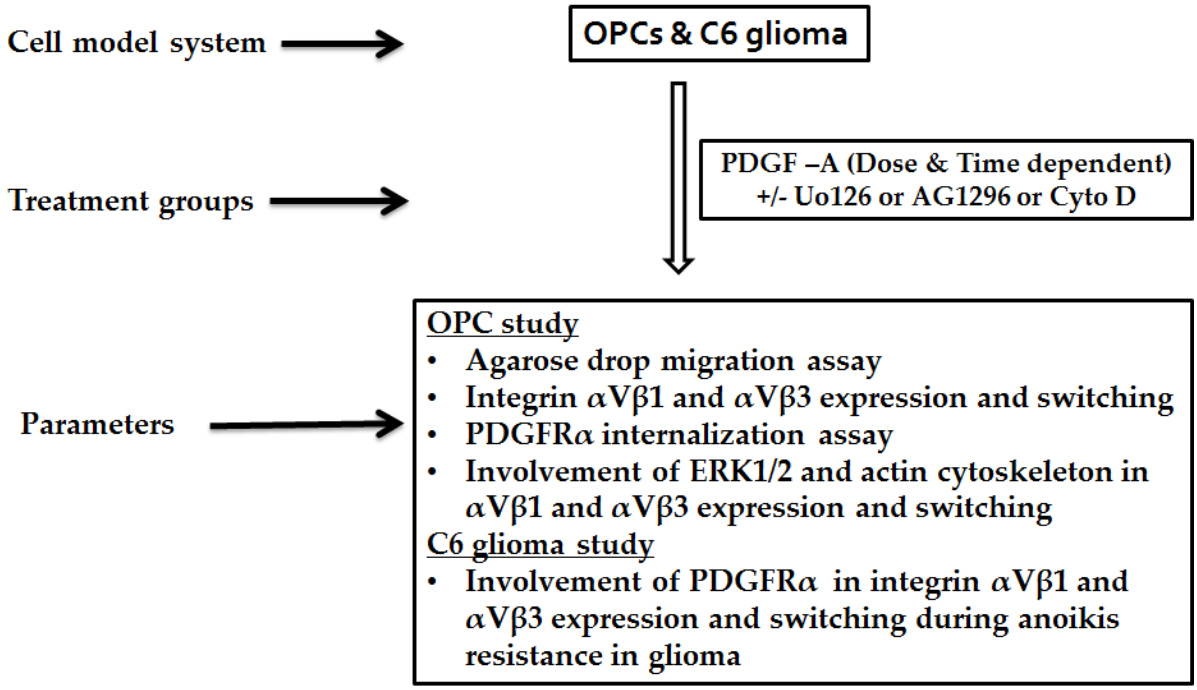
4.1 Introduction

Migration is critical for the normal dispersal of OPCs during brain development. Previous studies have shown significant roles for integrins in OLG lineage behaviors, as described above in introduction (Subheading; 1.4). Studies have shown a role for the β1 integrin in regulating OPC migration (Frost et al. 1996; Milner et al. 1996). The exposure to growth factors changes the way that the OPC react to the growth factors (Frost et al. 2009). Further, a recent study shows that PDGF activated Fyn plays a regulatory role in OPC migration (Miyamoto et al. 2008), providing evidence that PDGFRα/integrin interactions play an important role in regulating OPC migration. Other studies have shown that OPC proliferation requires PDGFRα interaction with αvβ3 integrin in lipid rafts (Baron et al. 2002). Moreover, PDGFRα and integrin interaction is required for OPC survival (Decker and ffrench-Constant , 2004). Most of the previous studies have used OPC exposed to exogenous growth factors prior to integrin analysis. In addition, data showed that OPCs respond to growth factors at different concentrations in different ways. Hence, the present study hypothesized that PDGFRα activation is responsible for the switches in integrin expression and further regulated oligodendrocyte behaviours. We expect to identify the nature of the interactions between PDGFRα and the specific integrins that would be involved in the regulation of OPC migration. The preliminary data from the present study showed the changes in integrin expression in OPCs resulting from PDGFRα activation. However, earlier studies have reported the role of both PDGFRα and integrin αvβ1 in OPC migration (Armstrong et al. 1990; Frost et al. 1996; Milner et al. 1996). However, we expect to identify a direct association between the activated PDGFRα and the αvβ1 integrin after growth factor treatment. In addition, the data clarified the role of PDGFRα transient and continuous activation on β1 and β3 integrin subunit in the regulation of OPC migration and proliferation. Finally, we have provided the substantive evidence to specify the role of integrin switching in OPC migration. Moreover, the study was extended to study the role PDGFRα in integrin switching during glioma anoikis resistance. PDGFR and integrin signaling are deregulated in the glioma. Findings from the present study will reveal correlation between integrin

expression and switching in anoikis resistance instructed by the PDGFRα/PDGF-A regulatory axis.

4.2 Plan of work



4.3 Results

4.3.1 Dose-dependent effect of PDGFRα activation on αvβ1 and αvβ3 integrins expression

As discussed earlier previous studies have shown that OLGs change their expression pattern of integrins as they progress through their lineage.However, the difference in our current study and the previous study is that PDGF and FGF were used to expand the population of OPCs prior to plating and analysis of the integrins. We used primary OPCs, isolated from neonatal brains, and not exposed to any exogenous growth factors prior to integrin analysis. Our results showed that in freshly isolated/ naïve OPCs, the expression of β1 integrin was very low as compared to β3 integrin (Fig.4.1). However, as the freshly isolated OPCs plated on PLL, the expression of β1 integrin increased

significantly (Fig.4.1). The present study shows that the expression of β3 integrin was high in freshly isolated/naïve OPCs than β1 integrin. As our data showed that freshly isolated OPCs significantly express αvβ3 subunit, but not the αvβ1 subunit (Fig.4.1). Data from the present study indicates that the previous study may not have been fully accurate.The current data suggested extracellular regulation of integrin expression in OPCs and therefore, we proposed to assess changes in integrin expression after treatment with the 1ng/ml and 10ng/ml PDGF-A (previously used concentration & described in methodology section). PLL was used as control substratum which supports cell adhesion and spreading without engaging integrins. Here we show that 1ng/ml PDGF-A concentration was unable to increase the expression of integrins, αvβ1 and αvβ3. However, 10ng/ml PDGF-A concentration significantly increased the expression of both the integrins (Fig.4.2). Moreover, there was no partially glycosylated band of αvβ1 subunit in the OPCs treated with 10ng/ml PDGF-A. Partially glycosylated integrins are present in the cytoplasm and fully glycosylated integrins are the membrane recruited. The anti- αvβ1 antibody used in the present study detects both partially and fully glycosylated bands in western blot analysis. This suggest that higher concentration of PDGF-A may induce glycosylation of αvβ1 integrin and therefore, increase its recruitment in the membrane.

4.3.2Time-dependent effects of PDGFA on αvβ1 and αvβ3 integrins expression

Further, the data shows that the αvβ3 subunit expression level appears to increase after PDGF-A continuous treatment (Fig.4.3), which was not the case with αvβ1. This finding fits very well with the knowledge that the αvβ3 integrin is required for PDGF induced OPC proliferation (Baron et al., 2002). Next, the internalization of PDGFRα was studied in continuous and transient exposure of PDGF-A. For the PDGFRα internalization study, biotinylation followed by immunoprecipitation was done. Fig.4.4A, shows the optimization of biotinylation and immunoprecipitation followed by western blot analysis of PDGFRα with all the controls. It was observed that PDGFRα internalized significantly increased in the continuous exposure (Fig.4.4B,C). Further studies were done using continuous exposure of PDGF-A. Next, the role of PDGFRα activation on integrin expression was confirmed by inhibiting PDGF

receptor tyrosine kinase using AG1295. Inhibition of PDGFRα by AG1295 significantly decreased the PDGFRα activation mediated integrin expression (Fig.4.5A,B,C). Moreover, the role of αvβ1 integrin was checked in the PDGFRα activation mediated OPC migration. Anti- αvβ1 antibody was used specifically to block the integrin αvβ1, whereas, RGD peptide was used to block all the classes of integrins. Along with that there was PDGFRα inhibition group (AG1295). Results showed that in all the treatment groups (anti- αvβ1 antibody, RGD peptide blocking and AG1295), PDGFRα activation mediated OPC migration was significantly decreased (Fig.4.5D).

4.3.3 Role of pERK1/2 and actin cytoskeleton in PDGFRα activation mediated integrin switching (αvβ1 and αvβ3)

As shown earlier (chapter -3; result-3.3.4), the higher concentration of PDGF-A (10ng/ml) can significantly activate ERK1/2 and actin without prior activation of integrin. The data showed that inhibiting PDGFRα by AG1295 (10μM)significantly decreased the PDGF-A mediated ERK1/2 and actin activation (Fig.4.6A,B,C and Fig.4.7A,B). Next, the role of pERK1/2 in the PDGFRα mediated integrin expression was examined. Inhibiting ERK1/2 (using UO126) in PDGFA treated OPCs, significantly decreased the expression of integrins αvβ1 and αvβ3 (Fig.4.6D,E,F). Further, we studied the role of actin cytoskeleton in the PDGFRα mediated integrin expression. Inhibiting actin cytoskeleton Cytochalasin-D (Cyto-D) significantly decreased the PDGFRα activation mediated expression of αvβ1 and αvβ3 integrins (Fig.4.7C,D,E). The data suggests involvement of ERK and actin in integrin switching.

