# 2.Materials and Methods

## 2.1 Oligodendrocyte Progenitor Cells Isolation and Culture

Oligodendrocyte Progenitor Cells Isolation and Culture OPCs were cultured by a previously described method (Chen et al. 2007; Frost et al. 2009; Vora et al. 2011; Tripathi et al. 2017). Briefly, OPCs were isolated from P0–P2 day rat cortices. The rat cerebra from the brains were dissected, minced, and digested at 37°C with 0.8 ml DNase I stock solution (0.2 mg /ml) and 0.6 ml trypsin stock solution (0.25%) in 13.6 ml HBSS, to generate a single-cell suspension. Dissociated cells were plated on poly-L-Lysine (PLL)-coated T75 cm2 flasks with high-glucose Dulbecco's modified Eagle's medium (DMEM-Gibco) containing 10 % fetal bovine serum (FBS-Gibco) and 1 % penicillin/streptomycin (1X-Invitrogen) then incubated at 37ºC in the presence 5 % CO2.. The cell suspension was spreaded over the entire flask surface. The media was changed every 2–3 days completely with 10 ml DFG for 10 days to obtain mixed glial cultures containing OPCs on an astrocyte monolayer. Purified OPCs were obtained by 18–20 h shaking method on an orbital shaker at 37ºC followed by differential adhesion for 1 h on the non-tissue culture plastic petri dish (Eppendorf) in the CO2 incubator at 37ºC for differential adhesion of contaminating microglia and astrocytes. The cell suspension was collected in a 50ml tube by passing the cell suspension slowly through 40µm pore size filter (Genetix). OPCs were collected by centrifugation at 10 min at 100g. The supernatant was discarded without disturbing the pellet. The cell pellet was dissociated in small amount of the remaining medium (0.5ml). The cells were counted using the Trypan blue exclusion assay with a hemocytometer. Living cells do not take up the dye and are colorless under brightfield. The OPCs were diluted in the DMEM+B27 Media (Invitrogen) according to the desired cell number. OPCs were cultured in Sato medium (Bottenstein & Gordon, 1979). PDGF-AA (Sigma) was added at 10ng/ml to Sato medium for proliferation of OPCs. Figure 2.1 shows the flow chart of derivation of oligodendrocyte progenitor cells(OPCs) from P0-P2 day rat pups.The isolated OPCs and OLGs were significantly enriched and confirmed using morphological and immunocytochemical analyses. Use, care and housing of all animals were done in compliance with Institutional Animal Ethical Committee (IAEC), Biochemistry Department, Faculty of Science, The Maharaja Sayajirao University of

Baroda. The protocol numbers are mentioned below: BC/17/2012, ZD/18/2013, ZD/02/2013, ZD/28/2014, ZD/01/2016 and ZD/03/2017

