Published in final edited form as:
Glia. 2010 January 15; 58(2): 157–168. doi:10.1002/glia.20909.

TUBULIN POLYMERIZATION PROMOTING PROTEIN (TPPP/p25) IS CRITICAL FOR OLIGODENDROCYTE DIFFERENTIATION

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Abstract

TPPP/p25, a recently identified tubulin polymerization promoting protein (TPPP), is expressed mainly in myelinating oligodendrocytes of the CNS. Here we show that TPPP/p25 is strongly up-regulated during the differentiation of primary oligodendrocyte cells as well as the CG-4 cell line. The microRNA expression profile of CG-4 cells before and after induction of differentiation was established and revealed differential regulation of a limited subset of microRNAs. miR-206, a microRNA predicted to target TPPP/p25, was not detected in oligodendrocytes. Over-expression of miR-206 led to down-regulation of TPPP/p25 resulting in inhibition of differentiation. Transfection of siRNAs against TPPP/p25 also inhibited cell differentiation and promoted cell proliferation, providing evidence for an important role of TPPP/p25 during oligodendrogenesis. These results support an essential role for TPPP/p25 in oligodendrocyte differentiation likely via rearrangement of the microtubule system during the process elongation prior to the onset of myelination.

Keywords

TPPP/p25; oligodendrocytes; cytoskeleton; microRNA

INTRODUCTION

Oligodendrocytes are essential for the proper development and function of axonal networks in the central nervous system (CNS). During development, these myelin-forming cells are metabolically the most active cells in the CNS (Jiang et al., 2007); as they extend filopodial processes and contact axons for myelin ensheathment. Cytoskeleton of oligodendrocytes has some unique characteristics. Main proteins of myelin, like myelin basic protein (MBP) and 2',3'-cyclic nucleotide 3'-phosphohydrolase are interacting with microtubules and microfilaments *in vitro* and in oligodendrocytes; flattened membranes contain mostly microtubules in cultured oligodendrocytes (Richter-Landsberg, 2008). TPPP/p25 is a prototypical member of a new and unique family of tubulin-binding proteins (Vincze et al., 2006) that are implicated in the stabilization of the microtubule network. TPPP/p25, in contrast to its homologues p18 and p20, has a well-established unstructured motif: the disordered tail. This protein domain has been proposed to function as a signalling sequence

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that affects the Tubulin Polymerization Promoting (TPP) activity upon Ser/Thr phosphorylation (Hlavanda et al., 2007). The primary intracellular target of TPPP/p25 is tubulin/microtubules. As visualized by electron and atomic force microscopy, TPPP/p25 induces microtubule assembly *in vitro* (Vincze et al., 2006) and at substoichiometric concentrations, TPPP/p25 promotes the polymerization of tubulin into aberrant forms, such as double-walled tubules and aggregates (Tirian et al., 2003). TPPP/p25 displays extensive microtubule bundling activity independently of whether the microtubules are stabilized by paclitaxel or promoted by TPPP/p25.

TPPP/p25 was originally identified within the oligodendrocytes and neuropil in rat brain (Takahashi et al., 1993). More recently, TPPP/p25 was shown to be specifically expressed in myelinating oligodendrocytes which are derived from oligodendrocyte progenitor cells (OPCs) during CNS development (Skjoerringe et al., 2006; Lehotzky et al., 2008). Notably, TPPP/p25 was found in oligodendrocytes within white matter tracts as well as in perineuronal oligodendrocytes cells of the human cortex (Kovacs et al., 2007). *In silico* comparative genomic studies as well as proteomic experiments showed that *TPPP* genes are conserved in the genomes of ciliated organisms but are lacking from non-ciliated ones (Orosz and Ovadi, 2008). Because ciliary structures are microtubule-based cellular extensions of sensory and/or motile function, intimate relationship between TPPP/p25 expression and cilia formation was propounded. The regulation of TPPP/p25 expression is important since abnormal accumulation of TPPP/p25 is associated with neuronal and glial cytoplasmic inclusions in neuropathological conditions such as Parkinson's disease, Multiple System Atrophy (MSA) and other α-synucleinopathies (Kovacs et al., 2004). While TPPP/p25 mRNA is present in oligodendrocytes and neurons but not in astrocytes (Cahoy et al., 2008), TPPP/p25 protein is only found in oligodendrocytes in non pathological conditions.

