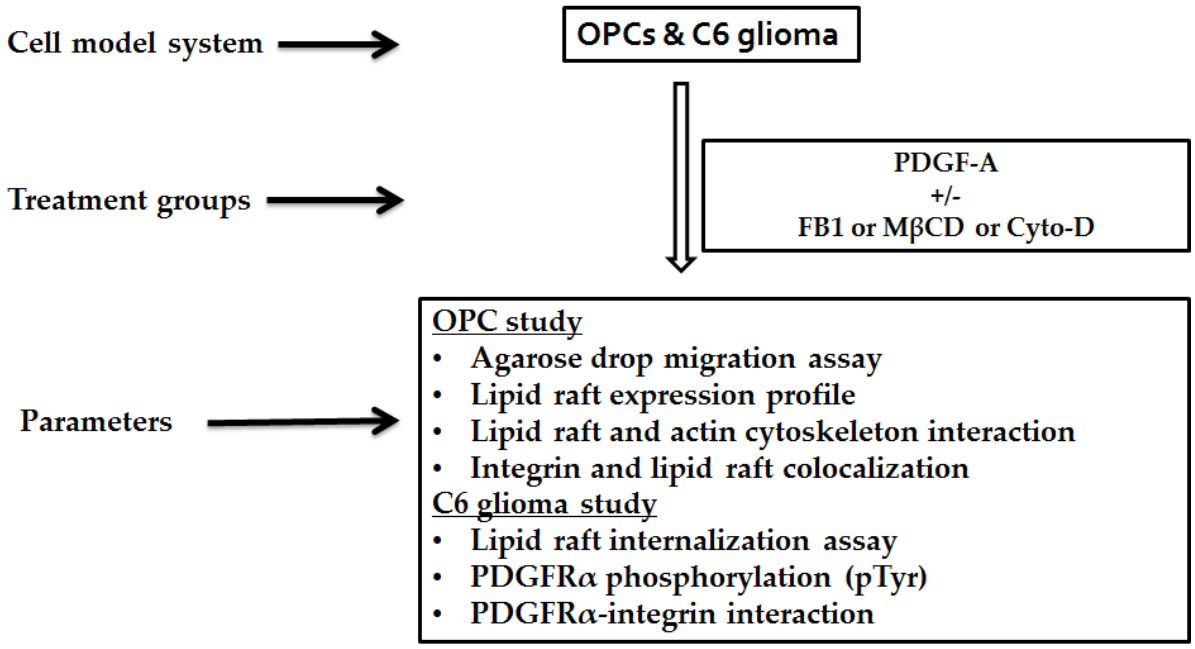
5.1 Introduction

As discussed in **Chapter-1,** lipid rafts are defined as dynamic membrane microdomains enriched in cholesterol and sphingolipids, in which proteins and lipids are orderly assembled (Kramer et al. 1997; Gielen et al. 2006; Hoetzl et al. 2007; Simons and Gerl 2010). These structures are more organized than the typical bilayer, but are free floating within it. Lipid rafts ensure that signaling cascade constituents are within appropriate molecular distance to facilitate signal transduction by raft-bound receptors (Lingwood and Simons 2010). We expect to identify the nature of the interactions between PDGFRα and integrins that regulate OPC migration. Further, we expect to prove that lipid raft microenvironments are critical for the regulation of PDGFRα mediated integrin expression and switching during OPC migration. In addition, the elucidation of the mechanism underlying the unregulated PDGFRα activation and lipid raft- mediated regulation of glioma anoikis resistance will provide new insights into the mechanism of invasion/metastasis and also provide new targets for cancer prevention and therapy. As discussed above lipid rafts are the sites of receptor activation and initiation of signaling molecules. Data from the current proposed study will help to better understand, how lipid rafts modulate cell fate and how these lipid rafts can be modulated, which will lead to the development of novel strategies in the treatment of glioma metastasis.

5.2 Plan of work



5.3 Results

5.3.1 PDGF-A increased lipid raft in OPCs

Since lipid rafts are known to be involved in the efficacy of the assembly of signaling cascade, lipid rafts formation was monitored in response to the exposure of PDGF-A. Immuno florescent staining (Fig.5.1A,B) as well as western blot (Fig.5.1C,D) analysis of GM1 (a constituent of lipid raft) demonstrated that the accumulation of lipid rafts after the treatment of PDGF-A in OPCs was significantly more as compared to that of control cells. Also, cells treated with higher dose of 10ng/ml PDGF-A accumulated more pronounced lipid raft expression as compared to that of 1ng/ml of PDGF-A signifying the efficacy of higher dose. Higher concentration of PDGF-A, not only significantly increased the expression levels but also the aggregation or coalescence of lipid rafts (Fig.5.1Ac) (white arrows represents the aggregation or coalescence of lipid rafts).

5.3.2PDGFRα activation mediated lipid raft-actin interaction

As shown in Fig.5.2A, higher concentration of PDGF-A treatment significantly increased the OPCs cell process formation as compared to 1ng/ml PDGF-A and PLL. We have further confirmed activation of actin filament as evident by F actin staining in a dose dependent manner (Fig.5.2B). The length of cellular processes formed were also higher in PDGF activated group as compared to control (Fig.5.2B). Next we have examined that whether the PDGF-A (10ng/ml) dependent increased lipid raft and F-actin are interacting or not. For that we have checked the levels of lipid rafts in actin-cytoskeletal fraction. Our data shows that lipid raft expression was increased in the PDGF-A(10ng/ml) treated OPCs actin-cytoskeletal fraction(Fig.5.3A,B).We have further confirmed PDGF-A (10ng/ml) dependent lipid raft and actin cytoskeleton interaction by confocal microscopy co-localization studies as compared to PLL control (Fig.5.3C).Further we have confirm the role of PDGFRα activation on the lipid raft and actin cytoskeleton interaction. For that we have used AG1295 inhibitor (10μM) to inhibit the PDGFRα-RTK activation. AG1295 significantly decreased the PDGF-A (10ng/ml) dependent lipid raft and actin cytoskeleton interaction (Fig.5.3D,E). In Chapter-4 our data showed the PDGFRα activation by PDGF-A (10ng/ml) significantly increased the integrin αvβ1 expression and aggregation (Fig.4.2D). Here we show that integrin αvβ1 was found co-localized with lipid rafts after PDGF-A (10ng/ml) treatment (Fig.5.3F).

5.3.3 Lipid raft depletion by FB1 decreased the PDGF-A mediated effects on OPCs

Next we have confirmed the role of lipid rafts in PDGF-A(10ng/ml) mediated effects on OPCs. Lipid rafts were depleted using FB1(conc.) remarkably decreased the formation of cellular processes indicating that lipid rafts are mandatory for this phenomenon . However, treatment with PDGF could not rescue the process formation even after FB1 insult (Fig.5.4A,B). Rounding up and loss of cell processare characteristic feature of the apoptotic cell. To explore possible altered cell viability, cell viability assay by MTT was performed. It appears that the morphological changes induced by FB1 were not due to altered cell viability. Further, FB1 significantly

decreased the PDGF-A(10ng/ml) dependent lipid raft expression level and OPC migration (Fig.5.4C,D,E,F).

5.3.4 PDGF-A revert the effects of MβCD on OPCs

In contrast to the mode of action of FB1 which inhibits total sphingolipid biosynthesis thereby depleting completely the lipid raft content, MβCD on other hand only deplete the lipid raft from the cell membrane (Merrill et al. 1993) (Klein et al., 2002; Sanchez et al., 2011). As shown in Fig.5.5, MβCD insult could be reverted by the treatment of PDGF-A(10ng/ml) in the OPCs. Thus, PDGF signaling is instrumental in recruitment of lipid rafts on the membrane.

