CHAPTER 2

EVALUATION OF MECP2 MEDIATED REGULATION OF MYELIN RELATED GENE EXPRESSION AND OLIGODENDROCYTE CELL BEHAVIOR

2.1. INTRODUCTION

Myelination is crucial for rapid propagation of nerve impulses and in providing trophic support to axons. Oligodendrocytes form the myelin sheath around the axons in the central nervous system (CNS). Oligodendrocyte development progress through several stages which includes oligodendrocyte precursor, immature oligodendrocyte and finally mature myelinating oligodendrocytes (Baumann and Pham-Dinh, 2001; Bradl and Lassmann, 2010). Oligodendrocytes precursor cells (OPCs) migrate away from the site of their origin to populate the white matter tracts of the brain, including the corpus callosum and the cortex. Thus, OPC migration is a pre-requisite for myelination in the brain. Several growth factors regulate oligodendrocyte progenitor behavior, including platelet derived growth factor A (PDGFA) (Vora et al., 2011) and fibroblast growth factor 2 (FGF2) (Baron et al., 2000). PDGF-A has been shown to be critical for the formation of normal myelin during development (Soriano 1997). PDGF is known to modulate several different OPC behaviors, including proliferation (Baron et al., 2000, 2002; Ebner et al., 2000; Frost et al., 2003), migration (Armstrong et al., 1991; Frost et al., 1996, 2009), and survival (Barres et al., 1992; Ebner et al., 2000). PDGF regulates the oligodendrocyte progenitor migration via activation of ERK1/2 signaling (Frost et al., 2009).

The progression of oligodendrocyte development is tightly controlled by both extracellular signals and intrinsic determinants in a specific spatial and temporal manner. In the CNS, myelin proteins include myelin PLP (Proteolipid protein), the related product DM20, MBP (myelin basic protein), MAG (myelin-associated glycoprotein), CNP (29, 39-cyclic nucleotide 39-phosphodiesterase), MOG (myelin oligodendrocyte glycoprotein) and MOBP (myelin-associated oligodendrocyte basic protein). The progression of oligodendrocyte differentiation is marked by expression of myelin proteins and is regulated by several transcription factors that include negative factors such as Hes5, ID2/4, Sox5/Sox6 and the positive factors such as Sox10, Nkx2.2, MRF, Zfp191, YY1, Zfp488 and Sip1. Regulation of myelin protein such as PLP1, MBP, MAG, MOG, and CNP is crucial for oligodendrocytes development and myelination (Emery, 2010 a, 2010 b; He & Lu, 2013; Huang et al., 2013). Several diseases are characterized by abnormal myelin development includes leukodystrophies, schizophrenia, autism and Pelizaeus–Merzbacher disease (Baumann & Pham-Dinh, 2001; Nagarajan et al., 2009; Peirce et al., 2006).

MeCP2 (Methyl-CpG binding protein 2) is a transcriptional regulator, which preferentially binds to methylated CpG dinucleotide in DNA. MeCP2 mutations have been linked to Rett syndrome an autism spectrum disorder (Meehan et al.,1992; Amir et al., 1999). The classical function of MeCP2 is repression of target genes through recruitment of HDAC and mSIN3 but recently Zoghbi and colleagues (Ben-Shachar et al., 2009; Chahrour et al., 2008) found that MeCP2 can also act as activator. It was believed that loss of MeCP2 solely in neurons cause Rett syndrome. However, MeCP2 is also expressed in glial cells and its loss in glial cells also contributes to Rett syndrome pathology (Ballas et al., 2009; Maezawa and Jin, 2010; Tochiki et al., 2012; Vora et al., 2010; Nguyen et al., 2013). BDNF (Brain derived neurotrophic factor) is one of the most studied target gene whose expression is modulated by MeCP2 and is shown to be involved in neuronal survival, differentiation, synaptic plasticity and myelination (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006; Khorshid Ahmad et al., 2015; Xiao et al., 2010). Brain magnetic resonance study in *Mecp2-/y* mice detected a significant reduction in the thickness of the corpus callosum which shows that white matter is affected in Rett syndrome (Saywell et al., 2006). Furthermore, it has been shown that myelin gene expression are altered in MeCP2-null mice (Nguyen et al., 2013; Vora et al., 2010).

Since MeCP2 is known to act as a global transcriptional modulator, the focus of the present study was to evaluate the role of MeCP2 on myelin genes regulation in cultured rat oligodendrocytes. Expression of myelin genes (MBP, PLP, MAG, MOG, and MOBP), BDNF and transcriptional regulators of oligodendrocytes (YY1 and Id4) were analyzed in MeCP2 knockdown oligodendrocytes following *in vitro* primary culture of rat oligodendrocytes. Further, the effects of MeCP2 on the regulation of oligodendrocytes precursor cells migration were also assessed.

2.2. MATERIALS AND METHODS

2.2.1 Oligodendrocytes Culture and Isolation

Mixed glial cultures were generated from 1-2 day old rat pups (Charles foster) as described previously (Chen et al., 2007). Briefly, the cerebra of rat pups 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. Cells were plated into

75 cm^2 flasks and grown in DMEM with 10% fetal bovine serum (FBS) at 37°C and 5% CO2 for next 10 days. After 6-8 days, OPCs (Oligodendrocytes Precursor Cells) can be visualized on the mesh of astrocytes. On the 10th day, OPCs were purified from mixed glial cells by a shake-off procedure. Cells were shaken initially for 1 h at 200 rpm to remove microglia , refed and shaken for 18 –20 h at 37°C at 200 rpm. OPCs were collected by centrifugation at 10 min at 100g. OPCs were cultured in Sato medium (Bottenstein & Gordon, 1979). PDGF-AA (Sigma) was added at 10ng/ml to Sato medium for proliferation of OPCs. Animal protocol was duly approved by institutional animal ethical committee (IAEC), Faculty of Science, The M.S University of Baroda (Ref. No. ZD/01/2013; ZD/13/2014; ZD/31/2014; ZD/01/2016).

2.2.2 Immunocytochemistry

Cells were grown on coverslips were fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 0.25% Triton X-100 in PBS for 10 min, blocked by 1% (wt/vol) BSA in PBS containing 0.2% (vol/vol) Tween -20 and incubated overnight at 4°C with the following primary antibodies: anti-MBP (Santa Cruz), anti-PDGFRα (Santa Cruz), anti-O4 (R&D) and anti-MeCP2 (Milipore). After washing, corresponding secondary antibodies conjugated with either FITC or TRITC were incubated with the cells for 1hr at room temperature. Fluorescence signals were detected using confocal microscopic imaging system (Carl Zeiss, Germany, Model LSM- 710).

2.2.3 Small Interfering RNA (siRNA) Transfection

OPCs 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 hrs of first transfection for optimal silencing. RNA and Proteins were isolated after 24 hrs of second transfection.

2.2.4 Plasmid transfection

Plasmid construct (pMeCP2Y) expressing rat MeCP2 ORF fused with YFP (Yellow fluorescent Protein) in pEYFP-N1 (Figure 2.9) was generous gift from Prof. M. Cristina Cardoso (Cell Biology and Epigenetics, Dept. Biology, Technische Universität Darmstadt, Germany).OPCs were transfected with plasmids expressing MeCP2-YFP (pMeCP2Y) or mock plasmid

expressing YFP (pEYFP) using primary cell transfection kit in 4D-Nucleofector (Lonza) following manufacturer instructions. The Plasmids used in the study were duly approved by Institutional Biosafety Committee (IBSC).

2.2.5 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 37^0C. The 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.

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), 0.5-1.0U and sterile double distilled water to make up the volume. Ligation reaction was carried out at 16^0C for 12-16h.