Small non-coding RNAs such as microRNAs (miRNAs) were recently discovered as regulators of gene expression at the post-transcriptional level. Mammalian miRNAs are transcribed as long primary transcripts (pri-miRNAs) and further processed in the nucleus by the Drosha complex, yielding the precursor miRNAs (pre-miRNAs). After nuclear export by Exportin 5, the RNAse III type enzyme Dicer cleaves the pre-miRNA to release the mature form of the miRNA. The miRNAs act either by degradation of target mRNAs or by repression of translation through base pairing within the 3' Untranslated Region (3'UTR) (Valencia-Sanchez et al., 2006). *In silico* analysis suggested that miRNA genes, which contribute about 3-4% of all human genes, regulate the protein synthesis of about a third of all human genes (Lewis et al., 2003). An initial target prediction analysis suggested that four miRNAs (miR-206, miR-1, miR-34a and miR-449) could regulate the expression of human TPPP/p25 (John et al., 2004) and currently, the miRBase Release 12.0, Sept 2008, predicts human TPPP/p25 as a target of 23 miRNAs (Griffiths-Jones et al., 2008). Among all those predicted binding sites, only the miR-1/miR-206 binding site shows cross-species conservation.

In this study, we established the miRNA expression profiles of CG-4 cells and confirmed the absence of miR-206 in this oligodendrocyte cell line, consistent with what previously observed in oligodendrocytes purified from postnatal rat brains (Lau et al., 2008). After over-expression of miR-206 in oligodendrocytes, differentiation of progenitor cells was blocked and TPPP/p25 expression was not detected, thus validating TPPP/p25 as target of miR-206. The regulation of TPPP/p25 expression during oligodendrocyte maturation was further studied using CG-4 cells as well as primary oligodendrocytes. As seen with miR-206, we showed herein that down-regulation of TPPP/p25 expression by siRNAs also inhibited the maturation of progenitor cells, thus reinforcing the important role of TPPP/p25 in the dynamics of cytoskeletal rearrangement during the process elongation of oligodendrocytes.

MATERIALS AND METHODS

Cell culture

The CG-4 cell line with passage number 17 was kindly provided by Dr. Jean-Claude Louis (Amgen, CA). The cells were propagated in DMEM-N1 containing 30% of the same medium conditioned by the neuroblastoma cell line B104 (70/30 media) (Louis et al., 1992). To promote the maturation of progenitor cells into differentiated oligodendrocytes, the cells were grown in DMEM-N1 with 40 ng/ml T3 (Sigma) and 20 ng/ml CNTF (Sigma) (Oligo media) (Wang et al., 2006). For immunofluorescence microscopy, 2×10^4 cells were plated onto poly-L-ornithine (PLO)-coated glass coverslips in 70/30 or Oligo medium. For immunoblotting analysis, the cells were plated onto PLO-coated 6-well plate (10^5 cells / well). For rat primary oligodendroglial culture, hemispheres from 7 day old Sprague-Dawley rat pups were mechanically triturated. Single cells were resuspended in DMEM/10% FCS and seeded into Falcon T75 flasks that have been precoated with PDL (Sigma). When astrocyte monolayer became confluent, microglial cells were removed by shaking for 1h at 37°C. The flasks were then placed under constant shaking at 200 rpm. After overnight shaking, the supernatant was seeded onto uncoated dishes for 35 min. The supernatant was then collected and after centrifugation, the cell pellet was resuspended in DMEM, 5 μg/ml insulin (Sigma), 50 μg/ml transferrin, 30 nM selenium, 20 ng/ml PDGF-AA (PeproTech) and 20 ng/ml bFGF (PeproTech). To induce differentiation, primary oligodendroglial progenitor cells were grown in the same media as described above, without growth factors and with 30 nM triiodothyronine (Sigma) and 0.5% FCS (Amstrong, 1998).

Transfection of CG-4 cell line and viral transduction of primary oligodendrocytes

CG-4 cells were transfected with 50 ng of plasmid DNA or 20 ng of indicated siRNA using Fugene HD reagent (Roche) according to the manufacturer's recommendations. For analysis of oligodendrocyte progenitor cells, cells were kept in the 70/30 medium after transfection. To induce differentiation, transfected cells were transferred to the Oligo medium 6 hours after transfection and then incubated for 4 additional days. A second round of siRNA transfection was performed in the Oligo medium as indicated. Viruses were produced in 293T cells by calcium phosphate-mediated transient transfection of the siRNA-T1, siRNA-T3 or miR-206 constructs together with psPAX2 (Addgene 12260) and pMD2.G (Addgene 12259) plasmids. Rat primary oligodendrocytes were transduced after overnight shaking, plated in the media depleted of growth factors and allowed to differentiate for 4 days.

