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Differential Regulation of MeCP2 Phosphorylation by Laminin in Oligodendrocytes

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Abstract Oligodendrocytes (OLGs) are the myelinating cells of the central nervous system (CNS), and its proper differentiation is crucial for normal functioning of neurons. Methyl-CpG-binding protein 2 (MeCP2) is a multifunctional methylated DNA binding protein; mutation of which causes Rett syndrome, a severe neurodevelopmental disorder. Previously, we reported that MeCP2 is expressed in all the stages of oligodendrocyte development, and also shown the role of MeCP2 as a transcription regulator of myelin genes in OLGs. The expression and function of MeCP2 phosphorylation at S80 (pS80MeCP2) has been well studied in neurons and astrocytes; however, there is no data so far available in OLGs regarding pS80MeCP2. Certain developmental stimuli such as growth factors and extracellular matrix (ECM) protein play important role in OLG development. In the present study, we have examined the effects of external stimuli (growth factors (GFs) and extracellular matrix (ECMs)) on S80 phosphorylation of MeCP2 in N19 oligodendroglial cells (N19 OLGs). This study provides the first evidence that laminin (LN) differentially regulates the expression of pS80MeCP2 in immature and mature N19 OLGs. Thus, MeCP2 is phosphorylated in a stimulus-dependent manner during oligodendrocyte development, and thereby, it may regulate the oligodendrocyte behavior.

Keywords pS80MeCP2 · Laminin · Oligodendrocyte · Extracellular stimuli

Introduction

Methyl-CpG-binding protein 2 (MeCP2) in recent times have been shown to be a well-known link between epigenetics and human disease; mutations in which lead to the neurological disorders, such as Rett syndrome(Xu et al. 2014; Chahrour et al. 2008; Zachariah and Rastegar 2012). However, till now, there are no reports on expression and function of phosphorylated MeCP2-S80 in oligodendrocytes (OLGs). The well-documented MeCP2 phosphorylation sites are S421 and S80; phosphorylation of which modulates the transcription in opposite manners leading to gene silencing or activation (Tao et al. 2009; Zhou et al. 2006; Chao and Zoghbi 2009; Rutlin and Nelson 2011). Animal model studies have further shown that even the loss of a specific phosphorylation site of MeCP2 (S80, S421) disturbs normal maturation of the mammalian brain (Tao et al. 2009). The expression and function of MeCP2 phosphorylation at S80 (pS80MeCP2) has been well studied in neuron (Gonzales et al. 2012). Recently, MeCP2 and its phosphorylation studies were done in astrocytes (Liu et al. 2017). However, there is no data so far available in OLGs regarding MeCP2 phosphorylation status.

OLGs are glial cells that produce and maintain myelin, which is essential for saltatory conduction of action potentials in the central nervous system (CNS). Various growth factors (GFs) and extracellular matrix (ECM) have been reported to influence the proliferation, differentiation, and survival of OLGs in vitro and in vivo (Bogler et al. 1990; Baron et al. 2005; Milner et al. 1997; Novak and Kaye 2000; Frost et al. 2009; Vora et al. 2011; O'Meara et al. 2011). In the normal cellular environment, both GFs and ECM proteins coexist, but

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the underlying mechanism by which they regulate cell behavior is poorly understood. Oligodendrocyte progenitor cell (OPC) migration is critical not only during normal myelination but also during remyelination (Keough and Yong 2013). Moreover, studies have shown the altered levels of GFs and ECM in the demyelinating lesions (Hughes et al. 2013).

One critical aspect of these biological processes is the mechanism by which OLG transcription factors/regulators integrate the external stimuli to trigger changes in gene expression. It has been studied that environmental factors can regulate the gene expression by epigenetic modifications (Vigetti et al. 2014). To the best of our knowledge, there is no information available about the stimulus-dependent expression and regulation of phosphorylated MeCP2 in OLGs. The present study focuses on the role of developmental stimuli in regulating MeCP2 phosphorylation in OLGs. We have checked the expression of MeCP2 phosphorylation in OLGs at S80. This is the first report to show that MeCP2 is phosphorylated at S80 site in immature and mature OLGs in a differentially regulated manner by laminin (LN).

