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Involvement of MeCP2 in Regulation of Myelin-Related Gene Expression in Cultured Rat Oligodendrocytes

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Abstract Methyl CpG binding protein 2 (MeCP2) is a multifunctional protein which binds to methylated CpG, mutation of which cause a neurodevelopmental disorder, Rett syndrome. MeCP2 can function as both transcriptional activator and repressor of target gene. MeCP2 regulate gene expression in both neuron and glial cells in central nervous system (CNS). Oligodendrocytes, the myelinating cells of CNS, are required for normal functioning of neurons and are regulated by several transcription factors during their differentiation. In current study, we focused on the role of MeCP2 as transcription regulator of myelin genes in cultured rat oligodendrocytes. We have observed expression of MeCP2 at all stages of oligodendrocyte development. MeCP2 knockdown in cultured oligodendrocytes by small interference RNA (siRNA) has shown increase in myelin genes (myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin-associated oligodendrocyte basic protein (MOBP)), neurotrophin (brain-derived neurotrophic factor (BDNF)), and transcriptional regulator (YY1) transcripts level, which are involved in regulation of oligodendrocyte differentiation and myelination. Further, we also found that protein levels of MBP, PLP, DM-20, and BDNF also significantly upregulated in MeCP2 knockdown oligodendrocytes. Our study suggests that the MeCP2 acts as a negative regulator of myelin protein expression.

Keywords MeCP2 · Myelination · Rett syndrome · Oligodendrocytes · MBP · PLP · MOG · MOBP · BDNF · YY1

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 CNS. Oligodendrocyte development progress through the several phases includes oligodendrocyte precursor, immature and to finally mature myelinating oligodendrocytes (Baumann and Pham-Dinh 2001; Bradl and Lassmann 2010). 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 proteolipid protein (PLP), the related product DM20, myelin basic protein (MBP), myelin-associated glycoprotein (MAG), 29,39-cyclic nucleotide 39-phosphodiesterase (CNP), myelin oligodendrocyte glycoprotein (MOG), and myelin-associated oligodendrocyte basic protein (MOBP).The progression of oligodendrocyte differentiation is marked by expression of myelin proteins and is regulated by several transcription factors including negative factors Hes5, ID2/4, Sox5/Sox6 and the positive factors Sox10, Nkx2.2, MRF, Zfp191, Yin Yang 1 (YY1), Zfp488, and Sip1. Regulation of myelin protein such as PLP1, MBP, MAG, MOG, and CNP is crucial for oligodendrocyte development and myelination (Emery 2010a, 2010b; He and Lu 2013; Huang et al. 2013).Several diseases are characterized by abnormal myelin development that

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includes leukodystrophies, schizophrenia, autism, and Pelizaeus–Merzbacher disease (Baumann and Pham-Dinh 2001; Nagarajan et al. 2008; Peirce et al. 2006).

Methyl-CpG binding protein 2 (MeCP2) is a transcriptional regulator, which preferentially bind 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 gene 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, current studies show that MeCP2 is also expressed in glial cells, and its loss in glial cells also contributes Rett syndrome pathology. Brain-derived neurotrophic factor (BDNF) is one of the most studied target genes whose expression is modulated by MeCP2 and involved in neuronal survival, differentiation, synaptic plasticity, and myelination. 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 act as global transcriptional modulator, in the present study, we evaluated the role of MeCP2 on myelin gene regulation in cultured rat oligodendrocytes. We analyzed transcript level of myelin genes, BDNF, and YY1, a transcriptional regulator of oligodendrocyte differentiation and found to be induced in MeCP2 small interference RNA (siRNA)-treated oligodendrocytes. Moreover, we also found that protein levels of MBP, PLP, DM-20 (an isoform of PLP), and BDNF correlate with transcript level in MeCP2 knockdown cells. Our results indicate that MeCP2 regulates the myelination by negatively regulating the expression of crucial proteins involved in synthesis and promotion of myelination.

Materials and Methods

Oligodendrocyte Culture and Isolation

Mixed glial cultures were generated from 1to 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 % CO_2 for next 10 days. After 6–8 days, oligodendrocyte precursor cells (OPCs) 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 100×*g*. OPCs were cultured in Sato medium (Bottenstein and Sato 1979). PDGF-AA (Sigma) was added at 10 ng/ml to Sato medium for proliferation of OPCs.

