

PDGFR inhibition mediated intracellular signalling in C6 glioma growth and migration: role of ERK and ROCK pathway

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Abstract Aberrant PDGFR (Platelet derived growth factor receptor) signalling in brain tumors and gliomas is one of the primary cause of tumor progression. PDGFR stimulation by its ligand and the role of its downstream mediators such as extracellular regulated kinases (ERK1/2), PI3K and ROCK pathways have not been thoroughly investigated. The present study sought to investigate the role of PDGF receptor signalling inhibition on suppression of rat C6 glioma growth and migration. Treatment of C6 cells with PDGFR inhibitor, AG1295 caused a significant reduction in migration and proliferation by regulating the ERK and ROCK signalling. Subsequently, PDGFR blocking was demonstrated to regulate cytoskeleton reorganization by modulating the Actin-pMLC reorganization and pERK-FAK-Paxillin complex formation which may further regulate the C6 glioma migration. Further, other malignant behaviour of C6 glioma such as anchorage independent growth, adhesion, invasion and sphere forming abilities were found to be impaired by PDGFR blocking. PDGFR inhibition further regulates the C6 glioma tumor behaviour by inducing gene expression of GFAP, BDNF, and MECP2 and down regulating FAK expression. In conclusion, our data elucidate novel mechanisms involve in PDGFR inhibition mediated inhibition of

C6 glioma growth and migration which can be a future potential target for the treatment of glioma.

Keywords C6 glioma · Migration · PDGFR · ERK · ROCK

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 which makes glioma resistant to chemotherapy and surgery (G Gritsenko et al. 2012; Grobбен et al. 2002; Hoelzinger et al. 2007). Genetic alterations occurring inside these gliomas lead to the aberrant elevation of several receptor tyrosine kinases and their secreted factors. PDGFR α (Platelet-derived growth factor receptor- α) is a receptor tyrosine kinase (RTK) which is commonly over expressed and amplified in gliomas (Lokker et al. 2002). Expression of PDGFR α and its ligand, PDGF-A, enhances GBM tumor growth and invasion in the brain (Liu et al. 2011; Feng et al. 2011). Presence of autocrine and paracrine PDGF signaling in C6 glioma regulate their survival, proliferation, and differentiation (Chen et al. 2014; Grobбен et al. 2002; Lokker et al. 2002; Popescu et al. 2015). PDGFR signalling leads to the activation of intracellular signalling pathways such as RAS/MAPK and PI3K/AKT (Nazarenko et al. 2012; Roberts and Der

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2007). However, the molecular mechanisms governing PDGFR activation and downstream signalling 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, it has been demonstrated that Ras-ERK with Rho-Rho associated protein kinase (ROCK) pathway function in a co-operative manner in cancer cell migration and growth (Zohrabian et al. 2009; Liu et al. 2011). Rho kinase and its downstream effector ROCK are small GTPase proteins involved in several functions such as cytoskeletal reorganisation, cell motility and phosphorylation of myosin light chain (MLC) (Nobes and Hall 1995). Rho kinases elevate the phosphorylation of myosin and cause acto-myosin contraction of tumor cells which further leads to its migration (Matsuoka and 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 the present study is to investigate the role of PDGF receptor signalling inhibition on growth, migration and other malignant phenotypes of C6 glioma cells.

Materials and methods

Cell culture

The rat C6 glioma cell line was obtained from the National Centre for Cell Science (Pune, India). Cells were maintained at 5% CO₂ and 37 °C in DMEM/F12 medium (Gibco-Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS and 1% Penicillin and Streptomycin (Himedia, Mumbai, India). The following pharmacological inhibitors were used in this

study at 10 µM concentration each- PDGFR inhibitor-AG1295 (Millipore), MEK inhibitor-U0126 (Santa Cruz) and Rock inhibitor-Y-27632 (Santa Cruz).

MTT assay

Cell viability was assessed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (SRL, Mumbai, India). This assay is based on the cellular conversion of a tetrazolium salt into a formazan product that can be easily detected using 96 well plate reader. Approximately 5000 cells per well were plated into 96 well plates and treated alone with 10 µM AG1295 (Millipore, Billerica, MA, USA), 10 µM U0126 (Santa Cruz Biotech, Santa Cruz, CA, USA) and 10 µM Y-27632 (Santa Cruz) for 24 h. MTT (5 mg/ml) dissolved in PBS was added to the wells and incubated for 3–4 h at 37 °C. Later, DMSO was added to all wells and mixed thoroughly to dissolve the dark blue formazan crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570–630 nm. To minimise the variation among different assays, the data were corrected against the control and were plotted using the optical density of the control wells as 100% survival. The experiments were performed in triplicate and repeated at least three times.

BrdU incorporation assay

Cells were seeded on coverslips and treated alone with 10 µM each of AG1295, Y-27632 and U0126 for 24 h, then incubated with 10 µM of BrdU (SRL) for 2 h, washed twice with PBS and fixed with ice cold methanol for 10 min at 4 °C. Later, cells were exposed to 2M HCl for 60 min at 37 °C to denature DNA followed by incubation with anti-BrdU antibody (Thermo Fisher, Waltham, MA, USA) overnight at 4 °C. Then, BrdU incorporation was visualised using fluorescence microscope (The FLoid Cell Imaging Station, Thermo Fisher) following staining with anti-mouse FITC (Bangalore Genei, Bangalore, India) and four frames of cells were photographed. Percentage of BrdU-positive cells were analysed and calculated by Image J software.