Materials and Methods

Cell Culture

Cell culture reagents were purchased from Gibco/Invitrogen. Mouse oligodendroglial cell line N19 was a kind gift from Dr. Pablo Paez (Department of Pharmacology and Toxicology, Hunter James Kelly Research Institute, School of Medicine and Biomedical Sciences, SUNY, University at Buffalo, NYS Center of Excellence, 701 Ellicott St., Buffalo, New York. USA). These cells were conditionally immortalized OLGs. The N19 OLGs were grown up to 70% confluence at 36 °C, in 75-cm^2 flasks (2–3 × 10^6 cells/flasks) or on poly-L-lysine-coated coverslips (2 × 10^5 cells/ml; for immunocytochemical studies) in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. (Silvestroff et al. 2013). For the differentiation studies, the N19 cells were placed at 39 °C in differentiating medium DMEM/F12/1% FBS and 1% penicillin/streptomycin with apotransferrin (100 μg/ml) for 7 days (Paez et al. 2004).

Treatment Groups

N19 immature and mature cells were plated in DMEM/F12 and 1% penicillin/streptomycin (Gibco) on poly-L-lysine (PLL; Sigma)-coated dishes or glass coverslips, and were serum starved overnight prior to any treatment. Cells were exposed to platelet-derived growth factor alpha (PDGF-A) (Sigma), fibroblast growth factor (FGF) (Sigma), fibronectin (FN) (Invitrogen), and laminin (LN) (Invitrogen) for 1 h. PLL was used as control substratum which supports cell adhesion and spreading without engaging integrins. In all the experiments, the PLL, FN, and LN were used at the concentration of 10 μg/ml. PDGF-A and FGF were used at the concentration of 10 ng/ml. For α6β1 integrin blocking studies, cells were kept with anti-α6 blocking antibody Cat. no. MAB13501 from R&D systems and anti-β1blocking antibody Cat. no. MA2910 from Thermo Fisher for 1 h prior to LN exposure.

Immunocytochemistry

In this study, the following primary antibodies were used: rabbit anti-MeCP2 (total) (diluted at 1:1000) Cat. no. MP4591 from ECM Biosciences, rabbit anti-phosphorylated MeCP2-S80 (diluted at 1:1000) Cat. no. MP4601 from ECM Biosciences, mouse anti-myelin basic protein (MBP) (diluted at 1:1000) Cat. no. ab62631 from Abcam, and goat anti-PDGFRα (diluted at 1:1000) Cat. no. sc31178 from Santa Cruz. N19 cells (immature and mature) grown on coverslips were then fixed with 4% paraformaldehyde in phosphate buffer saline pH 7.5 (PBS), washed three times for 5 min with PBS, and followed by incubation in blocking solution (3% bovine serum albumin in PBS). The cultures were incubated with the appropriate primary antibodies in blocking solution overnight at 4 °C, followed by incubation with secondary antibodies (1/400 in blocking solution) in 1-ml blocking solution (Invitrogen) for 1 h. Coverslips were rinsed and mounted with anti-fade mounting medium (Invitrogen) and sealed with nail polish. Images were visualized and captured using a Carl-Zeiss confocal microscope.

Immunoblotting

Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose. Primary antibodies used were total rabbit anti-MeCP2 (diluted at 1:1000) Cat. no. MP4591 from ECM Biosciences, rabbit anti-phosphorylated MeCP2-S80 (diluted at 1:1000) Cat. no. MP4601 from ECM Biosciences, mouse anti-myelin basic protein (MBP) (diluted at 1:1000) Cat. no. ab62631 from Abcam, mouse anti-β-actin (diluted at 1:5000) Cat. no. MA5-15739 from Thermo Fisher, and goat anti-PDGFRα (diluted at 1:1000) Cat. no. sc31178 from Santa Cruz. Appropriate secondary antibodies were used. Membranes were developed using the ECL Plus chemiluminescent kit (Invitrogen).