Immunocytochemistry

Cells were grown on coverslips that were fixed with 4 % (*w*/*v*) paraformaldehyde, permeabilized with 0.25 % Triton X-100 in phosphate-buffered saline (PBS) for 10 min, blocked by 1 % (*w*/*v*) BSA in PBS containing 0.2 % (v/v) 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 1 h at room temperature. Fluorescence signals were detected using confocal microscopic imaging system (Carl Zeiss, Germany, Model LSM-710).

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 h of first transfection for optimal silencing. RNA and proteins were isolated after 24 h of second transfection.

Western Blotting

Cells were lysed in Laemmli buffer and stored at −20 °C until analyzed. Protein was quantified using Qubit protein assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen). Cell lysates with equal protein loads (40 ug) 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 °C. The 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).

Table 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)

Images of specific protein bands on X-ray films were digitally scanned and quantitatively analyzed using Image J software.

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). Total RNA (1.0 μg) was used for a 20 ul 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 messenger RNA (mRNA) targets. The melting curve of each sample was measured to ensure the specificity of the products. Data were normalized to

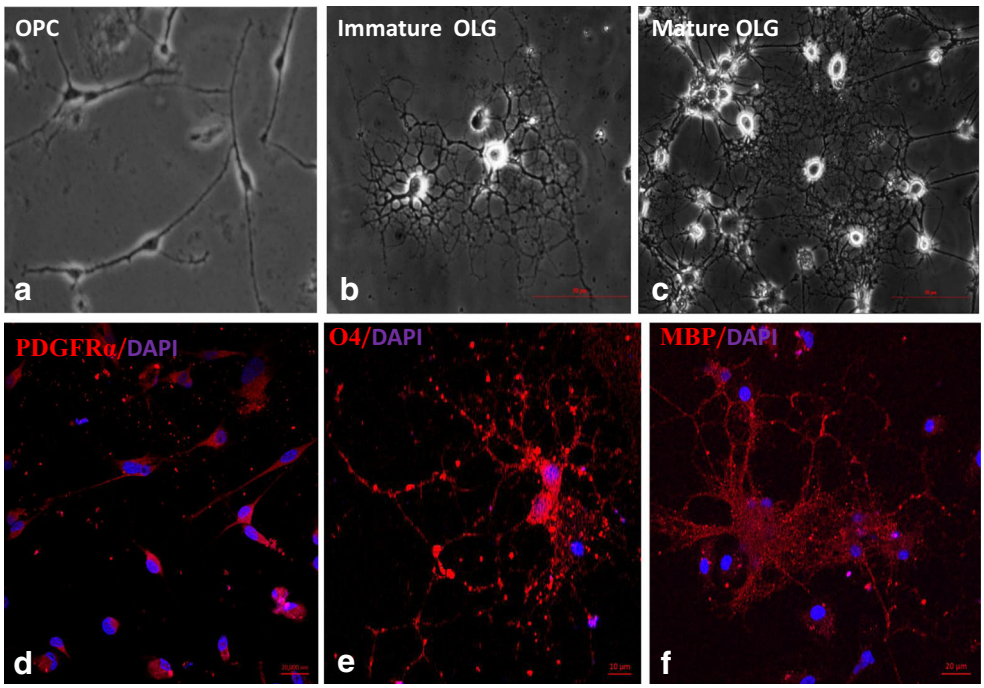

Fig. 1 Morphological and immunocytochemical characterization of oligodendrocyte developmental stages: oligodendrocyte precursor cells (OPCs) were plated and allowed to grow in Sato media containing PDGF-A (proliferation medium). After 24 h post-plating, cultures were entirely switched to Sato media without PDGF-A (differentiation medium). Cells were then fixed 24 h after differentiation day -1 (**a**, **d**), Day-5 (**b**, **e**), and day- 10 (**c**, **f**) for OPCs, immature, and mature oligodendrocyte (OLG) stages, respectively. OPCs were detected by stage specific marker PDGFRα (*red*). O4 (*red*) marker was used for immature and MBP (*red*) for mature oligodendrocytes. Nuclear was stained blue with 4′,6-diamidino-2-phenylindole (DAPI)

