CHAPTER TWO

2. MATERIAL AND METHODS

2.1 Oligodendrocyte Isolation and Culture

OPCs were isolated from mixed glial cultures generated from the 1-2 day old rat pups (Charles Foster) as described previously (Y. Chen et al., 2007; Sharma et al., 2015). Mixed glial cultures were maintained at 5 % CO2 and 37ºC in DMEM supplemented with 10% FBS and 1 % Penicillin and streptomycin on PLL (Poly-L-Lysine) coated tissue culture flasks for 7-10 days. OPCs were removed by shaking mixed glial flasks overnight in an orbital shaker. The supernatant, containing cells shaken off the monolayer is placed in a non-tissue culture plastic petri dish to allow the differential adhesion of microglia cells. After the "subtraction" process, the cells obtained are greater than 95% OPCs which were used for further experiments (Figure 2.1).

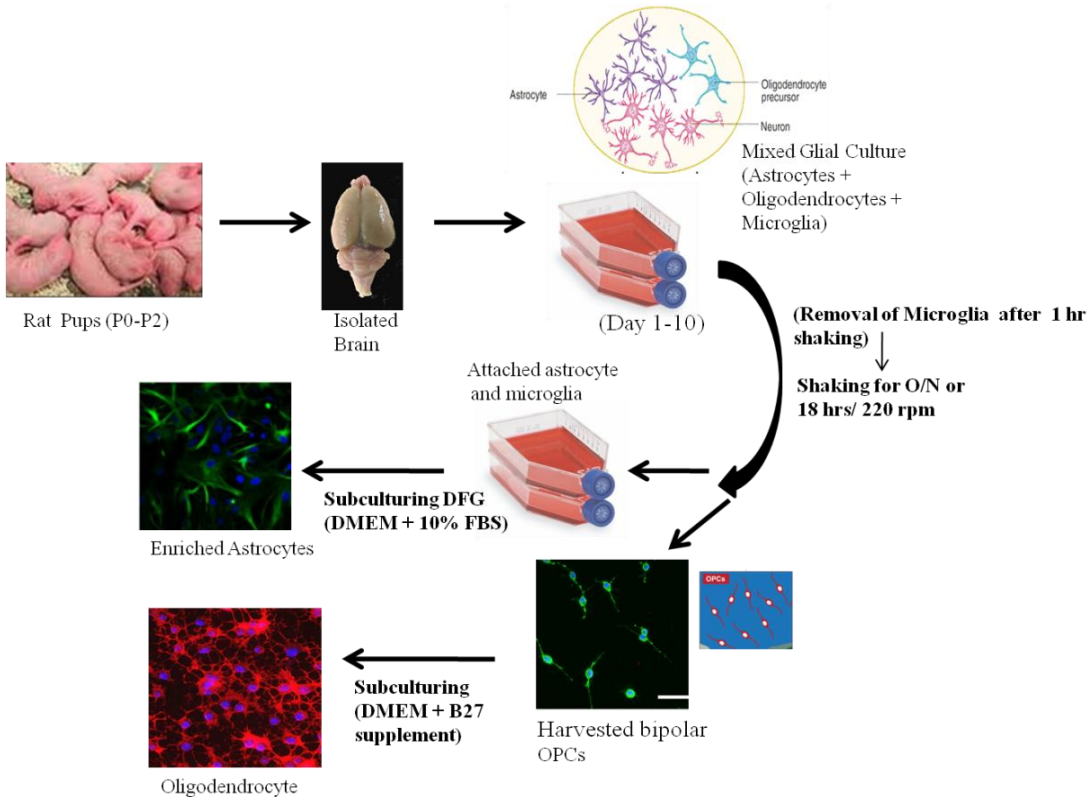

Figure 2.1: Oligodendrocyte Isolation and culture

2.2 Cell Line Culture and Maintenance

The Rat C6 glioma cell line was obtained from the National Centre for Cell Science, Pune, India**.** Cells were maintained at 5% CO_2 and 37°C in DMEM/F12 medium (Gibco) supplemented with 10% FBS and 1% Penicillin and Streptomycin.

2.3 Chemicals and Inhibitors

The following growth factors and pharmacological inhibitors at the given concentration each were used for the inhibition studies of signaling pathways and membrane receptors. Concentration and exposure time were determined using previous studies (See table 2.1).