2.2.6 Plasmid transformation and isolation

Plasmid was transformed into competent *E.coli DH5α* strain using calcium chloride method (Sambrook and Russell, 2001). Transformants were selected on kanamycin containing Luria agar plates. Plasmid was isolated from transformants using Plasmid kit (Qiagene) 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).

2.2.7 Western Blotting

Cells were lysed in laemmli buffer and stored at -20^0C until analyzed. Protein was quantified using Qubit protein assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen). Cell lysates with equal protein loads (40 µg) were resolved by SDS polyacrylamide gel electrophoresis (10%) and

transferred to nitrocellulose membrane. The membranes were blocked using 2% skim milk in Tris buffered saline and Tween 20 mixture (0.2%), before incubation in primary antibodies overnight at 4^0C. Following primary antibodies were used, anti-MeCP2 (Milipore), anti-Beta actin (Thermo, pierce), anti-myelin basic protein (MBP) (Santa Cruz), anti-myelin PLP antibody (Abcam) and anti-BDNF (Abcam). 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 quantitatively analyzed using Image J software.

2.2.8 RNA Isolation and qPCR

Total RNA was harvested from cells by Trizol reagent (Sigma). The concentration of RNA was quantified using a Qubit RNA assay kit (Invitrogen) in Qubit 2.0 Fluorometer (Invitrogen). 1.0 µg of total RNA was used for a 20 µl reverse transcription (RT) reaction using verso cDNA synthesis kit (Thermo Scientific). Quantitative RT-PCR was performed using SYBR Select master mix (Applied Biosystems) in QuantStudio12K (Life Technology) real-time PCR machine with primers to detect selected mRNA targets. The melting curve of each sample was measured to ensure the specificity of the products. Data were normalized to the internal control *Gapdh* and analyzed using $2^{-\Delta\Delta CT}$ Method (Livak & Schmittgen, 2001). Primers used for qPCR are listed in Table 2.1.

2.2.9 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. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

2.3. RESULTS

2.3.1 Oligodendrocytes isolation and characterization

Cells were allowed to differentiate in Sato medium without growth factors. Morphological changes were observed at each stage of oligodendrocyte development from oligodendrocyte precursor cells (OPCs) to immature oligodendrocytes and then finally mature oligodendrocytes (OLG). Cells at each stage were also fixed for immunocytochemical characterization by stage

specific markers PDGFRα (OPCs), O4 (immature OLG) and MBP (mature OLG) (Figure 2.1). Cells were characterized morphologically as well as immunocytochemically at each stages of development. Further, to confirm that morphological maturation is paralleled by biochemical maturation in culture condition, MBP marker protein expression levels were determined at distinct stages of OPCs maturation and found to be increased as OPCs differentiated into mature oligodendrocytes (Figure 2.1).

2.3.2 MeCP2 expression in Oligodendrocytes

MeCP2 expression were detected in oligodendrocytes by immunocytochemistry. MeCP2 expression was confirmed at each stage of oligodendrocytes development which includes OPC (oligodendrocyte precursor cells), immature oligodendrocytes and mature oligodendrocytes by double immunostaining with stage specific markers (PDGFRα (OPCs), O4 (immature OLG) and MBP (mature OLG)) and MeCP2. MeCP2 shows both nuclear and cytoplasmic localization. MeCP2 nuclear localization increases as oligodendrocytes differentiate into mature oligodendrocytes (Figure 2.2).

2.3.3 MeCP2 down regulation in oligodendrocytes induce the myelin genes, BDNF and YY1 transcript level

To investigate the precise role of MeCP2 on myelin gene expression, we suppressed the MeCP2 expression in oligodendrocytes using specific siRNA. Cells were transfected twice for optimal silencing of MeCP2. Oligodendrocytes cells transfected with MeCP2 siRNAs exhibited 44 to 70 % reduction in MeCP2 mRNA compared with the cells treated with a scrambled negative control siRNA (Figure 2.3B). Suppression of MeCP2 in oligodendrocytes alter the cell process growth, the number of cell processes increases in MeCP2 siRNA treated oligodendrocytes compared to control(Figure 2.3A),indicate the that MeCP2 knock down induce the morphological differentiation of oligodendrocytes.

Next, the expression of myelin genes transcripts were assessed by qPCR in MeCP2 siRNA treated oligodendrocytes compared to negative control siRNA. There were significant up regulation of PLP (1.71 fold, *P<0.05) and MOG (1.41 fold, *P<0.05) mRNA levels in MeCP2 knock down oligodendrocytes. Other myelin genes MBP, MOBP mRNA level were also found to be increased by 1.68 and 1.43 fold respectively, while MAG expression was found to be

reduced (Figure 2.3B). Apart from myelin genes, neurotrophin (BDNF) and transcriptional regulator (YY1 and ID4) mRNA level were also analyzed and found 1.97 and 2.81 fold increase in BDNF and YY1 respectively, while no change was observed in transcript level of differentiation inhibitor ID4 (Figure 2.4).

2.3.4 MeCP2 down regulation in oligodendrocytes induce the myelin proteins (MBP, PLP and DM-20) and BDNF protein level

To verify whether increase in MBP, PLP and BDNF transcripts level were also accompanied by an increase in the corresponding proteins, western blot analysis were carried out to analyze the protein expression level. MeCP2 knock down in oligodendrocytes was also confirmed by western blot and found ~ 40% reduction in MeCP2 protein level compared to negative control treated oligodendrocytes (Figure 2.5). Further, expression of three myelin related proteins: MBP, PLP and DM-20 were investigated and found significant up regulation of MBP (1.61 fold,*P<0.05), PLP (2.26 fold, ***P<0.001) and DM-20 (1.78 fold, *P<0.05) in MeCP2 knock down oligodendrocytes (Figure 2.6). BDNF protein level was also correlated with transcript level and it was found significantly up regulated (1.70 fold, **P<0.01) (Figure 2.7).

2.3.5 MeCP2 regulate the oligodendrocytes precursor cells (OPCs) migration

To further assess the role of MeCP2 in early oligodendrocytes developmental stage, OPC migration was studied. Migration was studied using scratch assay in OPCs treated with MeCP2 siRNA or Negative control siRNA in presence or absence of PDGF-A. PDGF-A is a potent motogen, which is essential to drive OPC migration. Our results indicate that MeCP2 knock down reduces the migration of OPCs in scratch compared to negative control. MeCP2 knock down also reduce the PDGF-A induced migration in OPCs (Figure 2.8). Thus, the data indicate that MeCP2 regulates the PDGF-A induced migration of OPCs.

2.3.6 Plasmid isolation and confirmation

Plasmids were isolated from *E.coli DH5α* harboring pMeCP2Y or pEYFP and visualized by ethidium bromide (EtBr) staining after electrophoresis on 0.8% agarose gels (Figure 2.10 A and D). pMeCP2Y plasmid was confirmed by restriction digestion and PCR. pMeCP2Y plasmid was subjected to double digestion with BglII and EcoRI which result in the release of 1515bp MeCP2

insert (Figure 2.10 B). Further, pMECP2 plasmid was confirmed by amplification using MeCP2 specific primers (Figure 2.10 C).

Control plasmid pEYFP was prepared from pMeCP2Y by excising the MeCP2 ORF using XhoI and EcoRI restriction enzyme; blunt ends were generated by end filling with Klenow fragment followed by blunt end ligation. pEYFP plasmid was confirmed by absence of MeCP2 amplification using gene specific primers (Figure 2.10 D).

