Dynamics of Ten-Eleven Translocation Hydroxylase Family Proteins and 5-Hydroxymethylcytosine in Oligodendrocyte Differentiation

Xianghui Zhao,1* Jinxiang Dai,2,3* Yue Ma,2 Yajing Mi,4 Daxiang Cui,2 Gong Ju,1 Wendy B. Macklin,3 and Weilin Jin2,4

The ten-eleven translocation (TET) family of methylcytosine dioxygenases catalyze oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and promote DNA demethylation. Despite the abundance of 5hmC and TET proteins in the brain, little is known about their role in oligodendrocytes (OLs). Here, we analyzed TET expression during OL development in vivo and in vitro, and found that three TET family members possess unique subcellular and temporal expression patterns. Furthermore, the level of 5hmC exhibits dynamic changes during OL maturation, which implies that 5hmC modification may play a role in the expression of critical genes necessary for OL maturation. siRNA-mediated silencing of the TET family proteins in OLs demonstrated that each of the TET proteins is required for OL differentiation. However, based on their unique domain structures, we speculate that the three TET members may function by different mechanisms. In summary, we have established the temporal expression of TET proteins and the dynamic level of 5hmC during OL development and demonstrate that all three TET members are necessary for OL differentiation.

Key words: epigenetic regulation, 5hmC, oligodendrocyte development, DNA hydroxylase

Introduction

The oligodendrocyte (OL) is the myelinating cell in the central nervous system and it plays important roles in brain development. Dysmyelination or remyelination failure in disease or after injury impairs rapid propagation of action potentials and leads to axon degeneration, which is associated with demyelinating disorders such as multiple sclerosis and leukodystrophy (Kalman and Leist, 2004). Extensive studies have focused on identifying the regulators of oligodendrocyte precursor cell (OPC) differentiation, to enhance remyelination in disease or injury.

OLs are derived from multipotential neural progenitor cells and the development of a functional OL includes cell fate commitment, OPC migration, proliferation, maturation, and ultimately myelination, and these processes are regulated by dynamic interplay between intrinsic and extrinsic signals, especially stage-specific transcription factors (Emery, 2010; He and Lu, 2013). Additionally, epigenetic events have also been shown to be critical for OL differentiation (Copray et al., 2009; Liu and Casaccia, 2010; Yu et al., 2010), including histone modification by HDACs and HATs, and post-translational silencing by small-noncoding RNAs. However,
how DNA modifications, or DNA cis-regulatory elements, are programmed during OL differentiation remains unclear, although Yu and Chen recently identified an important role for chromatin remodeling mediated by ATP-dependent SWI/SNF complex subunits in the initiation and promotion of OL lineage progression and maturation (Yu et al., 2013). Elucidating the key elements and factors that control the corresponding target gene expression would help us understand important elements of the regulation of OL development.

Methylation at the 5-position of cytosine (5mC) is one of the major epigenetic modifications in the genome, and the 5mC methylation pattern is accurately preserved during mitotic inheritance through the action of DNA methyltransferases (DNMTs; Goll and Bestor, 2005). Cytosine methylation plays an important role in key biological processes, such as X-chromosome inactivation in females, allele-specific silencing of imprinted genes, and transcriptional repression of transposons. The recent rediscovery of 5hmC (hydroxymethylcytosine), an intermediate in the demethylation of cytosines, in mammals reveals that covalent DNA modifications are more dynamic than previously believed (Blutani et al., 2011; Dahl et al., 2011). 5hmC is abundant in embryonic stem cells (ESCs) and adult brain cells (Ito et al., 2010; Jin et al., 2011; Koh et al., 2011; Suzlach et al., 2011b; Wu et al., 2011a), and extensive studies on 5hmC genome-wide mapping suggest its role in transcriptional regulation as an novel epigenetic mark (Jin et al., 2011; Shen and Zhang, 2013; Wang et al., 2012; Wu et al., 2011a; Xu et al., 2011).

Although not rigorously confirmed, the ten-eleven translocation (TET) family protein-mediated oxidation of 5mC is apparently the only source of 5hmC in mammalian cells (Ito et al., 2010; Tahiliani et al., 2009). TET family contains three members, TET1, TET2, and TET3, all of which share high homology in their C-terminal catalytic domains (Tan and Shi, 2012). The discovery of this family of enzymes suggested a potentially novel mechanism for the regulation of DNA methylation, with 5hmC acting as an intermediate during DNA demethylation, although the biology and regulation of 5hmC and TET family enzymes during development remain elusive.