Chemical	Concentration	Purpose	Manufacturer	References
U0126	10μM	MEK inhibitor	Santa Cruz	(E. E. Frost et al., 2009)
Y-27632	10μM	ROCK inhibitor	Santa Cruz	(Narumiya et al., 2000)
PDGF-A	10ng/ml	Growth factor	Sigma	(E. E. Frost et al., 2009)
AG1295	10μM	PDGFR Inhibitor	EMD	(Richard Milner et al., 1996)
Aphidicolin	20 μM	Inhibitor of DNA replication	Sigma	(Richard Milner et al., 1996)

Table 2.1: List of Chemical and Inhibitors

2.4 Migration Assays

A) Agarose drop assay

Migration was assessed by agarose drop migration assay as described previously (E. E. Frost et al., 2000). The working solution were made for resuspending the isolated cells consisted of 1:2 ratio of low melting point agarose and complete media (Sato's Media with 10% FBS) respectively. It was warm to 37°C in the water bath prior to the experiment. Approximately 35,000-50,000 cells were resuspended in a small drop (1.5μl) of Low Melting Point Agarose and plated onto a 24 well plate. The plate was incubated at

4°C for 15 mins to allow the drops to set. The agarose drops were later exposed to the given concentration of PDGF-A or inhibitors for the stipulated time as mentioned in Table 2.1. The migratory properties of cells were evaluated by their ability to migrate out of the Agarose Drop. Eventually, migration away from the edge of the drop was measured in micrometer using NIS Element Software (Nikon microscope) on four sides of the drop. To distinguish between active migration from the agarose drop and dividing cells in response to growth factors, the inhibitor of DNA replication i.e., Aphidicolin was added to the media in all the treatment groups(McKinnon et al., 1993; Richard Milner et al., 1996). (See Fig 2.2)

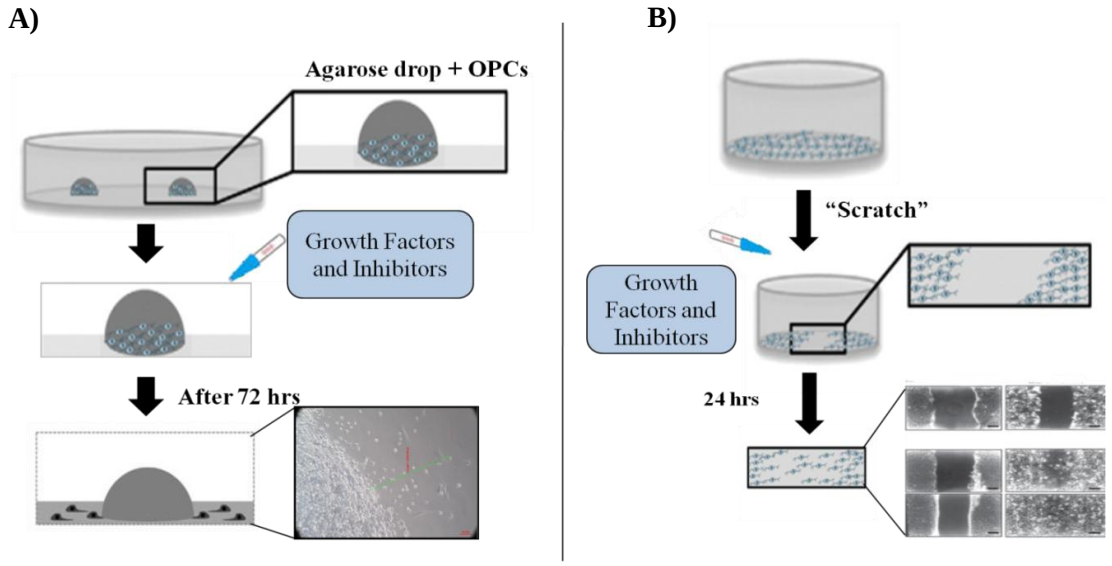

Figure 2.2: Migration assays A) Agarose drop assay and B) Scratch migration assays

B) Scratch assay

Cells were grown to confluence in 24 well plate, a scratch was made on the monolayer using the sterile 200µl pipette tip. After scratch, cells were treated with prescribed concentration of PDGF-A and inhibitors. The treatments groups were exposed to PDGF-A and inhibitors according to the experimental time points and media was replenished with serum free DMEM/F12 and photographed. For OPC study, Images of same scratch region were taken at 0 hrs, 24 hrs and 72 hrs until it was covered with cells. For glioma study, images of the scratch was taken similarly at 0hrs and 24 hrs and quantified by

measuring distance covered by cells between 0 hr to 24 hrs using Image J software. Relative migration distance = (A-B)/A, where A represents the mean width of the cell scratch at 0 hrs and B represents the mean width of the cell scratch at 24 hrs. Results are expressed as the means ± SEM. (See Fig 2.2)