2.3.7 MeCP2 overexpression in oligodendrocytes

To complement the knock down study, attempts were made to over express MeCP2 in primary oligodendrocytes. Primary oligodendrocytes are very hard to transfect cells, 4D-nucleofector (Lonza) system was employed for transfection. Several attempts were made for oligodendrocytes transfection with pMECP2Y or pEYFP but very low 10-15% transfection was observed with high mortality. It was very difficult to study the myelin gene expression following very low transfection efficiency (10-15%) and high mortality, so only morphology of oligodendrocytes were analyzed following transfection. Since MECP2 is a nuclear protein, it was found to be localized to nucleus in cells expressing pMeCP2 compared to pEYFP transfected cells. Further, MeCP2 over expressing oligodendrocytes were found to show less process compared to control plasmid following differentiation in Sato medium, suggesting that regulation of morphological differentiation of oligodendrocytes is MeCP2 dependent (Figure 2.11).

2.4. DISCUSSION

Oligodendrocytes developmental stages from oligodendrocyte precursor to myelin forming oligodendrocytes are regulated by several transcriptional factors which comprises of positive and negative regulators. Some of the transcription factors are stage specific and express at particular stage of development while others continue to express at all stages of development and may also be involved in later stage of terminal differentiation and myelin maintenance (L.He and Lu, 2013;Wegner, 2008). Oligodendrocytes differentiation is regulated by several transcription factors which play important role in promoting (YY1, Sox10, MRF, Olig1and Nkx2.2) and repressing (Id2, Id4, Hes5, and Sox6) myelin genes expression (Emery, 2010a).

Till date, there exist many reports indicating the role of MeCP2 in neuronal cells and further linked to Rett syndrome. Earlier studies have shown that MeCP2 is expressed only in neurons

and absent in other glial cells (Shahbazian et al., 2002; Jung et al., 2003; Kishi and Macklis 2004) but recent studies have shown MeCP2 expression in all glial cells including astrocytes, oligodendrocytes, and microglia (Ballas et al., 2009; Maezawa & Jin, 2010; Tochiki et al., 2012; Vora et al., 2010). The current study has also clearly confirmed the MeCP2 expression at all stages of development in cultured rat oligodendrocytes, which further supports few reports regarding MeCP2 expression in oligodendrocytes. MeCP2 localization to nucleus increases in maturing oligodendrocytes compared to OPCs.

MBP and PLP (and isoform DM-20) are the major CNS myelin proteins which constitutes 80% of total protein. MBP is essential for myelin compaction and loss of MBP results in absence of major dense line. PLP/DM-20 has important role in stabilizing the intraperiodic line of myelin and whose deficiency leads to abnormal condensation of it. Besides its role as a structural protein of myelin, PLP also plays a role in regulating cellular processes like ion exchange, cell migration and programmed cell death (Baumann and Pham-Dinh, 2001; Fulton et al., 2010; Quarles et al., 2006). In this study, there was significant induction in myelin genes (MBP, PLP, MOG, and MOBP) mRNA expression in MeCP2 deficient oligodendrocytes. Similarly, MBP and PLP protein level were also found up regulated in MeCP2 deficient oligodendrocytes. The results suggest that MeCP2 negatively regulates the expression of myelin genes. Since MeCP2 is expressed at all stages of development, it may be involved in maintenance of myelin by repressing myelin genes expression. Myelin-associated oligodendrocyte basic protein (MOBP) is one of the myelin protein, and is thought to be involved in compaction or stabilization of myelin but exact function of MOBP is still unclear (Montague et al., 2006). MOBP was found to be up regulated in *Mecp2*- null mice and directly regulated by MeCP2 binding through its promoter (Urdinguio et al., 2008). Myelin genes such as MBP (myelin basic protein), MAG (myelin associated glycoprotein) and PLP mRNA levels were found to be increased in the corpus callosum and forebrain of *Mecp2* null mouse brain (Vora et al., 2010). Recently, Nurit Ballas group has shown a significant contribution of oligodendrocytes MeCP2 expression in Rett syndrome pathology. Mice lacking MeCP2 in oligodendrocytes lineage developed severe hind limb clasping phenotype and restoration of MeCP2 solely in oligodendrocytes resulted in significant improvement of some RTT phenotype like significant improvement of hind limb clasping phenotype and motor activity deficits. Further, myelin genes MBP and PLP were found to be impaired in brain lacking MeCP2. MBP level was partially restored upon expression of

MeCP2 in oligodendrocytes while level of PLP remain unchanged, suggesting possibility of non cell autonomous effect of other cell on expression of myelin proteins (Nguyen et al., 2013).

BDNF is one of the predominant target of MeCP2 in neurons (Guyet al., 2011). In addition to survival and growth promoting action on neurons, BDNF also promotes myelination and remyelination or myelin repair by oligodendrocytes (Khorshid Ahmad et al., 2015; Xiao et al., 2011). MeCP2 may affect myelination indirectly through the regulation of BDNF expression. In this study, it was observed that BDNF expression is induced in MeCP2 knock down oligodendrocytes. BDNF derived from oligodendrocytes have been shown to support survival and function of nearby neurons (Dai et al., 2003). Present study suggests that oligodendrocytes derived BDNF is also under the regulation of MeCP2.

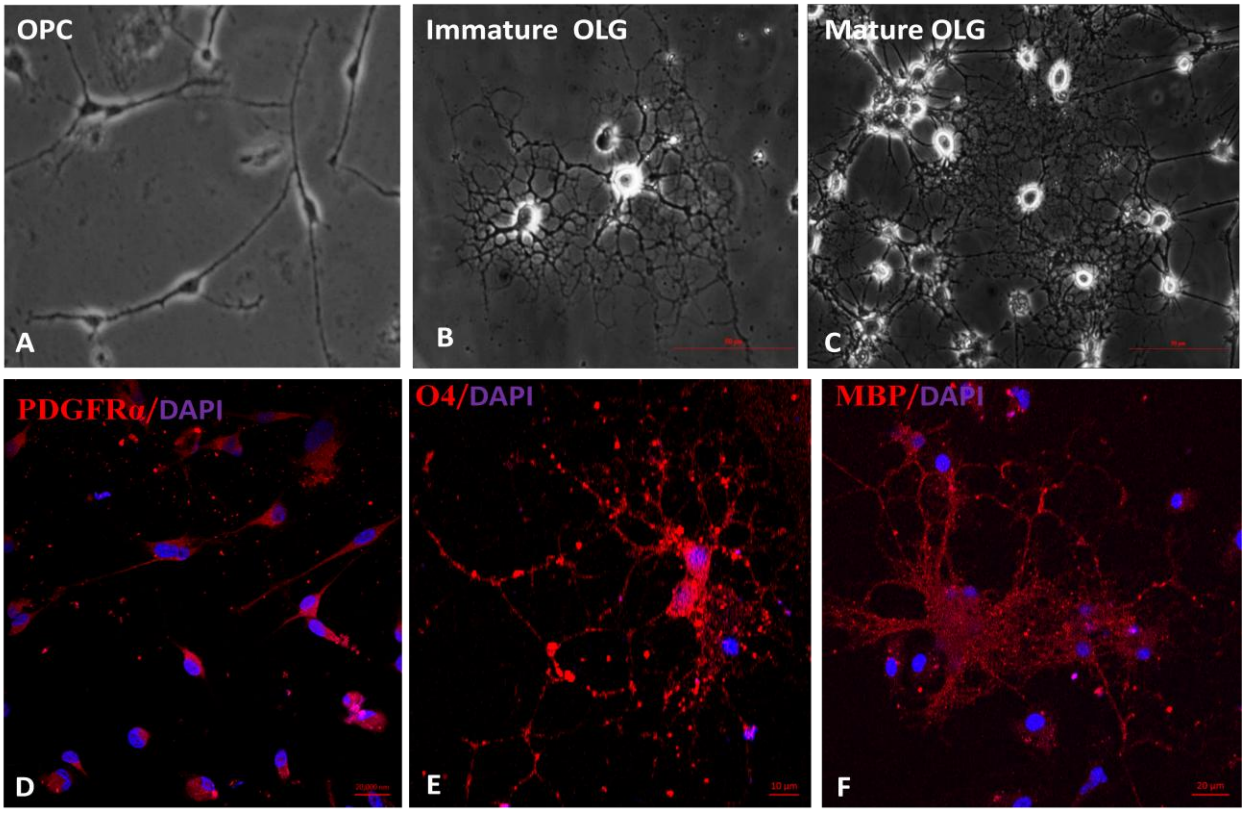
YY1 (Yin Yang 1), a transcriptional regulator of oligodendrocytes differentiation, promotes the oligodendrocytes differentiation through the repression of transcriptional inhibitors (Tcf4 and Id4) of myelin gene expression (He et al., 2007). The results of the present study show that transcript level of YY1 increases in MeCP2 deficient oligodendrocytes which may also contribute to increased myelin gene expression observed in MeCP2 deficient oligodendrocytes.