Scratch assay

C6 cells were grown to confluence in 24 well plates, a scratch was made on the monolayer using the sterile

200 µl pipette tip. After scratch, cells were treated alone with the following inhibitors 10 µM AG1295, 10 µM U0126 and 10 µM Y-27632 for 24 h. After 24 h, media were replenished with serum free DMEM/F12. Images of same scratch region were taken at 0 and 24 h and quantified by measuring distance covered by cells between 0 and 24 h using Image J software. Relative migration distance = $(A-B)/A$, where A represents the mean width of the cell scratch at 0 h and B represents the mean width of the cell scratch at 24 h. Results are expressed as the mean \pm SE.

Immunocytochemistry

Cells were plated on glass coverslips and treated with 10 µM AG1295 or Y-27632 for 24 h. Then, Cells were fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 0.25% (vol/vol) Triton X-100 in PBS for 10 min, blocked by 1% (wt/vol) BSA in PBST, and incubated overnight at 4 °C with anti-pMLC (Abcam, Cambridge, MA, USA). After washing, cells were incubated in corresponding fluorescent conjugated secondary antibody (Bangalore Genei) for 1 h at room temperature. For actin staining, cells were additionally incubated with phalloidin conjugated Alexa-Fluor 488 (Invitrogen) for 20 min to visualise F-Actin cytoskeleton. Fluorescence signals were detected using confocal microscopic imaging system (Carl Zeiss, Oberkochen, Germany, Model LSM-710).

Co-Immunoprecipitation

Cells were grown on 90 mm plates in presence or absence of 10 µM AG1295 for 24 h. For Co-immunoprecipitation, cells were lysed in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 50 mM Tris-HCL (pH 8.0), 150 mM sodium chloride) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor (Roche, Mannheim, Germany). Immunoprecipitation was carried with Anti-FAK (Santa Cruz, Dallas, TX, USA) antibody using Dynabeads Protein G immunoprecipitation Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Immunoprecipitated protein was resolved by SDS-PAGE followed by western blot analysis using the following antibodies: Anti-Paxillin (Santa Cruz), Anti-pERK (R&D systems,

Minneapolis, MN, USA) and Anti-Total ERK 1/2 (Sigma).

Colony formation assay

Approximately, 1×10^3 cells were seeded in DMEM-F12 containing 0.5% low melting agarose and 10% FBS (top agar medium) and overlaid onto bottom agar medium (DMEM-F12 containing 1% low melting agarose (Himedia) and 10% FBS (Gibco-Life Technologies)). Colonies were allowed to form in presence or absence of 10 µM AG1295. After 10–12 days, viable cell colonies > 0.1 mm in size were counted and photographed.

Adhesion assay

The efficiency of cell adhesion was determined by measuring the number of cells that adhered to extracellular matrix (ECM) Fibronectin (Invitrogen, Carlsbad, CA, USA). Cell culture plates were coated with Fibronectin (10 µg/ml) overnight at 4 °C, followed by washing with PBS and blocking with DMEM containing 10% FBS. Cells treated with or without AG1295 were seeded onto ECM-coated 96-well plates (1×10^5 cells/well) in serum free DMEM/F12 medium. After 30 min of incubation, the non adherent cells were removed by PBS wash, followed by fixation in 4% formaldehyde and staining with 0.5% crystal violet (SRL) dissolved in 20% methanol. Later on, the stain was eluted out by 100% methanol and optical density was measured at 490 nm in a microplate reader (Biotek, Winooski, VT, USA).

Invasion assay

Invasion assay was carried out using growth factor reduced matrigel invasion chambers (Corning, NY, USA) according to the manufacturer's protocol. In brief, C6 cells were harvested in serum-free medium and transferred to the hydrated matrigel chambers ($\sim 10,000$ cells per well) in presence or absence of 10 µM AG1295. The chambers were incubated for 24 h in culture medium with 10% FBS in the bottom chambers before examination. The cells on the upper surface were scraped and washed away, whereas the invaded cells on the lower surface were fixed and stained with 0.05% crystal violet for 15 min. The

invaded cells were counted and the relative number was calculated.

Sphere formation assay

Cells were dissociated into single cells and seeded at a density of 200 cells/well in a 96 well plate, post treatment with AG1295 for 24 h. Cells were grown to form spheres in DMEM/F12 medium containing EGF (Invitrogen) (20 ng/ml) and FGF (Sigma) (20 ng/ml) for 7 days. After, 7 days photographs of spheres were taken under inverted phase contrast microscope (Nikon, Tokyo, Japan) and their numbers and sizes were calculated.

RNA isolation and qPCR

Total RNA from cells, was isolated using Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA concentrations were measured using a Qubit RNA assay kit (Invitrogen) in a Qubit 2.0 Fluorometer (Invitrogen) and 1 µg of total RNA was used for reverse transcription reaction using verso cDNA synthesis kit (Thermo Scientific). Quantitative RT-PCR was performed using SYBR select Master Mix (Applied Biosystems, Foster City, CA, USA) in QuantStudio 12K (Life Technologies) real-time PCR machine (QuantStudio 12K from Life Technologies) with primers to detect selected messenger RNA (mRNA) targets. The melting curve of each sample was measured to ensure the specificity of the products. The mean of housekeeping gene GAPDH was used as a control to normalise the variability in the expression levels and data were analysed using $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). The following Primers pair were used for qPCR—*Mecp2*—*Forward Primer*: *gaccggggacatgatga*, *Reverse Primer*: *caatcaattctactttagagcga*; *Bdnf*- *Forward primer*: *ccataag-gacgcggactgt* *Reverse Primer*: *gaggctccaaaggcacttga*; *Gapdh* - *Forward Primer*: *agacagccgcatcttctgt*, *Reverse Primer*: *ctgccgtggtagtcat* (Sharma et al. 2015) and *Gfap*—*Forward Primer*: *gcctctccctgtctcgaatg*, *Reverse Primer*: *cgcttgttttctgttcca*.