2.5 Cell Proliferation Assay

Cells were treated with 10 µM BrdU for 4 hrs (for OPCs study) and 2 hrs (for C6 glioma study) prior to fixation with ice-cold methanol for 10 min at 4 °C. Then in order to denature DNA, cells were treated with 2M HCL for 60 mins at 37°C followed by incubation with anti-BrdU antibody for overnight at 4ºC. BrdU incorporation was visualized using fluorescence microscope following staining with anti-mouse FITC and Nuclei with DAPI. Fluorescent staining was observed and photographed at four frames using the Floid cell imaging system (Thermo fisher scientific). BrdU positive cells were calculated as percentage to total cells (labeled by DAPI). Percentage of BrdU-positive cells were analyzed and calculated by Image J software.

2.6 Small Interfering RNA (siRNA) Transfection

Cells were transfected with siRNA duplexes (predesigned and synthesized by Sigma) specific for rat ERK1 (siRNA ID: SASI_Rn02_00261822), ERK2 (siRNA ID: SASI_Rn01_00107865) or with a universal negative control siRNA (SIC001), using Hiperfect transfection reagent (Qiagen) following the manufacturers protocol. Gene silencing was observed after 24 hrs of transfection.

2.7 Plasmid Transfection

Plasmid construct (pEGFP-ERK1) expressing rat ERK1 ORF fused with EGFP (Green fluorescent protein) in pEYFP-N1 (Figure 2.9) was generous gift from Prof. Phillipe Lenormand (Institute of Research on Cancer and Aging, Nice, France). C6 glioma cells were transfected with plasmids expressing ERK1-EGFP (pEGFPERK1) or mock plasmid expressing GFP (pEGFP) for transient transfection using Attractene reagent (Qiagen) following manufacturer instructions.

2.8 Restriction digestion, End filling by Klenow fragment and blunt end ligation

0.5-1.0µg DNA sample was used for each restriction enzyme digestion. 1-3 U of the restriction enzymes (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10µl. The reaction mixture was incubated overnight at 37°C. DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently photographed. In case of double digestion, a compatible buffer for the two Res was essentially checked, if not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers (See Fig 2.3).

Blunt ends were generated from sticky ends produced by restriction digestion prior to ligation using Klenow fragment (NEB) as per manufacturer protocol. Ligation was carried out in 10µl volume containing the following constituents: blunt end vector generated by klenow fragment, 10X T4 DNA ligase buffer,1µl; T4 DNA ligase (MBI Fermentas), 0.5-1.0 U and sterile double distilled water to ake up the volume. Ligation reaction was carried out at 16°C for 12hr – 16hr.

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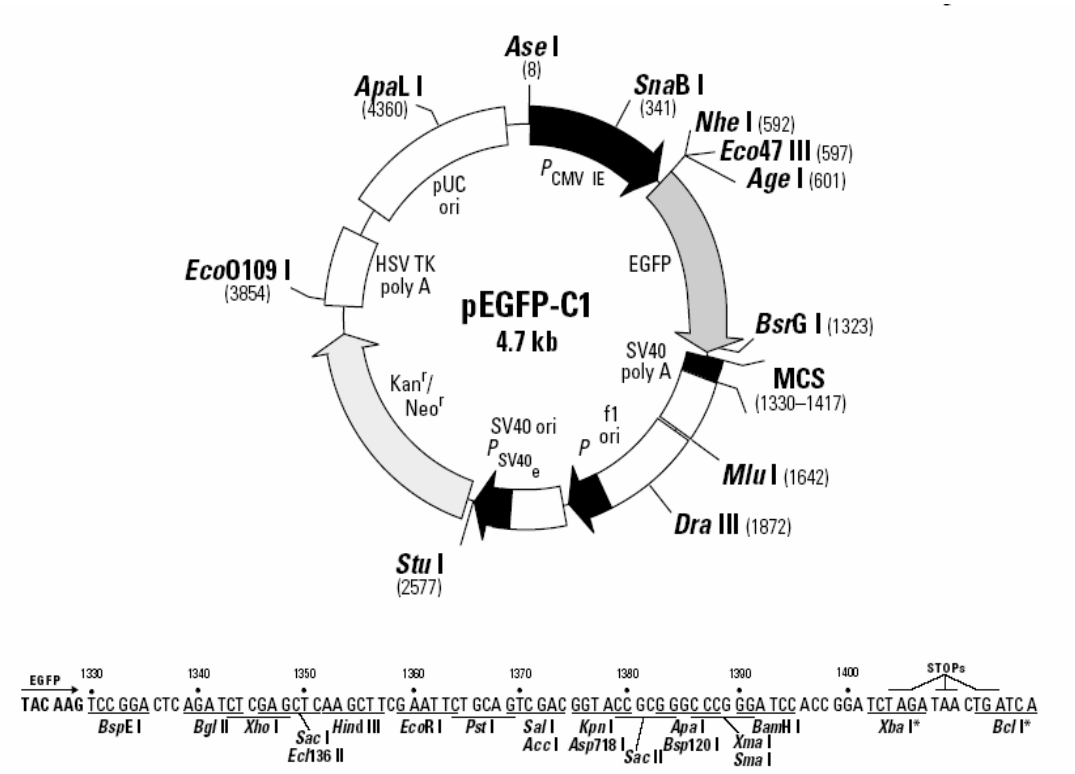

