CHAPTER FIVE

5. To determine the role of ERK and ROCK signaling in PDGFR inhibition mediated intracellular signaling in c6 glioma growth and migration.

5.1 INTRODUCTION

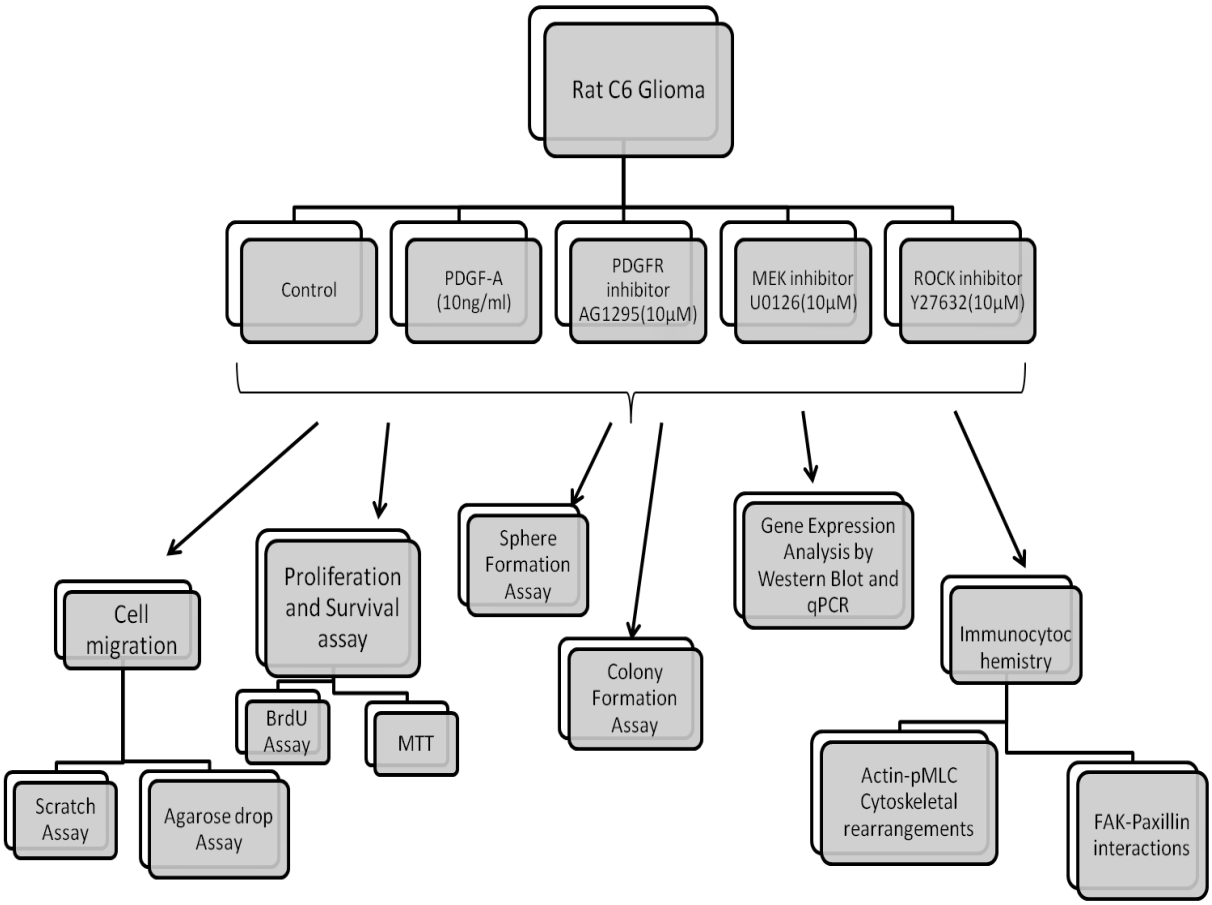
Gliomas are among the most rapid, aggressive and highly invasive brain tumors. Migration, adhesion and invasion to the other brain tissue contribute to its metastasis. It responds poorly to conventional operation and chemistry treatments (G Gritsenko et al., 2012; Grobben et al., 2002). Genetic alterations occurring inside these gliomas lead to the aberrant elevation of several receptor tyrosine kinases and their secreted factors. PDGFRα (Platelet-derived growth factor receptor-α) is a receptor tyrosine kinase (RTK) which commonly over expressed and amplified in gliomas (Lokker et al., 2002b). Expression of PDGFRα and its ligand, PDGF-A, enhances GBM tumor growth and invasion in the brain (Feng et al., 2011; K.-W. Liu et al., 2011). Autocrine and paracrine loops of PDGFR, in particular, is one of those RTKs affecting the survival, proliferation and differentiation of rat C6 glioma (D. Chen et al., 2014; Grobben et al., 2002; Lokker et al., 2002b; Popescu et al., 2015). PDGFR signaling leads to the activation of intracellular signaling pathways such as RAS/MAPK and PI3K/AKT (Nazarenko et al., 2012; Roberts & Der, 2007). However, the molecular mechanisms governing PDGFR activation and downstream signaling regulating glioma migration has not been fully understood.

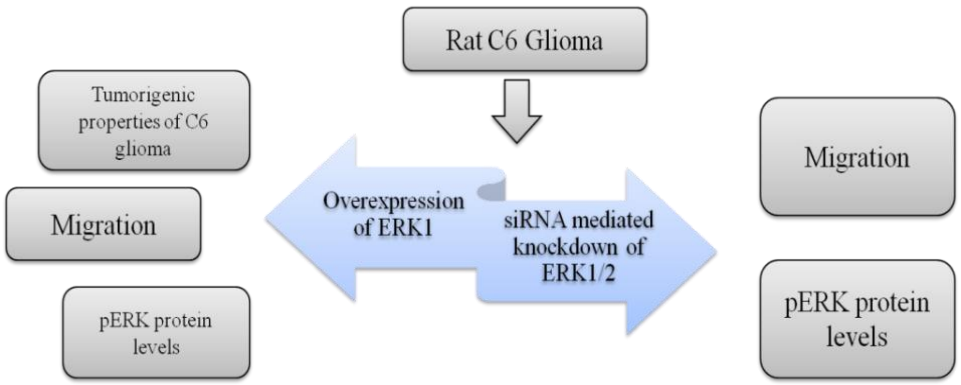
ERK 1/2, a downstream target of RAS/MAPK pathway, has been reported to be up regulated in glioma and involved in cell cycle progression, proliferation and migration (Lind et al., 2006). Pharmacological inhibition by U0126, a MEK1/2 protein kinase inhibitor has been shown to reduce the invasive property of the human T98G high-grade glioma by regulating the matrix metalloproteinase activity (Kunapuli et al., 2004). On the contrary, activation of ERK1/2 can lead to proliferation, differentiation or cell cycle arrest depending on the intensity and time of stimulation (Gentile et al., 2015). However, few studies have established Ras-ERK with Rho- Rho associated protein kinase (ROCK) pathway functioning in a co-operative manner in cancer cell migration and growth (K.-W. Liu et al., 2011; Zohrabian et al., 2009). Rho kinase and its downstream effector ROCK are small GTPase proteins involved in several functions such as cytoskeletal reorganization, cell motility and phosphorylation of myosin light chain (MLC) (Nobes & Hall, 1995). Rho kinases elevate the

phosphorylation of myosin and cause acto-myosin contraction of tumor cells which further leads to its migration (Matsuoka & Yashiro, 2014; Narumiya et al., 2009; Stice et al., 1999). Furthermore, ROCK and MLCK (Myosin light chain kinase) both contribute to the decrease in the turnover of the focal complexes and formation of focal adhesions and cell migration in fibroblast (Totsukawa et al., 2004).

Since the downstream molecular mechanism of PDGFR signaling involved in growth and migration is largely unknown, the aim of present study is to investigate the role of PDGF receptor signaling inhibition on growth, migration and other malignant phenotype of C6 glioma.