Western blotting

Cells were lysed in Laemmli buffer and stored at -20°C until analyzed. Cell lysates with equal protein

loads (40 µg) were resolved by SDS polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose membrane. Membrane was blocked in 3% BSA in Tris buffered saline (TBS) containing Tween 20 (0.2%), followed by overnight incubation with primary antibody at 4°C . The following primary antibodies were used, anti-pERK1/2 (R&D systems), Anti-ERK1/2 (Sigma), Anti-pMLC (Abcam), Anti-Beta actin (Thermo, Pierce, Waltham, MA, USA), Anti-FAK (Santa Cruz), Anti-GFAP (Thermo, Pierce). Bands were visualized using corresponding horseradish peroxidase-conjugated secondary antibodies (Sigma). Specific immunoreactivity was visualized using an ECL kit (Invitrogen). Images of specific protein bands on X-ray films were digitally scanned and intensity was calculated using Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

The data from more than two groups were analysed by one-way analysis of variance (ANOVA) followed by post-hoc analysis of multiple comparisons. Data from two groups were statistically analysed using two-tailed Student's *t* tests. Results are expressed as mean \pm standard error mean (SEM). A value of $P < 0.05$ was considered statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

Results

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

First, to elucidate whether inhibition of PDGFR, ERK and ROCK signalling affects the viability of the C6 glioma cells, cells were treated alone with each of specific inhibitors AG1295, U0126 and Y-27632 at 10 µM for 24 h and cell viability was measured by MTT assay. It was observed that inhibition of PDGFR, ERK and ROCK signalling for 24 h did not lead to any significant change in the cell viability (Fig. 1). Next, we investigated the effect of AG1295, U0126 and Y-27632 treatment on C6 cell proliferation by BrdU incorporation. A significant reduction in the percentage of BrdU positive cells was observed in cells treated with AG1295 ($***P < 0.001$) and U0126

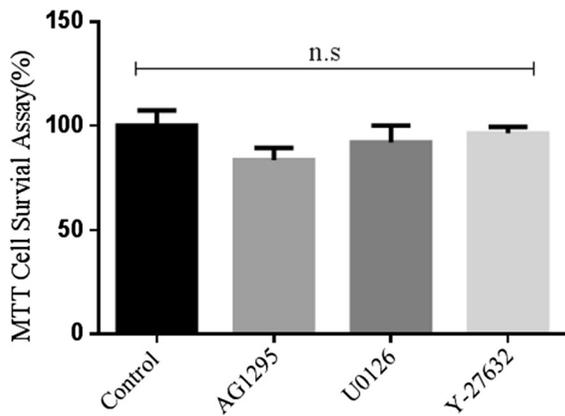


Fig. 1 Effect of PDGFR inhibition, ERK inhibition and ROCK inhibition on C6 cell survival Cell survival was determined by MTT assay in cells treated alone with following inhibitors—10 μ M AG1295, 10 μ M U0126 and 10 μ M Y-27632 for 24 h. The statistical data showed percentage of viable cells with respect to control. Values represent mean \pm SEM from 3 to 4 samples. *n.s.* not significant; * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 compared to control

(*** P < 0.001) compared to control (Fig. 2). However, no significant change was observed in C6 cells treated with Y-27632.

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. We found that PDGFR inhibition significantly reduced the migration of C6 glioma compared to control (Fig. 3a, b). 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. We found significant reduction of migration in cells treated with U0126 and Y-27632 compared to control (Fig. 3a, b). To confirm that ERK and ROCK signalling 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 cells treated with AG1295 compared to control (Fig. 3c, d), suggesting that PDGFR inhibition regulates the migration by modulating the downstream ERK and ROCK signalling.

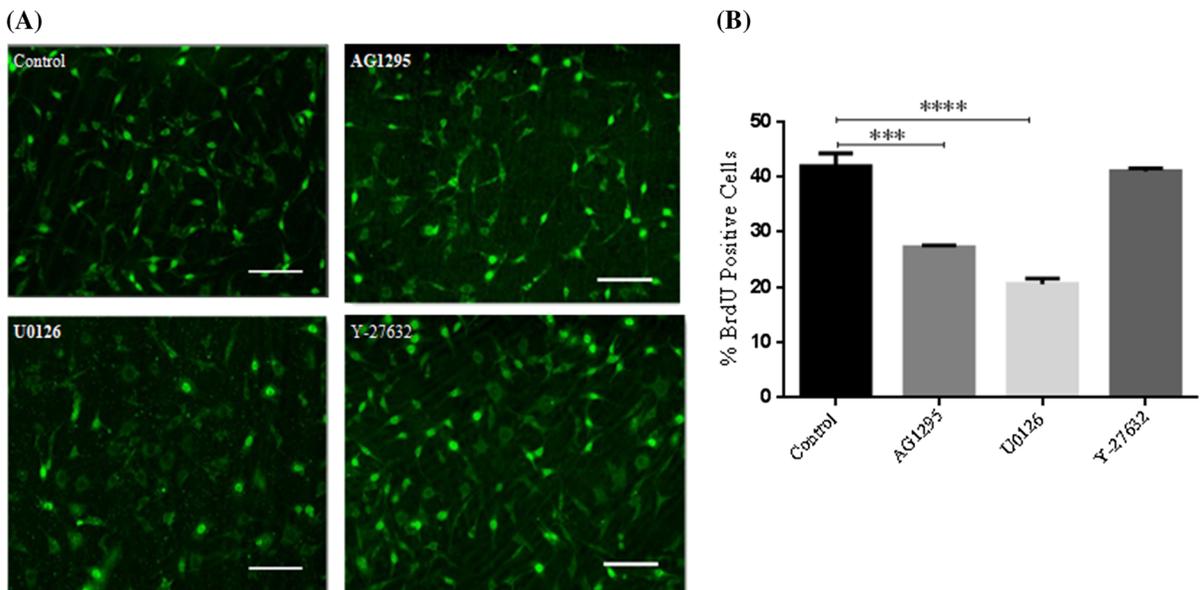


Fig. 2 Effect of PDGFR inhibition, ERK inhibition and ROCK inhibition on C6 glioma proliferation **a** Representative fluorescence images of BrdU-positive cells in C6 cells pre-treated for 24 h with PDGFR inhibitor AG1295 (10 μ M), MEK inhibitor U0126 (10 μ M) and ROCK inhibitor Y-27632