Co-immunoprecipitation

For co-immunoprecipitation, N19 OLGs were washed twice with ice cold PBS and lysed in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 1.5 m M $MgCl_2$, 1% NP-40, 15% glycerol,

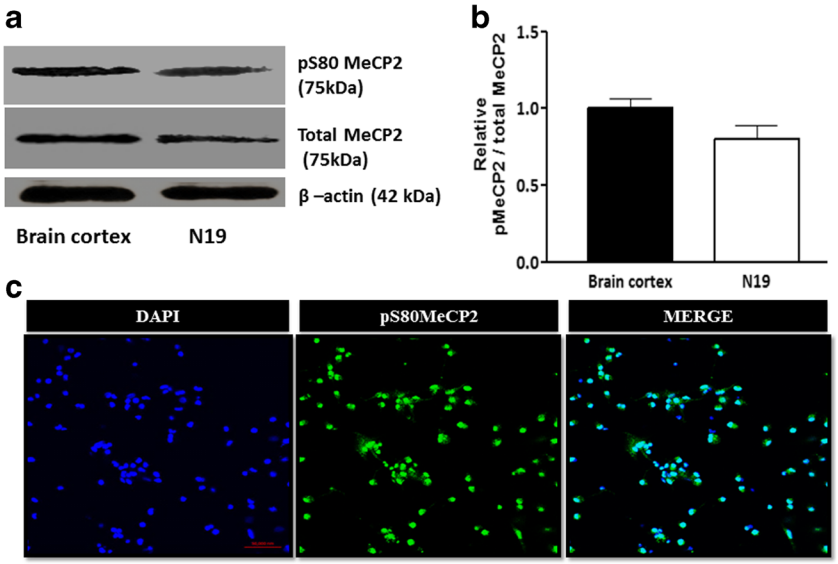

Fig. 1 pS80MeCP2 expression in N19 OLGs. **a** Representative Western blots for phosphorylated and total MeCP2 in lysates of brain cortex and N19 OLGs. **b** After densitometric analysis, pS80MeCP2 data were normalized to total MeCP2. Three different cell preparations were analyzed three times ($N = 3$). *Error bars* represent ±SEM. **c** Representative immunostaining images showing pS80MeCP2 expression in N19 OLGs (*green*). The nucleus was stained with DAPI (*blue*). *Scale bar* = 50 μm. Data were evaluated using Student's *t* test or one-way ANOVA parameter where appropriate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

2 mM EDTA) by a 30-min incubation at 4 °C, containing 0.5% bovine serum albumin and proteinase inhibitor cocktail (Sigma). Equal amounts of clarified cell lysates were incubated with 5 μg of anti-α6 (Cat. no. MAB13501 from R&D systems) antibody overnight at 4 °C. After adding protein A/G agarose beads (Santa Cruz) for 1 h, the suspension was centrifuged, and pellets were washed three times with lysis buffer, and once with PBS. Immunoprecipitated proteins were solubilized in Laemmli sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and blotted with antibodies to integrin α6 (Cat. no. MAB13501 from R&D systems) and β1 (Cat. no. MA2910 from Thermo Fisher) subunits. Bound antibodies were revealed by horseradish peroxidase-conjugated secondary antibodies and the ECL detection system.

Statistical Analysis

Confocal microscopy images were analyzed by the ZEN 2012 imaging software. For statistical analysis, a minimum of 150 randomly chosen cells per condition were analyzed ($N = 3$ independent experiments with three to four replicates). Differences between treatment groups were analyzed using Student's *t* test, or one-way analysis of variance with Bonferroni's post-test where appropriate. Statistical analysis was performed with the Prism 3 software (GraphPad Software Inc.). $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Result and Discussion

MeCP2 Is Phosphorylated at S80 in Response to Various Growth Factors and ECM in N19 Oligodendroglial Cells

MeCP2 undergoes various post-translational modifications, such as phosphorylation, ubiquitination, acetylation, and sumoylation (Bellini et al. 2014). However, the functional roles of these PTMs are largely unexplored. Accumulated evidences suggest that MeCP2 could be phosphorylated at multiple sites, of which S80 is well-documented phosphorylation site (Tao et al. 2009; Zhou et al. 2006). OLGs have been shown to express MeCP2 and play a significant role in Rett syndrome (RTT) pathogenesis; therefore, it has become increasingly important to study the phosphorylation status of MeCP2 in OLGs (Ballas et al. 2009; Nguyen et al. 2013; Sharma et al. 2015).