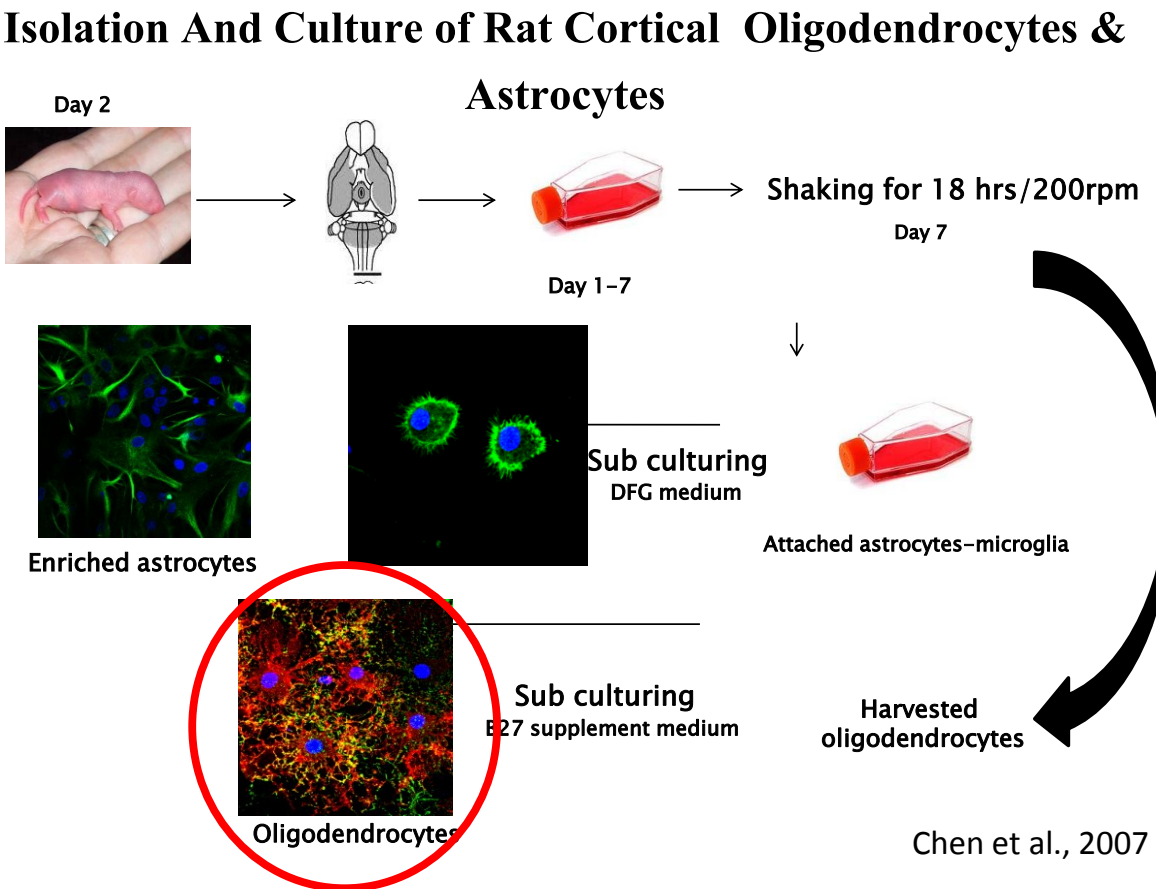


*Fig. 2.1 Flow diagram of isolation and culture of OPCs*

## 2.2 Glioma Cell line

Rat C6 glioma (NCCS, Pune) were grown as monolayer cultures in DMEM (GIBCO) supplemented with 10% heat-inactivated foetal bovine serum (FBS, GIBCO) and penicillin/streptomycin (Invitrogen). Cell lines were maintained at 37°C in 5% CO2.

## 2.3 Cell Migration Assay

OPC migration was assessed using the agarose drop migration assay (Varani et al. 1978; Frost et al. 2000, 2009). Briefly, the working solutions were made for resuspending the freshly isolated OPCs, e.g OPCs were resuspended in 40 µl Sato's medium plus 10% FBS, and warm to 37°C in the water bath prior to the experiment. . The cell density used varied between 20 and 100 × $10^6$ cells/ml. 20 µl of the 1% agarose was added to the cell suspension and mix thoroughly. Immediately after mixing, 1.5 µl drops of the cell suspension were placed at the centre of wells in a 24-well tissue culture plate. The plate was incubated at 4°C for 15 min to allow the agarose drop to set. After that 50 µL Sato's medium media (+/– ECM) was added carefully around the drop. After 2 h, a further 450 µL of Sato's medium was carefully added (down the side of the well) before returning the plates to the incubator. The extent of cell migration was measured at daily intervals for 1–5 d using a phase contrast microscope. Cells migrate out to form a uniform corona around the drop. At any one time point, the distance between the edge of the drop and the leading edge of migrating cells within the corona can be recorded 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 medium (Milner et al. 1996; Ikegami et al. 1978; McKinnon et al. 1993). In all the treatment groups, cells were allowed to migrate for 72 h in the presence of aphidicolin. Hence, the emergence of cells from agarose drop was not due to proliferation but reflected entirely due to cell migration. Photomicrographs of the agarose drop assays were taken on a Nikon TS100 inverted microscope using phase optics.