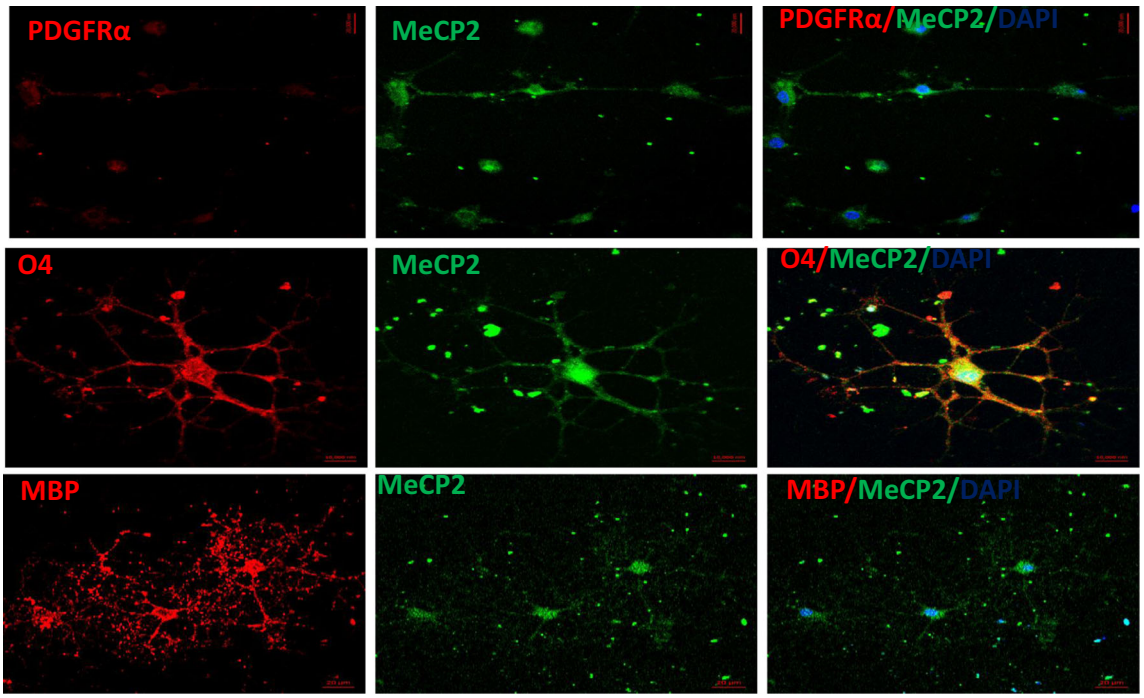

Fig. 2 MeCP2 expression in oligodendrocytes lineage cells: oligodendrocyte precursor cells (OPCs) were detected by stage-specific marker PDGFRα (*red*). O4 (*red*) marker for immature and MBP (*red*) marker for mature oligodendrocytes was used. MeCP2 (*green*) was detected at all stage of oligodendrocyte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

the internal control *Gapdh* and analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Primers used for qPCR are listed in Table 1.

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 (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

Results

Oligodendrocyte Isolation and Characterization

Cells were allowed to differentiate in Sato medium without growth factors. Morphological changes were observed at each stage of development from OPCs to immature oligodendrocytes and then finally mature oligodendrocytes (OLGs). Cells at each stage were also fixed for immunocytochemical characterization by stage specific markers PDGFRα (OPCs), O4 (immature OLG), and MBP (mature OLG) (Fig. 1). Cells were