(10 μ M). Scale bar = 50 μ m (20X). **b** The statistical data showed percentage BrdU-positive cells calculated from panels in (a). Values represent mean \pm SEM from 3 to 4 samples. * p < 0.05; ** p < 0.01; *** p < 0.001, **** p < 0.0001 compared to control

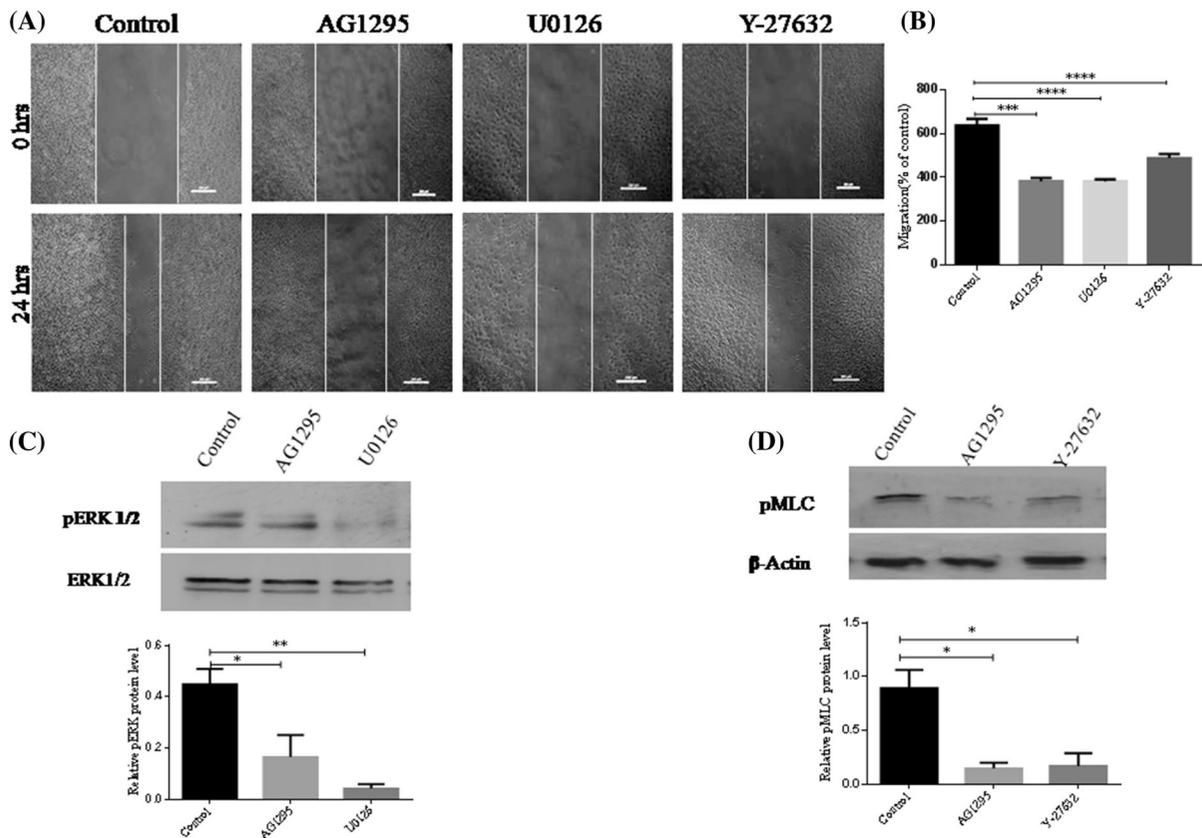


Fig. 3 PDGFR signaling regulates C6 glioma migration through ERK1/2 and ROCK pathway **a** Migration was analyzed by Scratch assay in the control, AG1295 (10 μ M), U0126 (10 μ M) and Y-27632 (10 μ M) groups treated for 24 h (Scale bar = 20 μ m). **b** Quantitative analysis of percent migration in scratch Assay. **c** Western blot analysis of pERK1/2 in C6 glioma cells in control, PDGFR

inhibitor AG1295 and MEK inhibitor U0126 groups treated for 24 h. **d** Western blot analysis of pMLC in control, PDGFR inhibitor AG1295 and Rock Inhibitor Y-27632 treated groups. Values represent mean \pm SEM from 3 to 4 samples. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 compared to the control group

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 whether PDGFR inhibition in C6 glioma regulates cytoskeleton reorganization, cells were treated with AG1295 or Y-27632 followed by immunostaining for actin and pMLC. Stress fiber formation was found to be reduced in cells treated with AG1295 and Y-27632 compared to control (Fig. 4). Also, we found that levels of pMLC were reduced and mostly concentrated around the nucleus in both AG1295 and Y-27632 treated cells as compared to control (Fig. 4). Thus, reduced actin stress fibers and

pMLC may further contribute to reduced migration observed in AG1295 and Y-27632 treated cells.

Next, we performed immunoprecipitation 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. We observed reduction in pERK–FAK–Paxillin interaction in cells exposed to AG1295, compared to control (Fig. 5), suggesting that PDGFR inhibition may regulate C6 glioma migration by regulating the pERK–FAK–Paxillin interaction.

