CHAPTER 4

EVALUATION OF MECP2 FUNCTION IN THE REGULATION OF GLIOMA GROWTH AND GENE EXPRESSION

4.1. INTRODUCTION

Epigenetic mechanisms include DNA methylation, histone modifications and non coding RNAs play an important role in development and function of central nervous system (Graff et al., 2011). Epigenetic abnormalities can lead to uncontrolled cell division, tumor initiation and growth, invasiveness and metastasis (Fouse and Costello, 2009). Glioblastoma multiforme (GBM) is a form of malignant and aggressive brain tumor (Burgess et al., 2008). Aberrant DNA methylations are widely reported in glioma and include global and gene-specific DNA hypomethylation, local DNA hypermethylation of gene promoters (Kondo et al., 2014).

Methyl CpG binding protein-2 (MeCP2) is an epigenetic regulator which binds to methylated CpG dinucleotide in DNA and essential for brain development. MeCP2 mutations are the major cause of the neuro-developmental disorder Rett syndrome (Meehan et al. 1992; Amir et al.1999). Previous studies have reported that MeCP2 is essential for proper functioning of neurons, but recent reports have shown that it is also important for functioning of glial subtypes in the brain (Yamamuro et al., 2015; Cronk et al., 2015; Sharma et al., 2015). Other than its role in neurological disorders, MeCP2 has also been shown to play an important role in many cancers such as breast, colorectal, lung, liver, and prostate cancer (Parry and Clark, 2011). Recently, it has been demonstrated that valproic acid which inhibits histone deacetylase (HDAC) enzyme activity, control the expression of the MeCP2 and HDAC1 to HDAC3 genes in C6 glioma cells (Kim et al., 2008). In gastric carcinoma cells, micro RNA miR-212 was shown to suppress translation of MECP2 transcripts, which in turn resulted in reduced depth of cellular invasion (Wada et al., 2010). In addition, MeCP2 has been linked to other cancers, such as myeloma (Wang et al., 2006), hematological malignancies (Meklat et al., 2007), ductal carcinomas (Xu et al., 2012), and cervical cancers (Wang et al., 2013).

However, far too little attention has been paid on MeCP2 function in glioma. Rat C6 glioma is used as a model system to study glioblastoma growth and invasion (Grobben et al., 2002). Also, C6 glioma was found to retain the characteristic of glial precursor, express some oligodendrocyte and astrocytes marker genes including PLP, MAG and GFAP. Earlier studies have shown that C6 glioma can be used as model system to study myelin genes expression (Salvati et al. 2004; Zhu et al. 1994; Ye et al 1992; Leisewitz et al. 2008).

The purpose of the present study was to assess the role of MeCP2 in C6 glioma malignant behavior and expression of oligodendrocytes and astrocytes marker genes. To gain better insights, MeCP2 was knockdown or stably expressed in C6 glioma followed by analysis of malignant phenotype and gene expression.

4.2. MATERIALS AND METHODS

4.2.1 Cell Culture

The Rat C6 glioma cell line was procured from NCCS (National Centre for cell science, Pune, India. Cells were maintained at 5% CO_2 and 37°C in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% Penicillin and Streptomycin.

4.2.2 MeCP2 Overexpression

Plasmid construct expressing rat MeCP2 ORF fused with YFP (Yellow fluorescent Protein) in pEYFP-N1 was generous gift from Prof. M. Cristina Cardoso (Cell Biology and Epigenetics, Dept. Biology, Technische Universität Darmstadt, Germany). For transient transfection, cells were transfected with plasmids expressing MeCP2-YFP (pMeCP2Y) or mock plasmid expressing YFP (pEYFP) using attractene reagent (Qiagene) following manufacturer instructions. For stable transfection, 48 hrs post transfection, cells were allowed to grow in medium containing 1mg/ml G418 (Invitrogen) for 2-3 weeks. Colonies of stable cells were picked and allowed to grow in medium containing 0.5 mg/ml G418.

4.2.3 Small Interfering RNA (siRNA) Transfection

Cells were transfected with siRNA duplexes (predesigned and synthesized by Sigma) specific for rat MeCP2 (siRNA ID: SASI_Rn01_00072926) or with a universal negative control siRNA (SIC001), using HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. The transfection was repeated after 48 h of first transfection for optimal silencing. RNA and proteins were isolated after 24 h of second transfection.

4.2.4 BrdU staining assay

Cells were treated with 10μM BrdU for 4h prior to fixation with ice-cold methanol for 10 min at 4 °C. Cells were incubated with 2M HCl for 60 min at 37 °C to denature DNA then washed with

PBS. BrdU incorporation was visualized by immunocytochemistry using a mouse anti-BrdU primary antibody (Thermo fisher) followed by anti-mouse FITC (Bangalore Genei). Cell nuclei were stained with DAPI. Fluorescent staining was observed and photographed using the Floid cell imaging system (Thermo fisher scientific). BrdU positive cells were calculated as percentage to total cells (labeled by DAPI).

4.2.5 Scratch assay

Cell migration was examined using scratch assay (Xiong et al., 2013b). A scratch was introduced by scraping confluent cell monolayer with a sterile 200 µl pipette tip and then washed gently twice with cell culture medium to remove cell debris. Migrations of cells were monitored after 24 hrs under inverted phase contrast microscope and percent migration rate (MR %) was calculated as: MR % = (A-B/A) X 100 %, where A represents the mean width of the cell scratch at 0 hr and B represents the mean width of the cell scratch after 24 hrs.

4.2.6 Soft agar assay

Anchorage-independent growth as a characteristic of *in vitro* tumorigenicity was assessed by soft agar clonogenic assay (Kakuguchi et al., 2010). Cells were detached and re-suspended (1.25 x 10^3 cells per well in 24 well plate) 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). After 10 days, colonies >0.1 mm in diameter were scored as positive and counted under inverted phase-contrast microscope.

4.2.7 Sphere formation assay

The sphere formation assay was performed (Johnson et al., 2013) by re-suspending C6 cells in serum-free medium NSC (Neural stem cell) media containing DMEM/F12 (Life Technologies), 20ng/ml recombinant EGF (Life Technologies), 20ng/ml bFGF (Life Technologies), 2% B27 (Life Technologies), and 1% penicillin–streptomycin. Cell were plated at a density of 1 cell/µl in 96-well plates and allowed to form spheres for 7-10 days. The number of spheres formed was counted under phase contrast microscope.

4.2.8 Adhesion assay

The efficiency of cell adhesion was determined by measuring the number of cells that adhered to Extracellular matrix (ECM) substrate fibronectin (FN) (De Aguiar et al., 2002). 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. Cells were seeded onto ECM-coated 96-well plates (1 x 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).

4.2.9 Invasion assay

Invasion assays were carried out using growth factor reduced matrigel invasion chambers (Corning) according to the manufacturer's protocol. In brief, transfected C6 cells were harvested, in serum-free medium and transferred to the hydrated matrigel chambers (~100000 cells per well). The chambers were then incubated for 24h 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. Finally, invaded cells were counted and the relative number was calculated.

4.2.10 Western blotting

Cells were harvested in Laemmli buffer and protein content was determined by Qubit protein assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen). Aliquots containing equal amount of protein (40 ug) were resolved by SDS polyacrylamide gel electrophoresis (10 %) and transferred to nitrocellulose membrane. The membranes were blocked using 3% BSA in Tris-buffered saline and Tween 20 mixture (0.2%), before incubation in primary antibodies overnight at 4 °C. The following primary antibodies were used to detect the target proteins: anti-MeCP2 (Milipore), anti-beta actin (Thermo, Pierce), anti-GFAP antibody (Abcam), anti-BDNF (Abcam), anti-FAK (Santacruz), anti-pERK1/2(R&D systems) and anti-ERK1/2 (Sigma). After being washed, membranes were incubated in the corresponding horseradish peroxidase conjugated secondary antibody. Bands were visualized by chemiluminescence with ECL Western Blotting Detection Kit (Invitrogen) on X-ray film. Films were digitally scanned and quantitatively analyzed using Image J software.

4.2.11 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. GAPDH was used as a control to normalize the variability in the expression levels and data was analysed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The PCR products were analyzed by 1.5% agarose gel electrophoresis. The primers used in study are shown in Table 4.1.

4.2.12 Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed using EZ-Magna ChIP™ HiSens kit (Millipore) following the manufacturer instructions. Briefly, C6 cells were cross-linked with 1% formaldehyde at 37 °C for 10 min and then rinsed with ice-cold PBS twice. Cells were lysed in nuclei isolation buffer and sonicated (20 sec on and 20 sec off for 10 min in sonics Vibra cell) to produce chromatin with average length of 200–1000 base pairs. Chromatin was pre-cleared by adding1 µg of rabbit IgG for 2-4 hrs at 4^0C. Pre-cleared chromatin was subjected to immunoprecipitation using anti-MeCP2 antibody (Millipore).The DNA was analyzed by PCR using promoter primers given in Table 4.2. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

4.2.13 Statistical analysis

Results are expressed as mean ± standard error mean (SEM) and statistically analyzed using two-tailed Student's t tests. A value of $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

4.3. RESULTS

4.3.1 MeCP2 expression in C6 glioma

Initially, MeCP2 expression in C6 glioma was determined by RT-PCR and immunocytochemistry. C6 glioma was found to express the MeCP2 at both transcript and protein level (Figure 4.1).

4.3.2 MeCP2 over expression in C6 glioma

Rat C6 glioma cell line was chosen as glioma model to investigate the role of MeCP2 in glioma progression and gene expression. To examine the role of MeCP2 in glioma development, plasmid expressing MeCP2-YFP fusion (pMeCP2Y) protein was transfected in C6 glioma. Since MeCP2 is a nuclear protein, MeCP2-YFP fusion protein exhibited nuclear localization in C6 glioma (Figure 4.2 A). Further, MeCP2 over-expression was confirmed by qPCR and RT-PCR analysis (Figure 4.2 B and D). Western blot analysis shows clear band of 102 kDa correspond to MeCP2-YFP fusion protein in C6 cells transfected with pMECP2Y and absent in cells transfected with pEYFP (Figure 4.2 C). C6 cells stably expressing the pMeCP2 and pEYFP were selected using G418. MeCP2 over expression induces the morphological transformation of C6 glioma from normal spindle shape cells to larger nuclei with extensive sheet like processes (Figure 4.3).

4.3.3 MeCP2 impairs C6 glioma proliferation, anchorage independent growth, adhesion and sphere forming ability

To further analyze the impact of MeCP2 on glioma growth, we evaluated the proliferation of C6 glioma cells stably expressing the pMeCP2 and pEYFP by immunofluorescence microscope for BrdU-positive cells. MeCP2 over expression significantly decrease the number of BrdU positive cells (11.63 ± 2.51%) compared to cells expressing mock control (49.23±2.58%) (Figure 4.4 A and B). In order to investigate that whether MeCP2 overexpression induces the C6 apoptosis, expression of cleaved procaspase-3 was analyzed by western blotting. No significant effect on procaspase-3 cleavage was observed between cells transfected with pMECP2Y and pEYFP, indicating that the MeCP2 over expression is not related to the activation of programmed cell death (Figure 4.4 C). MeCP2 over expression affect the anchorage independent growth of C6 glioma since MeCP2 over expression in C6 cells significantly reduced the number and size of colonies on soft agar compared to cells expressing pEYFP (Figure 4.5A and B). Next, to

examine the effect of MeCP2 on C6 sphere formation ability, cells were allowed to form sphere in serum free NSC medium. Ectopic expression of MeCP2 completely inhibits the sphere formation of C6 glioma stem cells (Figure 4.5 C and D). Further, MeCP2 was found to affect the adhesion of C6 glioma to extracellular matrix fibronectin. C6 cells expressing pMeCP2Y shows significantly reduction in adhesion to fibronectin compared to control (Figure 4.6)

4.3.4 MeCP2 inhibits the migration and invasion ability of C6 glioma

Cell migration and invasion are essential for cancer metastasis. To investigate the effect of MeCP2 on cell migration, scratch assay was performed in cells expressing the pMeCP2Y or pEYFP. Migration of cells in scratch was measured after 24 hrs. Ectopic expression of MeCP2 significantly suppresses the C6 migration, compared to pEYFP group, migration was found to be 32% reduced in MeCP2 over-expressing cells (Figure 4.7 A and B). Further, invasion ability of C6 cells expressing either vector control (pEYFP) or pMeCP2Y were examined by transwell assay. It was observed that MeCP2 over expression significantly impair the invasion ability of C6 glioma (Figure 4.7 C and D), indicating that crucial role of MeCP2 in invasion of glioma.