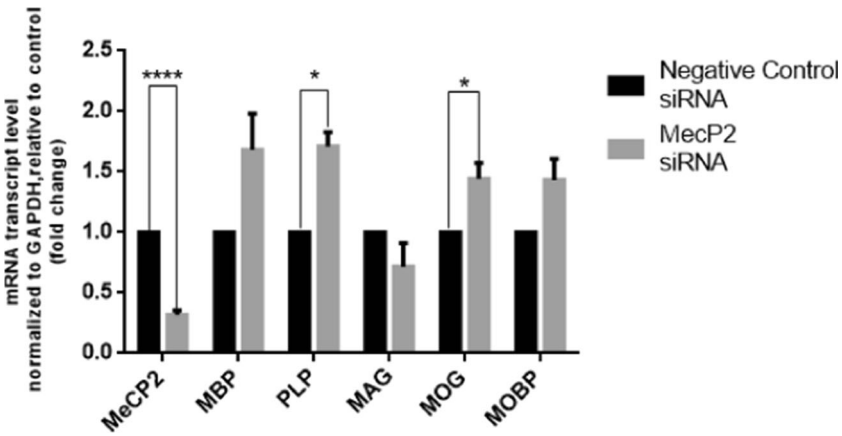

Fig. 3 Suppression of MeCP2 mRNA and increased myelin gene mRNA expression by MeCP2 siRNA in oligodendrocytes. The data reflect the results of qPCR results performed in duplicate from two to three independent biological replicates. Values are represented as mean±SEM. *$P<0.05$;**$P<0.01$; ***$P<0.001$; ****$P<0.0001$ (unpaired Student's *t* test)

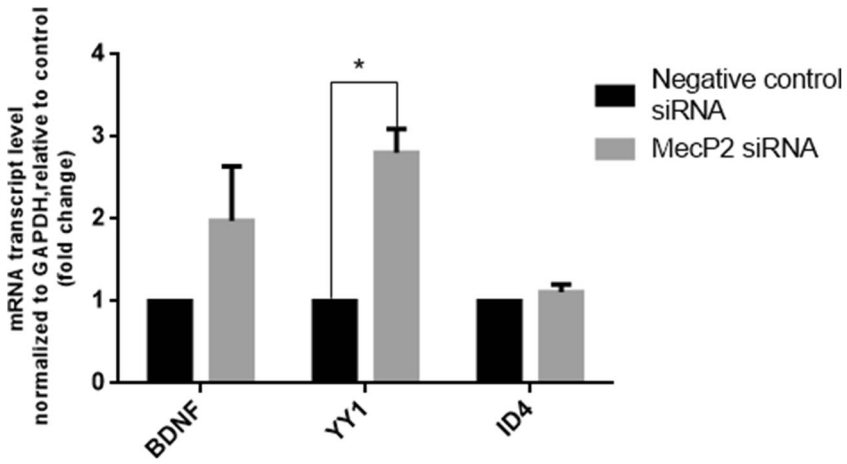

Fig. 4 Expression of mRNAs for BDNF, YY1, and ID4 in oligodendrocytes treated with MeCP2 siRNA. The data reflect the results of qPCR results performed in duplicate from two to three independent biological replicates. Values are represented as mean±SEM. *$P<0.05$;**$P<0.01$; ***$P<0.001$; ****$P<0.0001$ (unpaired Student's *t* test)

characterized morphologically as well as immunocytochemically at each stages of development.

MeCP2 Expression in Oligodendrocytes

We used immunocytochemistry to detect MeCP2 expression in oligodendrocytes. MeCP2 expression was confirmed at each stage of oligodendrocyte development which includes OPC, immature, and finally mature oligodendrocytes by double immunostaining with stage-specific markers and MeCP2. MeCP2 shows both nuclear and cytoplasmic localization (Fig. 2).

MeCP2 Downregulation 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 (Fig. 3). Next, we assessed the expression of myelin gene transcript by qPCR in MeCP2 siRNA-treated oligodendrocytes compared to negative control siRNA. We observed a significant upregulation of PLP (1.71-fold, *$P<0.05$) and MOG (1.41-fold, *$P<0.05$) mRNA level. 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 (Fig. 3). Further, we performed the gene expression study of neurotrophin (BDNF) and transcriptional regulator (YY1 and ID4). We detected a 1.97-fold increase in BDNF and 2.81-fold increase in YY1, and no change was observed in mRNA expression of differentiation inhibitor ID4 (Fig. 4).