5.3.5Lipid raft internalization regulate PDGFRα-αVβ3 interaction in C6 glioma cells

Next we have examined the role of lipid raft and actin cytoskeleton interaction during anoikis resistance in C6 glioma cells. It has been shown that growth factors support anoikis resistance during ECM detached condition. In order to further validate if lipid rafts are in turn responsible for the interaction of PDGF and integrin, cells were treated with cytochalasin D (inhibitor of actin polymerization). When kept in suspension the lipid rafts of the cells gets internalized (Fig.5.6Ab). After treatment with cytochalasin D, internalization of lipid rafts was reduced profoundly as shown in Fig.5.7Ac. The reduced internalization of lipid rafts modulated the interaction of PDGFRα-αVβ3 interaction (Fig.5.6B, C, D, and E)

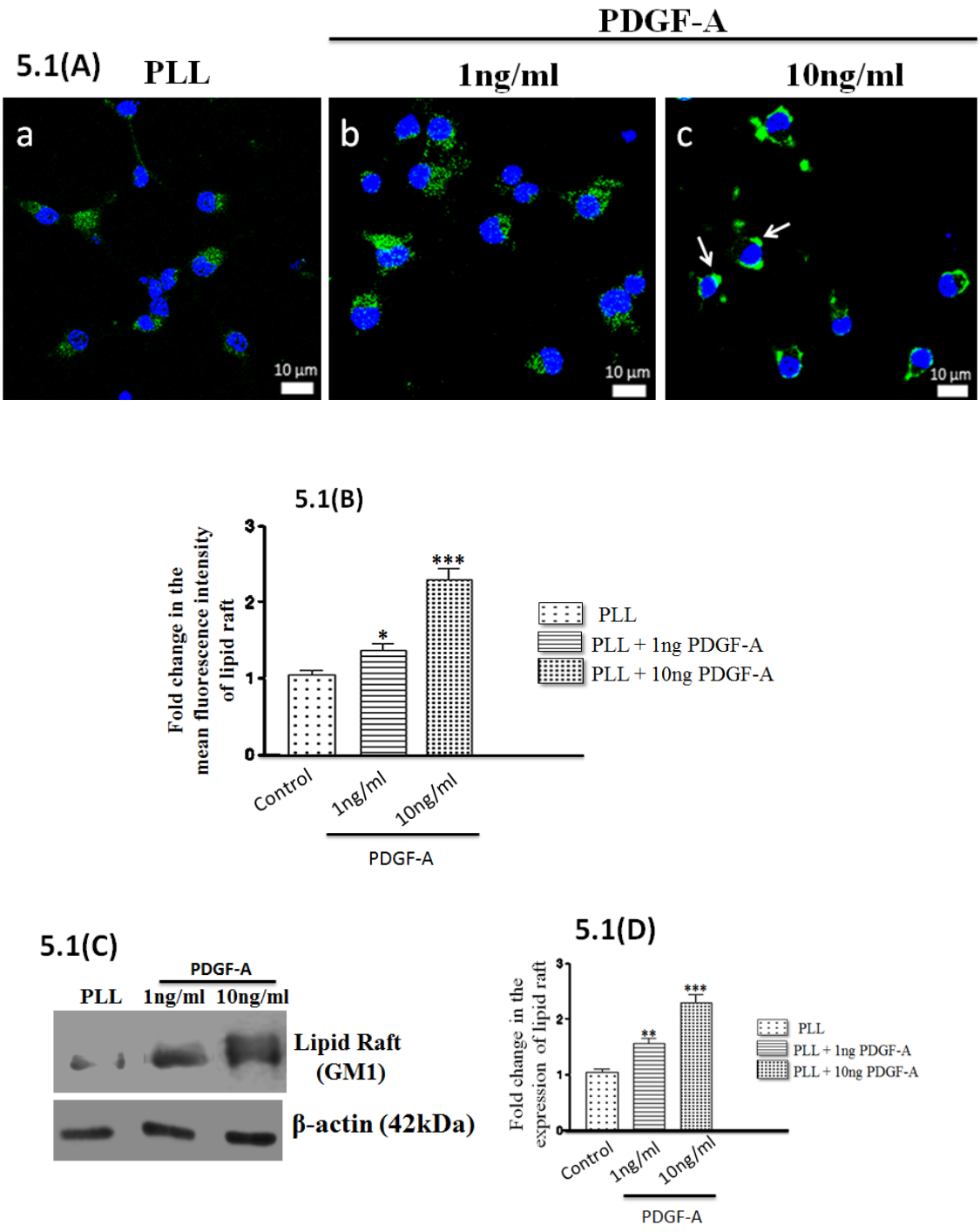
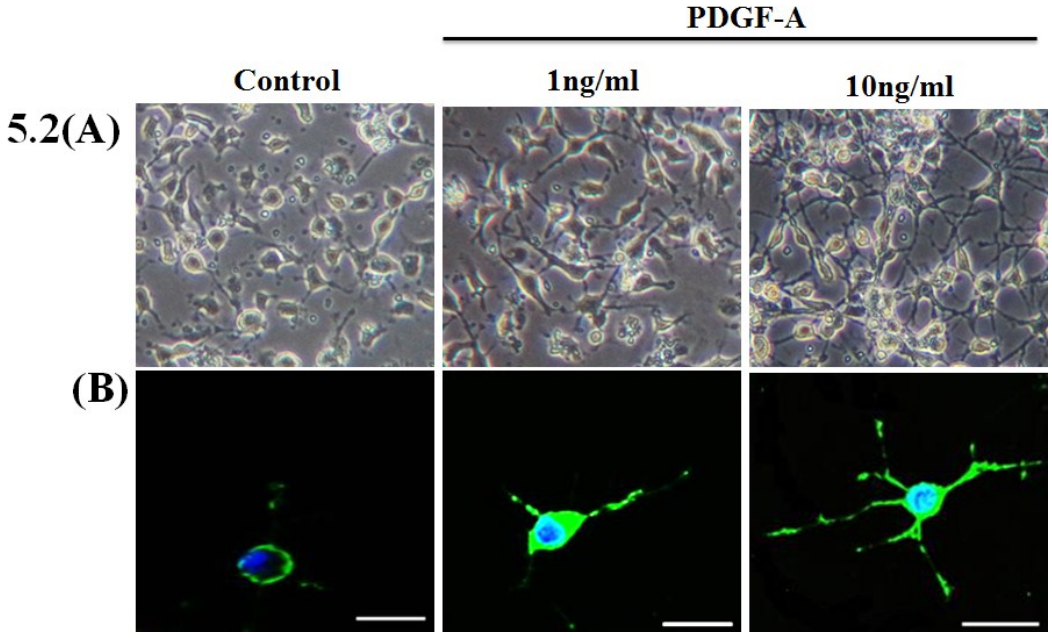


Fig 5.1 PDGF-A increased the lipid rafts in OPCs

(A) *OPCs were exposed to PDGF-A at 1ng/ml and 10ng/m dose. Nucleus was stained with DAPI (blue; a-c), Cholera toxin B (CTXB green; a-c). Scale bar=10µm.* ***(B)*** *Mean fluorescence intensity of lipid rafts in the OPCs 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)*** *Representative Western blot for Lipid raft after treatment of OPCs with PDGF-A 1ng/ml and 10ng/ml with PLL as a control. Cholera toxin B-HRP (CTXB-HRP) was used to detect the lipid raft (CTXB binds with the GM1 of lipid rafts).* ***(D)*** *After densitometric analysis, Lipid raft was normalized to actin. 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).*