Further, the role of MeCP2 in oligodendrocyte precursor cells (OPCs) migration was also examined. OPC migration is a pre-requisite for myelination in the brain. PDGF is known to modulate several different OP behaviors, including proliferation (Baron et al., 2000, 2002; Ebner et al., 2000; Frost et al., 2003), migration (Armstrong et al., 1991; Frost et al., 1996, 2009), and survival (Barres et al., 1992; Ebner et al., 2000). PDGF regulates the oligodendrocyte progenitor migration via activation of ERK1/2 signaling (Frost et al., 2009). A recent study has shown that MeCP2 dysfunction cause migration defect in iPSC-derived neurons in layered hydrogel (Zhang et al., 2016). Data from the current study show that MeCP2 knock down significantly reduce the OPC migration in presence or absence of PDGF-A, which therefore suggests MeCP2, may regulate the PDGF-A mediated OPC migration.

2.5. CONCLUSION

Thus, the present study focused on MeCP2 role in myelin genes regulation in oligodendrocytes. MeCP2 was found to negatively regulate the major myelin (MBP, PLP, MOG and MOBP) and related proteins (BDNF and YY1) expression, which is essential for oligodendrocytes differentiation. Further, MeCP2 deficiency was found to reduce the migration of OPCs. MeCP2 over expression inhibits the morphological differentiation of oligodendrocytes which may further confirm function of MeCP2 as regulator of oligodendrocytes differentiation.

A



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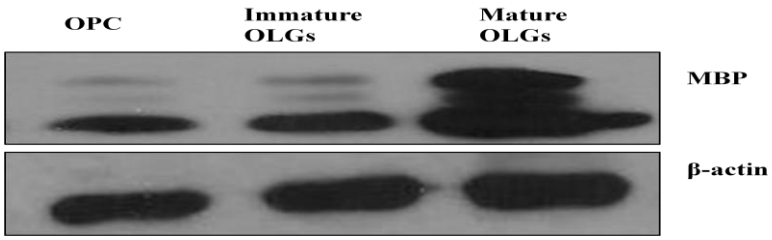


Figure 2.1: Morphological, Immunocytochemical and Biochemical characterization of oligodendrocytes developmental stages: (A) Oligodendrocytes precursor cells (OPCs) were plated and allowed to grow in Sato media containing PDGF-A (proliferation medium). After 24 hrs post plating, cultures were entirely switched to Sato media without PDGF-A (differentiation medium). Cells were then fixed 24 hrs after differentiation Day -1 (A and D), Day-5(B and E) and Day-10 (C and F) for OPCs, immature and mature oligodendrocytes (OLGs) stages respectively. OPCs were detected by stage specific marker PDGFRα (Red). O4 (Red) marker was used for immature and MBP (Red) for mature oligodendrocytes. Nucleus was stained blue with 4',6-diamidino-2-phenylindole (DAPI). **(B)** MBP protein expression in Oligodendrocytes developmental stages.

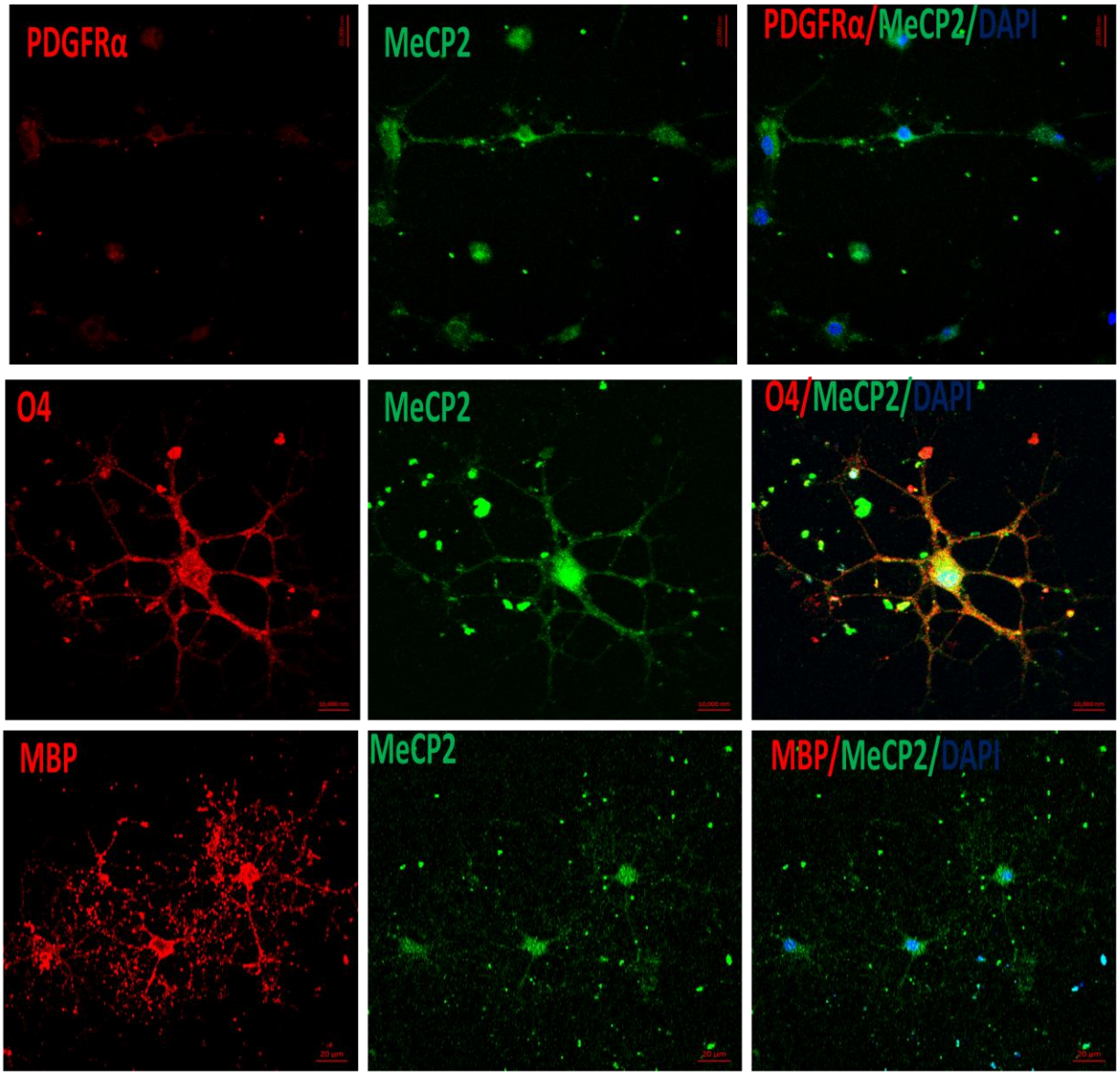


Figure 2.2: MeCP2 expression in oligodendrocytes lineage cells: Oligodendrocytes precursor cells (OPCs) were detected by stage specific marker PDGFRα (Red). O4 (Red) marker for immature and MBP (Red) marker for mature oligodendrocytes were used. MeCP2 (green) was detected at all stage of oligodendrocytes development. Nuclear was stained blue with 4',6-diamidino-2-phenylindole (DAPI).