Figure 2.3: Full-length cDNA of mouse ERK1 subcloned into pcDNA3 vector (Invitrogen)

2.9 Plasmid transformation and isolation

Plasmid was transformed into competent E.coli DH5α strain using calcium chloride method(Sambrook & Russell, 2001). Transformants were selected on kanamycin containing Luria agar plates. Plasmid was isolated from transformants using Plasmid kit (Qiagen) following manufacturer protocol. Plasmid was checked on 0.8% agarose gel and concentration was determined by Qubit double strand DNA assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen).

2.10 Western blotting

Cells were lysed in Laemmli buffer and stored at -20°C. Protein concentration was determined by Qubit protein assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen). Cell lysates with equal protein loads (40μg) 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. Bands were visualized using

corresponding horseradish peroxidase-conjugated secondary antibodies (Sigma) (See Table 2.5). 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.

Antibody	Dilution	Molecular Weight (kDa)	Purpose	Manufacturer
pERK1/2	1:750	42/44	Signaling Protein Kinase	R & D systems
Total ERK1/2	1:1000	42/44	Signaling protein kinase	Sigma
Paxillin	1: 500	68	Cytoskeletal Protein	Santa Cruz
FAK	1:500	125	Cytoskeletal Protein	Santa Cruz
pMLC	1:500	20	Cytoskeletal Protein	Abcam
β-Actin	1:1000	42	Housekeeping Protein	Pierce
GFAP	1:1000	55	Intermediate Filament protein	Pierce

Table 2.2 Primary Antibodies for Immunoblotting

2.11 Immunocytochemistry

Cells were seeded on PLL coated glass coverslips (for OPCs) and glass coverslips with no ECM (for Glioma study) followed by treated with PDGF-A and inhibitors as described. Further, cells were fixed with 4% (wt/vol) paraformaldehyde, permeabilize with 0.25% vol/vol) Triton X-100 in PBS for 10min followed by blocking in 1% (wt/vol) BSA in PBST for 30 min. Cells were incubated overnight at 4°C with primary antibodies (See Table 2.6). After 24 hrs, cells were washed and stained with corresponding fluorescent conjugated secondary antibody for 1hr at room temperature. For actin staining, cells were incubated with phalloidin conjugated Alexa-Fluor 488 (Invitrogen) for 20 min to visualize F-Actin cytoskeleton. Fluorescence signals were detected using confocal microscopic imaging system (Carl Zeiss, Model LSM-710).

Antibody	Dilution	Purpose	Manufacturer
pERK1/2	1:100	Signaling Protein Kinase	R& D Systems
PDGFRα	1:100	Cell Surface Receptor	Santa Cruz
F-Actin (Phalloidin)	1:20	Actin Marker	Invitrogen
pMLC	1:100	Cytoskeletal Protein	Santa Cruz

FAK	1:100	Cytoskeletal Protein	Santa Cruz
Paxillin	1:100	Cytoskeletal Protein	Santa Cruz
Anti-Mouse TRITC	1:200	Secondary Antibody	Sigma
Anti-Rabbit FITC	1:200	Secondary Antibody	Abcam
Anti-Goat FITC	1:200	Secondary Antibody	Bangalore Genei
Anti-Rabbit TRITC	1:200	Secondary Antibody	Bangalore Genei