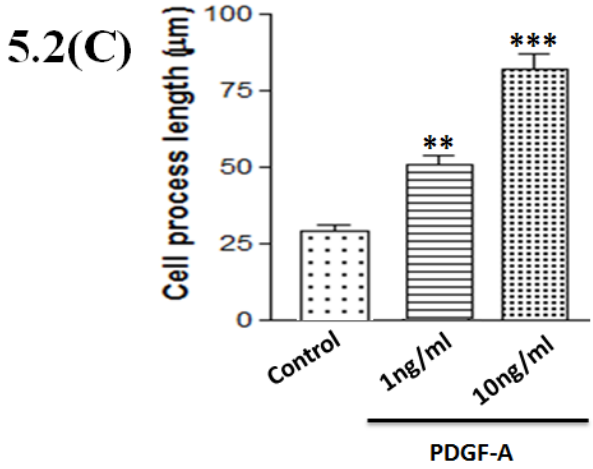
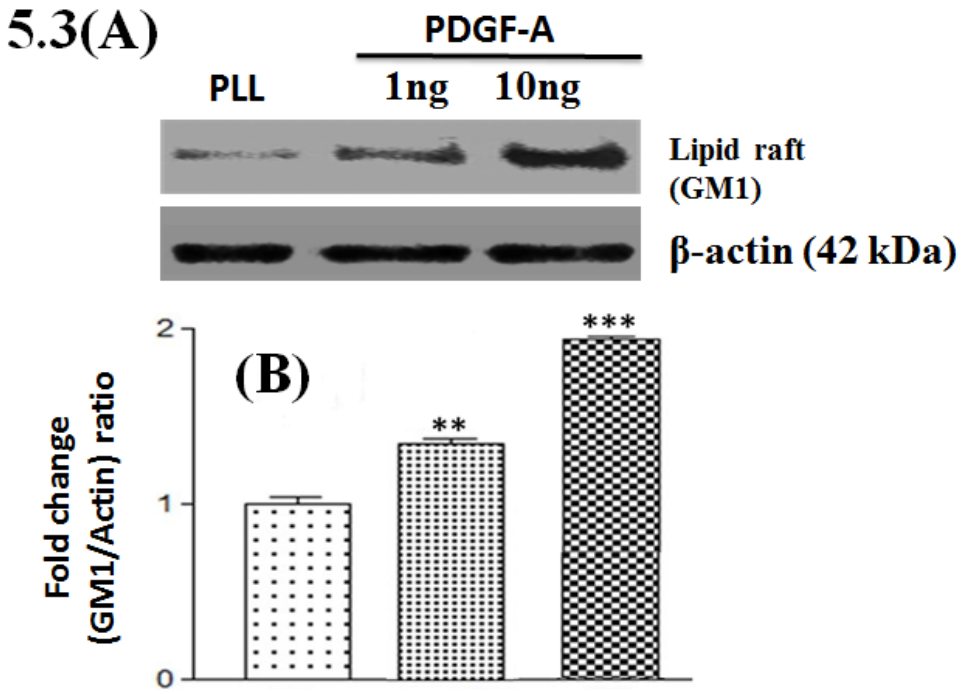
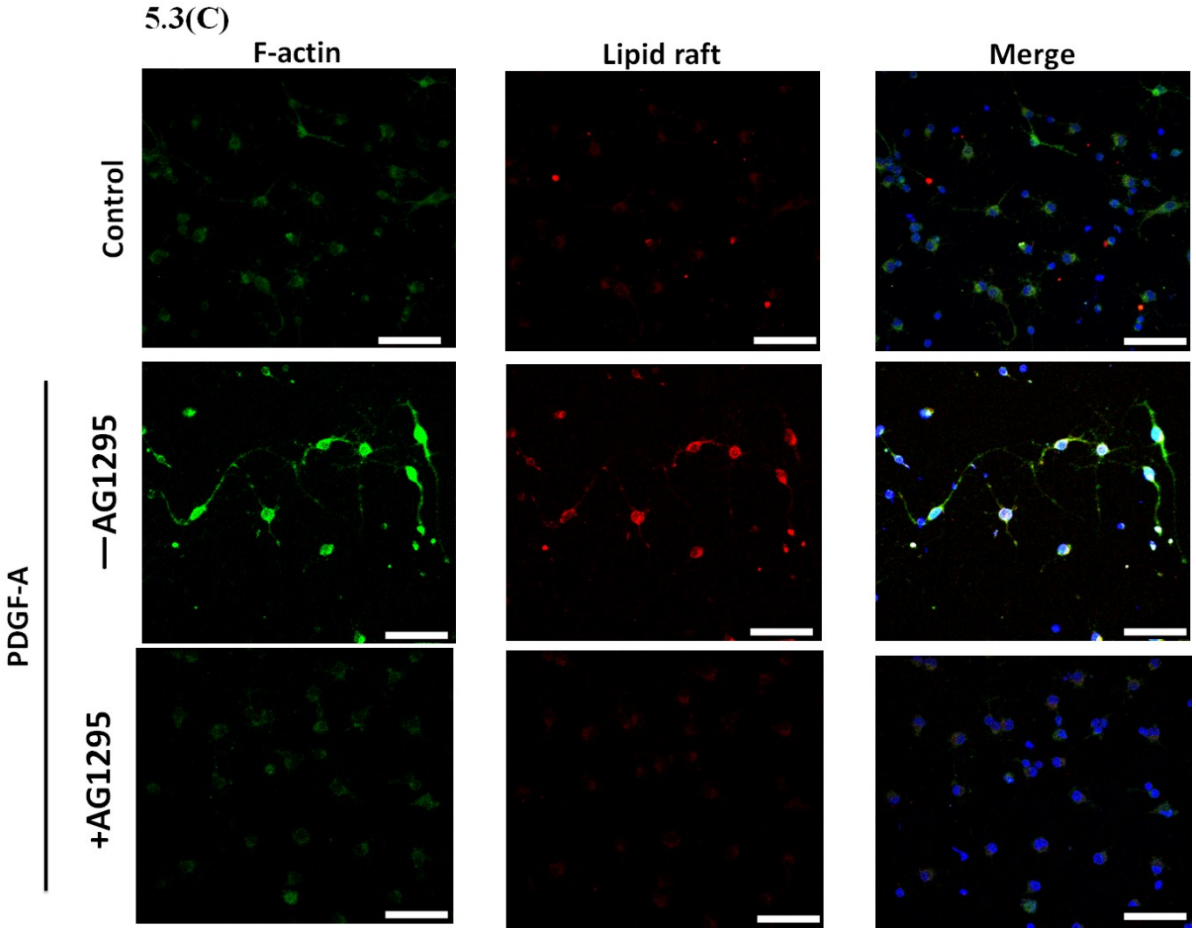
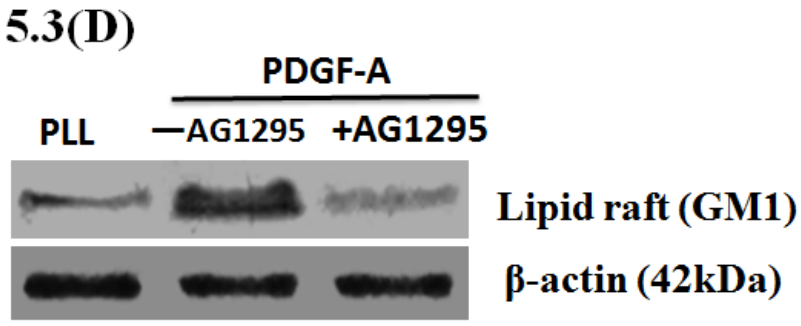