5.2 PLAN OF WORK





5.3 RESULTS

5.3.1 Effect of PDGFR, ERK and ROCK signaling inhibition on C6 cell survival and proliferation

First, the effect of AG1295, U0126 and Y-27632 treatment on C6 cell proliferation by BrdU incorporation was investigated. A significant reduction in the percentage of BrdU positive cells were observed in cells treated with AG1295 (***P<0.001) and U0126 (***P<0.001) compared to control (Figure 5.1 A & B). However, there were no significant change was observed in C6 cells treated with Y-27632. Next, to elucidate whether inhibition of PDGFR, ERK and ROCK signaling affects the viability of the C6 glioma cells, cells were treated alone with each of specific inhibitor AG1295,U0126 and Y-27632 at 10µM for 24hrand cell viability was measured by MTT assay. It was observed that inhibition of PDGFR, ERK and ROCK signaling for 24hr does not show any significant change in the cell viability (Figure 5.1 C).

5.3.2 Involvement of ERK and ROCK signaling in PDGFR mediated glioma migration

To examine the role of PDGFR downstream signaling involved in C6 glioma migration, cells were treated with PDGFR inhibitor (AG1295) followed by analysis of cell migration by scratch assay. It was found that PDGFR inhibition significantly reduces the migration of C6 glioma compared to control (Figure 5.2 A , B, and C). Further, to assess the role of PDGFR mediated ERK and ROCK signaling in C6 glioma migration, U0126 and Y-27632, inhibitors of ERK and ROCK signaling were used respectively. A significant reduction of migration in cells treated with U0126 and Y-27632 compared to control (Figure 5.2 A , B and C) was observed. To confirm that ERK and ROCK signaling are downstream of PDGFR, the protein levels of pERK and pMLC were determined in cells treated with AG1295 or U0126 and Y-27632 alone. The levels of both pERK and pMLC were found significantly reduced in cell treated with AG1295 compared to control (Figure 5.2 D & E), suggesting that PDGFR inhibition regulates the migration by modulating the downstream ERK and ROCK signaling.

5.3.3 PDGFR inhibition modulates cytoskeleton reorganization and pERK-FAK-Paxillin complex formation in C6 glioma

Cell migration comprises adhesion and cytoskeletal reorganization in response to chemical stimuli. In order to study that whether PDGFR inhibition in C6 glioma regulates cytoskeleton reorganization, cells were treated with AG1295 or Y-27632 followed by immunostaining for actin and pMLC. The stress fiber formation was found to be reduced in cells treated with AG1295 and Y-27632 compared to control (Figure 5.3).Also, the levels of pMLC were reduced and mostly concentrated around the nucleus in both AG1295 and Y-27632 treated cells as compared to control (Figure 5.3). Thus, reduced actin stress fibers and pMLC may further contribute to reduced migration observed in AG1295 and Y-27632 treated cells.

Next, immunoprecipitation was performed to investigate the effect of PDGFR blocking on pERK-FAK-Paxillin interaction in C6 cells. Interaction of pERK with focal adhesion proteins FAK and Paxillin has been shown to play an important role in cell migration. It was observed that pERK-FAK-Paxillin interaction reduces in cells exposed to AG1295, compared to control (Figure 5.4), which suggest that PDGFR inhibition may regulate the C6 glioma migration by regulating the pERK-FAK-Paxillin interaction.

5.3.4 PDGFR inhibition impairs anchorage independent growth, sphere forming ability, adhesion and invasion of C6 glioma

To examine the effect of PDGFR inhibition on anchorage-independent growth, one of the most reliable markers of malignant transformation, the C6 glioma cells were subjected to soft agar assay. It was found that PDGFR inhibitor significantly ($p < 0.05$) reduced the size and number of colonies formed on soft agar compared to control and PDGF-A treated cells (Figure 5.5 A & B) which suggests that PDGFR signaling inhibition suppress anchorage independent C6 glioma growth.

Another tumorigenic potential of glioma is its ability to grow as tumor sphere which is also considered a marker for stemness and self renewal ability. To determine whether PDGFR inhibition affects the stem-like properties, C6 cells were allowed to form sphere in presence or absence of AG1295. PDGFR inhibition significantly ($p< 0.05$) impairs sphere formation in C6 glioma, compared to control and PDGF-A

treated cells (Figure 5.5). However PDGF-A exposure led to the formation of large spheres as compared to control.

Next, in order to examine if PDGFR inhibition affect the glioma adhesion to extracellular matrix, the attachment of C6 cells to Fibronectin (10µg/ml) was analyzed. PDGFR inhibitor was found to significantly ($p < 0.0001$) reduce the adhesion ability of C6 glioma (Figure 5.6).

Lastly, invasion ability of C6 glioma treated with PDGFR inhibitor was examined by matrigel invasion assay. The results indicated that PDGFR inhibition significantly decrease the cell invasion compared to control (Figure 5.6). Over all, results suggest that blocking of PDGFR signaling suppress C6 glioma characteristic malignant behavior.

5.3.5 PDGFR inhibition regulates the expression of GFAP, FAK, BDNF and MeCP2

The impact of PDGFR signaling inhibition on expression of GFAP, FAK, BDNF and MeCP2 which are known to play important role in regulation of C6 glioma pathology was investigated. PDGFR inhibition significantly induced the transcript level of GFAP (1.5-fold), BDNF (3.92-fold) and MeCP2 (2.46-fold) in C6 glioma, compared to control (Figure 5.7 A Further, protein levels of GFAP and FAK were determined by western blot in cells exposed to PDGFR inhibitor. The results indicated that PDGFR inhibition significantly increase the GFAP level (2.88-fold) and reduce the FAK level (0.41-fold), compared to control (Figure 5.7 B, C & D).

5.3.6 Overexpression of ERK1 regulates C6 glioma migration

To determine the specific role of ERK1 among the two isoforms of ERK1/2 in C6 glioma migration, C6 glioma was transfected with plasmid expression pERK1-EGFP fusion (pERK1) protein. ERK1 overexpression was confirmed by fluroscence microscopy and western blot analysis, which showed clear band of 75kDa corresponding to ERK1-EGFP fusion protein in C6 cells transfected with pERK1-EGFP and absent in cells transfected with pEGFP (Fig 5.8 A & B). To assess the migration, scratch assay was performed in cells expressing the pERK1-EGFP and pEGFP. Migration of cells in scratch was measured after 24 hrs. ERK1 overexpression increases the C6 glioma migration compared to pEGFP group (5.8 C).

Next, the invasion ability of C6 cells expressing either control (pEGFP) or pERK1-EGFP was examined by transwell invasion assay. It was observed that ERK1 overexpression impairs the invasion ability of C6 glioma (Fig 5.9 A) which may suggest the role of ERK1 in invasion property of glioma. Further, ERK1 was found to affect the adhesion of C6 glioma to extracellular matrix fibronectin. C6 cells expressing pERK1-EGFP shows significant increase in adhesion to fibronectin compared to control (Fig 5.9 B)

5.3.7 ERK1/2 knockdown in C6 Glioma

To complement the overexpression studies, knockdown experiment was done using ERK1 specific siRNA in C6 glioma. Knockdown of ERK in C6 glioma was shown by western blotting (Figure 5.10 A). Further, the effect of single and combined knockdown of ERK isoforms (ERK1 and ERK2) on C6 migration was examined and found reduction in migration of cells in both treated with single or combined ERK1/2 siRNA compared to negative control (Figure 5.10 B). In contrast to ERK1 overexpression, ERK1 siRNA mediated a significant reduction (*$P<0.05$) in the migration (Fig 5.10 B).

5.3.8 Inhibition of Calcium signaling in C6 glioma

Apart from ERK and ROCK signaling, to understand the contribution of calcium signaling in C6 glioma growth, migration and tumorigenic potentials, C6 glioma cells were treated with calcium chelator MAPTAM (10μM) for 24 hrs. MAPTAM, calcium chelator, binds to the intracellular calcium and inhibits the other calcium signaling pathways. It was observed that the calcium chelation significantly (***$P<0.001$) impairs the migrational abilities of the C6 glioma cells compared to the control (Fig. 5.11 A & B). But, then survival abilities was also significant decreased in the MAPTAM chelated group for 30 mins and 24hrs as compared to the control (Fig 5.11 C) which suggests crucial role of intracellular calcium in C6 glioma growth. Further, the anchorage independent growth ability of the C6 glioma with MAPTAM was analyzed by soft agar assay. It was found that inhibition of calcium signaling significantly hampers the anchorage independent growth of C6 glioma cell.