To investigate the involvement of TET family proteins and 5hmC modification in OL differentiation, we first performed Western blot and immunostaining assays to examine TET protein expression, as well as the level of 5hmC in OLs at different developmental stages. Although all three TET family members are expressed in OLs, each of them reveals a unique expression pattern. The level of 5hmC is also highly correlated with OL differentiation, which implies that 5hmC modification might be critical for the switch on/off of certain genes that regulate OL development. Remarkably, we found that eliminating the function of any of the TET proteins by siRNA mediated-knockdown significantly inhibited OL maturation. These studies uncover a dynamic pattern of TET protein expression and DNA cytosine modification during OL differentiation, and provide evidence that TET family members are essential for the normal development of the OL lineage.

**Materials and Methods**

**Antibodies**

TET1 antibody was prepared in rabbit against a synthetic 14 amino acid antigen "PALGVKHSENDSPVC" of human TET1 (from 146aa to 159aa); TET3 antibody was prepared in rabbit against a short peptide antigen “GQQEAKLYGKKRC” of human TET3 (from 1598aa to 1610aa). These two peptides are conserved among human, mouse and rat TET1 and TET3 proteins, respectively. These antibodies were purified by antigen affinity column. Rabbit anti-TET2 antibody was purchased from PTGLAB. Rabbit anti-5hmC antibody (#39769), anti-5ac antibody (#61225), anti-5fc (#61223), and monoclonal 5mC antibodies (#39649) were all from Active Motif. Antibodies against OL markers are as follows: Monoclonal antibody against Nogo-A has been used in our previous study (Zhao et al., 2007), rabbit anti-PDGF-Rζ (Abcam, ab61219), goat anti-Sox10 antibody (Santa Cruz, sc-17342), rat Plp monoclonal antibody (AA3 clone; a gift from Dr. S. Pfieffer), mouse monoclonal CC1 antibody (Calbiochem, #OP80), mouse anti-CNPase (Sigma, C5922), and rat monoclonal anti-MBP (Abcam, ab7349).

**DNA Constructs and siRNAs**

Full length of TET1, 2, 3 constructs were obtained from Dr. Heinrich Leonhardt, Dr. Ross Levine, and Dr. Toshinobu Nakamura, respectively. TET1 specific siRNA (GCTCATGAGAGCTAAGGGTATGG), two TET2 specific siRNAs (TET2-1 GTGATGTAAGTT TGCCAGAAGC, TET2-2 CTCAGGGATGTCCTATTGCTAAA), two TET3 specific siRNAs (TET3-1 GTCTCAAGCAGAAGCTAT TTG, TET3-2 AAGCCAACTATCTTCGGAAT), and control scramble siRNA (GCCGGCGATCGAGCCCAT) were synthesized by Ribobio (Guangzhou Ribobio), based on published studies (Ito et al., 2010; Williams et al., 2011). TET2-1-shRNA was constructed by GenePharma (Shanghai GenePharma) into a lentivirus expression vector pGLVH1/GFP+Puro.

**Tissue Collection and Immunohistochemistry**

All animal experimental protocols were approved by the Animal Care and Use Committee of Fourth Military Medical University and University of Colorado Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Perfusion of mice and immunostaining for mouse tissue was performed generally as described previously (Trapp et al., 1997). On day 1, free-floating brain sections (30 μm) were washed three times in PBS and antigen retrieval was performed with 10 mM sodium citrate (pH = 6.0) at 65°C for 10min in a BioWave (Ted Pella). Sections were rinsed in PBS twice, blocked in blocking solution (200 mM NaCl, 50 mM Tris-HCl, 100 mM l-lysine, and 150 mM Glycine) containing 3% normal donkey serum (NDS) and 1% Triton X-100 for 1 h at RT;

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and incubated in primary antibodies in 3% NDS containing 0.5% Triton X-100 for 72 h at 4°C. All TET antibodies were used at 1:100, Sox10 and CC1 antibodies were used at 1:200. On day 4, sections were washed three times in PBS, incubated with secondary antibodies (1:800) at RT, and then counter stained with DAPI for 5 min. Finally, sections were washed three times in PBS and mounted in Vectashield (Vector Laboratories). Images were taken on a Zeiss Axios Imager M2 with Apotome module. For the staining of DNA cytosine derivatives (5hmC, 1:5000), before permeabilization, samples were subjected to DNA denaturation with 2N hydrochloric acid at 37°C for 20 min and then neutralized with 0.1 M sodium borate at pH 8.5 for 2 × 10 min.