4.3.4 PDGFRα inhibition modulate integrin switching during anoikis resistance in C6 glioma cells

Next a cell system was used which can survive in the ECM/attachment independent manner. The phenomenon is called as ‘anoikis resistance’. For that the C6 glioma cells were used. In that the role of PDGFRα was studied in the modulation of αvβ1 and αvβ3 integrins. C6 glioma were kept in two conditions; attached and anoikis induced condition. Anoikis condition was induced by using Poly-Hema. Poly-Hema doesn’t

allow the cells to attach and keep the cells in suspension. Attached C6 glioma were represented as 'att' and anoikis induced cells was represented as 'sus'. PDGFRα inhibition didn't affect the αvβ1 and αvβ3 integrins expression in 'att' C6 glioma (Fig.4.8A,B,C). However, in 'sus'C6 glioma, inhibition of PDGFRα significantly affected the integrins expression. It was observed that in 'sus'C6 glioma, integrin αvβ1 was significantly increased and integrin αvβ3 was significantly decreased in the PDGFRα inhibited condition (Fig.4.8D,E,F).

4.3.5 PDGFRα and αvβ3 integrin uncouples during anoikis resistance

Above result shows that PDGFRα can modulate the integrin expression in C6 glioma in anoikis resistance condition. Firstly, immunoprecipitation (IP) results showed that tyrosine phosphorylation (pTyr) of PDGFRα was increased in 'sus'C6 glioma as compared to'att' C6 glioma (Fig.4.9A,B). For that IP was done using anti-PDGFRα and immunoblotting (IB) was done using anti-pTyr. Secondly, PDGFRα and integrin αvβ3 direct interaction was studied. IP results showed that PDGFRα and integrin αvβ3 direct interaction was significantly decreased in 'sus'C6 glioma as compared to'att' C6 glioma (Fig.4.9C,D). IP was done using anti-PDGFRα and IB was done using anti-αvβ3.

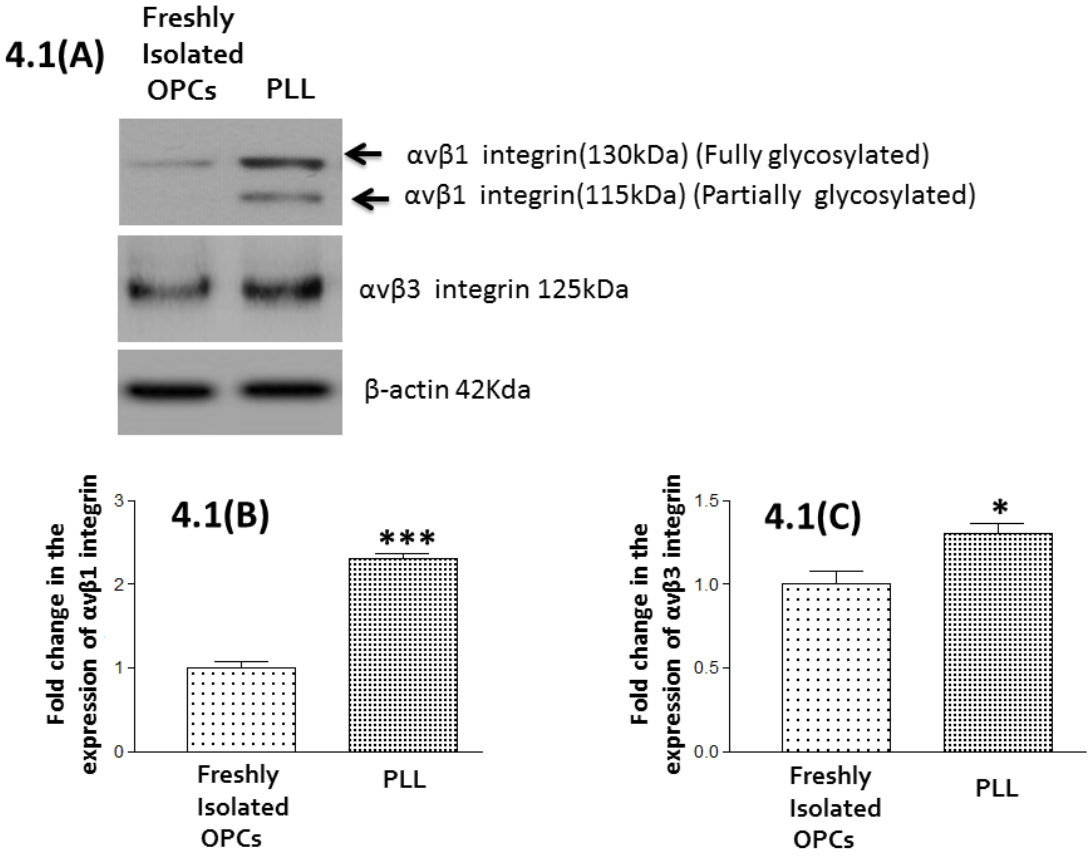
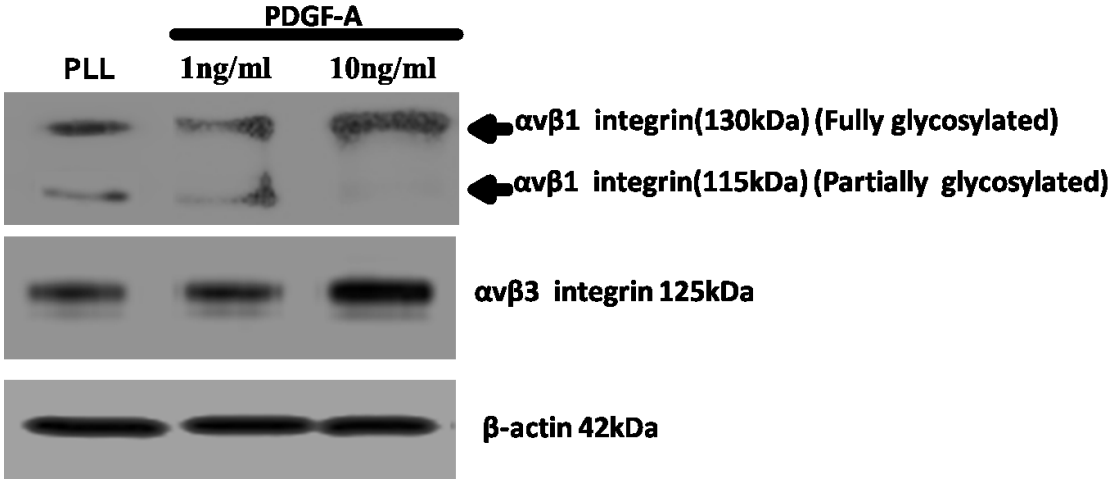
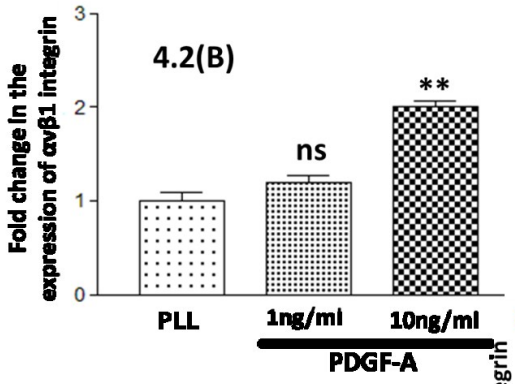


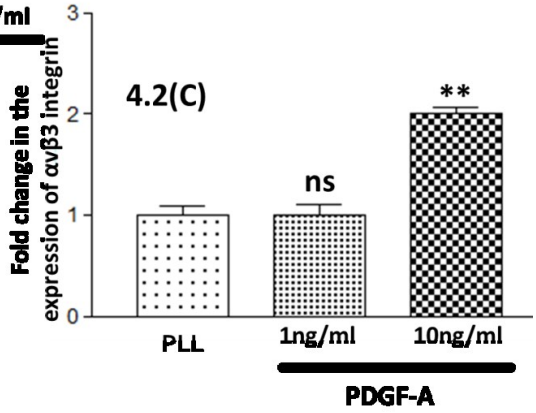
Fig.4.1-Integrin expression in OPCs

(A)*Representative Western blot for integrins αvβ1 and αvβ3 in freshly isolated OPCs and OPCs plated on PLL (1hr adhesion). There were two bands observed in PLL adhered OPCs. Anti- αvβ1 antibody detects two bands of αvβ1 integrin; one is fully glycosylated (130kDa) and another is partially glycosylated (115kDa) forms. (B) and (C) Graphical representation of integrins αvβ1 and αvβ3, after densitometric analysis. Integrins αvβ1 and αvβ3 data were normalized to β-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, ns= non significant).*

4.2(A)


PDGF-A
PLL
1ng/ml
10ng/ml
αvβ1 integrin(130kDa) (Fully glycosylated)
αvβ1 integrin(115kDa) (Partially glycosylated)
αvβ3 integrin 125kDa
β-actin 42kDa


4.2(B)
Fold change In the expression of αvβ1 integrin
3
2
1
0
**
ns
PLL
1ng/ml
10ng/ml
PDGF-A


4.2(C)
Fold change in the expression of αvβ3 integrin
3
2
1
0
**
ns
PLL
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10ng/ml
PDGF-A