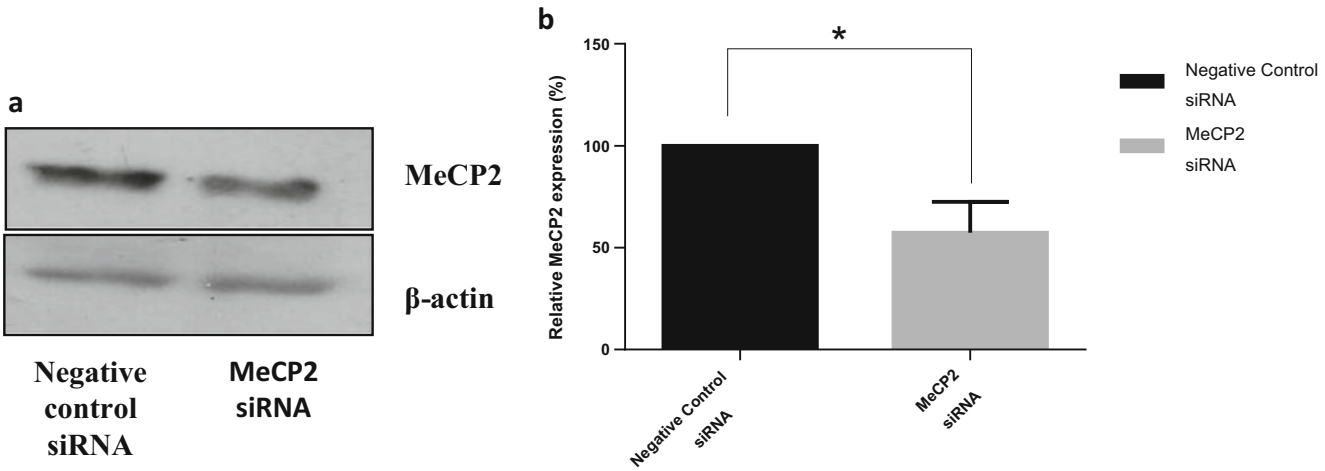

Fig. 5 Knockdown of MeCP2 expression in cultured oligodendrocytes. **a** Representative western blot of MeCP2. **b** Relative change in MeCP2 protein expression. Cells were treated with MeCP2 siRNA. Values represent mean±SEM from three to four samples. *$P<0.05$;**$P<0.01$; ***$P<0.001$, compared with cells treated with negative control siRNA, normalized to β-actin (unpaired Student's *t* test)

MeCP2 Downregulation 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. Protein levels were determined by Western blot analysis. 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 (Fig. 5). We further investigated expression of three myelin related proteins: MBP, PLP, and DM-20. We observed a significant upregulation 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 knockdown oligodendrocytes (Fig. 6). BDNF protein level was also correlated with transcript level and it was found significantly upregulated (1.70-fold, $**P<0.01$) (Fig. 7).

Discussion

Oligodendrocytes developmental stages from oligodendrocyte precursor to myelin forming oligodendrocytes