Oligodendrocyte Primary Culture
Isolation and culture of rat OPCs followed protocol as previously described (Zhao et al., 2010). Briefly, brains were removed from P2 Sprague Dawley rat pups, and the cortices were dissected. Cortical pieces were enzymatically digested followed by mechanical dissociation. Cells were resuspended in DMEM with 10% fetal bovine serum and plated onto T75 flasks. The resulting mixed glial cultures were maintained for 7–10 d. Purified OPCs were prepared by differential shaking and were seeded onto poly-l-ornithine–coated 35-mm dishes at the density of 3 × 10⁴ cells/cm² in OL growth medium (Sato medium supplemented with fibroblast growth factors, and plasmid-derived growth factor AA). OPCs were amplified in growth medium supplemented with fibroblast growth factors, and platelet-derived growth factor AA). OPCs were amplified in growth medium for ~4 d and passed with isolation medium (0.2% DNase I + 5 µg/ml insulin + 0.04% EDTA). To initiate differentiation, OPCs were seeded onto poly-l-lysine–coated dishes or coverslips in differentiation medium with triiodothyronine, ciliary neurotrophic factor (CNTF), and N-acetyl-L-cysteine (NAC). Experiments were replicated using cells from three different primary cultures.

Transient Transfection and Immunocytochemistry
For in vitro TET knockdown, OPCs were transfected with 50 nM duplex siRNA against individual genes or control nontargeting siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Six hours after transfection, the cultures were changed to differentiation medium. Three or four days later, cultures were harvested for immunocytochemistry and qRT-PCR assay as indicated. For TET2-shRNA transfection, OPCs were electroporated according to the manufacturer’s protocol (Amaxa, Roche Applied Science) and the progression of transfected cells was analyzed 3 or 5 d later.

To test the effect of TET2 on the regulation of 5hmC level, HEK293T cells were transfected with TET2 shRNA plasmid using Lipofectamine 2000. Forty-eight hours later, cells were analyzed by immunostaining for 5hmC.

For immunocytochemistry assay, OL cultures were fixed in 4% paraformaldehyde. After permeabilization for 15 min, samples were incubated with primary antibody for 1 h at room temperature followed by fluorescent secondary antibody for another hour. Samples were counter-stained with DAPI and visualized with an Olympus confocal microscope.

RNA Extraction and qRT-PCR
Total RNAs were purified from cell cultures using TRIzol reagent according to the manufacturer’s instruction (Invitrogen). RNA was transcribed to cDNA with the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). qRT-PCR was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA), and relative gene expression was normalized to internal control GAPDH. Primer sequences for target genes are as follows. Rat GAPDH: ACAAGATGCT GAAGGTCCCTGTGTA and AGGTTGCCATTTCTCAGCTT; rat MBP: GTACACTTGGCCTTCTAGT and CTGGAAATTCTAGCTCCCTCATC; rat PLP: TCTTTTGCGACTACAA GACCCCA and CAAAACATTGAGACACCGGCTCAAA; rat Hes1: AGAAAAATATTCTGTGCCCCG and TTTCAATTTATTTCTGGC CGC; rat ID2: ATGGAAATCTCTGAGCAGCAGCTAC and ACTGT TGGTTCGTCAGGGTCCTCT; rat PDGFβ: TGTGGACATT GACACGGGTACAT and ATCTCTGGTCATCCAGGCACTT; rat TET1: GAAGAGCAAAAGGTGTTGGTGGTTTG and CGTTGTT CGGTGTCACCTCCT; rat TET2: CCTCAACAGGTCTCATTCA TAC and GGTGACTCTTGGTCTTTTATATG; rat TET3: GGGAA GTAGTGTGGTTAGA and GTAGGACAGCAGATGAA.

Western Blot Assay and Nuclear/Cytoplasmic Fractionation
For Western blot analysis, whole cell lysates were prepared from tissue or cells using RIPA buffer. For nuclear and cytoplasmic extracts, subcortical white matter tissue was lysed in hypotonic buffer (10 mM HEPES (pH 8), 2 mM MgCl₂, 10 mM KCl, and 0.5%NP40). Nuclei were then isolated by centrifuging at 1500g for 5 min, and washed in hypotonic buffer 3 times, then resuspended in the same buffer with 500 mM NaCl plus protease inhibitor cocktail from Roche. Protein concentrations in centrifugation-clarified cell lysates were measured by the BCA Protein Assay Kit (Pierce) and equal amounts of protein were separated on SDSPAGE gel and transferred to Hybond PVDF (Amersham Biosciences). For protein blotting, primary antibody against Beta-tubulin (1:5000), TET1 (1:500), TET2 (1:1000), and TET3 (1:500) were used, respectively. Signals were developed with horseradish peroxidase–conjugated secondary antibodies, followed by ECL kit (Amersham Biosciences) or Li-Cor Odyssey system.