## 2.4 Immunocytochemistry (ICC)

Immunofluorescent staining was performed in order to characterize the cell populations and to assess the stages of differentiation and maturity of the cultured OPCs. Cells were harvested as above, and plated on glass coverslips in order to view them using confocal microscopy. Antibodies for proteins specific to OPCs and C6 glioma cells were used to determine the cell types present. The list of antibodies is mentioned in Table 2.1. Immunofluorescent staining was used to study the expression and distribution of different proteins. After OPCs/glioma were cultured as described above, they were seeded on glass coverslips coated with poly-L-lysine (PLL). OPCs were maintained in DMEM+B27 Media for 24hours prior to the cell treatments. After the cell treatment the cells were stained with fluorophore-linked antibodies and cell types confirmed based on specific antibody staining as visualized using confocal microscopy. Cells were fixed in ice cold 4% paraformaldehyde prepared in PBS pH 7.4 for 15 minutes at room temperature and then washed three times for 10 minutes each with ice cod PBS. For the proteins which were expressed intracellularly, permeabilization step was performed for which the cells were incubated in PBS containing 0.25% Triton X-100 for 10 minutes at room temperature and then three times for 5 minutes each with ice cod PBS. However, it is not appropriate to perform permeabilization for the membrane associated antigens since it destroys membranes. Further, blocking was done by incubating the cells with 1% BSA in PBST for 30 min to block unspecific binding of the antibodies. The cells were incubated in protein specific antibodies and their corresponding dilution (diluted in 1% BSA in PBST) in a humidified chamber for overnight at 4°C. As a control for the secondary fluorescence, one plate of cells was incubated without primary antibody. Cells were then washed three times for 5 minutes each with PBS. Cells were incubated in secondary antibodies specific for primary antibodies (diluted in 1% BSA in PBST) for 1 hour at room temperature in the dark. Cells were again washed three times for 5 minutes each with PBS as previously stated. The coverslips were mounted using the Gold Antifade Mountant with DAPI ((4',6-diamidino-2-phenylindole) and sealed with the nail polish to prevent drying and movement under the microscope. The slides were stored at -20°C and the images were taken within one week of staining. The images were acquired using a 60X/40X objective in confocal microscope. Images were analysed using ImageJ software.

**Table 2.1 Antibodies for Immunofluorescence**

| Antibody | Dilution | Purpose | Manufacturer |
|---|---|---|---|
| PDGFRα | 1:50 | OPC Marker | Santacruz |
| O4 | 1:100 | Immature OLG marker | R&D |
| MBP | 1:100 | Mature OLG marker | Abcam |
| PLP | 1:100 | Mature OLG marker | Abcam |
| pERK1/2 | 1:50 | Signaling Protein | Santacruz |
| F-Actin (Phalloidin) | 1:1000 | Actin marker | Invitrogen |
| Integrin αvβ1 | 1:100 | Membrane Receptor | Abcam |
| Integrin αvβ3 | 1:50 | Membrane Receptor | Abcam |
| Lipid raft (Cholera Toxin B) | 1:1000 | Lipid raft marker | Invitrogen |
| Anti-Rabbit FITC | 1:200 | Secondary Antibody | Abcam |
| Anti-Goat TRITC | 1:200 | Secondary Antibody | Abcam |
| Anti-Mouse TRITC | 1:100 | Secondary Antibody | Abcam |

## 2.5 Protein Extraction, Protein Quantification and Western Blotting

After exposure to various treatments, the cells were scraped off plates into 5mL tubes (Eppendorf) and spun at 5000rpm for 5 minutes. Pellets were resuspended and washed twice with ice cold PBS. Depending on the number of cells used for each treatment and the level of confluence, 100-200 µl of RIPA lysis buffer along with the protease inhibitor cocktail was added in order to lyse the cells. The cell lysates were stored at -20ºC until used for western blotting. The total protein was quantified using Qubit protein assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer's instructions.