4.3.5 MeCP2 inhibit the extracellular signal regulated kinase (ERK) signaling in C6 glioma

In order to identify the mechanism of MeCP2 mediated suppression of glioma growth and migration, component of ERK signaling were determined by western blot. The results indicated that MeCP2 over-expression significantly (***$p < 0.001$) reduced the phospho-ERK (pERK) levels in C6 cells. There was ~70% reduction in pERK level in C6 cells harboring the pMECPY compared to cells expressing pEYFP alone (Figure 4.8 A and B).

4.3.6 MeCP2 regulate the expression of GFAP, FAK and BDNF in C6 glioma

Next, we assessed the role of MeCP2 on GFAP, FAK and BDNF protein expression, which were found to be associated with C6 glioma differentiation and glioma development. We observed that MeCP2 over-expression promotes C6 glioma differentiation by inducing GFAP (Glial fibrillary acidic protein) protein level (4.69 fold, *$p < 0.05$), compared to control (Figure 4.9 A and B). In contrast, BDNF (Brain-derived neurotrophic factor) (0.3 fold, ****$P<0.0001$) (Figure 4.9 C and D) and FAK (Focal adhesion kinase) protein level (0.52 fold, **$P<0.01$) (Figure 4.9 E and F) were found to be reduced in MeCP2 over-expressing C6 cells.

4.3.7 MeCP2 directly binds to GFAP and BDNF promoter

To further confirm that whether MeCP2 is recruited to endogenous GFAP and BDNF promoters, chromatin immunoprecipitation (ChIP) assay was performed. We analyzed MeCP2 binding to GFAP promoter regions - GFAP Exon-1 (+478 TO -14 bp) and GFAP STAT 3 (-1320 to -1621 bp) and BDNF Promoter IV (-56 to-201bp) in C6 expressing pMECP2Y and pEYFP. MeCP2 occupancy to GFAP and BDNF promoter is enhanced in MeCP2 over expression condition, compared to control (Figure 4.10).

4.3.8 MeCP2 knock down in C6 glioma

To complement the over expression studies, knock down experiment was done using MeCP2 specific siRNA in C6 glioma. Inhibition of MeCP2 in C6 was shown by western blotting (43%, ***P<0.001) and RT-PCR (Figure 4.10). Real-time quantitative RT-PCR indicated that siRNA reduce the MeCP2 mRNA level (32%, ****P<0.0001) (Figure 4.11). Effect of inhibition of MeCP2 expression on C6 proliferation and migration was examined. In contrast to MeCP2 over expression, MeCP2 suppression was found to significantly induce the proliferation of C6 glioma (Figure 4.12), while there was no significant increase in migration was observed, compared to negative control (Figure 4.13). To investigate the effect of MeCP2 on C6 cell adhesion to extracellular matrix, adhesion assay was performed. The data indicate that MeCP2 suppression did not alter the C6 adhesion to extracellular matrix fibronectin (Figure 4.14). On the contrary to MeCP2 over expression, the levels of pERK were found to increase in MeCP2 knockdown C6 cells (Figure 4.15 A and B).Similarly, the BDNF and GFAP protein level were also found oppositely regulated in comparison to MeCP2 over expressing C6 cells (Figure 4.15 C, D, E, and F).

4.3.9 MeCP2 regulates myelin gene expression differentially in C6 glioma and oligodendrocytes

In earlier Chapter-2, MeCP2 has been shown to negatively regulate the myelin genes and BDNF expression in oligodendrocytes. Here in this study, since C6 glioma was found to express the oligodendrocytes and astrocytes marker, role of MeCP2 on expression of these genes were studied. First, C6 cells were examined for expression of myelin genes PLP and MAG by RT – PCR which confirm the presence of PLP and MAG transcript (Figure 4.16A). Unlike oligodendrocytes, MeCP2 was found to differentially regulate the myelin gene expression. MeCP2 over expression in C6 glioma induce the transcript level of MBP, PLP and MAG (Figure

4.16 B) while reduced levels were observed in MeCP2 knock down C6 glioma (Figure 4.16 A). Further, PLP expression was also confirmed at protein level by western blotting and found to be correlated with transcript level in MeCP2 over expression and knock down conditions (Figure 4.16 C and D: Figure 4.17 B and C). Oligodendrocytes were used as a positive control for western blotting of PLP in C6 glioma. MeCP2 positively regulates the expression of myelin genes in C6 glioma. Further, ChIP assay revealed that in contrast to oligodendrocytes, MeCP2 does not bind to promoter regions of MBP and PLP (Figure 4.18).

4.4 DISCUSSION

Epigenetic mechanisms such as DNA methylation and histone modifications play crucial role in cancer development (Esteller, 2008). Aberrant DNA methylation is an attribute of cancer which often leads to silencing of tumor suppressor genes (Lopez-Serra and Esteller, 2008). In addition to the role of de-methylating agent (5-aza-2′-deoxycytidine) (5-aza-CdR) as anti-cancer by activating the tumor suppressor genes, DNA methylation inhibitor could also de-methylate and activate the genes involve in invasion and metastasis and thus can promote the tumor invasion (Ateeq et al., 2008).

MeCP2 is an epigenetic protein which preferentially binds to methylated CpGs and is involved in transcriptional repression. In addition to MeCP2 function in neuro-developmental disorder, Rett syndrome, its role in several cancer types like myeloma, hematological malignancies, breast, colorectal, lung, liver and prostate cancer has been studied (Parry and Clark, 2011). However, understanding of MeCP2 contribution specifically in glioma is still unclear. In this study, the role of MeCP2 over-expression on C6 glioma progression has been investigated. Present study demonstrated that MeCP2 reduces proliferation, migration, invasion, colony formation, adhesion and sphere forming ability of C6 glioma. The result is in the lines of earlier studies that found MeCP2 regulates malignant behavior of pancreatic cancer cells by suppressing the expression of LIN28A. LIN28A over-expression was found to promote colony formation, stemness and invasion in pancreatic cancer cells (Xu et al., 2016). Moreover, LIN28A also promotes invasion, proliferation and clonogenecity in glioblastoma cell lines (Mao et al., 2013; Qin et al., 2014). Another study reported that in human breast cancer, MeCP2 expression was very low compared to adjacent normal breast tissue. In breast cancer, MeCP2 in association with Z-DNA and NF1 family of transcription factors was found to suppress the expression of

ADAM-12, a multifunctional protein which promotes cancer metastasis (Ray et al., 2013). Valproic acid was found to induce the HDAC and MeCP2 mRNA expression in C6 glioma (Kim et al., 2008).

Surprisingly, MeCP2 suppress the pERK levels which support the reduced proliferation and migration observed in MeCP2 over-expressed C6 cells. The results of the present study are consistent with the observations made in previous studies which demonstrated that ERK signaling has been implicated in glioma proliferation and invasion. ERK signaling inhibition in gliomas has shown potential anti tumor effects (Dudley et al., 1995; Favata et al., 1998). C6 glioma cells treated with U0126, a MEK1/2 inhibitor show dramatic reduction in proliferation and migration (Lind et al., 2006). These findings suggest that MeCP2 may inhibit the tumorigenic properties of C6 by regulating the ERK signaling. Present study demonstrated that MeCP2 promotes the C6 differentiation by inducing GFAP expression. MeCP2 induced GFAP expression in C6 glioma could be due to the reduced level of pERK. The result is in consistent with previous report by Lind et al., (2006) which showed that ERK inhibition promotes the C6 glioma differentiation by inducing the GFAP expression (Lind et al., 2006). GFAP levels were found to be inversely correlated with the invasiveness of human gliomas (Kajiwara et al., 1992; Murphy et al., 1998). Next, MeCP2 also reduce the mature BDNF level in C6 glioma. Recent studies have shown that proBDNF and mature BDNF exert opposite effects on C6 glioma growth and migration. In contrast to proBDNF, mature BNDF was found to promote the C6 glioma growth and migration in vitro (Xiong et al., 2013a; Xiong et al., 2013b). The results from ChIP assay indicated that MeCP2 regulates the GFAP and BDNF expression in C6 cells by directly binding to their promoter region. MeCP2 recruitment to these promoters is enhanced in cells expressing pMECP2Y compared to control. Previous reports have shown that MeCP2 bind to BDNF and GFAP gene promoters and suppress their expression in neurons (Chen et al., 2003; Martinowich et al., 2003; Namihira et al., 2004; Zhou et al., 2006; Setoguchi et al., 2006; Cheng et al., 2011; Andoh-Noda et al., 2015). In contrast to repression, MeCP2 was also found to activate the GFAP expression in the hypothalamus of a male transgenic mouse over-expressing MeCP2 (Chahrour et al., 2008).

FAK (Focal adhesion kinase) protein level is found to be elevated in many cancers (Gabarra-Niecko et al., 2003). The data showed that MeCP2 over-expression reduces the FAK level in C6 glioma which may lead to inhibition of the migration. FAK has been shown to promote

glioblastoma cell proliferation, survival and migration *in vitro*. Also elevated FAK protein leads to increase in ERK activity in astrocytoma and glioblastoma (Natarajan et al., 2003). Inhibition of Src-FAK pathway has been shown to decrease glioma migration and invasion (Oliveira-Ferrer et al., 2008).

In earlier chapters it was observed that MeCP2 negatively regulate the myelin gene expression by directly binding to their promoter regions in oligodendrocytes (Sharma et al., 2015). In contrast to oligodendrocytes, MeCP2 positively regulates myelin genes (MBP, PLP and MAG) expression in C6 glioma but does not bind to their promoter region. This suggests that MeCP2 may indirectly regulate the myelin gene expression in C6 glioma. MeCP2 mediated differential regulation of myelin genes in oligodendrocytes and C6 glioma could be due to different cell system or binding to promoter regions. For instance, Id1,Id2, Id3 and Id4 genes are preferentially expressed in astrocytes but they do not appear to be targets of MeCP2 but are targets in neuronal cells (Kitamura et al., 2001: Jordan et al., 2007).

4.5. CONCLUSION

Present study focused on two aspects, first role of MeCP2 in regulation of C6 glioma malignant phenotypes and related gene expression. MeCP2 was found to suppress tumorogenic characteristics of C6 glioma which includes proliferation, migration, invasion, adhesion, anchorage independent growth and sphere forming ability. Further, MeCP2 reduce the tumor behavior of C6 glioma by regulating the expression of GFAP, BDNF and FAK expression. MeCP2 regulates the expression of GFAP and BDNF by directly binding to their promoter regions. Second part focused on MeCP2 function in regulation of myelin genes expression in C6 glioma. In contrast to oligodendrocytes, MeCP2 positively regulate the myelin genes (MBP, PLP and MAG) expression in C6 glioma. Unlike oligodendrocytes, MeCP2 does not bind to promoter regions of MBP and PLP genes which suggest that MeCP2 differentially regulates its target genes in different cell types.