Statistical Analysis
Quantifications were performed from at least three independent experiments, and data were presented as mean±SEM in the graphs. Student’s t test was used to compare two sets of data, and a value of P < 0.05 was considered statistically significant.

Results
Antibody Preparation and Characterization
We generated polyclonal antibodies against TET1 and TET3 protein (Fig. 1A). To characterize these antibodies, GFP or Flag-tagged full length mouse TET cDNAs were constructed and transfected into HEK293T cells. Western blot showed that both TET1 and TET3 antibody specifically recognized an appropriately sized band (Fig. 1B). For TET2, we used a commercial antibody, which also identified the expected band in Flag-TET2 transfection group. Given the appearance of weaker bands with similar size in parallel transfections, we
speculated that HEK293T cells expressed endogenous TET2, and there was a smaller TET2 isoform in this cell line that could be recognized by this antibody (Fig. 1B). Meanwhile, double-immunocytochemistry with TET and tag antibodies in transfected HEK293T cells confirmed the reactivity of these antibodies (Fig. 1C). The three TET antibodies were also tested on Western blots of rodent brain or cell culture samples, which identified isoforms of these proteins (Supp. Info. Fig. S1).

Expression of TET1-3 During Differentiation of OL Cultures

Western blot and quantitative real-time PCR (qRT-PCR) analyses were done on purified OL cultures to investigate TET family gene expression. OPCs were induced to differentiate by mitogen withdrawal as well as addition of CNTF, NAC, and triiodothyronine (T3). TET1 and TET3 protein expression decreased as OLs differentiated (Fig. 2A,B,D), but not for that of TET2, which showed a rather constant level (Fig. 2A,C) and appeared a ~130 KD band in Western blot assay initially as OLs began to differentiate (Fig. 2A, red star). From analysis of subcellular extractions from P14 cortex (Fig. 2E), we speculated that this ~130 KD band represented the nuclear isoform of TET2. By contrast to its protein expression, TET1 mRNA increased robustly during early OPC differentiation, and then decreased with later maturation (Fig. 2F); TET2 and TET3 mRNA levels were comparable to their protein expression, respectively (Fig. 2F).

TET1 is Downregulated in Mature OL

In the developing brain, the number of TET1/Sox10 double-positive cells was highest in P1 and P4 corpus callosum (Fig. 3A,B), at which time most OLs are in their early or later progenitor stage respectively. TET1 in Sox10+ cells was dispersed in the cytoplasm at P1 [Fig. 3A(c,d)] and then concentrated into the nucleus at P4 [Fig. 3A(g,h)]. From P10, TET1 expression in Sox10+ cells decreased markedly compared with that of early stages (Fig. 3A,B). Notably, TET1/Sox10 double-positive cells were barely detectable at P30, the peak of myelinogenesis, despite the presence of Sox10-expressing cells. To confirm this, P7-P25 corpus callosum protein samples were analyzed by Western blot, which showed that the peak of TET1 expression was P10, with reductions thereafter (Fig. 3C). This age-dependent expression pattern of TET1 was further tested in cell cultures: TET1 immunoreactivity was detected throughout the OPC, that is, in the cytoplasm, the nucleus and the cell processes, which was similar to hippocampal neurons (Kaas et al., 2013); its expression then fell and TET1 was barely detectable in differentiating CNPase+ and mature MBP+ cells (Fig. 3D).