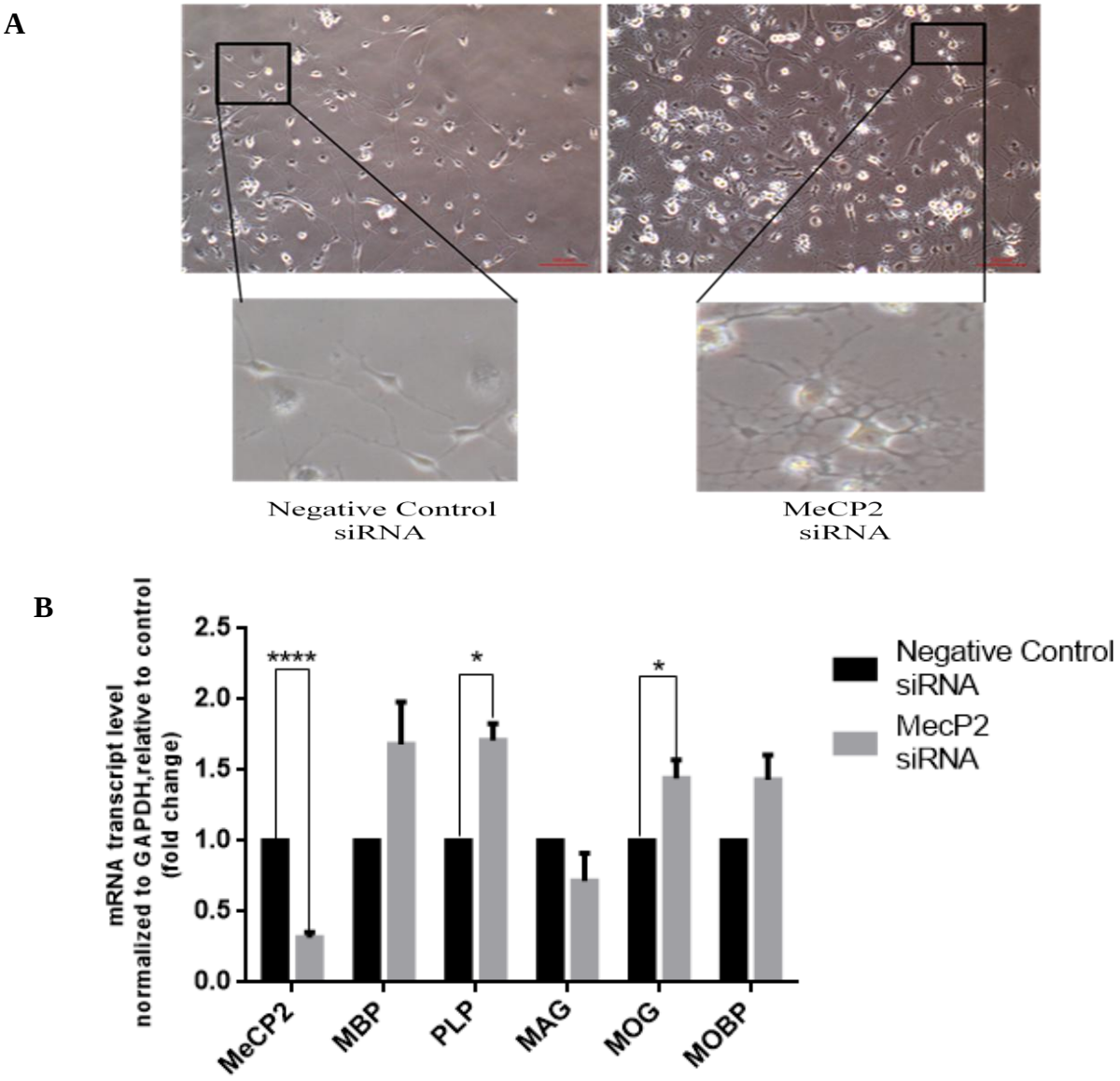


Figure 2.3: Change in cell morphology and increased myelin genes mRNA expression by MeCP2 siRNA in oligodendrocytes. (A) MeCP2 siRNA induce the morphological changes in oligodendrocytes. The number of cell process in oligodendrocytes treated with MeCP2 siRNA are more than control. **(B)** Myelin genes expression in MeCP2 siRNA treated oligodendrocytes. The data reflect the results of qPCR performed in duplicate from 2-3 independent biological replicate.Values are represented as mean ± SEM. *P<0.05;**P<0.01; ***P<0.001; ****P<0.0001 (unpaired Student's t-test).

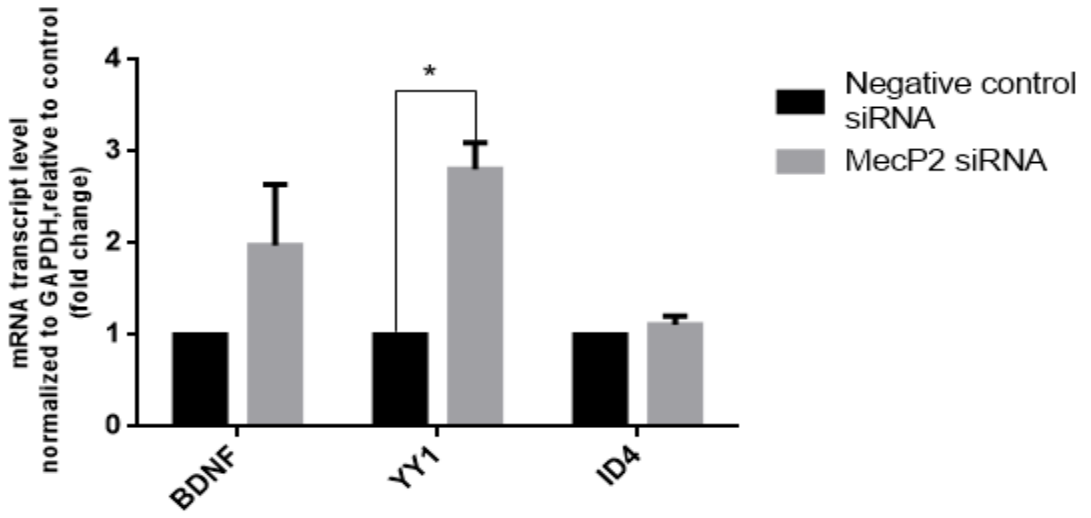


Figure 2.4: Expression of mRNAs for BDNF, YY1 and ID4 in oligodendrocytes treated with MeCP2 siRNA. The data reflect the results of qPCR performed in duplicate from 2-3 independent biological replicate. Values are represented as mean ± SEM. *P<0.05;**P<0.01; ***P<0.001; ****P<0.0001 (unpaired Student's t-test).