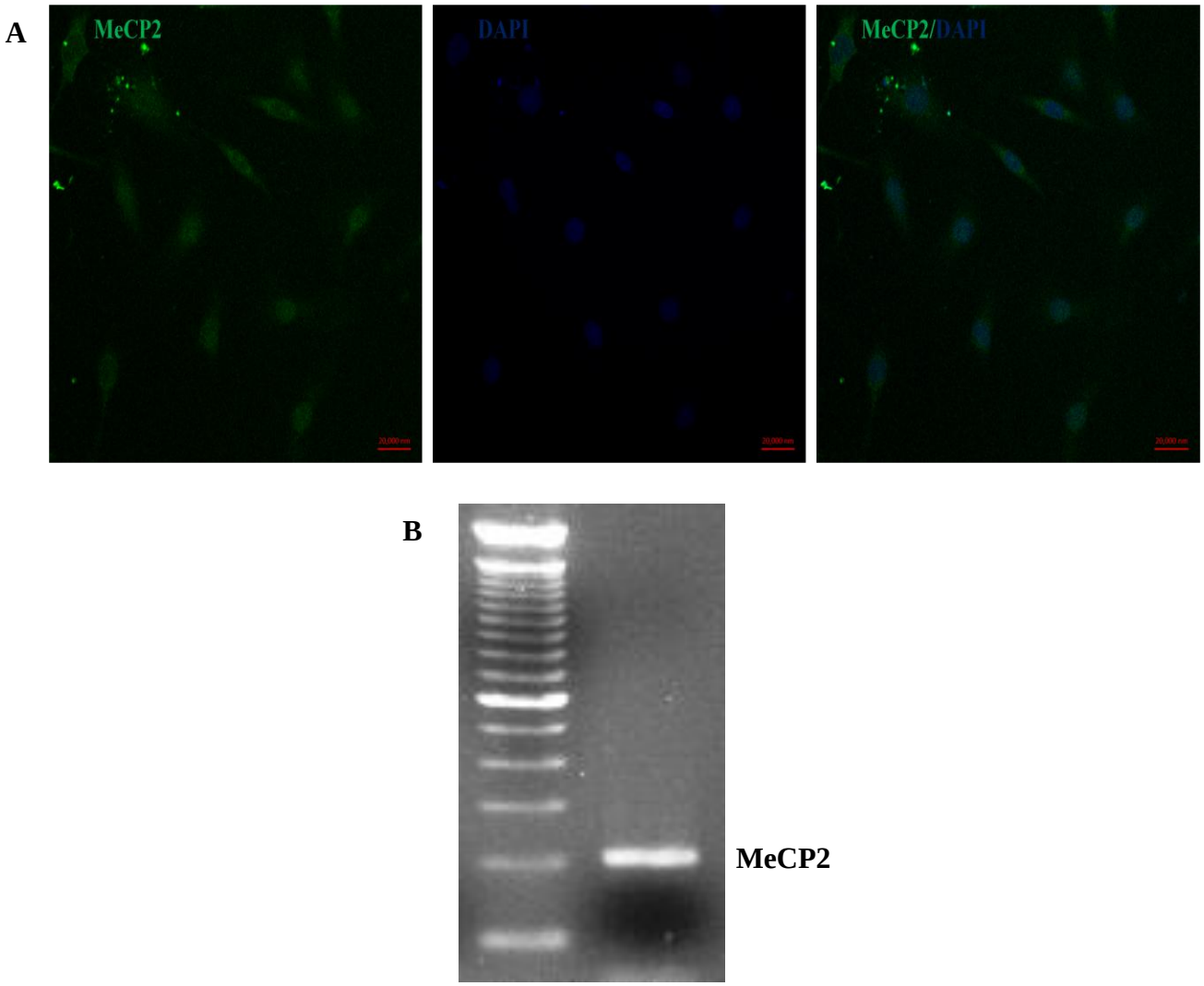


Figure 4.1: MeCP2 expression in C6 glioma. (A) MeCP2 (green) expression was detected by immunocytochemistry in C6 glioma. Nucleus was stained blue with 4',6-diamidino-2-phenylindole (DAPI). **(B)** MeCP2 expression in C6 glioma by RT-PCR, Lane -1 100 bp DNA ladder and Lane 2 MeCP2 amplification using specific primer.

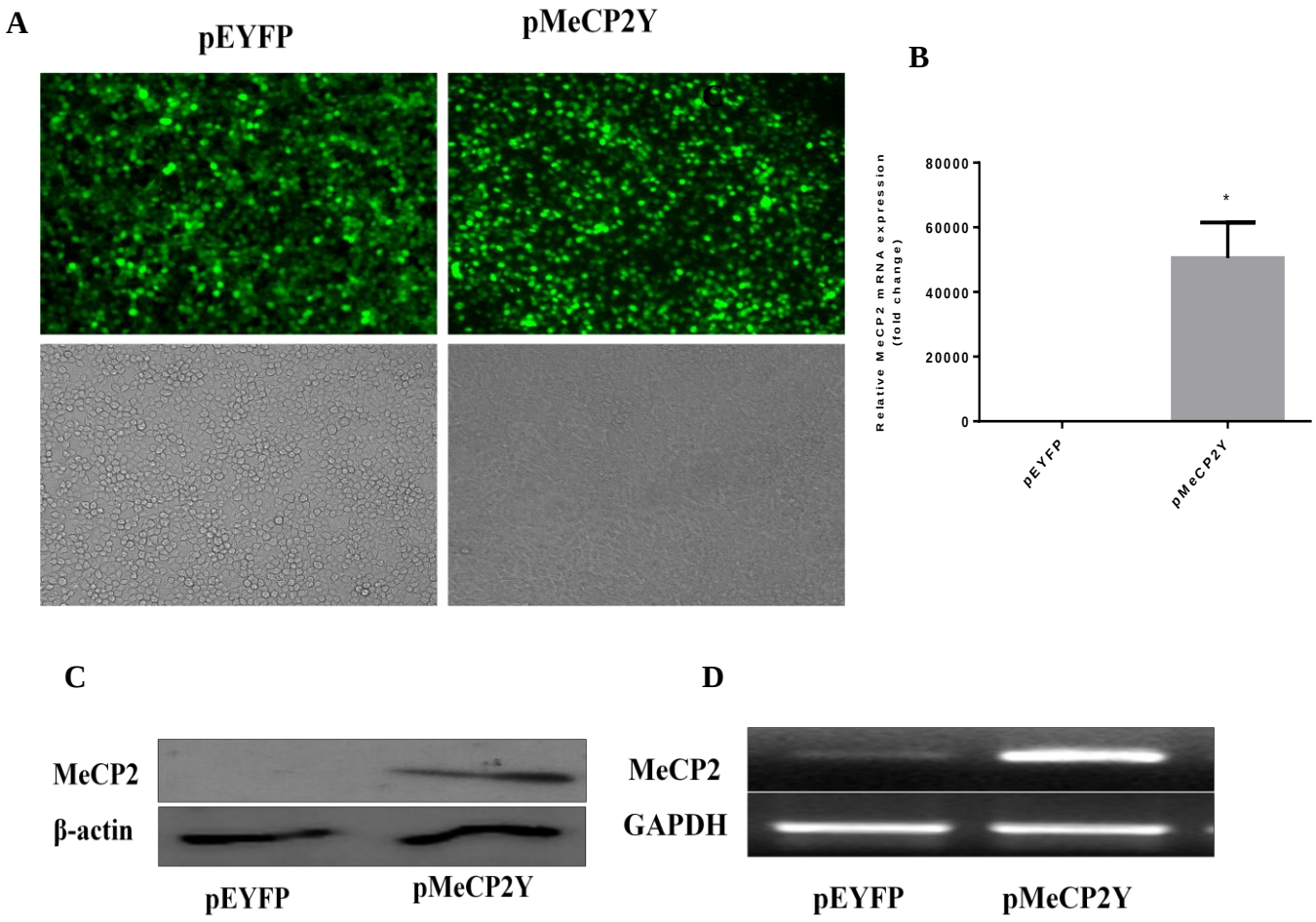


Figure 4.2: **MeCP2 over expression in C6 glioma**. C6 cells were transfected with pMECP2Y or control plasmid pEYFP**. (A)** MeCP2 over expression was confirmed by nuclear localization of MeCP2-YFP fusion protein in pMECP2Y transfected C6 cells, compared to pEYFP under fluorescence microscopy (Upper Panel). Phase contrast image of pEYFP or pMeCP2Y transfected C6 cells (Lower Panel). Confirmation of MeCP2over expression at mRNA level by qPCR **(B)** and RT-PCR **(D)** and protein level **(C)** by western blotting.

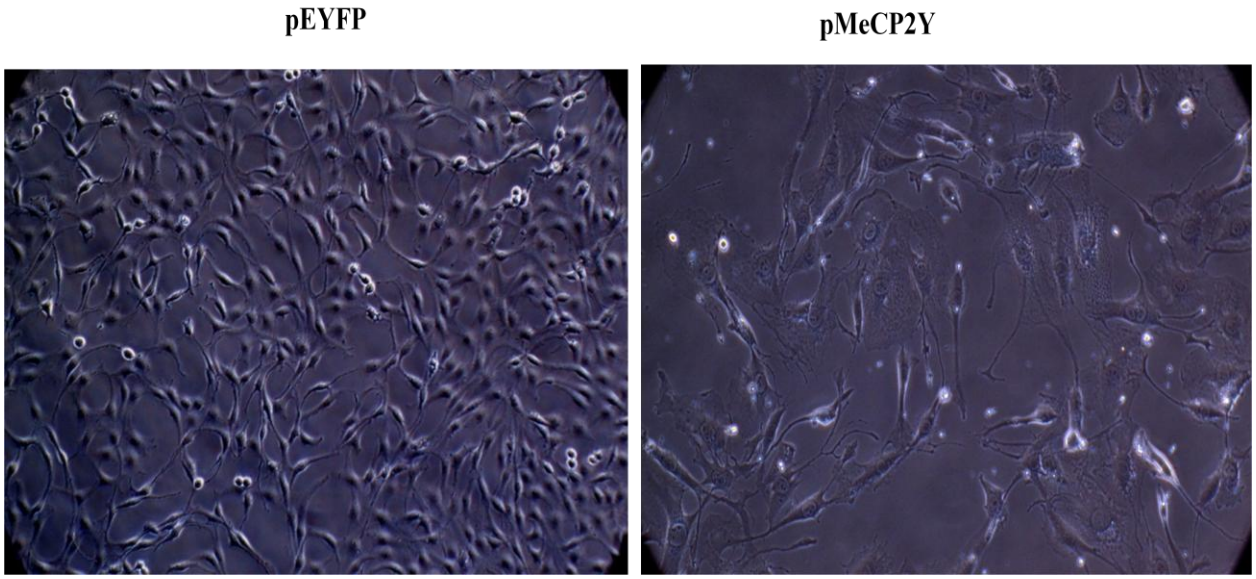


Figure 4.3: Stable MeCP2 over expression in C6 glioma induce the morphological transformation. C6 glioma cells expressing pYFP vector shows uniform spindle shape **(**Left panel), while C6 glioma expressing pMeCP2-YFP form large sheet like process and large nucleus (Right panel).