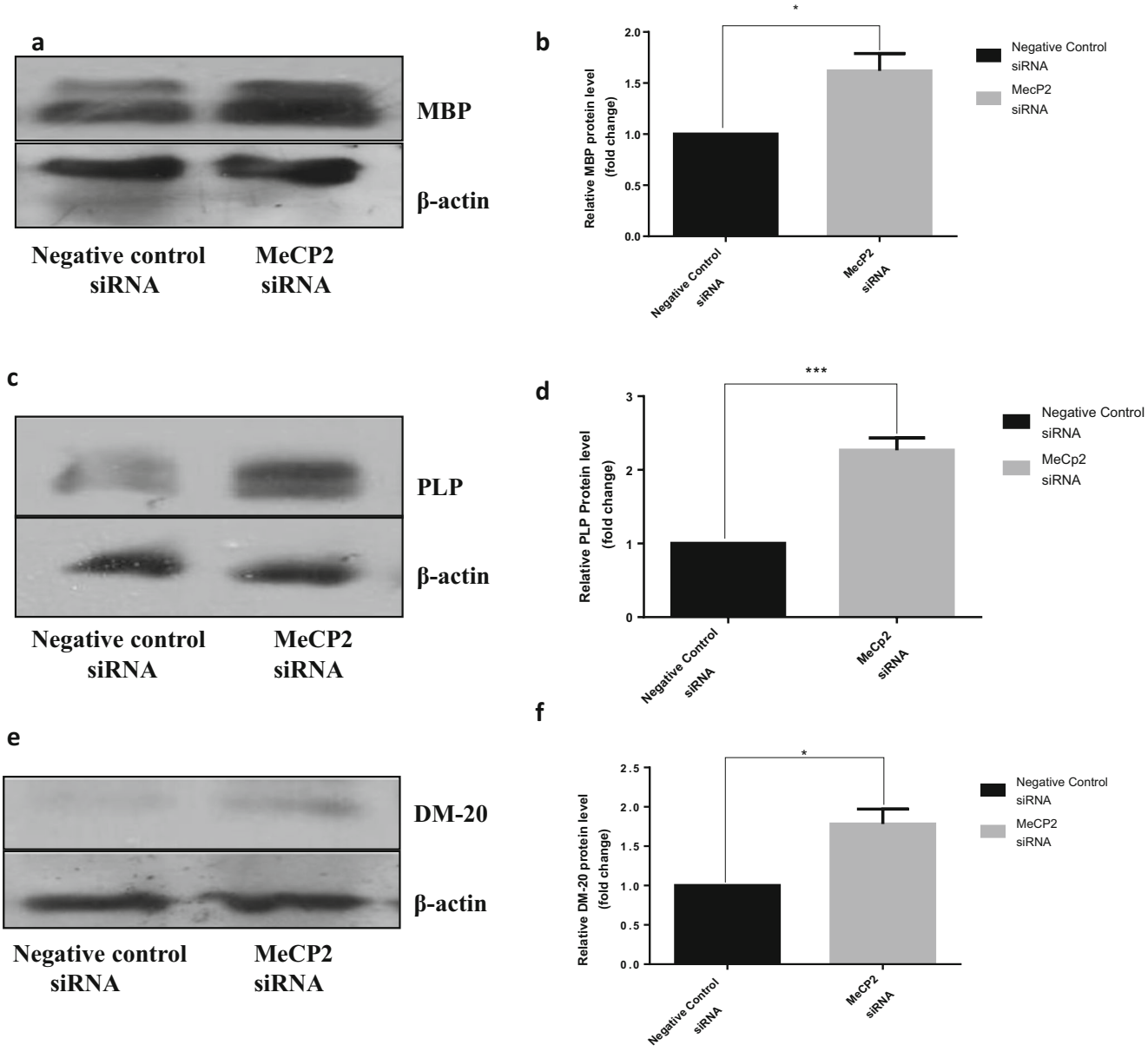

Fig. 6 Myelin proteins expression in MeCP2 deficient oligodendrocytes. Change in myelin protein levels were measured and compared between cells treated with MeCP2 siRNA and negative control siRNA. **a**, **c**, **e** Representative Western blots of myelin proteins MBP, PLP, and DM-20, respectively. **b**, **d**, **f** Quantitative analysis of MBP, PLP, and DM-20 proteins, respectively, normalized to β-actin. Values are represented as mean±SEM from three to four samples. $*P<0.05$;$**P<0.01$; $***P<0.001$ (unpaired Student's *t* test)