5.4 DISCUSSION

Aberrant expression of PDGFR signaling is one of the major signaling defects observed in glioblastoma (Martinho et al., 2009; Nazarenko et al., 2012; Bengt Westermark et al., 1995). The current study demonstrates that inhibition of PDGFR

signaling regulates glioma growth and migration through ERK and ROCK signaling pathways which could be correlated by reduced levels of pERK and pMLC observed in cells treated with PDGFR inhibitor. These results are in consistent with previous reports which show that PDGFR signaling activates ERK and ROCK protein kinase which are reported to be involved in migration, survival and proliferation (Cui et al., 2014; Mitsutoshi Nakada et al., 2011; Stice et al., 1999; Zohrabian et al., 2009). PDGFR autocrine signaling regulates the ERK phosphorylation in glioblastoma cell lines and PDGFR inhibition impairs soft agar colony formation and C6 glioma tumor growth in nude mice (Lokker et al., 2002b).Inhibition of ERK signaling using U0126 leads to decreased migration and proliferation of C6 glioma (Lind et al., 2006).

The primary requirement of cell migration begins with propulsion of leading edge which is formed after the complex process of polymerization of F-actin, formation of stress fibers and lamellipodia. Polymerization of F-Actin is one of the basic mechanisms of cell membrane propulsion which can generate significant force to move the leading edge (Ananthakrishnan & Ehrlicher, 2007; Rědowicz, 1999; Sahai & Marshall, 2002). Rho/ROCK pathway has been involved in stress fibers assembly and formation of actin rich filopodia and lamellipodia at the leading edge of the cells (Ying et al., 2006). Thus, inhibition of ROCK signaling leads to the reduction in actin stress fibers formation in cells treated with PDGFR inhibitor AG1295, which suggests that PDGFR regulates the stress fibers formation via ROCK signaling. ROCK pathway also regulates the cell migration by phosphorylation of MLCK (myosin light chain kinase) and MLC (myosin light chain) (Bogatcheva et al., 2011). pMLC at the cell periphery restricts membrane protrusions by counteracting the protrusive activity powered by actin polymerization. Loss of pMLC at the periphery and its accumulation at the centre lead to less matured adhesive structures which explains the less efficient migration (Salhia et al., 2008; Totsukawa et al., 2004). Similarly, the data from present study demonstrated reduced phosphorylation of myosin and its presence mostly near the nucleus or centre due to PDGFR inhibition suggests actin monomers retraction from the cell periphery and formation of adhesive structures which lead to inhibition of C6 glioma migration.

Cell migration involves the process of constant assembly and disassembly of focal adhesions. These focal adhesions contain aggregation of cytoskeletal proteins such as FAK (focal adhesion kinase), Paxillin, Vinculin etc (Nagano et al., 2012; Parsons et

al., 2010; Petit & Thiery, 2000; Truong & Danen, 2009). ERK influences the FAK-Paxillin interactions by phosphorylation of FAK or Paxillin which leads to regulation of focal adhesion dynamics (Hayakawa et al., 2011; Huang et al., 2004; Igishi et al., 1999; Teranishi et al., 2009). Alteration of FAK and Paxillin dynamics results in decreased migration by reduction in Focal adhesion turnover and lamellipodia formation (Deramaudt et al., 2014). Previous reports on ERK modulated disassembly of FAK-Paxillin complex suggests its role in assembly and disassembly of focal adhesions (Hunger-Glaser et al., 2003). In present study, PDGFR inhibition reduces the interactions of activated ERK and focal adhesion proteins FAK and paxillin, suggests a novel regulatory mechanism of C6 glioma migration by PDGFR inhibitor.

In order to study the effect of PDGFR inhibition on malignant behavior of C6 glioma, soft agar assay, adhesion assay, transwell invasion assay and sphere assay were employed in this study. The results of present study indicate that PDGR inhibition significantly affect this malignant phenotype of C6 glioma. In PDGF transformed 3T3 and human astrocytoma cells, expression of PDGF dominant negative mutants break the PDGF autocrine loop and impair their anchorage independent growth and colonies formation (Shamah et al., 1993); Shamah et al., 1993).

Similarly, introduction of a truncated PDGFβ receptor in rat c6 glioma cell line showed a significant reduction in cell growth (Strawn et al., 1994). Moreover, recently it has been reported that inhibition of MEK-ERK pathway prevents the stem-like phenotype and sphere formation in rhabdomyosarcoma cell line (Ciccarelli et al., 2016). PDGFRA and downstream members of MAPK (RAS/mitogen-activated protein kinase) were found to be up regulated in metastatic medullablastoma tumors. In vitro study shows that PDGF-A enhance the medullablastoma migration and increases the downstream ERK1/2 phosphorylation in dose dependent manner (MacDonald et al., 2001). The present study further extends the role of PDGR inhibition on malignant phenotype of C6 glioma which was not studied earlier.

In addition, PDGFR inhibition was also found to regulate the expression FAK, GFAP and MeCP2 in C6 glioma. Focal adhesion kinases (FAK), a non-receptor tyrosine protein kinase, is upregulated in glioblastoma and found to promote proliferation, survival, adhesion and migration of glioblastoma (Mei et al., 2014; Mitra & Schlaepfer, 2006; Natarajan et al., 2003). More specifically, FAK is recruited to the

site of the adhesions and is reported to be the regulator of focal adhesion assembly and disassembly (Sieg et al., 2000). Here, PDGFR inhibition reduces the FAK level in C6 glioma which may contribute to reduced migration and adhesion of cells. PDGFR inhibition also has found to promote the C6 glioma differentiation by inducing GFAP levels. This increase in GFAP level is consistent with previous studies which shows inhibition of ERK signaling increases the GFAP level in C6 glioma (Lind et al., 2006). Moreover, in human glioma increase in amount of GFAP has been shown to reduce the invasiveness (Kajiwara et al., 1992; Murphy et al., 1998). Further, treatment of neuroprotective valproic acid to C6 glioma was found to induce the MeCP2 transcript levels (B. Kim et al., 2008). Similarly in present study also MeCP2 mRNA levels were increased in C6 glioma following PDGFR inhibition. MeCP2 has been also shown to repress the expression of the genes involved in metastatic behavior in breast and pancreatic cancer cells (Ray et al., 2013; M. Xu et al., 2016). Similarly, significant increase in BDNF transcript levels which suggests its neuroprotective effect in C6 glioma cells was found. Valproic acid as neuroprotective has been shown to upregulate the BDNF level in C6 glioma (Rincón Castro et al., 2005). Specifically, increased in level of proBDNF was found to inhibit the growth and migration and increase apoptosis and differentiation of C6 glioma by inducing GFAP expression (Xiong et al., 2013).

Pharmacological manipulations to inhibit MEK kinases links the receptor mediated activation of Ras-GTPase proteins and their cytoplasmic effectors ERK1 and ERK2 (Mazzucchelli et al., 2002). Both these isoforms display differential functions in the cell signaling and brain physiology(Christen et al., 2015). Mice lacking ERK1 demonstrates abnormal signaling responses which are linked to the upregulation of ERK2 activity in the brain (Mazzucchelli et al., 2002; Nekrasova et al., 2005). Further, ERK deficient mice exhibits normal T cell effector function and increased susceptibility to experimental autoimmune encephalomyelitis (Nekrasova et al., 2005). Individual loss of either ERK1 and ERK2 slows down the proliferation rate of genetically matched primary embryonic fibroblast , and their combined loss resulted in a complete arrest of cell proliferation associated with G1 arrest (Lefloch et al., 2008; Voisin et al., 2010). Similarly, in the present study overexpression of ERK1 in C6 glioma was carried out which shows increased migration and adhesion, but a decrease in the invasion abilities of C6 glioma cells while ERK1/2 knockdown studies

showed reduction in the migration of C6 cells. Overall, the overexpression and knockdown study gives an unclear picture about the role of ERK signaling mechanisms during glioma progression and further studies need to be carried out.

Among the various signaling pathways activated during cellular development, voltage dependent calcium influx plays a key role in regulating several essential processes, such as proliferation, apoptosis and cell migration (Berridge, 2004; Paez et al., 2010; Wei et al., 2009). Recent studies suggests that calcium as the positive regulator of tumorigenesis in glioblastoma, and deregulation of which contributes to the progression of the disease its progression (Leclerc et al., 2016). In the present study, the calcium chelation affects the survival, anchorage independent growth and migrational properties of rat C6 glioma which elucidates the importance and requirement of calcium signaling in glioma progression.