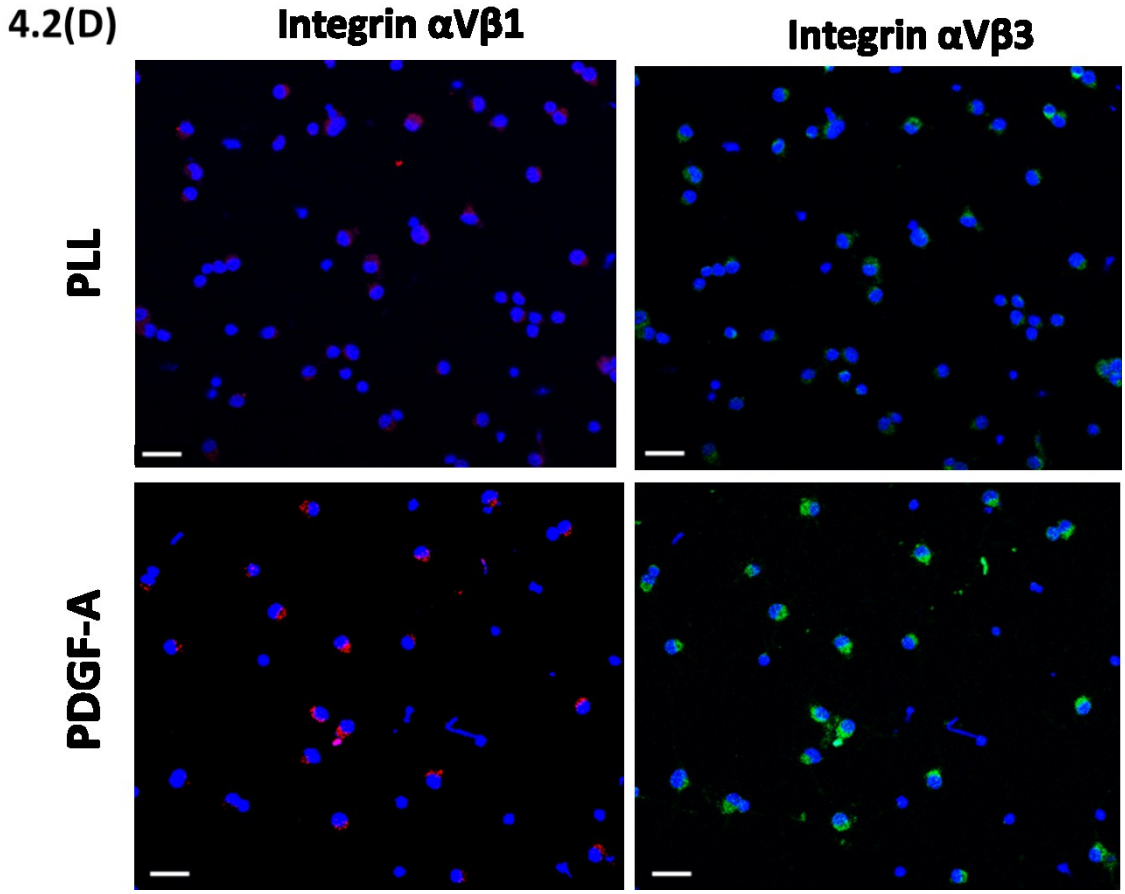


Fig.4.2-Dose dependent effect of PDGF-A on integrin expression

(A)*Representative Western blot for integrins αvβ1 and αvβ3 in OPCs treated with 1ng/ml and 10ng/ml PDGF-A for 1 hr. PLL was control. Anti- αvβ1 antibody detects two bands of αvβ1 integrin; one is fully glycosylated (130kDa) and another is partially glycosylated (115kDa) forms.* ***(B-C)****Graphical representation of integrins αvβ1 and αvβ3, after densitometricanalysis.Integrins αvβ1 and αvβ3 data were normalized to β-actin.* ***(D)*** *Immunocytochemistry showing staining of integrins αvβ1 (red) and αvβ3 (green) in OPCs treated with 10ng/ml PDGF-A for. Scale bar=20µm. 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, ns= non significant).*

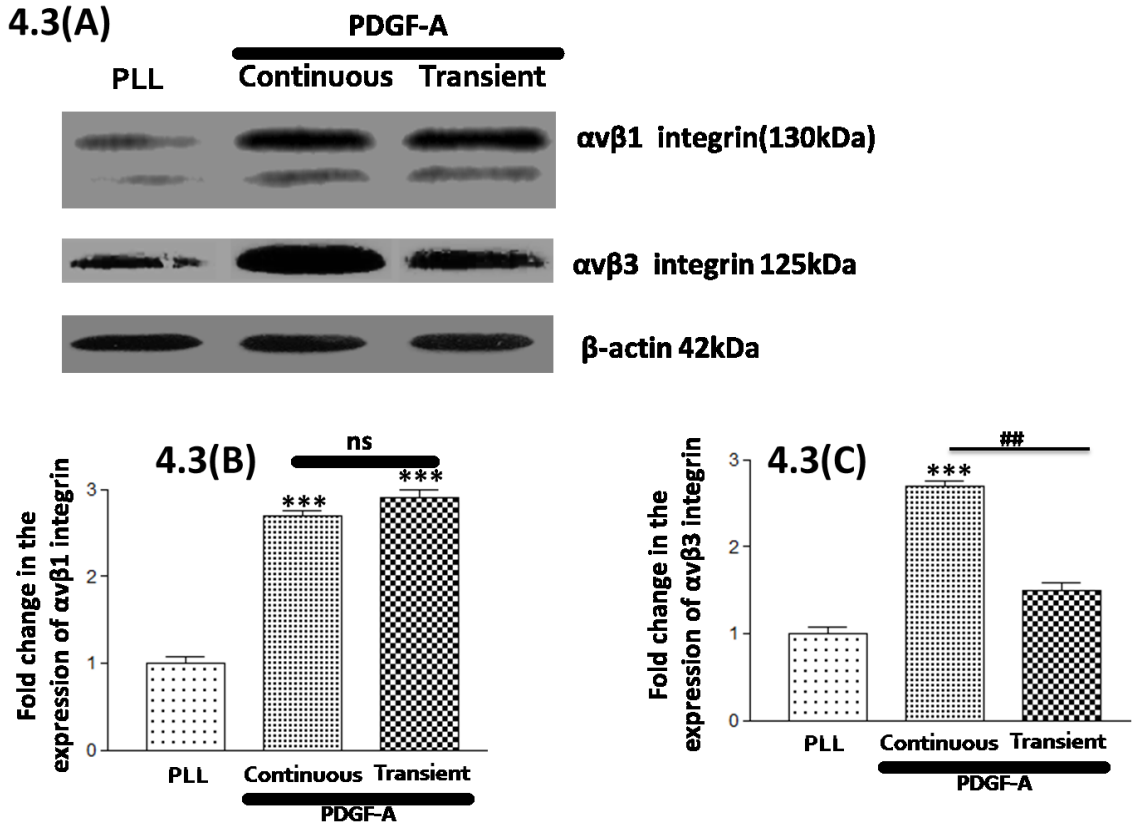


Fig.4.3 Time dependent effect of PDGF-A(10ng/ml) on integrin expression

(A)-*Representative Western blot for integrins αvβ1 and αvβ3 in OPCs treated transiently and continuously with 10ng/ml PDGF-A. In case of transient exposure, OPCs were treated with 10ng/ml for 30min then the medium was replaced with serum free medium and the OPCs were incubated for 24 hrs. And in continuous treatment, OPCs were treated continuously with 10ng/ml medium for 24hrs. PLL was control. Anti- αvβ1 antibody detects two bands of αvβ1 integrin; one is fully glycosylated (130kDa) and another is partially glycosylated (115kDa) forms.* ***(B) and (C)*** *Graphical representation of integrins αvβ1 and αvβ3, after densitometric analysis. Integrins αvβ1 and αvβ3 data were normalized to β-actin. Three different cell preparations were analyzed three times N= 3. Error bars represent ± SEM. Data were evaluated using One way ANOVA -parameter. (Transient and continuous treatment compared to PLL control *P<0.05, **P<0.01, ***P<0.001, ns= non significant). (Transient compared to continuous #P<0.05, # # P<0.01, # # # P<0.001, ns= non significant).*