TET2 is Translocated Between Cytoplasm and Nucleus During OL Differentiation

In the developing brain, TET2/Sox10 double-positive cells were prominent up to P30 in the corpus callosum (Fig. 4A,B), consistent with the Western blot results for corpus callosum samples.
At P1 and P4, TET2 immunoreactivity in Sox10+ cells was mainly dispersed in the cytoplasm. From P7, the initiating stage of myelinogenesis, TET2 was expressed in both the cytoplasm and the nucleus of Sox10+ cells. Interestingly, as the brain developed, the immunoreactivity in the nucleus aggregated into smaller speckles [Fig. 4A(o,p)]. These speckles appear to be specific, since the TET protein oxidation product was also noted to have this distribution (see below). At p20, a diffusely punctated staining of TET2 that is not related to cells was observed, which also appeared in p10 and p15. We believe that this is due to the nonspecific staining and is different from the nuclear staining in p10. Meanwhile, the developmental shift in TET2 distribution was consistent with our Western blot results showing the appearance of novel nuclear isoforms of TET2 as OLs differentiate (Fig. 2A). This was further confirmed in cell culture staining. OPCs were expressed TET2 in the cytoplasm and the processes, but as the cells differentiated to a CNPase+ immature stage, distinct nuclear staining of TET2 was observed (Fig. 4D). Then in MBP+ mature cells, TET2 was partially translocated back to the cytoplasm, retaining major expression in the nucleus. The stage-dependent appearance of nuclear isoforms implies that TET2 may be involved in initiating OL maturation.

**TET3 is Exclusively Expressed in the Nucleus of Developing OLs**

Compared with its family members, TET3 displayed rather steady expression in the nucleus during the OL differentiation (Fig. 5). In the developing brain, TET3 was located in the nucleus of Sox10+ cells, and the nuclear punctate staining of TET3 could be detected at P10, but was most prominent at P15 and P20 (Fig. 5B). TET3 was no longer detectable in OLs at P30 (Fig. 5A,C). Western blots of corpus callosum samples showed high expression of TET3 at early time points, which was reduced as animals developed, consistent with the immunostaining results (Fig. 5D). The striking nuclear location of TET3 was seen at all differentiation stages in cultured OLs (Fig. 5E). These observations suggest that nuclear TET3 might be important for the normal development of OL.

**The Expression of 5-Hydroxymethylcytosine (5hmC), the TET Protein Oxidization Product, in OL Lineage**

In the developing brain, all Sox10+ and CC1+ cell nuclei were labeled with 5hmC and showed an increasing signal as OLs differentiated (Fig. 6A,B). P1 and P4 corpus callosum OLs were weak for 5hmC immunostaining, whereas maturing OLs from P7 to P30 had increased signals. We also noticed that numerous cells positive for 5hmC do not demonstrate a staining for Sox10 or CC1. We presume that the 5hmC+/Sox10− cells in neonatal corpus callosum are in their precursor/progenitor stage that are negative or weak for Sox10 expression. Similarly, CC1 is a later marker for OL lineage, and 5hmC+/CC1− cells in corpus callosum
are either OPCs or immatures OLs in respective panels. In purified cell cultures, the 5hmC signal was strongest in immature CNPase\textsuperscript{1} cells (Fig. 6C).

In contrast to the pattern of 5hmC expression in differentiating OLs, the signal for 5-methylcytosine (5mC), which is believed to label silenced genes (Dahl et al., 2011), was expressed in immature cells and reduced upon cell maturation. In MBP\textsuperscript{1} cells, 5mC was hardly detectable (Supp. Info. Fig. S3A).

TET proteins can oxidize 5mC or 5hmC further and convert them to 5-formylcytosine (5fC) and/or 5-carboxylcytosine (5caC; He et al., 2011; Ito et al., 2011), although at much lower levels than 5hmC, and their functions have not been established. In this study, we examined the expression of 5fC and 5caC in OL cultures by immunocytochemistry. Both 5fC and 5caC were weaker in OL lineage compared to their 5hmC derivates (Supp. Info. Fig. S3B,C). In particular, 5fC was undetectable in CNPase\textsuperscript{1} immature cells (Supp. Info. Fig. S3B), which was consistent with the fact that 5hmC is very high at this stage.

**TET Proteins Are Required for OL Maturation**

To determine the effects of TET proteins on OL differentiation in vitro, we transfected OPCs with siRNAs against individual TET1-3 members and control nontargeting siRNA. We first tested the efficiency of several reported TET1-3 sequences in knocking down TET expression in OL cultures. qRT-PCR indicated that three sequences (TET1-siRNA, TET2-siRNA-1, and TET3-siRNA-1) decreased TET mRNA expression (Fig. 7A), but not other family members (data not shown). These were then used in subsequent studies. TET mRNA knockdown in OL cultures increased the mRNAs encoding several myelination inhibitors, such as Hes1, Id2, and the OPC marker Pdgfr\alpha; at the same time, the expression of myelin genes\textit{Mbp} and\textit{Plp} was inhibited (Fig. 7B). At 3 d post-transfection, 43.5 ± 3.99% control transfected cells had differentiated into immature OLs with complex processes, expressing CNPase (Fig. 7C,D). In contrast, all three TET siRNA-transfected cultures showed lower branched morphology and smaller outgrowth area. The number of CNPase\textsuperscript{+} cells decreased to 31.5 ± 2.59%, 23.0 ± 2.68%, and
25.36 ± 4.21% in TET1, TET2, and TET3 siRNA transfection groups, respectively (Fig. 7D). At 4 d post-treatment, ~80% cells in the control group expressed the mature OL marker MBP, but that number decreased to 30–40% in TET siRNA transfected cultures, suggesting that most cells were arrested at their late precursor stage (Fig. 7C,D). Meanwhile, we have also tested the effect of two siRNA-2 (ineffective sequences) on the differentiation of OLs, but did not see their ability to reduce the percentage of mature OLs (data not shown).