5.5 CONCLUSION

The present study focused on different signaling aspects which governs C6 glioma migration. The role of PDGF activated ERK and ROCK signaling pathway in C6 glioma migration was determined. Inhibition of PDGFR in C6 glioma reduces the activation of ERK and ROCK signaling in C6 glioma. Further, Similar to OPCs, PDGFR inhibition was found to suppress the C6 glioma migration by reducing the pERK-FAK-Paxillin and actin reorganization. Further, Inhibition of the PDGF signaling was found to suppress proliferation, invasion, adhesion, anchorage independent growth and sphere forming ability. Further, PDGFR inhibition was shown to reduce the tumor behavior of C6 glioma by regulating the expression of GFAP, MeCP2, BDNF and FAK expression. Overexpression of ERK1 showed increase in migration and changes in invasion and adhesion properties of C6 glioma, and ERK1/2 knockdown was found to reduce the migration. Thus, ERK signaling was found to play an important role in C6 glioma migration. Lastly, inhibition of calcium signaling was also found to reduce the migration and anchorage independent growth of C6 glioma.

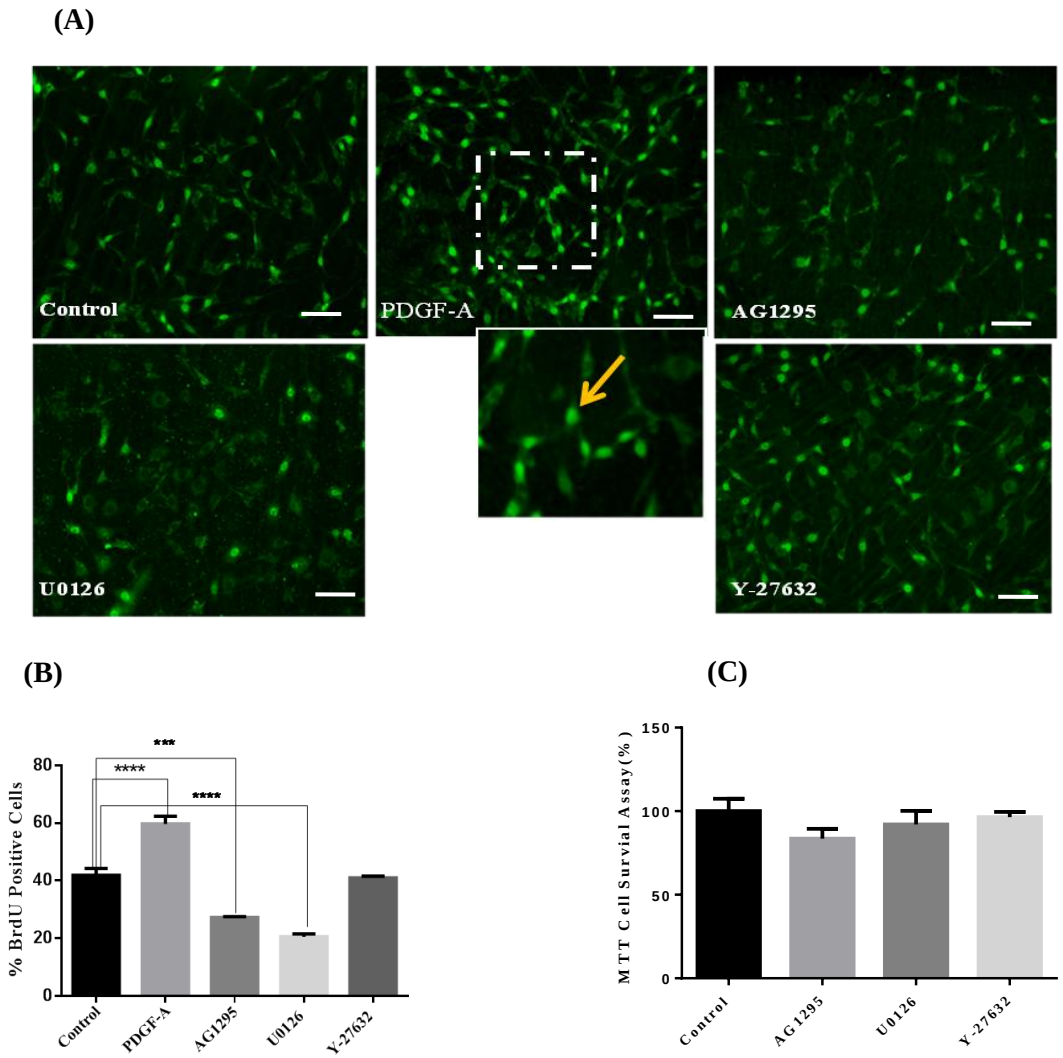


Figure 5.1: PDGFR signaling regulates C6 glioma proliferation and survival : (A)Representative fluorescence images of BrdU-positive cells in C6 cells pre-treated for 24hrs with PDGF-A (10ng/ml), PDGFR Inhibitor AG1295(10µM) ,MEK inhibitor U0126 (10µM) and ROCK inhibitor Y-27632 (10µM) .Scale bar=50 µm(B) The statistical data showed percentage BrdU-positive cells calculated from panel A.(C) Effect of PDGFR inhibition, ERK inhibition and ROCK inhibition on C6 cell survival was determined by MTT assay in cells treated alone with following inhibitors : 10µM AG1295, 10µM U0126 and 10µM Y-27632 for 24 hrs. The statistical data showed percentage of viable cells with respect to control. Values represent mean ± SEM from 3-4 samples. *p<0.05;**p<0.01; ***p<0.001;****p<0.0001 compared to control.

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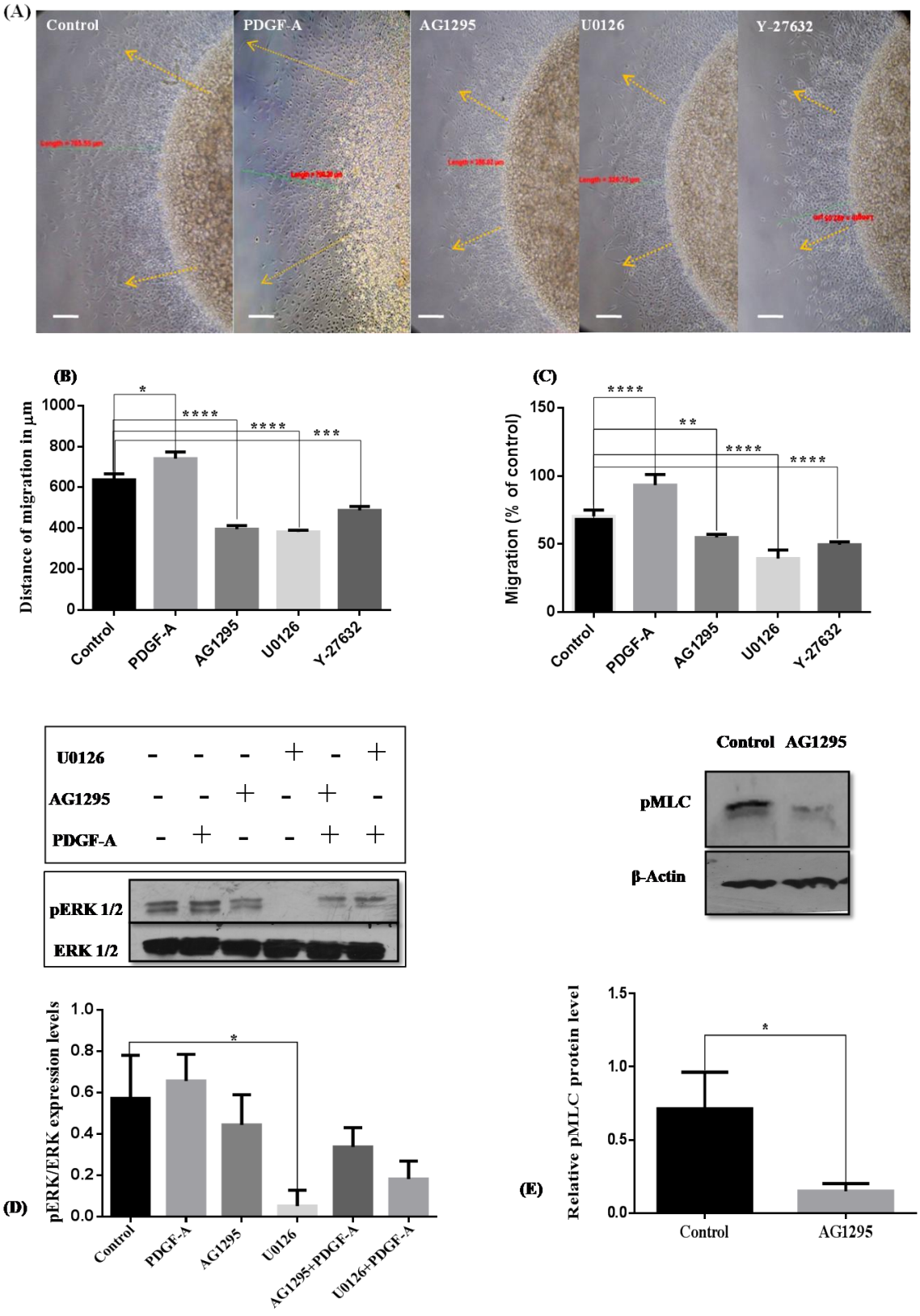