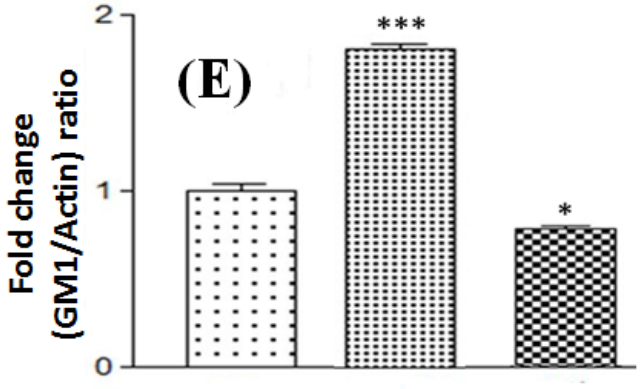
Fig. 5.2 Dose dependent effects of PDGF-A on F-actin staining and cell process formation

(A) *Phase contrast photomicrograph showing OPCs treated with PDGF-A 1ng/ml and 10ng/ml.* ***(B)*** *OPCs were exposed to PDGF-A at 1ng/ml and 10ng/ml dose. PLL was used as control. Nucleus was stained with DAPI (blue; d-f), F-actin (Alexafluor 488; green; d-f). Scale bar=50µm.* ***(C)*** *Morphological analysis using phase contrast microscopy shows that cell process length increases in a dose dependent manner. Process length was significantly more in (PDGF-A-10ng/ml) than compared to PDGF-A-1ng/ml and PLL (arrows). Graph shows the effect of PDGF-A in a dose dependent manner 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).*




5.3(C)
F-actin
Lipid raft
Merge
Control
PDGF-A
—AG1295
+AG1295


5.3(D)
PDGF-A
PLL
—AG1295
+AG1295
Lipid raft (GM1)
β-actin (42kDa)


(E)
Fold change (GM1/Actin) ratio
2
1
0
***
*

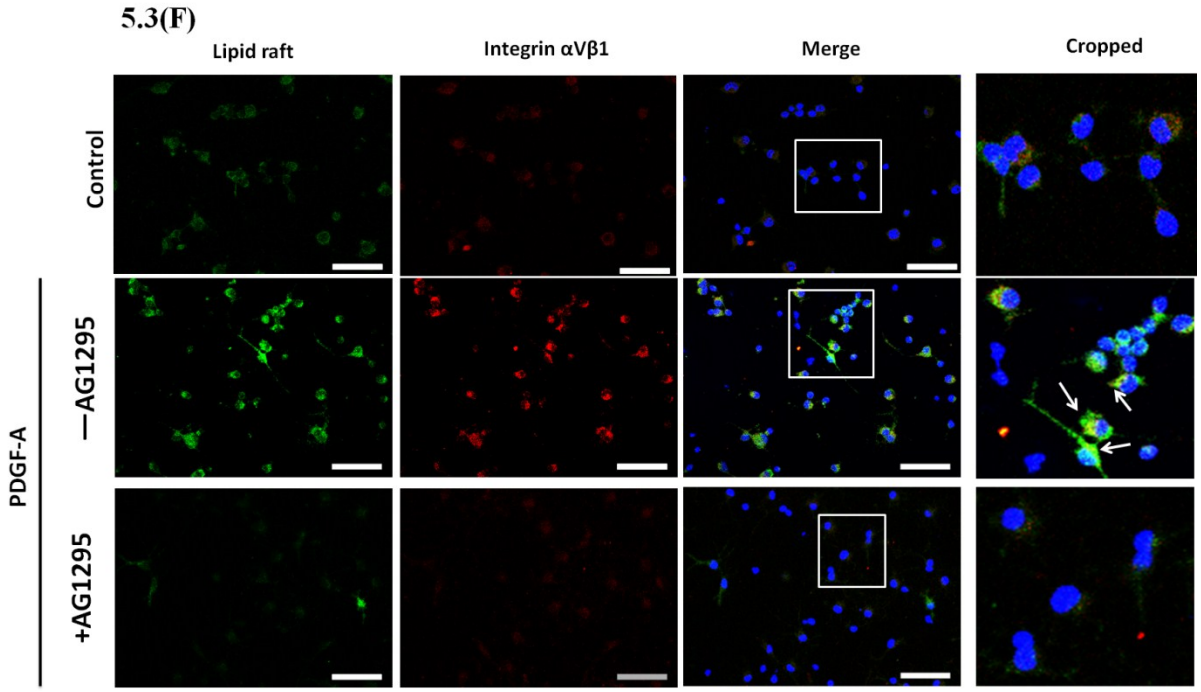
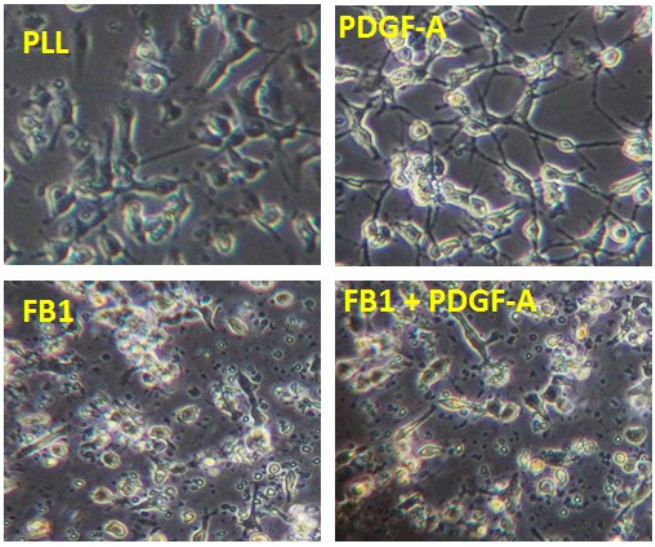


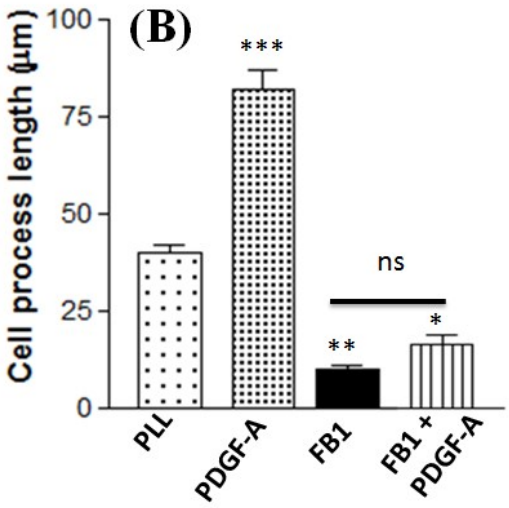
Fig.5.3 Effect of PDGF-A on lipid raft and actin cytoskeleton interaction

(A) *Representative Western blot for GM1 after treatment of OPCs with PDGF-A - 1ng/ml and 10ng/ml in actin cytoskeletal fraction.* ***(B)*** *After densitometric analysis, GM1 data were normalized to actin. 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).* ***(C)*** *Immunocytochemistry showing the co-localization of F-actin and Lipid rafts. OPCs were exposed to (PDGF-A-10ng/ml) .The cells were studied for the formation of microfilament extensions (filopodia). Cells were stained for F-Actin (phalloidin 488-green) and Lipid raft (red). Co-localization of F-actin and Lipid raft as shown in the merge panel. Scale bar:50µm.* ***(D)*** *Representative Western blot for GM1 after treatment of OPCs with PDGF-A with or without PDGF-A inhibitor(AG1295) treatment.* ***(E)*** *After densitometric analysis, GM1 data were normalized to actin. 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).* **(F)** *Immunocytochemistry showing the co-localization of Lipid raft and Integrin αVβ1 . OPCs were exposed to (PDGF-A-10ng/ml) and were stained for the Lipid raft (green)*