The following procedure was adapted from the Bio-Rad Mini-PROTEAN system reference guide (Bio-Rad). Stored protein samples were thawed on ice and 40µg of protein was aliquoted from each sample. 5x Loading dye and reducing agent were added in the correct dilutions, based on the desired final volume (10-25µL). The samples were mixed, and heated at 100°C for 5 minutes using a water bath and then briefly centrifuged (pulse spin). Thermo PAGERULER Plus Ladder was loaded into one lane of the gel as a

molecular weight marker and protein samples were loaded in successive lanes of the gel. Proteins were resolved by SDS polyacrylamide gel electrophoresis (10%) and separated by electrophoresis using a constant voltage (100V) for 90 minutes. After separation, the gel was removed and placed in 1x Transfer buffer (0.190mM glycine, 25µM tris-base, 200ml/l methanol) at room temperature. From anode to cathode side, the sandwich was comprised of a transfer sponge, filter paper, gel, 0.2µM nitrocellulose membrane, filter paper, and a transfer sponge clamped in a cassette. The cassette was placed in a Bio-Rad Mini Trans-Blot apparatus containing transfer buffer and ice pack. Proteins were then transferred to nitrocellulose membrane at 100 volts for 90 minutes.

After transfer, the nitrocellulose membrane was placed in a small plastic container and covered with 5% BSA (Bovine Serum Albumin) in TBS-T (20mM Tris, 500mM NaCl and 0.05% v/v Tween 20) and then placed on an orbital shaker at room temperature for 60 minutes to block nonspecific antibody binding. The blot was then placed primary antibody (made in 5ml of 5% BSA) and continuously rotated overnight at 4ºC. Concentrations of all primary antibodies used can be found in Table 2.2. After primary antibody incubation, the membrane was washed 6 times with TBS-T for 10 minutes each, at room temperature on an orbital shaker. The blot was then incubated on an orbital shaker for one hour at room temperature in 5ml of secondary antibody conjugated with horseradish peroxidase (HRP) in 10mL of 5% BSA solution (dilutions found in Table 2.2). After incubation with secondary antibody, the membrane was once again washed 6 times as above. The blots were developed using specific immunoreactivity was visualized using an ECL kit (Invitrogen).The following steps were performed in a dark room. The X-ray cassette was opened and film was placed inside for exposure. Separate pieces of film were exposed for 30 seconds, 2 minutes and 5 minutes. The film was developed and the immunoreactive bands were digitally scanned quantitated by densitometry using ImageJ.

Table.2.2 Antibodies for Immunoblotting

| Antibody | Dilution | Purpose | Manufacturer |
|---|---|---|---|
| PDGFRα | 1:1000 | PDGFA Receptor | Santacruz |
| β-Actin | 1:1000 | Housekeeping gene | Sigma |
| Paxillin | 1:1000 | Cytoskeleton protein | R&D |
| Total ERK ½ | 1:1000 | Signaling Protein | Abcam |
| pERK1/2 | 1:1000 | Signaling Protein | Santacruz |
| Integrin αvβ1 | 1:1000 | Membrane Receptor | Abcam |
| Integrin αvβ3 | 1:1000 | Membrane Receptor | Abcam |
| Lipid raft (Cholera Toxin B HRP) | 1:1000 | Lipid raft marker | Invitrogen |