Figure 5.2: PDGFR signaling regulates C6 glioma migration through ERK1/2 and ROCK pathway. (A) Migration was analyzed by agarose drop assay in the Control, PDGF-A (10 ng/ml) for 24hrs, AG1295 (10µM) for 2 hrs,U0126 (10µM) for 2 hrs and Y-27632 (10µM) for 2 hrs (Scale bar = 20,000 nm)**(B)** Quantitative analysis

of distance of migration in agarose drop assay in the above groups, **(C)** Quantitative analysis of percentage migration in scratch assay in the above groups **(D)** Western blot analysis of pERK1/2 in C6 glioma in Control, PDGF-A for 24hrs , PDGFR inhibitor for 2 hrs, MEK inhibitor for 2hrs, PDGFR inhibitor (2hrs) followed by PDGF-A(24hrs), MEK inhibitor (2hrs)followed by PDGF-A(24 hrs) respectively from left to right lanes **(E**) Western blot and statistical analysis of pMLC in control and PDGFR inhibitor AG1295 group.*p<0.05; **p<0.01;***p<0.001;****p<0.0001 compared to the control group.(One way ANOVA followed by Dunnett test for multiple comparison)

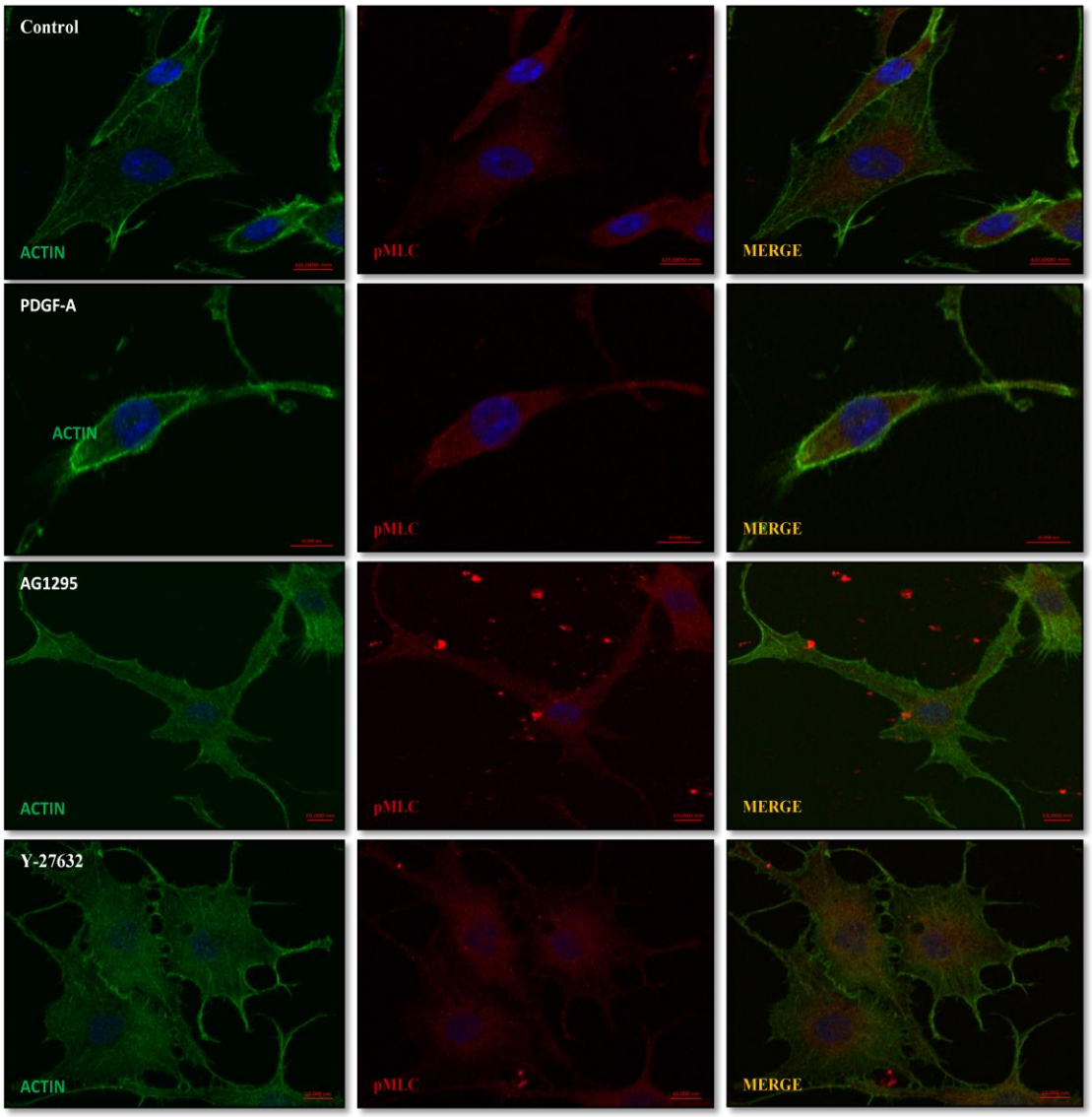


Figure 5.3: Effect of PDGFR and ROCK inhibition on actin-myosin rearrangements in Rat C6 glioma. The formation of stress fibres in PDGF-A treated glioma cells were blocked by AG1295 and Y-27632. The PDGF-A treated cells clearly showed polymerization of actin monomers at the leading edges as compared to the control. Also, Phosphorylation of pMLC was reduced and mostly concentrated near

the nucleus in both the AG1295 and Y-27632 treated glioma as compared to the control and PDGF-A treated glioma. Immunoflorescence staining of Actin with phalloidin and pMLC in Control , PDGF-A , AG1295 and Y-27632 treated glioma. (Scale Bar: 10000nm)

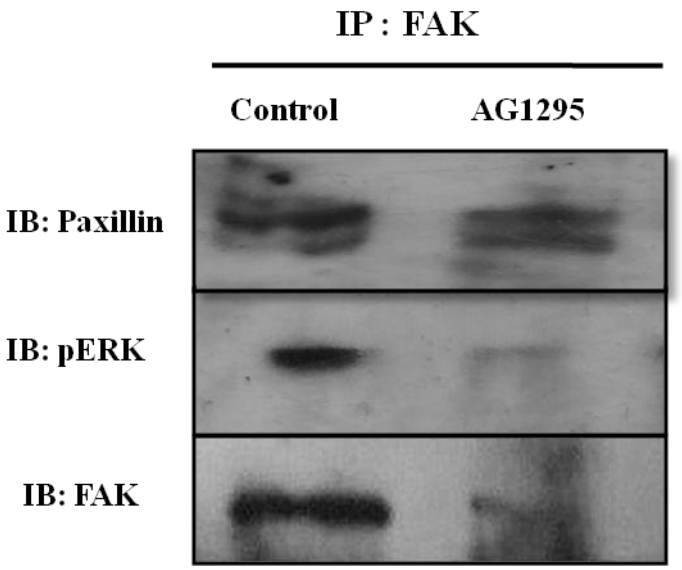


Figure 5.4: Association of FAK with Paxillin and phosphorylated ERK1/2 in C6 glioma cells and its inhibition by AG1295. (**A**) Cell lysates were prepared after treatment with AG1295 for 24 hrs and subjected to immunoprecipitation (IP) with antibodies to FAK. The resulting precipitates were then subjected to immunoblot analysis (IB) with antibodies to phosphorylated ERK1/2, to Paxillin, or to FAK.