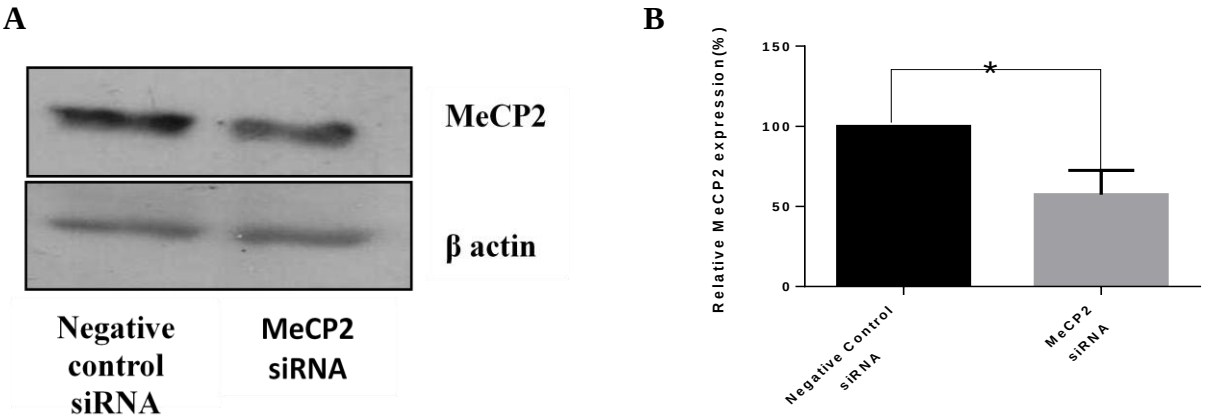


Figure 2.5: Knock Down of MeCP2 expression in cultured oligodendrocytes. **(A)** Representative western blot of MeCP2. **(B)** Relative change in MeCP2 protein expression. Cells were treated with MeCP2 siRNA. Values represent mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001, compared with cells treated with negative control siRNA, normalized to β-actin (unpaired Student's t-test).

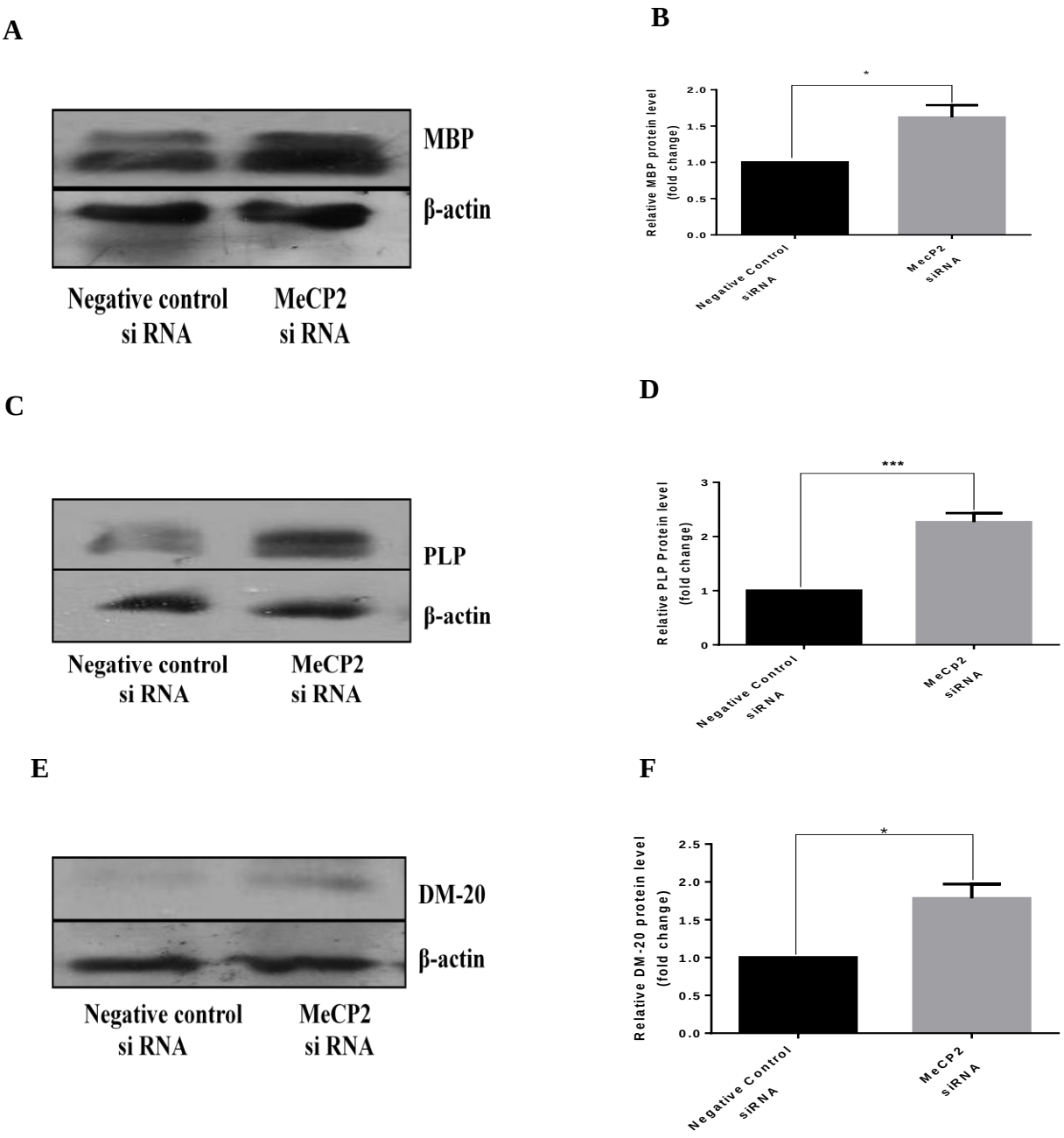


Figure 2.6: Myelin proteins expression in MeCP2 deficient oligodendrocytes. Change in myelin proteins level were measured and compared between cells treated with MeCP2 siRNA and Negative control siRNA. (A), (C) and (E) are representative western blots of myelin proteins MBP, PLP and DM-20 respectively. (B), (D) and (F) are quantitative analysis of MBP, PLP and DM-20 proteins respectively, normalized to β-actin. Values are represented as mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001 (unpaired Student's t-test).

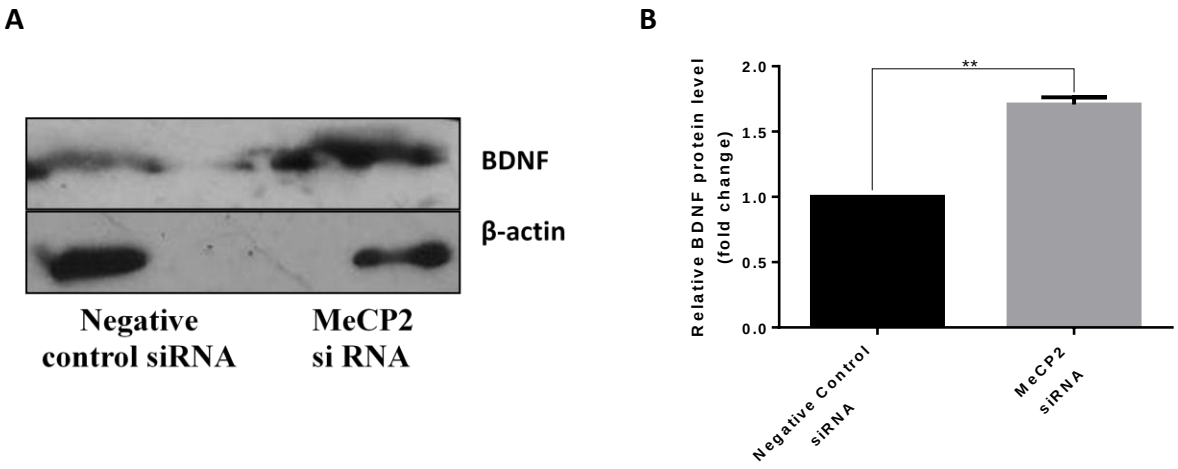


Figure 2.7: MeCP2 regulate the BDNF level in oligodendrocytes. (A) Representative western blot of BDNF in MeCP2 siRNA and Negative control siRNA treated cells (B) Quantitative analysis of western blot. Values are represented as mean ± SEM from 3–4 samples. *P<0.05;**P<0.01; ***P<0.001 (unpaired Student's t-test).