## 2.6 Immunoprecipitation

The cultured cells were incubated (80-90% confluent monolayer) in 100 mm cell culture plate or approximately 2-5 x $10^7$ suspension cells in flask). 1–3 ml ice cold RIPA buffer (Milner et al., 1994) was added to subconfluent cell monolayer and incubated at 4º C for 10 minutes. The cellular debris was pelleted by centrifugation at 10,000xg for 10 minutes at 4º C. Supernatant was transferred to a fresh tube. Lysate was precleared by adding 1.0 µg of the appropriate control IgG together with 20 µl of appropriate suspended (25% v/v) agarose conjugate (Protein A/G-Agarose- SantaCruz). After 30 min of incubation it was centrifuged for 3000rom for 30seconds at 4º C. From the supernatant 100–1000 µg of total cellular protein was transferred to a microcentrifuge tube and 1–10 µl (i.e., 0.2–2 µg) primary antibody anti-PDGRα, anti αvβ1 was incubated for 1–2 hours at 4º C. 20 µl of the Protein A/G-Agarose conjugate suspension was added to the above mixture and kept on a rotating device for 1 hour to overnight at 4º C. The immunoprecipitates were collected by at 3000 rpm for 30 seconds at 4º C. Pellet was gently washed with 1.0 ml PBS buffer 2–4 times and resuspended in Laemmli buffer and stored at -20º C till used.

## 2.7 Cell surface receptor biotinylation and internalization assay

For cell surface labelling experiment ts biotinylation was done by following Baron et al., 2002; Vassilieva et al., 2008, cell surface molecules were labelled with 0.1 mg/ml NHS-LC-biotin (Pierce) for 25-30 min at 37°C. Labeled cells were washed three times in cold PBS and then incubated at 37°C in prewarmed serum-free DMEM media (control and treatments) for different time points. Then the cells were washed three times in cell wash buffer (50 mM Tris-HCl, 150mMNaCl, 1 mMCaCl2 and 1 mMMgCl2 pH 7.5), scraped and lysed as described above. Extracts, i.e. supernatant and pellet, were collected by centrifugation at 14 000 r.p.m. for 10 min at 4°C. The insoluble pellet was washed once with lysis buffer and solubilized in a small volume of solubilization buffer (50 mM Tris-HCl, 5 mM EDTA,1% SDS) by passage through a 21-gauge needle, and diluted with extraction buffer to the same volume as the supernatant. Supernatants were also adjusted to an SDS concentration equal to that of the solubilized pellets. The amount of total protein in the extracts was determined with the Bio-Rad detergent-compatible protein assay with BSA as standard. As indicated, equal amounts of protein or equal volumes were then subjected to immunoprecipitation. For the identification of internalized receptors, precipitated biotin-labelled cell surface integrin or PDGFRα was analysed by SDS-PAGE (7.5%) under non-reducing conditions, followed by immunoblot ECL detection with streptavidin- peroxidase (Amersham).

## 2.8 Lipid raft staining

Lipid raft staining was performed according to Janes et al. 1999. Aliquots of $1x10^6$ cells (in 100 ml) were labelled in DMEM medium with Alexa Fluor-conjugated cholera toxin B (Invitrogen), which binds to the ganglioside GM1 on the cell surface, for 30 minutes on ice. After three washes, cells were treated with PDGF-A or kept in suspension for anoikis assay. After three washes, cells were fixed in 1% paraformaldehyde for 30 minutes on ice, mounted onto slides and studied by confocal microscopy.

## 2.9 Anoikis assay

Anoikis was assayed (Liau et al., 2007; Cai et al., 2015) by plating cells on polyHEMA-coated plates. A solution of 120 mg /ml poly-HEMA (Sigma) in 100% ethanol was made and diluted 1:10 in 95% ethanol; 0.95 ml/mm of this solution was overlaid onto 35-mm wells and left to dry in a heated dryer system for 12 h. Before use, wells were washed twice with PBS and once with DMEM. In all, $1 \times 10^6$ cells, suspended in specified medium (Control and treatment) were incubated in the polyHEMA-coated wells for a specified time in a humidified (37°C, 5% CO2) incubator.

## 2.10 Actin cytoskeleton fraction

Actin cytoskeleton fraction was prepared following Bodin et al., 2005. Briefly, $2\times10^9$ cells/ml were lysed by addition of 0.5 ml of a 3× ice-cold cytoskeleton (CSK) lysis buffer [1.5% Triton X-100 (v/v), 3 mM PMSF, 3 mM Na3VO4, 6 μg/ml each of leupeptin and aprotinin, 60 mM EGTA and 300 mM Tris, pH 7.4] incubated for 10 minutes and centrifuged (15,000 g, 15 minutes, 4°C) to obtain the Triton X- 100 insoluble low-speed pellet. This fraction was washed with 1× CSK lysis buffer and then with CSK lysis buffer without Triton X-100 to obtain the cytoskeleton.