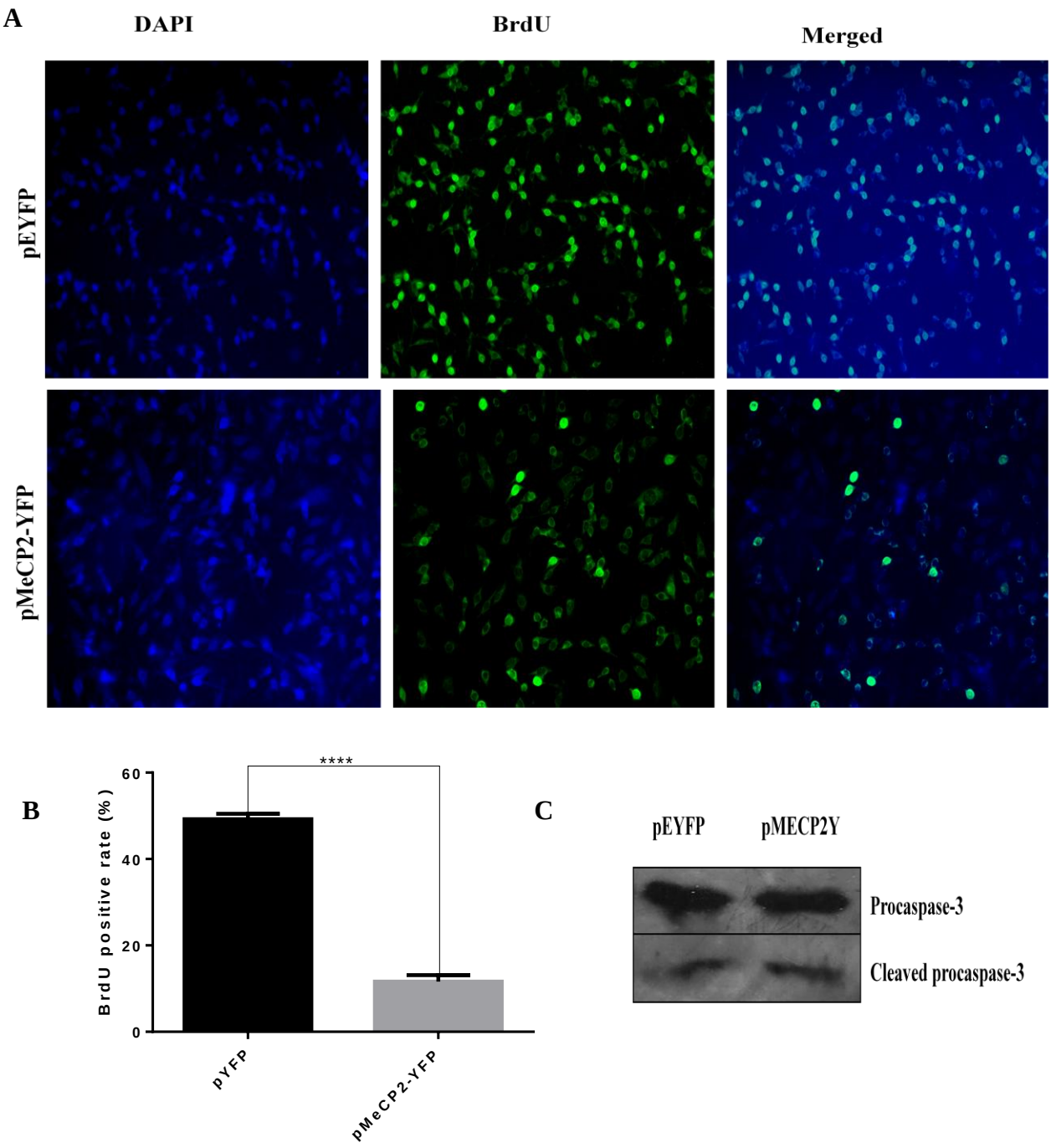


Figure 4.4: MeCP2 over expression inhibit C6 glioma growth proliferation. **(A)** Immunofluorescence staining of BrdU in C6 cells expressing pEYFP or pMeCP2Y. **(B)** The percentage of BrdU positive cells was calculated. Data are derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001; ****P<0.0001 (unpaired Student's t-test). **(C)** Caspase-3 protein was determined by western blotting in C6 expressing pEYFP or pMeCP2Y.

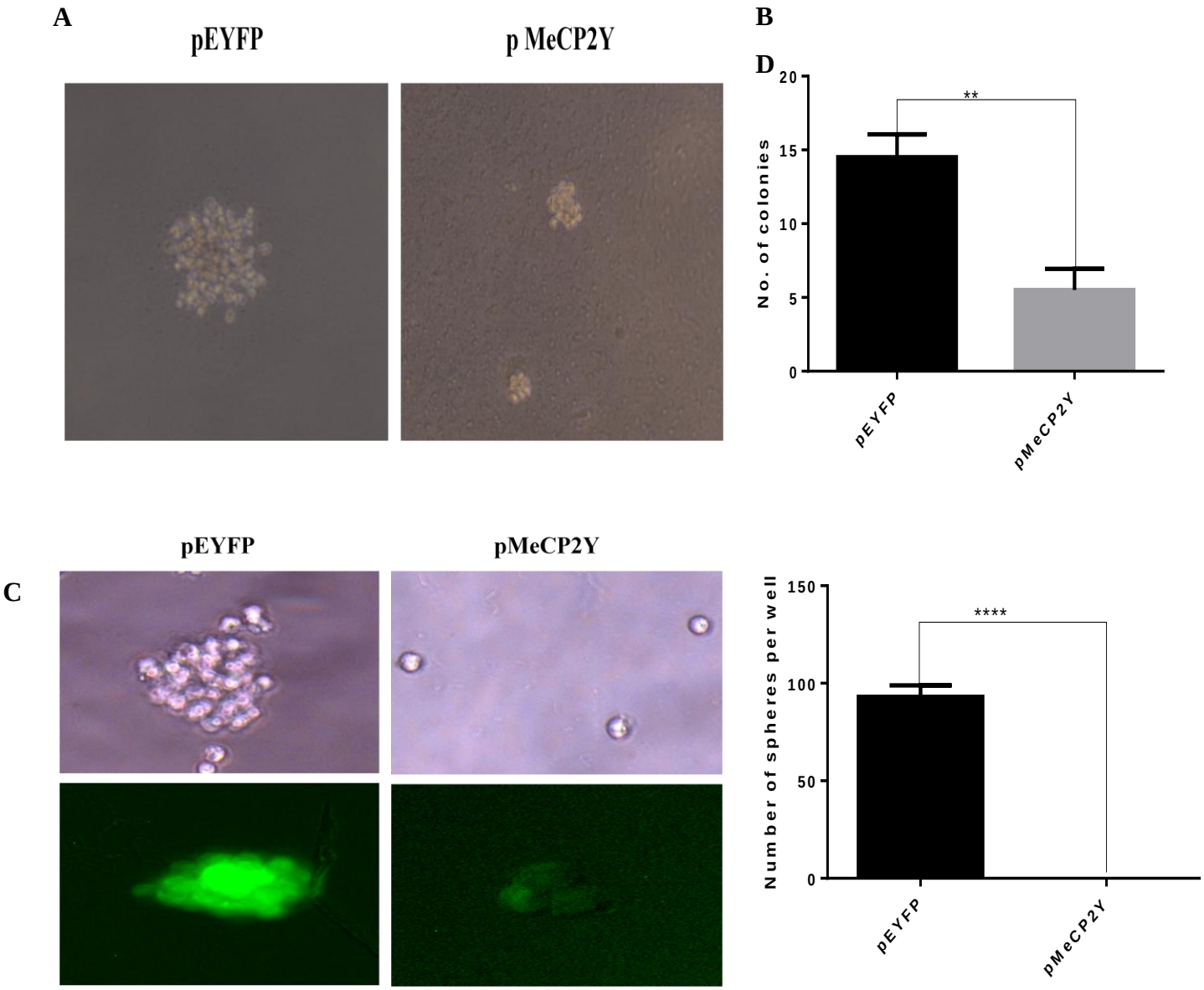


Figure 4.5: MeCP2 over expression in C6 glioma suppress anchorage independent growth and sphere formation. (A) MeCP2 over expression suppresses the anchorage independent growth of C6 glioma. pMeCP2Y expressing cells shows small size colonies on soft agar compared to pEYFP. **(B)** Quantitative analysis of number of colonies formed. **(C)** MeCP2 over expression reduces sphere formation ability of C6 glioma expressing pMeCP2Y compared to pEYFP only. Representative phase contrast and fluorescence images of spheres are shown. **(D)** Quantitative analysis of number of spheres formed. Data are derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001; ****P<0.0001 (unpaired Student's t-test).

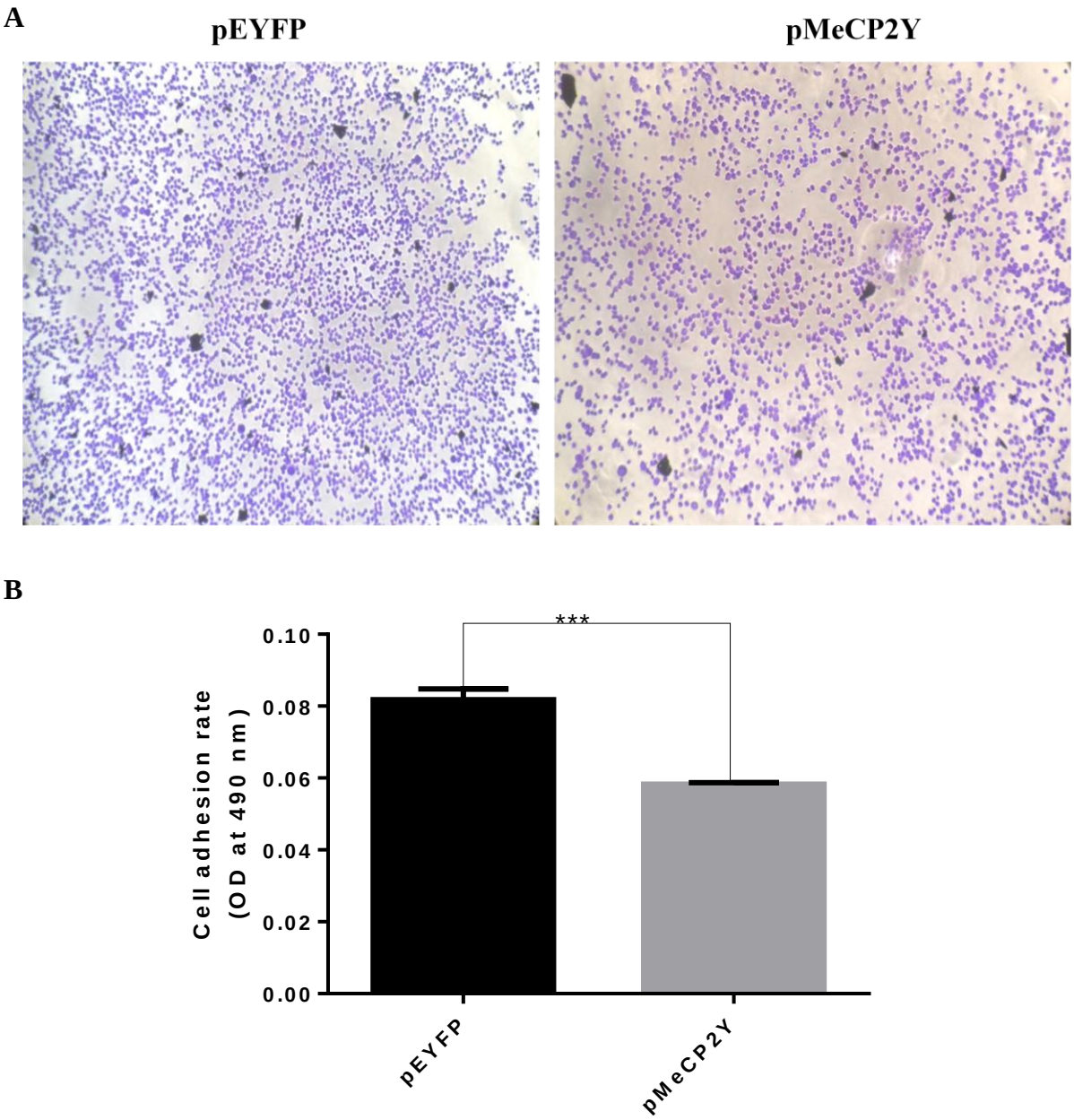


Figure 4.6: MeCP2 overexpression reduces the C6 glioma adhesion to extracellular matrix fibronectin. (A) MeCP2 overexpression impairs the adhesion property of C6 glioma as compared to the control pEYFP. Adhered cells were stained with crystal violet. **(B)** Quantitative analysis of adhesion by measuring the optical density (OD) of extracted crystal violet from adhered cells at 490nm.Data derived from three experiments and presented as means ± SEM *p < 0.05;**p < 0.01; ***p < 0.001 (unpaired Student's t-test).