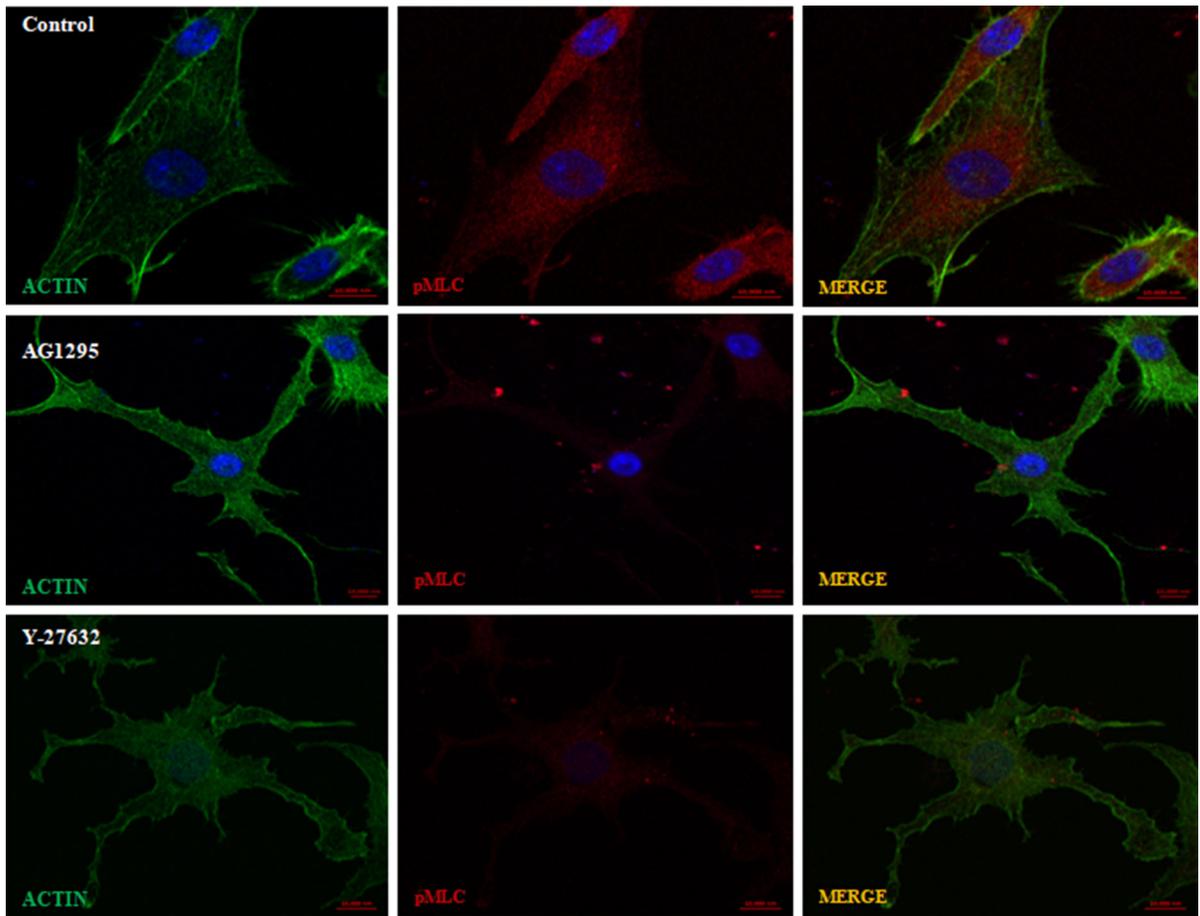


Fig. 4 Effect of PDGFR and ROCK inhibition on Actin-myosin rearrangements in rat C6 glioma C6 Glioma cells were treated with AG1295 or Y-27632. The formation of stress fibres and pMLC localizations were visualised in C6 glioma treated with AG1295 or Y-27632 by immunofluorescence

staining of pMLC (Red) and actin (Green) (Scale Bar: 10 μ m). Phalloidin Alexa 488 was used for F-actin staining. The experiment was performed three times with similar results. (Color figure online)

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 (Fig. 6a, b) which suggests that PDGFR signalling 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 spheres in presence or absence of AG1295. We found a significant reduction in size of the spheres in PDGFR inhibited C6 glioma ($41.74 \pm 11.64 \mu\text{m}$) as compared to control ($116.37 \pm 36.55 \mu\text{m}$). Further, we found a significant decrease in the number of spheres in PDGFR inhibited glioma (5 spheres \pm 1) as compared to the control (25 spheres \pm 8). Overall, PDGFR inhibition significantly impaired the number ($p < 0.001$) and size ($p < 0.01$) of sphere formation in C6 glioma, compared to control (Fig. 6c, d).

Next, In order to examine if PDGFR inhibition affect the glioma adhesion to extracellular matrix, we analysed

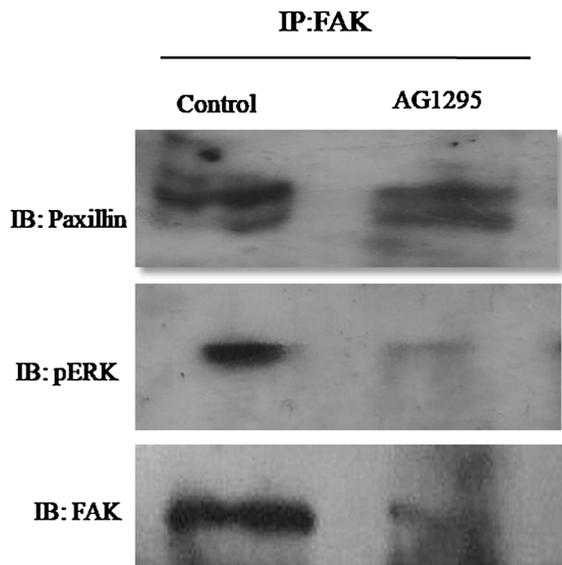


Fig. 5 Effect of PDGFR inhibition on FAK-Paxillin -pERK interaction in C6 glioma Association of FAK with Paxillin and phosphorylated ERK1/2 in C6 glioma cells and its inhibition by AG1295 PDGFR inhibitor. Cell lysates were prepared after treatment with AG1295 for 24 h and subjected to immunoprecipitation (IP) with antibodies to FAK. The resulting precipitates were then subjected to immunoblot analysis (IB) with antibodies to phosphorylated ERK1/2, Paxillin, or to FAK respectively. The experiment was performed two times