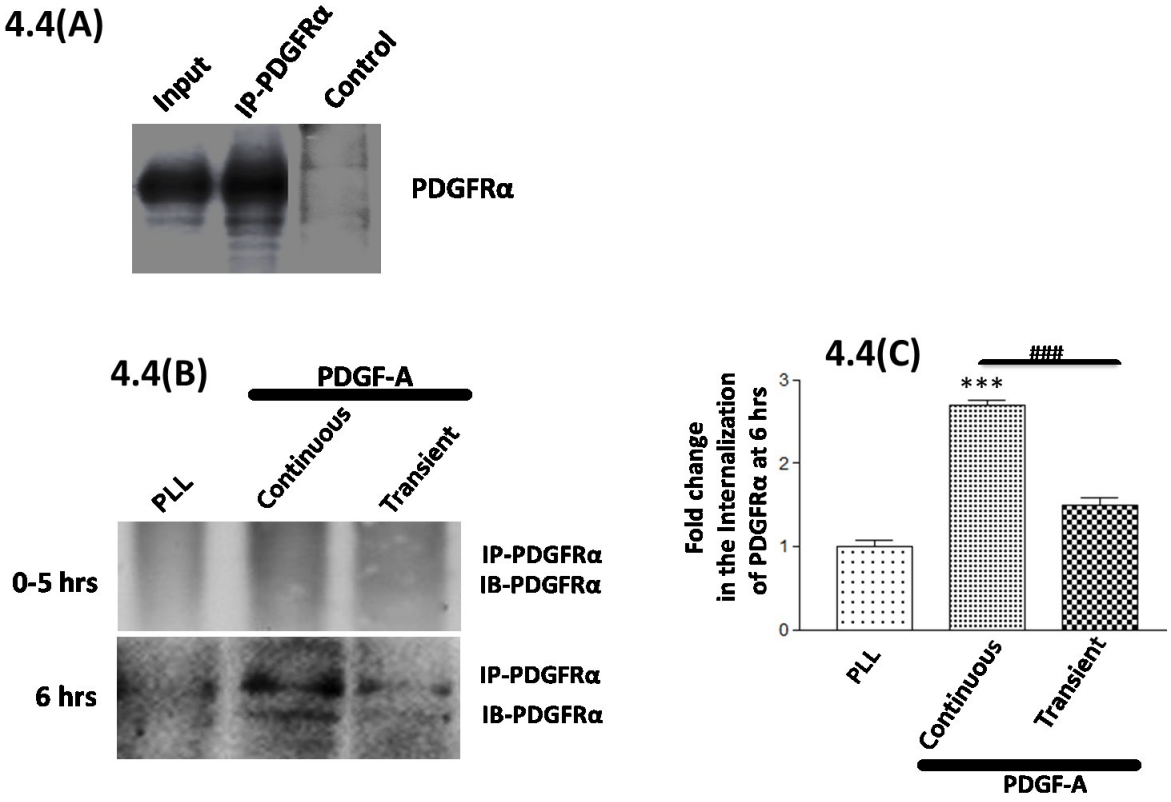
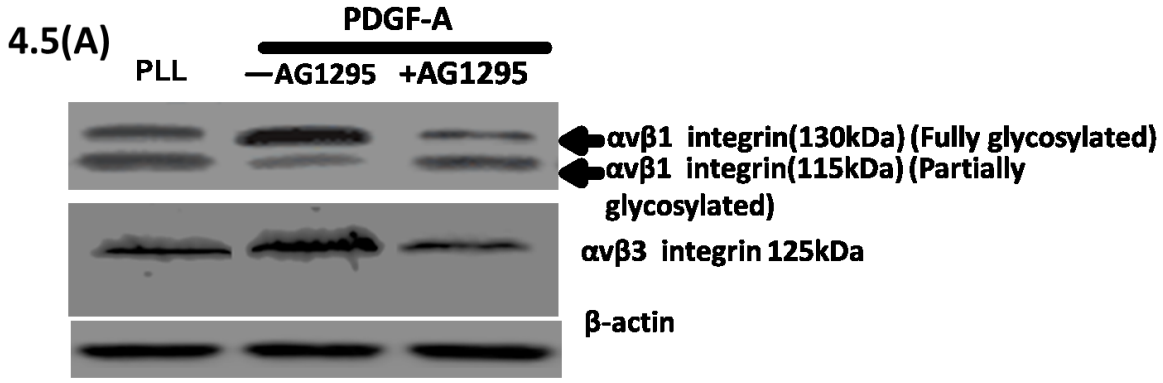
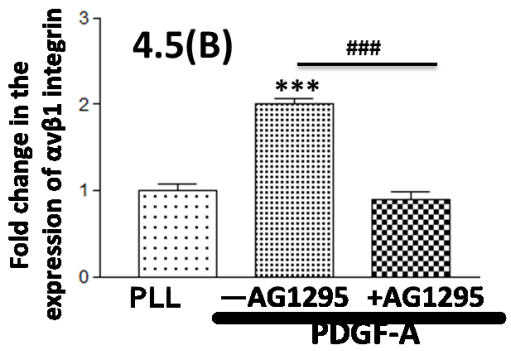


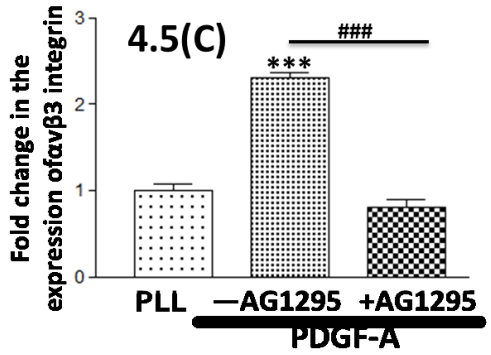
Fig.4.4 Time dependent effect of PDGF-A(10ng/ml) on PDGFRα internalization

(A)*-Representative Western blot for PDGFRα after biotinylation followed by immunoprecipitaion (IP). This represents all the controls and optimization of biotinylation and IP of PDGFRα in OPCs. Lane 1-'Input' represents the sample just before the IP. Lane-2 represents the IP of PDGFRα and Lane 3- represents the sepharose beads control without the anti- PDGFRα antibody for IP.* ***(B)*** *Representative PDGFRα internalization assay: Western blot for PDGFRα after biotinylation followed by immunoprecipitaion (IP) of the OPCs treated transiently and continuously with 10ng/ml PDGF-A . OPCs were treated transiently and continuously for different period of time. But till 5 hrs there were no internalization of PDGFRα observed. Only after 6 hrs the internalization of PDGFRα was observed.* ***(C)*** *Graphical representation of PDGFRα internalization. Three different cell preparations were analyzed three times N= 3. Error bars represent ± SEM. Data were evaluated using One way ANOVA -parameter . (Transient and continuous treatment*

*compared to PLL control *P<0.05, **P<0.01, ***P<0.001, ns= non significant). (Transient compared to continuous #P<0.05, # # P<0.01, # # # P<0.001, ns= non significant).*







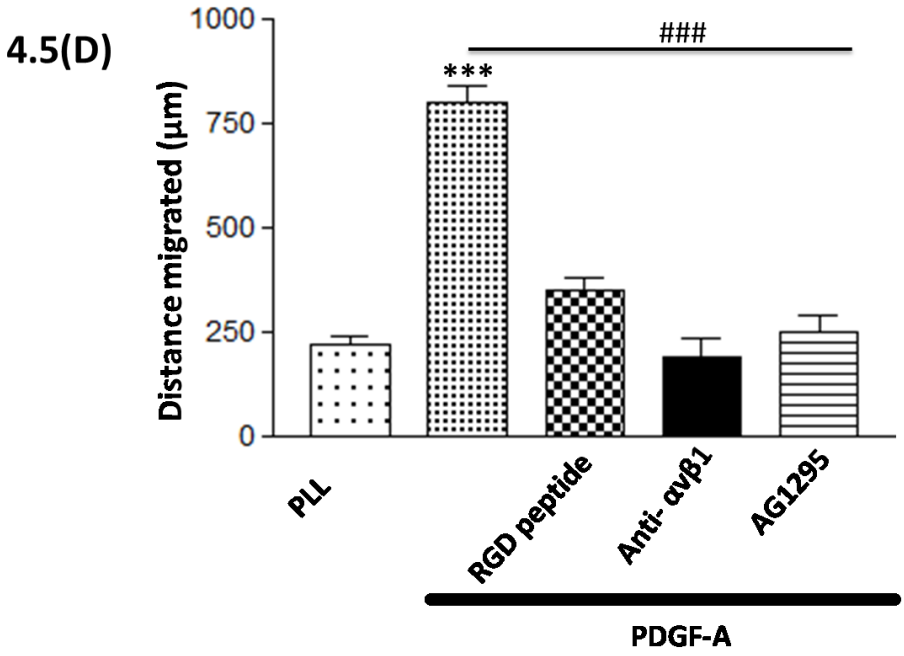
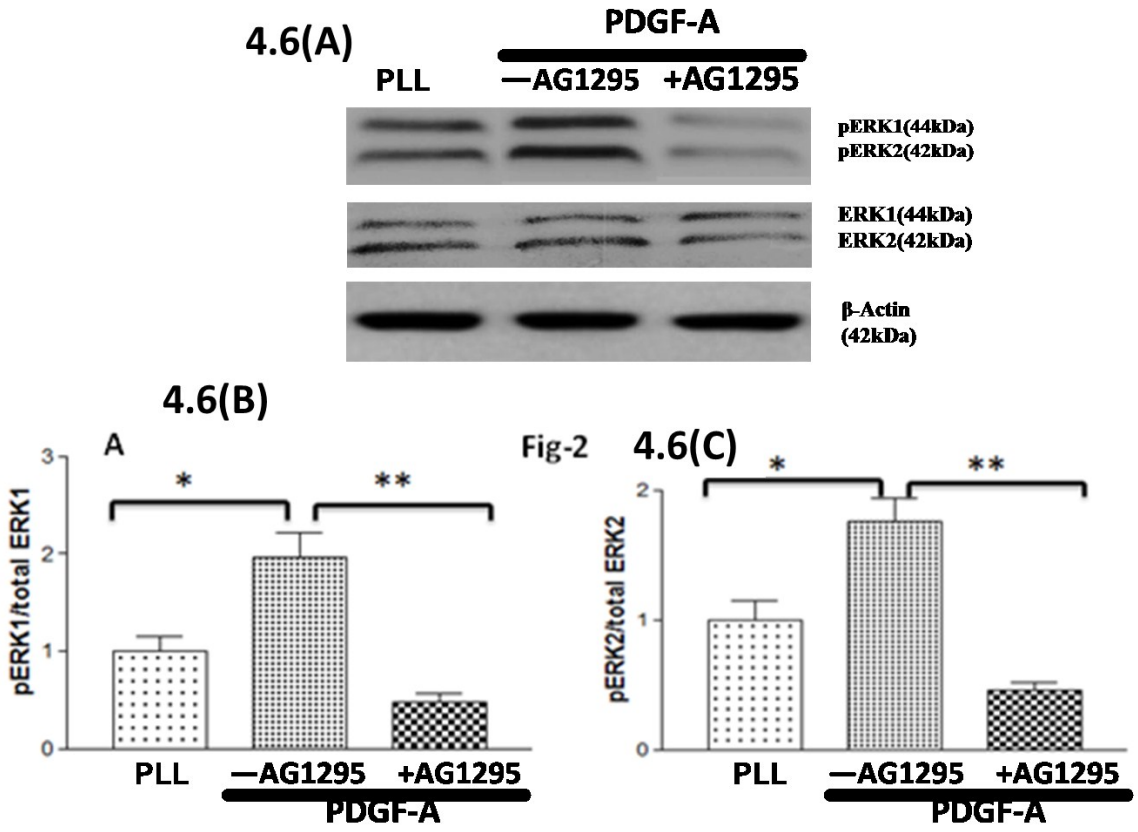


Fig.4.5-Effect of PDGF receptor kinase inhibition on integrin expression and OPC migration