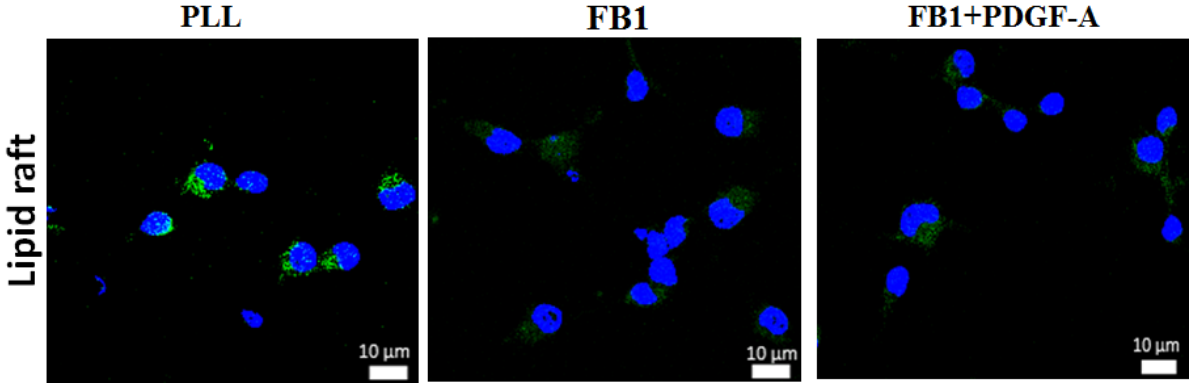
and integrin αVβ1(red). Co-localization of lipid rafts and integrin αVβ1 as shown in the merge panel and the cropped images. Scale Bar = 50μm

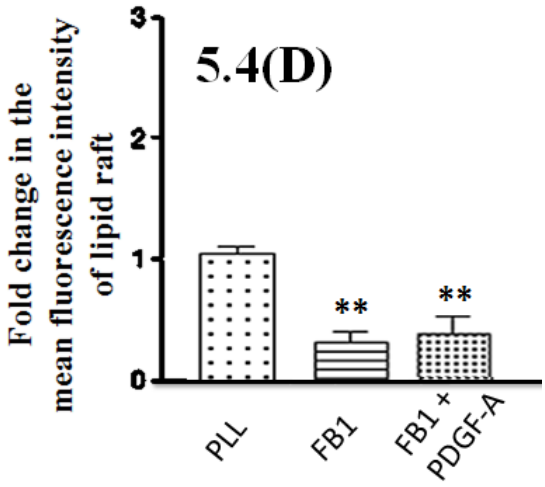
5.4(A)



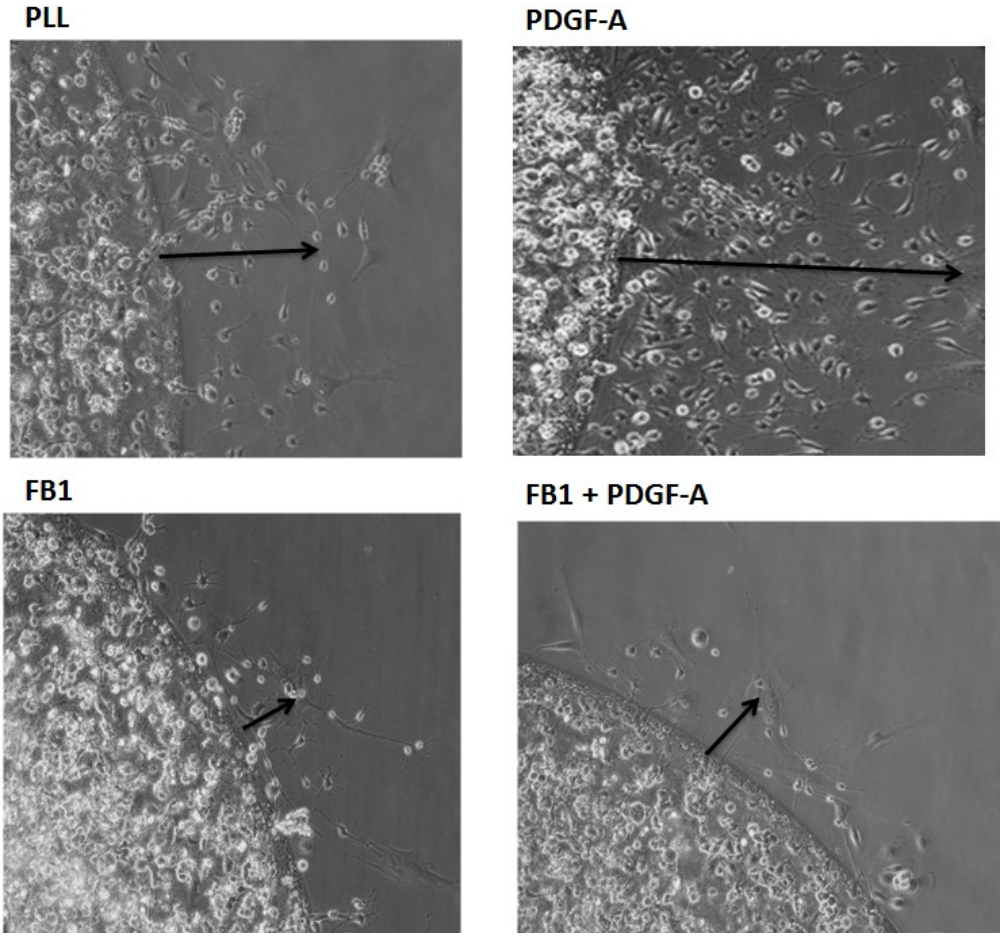


5.4(C)




5.4(D)
Fold change in the mean fluorescence intensity of lipid raft
3
2
1
0
**
**
PLL
FB1
FB1 + PDGF-A

5.4(E)


PLL
PDGF-A
FB1
FB1 + PDGF-A

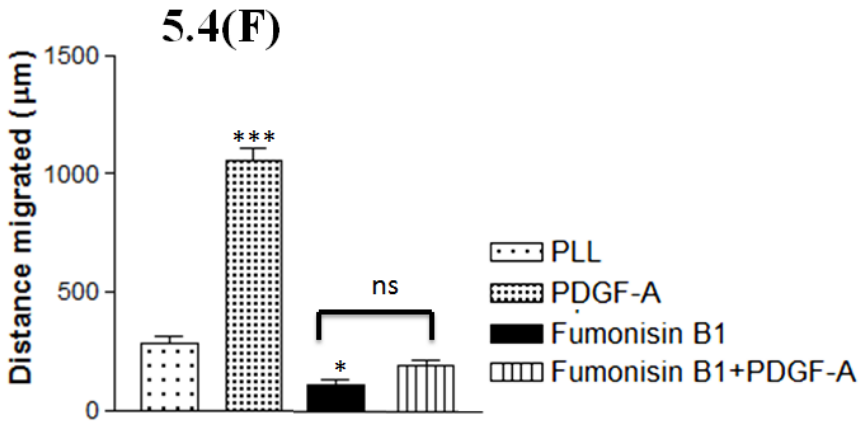
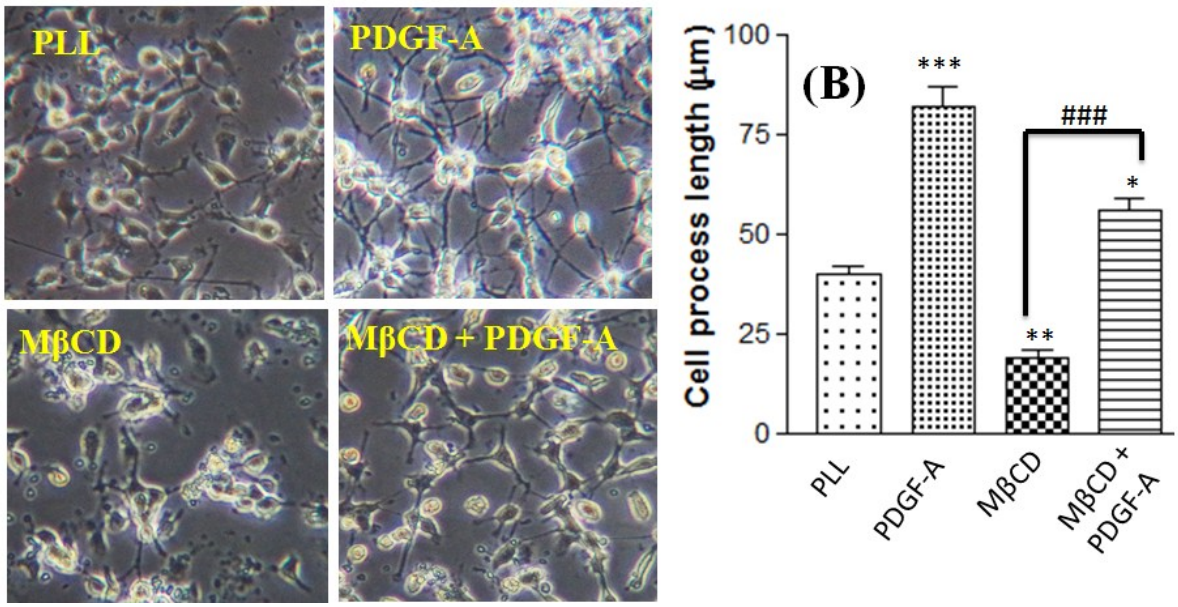