the attachment of C6 cells to Fibronectin (10 $\mu\text{g}/\text{ml}$). PDGFR inhibitor was found to significantly ($p < 0.0001$) reduce the adhesion ability of C6 glioma (Fig. 7a, b).

Lastly, invasion ability of C6 glioma treated with PDGFR inhibitor was examined by matrigel invasion assay. The results indicated that PDGFR inhibition significantly decrease cell invasion compared to control (Fig. 8a, b). Over all, results suggest that blocking of PDGFR signalling suppresses C6 glioma characteristic malignant behaviour.

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

We investigated the impact of PDGFR signaling inhibition on expression of GFAP, FAK, BDNF and MeCP2 which are known to play an important role in regulation of C6 glioma pathology. 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 (Fig. 9a). 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 increases the GFAP level (2.88-fold) and reduce the FAK level (0.41-fold), compared to control (Fig. 9b-d).

Discussion

Aberrant expression of PDGFR signaling is one of the major signaling defects observed in glioblastoma (Nazarenko et al. 2012; Westermarck et al. 1995; Martinho et al. 2009). In the current study, we demonstrated that inhibition of PDGFR signaling regulates glioma growth and migration through ERK and ROCK signaling pathways which could be correlated with reduced levels of pERK and pMLC observed in cells treated with PDGFR inhibitor. These results are 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 (Stice et al. 1999; Zohrabian et al. 2009; Cui et al. 2014; Nakada et al. 2011). 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. 2002). 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 fibres and lamellipodia. Polymerisation of F-actin is one of the basic mechanisms of cell membrane propulsion which can generate significant force to move the leading edge (Ananthkrishnan and Ehrlicher 2007; Redowicz 1999; Sahai et al. 2002). Rho/ROCK pathway has been involved in stress fibres assembly and formation of actin rich filopodia and lamellipodia at the leading edge of the cells (Ying et al. 2006). Similar to ROCK signaling inhibition, we also observed reduction in actin stress fibres formation in cells treated with PDGFR inhibitor AG1295 which suggests that PDGFR regulating the stress fibres formation via ROCK signaling. ROCK pathway also regulates the cell migration by phosphorylation of MLC (myosin light chain kinase) and MLC (myosin light chain) (Bogatcheva et al. 2011). pMLC at the cell