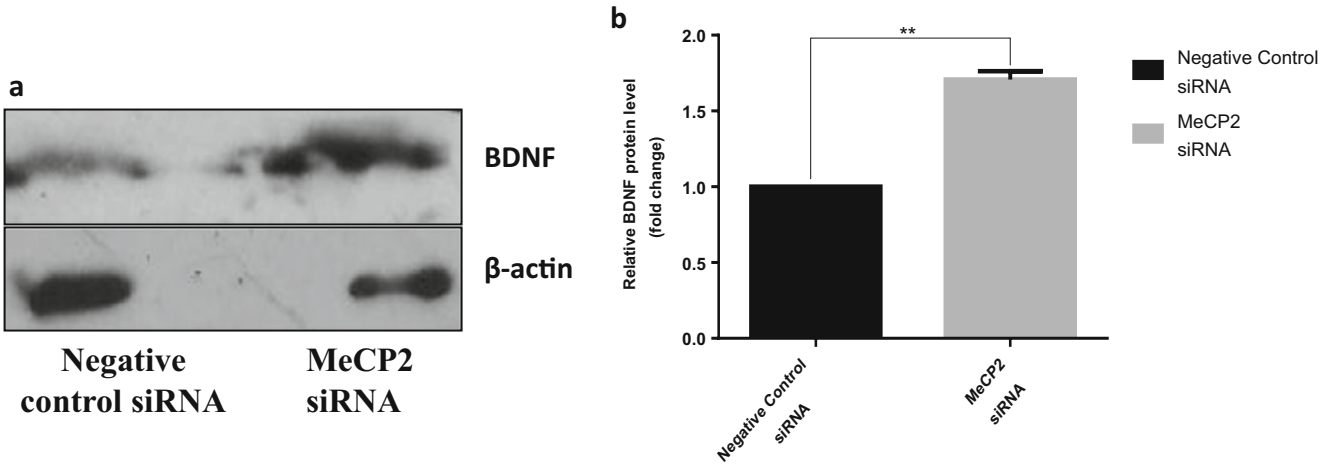

Fig. 7 MeCP2 regulate the BDNF level in oligodendrocytes. **a** Representative Western blot of BDNF in MeCP2 siRNA and negative control siRNA-treated cells. **b** Quantitative analysis of Western blot. Values are represented as mean±SEM from three to four samples. *$P<0.05$;**$P<0.01$; ***$P<0.001$ (unpaired Student's *t* test)

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). Oligodendrocyte differentiation is regulated by several transcription factors by promoting (YY1, Sox 10, MRF, Olig1and Nkx2.2) and repressing (Id2, Id4, Hes5, and Sox6) myelin gene expression (Emery 2010a).

In the present study, we focused on role of MeCP2 in regulation of myelin gene expression. Till date, many studies has been carried out on the role of MeCP2 in neuronal cells and 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 current studies have shown MeCP2 expression in all glial cells including astrocytes, oligodendrocytes, and microglia (Ballas, Lioy, Grunseich, and Mandel 2009; Maezawa and Jin 2010; Tochiki et al. 2012; Vora et al. 2010). We have also clearly shown MeCP2 expression in cultured rat oligodendrocytes at all stages of development, which further supports few reports regarding MeCP2 expression in oligodendrocytes.

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. Beside 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. 2009; Quarles et al. 2006).

In our study, we found significant induction in myelin gene (MBP, PLP, MOG, MOBP) mRNA expression in MeCP2-deficient oligodendrocytes. Similarly, MBP and PLP proteins level were also found upregulated in MeCP2-deficient oligodendrocytes. Our result suggests 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. MOBP is one of myelin protein involved in compaction or stabilization of myelin, but exact function of MOBP is still unclear (Montague et al. 2005). MOBP was found to be upregulated in *Mecp2*-null mice and directly regulated by MeCP2 binding through its promoter (Urdinguio et al. 2008). Myelin genes MBP, MAG, and PLP mRNA levels have been found to increase in 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 targets of MeCP2 in neurons (Guy et 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. 2010). MeCP2 may affect myelination indirectly through the regulation of BDNF expression. In our study, we observed that BDNF mRNA and protein expression is induced in MeCP2 knockdown oligodendrocytes. BDNF derived from oligodendrocytes have been shown to support survival and function of nearby neurons (Dai et al. 2003). Our study suggest that oligodendrocyte-derived BDNF is also regulated by MeCP2.

YY1 is a transcriptional regulator of oligodendrocyte differentiation. YY1 promotes the oligodendrocyte differentiation through the repression of transcriptional inhibitors (Tcf4 and Id4) of myelin gene expression (He et al. 2007). We found that transcript level of YY1 increases in MeCP2-deficient oligodendrocytes.

Our data conclude that MeCP2 plays an important role in regulation of myelin genes and other factors (YY1 and BDNF) which promote the myelination. Further, studies on MeCP2 overexpression and myelin gene expression are underway.

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Conflict of Interest The authors declare that they have no conflict of interest.

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