In the present study, we first checked the expression of pS80MeCP2 in N19 OLGs. We used Western blot analysis to study the expression levels of pS80MeCP2 in N19 OLGs. pS80 MeCP2 was found to be expressed in N19 OLGs (Fig. 1a, b). Brain lysate was taken as a positive control. Confocal microscopy imaging showed the nuclear localization of the pS80MeCP2 in N19 OLGs (Fig. 1c). Earlier, reports have also shown that, unlike S421 phosphorylation, S80 phosphorylation not only occurs in neuronal cells but also seen in non-neuronal cells such as Hela cells and human fibroblasts (Bracaglia et al. 2009; Tao et al. 2009). Tao et al.

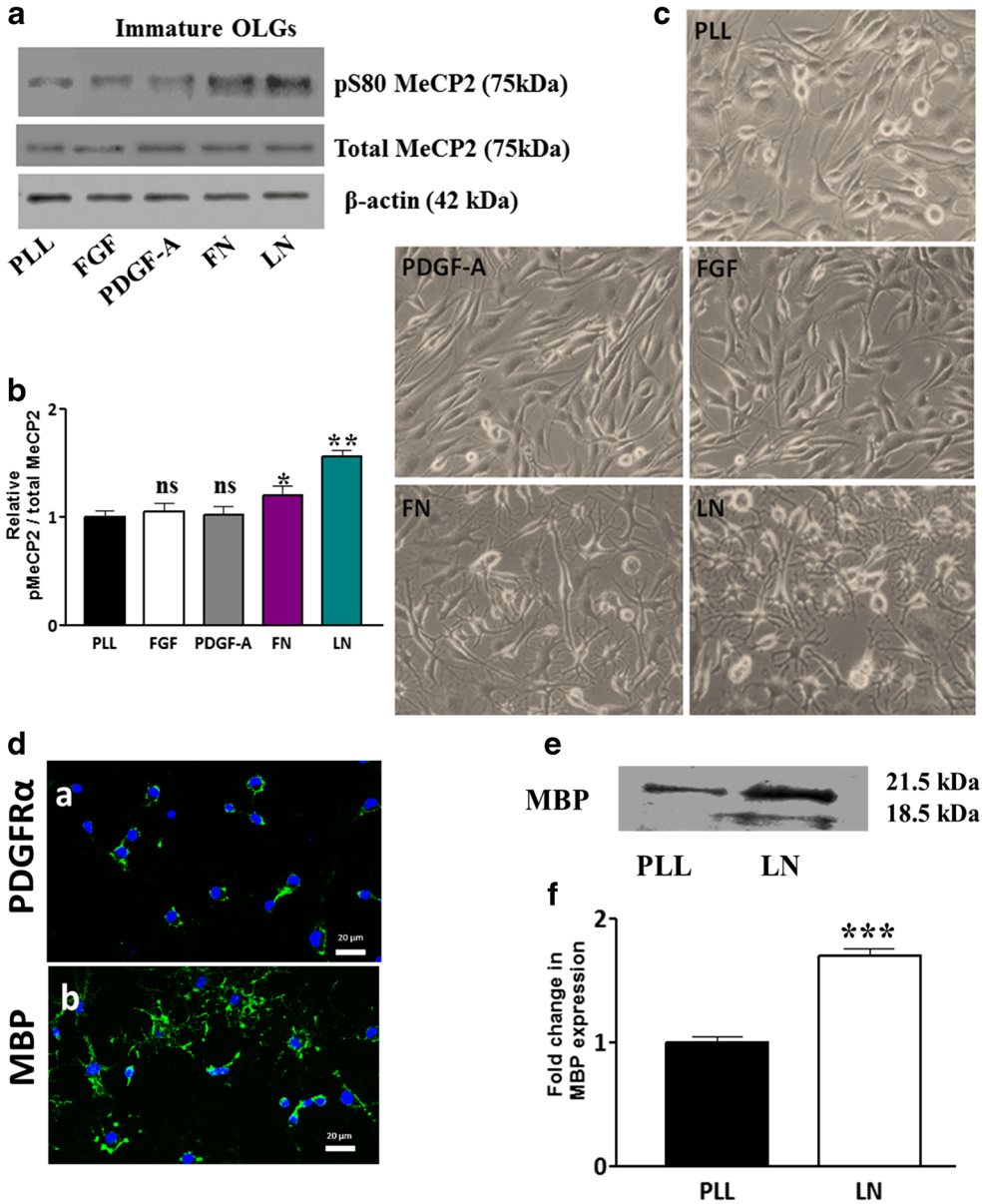

Fig. 2 MeCP2 S80 phosphorylation by growth factors and ECM. **a** Representative Western blot for phosphorylated (MeCP2 S80) and total MeCP2 in immature N19 OLGs exposed to FGF, PDGF-A, FN, and LN for 1 h. **b** Graph shows increased levels of pS80MeCP2 in LN-treated group. Values represent mean ± SEM. **c** Representative phase contrast images of N19 OLGs exposed to FGF, PDGF-A, FN, and LN for 24 h for morphological analysis. **d** Characterization of immature and mature N19 OLGs with cell-specific markers. Representative immunostaining images showing immature N19 OLGs with anti-PDGFRα (*green*—(*a*)) and mature N19 OLGs with anti-MBP (*green*—(*b*)). The nucleus was stained with DAPI (*blue*). *Scale bar* = 20 μm. **e** Representative Western blot for MBP in N19 OLGs exposed to PLL control and LN. **f** Graph shows increased levels of MBP in LN-treated group. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's *t* test or one-way ANOVA parameter where appropriate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

(2009) found that S80 phosphorylation is the highly conserved phosphorylation site of MeCP2.

During normal OLG development in the CNS, both GFs and ECM coexist in the cellular microenvironment, but the underlying mechanism by which they regulate OLGs behavior is poorly understood. Studies have shown the altered levels of GFs and ECM in the demyelinating lesions (Hughes et al. 2013). During development, extrinsic and intrinsic stimuli create epigenetic marks (Delcuve et al. 2009). These epigenetic modifications thereby alter the chromatin structure and modulate the transcription capability (Olynik and Rastegar 2012). It has been studied that MeCP2 is expressed at the level of histones and widely distributed across the genome. MeCP2 not only competes with the histones but also displaces them for chromatin binding sites (Ghosh et al. 2010; Nan et al. 1997). Studies in neurons have shown that diverse environmental stimuli affect MeCP2 function by differential phosphorylation (Zhou et al. 2006). So far, no study has been