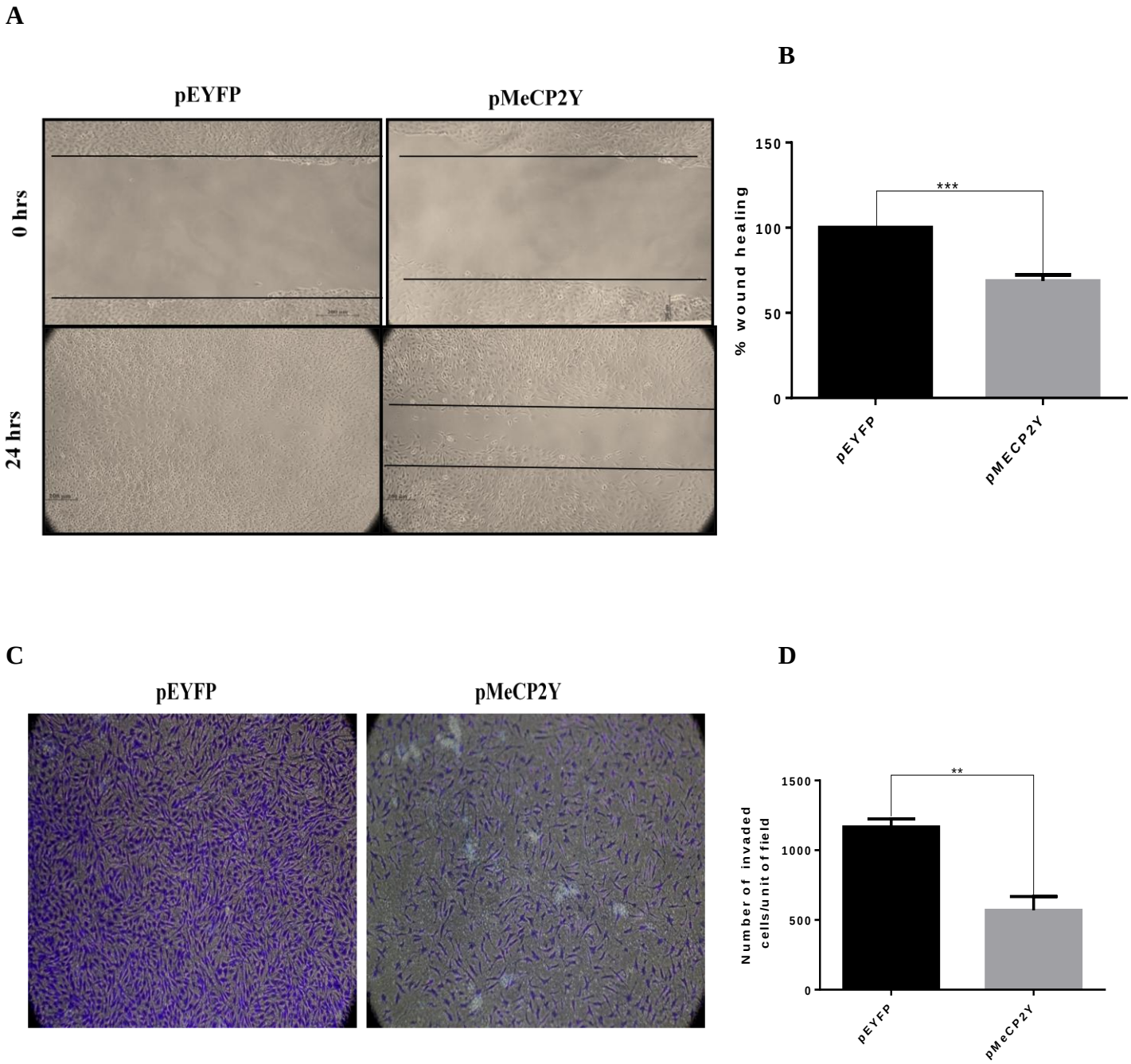
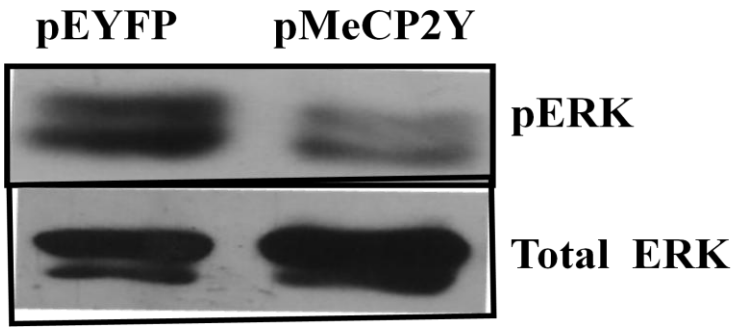


Figure 4.7: MeCP2 over expression reduce C6 glioma migration and invasion. Migration and invasion of C6 glioma transfected with pMeCP2 or pEYFP were studied by scratch **(A)** and transwell assay **(C)** respectively. Quantitative analysis of percent wound healing **(B).** Invaded cells were counted and analyzed **(D).** Data are derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001 (unpaired Student's t-test).

A



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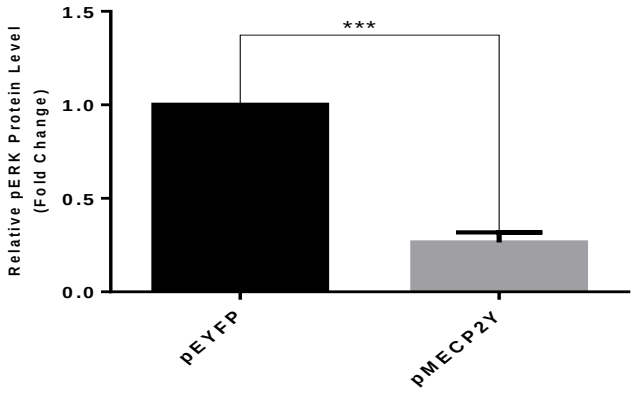

Figure 4.8: MeCP2 over expression suppress the pERK levels in C6 glioma (A) Western blot analysis of pERK level in C6 cells expressing pEYFP vector and pMeCP2Y. **(B)** Quantitative analysis of pERK protein level. Data are derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001 (unpaired Student's t-test).

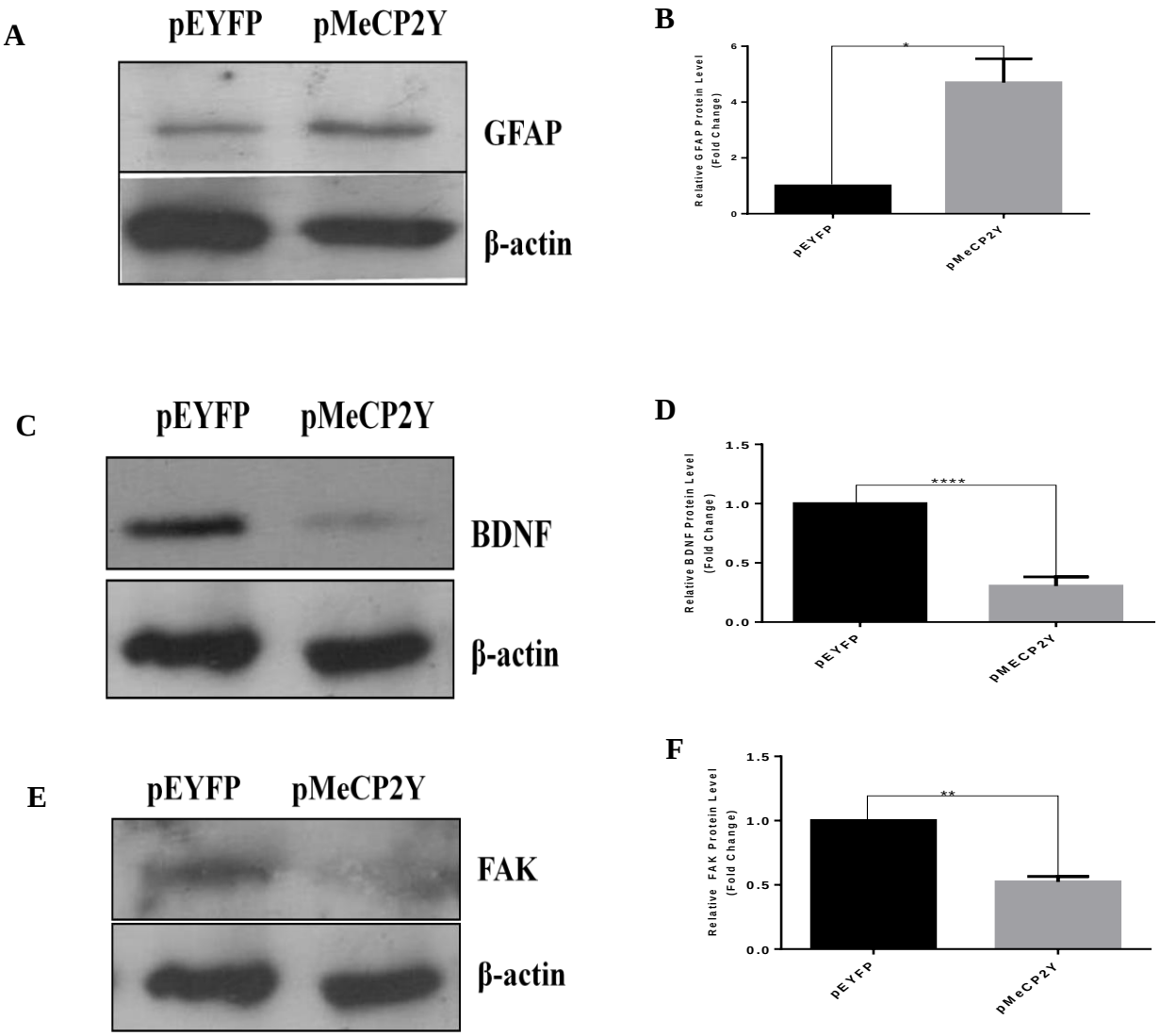

Figure 4.9: MeCP2 over expression in C6 glioma alter GFAP, BDNF and FAK expression. Western blot analysis of GFAF, BDNF and FAK **(A, C and E)** in C6 glioma expressing pEYFP or pMeCP2Y.Quantitative analysis of GFAP, BDNF and FAK **(B, D and F).**Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001, ****P<0.0001 compared with cells expressing pEYFP (unpaired Student's t-test**).**