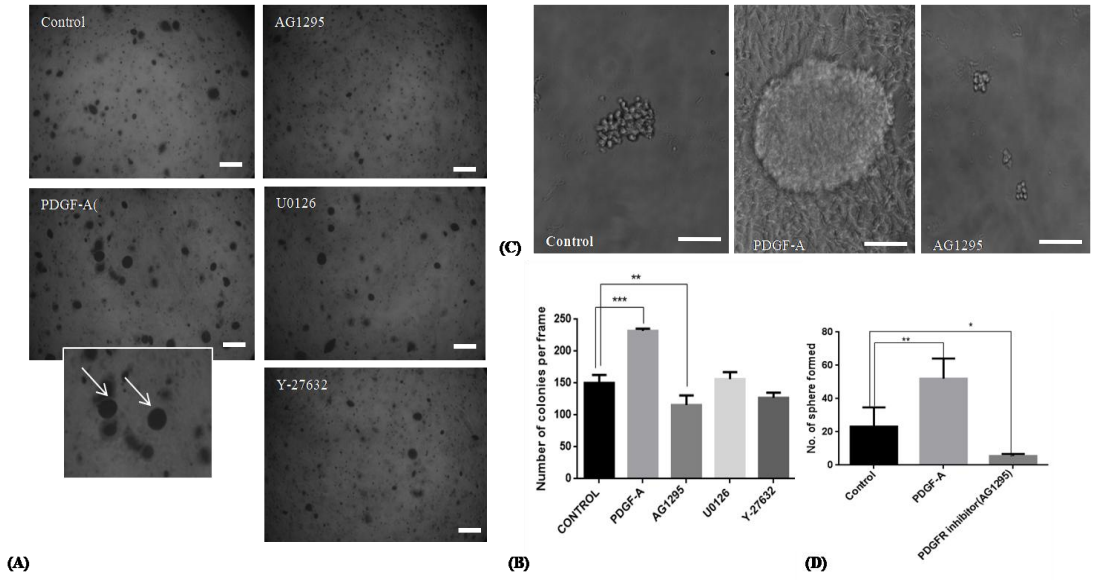
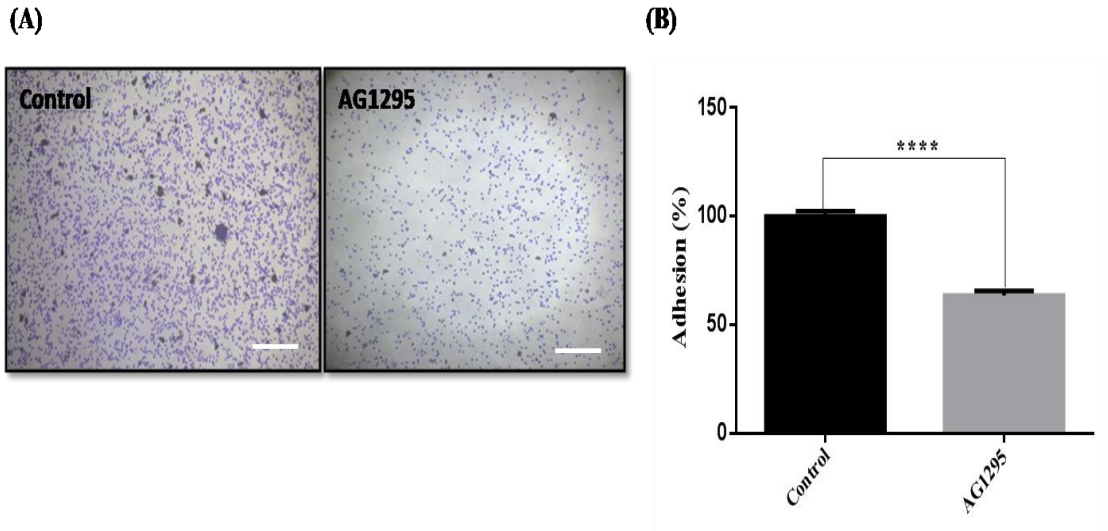


Figure 5.5: PDGFR inhibition in C6 glioma suppress sphere formation and colony formation in soft agar. (A) PDGF inhibition suppresses the anchorage independent growth of C6 glioma. PDGFR inhibitor group showed smaller and lesser of colonies as compared to the PDGF-A and Control group**. The number of colonies > 0.1 mm in size was counted**. (B) Quantitative analysis of number of colonies formed in respective groups.(C)PDGFR inhibition reduces sphere formation ability of C6 glioma as compared to PDGF-A and Control group. (D) Quantitative analysis of number of sphere formed. Data are derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001



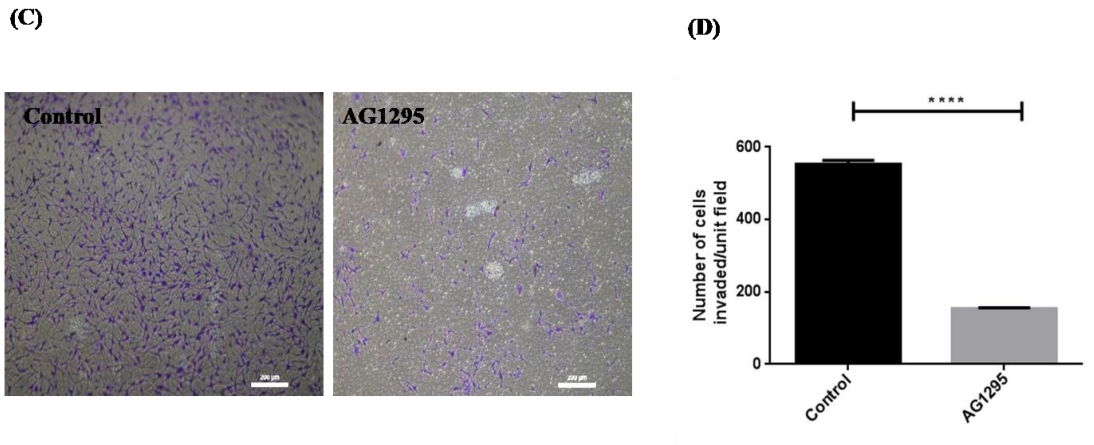


Figure 5.6: Effect of AG1295 on adhesion and invasion property of C6 glioma cells: PDGF inhibition impairs the adhesion property of C6 glioma as compared to the control****p<0.0001. **(A & B)** Crystal violet staining of the adhered cells to the fibronectin in control and PDGFR inhibitor treated groups. The PDGFR inhibited group showed less number of attached cells. PDGF inhibition impairs the invasive property of C6 glioma as compared to the control****p<0.0001**. (C & D)** Crystal violet staining of the invaded cells from the matrigel coated membrane in control and AG1295 treated groups. The AG1295 group showed less number of invaded cells. Data derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001