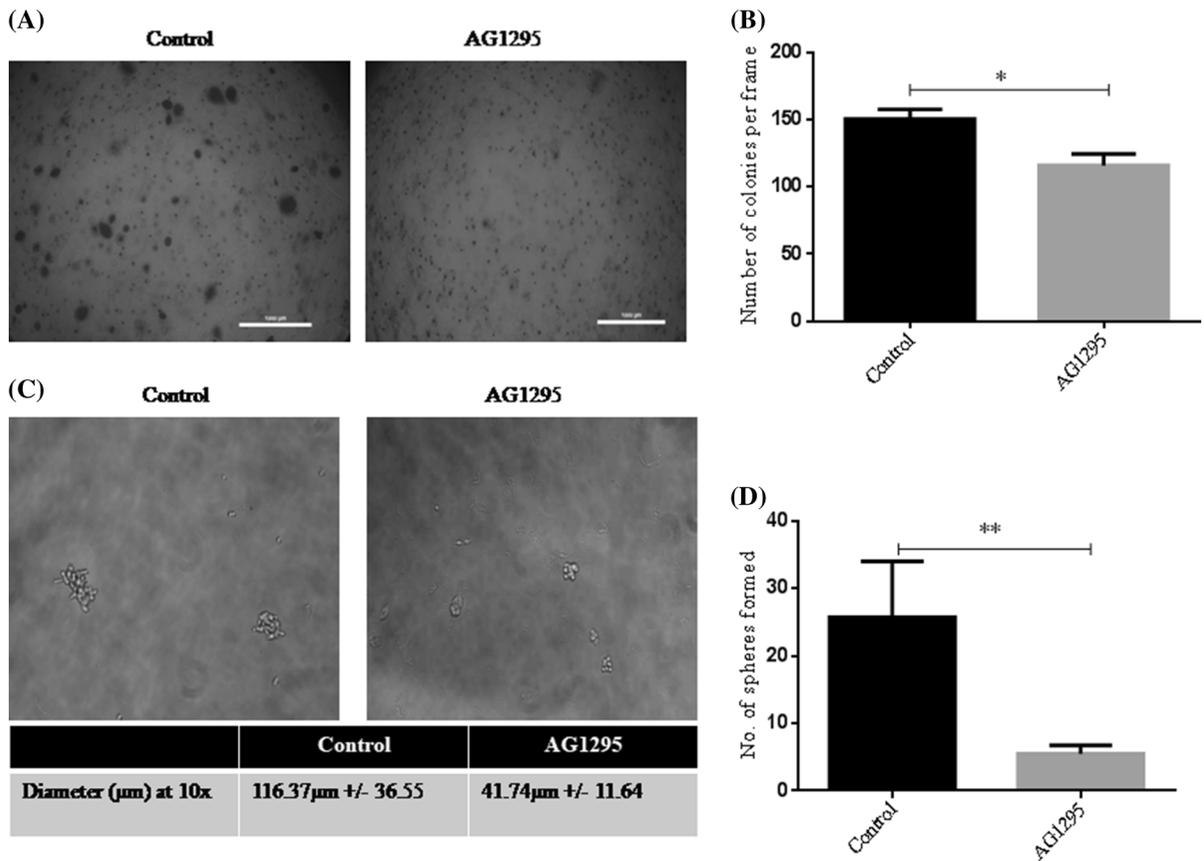


Fig. 6 PDGFR inhibition in C6 glioma suppresses colony formation in soft agar and sphere formation **a** PDGF inhibition suppresses the anchorage independent growth of C6 glioma. AG1295 treated cells show reduced number and size of colonies on soft agar compared to the control. The number of colonies > 0.1 mm in size was counted. Scale bar:1000 μm .

b Quantitative analysis of number of colonies formed in the respective groups. **c** PDGFR inhibition reduces sphere formation ability of C6 glioma as compared to control. **d** Quantitative analysis of number of spheres and average diameter of tumor spheres. Data are derived from three experiments and presented as mean \pm SEM * p < 0.05; ** p < 0.01; *** p < 0.001

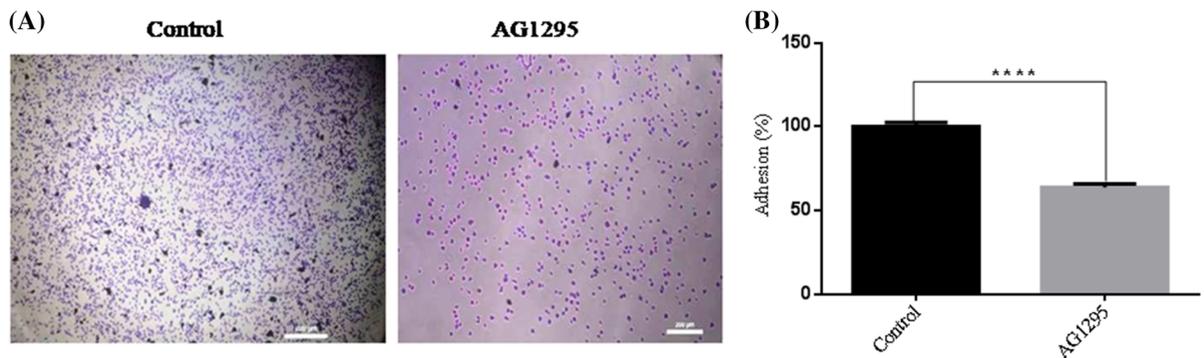


Fig. 7 Effect of AG1295 on adhesion ability of C6 glioma cells **a** PDGF inhibition impairs the adhesion property of C6 glioma as compared to the control. Adhered cells were stained with crystal violet. Scale bar: 200 μm . **b** Quantitative analysis of adhesion by measuring the optical density (OD) of extracted

crystal violet from adhered cells at 490 nm. The results are derived from at least three independent experiments and presented as mean \pm SEM * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001

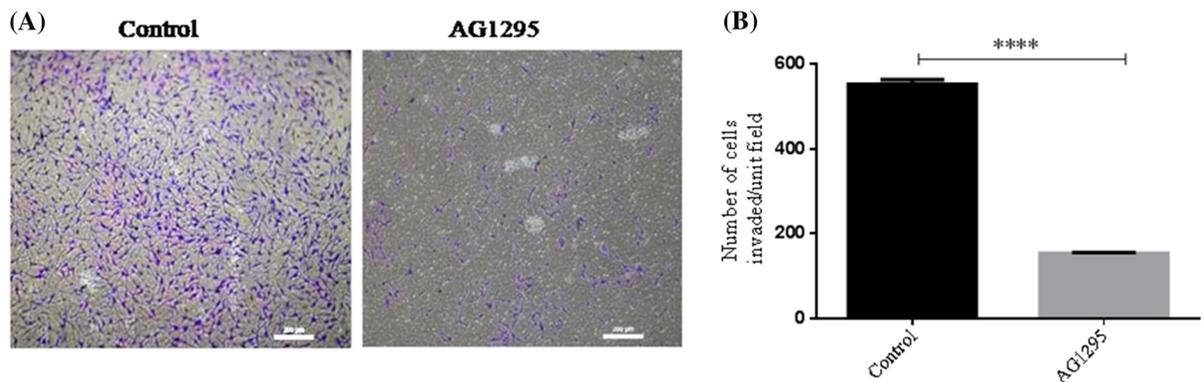


Fig. 8 PDGFR inhibition reduces the invasion of C6 glioma cells **a** Cell invasion was determined by the matrigel invasion assay. PDGFR inhibitor AG1295 suppresses the invasion of C6 glioma as compared to the control. Scale Bar: 200 μm . **b** Invaded

cells were counted and analyzed. Data are derived from three experiments and presented as mean \pm SEM * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ as compared to control