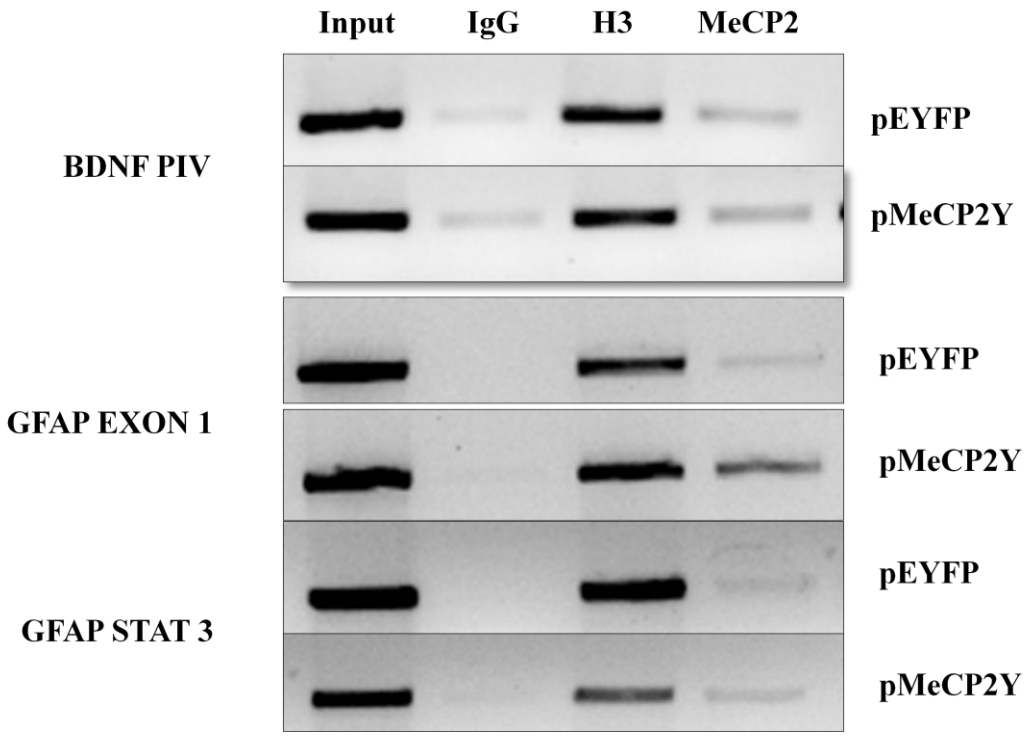

Figure 4.10: MeCP2 directly bind to GFAP and BDNF gene Promoters. Chromatin immunoprecipitation (ChIP) assay was performed in C6 glioma transfected with pEYFP or pMECP2Y with MeCP2 antibody. MeCP2 binding to GFAP (Exon 1 and STAT3) and BDNF promoter IV sequences were analyzed by PCR using specific primers. Normal Rabbit IgG and Anti-Trimethyl-Histone H3 (Lys4) antibodies were used as negative and positive control respectively. The input sample was also used as positive control.

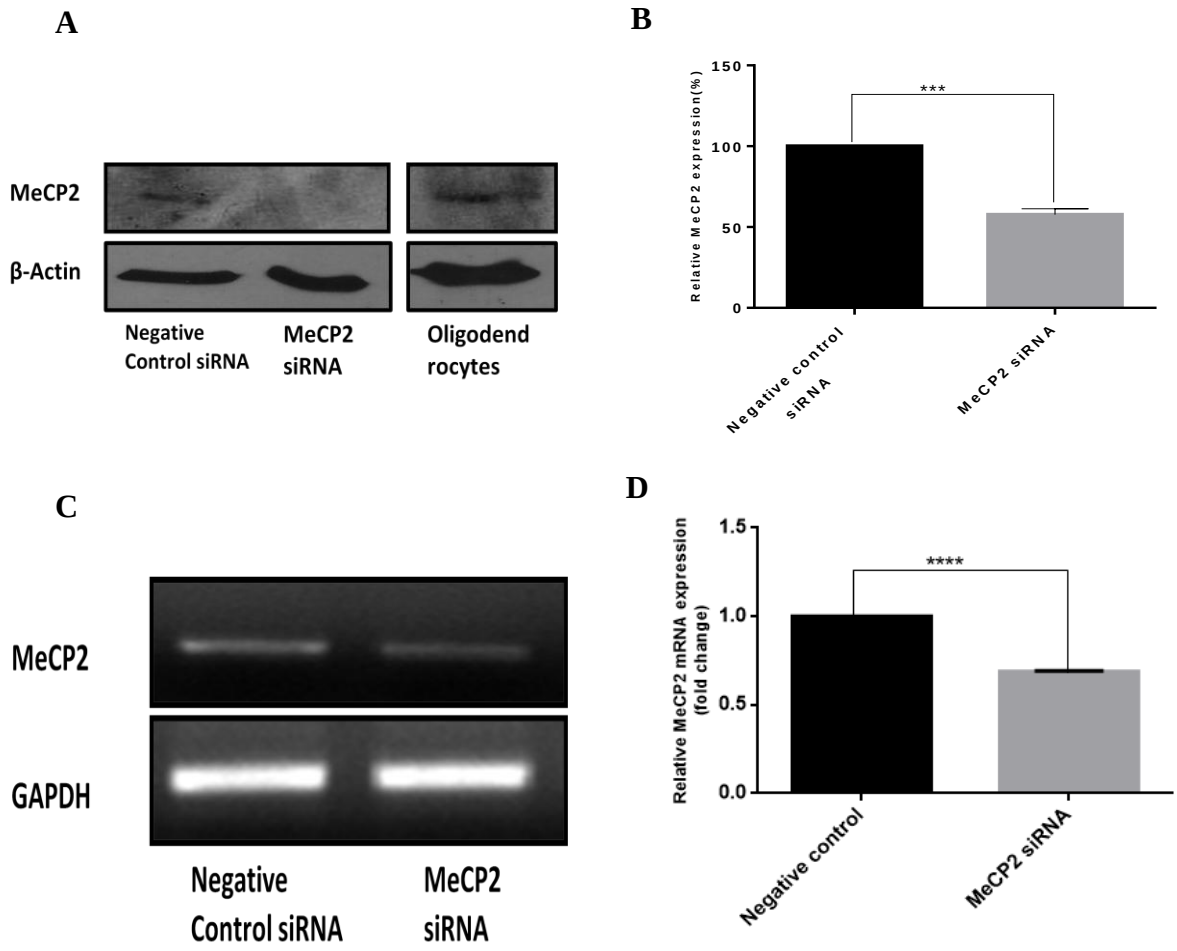

Figure 4.11: **MeCP2 Knock down in C6 glioma**.C6 glioma was transfected with MeCP2 siRNA and Negative control siRNA. MeCP2 levels were detected at protein (**A** and **B**) and mRNA level (**C** and **D).** oligodendrocytes were used as positive control for MeCP2 expression. Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01;***P<0.001, ****P<0.0001 compared with cells expressing pMeCP2-YFP (unpaired Student's t-test**).**

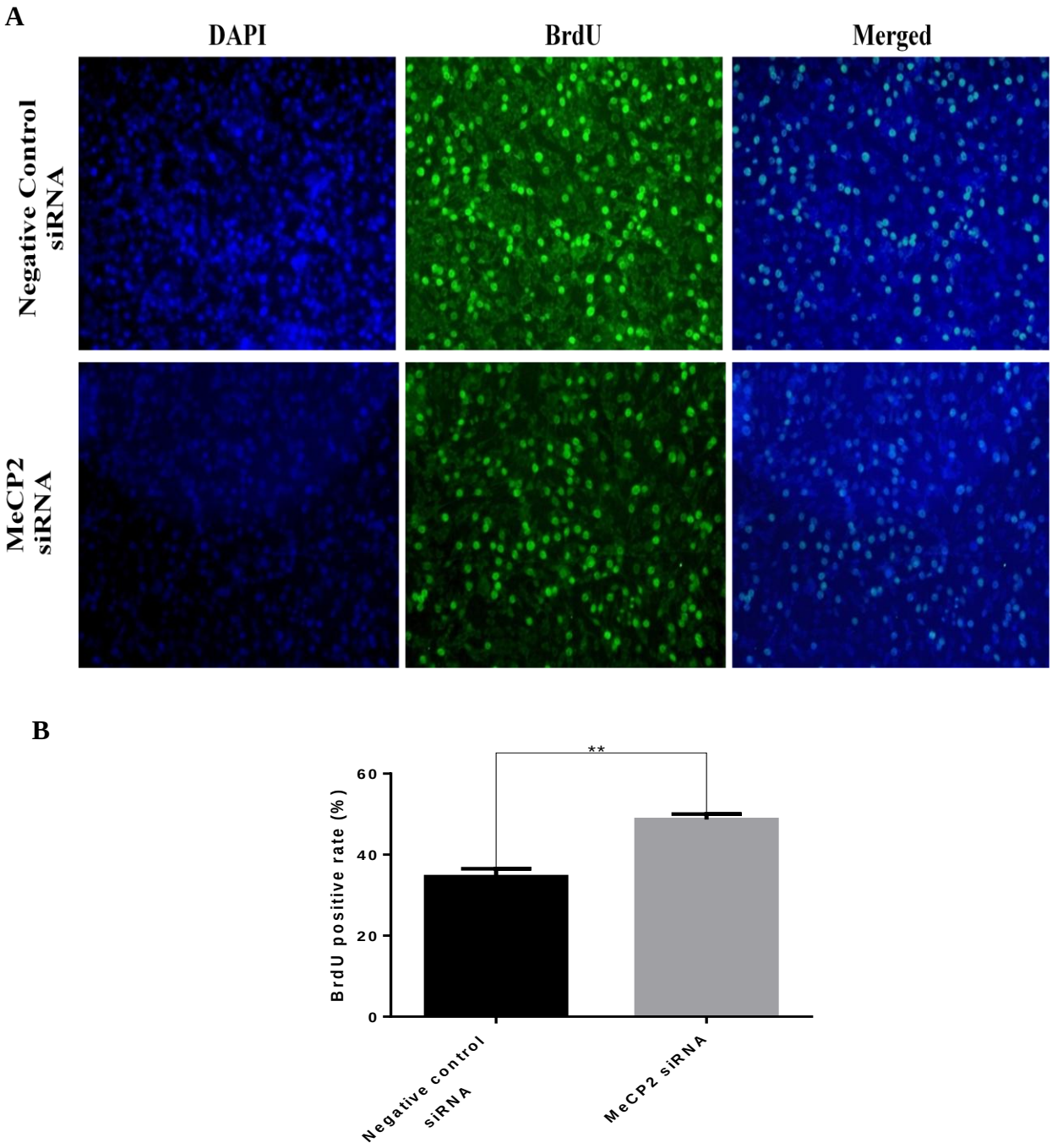

Figure 4.12: MeCP2 knockdown increase the C6 glioma proliferation. **(A)** Immunofluorescence staining of BrdU in C6 transfected with MeCP2 siRNA or Negative control siRNA. **(B)** The percentage of BrdU positive cells was calculated. Data are derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001; ****P<0.0001 (unpaired Student's t-test).