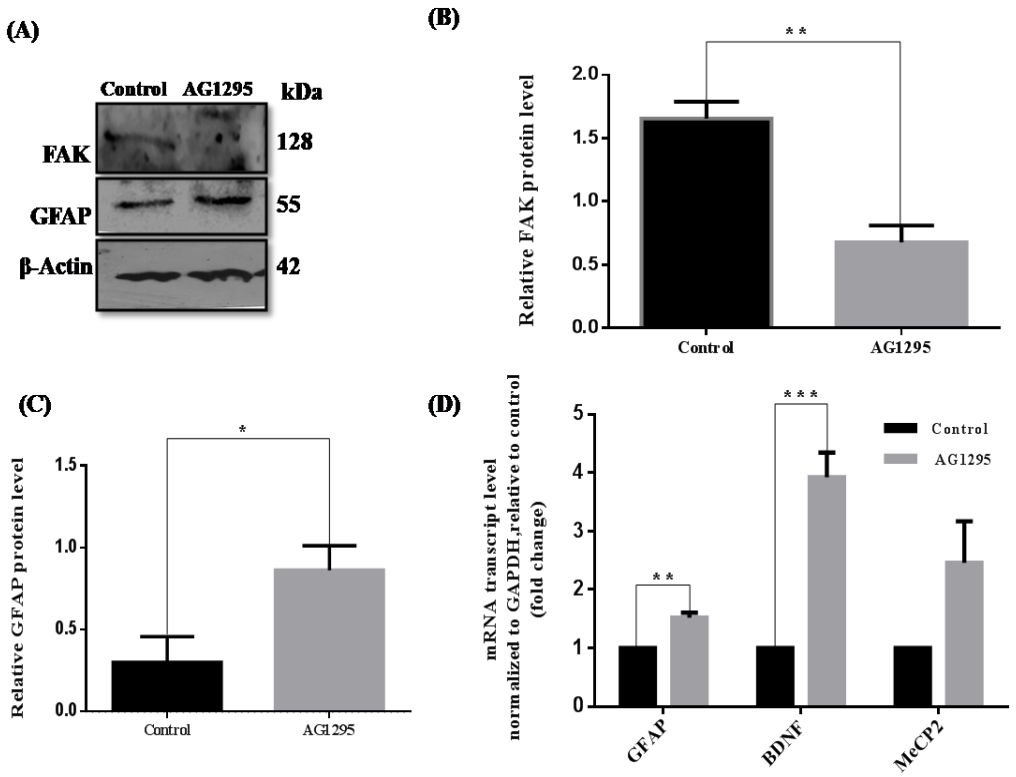


Figure 5.7: PDGFR Inhibition regulates gene expression in C6 glioma. (A) Western blot analysis of FAK and GFAP in C6 glioma control and PDGFR inhibitor treated groups. Quantitative analysis of FAK and GFAP **(B, C, D)**. **(D)** Change in mRNA expression of BDNF, MeCP2 and GFAP in C6 glioma in the presence and absence of PDGFR inhibitor. Values represent mean ± SEM from 3-4 samples. *P<0.05;**P<0.01; ***P<0.001,****P<0.0001 compared with control(unpaired Student's t-test**)** .

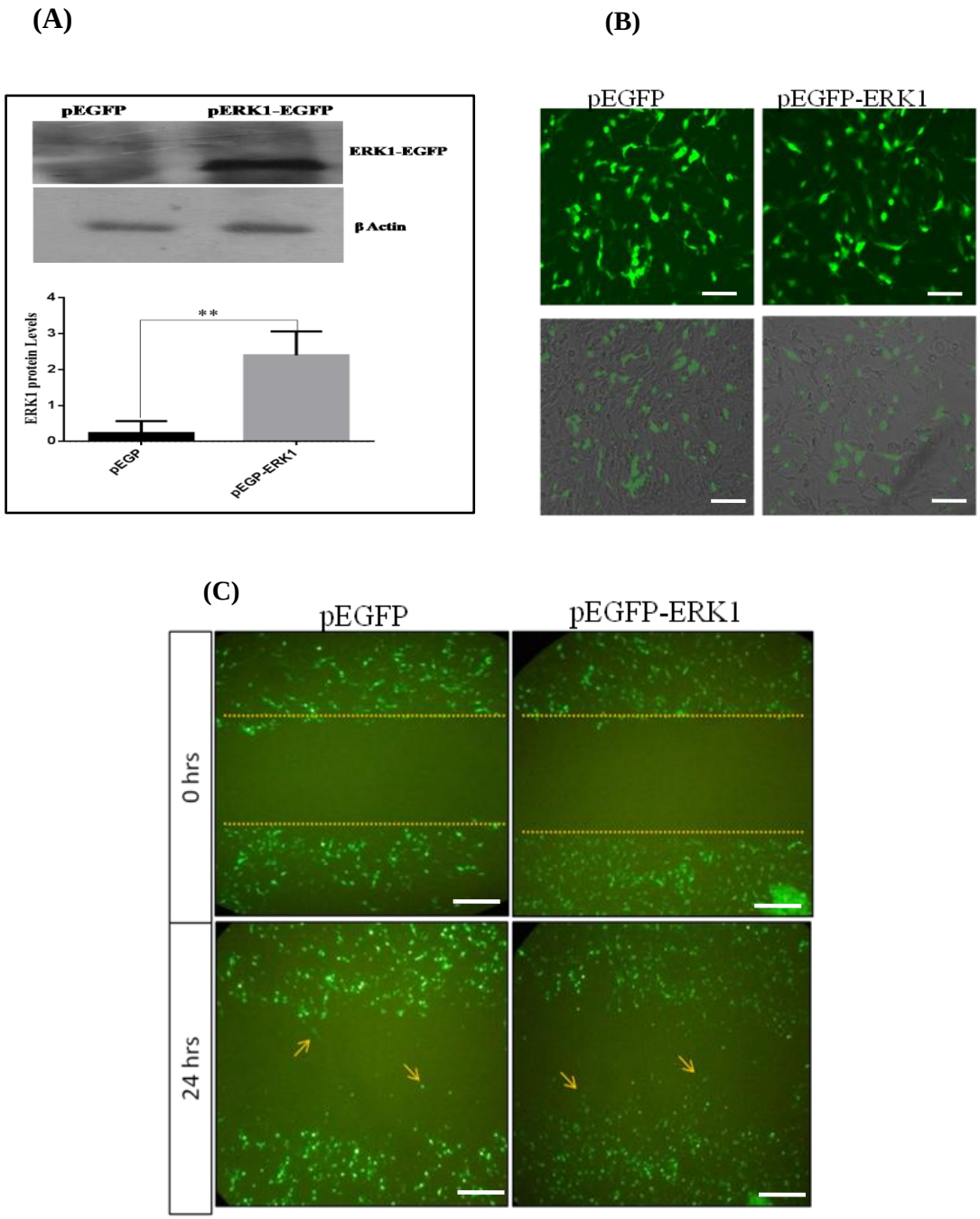
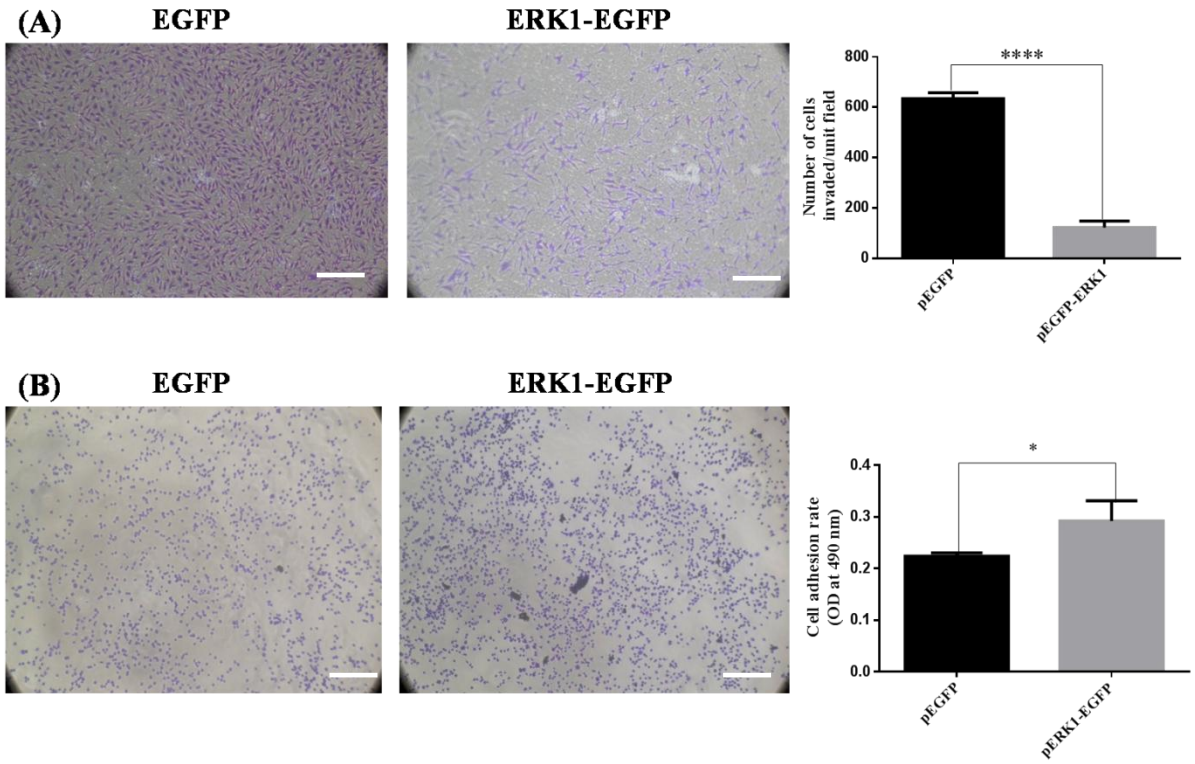