To further confirm the essential function of TET proteins in OL development, we constructed shRNA vector against TET2 with a GFP tag, to observe individual transfected cells. Purified OPCs were electroporated with TET2-shRNA plasmid and control scrambled shRNA plasmid. Three days later, cells were analyzed by Western blot, which demonstrated that TET2 protein expression was reduced to ~30% in the TET2-shRNA transfection group compared with control (Supp. Info. Fig. S4A). Meanwhile, transfection of TET2-shRNA in HEK293T cells, which do not express either of the other two TET family members (Fig. 1B), was effective in reducing the level of 5hmC (Supp. Info. Fig. S4B). At 5 d post-transfection in OPCs, the cultures were stained for MBP to analyze the differentiation of GFP-expressing cells. Most scramble-shRNA expressing cells in control group were immunopositive for MBP, but almost none of the TET2-shRNA-treated cells expressed MBP, despite the fact that some had highly branched processes (Fig. 7E,F). These results indicated that although all three TET family members regulated the differentiation of OLs, TET2 is particularly critical for the expression of some important myelin genes, such as MBP.

**Discussion**

Since the discovery that the enzymes TET1, TET2, and TET3 catalyze 5mC to 5hmC conversion in genomic DNA (Ito et al., 2010; Tahiliani et al., 2009), extensive research has
focused on their role in regulating active DNA demethylation. TET proteins have roles in diverse biological processes, including epigenetic regulation of gene transcription, embryonic development, stem cell function, haematopoietic differentiation and cancer (Cimmino et al., 2011; He et al., 2011; Mercher et al., 2012; Pastor et al., 2013; Tan and Shi, 2012), but their role in brain development, which has the highest 5hmC level of any mammalian tissue (Globisch et al., 2010; Ito et al., 2011; Szwagierczak et al., 2010), remains largely unknown. In our study, a correlation between the progression of OL maturation and the expression of TET family members, as well as 5hmC, was established. The effect of TET knockdown on the development of OLs was also tested.

TET enzymes are present in all metazoans that have retained cytosine methylation (Iyer et al., 2009), but the dynamic and overlapping expression of the family members in various cell types of the embryo and adult poses a challenge to dissecting the function of each of the three proteins. In OLs, three TET proteins show distinctive expression patterns during development (Summarized in Fig. 7G). For TET1, Western blot and immunostaining revealed that its protein level is highest in OPCs and declines with OL maturation. We speculated that TET1 might exert its functions mainly in the early or later OPCs, and this presumption was further strengthened by the almost undetectable immunoreactive signal for TET1 in immature cultured OLs. siRNA downregulation of TET1 mRNA was quite effective in cultured OLs (Fig. 7). The reduction in TET1 mRNA had a small effect upregulating OL inhibitor mRNAs and downregulating myelin gene expression, in particular MBP. It reduced the number of CNPase+ cells slightly, but had a much greater impact on the number of MBP+ cells. Thus, it appears to have a small but positive effect on OL differentiation.

TET2 showed a totally different expression pattern in OLs. Both Western blot and qRT-PCR indicated a rather
constant level of TET2, and to our surprise, immunostaining in tissue and cell samples demonstrated subcellular translocation of TET2 from the cytoplasm to the nucleus at the initiation of myelinogenesis. Consistent with this, Western blot of the nuclear/cytoplasmic fractionation from P14 cortex distinguished the nuclear isoform of TET2 that appeared at the beginning of OL differentiation in vitro. Only 40% downregulation of the TET2 mRNA resulted in significant increases in the expression of OL differentiation inhibitors and downregulation of PLP and MBP mRNAs. The number of CNPase or MBP cells was more dramatically reduced than for TET1 downregulation. Thus its expression appears to be a strong driver of OL differentiation.