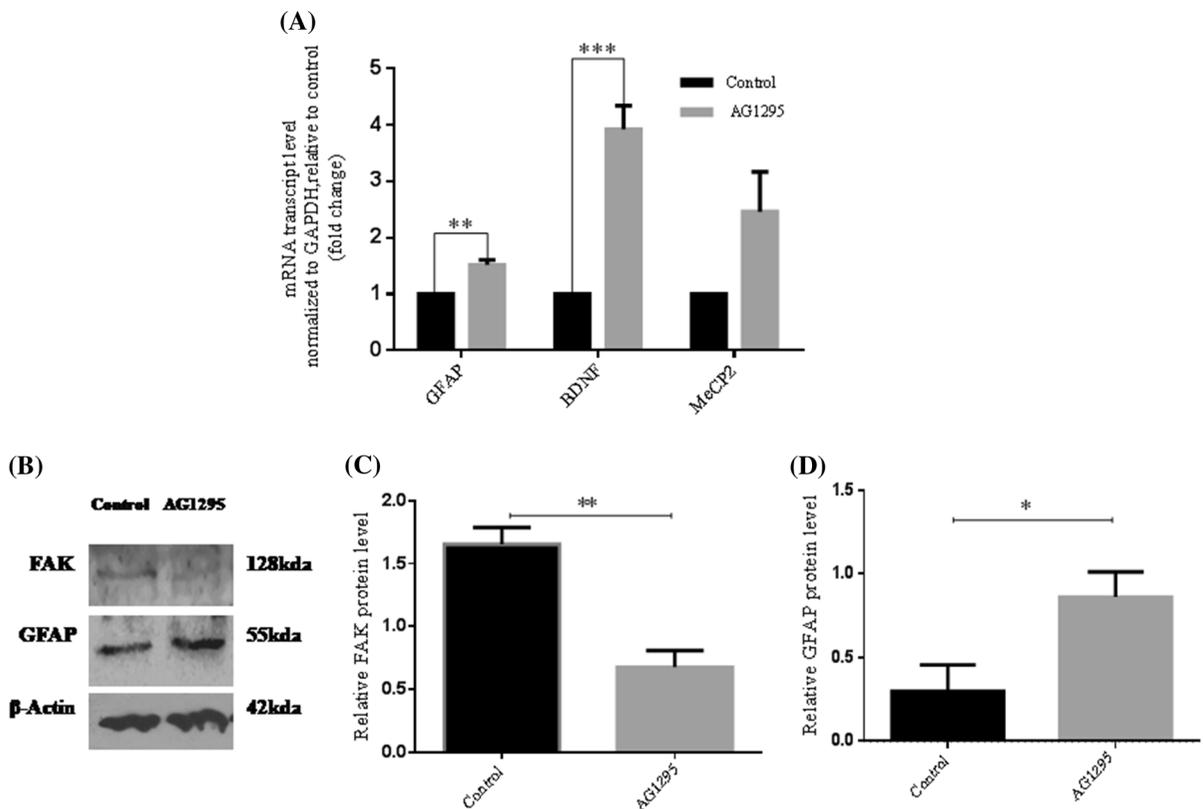


Fig. 9 PDGFR inhibition regulates gene expression in C6 glioma **a** Change in mRNA expression of BDNF, MeCP2 and GFAP in C6 glioma in the presence and absence of AG1295. **b** Western blot analysis of FAK and GFAP in C6 glioma control and AG1295 treated groups. Quantitative analysis of FAK and

GFAP (c, d). Values represent mean \pm SEM from 3 to 4 samples. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared with control. (Unpaired Student's t test)

periphery restricts membrane protrusions by counteracting the protrusive activity powered by actin polymerization. Loss of pMLC at the periphery and its

accumulation at the center lead to less matured adhesive structures which explains the less efficient migration (Salhia et al. 2008; Totsukawa et al. 2004).

Similarly, the data from the present study demonstrated reduced phosphorylation of myosin and its presence mostly near the nucleus or center 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. (Parsons et al. 2010; Truong and Danen 2009; Petit and Thiery 2000; Nagano et al. 2012). ERK influences the FAK–Paxillin interactions by phosphorylation of FAK or Paxillin which leads to regulation of focal adhesion dynamics (Huang et al. 2004; Igishi et al. 1999; Teranishi et al. 2009; Hayakawa et al. 2011). Alteration of FAK and Paxillin dynamics results in decreased migration by reduction in focal adhesion turnover and lamellipodia formation (Deraudt et al. 2014). Previous reports on ERK modulated disassembly of FAK–Paxillin complex suggest its role in assembly and disassembly of focal adhesions (Hunger-Glaser et al. 2003). In the present study we observed that PDGFR inhibition reduces the interactions of activated ERK and focal adhesion proteins. FAK and Paxillin, suggesting a novel regulatory mechanism of C6 glioma migration.

In order to study the effect of PDGFR inhibition on the malignant behaviour of C6 glioma, soft agar assay, adhesion assay, transwell invasion assay and sphere assay were employed in this study. The results of the present study indicate that PDGR inhibition significantly affect these malignant phenotypes 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).

Similarly, introduction of a truncated PDGF β 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 medulloblastoma tumors. An in vitro study showed

that PDGF-A enhances the medulloblastoma 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 of 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 (Natarajan et al. 2003; Mitra and Schlaepfer 2006; Mei et al. 2014). 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, we demonstrate that 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 showed that 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 (Kajimora et al. Kajiwara et al. 1992; Murphy et al. 1998). Further, treatment of C6 glioma with neuroprotective valproic acid was found to induce the MeCP2 transcript levels (Kim et al. 2008). Similarly in the 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; Xu et al. 2016). Similarly, we found significant increase in BDNF transcript levels which suggests its neuroprotective effect in C6 glioma cells. Valproic acid as neuroprotective agent has been shown to upregulate the BDNF level in C6 glioma (Rincón Castro et al. 2005). Specifically, an increase in levels of proBDNF was found to inhibit growth and migration and increase apoptosis and differentiation of C6 glioma by inducing GFAP expression (Xiong et al. 2013).

In conclusion, our study extended the earlier role of PDGFR inhibitor and demonstrated novel downstream mechanisms of PDGFR signaling inhibition on tumor

behavior of C6 glioma which could be targeted for future therapy.

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Compliance with ethical standards

Conflict of interest The Authors report no conflict of interest.

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