## 2.11 Cell Treatments

### 2.11.1Growth Factor and ECM treatments

Purified OPCs were plated in DMEM/F12 containing B27 supplement (Gibco), and 1 % penicillin/streptomycin (Gibco) on PLL (Sigma)-coated dishes or glass coverslips and were serum starved overnight prior to any treatment. These naïve OPCs were transiently exposed to PDGF-A (Sigma) alone and in combination with FN (Invitrogen) for 30 min (transient exposure activates signaling pathway/s that drive OPC migration behavior selectively without activating the cell proliferation) (Frost et al. 2009; Vora et al. 2011). For transient exposure studies, after 30min the medium containing PDGF-A was replaced with serum free medium. For continuous exposure experiments, medium containing PDGF-A was not replaced by serum free medium. PLL was used as control substratum which supports cell adhesion and spreading without engaging integrins. PLL is unable to activate the ERK1/2 (Chen et al. 1994; Schlaepfer et al. 1994;

Miyamoto et al. 1995; Zhu and Assoian 1995). In all the experiments, the PLL and FN were used at the concentration of 10 µg/ml. For mimicking near physiological PDGF-A, 1 ng/ml concentration was used and 10ng/ml was used as a higher concentration (Baron et al. 2002; Brunmark et al. 2002; Vora et al. 2011).

### 2.11.2Inhibitors/blockers

For the inhibition studies of signalling pathways, membrane receptors, lipid rafts and actin polymerization, following inhibitors/blockers were used (Table2.2). Concentration and exposure time were determined using previous studies. When treating with a cell signaling inhibitor, the inhibitor was added to the cultures 30 minutes prior to treatment. For blocking studies of integrin, blocking antibody was added 1hour prior to the experiment. For lipid raft disruption, cells were MβCD for 30 minutes and FB1 for 24 hours prior to the addition of growth factors.

**Table.2.3 Antagonists and Concentration for Cell treatments**

| Antagonist/ Inhibitor | Concentration | Function | Manufacturer | References |
|---|---|---|---|---|
| UO126 | 10µM | MAPK/ERK inhibitor | EMD | Frost et al. 2009 |
| AG1295 | 10µM | Selective inhibitor of PDGFR | EMD | Milner et al., 1996 |
| Cyto D | 2µM | Inhibitor of actin polymerization | EMD | Rivas et al., 2004 |
| MβCD | 5mM | lipid raft disrupting agent | Sigma | Sanchez et al., 2011 |
| Fumonisin B1 | 50µM | Inhibit ipid raft synthesis | Sigma | Merrill et al., 1993; Klein et al., 2002 |
| Aphidicolin | 10µM | Specific inhibitor of Nuclear DNA replcation | Sigma | Milner et al., 1996 |
| Blocking antibody αvβ1 | 10µg/ml | Block the αvβ1 integrin | Santacruz | Summers et al., 2010 |
| RGD Peptide | 10µg/ml | Inhibitor of integrin ligand interactions | Sigma | Summers et al., 2010 |
| Poly-Hema | 1% | Anoikis inducer | Sigma | Otero et al., 2014 |

## 2.12 Microscopy, quantification 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 results of migration assay are expressed as the mean ± standard error of the mean. For filopodia counting, a minimum of 50 randomly chosen OPCs for each experimental group were analyzed, and the total number of filopodia at the growing tips per cell were counted (n = 3 independent experiments). Data are reported as the mean number of filopodia per cell (Eyermann et al. 2012). Differences between treatment groups were analyzed using Student's t test, or one way analysis of variance with Bonferroni's post-test where appropriate. Statistical analysis was performed with Prism 3 software (GraphPad Software Inc.). P\0.05 was considered significant (*P<0.05, **P<0.01, ***P<0.001).