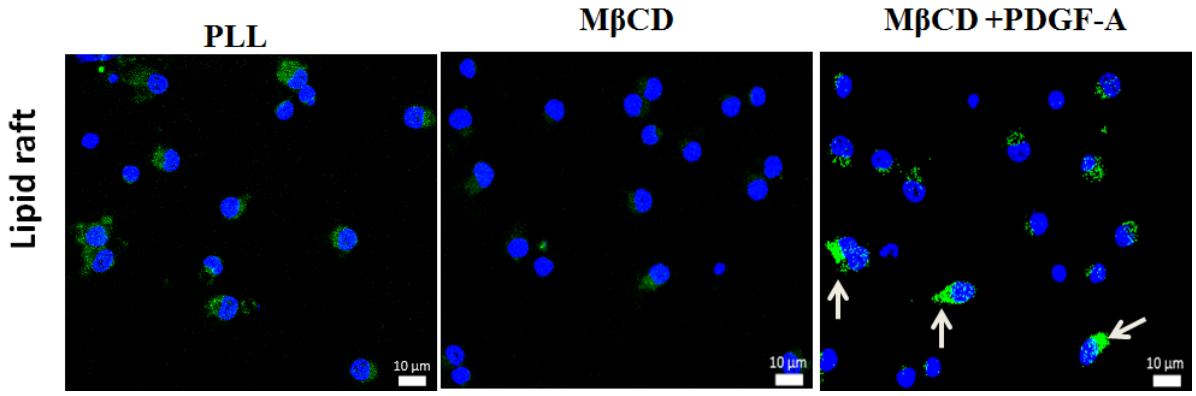
Fig. 5.4 Role of lipid raft inhibition by FB1 on PDGF-A mediated effects on OPCs

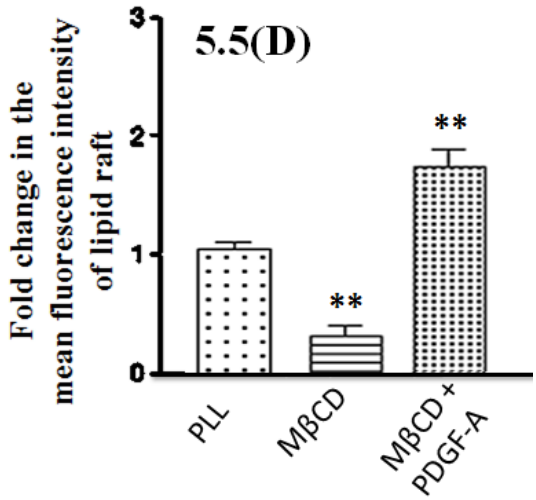
(A) *OPCs were exposed to PDGF-A (10ng/ml) in presence and absence of FB1. PLL was used as control.* ***(B)*** *Morphological analysis using phase contrast microscopy shows that cell process length decreases in FB1 and FB1+PDGF-A. Cell process length was significantly more in (PDGF-A) than compared to PLL. Graph shows the effect of FB1 on OPCs length and 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)*** *Immunocytochemistry showing Lipid raft staining (green) and DAPI (blue). OPCs were exposed to (PDGF-A-10ng/ml) in presence and absence of FB1. Scale Bar = 10µm* ***(D)*** *Graph showing the mean fluorescence intensity of lipid raft in OPCs exposed to (PDGF-A-10ng/ml) in presence and absence of FB1* ***(E)*** *Agarose drop migration assay (Frost et al., 2000 and Vora et al., 2011) in response to PDGF-A and FB1 treatment. Phase contrast photomicrograph showing PLL, PDGF-A, FB1and FB1+ PDGF-A. 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.* ***(F)*** *Graph showing the effects of PLL, PDGF-A, FB1and FB1+ PDGF-A 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).*

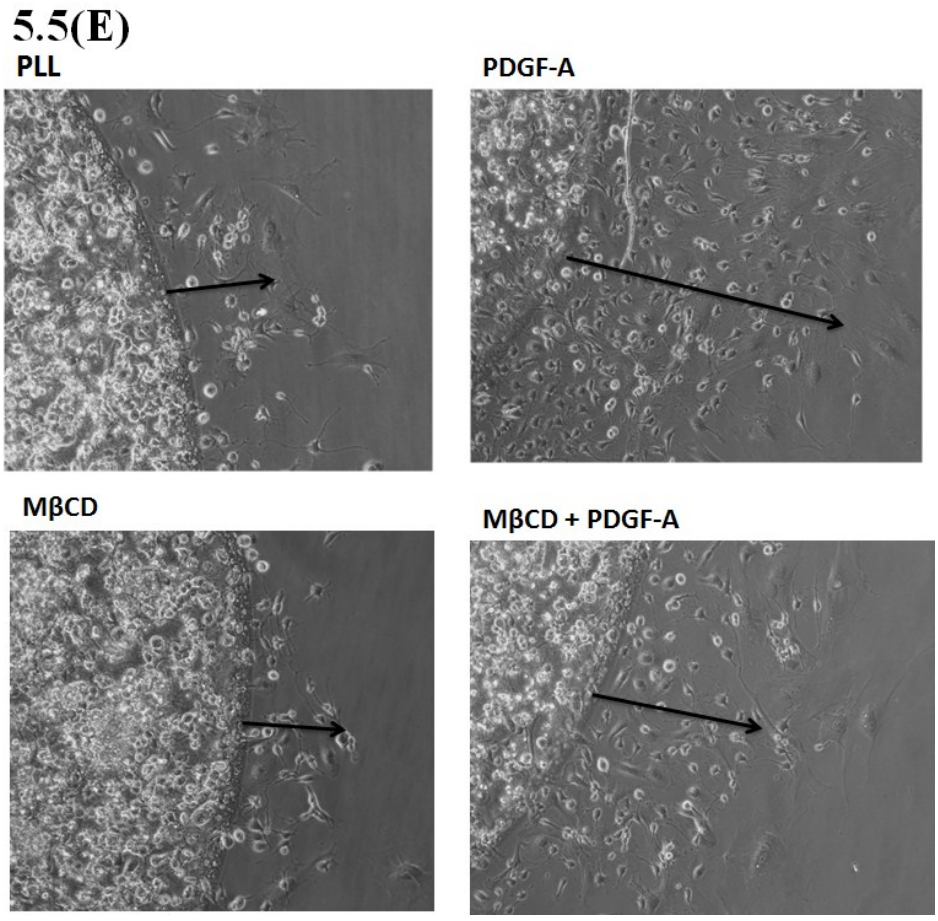
5.5(A)