(A)*Representative Western blot for integrins αvβ1 and αvβ3 in OPCs exposed to 10ng/ml PDGF-A with or without PDGF receptor kinase inhibitor (AG1295) for 24hrs. PLL was control. Anti- αvβ1 antibody detects two bands of αvβ1 integrin; one is fully glycosylated (130kDa) and another is partially glycosylated (115kDa) forms.* ***(B) and (C)*** *Graphical representation of integrins αvβ1 and αvβ3, after densitometric analysis. Integrins αvβ1 and αvβ3 data were normalized to β-actin. Three different cell preparations were analyzed three times N= 3. Error bars represent ± SEM. Data were evaluated using One way ANOVA –parameter. (Treatments compared to PLL control *P<0.05, **P<0.01, ***P<0.001, ns= non significant). (PDGF-A without AG1295 compared to PDGF-A with AG1295 #P<0.05, # # P<0.01, # # # P<0.001, ns= non significant).* ***(D)-****Graph represents the agarose drop migration assay in response to PDGF-A treatment with or without RGD blocking peptide, anti-αvβ1 antibody and AG1295. OPCs migrate away from the edge of the agarose drop. Migration of OPCs in suspension (40,000 cells per well). Migration was measured upto 72 h. PLL set was used as control. Each point represents the mean ± SEM of three separate experiments. N=3 with 3 replicates each N. Data were evaluated using One way ANOVA - parameter (PDGF-A, RGD blocking peptide, anti-αvβ1 antibody and PDGF receptor kinase inhibitor (AG1295) compared to PLL control *P<0.05, **P<0.01, ***P<0.001, ns= non significant). (RGD bloking peptide, anti-αvβ1 antibody and PDGF receptor kinase inhibitor (AG1295) compared to PDGF-A #P<0.05, # # P<0.01, # # # P<0.001, ns= non significant).*


4.6(A)
PDGF-A
PLL
—AG1295
+AG1295
pERK1(44kDa)
pERK2(42kDa)
ERK1(44kDa)
ERK2(42kDa)
β-Actin
(42kDa)
4.6(B)
A
pERK1/total ERK1
PLL
—AG1295
+AG1295
PDGF-A
Fig-2
4.6(C)
pERK2/total ERK2
PLL
—AG1295
+AG1295
PDGF-A

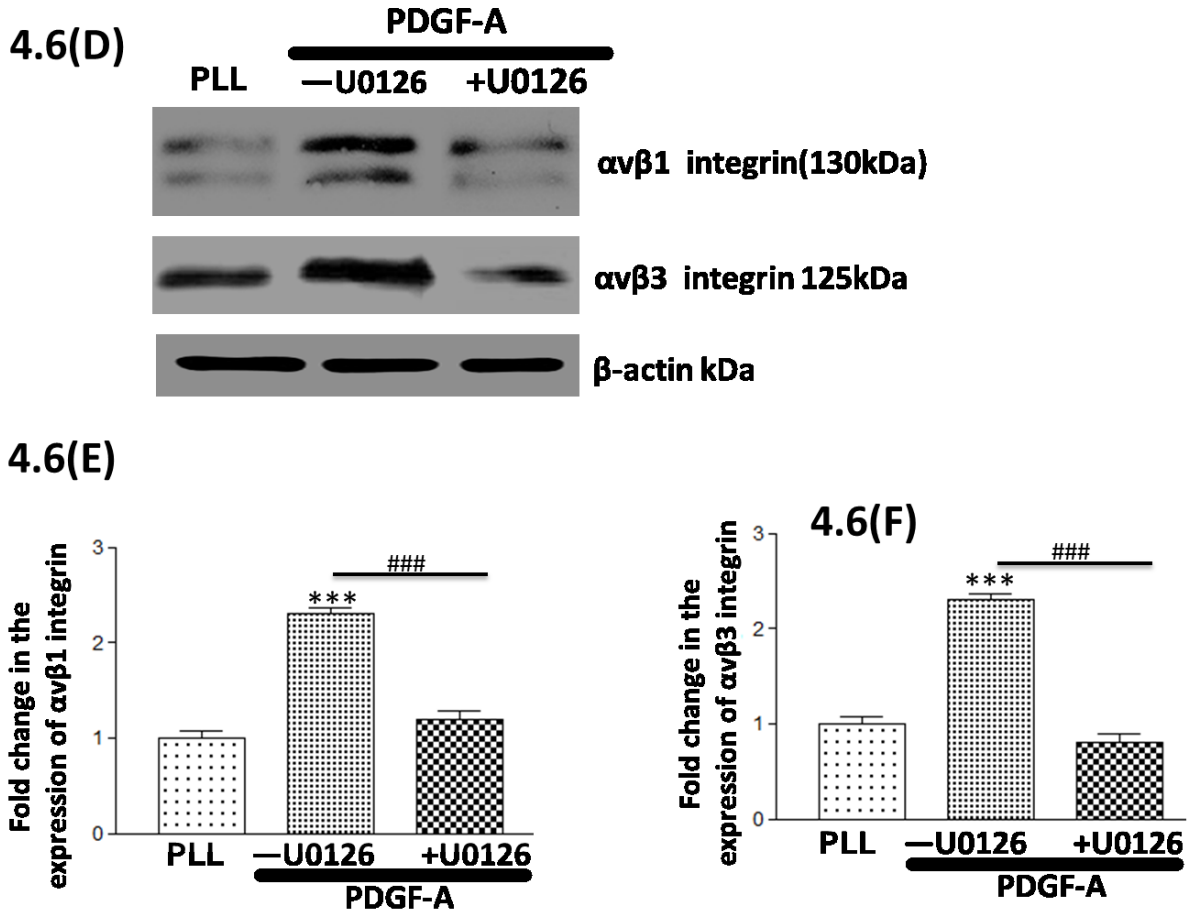
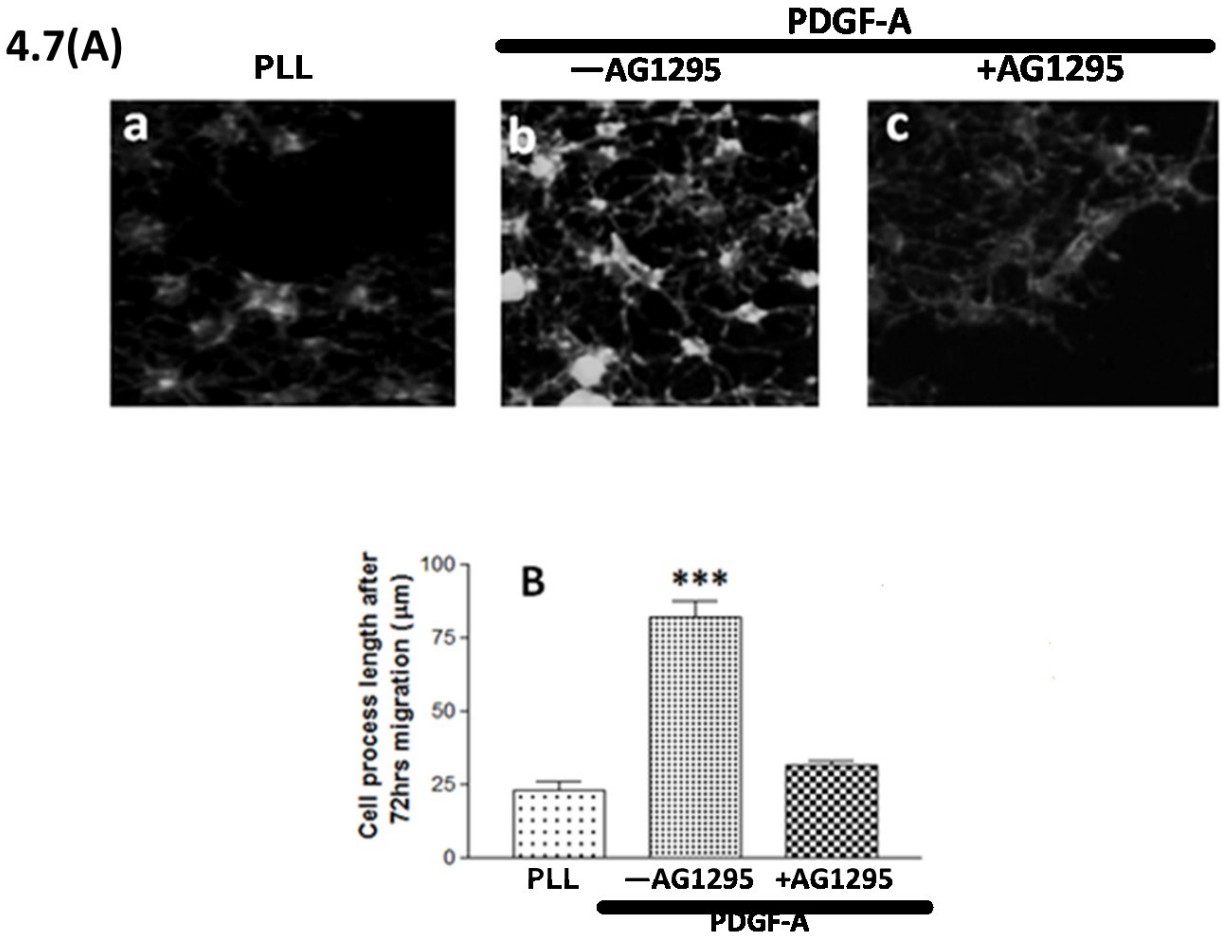


Fig. 4.6 - Effect of ERK1/2 inhibition on PDGFRα activation mediated integrin expression