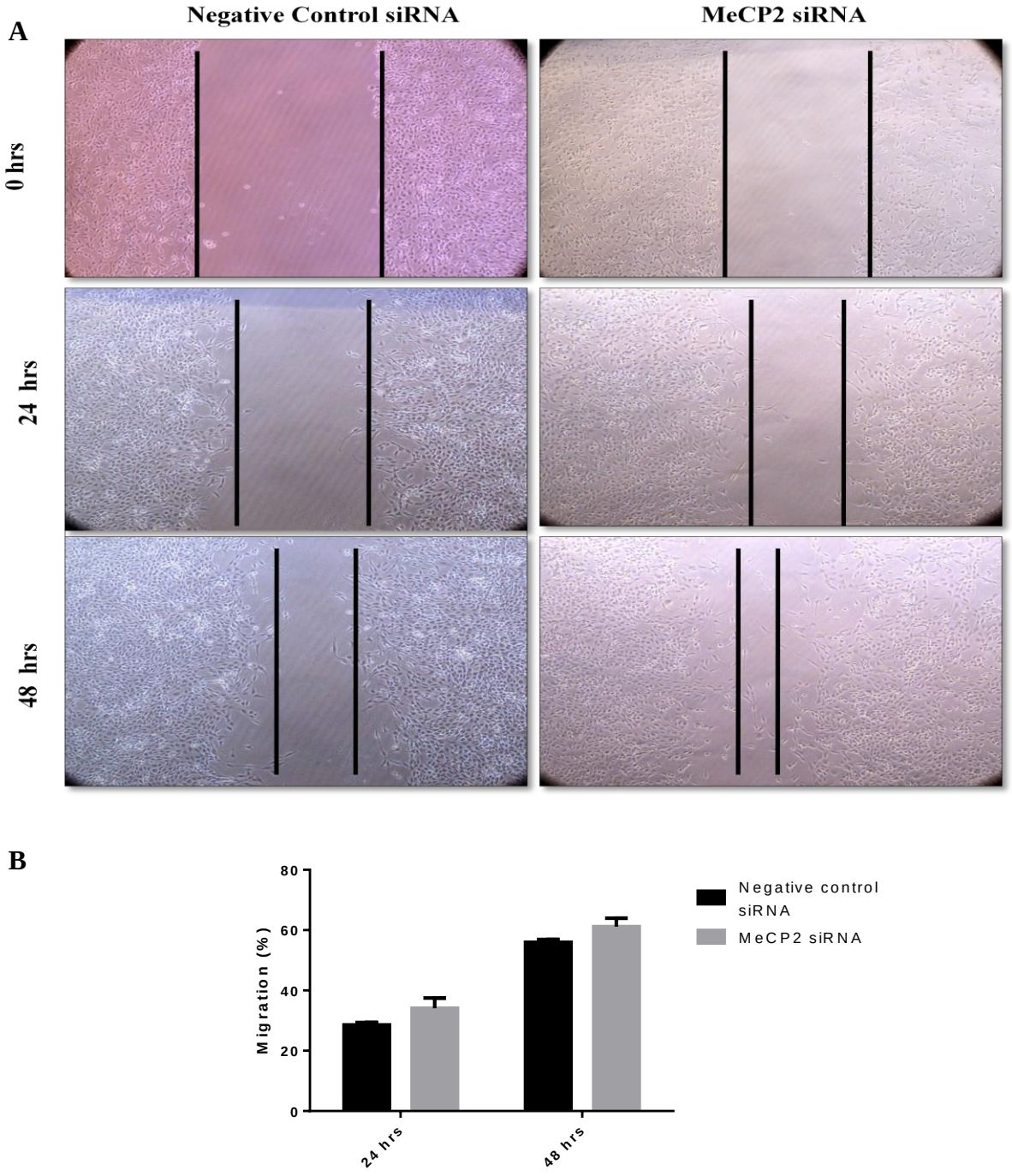

Figure 4.13: MeCP2 knock down regulates C6 glioma migration. (A) Migration was analyzed by scratch assay in C6 transfected with MeCP2 siRNA or negative control. **(B)** Percent wound healing was calculated at 24hrs and 48 hrs following scratch. Data are derived from three experiments and presented as means ± SEM *p < 0.05; **p < 0.01; ***p < 0.001 (unpaired Student's t-test).

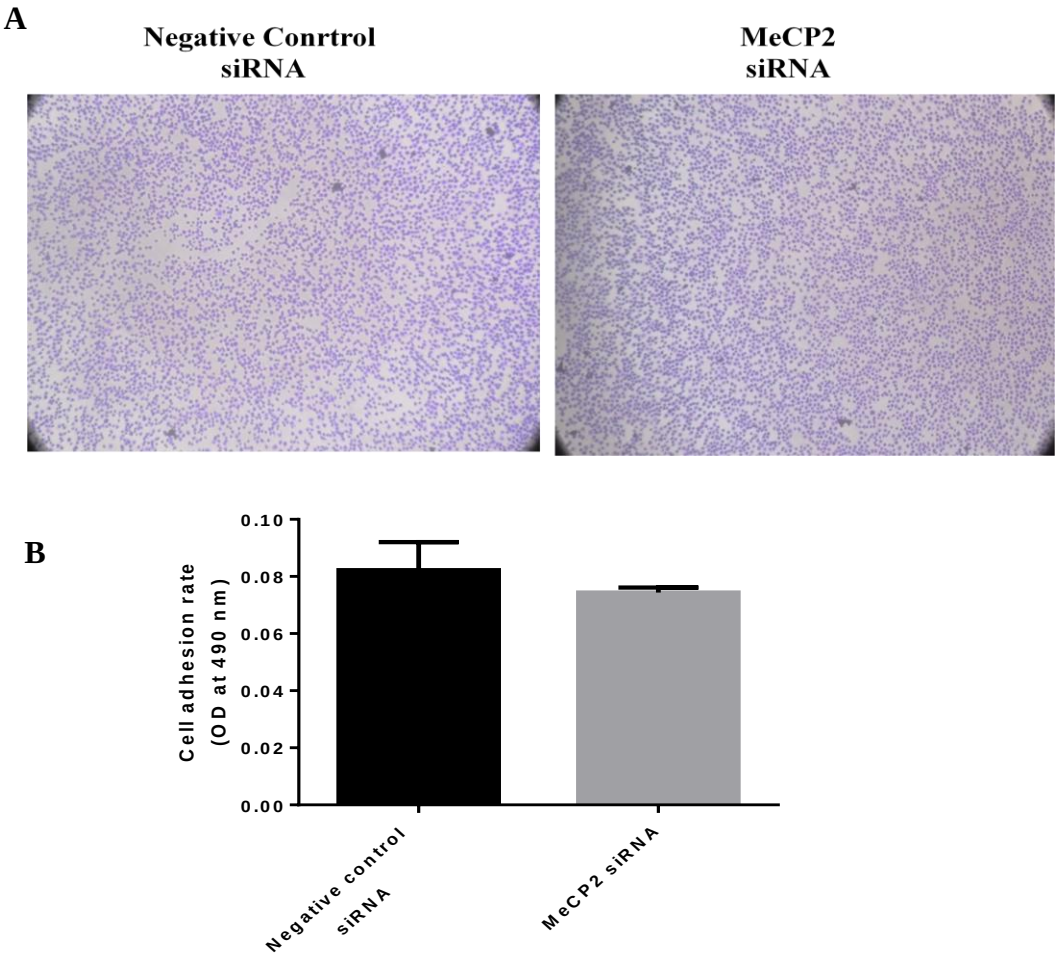

Figure 4.14: MeCP2 knock down do not alter the C6 glioma adhesion to extracellular matrix fibronectin. (A) Adhesion of MeCP2 knock down or negative control treated C6 glioma to fibronectin. Adhered cells were stained with crystal violet. **(B)** Quantitative analysis of adhesion by measuring the optical density (OD) of extracted crystal violet from adhered cells at 490 nm. Data derived from three experiments and presented as means ± SEM *p < 0.05;**p < 0.01; ***p < 0.001 (unpaired Student's t-test).

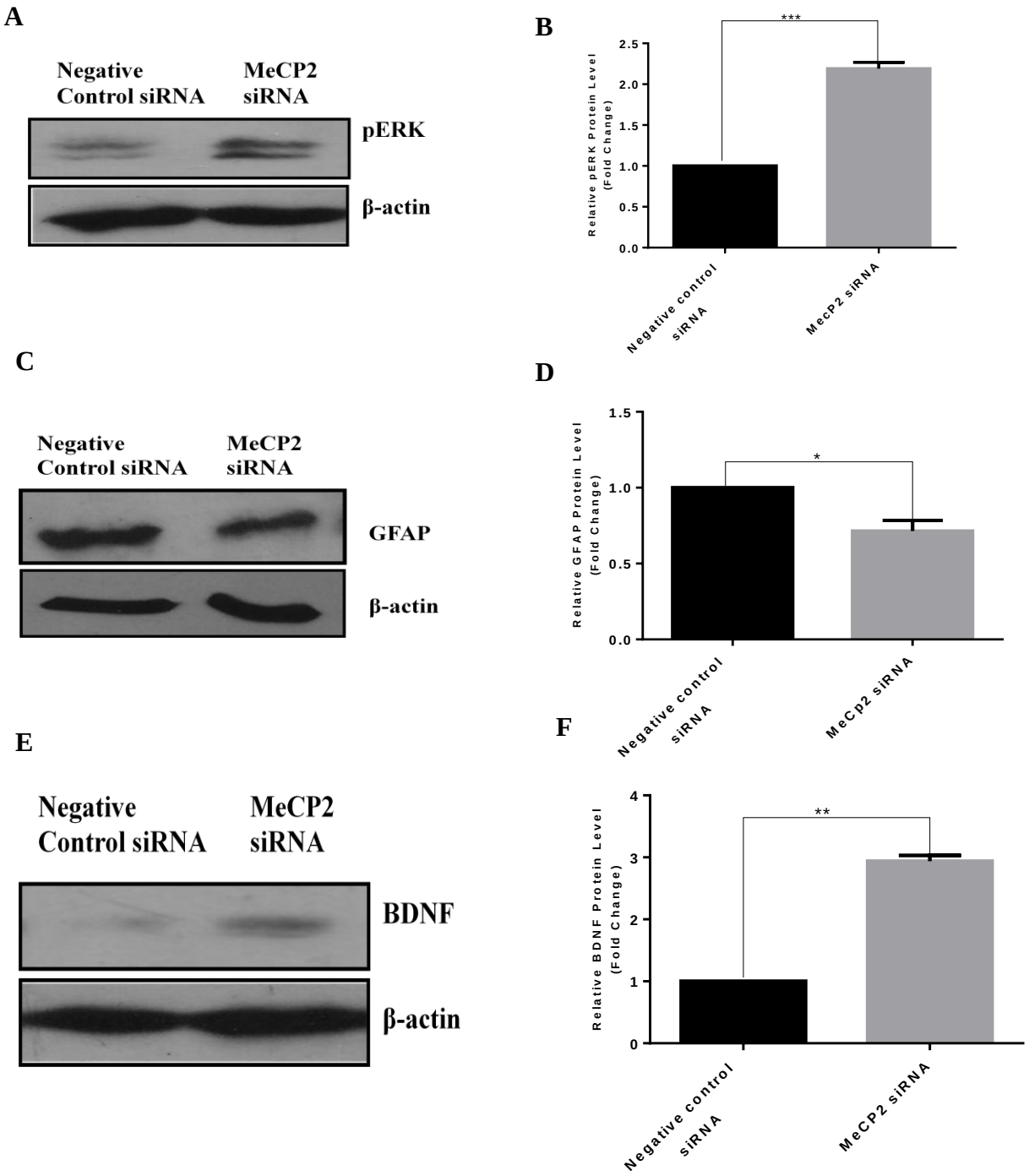

Figure 4.15: MeCP2 knock down alter pERK, GFAP and BDNF proteins level in C6 glioma. Western blot analysis of pERK, GFAP and BDNF**(A, C and E)** in C6 glioma transfected with MeCP2 siRNA or Negative control siRNA. Quantitative analysis of pERK, GFAP and BDNF **(B, D and F).** Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001, ****P<0.0001 (unpaired Student's t-test**).**