Western blot and qRT-PCR analysis of TET3 suggested that it decreased slightly with OL maturation, but this trend was not obvious by immunocytochemistry, where it appeared relatively constant at all stages. This might result from low sensitivity of immunohistochemistry in determining a potentially subtle change. Strikingly, TET3 is the only TET family member that localizes only to the nucleus in cells of the OL lineage, as has been seen in other cell types (Gu et al., 2011; Ito et al., 2010; Nakamura et al., 2012; Xu et al., 2012). The mouse TET3 protein contains five nuclear localization signals (NLS), and one of them has recently been identified to embed within the C-terminal catalytic domain (Xiao et al., 2013), which is conserved for TET3 orthologous protein among other species, but not conserved among the TET family. Thus, its nuclear localization appears to be a consistent characteristic of TET3 proteins, likely resulting from its primary structure. As with TET2, its downregulation had dramatic impact reducing OL differentiation, suggesting that TET3 is also an important positive regulator of OL differentiation.

Depletion of TET2 and TET3 in the developing mouse cortex, by in utero electroporation of shRNA, blocks differentiation of neural progenitor cells into neurons (Hahn et al., 2013). Our in vitro knockdown data demonstrate that every member of TET family enzymes is necessary for OL differentiation. However, due to the distinct expression pattern of

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**FIGURE 6:** Immunostaining of 5hmC in OLs during development. A: 5hmC specific immunostaining in OLs of developmental corpus callosum from P1 to P30 mice. Grid in (x) and (b’) is 5hmC+ cell with nuclear punctate staining. Scale bar, 50 μm in (a), 25 μm in (b). B: Histograms depict the percentage of 5hmC/Sox10 double positive cells among Sox10-expressing cells in corpus callosum of brain sections as indicated (n = 3). C: Purified OLs at indicated stages were double-immunostained with 5hmC and OL markers. Arrows indicate OLs with 5hmC staining. Scale bar, 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
FIGURE 7: TET family members are required for the differentiation of OLs in vitro. A: OPCs were transfected with TET siRNA and a negative control siRNA. qRT-PCR was assayed 48 h after transfection and the decreasing expression of individual TET mRNA was observed. Data represented as mean ± SEM. *P < 0.05, Student t test. B: Relative expression level of Hes1, Id2, Pdgfr, Pip, and Mbp was analyzed by qRT-PCR from TET siRNA transfected differentiating OL cultures. GAPDH was used as internal control. Data represent mean ± SEM. *P < 0.05, Student t test. C: TET siRNA transfected OPCs were induced to differentiate for 3–4 days and subjected to immunostaining for CNPase and MBP subsequently. Scale bar, 50 μm. D: Histogram depicts the percentage of CNPase+ and MBP+ cells from individual treatment. Data represented as mean ± SEM. *P < 0.05, one-way ANOVA. E and F: OPCs were electroporated with TET2-shRNA-GFP or control scrambled shRNA-GFP plasmid. After differentiation for 5 days, the cultures were immunostained with MBP antibody and the expression of MBP in GFP+ cells was analyzed. Representative images are GFP+ cells that positive for MBP in scrambled shRNA group (E) or negative for MBP in TET2-shRNA group (F). G Schematic diagram summaries the expression of TET family members during the differentiation of OLs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
each TET protein in OLs and the characteristic domain structure of the individual proteins, we speculate that they execute their actions through different mechanisms. Clearly, downregulation of only one of the TET family proteins reduces OL differentiation. Thus, they appear not to be able to compensate for each other.

All TET proteins contain a catalytic C-terminal CD domain (Cys-rich and DSBH regions) that belongs to the Cupin-like dioxygenase superfamily and exhibits 2-oxoglutarate- and iron (II)-dependent dioxygenase activity. TET proteins oxidize 5mC into 5hmC via these CD domains and require α-ketoglutarate as a cosubstrate for enzymatic activity (Tahliliani et al., 2009). Another distinct feature of TET family proteins is the CXXC zinc-finger domain, which can be found in the N-terminus of TET1 and TET3 but not in TET2, due to a chromosomal inversion event in which the exon containing the CXXC domain was detached and became a separate gene.