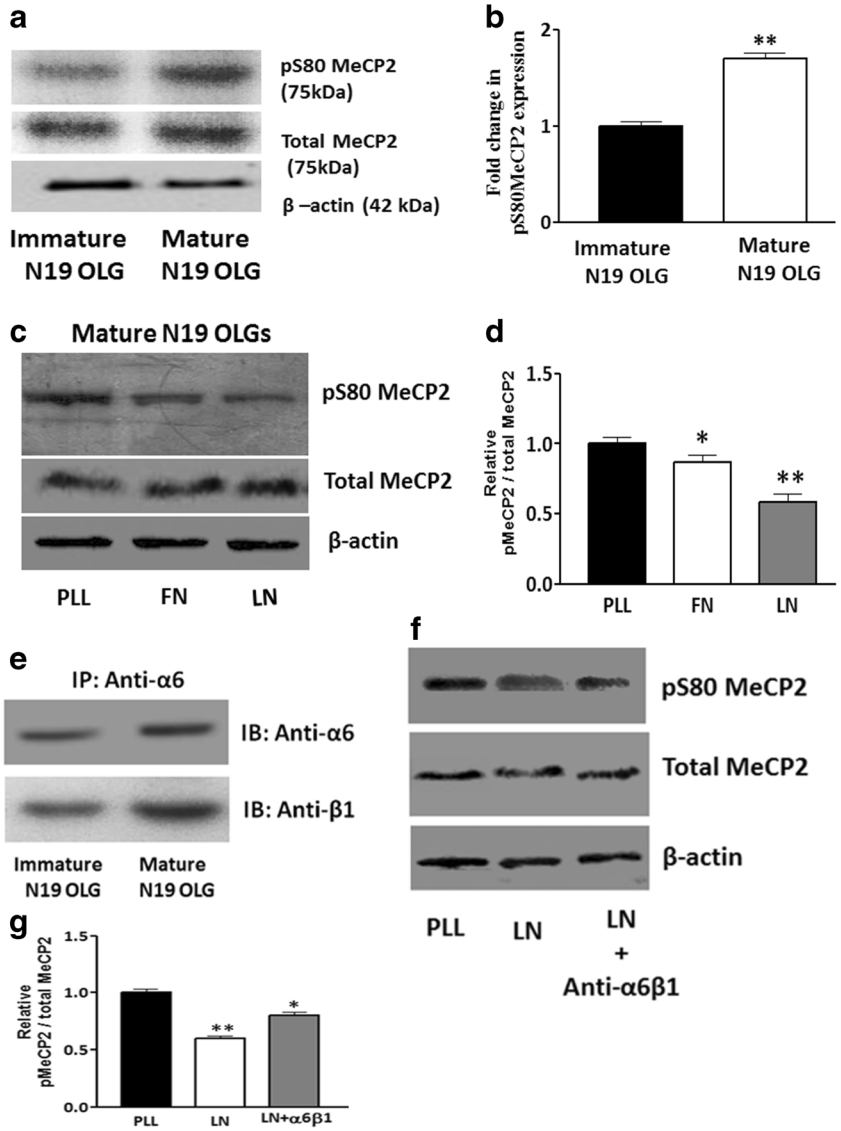

Fig. 3 Laminin decreases pS80MeCP2 in mature N19 OLGs. **a** Representative Western blot for pS80MeCP2 in immature and mature N19 OLGs. **b** Graph shows increased levels of pS80MeCP2 in mature N19 OLGs. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's *t* test or one-way ANOVA parameter where appropriate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **c** Representative Western blot for phosphorylated (MeCP2 S80) and total MeCP2 in mature N19 OLGs exposed to PLL control, FN, and LN for 1 h. **d** Graph shows decreased levels of pS80MeCP2 in LN-treated group. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's *t* test or one-way ANOVA parameter where appropriate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **E** Expression of α6β1 integrin in immature and mature N19 OLGs. Cell lysates from immature and mature N19 OLGs were incubated with α6 antibody. After immunoprecipitation, proteins were separated by SDS-PAGE under the non-reducing conditions and immunoblotted with indicated anti-α6 and anti-β1 antibodies. *IP* immunoprecipitation, *IB* immunoblot. **f** Representative Western blot for phosphorylated (MeCP2 S80) and total MeCP2 in mature N19 OLGs exposed to PLL control, LN, and anti-α6β1 integrin antibody. **g** Graph shows increased levels of pS80MeCP2 in anti-α6β1 integrin antibody-treated group. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's *t* test or one-way ANOVA parameter where appropriate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

carried out to demonstrate stimulus-dependent MeCP2 phosphorylation in OLGs. Two growth factors generally considered to play significant roles in oligodendrocyte development and myelination are PDGF and FGF2 (Armstrong et al. 1991; Fruttiger et al. 1999; Milner et al. 1997; Frost et al. 2009; Vora et al. 2011; Messersmith et al. 2000; Fortin et al. 2005; Bansal 2002; Bansal et al. 2003). Not only GFs but ECM like FN and LN also regulate the OLG behavior (Milner and Ffrench-Constant 1994; Milner et al. 1997; Milner et al. 1996; Tripathi et al. 2016). Hence, in the present study, we have investigated the individual effects of PDGF-A, FGF, FN, and LN on the MeCP2 phosphorylation at pS80 in N19 OLGs. PLL was used as control matrix. Our data shows that immature N19 OLGs exposed to LN resulted in enhanced