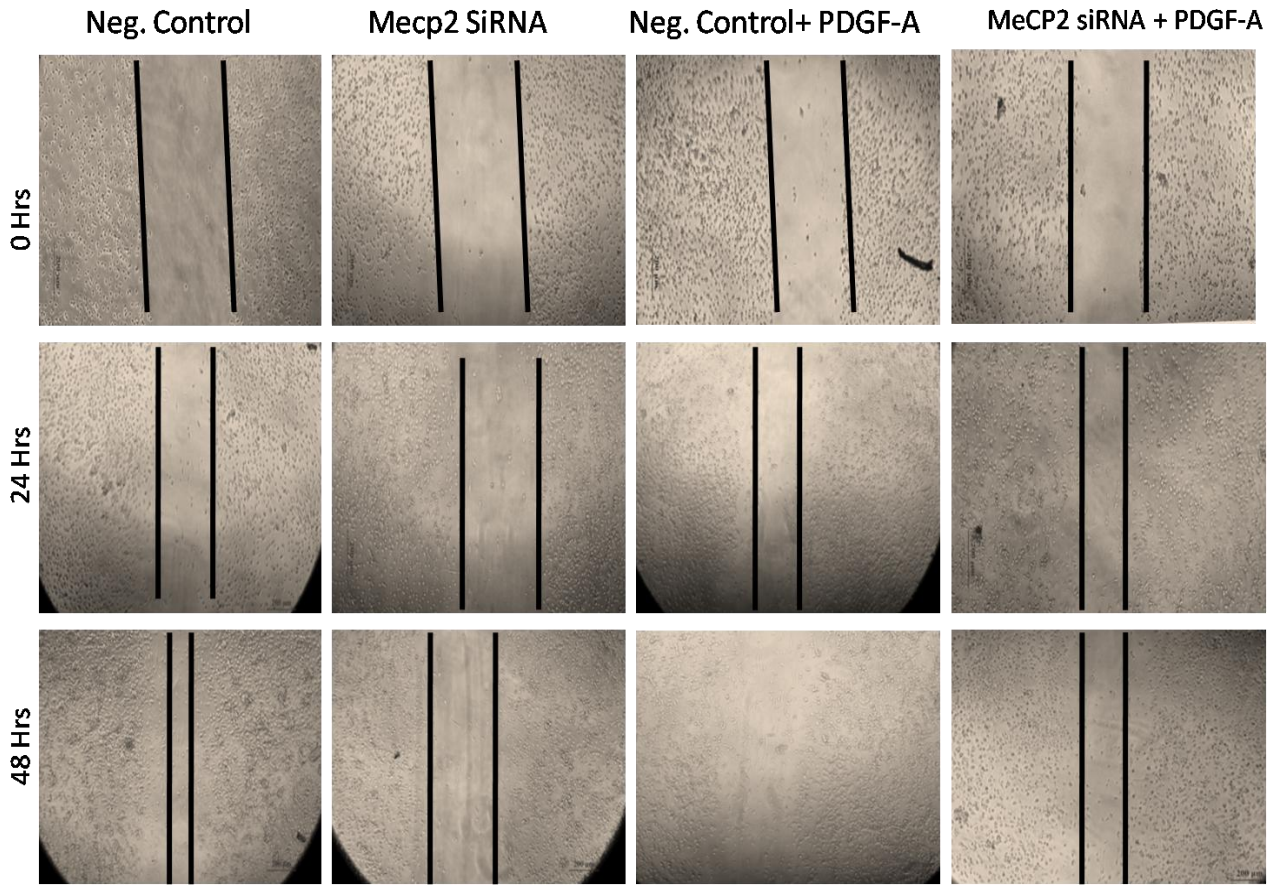


Figure 2.8: MeCP2 regulate the oligodendrocytes precursor cells (OPCs) migration. Scratch assay was performed in OPCs treated with MeCP2 siRNA or Negative control siRNA in presence or absence of PDGF-A (10ng/ml). Migration of cells in scratch was observed at 24hrs and 48hrs. Area between two vertical black lines is denoted as scratch.

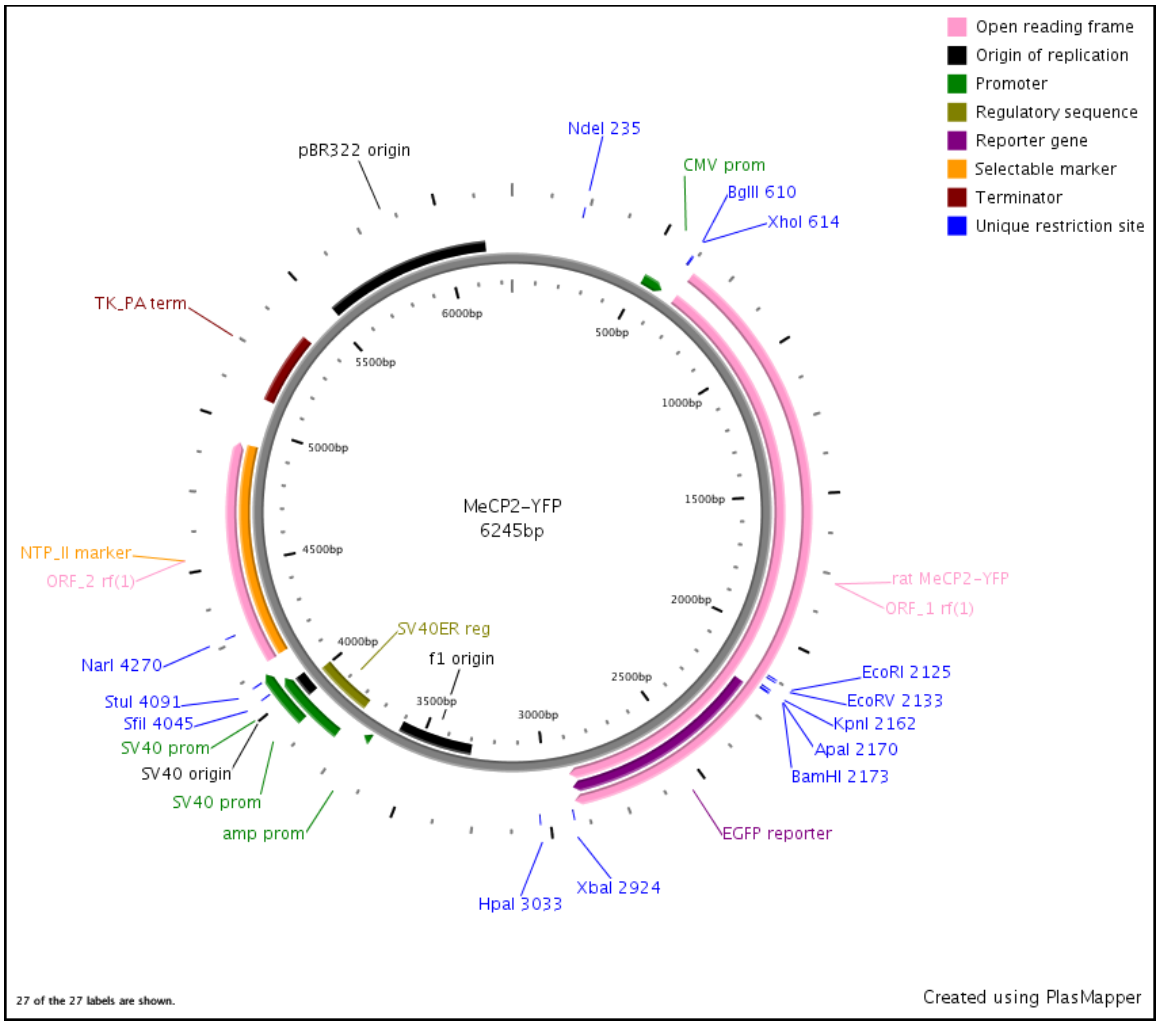
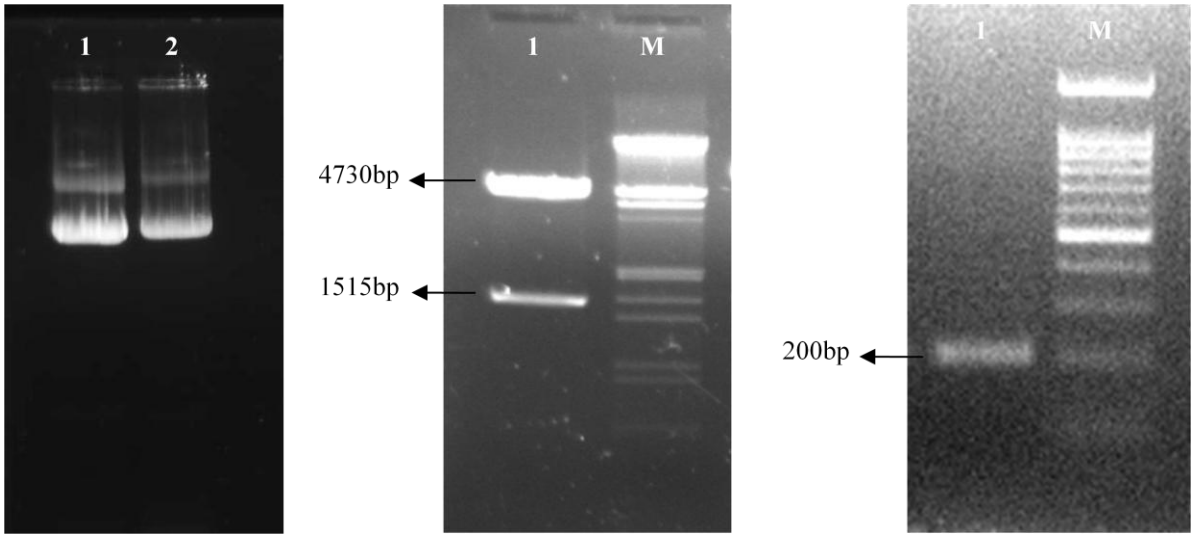
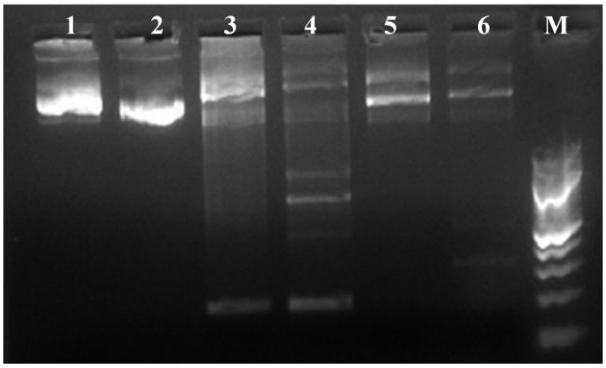