The CXXC domain of TET1 binds CpG sequences regardless of whether the cytosine is modified or unmodified (Xu et al., 2011; Zhang et al., 2010). Consistent with this, genome-wide mapping of TET1 binding by ChIP-seq approaches revealed its enrichment around transcription start sites in mouse ES cells (Williams et al., 2011; Wu et al., 2011b; Xu et al., 2011). There is a strong statistical correlation between the physical presence of TET1 at a promoter and increased gene expression from this promoter upon TET1 knockdown. Thus, it has been proposed that TET1 has a repressive role in target gene expression (Williams et al., 2011; Wu et al., 2011b; Xu et al., 2011). However, a recent study suggests that TET1 regulates the expression of some neuronal activity-dependent, immediate early genes in hippocampal pyramidal cells independently of its activity in 5mC to 5hmC conversion (Kaas et al., 2013), which implies that TET1 can regulate gene expression in a hydroxylase-independent way. Given its high expression in OPCs and the fact that its downregulation reduces differentiation, it may be required to induce expression of molecules needed for differentiation, potentially by some hydroxylase-independent pathway. Affinity measurements and X-ray crystallography demonstrate that the TET3 CXXC domain binds unmodified cytosine irrespective of whether it is followed by a guanine, (Xu et al., 2012). TET3 acts as a transcription factor at least partially by regulating the 5mC/5hmC status at target gene promoters and it is crucial for the expression of a set of key developmental genes (Xu et al., 2012). Since TET3 is localized in OL nuclei and silencing its expression results in the arrest of OL differentiation, we speculate that a set of OL developmental genes may act as TET3 targets.

Recently, Ko et al. suggested that like other TET proteins, TET2 also binds DNA through the Cys-rich region of its catalytic domain, and binds different genomic regions depending on the presence of another CXXC domain protein IDAX (Ko et al., 2013). In contrast to TET1, the physical presence of TET2 at a promoter is correlated with decreased gene expression from this promoter upon TET2 depletion, which indicates that TET2 is generally a positive regulator of gene expression (Chen et al., 2013). Thus, the nuclear aggregation of TET2 in the initiation stage of OL differentiation, together with the loss of function data, implies that the nuclear isoform of TET2 may be involved in the switching on of critical genes that initiate OL differentiation. However, the immunostaining signal for 5hmC is increasing at the same time that TET2 translocates to OL nuclei, which suggests another pathway for TET2 in transcriptional regulation.

5hmC has been reported showing the highest levels in the brain (Globisch et al., 2010; Ito et al., 2011; Szewagierczak et al., 2010), as high as 40% of 5mC in Purkinje neurons (Kriaucionis and Heintz, 2009). However, its functions in the nervous system remain largely unrevealed. Ruzov et al. observed that differentiation of neural progenitors towards OL lineages is characterized by the progressive loss of 5hmC staining (Ruzov et al., 2011). Brent et al. reported that the adult and pediatric white matter in human brain samples showed relatively high 5hmC staining, but it was variable within the oligodendroglial population and some cells showed almost no staining, suggesting epigenetic plasticity in oligodendroglial populations (Orr et al., 2012). Consistent with these studies, we also observed dynamic changes in 5hmC level in OL development.

Large-scale mapping has revealed the mammalian genomic distribution of 5hmC. In human and mouse ES cells, mouse neural progenitor cells and mouse neurons, 5hmC is enriched at promoters, and genes with hydroxymethylated promoters are expressed at lower levels than other genes (Hahn et al., 2013; Pastor et al., 2011; Stroud et al., 2011; Szulwach et al., 2011a; Wu and Zhang, 2011), suggesting a negative relationship between promoter hydroxymethylation and gene expression. However, in virtually all mammalian cell types studied, including mouse brain, 5hmC is enriched in gene bodies (Hahn et al., 2013; Lian et al., 2012; Szulwach et al., 2011a, 2011b; Thomson et al., 2012) and gene expression correlates positively with gene body hydroxymethylation (Mellen et al., 2012; Szulwach et al., 2011b; Wu et al., 2011a), although the reason for this correlation is unclear. In all mammalian cells, 5hmC is strongly enriched at enhancers (Stadler et al., 2011; Stroud et al., 2011; Szulwach et al., 2011a; Thomson et al., 2012; Yu et al., 2012), which are often regions of low CpG density with reduced levels of DNA methylation compared with neighboring regions. Notable, in differentiating ES cells, 5hmC levels increase sharply at activated enhancers, which rise almost immediately with the onset of differentiation
(Serandour et al., 2012). These different locations and apparent function indicate that further genomic mapping studies for 5hmC and its derivatives during OL differentiation is critical for understanding their function and mechanisms, as well as the role of the TET family members in OL development.

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References