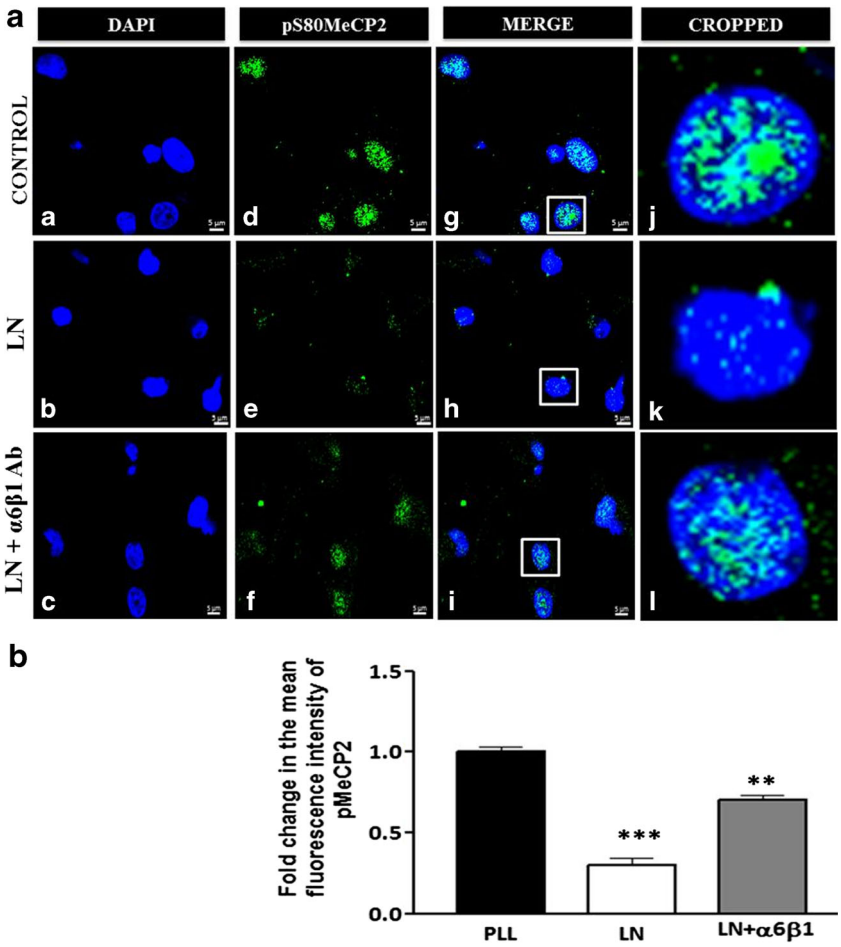

Fig. 4 Immunocytochemistry showing anti-α6β1 integrin antibody increases the pS80MeCP2 expression in LN-treated N19 OLGs. **a** Representative immunostaining images showing pS80MeCP2 expression in N19 OLGs (*green*; *d–f*). The nucleus was stained with DAPI (*blue*; *a–c*). Colocalization of pS80MeCP2 and DAPI shown in merge images (*g–i*). (*j–l*) are cropped images of (*g–i*). *Scale bar* = 5 μm. **b** Graph shows increased levels of pS80MeCP2 in α6β1 integrin blocking group. Values represent mean ± SEM

expression of pS80MeCP2 compared to other treatment groups (Fig. 2a, b). The data shows the importance of ECM more than GFs in the regulation of MeCP2 phosphorylation at S80 site.

Laminin Differentially Regulates the Expression of pS80MeCP2 in Immature and Mature N19 OLGs

Above results show that LN significantly increased the pS80 expression in immature N19 OLGs. But OLG shows different phases during development like early PDGF receptor alpha (PDGFRα)-positive OPCs, followed by O4-positive immature OLGs, and lastly differentiated MBP-positive mature OLGs (Yang et al. 2011). OPC differentiation is dependent on the external regulation. OPCs continue to proliferate but fail to differentiate in the presence of FGF and PDGF. Removal of GFs is essential for the proper differentiation of the OPCs to OLGs (Noble et al. 1988; Raff et al. 1988). Our results along with the others have shown that OPC migration also occurs in the absence of GFs, but OPCs must be plated on a permissive substratum (Kakita and Goldman 1999; Frost et al. 1996; Fruttiger et al. 1999; Tripathi et al. 2016). Not only during normal myelination but also during remyelination, ECM plays a critical role. The extracellular matrix is extensively altered during CNS injury (Gutowski et al. 1999; Sobel and Mitchell 1989; van Horssen et al. 2006; van Horssen et al. 2005; Satoh et al. 2009; Back et al. 2005). FN promotes OLG migration and proliferation, but reduces myelin-like membrane formation (Baron et al. 2005; Tripathi et al. 2016; Buttery and Ffrench-Constant 1999; Maier et al. 2005; Siskova et al. 2006). LN is a well-characterized ECM for OLG survival (Baron et al. 2003; Colognato et al. 2002) and myelination (Buttery and Ffrench-Constant 1999; Relvas et al. 2001; Siskova et al. 2006). LN is present on the surface of neurons, and OLGs contact neuronal LN through α6β1 integrin (O'Meara et al. 2011; Simons and Trajkovic 2006).