PLL
PDGF-A
MβCD
MβCD + PDGF-A
(B)
Cell process length (µm)
100
75
50
25
0
***
###
*
**
PLL
PDGF-A
MβCD
MβCD + PDGF-A

5.5(C)


PLL
MβCD
MβCD +PDGF-A
Lipid raft
10 µm
10 µm
10 µm


5.5(D)
Fold change in the mean fluorescence intensity of lipid raft
3
2
1
0
**
**
PLL
MβCD
MβCD + PDGF-A



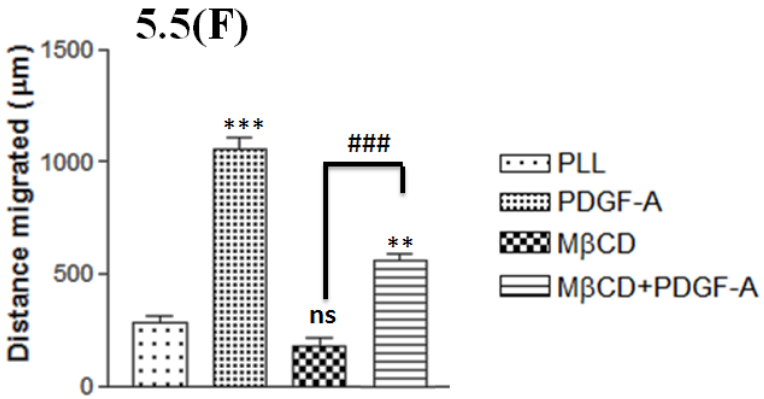
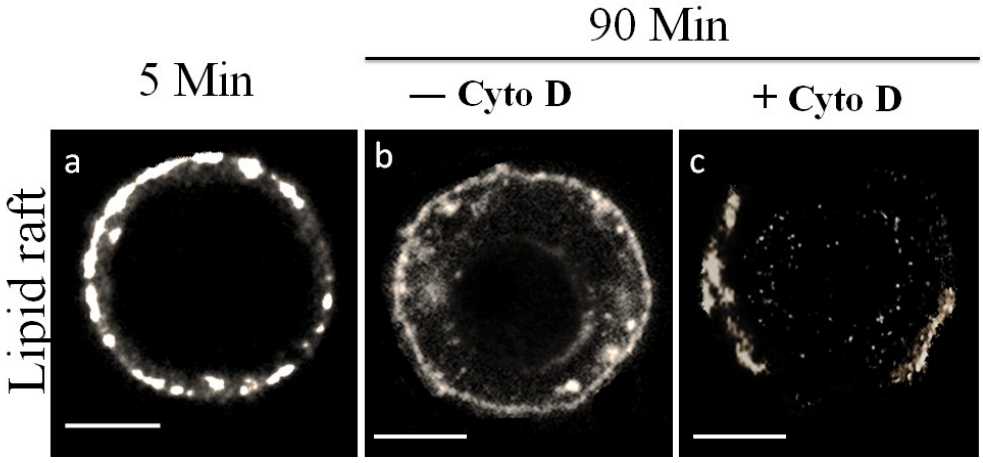


Fig. 5.5 PDGF-A revert the effects of MβCD on OPCs

(A) *OPCs were exposed to PDGF-A at in presence and absence of MβCD. PLL was used as control.* ***(B)*** *Morphological analysis using phase contrast microscopy shows that cell process length decreases in MβCD treated OPCs. Cell process length was*

*significantly more in (PDGF-A) than compared to PLL. Graph shows the effect of MβCD on OPCs length and 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)*** *Immunocytochemistry showing Lipid raft staining (green) and DAPI (blue) . OPCs were exposed to (PDGF-A-10ng/ml) in presence and absence of MβCD. Scale Bar = 10µm* ***(D)*** *Graph showing the mean fluorescence intensity of lipid raft in OPCs exposed to (PDGF-A-10ng/ml) in presence and absence of MβCD* ***(E)*** *Agarose drop migration assay (Frost et al., 2000 and Vora et al., 2011) was used to study the OPC migration. Phase contrast photomicrograph showing PLL, PDGF-A, MβCDand MβCD + PDGF-A. 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.* ***(F)*** *Graph showing the effects of PLL, PDGF-A, MβCDand MβCD + PDGF-A 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)*

5.6(A)



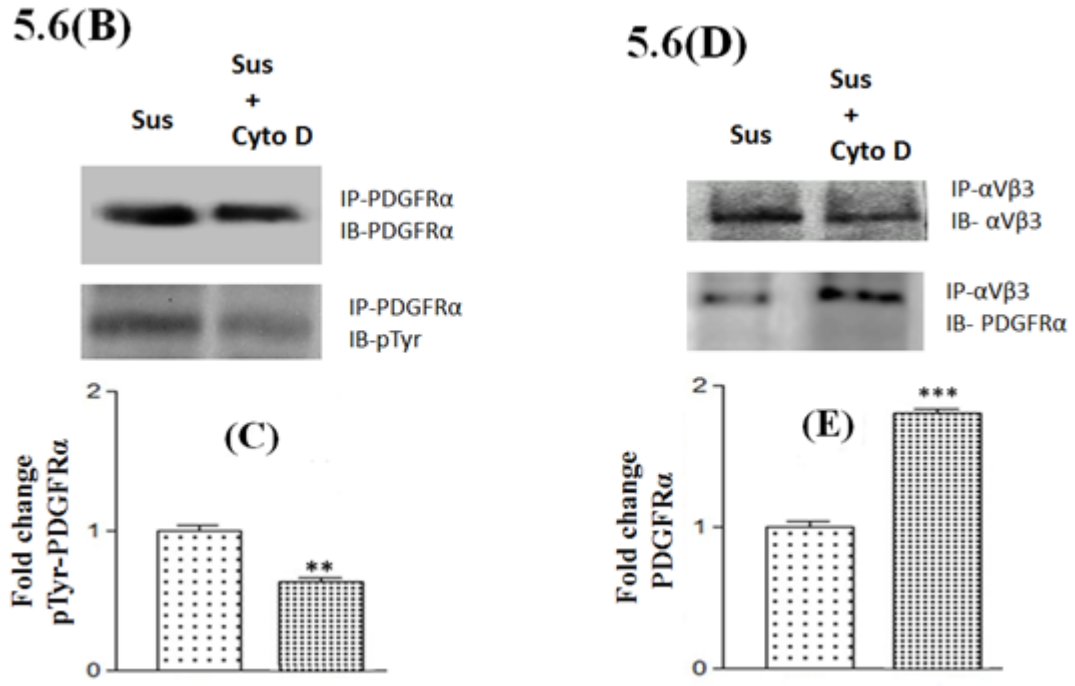


Fig. 5.6 Lipid raft internalization and PDGFRα-αvβ3 interactions in C6 glioma

(A) *Lipid raft internalization assay was performed by keeping C6 cells in suspension for 90mins. Lipid rafts gets internalized when cells detached from ECM. We have studied the effects of actin polymerization inhibitor (Cyto-D) on lipid raft internalization.* ***(B)*** *Effect of Cyto-D on PDGFRα phosphorylation was checked using anti-pTyr antibody. For that first the immunoprecipitation of PDGFRα was done. Then the pTyr was checked using western blot method.* ***(C)*** *Graph showing the effects of Cyto-D on PDGFRα phosphorylation. 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).* ***(D)****Effect of Cyto-D on PDGFRα-αvβ3 interaction was checked. For that first the immunoprecipitation of αvβ3 was done. Then the PDGFRα was checked using western blot method.* ***(E)*** *Graph showing the effects of Cyto-D on PDGFRα-αvβ3 interaction. 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).*

5.4 Discussion

Lipid rafts are specialized microdomains within the plasma membrane and intracellular membranes that possess a liquid-ordered structure resulting from enrichment in specific lipid components such as cholesterol and sphingolipids (Galbiati et al. 2001). Lipid rafts have been implicated in a range of cellular processes including regulation of signal transduction and trafficking of macromolecules (Simons and Toomre 2000; Schlegel et al. 1998). Rafts are believed to function as signaling platforms in which interactions between receptor proteins, adapter molecules, and effectors are tightly regulated to modulate signal generation. They are also thought to serve as sites of signal amplification and diversification.