Figure 5.8: Effect of ERK 1 over expression and ERK1/2 downregulation in C6 glioma. C6 cells were transfected with pERK1-EGFP or control plasmin EGFP **(A)**

ERK1 overexpression was confirmed by Western blot and statistical analysis of ERK1 in C6 cells transfected with plasmids pEGFP and pEGFP-ERK1 **(B)** Transfection of C6 glioma with pEGFP (Vector) and pEGP-ERK1 plasmid. Images were taken at EVOS FLiod Fluorescence microscope. **(C)** Scratch assay of C6 glioma expressing pEGP and pEGP-ERK1 in C6 glioma (20X) Yellow arrow indicates the cells migrating towards the scratch. Data derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001.



5.9 Effect of ERK1 overexpression of invasion and adhesion properties of C6 Glioma:(A) ERK1 overexpression reduces the invasive property of C6 glioma as compared to the control**p<0.01**.**Crystal violet staining and statistical analysis of the invaded cells from the matrigel coated membrane in control and ERK1 over expressed group. **(B)** ERK1 overexpression increases the adhesion property of C6 glioma as compared to the control****p<0.0001**.**Crystal violet staining and statistical analysis of the adhered cells to the fibronectin in control and ERK1 overexpressed group. Data derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001

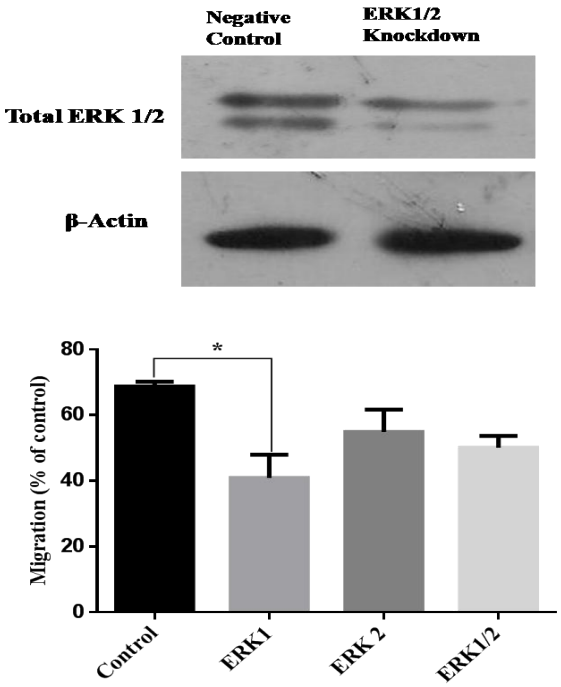
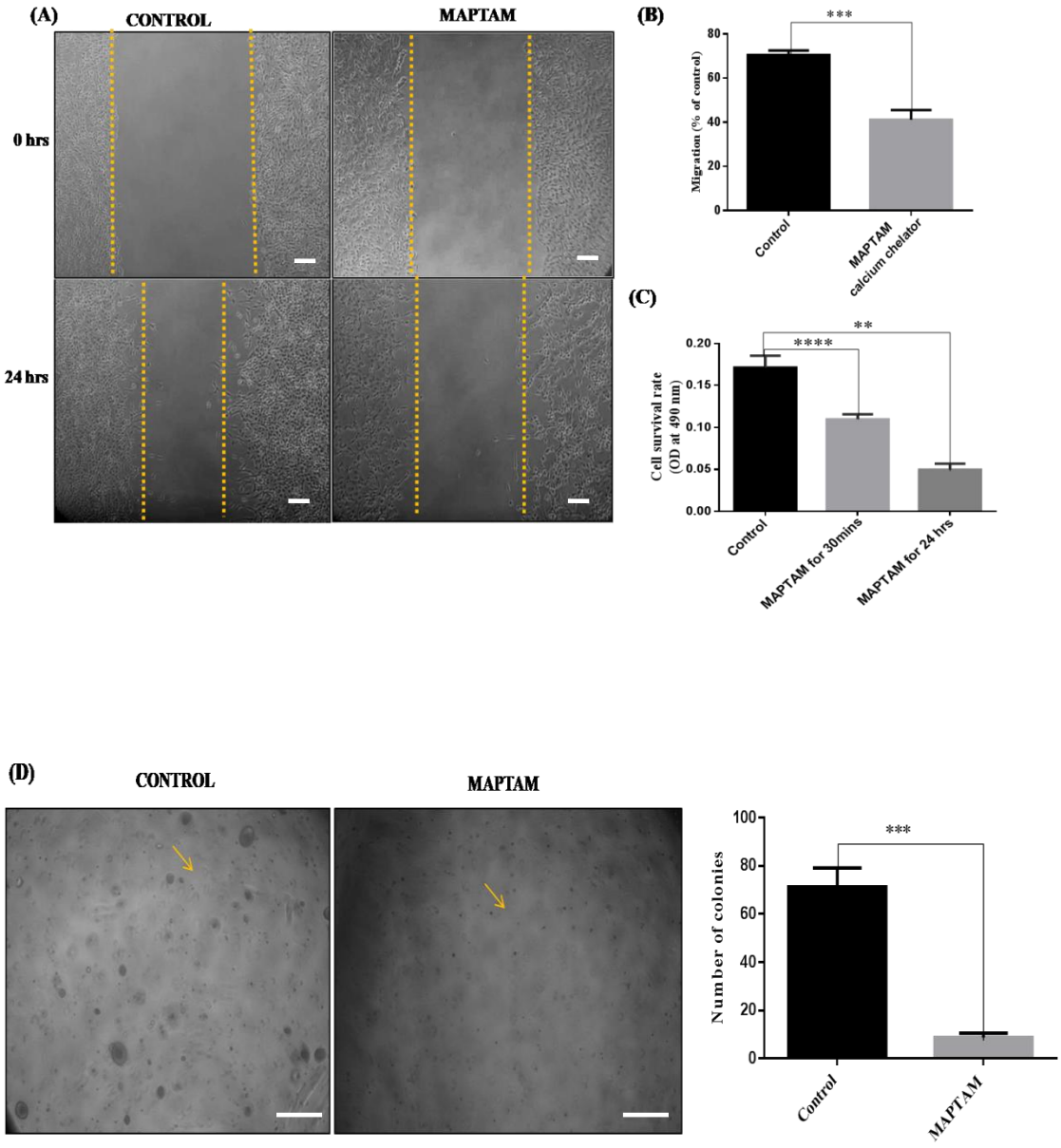


Fig 5.10: ERK1/2 knockdown reduce the C6 glioma migration: (A) Western Blot of ERK1/2 siRNA knockdown in C6 glioma cells and **(B)** Scratch migration assay in Negative control, ERK1 siRNA, ERK2 siRNA and ERK1/2(combined)siRNA knockdown groups. Data derived from three experiments and presented as means ± SEM *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$



5.11 Effect of Calcium Chelation in C6 glioma cell migration :(A) Calcium chelation leads to decreased C6 glioma migration .Representative phase contrast image of scratch assay in control and Calcium chelated MAPTAM C6 glioma**. (B)** Quantitative analysis of scratch assay showing percentage of migration as compared to control. **(C)**Calcium chelation with MAPTAM in C6 glioma cells lead to the decrease in the cell number. MTT survival assay of C6 glioma cells treated with MAPTAM (calcium chelator) for 30 mins and 24 hrs. **(D)** Calcium chelator MAPTAM suppresses the anchorage independent growth of C6 glioma. MAPTAM treated C6 cells showed smaller and lesser number of colonies as compared to the Control group**.** The number of colonies > 0.1 mm in size was counted. Quantitative analysis of number of colonies formed in respective groups.