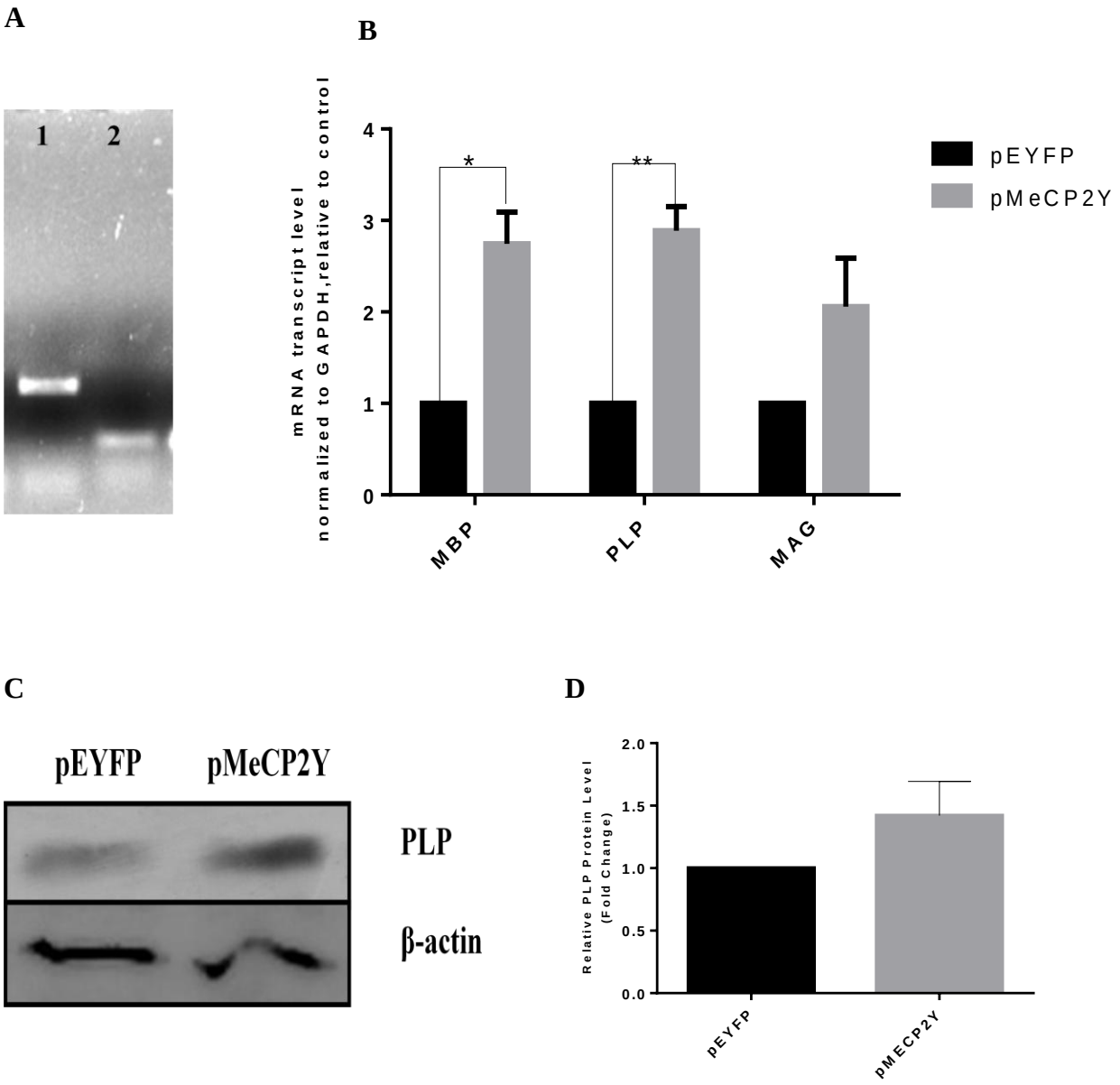

Figure 4.16: Myelin gene expression in MeCP2 overexpressing C6 glioma. (A) Expression of Myelin genes PLP (lane 1) and MAG (lane 2) were confirmed in C6 cells by RT-PCR. **(B)** MBP, PLP and MAG transcript levels in C6 glioma expressing pEYFP or pMeCP2Y. **(C)** Protein level of PLP was determined in C6 glioma expressing pEYFP or pMeCP2Y. **(D)** Quantitative analysis of PLP protein level. Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001, ****P<0.0001 (unpaired Student's t-test**).**

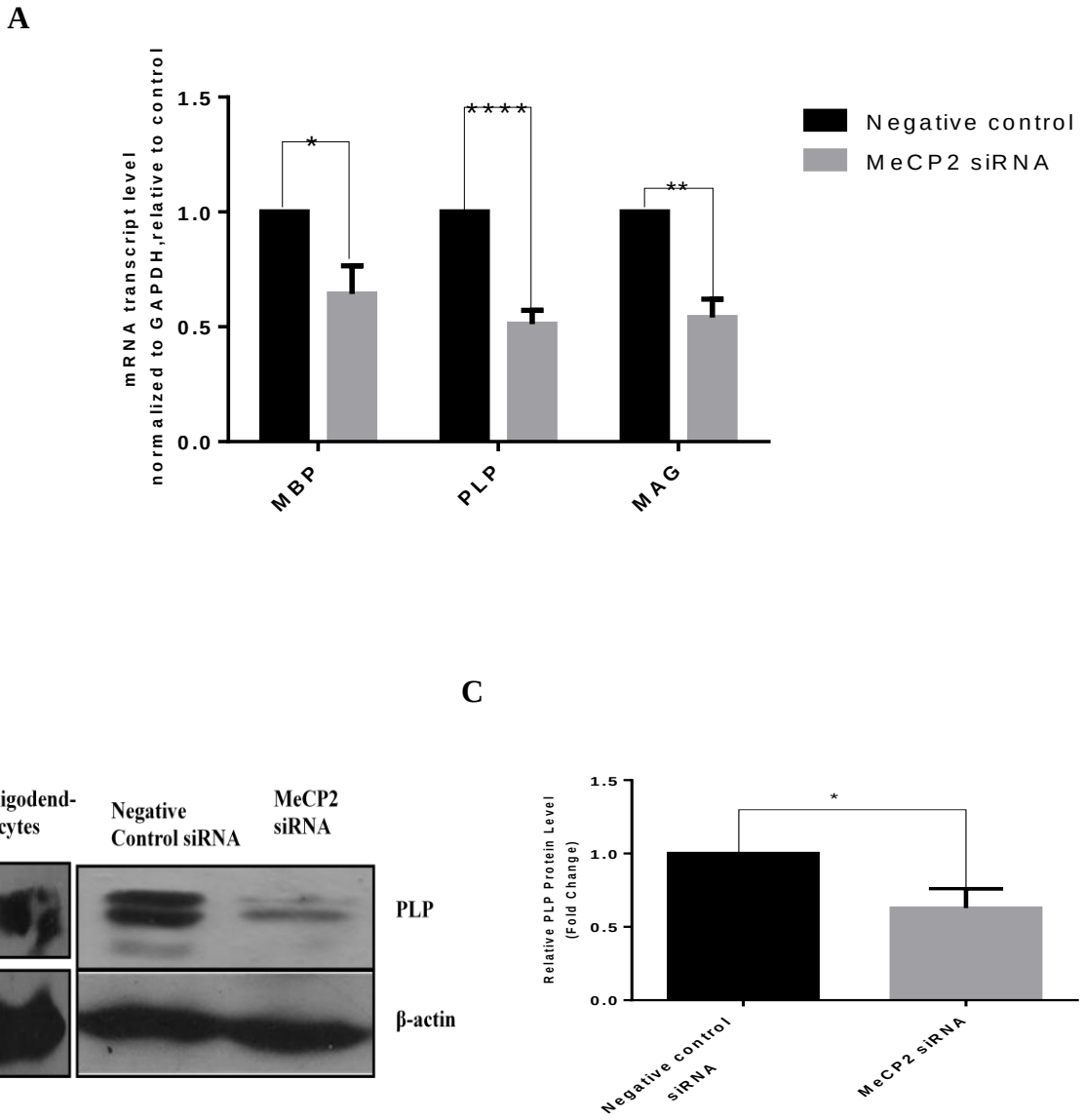

Figure 4.17: Myelin gene expression in MeCP2 Knock down C6 glioma. (A) MBP, PLP and MAG transcript levels in C6 glioma treated with MeCP2 siRNA or Negative control siRNA. **(B)** Protein level of PLP was determined in C6 glioma treated with MeCP2 siRNA or Negative control siRNA. **(C)** Quantitative analysis of PLP protein level. Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001, ****P<0.0001 (unpaired Student's t-test**).**

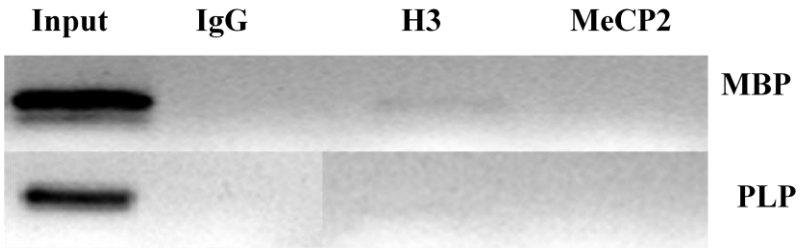

Figure 4.18: MeCP2 does not bind to MBP and PLP gene Promoters in C6 glioma.

Chromatin immunoprecipitation (ChIP) assay was performed in C6 glioma transfected with pEYFP or pMECP2Y with MeCP2 antibody. MeCP2 binds to MBP and PLP promoter sequences was analyzed by PCR using specific primers. Normal Rabbit IgG and Anti-Trimethyl-Histone H3 (Lys4) antibodies were used as negative and positive control respectively. The input sample was also used as positive control.

Table 4.1 Primers list used for real time PCR

Gene	Sequence	Reference
Mecp2	Forward: CAT GGT AGC TGG GAT GTT AG Reverse: GAG CTT TCT GAT GTC TCT GC	Present study
Mbp	Forward: CTC TGG CAA GGA CTC ACA CAC Reverse: TCT GCT GAG GGA CAG GCC TCT C	(Paintlia et al., 2004)
Plp	Forward: GTGTTCTCCCATGGAATGCT Reverse: TGA AGG TGA GCA GGG AAA CT	(Ueno et al., 2012)
Mag	Forward: TGT GTA GCT GAG AAG GAG TAT GG Reverse: ACA GTG CGA TTC CAG AAG GAT TAT	(Ghiani et al., 2007)
Gapdh	Forward: AGA CAG CCG CAT CTT CTT GT Reverse: CTT GCC GTG GGT AGA GTC AT	(Swiss et al., 2011)

Table 4.2 Primers list used for ChIP PCR

Symbol	**Primer sequence (location from transcription start site)**	**Gene promoter**
MBP F MBPR	5'-AGGCAGATGCGATCCAGAAC-3' 5'- CTGCTGTGGGATCTTCTTGGA- 3' (+ 27 ~ -210 bp)	MBP promoter
PLP F PLP R	5'-AGGGTT TAT GATCCAGATCCC-3' 5,- CCACCAAAGCTTCTCTAGAAT-3' (- 211 ~ -410 bp)	PLP promoter
BDNFPIV F BDNFPIV R	5'-GTTCGCTAGGACTGGAAGTGG-3' 5'-CCTCTGCCTCGAAATAGACAC-3' (- 56 ~ -201 bp)	BDNF Promoter IV (He et al., 2010)
GFAP Exon 1	5'-AGGCCCTGACATCCCAGGA-3' 5'- CTGCCTCAGGGTGCCGAG-3' (+478 TO -14 bp)	GFAP promoter
GFAP STAT 3	5'- ATGGCTTGAGTGCTGGGAGA-3' 5'-GCCAGTGAGGCATACGGC-3' (-1320 to -1621 bp)	