(A)*Representative Western blot of PDGF-A alone and in presence of AG1295 on ERK1/2 phosphorylation. PLL was used as control.* ***(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) *Representative Western blot for integrins αvβ1 and αvβ3 in OPCs exposed to 10ng/ml PDGF-A with or without ERK1/2 inhibitor (U0126) for 24hrs. PLL was control. (E-F) Graphical representation of integrins αvβ1 and αvβ3, after densitometric analysis. Integrins αvβ1 and αvβ3 data were normalized to β-actin. Three different cell preparations were analyzed three times N= 3. Error bars represent ± SEM. Data were evaluated using One way ANOVA -parameter.*

*(Treatments compared to PLL control *P<0.05, **P<0.01, ***P<0.001, ns= non significant). (PDGF-A without U0126 compared to PDGF-A with U0126 #P<0.05, # # P<0.01, # # # P<0.001, ns= non significant).*



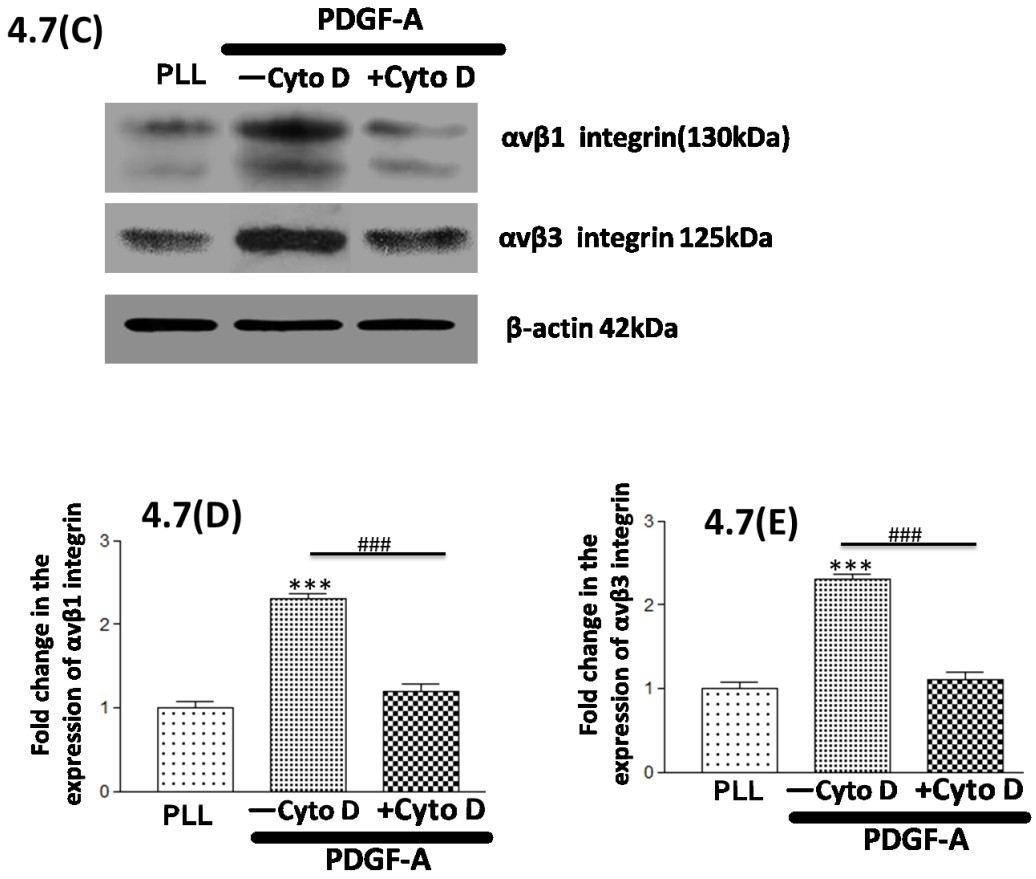
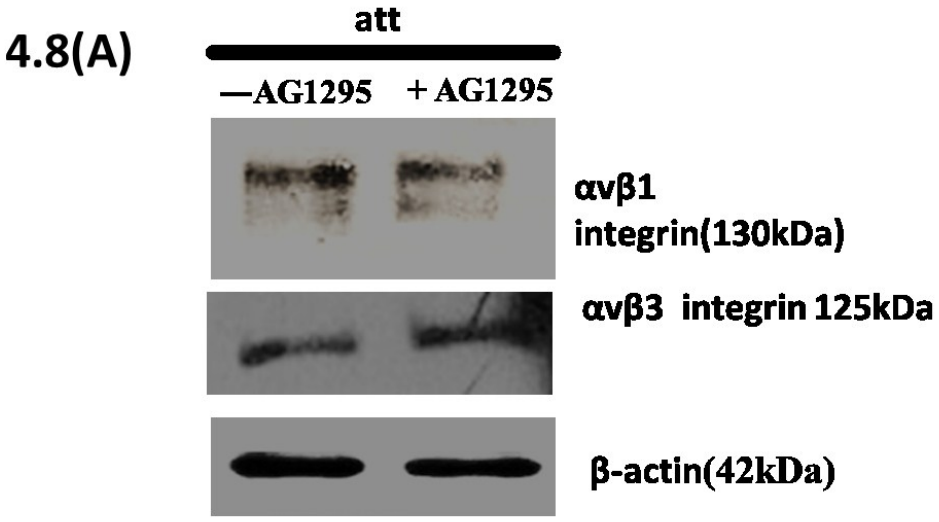
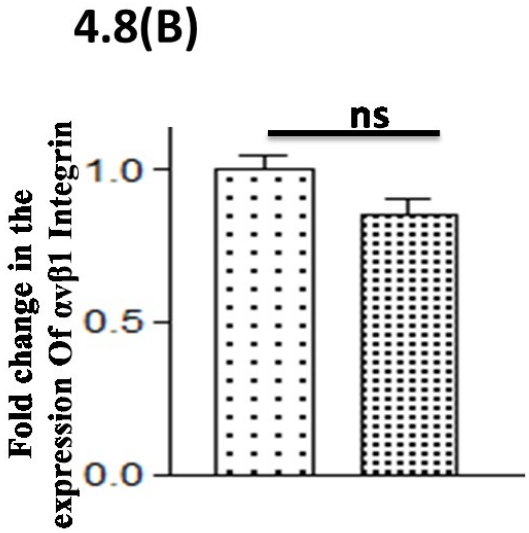


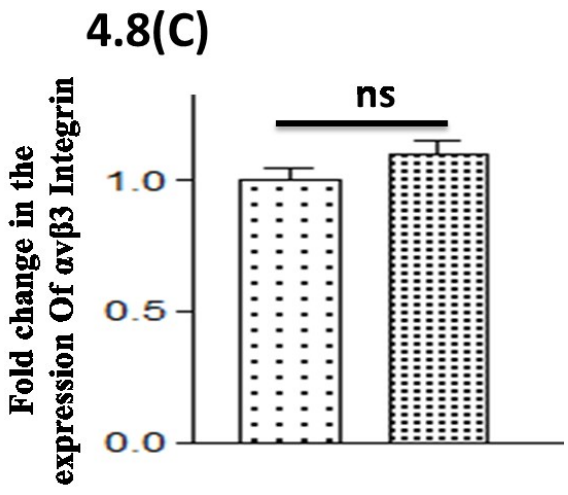
Fig. 4.7- Effect of actin polymerization inhibition on PDGFRα activation mediated integrin expression

(A) *OPCs were exposed to (a) PLL (b) PDGF-A and (c) PDGF-A with AG1295. OPCs migrate away from the edge of the agarose drop. After 72hrs cells were stained for Actin (phalloidin 488-green) and studied for the length of cell processes. Images are represented in grey scale. Scale bar (A-a, b, c) =20µm. Process length was significantly more in PDGF-A treatment than compared to PLL and in presence of AG1295. (B)- Graph showing the effect of PDGF-A (10ng/ml) with or without AG1295 on length of cell processes. The cell process length was determined by counting of 30 cells from three coverslips of each culture group that were randomly selected. Each point represents the mean ± SEM of three separate experiments. N=3 with 3 replicates each N. Data were evaluated using One way ANOVA -parameter (*P<0.05, **P<0.01, ***P<0.001).* ***(C)*** *Representative Western blot for integrins αvβ1 and αvβ3 in OPCs exposed to 10ng/ml PDGF-A with or without actin cytoskeletal inhibitor (Cyto D) for 24hrs. PLL was control. Anti- αvβ1 antibody detects two bands of αvβ1*

integrin; one is fully glycosylated (130kDa) and another is partially glycosylated (115kDa) forms. ***(D-E)****Graphical representation of integrins αvβ1 and αvβ3, after densitometricanalysis.Integrins αvβ1 and αvβ3 data were normalized to β-actin. Three different cell preparations were analyzed three times N= 3. Error bars represent ± SEM. Data were evaluated using One way ANOVA -parameter . (Treatments compared to PLL control *P<0.05, **P<0.01, ***P<0.001, ns= non significant). (PDGF-A without Cyto D compared to PDGF-A with Cyto D #P<0.05, # # P<0.01, # # # P<0.001, ns= non significant).*