Morphological analysis of N19 OLGs showed that LN significantly increased the differentiation of immature N19 OLGs to mature N19 OLGs (Fig. 2c). Hence, we examined the effects of FN and LN on mature N19 OLGs. Following standard protocols, N19 OLGs were allowed to mature (described in "Materials and Methods" section). Maturation of N19 OLGs was confirmed by the MBP expression study using ICC (Fig. 2d (a, b)). We have checked the pS80MeCP2 expression in immature and mature N19 OLGs. The data

showed that pS80MeCP2 levels were higher in mature N19 OLGs compared to those in immature N19 OLGs (Fig. 3a, b). Then, the mature N19 OLGs were exposed to FN and LN. PLL was used as a control matrix. Western blot analysis showed significant decrease in pS80MeCP2 expression levels in the mature N19 OLGs exposed to LN compared to those exposed to FN and PLL (control) (Fig. 3c, d). Our data also confirmed that LN enhanced the expression of MBP in mature N19 OLGs (Fig. 2e, f). Earlier, we have shown that MeCP2 regulates MBP expression in OLGs (Sharma et al. 2015). Data from the present study shows that LN regulates MeCP2 phosphorylation at S80 site and MBP, both. Hence, pS80MeCP2 may be a link between LN-dependent MBP expressions.

We further verified the LN-dependent pS80MeCP2 phosphorylation by blocking LN receptor, α6β1-integrin, which is the only known LN receptor (Baron et al. 2003). The expression of α6β1-integrin in immature and mature N19 OLGs was checked by co-immunoprecipitation studies (Fig. 3e). Blocking α6β1 significantly increased the expression of pS80MeCP2 (Fig. 3f, g). We further confirmed these observations by pS80MeCP2 nuclear localization studies using confocal microscopy imaging. The data showed significant decrease in the LN-dependent nuclear pS80MeCP2 expression and colocalization as compared to the PLL control. However, blocking the LN receptor (α6β1) significantly increased the LN-dependent decrease in the nuclear pS80MeCP2 expression and colocalization (Fig. 4a). Nuclear colocalization was further confirmed by the study of the mean fluorescence intensity of the nuclear pMeCP2 in the LN and LN receptor blocking groups (Fig. 4b). Our data shows that LN exposure increased the pS80 MeCP2 expression in the immature N19 OLGs but decreased in the mature N19 OLGs. This shows LN-dependent differential regulation of the pS80 MeCP2 in OLG lineage progression. Our data along with others have shown the regulation of myelin proteins by MeCP2 (Ballas et al. 2009; Nguyen et al. 2013; Sharma et al. 2015). Studies have shown that MeCP2-S80 phosphorylation is associated with inhibition of certain genes and MeCP2-S421 phosphorylation is linked to gene activation (Tao et al. 2009). Our results suggest that LN-dependent phosphorylation dynamics of MeCP2 may play a critical role in the gene regulation during OLG development, and currently, the ongoing research in our laboratory is focused on determining the functional role of MeCP2 pS80 during OLG development.

In summary, our data provide the first evidence that MeCP2 is phosphorylated in a stimulus-dependent manner during oligodendrocyte development, and thereby, it may regulate the oligodendrocyte behavior. Overall, the data also pinpoints the importance of ECM more than GFs in the regulation of MeCP2 phosphorylation at S80 site. However, further efforts to better understand the mechanisms by which S80 phosphorylation regulates MeCP2 function in OLGs may further our understanding of how mutations in MeCP2 lead to the neurological phenotype of RTT. In-depth understanding of the importance of ECM-dependent signaling in epigenetic regulation will help in the development of well-targeted therapies for the pathobiology of RTT.

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Compliance with Ethical Standards

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

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