Previous studies have reported that growth factors such as platelet derived growth factor or epidermal growth factor may use these lipid rafts as platforms for signaling (Paratcha and Ibanez 2002; Abulrob et al. 2004; Gielen et al. 2006). Studies have also shown that signaling through the PDGFR axis is mediated by caveolar and non-caveolar lipid raft microdomains in diverse cell types, including endothelial cells, fibroblasts, osteoblasts, and SMC (Liu et al. 1997a; Liu et al. 1997b; Liu et al. 1996; Solomon et al. 2000; Stehr et al. 2003). As discussed in Chapter-4, studies on OPCs showed that integrins αvβ3 and α6β1 interaction with PDGFRα is required for the proliferation and survival, respectively. For this interaction of PDGFRα and integrin, lipid raft microdomains is very much essential (Baron et al. 2002; Baron et al. 2003) (Decker and ffrench-Constant 2004). It was shown that at physiological concentration of PDGF-A (1ng/ml), PDGFRα-αvβ3 interaction is induced within lipid rafts when placed on vitronectin, for OPC proliferation. Interestingly, higher concentration of PDGF-A (10ng/ml) enhanced the OPC proliferation, without PDGFRα-αvβ3 interaction (Baron et al. 2002). However, no such reports are available regarding PDGFRα-αvβ1 interaction during OPC migration. Following co-immunoprecipitation studies, results show that at both the concentration of PDGF-A [i.e, physiological (1ng/ml) and higher concentration of PDGF-A(10ng/ml)], PDGFRα was not interacting with αvβ1 integrin in OPCs. However, the higher concentration of PDGF-A significantly enhanced the αvβ1 expression (Chapter-4; Fig.1). *Hence we*

hypothesized that PDGF activation of the PDGFRα leads to lipid raft microenvironment formation and the recruitment of integrins prior to cytoskeletal rearrangement.

PDGFRα mediated modulation of lipid rafts in membrane is not known. Here we show that PDGFRα activation increase the lipid rafts in OPCs (Fig.5.1). PDGFRα activation not only increased the expression levels but also the aggregation or coalescence of lipid rafts (Fig.5.1 (A) c). This coalescence of lipid rafts allows the entry of crucial signaling molecules into these microdomains upon ligand-mediated cross-linking of the membrane receptors. Lipid rafts serve as a site for the clustering and concentration of signaling proteins. LINGO-1 suppresses OLG differentiation by ErB2 translocation and activation in lipid rafts (Lee et al. 2014). Studies have shown that lipid rafts can cluster and this clustering may depend upon cholesterol and actin tethering to the membrane(Baron et al. 2003). Data from the present study also shows that PDGFRα activation increased the F-actin staining (Fig.5.2). Interactions between lipid raft and cytoskeletal components can contribute to the regulation of lipid raft assembly/clustering and cytoskeletal dynamics(Head et al. 2006; Suzuki et al. 2011). As shown in Chapter-3, 10ng/ml PDGF-A alone was sufficient to increase the cell process length, F-actin staining and filopodia formation (Fig.3.8). Plasma membrane is stretched by the actin cytoskeleton or microfilament to increase the cell process length and filopodia formation. Lipid rafts are the sites in membrane where microfilaments can get anchored, which is essential for the tethering (Ritchie et al. 2003; Kusumi et al. 2004). Our data shows that PDGFRα activation increased the lipid raft and actin cytoskeleton interaction in OPCs (Fig.5.3). Cytoskeleton can regulate the size of the lipid raft (Jacobson and Dietrich 1999). In addition to modulation of intracellular signaling cascades, emerging evidence suggests a role for lipid rafts in regulation of cell motility and changes in cell shape, including the formation of lamellipodia and filopodia(Rodgers and Zavzavadjian 2001; Guan 2004; Leitinger and Hogg 2002; Valensin et al. 2002; Simpson-Holley et al. 2002). Interestingly, laminin-2-dependent clustering of the α6b1 integrin within PDGFRα-containing lipid rafts was recently demonstrated in oligodendrocytes (Baron et al. 2003), suggesting a functional link between growth factor signaling, lipid rafts, and regulation of the cytoskeleton.

Moreover, co-localization studies by ICC showed that PDGFRα activation increased the integrin αvβ1 and lipid raft co-localization (Fig.5.3). Many proteins that directly regulate the cytoskeleton such as integrins, extracellular matrix proteins, and filament proteins have been shown to associate with rafts reviewed in (van Deurs et al. 2003). These unique lipid microdomains provide a spatial microenvironment for the aggregation of specific sets of proteins providing for enhanced efficacy and specificity of interactions between enzymes involved in signal transduction (Cremesti et al. 2001).

To confirm the role of lipid raft in PDGFRα mediated OPC migration, we have used Fumonosin B1 (FB1) (inhibitor of lipid raft synthesis). FB1 significantly decreased the PDGFRα activation mediated OPC migration, cell process formation and lipid rafts levels (Fig.5.4). Next, the PDGFRα activation mediated lipid raft recruitment to the cell membrane was analyzed using methyl-β-cyclodextrin (MβCD) to specifically remove the membrane lipid raft. The data shows that PDGFRα activation revert the effects of MβCD (Fig.5.5). Glial cells produce up to 90 % of neural cholesterol and therefore glial cells are relevant mediators to cholesterol homeostasis in the CNS (Dietschy and Turley 2004). Oligodendrocytes can synthesize cholesterol which is important during myelinogenesis (Dietschy and Turley 2004). Our results suggest that PDGFRα activation may regulate the OPC dependent cholesterol homeostasis in CNS.

The importance of lipids rafts in glioma progression has been well documented in the last decade and several studies indicate the involvement of lipid rafts in modulating the glioma biology and hence the current study was designed to clarify further the mechanistic role of lipid rafts during anoikis resistance in C6 glioma cells (Li et al. 2006). During ‘anoikis resistance’, glioma survives in the ECM detached condition. Results from the present study showed that lipid rafts are internalized in the C6 glioma cells kept in suspension [Fig.5.6(A) b]. Internalization of lipids rafts are shown be dependent on actin cytoskeleton (Head et al. 2014). Cytoskeleton is usually localized to lipid rafts and help in the regulation of lateral diffusion of membrane proteins and lipids in response to extracellular events like receptor activation (Head et al. 2014). As shown in chapter 4, PDGFRα uncoupling from the αvβ3 is essential for enhanced PDGFRα activity during anoikis resistance in C6 glioma cells. However, when the

actin polymerization is disrupted by cytochalasin D (Cyto D), the internalization of lipid raft got affected [Fig.5.6(A)c] and due to which the PDGFRα didn't uncouple from the αvβ3. These observations are in line with earlier reports which showed PDGFRα activation is essential for the pro-survival signals during ECM detached condition (Fleming et al. 1992; Hermanson et al. 1996; Heldin and Westermark 1999; Cenciarelli et al. 2016).

In conclusion, our study demonstrates for the first time the existence of a strong and specific PDGFRα-dependent interaction of lipid rafts with the actin cytoskeleton in OPCs. Moreover, the actin and lipid raft interaction is important for the PDGFRα and αvβ3 interactions during anoikis resistance.