Table 2.3 Antibodies used for Immunoflorescence

2.12 Immunoprecipitation

Cells were grown in 90 mm plates for described experimental groups for the given stipulated time. For co-immunoprecipitation, cells were lysed in RIPA buffer (0.1% SDS,0.5% Sodium deoxycholate,1% NP40, 50mM Tris-HCL(pH 8.0), 150 mM Sodium Chloride) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor (Roche). Immunoprecipitation was carried with Anti –FAK antibody [(1-2µg per 100-500µg of total protein(1ml of cell lysates)] using Dynabeads Protein G immunoprecipitation Kit(Life Technologies) following manufacturer protocol. Immunoprecipitated protein was resolved by SDS-PAGE followed by western blot analysis using following antibodies: Anti-Paxillin (Santa Cruz), Anti-pERK (R&D systems) and Anti- FAK (Santa Cruz).

2.13 Cell Survival Assay

Cell viability was assessed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. 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 followed by the treatment of PDGF-A and Inhibitors. MTT (5mg/ml) dissolved in PBS was added to the wells and incubated for 3-4 hrs at 37ºC. 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 minimize 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.

2.14 Colony Formation Assay

Approximately, $1x10^3$ 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 and 10% FBS).Colonies were allowed to form in the treatment and transfected groups in C6 glioma as mentioned. After 10-12 days, viable cell colonies > 0.1 mm in size were counted and photographed.

2.15 Adhesion Assay

The efficiency of cell adhesion was determined by measuring the number of cells that adhered to extracellular matrix (ECM) Fibronectin. Cell culture plates were coated with Fibronectin (10 μg/ml) for overnight at 4ºC, followed by washing with PBS and blocking with DMEM containing 10% FBS. After 24 hrs of treatments with growth and inhibitors and 48 hrs in case of plasmid transfection, cells were seeded onto ECM-coated 96-well plates (1×10^5 cells/ well) in serum free DMEM/F12 medium. After 30 mins of incubation, the non adherent cells were removed by PBS wash, followed by fixed in 4% formaldehyde and stained with 0.5% crystal violet dissolved in 20% methanol. Later on, the stain was eluted out by 100% methanol and optical density was measured at 490 nm in microplate reader (Biotek).

2.16 Invasion Assay

Invasion assay was carried out using Growth factor reduced matrigel invasion chambers (Corning) according to the manufacturer's protocol. In brief, C6 cells were harvested in serum- free medium and transferred to the hydrated matrigel chambers (~ 100000 cells per well) containing the PDGF-A or inhibitors. In case of assessing invasion in plasmid transfected cells, cells were harvested in transferred to chambers 48 hrs after the transfection. The chambers were incubated for 24hrs 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 mins. The invaded cells were counted and the relative number was calculated.

2.17 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 PDGF-A and inhibitors for 24 hrs and post transfection for 48 hr. Cells were grown to form spheres in DMEM /F12 media containing EGF (20 ng/ml)(Invitrogen) and FGF (20 ng/ml)(Invitrogen) for 7 days. After, 7 days spheres photographs were taken under inverted phase contrast microscope (Nikon) and their numbers and sizes were calculated.

2.18 RNA Isolation and qPCR

Total RNA from cells, was isolated using Trizol reagent (Invitrogen) following manufacturers protocol. RNA concentrations were measured using a Qubit RNA assay kit (Invitrogen) in 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) in QuantStudio 12K (Life technology) real-time PCR machine 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 normalize the variability in the expression levels and data was analyzed using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Following Primers pairs were used for qPCR (Table 2.7).

MeCP2	*Forward Primer: gaccggggacctatgtatga* *Reverse Primer: caatcaattctactttagagcga*
BDNF	*Forward primer: ccataaggacgcggacttgt* *Reverse Primer: gaggctccaaaggcacttga;*
GAPDH	*Forward Primer: agacagccgcatcttcttgt* *Reverse Primer: cttgccgtgggtagagtcat*
GFAP	*Forward Primer: gcctctccctgtctcgaatg* *Reverse Primer: cgccttgttttgctgttcca*

Table 2.7 List of Primers pairs

2.19 Microscopy and Statistical Analysis

Confocal microscopy images were analyzed by ZEN 2012 imaging software. For statistical analysis, a minimum of 150 randomly chosen cells per condition were analyzed (N=3) independent experiments with 3–4 replicates. The data from more than two groups were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc analysis of multiple comparisons. Data from two groups were statistically analyzed using two-tailed Student's *t* tests. Results are expressed as mean standard error mean (SEM). A value of $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). Statistical analysis was performed with Prism 6 software (GraphPad Software Inc.).