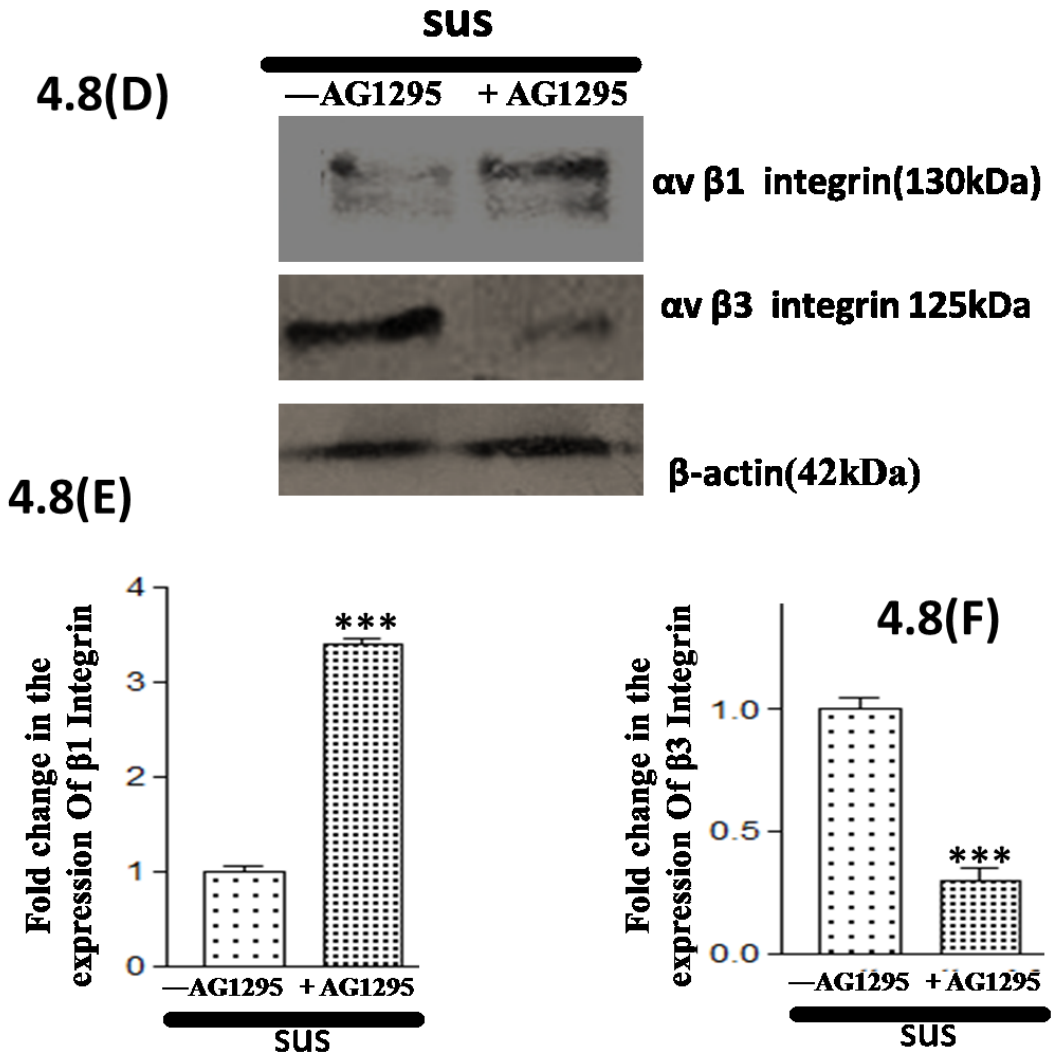


Fig. 4.8Effect of PDGF receptor kinase inhibition on integrin expression in C6 glioma

(A) *Representative Western blot for integrins αvβ1 and αvβ3 in attached (att) C6 glioma with or without PDGF receptor kinase inhibitor (AG1295) for 24hrs. PLL was control. Anti- αvβ1 antibody detects two bands of αvβ1 integrin; one is fully glycosylated (130kDa) and another is partially glycosylated (115kDa) forms.* ***(B-C)****Graphical representation of integrins αvβ1 and αvβ3 following densitometric analysis. Integrins αvβ1 and αvβ3 data were normalized to β-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, ns= non significant).* ***(D)*** *Representative Western blot for integrins αvβ1 and αvβ3 in*

suspended (sus) C6 glioma with or without PDGF receptor kinase inhibitor (AG1295) for 24hrs. PLL was control. ***(E-F)****Graphical representation of integrins αvβ1 and αvβ3, after densitometricanalysis.Integrins αvβ1 and αvβ3 data were normalized to β-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, ns= non significant).*

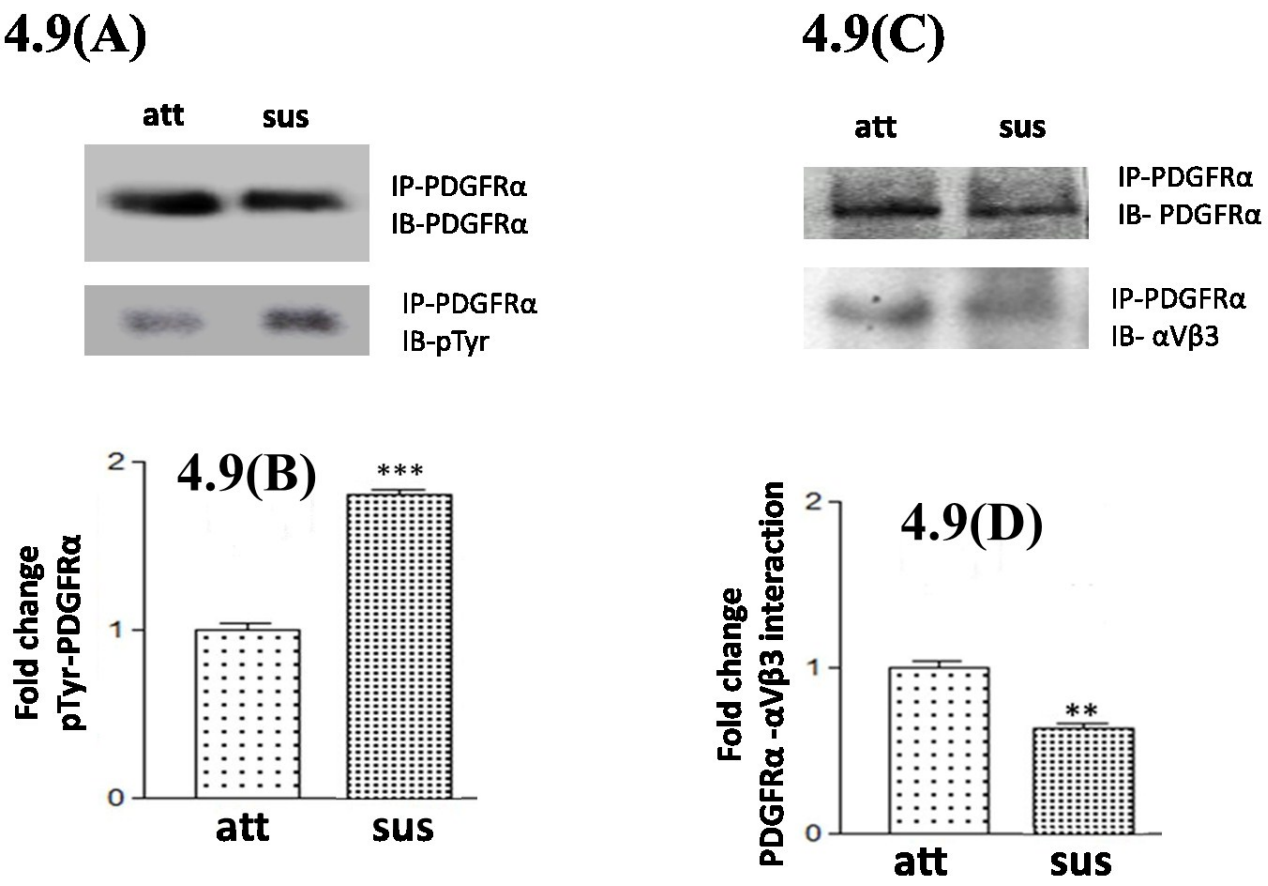


Fig. 4.9 Effect of attached (att) and suspended (sus) conditions on PDGFRα phosphorylation and PDGFRα-αvβ3 interaction

(A) *Effect of attached (att) and suspended (sus) conditions on PDGFRα phosphorylation in C6 glioma was checked using anti-pTyr antibody. For that first the immunoprecipitation of PDGFRα was done. Then the pTyr was checked using western blot method.* ***(B)*** *Graph showing the effects of att and sus on PDGFRα phosphorylation in C6 glioma. 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)*** *Effect of attached (att) and suspended (sus) conditions on PDGFRα-αvβ3 interaction in C6 glioma. For that first the immunoprecipitation of αvβ3 was done. Then the PDGFRα was checked using western blot method.* ***(D)*** *Graph showing the effects of att and sus 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).*

4.4 Discussion

While the primary role of integrins was thought to be as mediators of adhesive function, integrins also regulate cellular biological processes related to cell morphology, proliferation, survival, migration, and invasion (Hynes 2002; Aplin et al. 1999). In other words, integrins relay cues from the ECM to intracellular signaling machinery upon ligand binding, a process called "Outside-in Signaling" (Plow et al. 2000). On the other hand, intracellular signaling activated by other receptors could induce conformational changes in integrins, thus altering their functional activity, a process called "Inside-out Signaling". Therefore, integrins and RTKs can exchange or amplify their signaling pathways via both "Outside-in" and "Inside-out" signaling (Shen et al. 2012). 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). αvβ1 and αvβ3 play significant role in OPC migration and proliferation respectively (Milner et al. 1996; Baron et al. 2002). Studies on OPCs have shown 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). Recently, the role of PDGF-A has been shown in the tissue repair after spinal cord injury (Yao et al. 2017). Growth factors (GFs) can influence integrin function; one such mechanism is by altering the expression of integrin α/β pairs or adhesome components (Collo and Pepper 1999; Zambruno et al. 1995). However, there have been no reports, till date, detailing the relationship between PDGFRα activation mediated integrin expressions

in OPCs. The present data, along with the others have shown that the higher concentration of PDGF-A (10ng/ml) significantly enhanced the OPC migration without engagement of ECM (Chapter-3 result-3.3.4;(Vora et al. 2011; Frost et al. 2009).