Figure 2.9: Map of pMeCP2Y plasmid expressing MeCP2 fused with YFP (Generous gift from Prof. Cristina M. Cardoso, TU Darmstadt, Germany).



(A) **(B)** **(C)**



(D)

Fig 2.10: Plasmid isolation and confirmation. (A) Plasmid isolation from pMECP2Y transformed *E.coli DH5-α* (lane 1&2).**(B)** Restriction digestion of pMeCP2Y with BglII and EcoRI (Lane 1).**(C)** PCR amplification of MeCP2 from pMeCP2Y plasmid using specific primers. **(D)** Confirmation of control plasmid pEYFP by PCR: Lane 1 &2 -pMeCP2Y and pEYFP plasmids respectively, Lane3&4-MeCP2 amplification from pMeCP2Y (positive control), Lane 5&6- showing no MeCP2 amplification from pEYFP plasmid. M denotes DNA Marker (100bp ladder).

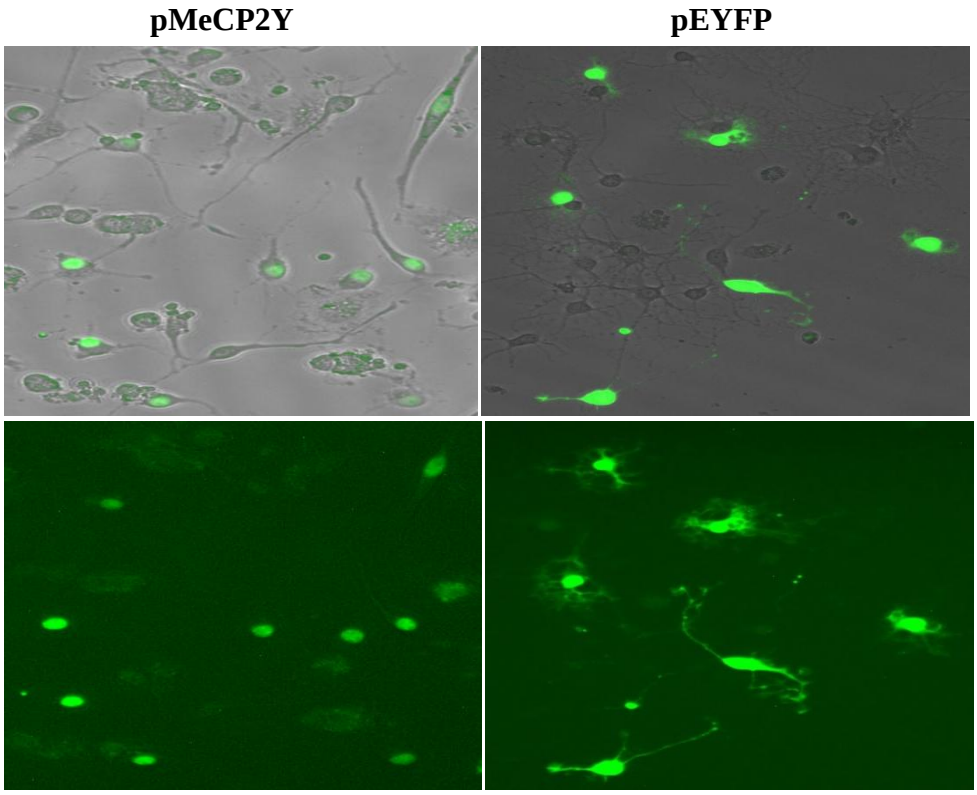


Figure 2.11:MeCP2 over expression in primary oligodendrocytes. Oligodendrocytes were transfected with pMeCP2Y or control plasmid pEYFP using 4-D nucleofector. Fluorescence images of transfected oliogodendrocytes expressing MeCP2-YFP or only YFP protein. MeCP2 was found to localize into nucleus.

Table 2.1: Primers used for Real time PCR

Gene	Sequence	Reference
Mecp2	Forward: GAC CGG GGA CCT ATG TAT GA Reverse: CAA TCA ATT CTA CTT TAG AGC GA	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)
Mog	Forward: GAG GGA CAG AAG AAC CCA CA Reverse: CAG TTC TCG ACC CTT GCT TC	(Swiss et al., 2011)
Mobp	Forward: ATA GGA GCA CAC AGT AGC CC Reverse: AGA CAA GCA AGC ACT CAG G	(Matsuoka et al., 2008)
Bdnf	Forward: CCATAAGGACGCGGACTTGT Reverse: GAGGCTCCAAAGGCACTTGA	(Fuchikami et al., 2009)
Yy1	Forward: ATG AGA AAG CAT CTG CAC ACC Reverse: CCA GCT GGT GGT CGT TTT AGC	(Dewald et al., 2011)
Id4	Forward: GTG CGA TAT GAA CGA CTG CT Reverse: CTG CAG GTC CAG GAT GTA GTC	(Dewald et al., 2011)
Gapdh	Forward: AGA CAG CCG CAT CTT CTT GT Reverse: CTT GCC GTG GGT AGA GTC AT	(Swiss et al., 2011)