So it was interesting to probe the underlying mechanism by which the higher concentration of PDGF-A modulate integrins following "Inside-out" signalling so as to enhance OPC migration? *Hence, we hypothesized that PDGFRα activation causes switches in integrin expression.* Previous studies have shown that OLG change their expression pattern of integrins as they progress through their lineage. OPCs express integrin αvβ1, which is essential for the migration (Milner et al. 1996; Milner and Ffrench-Constant 1994). However, our data (Fig.4.1) shows that freshly isolated OPCs express significantly low levels of integrin αvβ1 as compared to integrin αvβ3. Adhesion to the PLL significantly increased the αvβ1 levels (***P<0.001) as compared to the freshly isolated OPCs (Fig.4.1A, B). In the same experiment, increase in αvβ3 level (*P<0.05) was not as high as αvβ1 (Fig.4.1A, C). These observations suggest involvement of some extracellular regulation of integrin αvβ1 expression. Moreover, it is already shown that PDGFA significantly contributes to OPC migration (Armstrong et al. 1990; Frost et al. 1996; Frost et al. 2009; Vora et al. 2011) and thus it was interesting to examine the role of PDGFRα activation in integrin expression and switching. Physiological concentration of PDGF-A (1ng/ml) was unable to increase the expression of integrins αvβ1 and αvβ3. However, 10ng/ml PDGF-A concentration significantly increased the expression of both the integrins **(Fig.4.2)**. There have been several previous studies that have shown that RTK/Ligand binding duration is critical for the activation of different downstream signaling pathways (Heldin et al. 1998; Heldin and Ericsson 2001; Stork 2002). Earlier study supports this hypothesis, with transient activation of the PDGFRα resulting in migration but not proliferation of OPC (Frost et al. 2009; Vora et al. 2011). Hence, it was essential to clarify the role of higher concentration of PDGF-A (10ng/ml). The effects of continuous and transient exposure of higher concentration of PDGF-A (10ng/ml) on integrin expression and switching was therefore analyzed. The data shows that the αvβ3 subunit expression level appears to increase after PDGF-A continuous treatment (Fig.4.3), which was not the case with αvβ1. This finding fits

very well with the knowledge that the αvβ3 integrin is required for PDGF induced OPC proliferation (Baron et al. 2002). 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). Data from the current study confirms that continuous exposure to PDGF-A is required for PDGFRα internalization (Fig.4.4B, C). These findings confirm the relationship between PDGF-A continuous exposure, PDGFRα internalization, αvβ3 integrin expression and OPC proliferation.

Integrin analysis following western blotting showed presence of two bands, one is fully glycosylated (130 kDa) and another is partially glycosylated (115 kDa). Anti-β1 integrin antibody (Santacruz) can recognize both fully and partially glycosylated β1 integrin bands in western blot. Full glycosylation represents the pool of β1integrin which is recruited in the membrane and the partial glycosylation represents the pool of cytoplasmic β1integrin (She et al., 2010). Our data shows that when freshly isolated OPCs adhered to the PLL coated surface, the two prominent bands of β1integrin were seen suggesting both synthesis and recruitment of β1integrin. But when these adhered OPCs were exposed to PDGF-A, the fully glycosylated β1integrin level was significantly higher than the partially glycosylated one and this indicates the involvement of PDGFRα activation in the glycosylation and membrane recruitment of β1integrin. These results are in corroboration with the earlier reports on PDGF mediated enhanced glycosylation of the membrane receptors(Carlberg and Larsson 1996). Studies have also shown that PDGF-A regulate the OPC migration in dose and time dependent manner (Frost et al. 2009; Vora et al. 2011).

PDGF-A continuous exposure significantly increased the β3 integrin levels in comparison to the transient exposure without altering the β1 integrin levels. These data suggest that the activation of PDGFRα modulates the integrin 'in-side out' signaling. Studies have shown that integrin in-side out signaling is regulated by actin cytoskeleton activation (Calderwood et al. 2000). There are different proteins involved in the activation of actin cytoskeleton. The data from the previous (chapter -3) shows that pERK1/2 directly interacts with the F-actin, which was confirmed by ERK

inhibition studies. Inhibition of PDGF receptor kinase using AG1295 significantly reduced the PDGF-dependent pERK1/2 activation and also reduced the F-actin cytoskeleton organization and cell process formation. Moreover, inhibition of PDGF receptor kinase significantly reduced the PDGF-A dependent modulation of β1and β3 integrins. As shown earlier (chapter -3; result-3.3.4), the higher concentration of PDGF-A significantly activated ERK1/2 and actin without the FN engagement. PDGFRα inhibition significantly decreased the PDGF-A mediated ERK1/2 and actin activation (Fig.4.6A,B,C and Fig.4.7A,B). *We next hypothesized the role of pERK1/2 and actin activation in the PDGFRα activation mediated integrin expression and switching.* ERK1/2 and actin inhibition significantly decreased the expression of integrins αvβ1 and αvβ3(Fig.4.6D,E,F and Fig.4.7C,D,E). Earlier study has shown that sustained activation of ERK induced the β3 integrin gene expression (Woods et al. 2001). Recently it has been shown that induction of integrin β3 by sustained ERK activity promotes the invasiveness of TGFβ-induced mesenchymal tumor cells (Hong et al. 2016). Density of ligand, receptor concentration, ligand-binding affinity and cytoskeletal associations are all key determinants of cell migration speed (Huttenlocher et al. 1996; Palecek et al. 1997). These data suggest that activation of PDGFRα modulates the integrin 'in-side out' signaling and switch between β1and β3 integrins. Integrins activate signalling downstream of Ras to extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) through SHC or FAK, and they also activate Jun amino-terminal kinase (JNK) through FAK (Giancotti and Ruoslahti 1999; Miranti and Brugge 2002). FAK seems to integrate pro-migratory signals from integrins and RTKs, as cell migration that is induced by platelet derived growth factor (PDGF) or epidermal growth factor (EGF) requires FAK to associate with both RTKs and integrin-containing focal complexes (Sieg et al. 2000). Interestingly, whereas FAK signaling is necessary for directional cell movement, SHC promotes random cell motility (Gu, J. et al. 1999). It is increasingly clear that ERK/MAPK and JNK regulate cell migration by phosphorylating cytoskeletal components as well as by modifying gene expression. ERK/MAPK phosphorylates and activates the myosin light chain (MLC) kinase (MLCK), which induces the contraction of actomyosin fibres through phosphorylation of MLC (Klemke et al. 1997). JNK induces phosphorylation of paxillin, a component of focal adhesions. Paxillin phosphorylation might regulate cell migration by promoting the turnover of

focal adhesions (Huang et al. 2003). As paxillin binds to, and induces activation of, ERK/MAPK during the cell morphogenesis that is induced by hepatocyte growth factor (HGF) (Ishibe et al. 2003) which might cooperate with FAK to mediate joint integrin–RTK control of cell migration. Recently, role of actin associated with β1 integrin has been shown to regulate OPC directional migration (Zhu et al. 2016). The data from the present study confirms the above reports where it shows that PDGFRα activation results in the ERK and actin activation and their interaction acts as mediators for PDGFRα mediated integrin "Inside out" signaling.

Many in vivo and in vitro studies have reported surface modifications of integrin levels, or even neo-expression of some integrins, in carcinoma versus normal cells (Plantefaber and Hynes 1989; Zutter et al. 1995; Serini et al. 1996; Ben-Ze'ev 1997). Such modifications might be driven either by the ECM itself (Langhofer et al. 1993; Rabinovitz and Mercurio 1996) by GFs secreted by stromal cells and stimulating the invasive neoplastic elements in a paracrine fashion (Klemke et al. 1994; Doerr and Jones 1996), or even by cytokines synthesized de novo by carcinoma cells and acting back on the tumor mass via an autocrine circuit (Aasland et al. 1988; Bachrach et al. 1988; Mizukami et al. 1991). Hence, the role of PDGFRα activation mediated integrin switching was tested in a unique cell system which could survive in ECM /attachment independent manner, the phenomenon known as "anoikis resistance", using C6 glioma cells as a model system. Invasive and metastatic cancer cells usually acquire anoikis resistance and thereby reduce their dependency on cell–ECM adhesion; these cells can grow in suspension and metastasize from the primary tumor (Buchheit et al. 2014; Slattum and Rosenblatt 2014; Paoli et al. 2013; Douma et al. 2004; Yoshino et al. 2016). As anoikis resistance is unnecessary for the maintenance of organs by normal cells, this phenomenon is characteristic of malignant cancer cells and therefore constitutes a possible therapeutic target. However, the detailed mechanisms of anoikis resistance are not fully understood and moreover anoikis resistance-targeting drugs are not currently available. RGD integrin β1 is associated with inferior tumours, whereas, β3 integrin, increases with the WHO grade of malignancy (Uhm et al. 1999; Roth et al. 2013; Gladson and Cheresh 1991). Hence, in the present study the role of PDGFRα activation was studied in the modulation of αvβ1 and αvβ3 integrins in C6 glioma. PDGFRα inhibition did not affect the αvβ1 and αvβ3 expression in attached C6 glioma

cells. However, in suspended C6 glioma, inhibition of PDGFRα activation significantly decreased the αvβ3 expression levels. Further, the data showed that tyrosine phosphorylation (pTyr) of PDGFRα was increased in suspended C6 glioma as compared to attached condition. Moreover, the direct interaction of PDGFRα and integrin αvβ3 was significantly decreased in suspended C6 glioma as compared to attached C6 glioma. The overall results suggest the uncoupling of PDGFRα and integrin αvβ3 in C6 glioma anoikis resistance during metastasis.

In conclusion, data from the OPC study clarified the role of PDGFRα transient and continuous activation on β1 and β3 integrin subunit expression and switching in the regulation of OPC migration. It also provided the substantive evidence to specify the role of integrins in the behavioural switches during OPC migration. Moreover, data from C6 glioma studies, demonstrated the role of PDGFRα in integrin switching during anoikis resistance leading to glioma metastasis.