miRNA and siRNA experiments

Three synthetic small interfering RNAs (siRNAs) were purchased from Invitrogen and designed to target mouse TPPP/p25. The sequence of siRNA1 was: 5’-CCAAUCAGGAAAGGGCAAGGGCAAA-3’. The sequence of siRNA3 was: 5’-GCCACGUGAUCGAUGGGAAGAAUGU-3’ and the sequence of siRNA5 was: 5’-GGACAUUGUCUUCAGCAAGAUCAAA-3’. The transfection of the siRNA duplexes into freshly plated CG-4 cells was carried out using Fugene HD reagent (Roche). Fluorescently labelled siRNA negative control was obtained from Santa Cruz Biotechnology. For siRNA experiments in primary oligodendrocytes, siRNA-T1 and siRNA-T3 were designed to target respectively the following sequences: 5’-CCUGCAGGACCAUCACCUUUG-3’ and 5’-AGGCUGCUAAGAGGUUGUCGC-3’ of rat TPPP/p25. The siRNAs were cloned behind the H1 promoter found in the pGreenPuro plasmid (System Biosciences) using BamH I and EcoR I as restriction sites. The miR-206 expressing plasmid was constructed by inserting the miR-206 containing sequence into pcDNA 6.2-GW/EmGFP-miR (Invitrogen) following the manufacturer's instructions. The sequence of the oligonucleotide containing miR-206 was: 5’-
TGCTGTGGAATGTAAGGAAGTGTGTGGGTTTTGGCCACTGACTGACCCACACAC

CCTTACATTCCA-3'. For over-expression of miR-206 in primary oligodendrocytes, a genomic sequence containing miR-206 was amplified by PCR using 5'-AAAAAAGCAGGCTTTTCAAATCCACCCTGCAAC-3' and 5'-CAAGAAAGCTGGGTGACAGTGTGACCCAGAAGCA-3'as primers. The product was subjected to a second round of PCR using UNI ATTB1 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3' and UNI ATTB2 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3' as Gateway primers (Invitrogen). After BP cloning, LR recombination reaction was done with pCDH-CEF1-EGFP-ATTR plasmid. The vector was constructed from pCDH-CMV-MCS-EF1-copGFP (System Biosciences) using standard cloning techniques and contained attR sites downstream the EGFP sequence, allowing co-expression of EGFP and miRNA.

Determination of proliferation index in transfected CG-4 cells

The CG-4 cells were transfected with the siRNAs and incubated with 10 μM 5'-ethynyl-2'-deoxyuridine (EdU) for 12 hours before detection of incorporated nucleotide analogue by a click reaction with Alexa 596 Azide (Salic and Mitchison, 2008) (Invitrogen). Proliferating cells were detected as having red nuclei. All nuclei were counterstained with DAPI, and both types of nuclei counted using ImageJ. Red nuclei/ total nuclei ratio was calculated for each sample. Counting was done for over 1,000 cells.

Luciferase assays

The full-length cDNA of the mouse TPPP/p25 (clone IMAGE: 6837919) was obtained from the NIH Mammalian Gene Collection (MGC). After PCR amplification of the 3'UTR using the primers forward 5'-GAGACTCGAGCCTCATAGGCTGACGCTCTGGACACTATAGGTGGC-3' and reverse 5'-GAGAGCGGCCGCCCATATTTTAAAACCTTTATTCATC-3', the PCR product was cloned into pSICHECK2 plasmid (Promega) using Xho I and Not I. The 88 bp sequence derived from the 3'UTR of rat TPPP/p25, and containing the predicted binding site of miR-206, was cloned into pSICHECK2 using two overlapping oligonucleotides. The sequence of the top oligonucleotide was 5'-TCGAGCCTTACGGGCTGGAGCTCCGGACACTACAGGTGGCAGGCACAGGACTTACACCCATTGCACTTCATTACATTCCTATGTTAACTGGGC-3'. The sequence of the bottom oligonucleotide was: 5'-GGCCGCCCAGTTAACATAGGAATGTAATGAAGTGCAATGGGTGTAAGTCCTGTGCCTGCCACCTGTAGTGTCCGGAGCTCCAGCCCGTAAGGC-3'. After phosphorylation and annealing, the duplex was inserted into pSICHECK2 plasmid using Xho I and Not I.

For transient transfection, HeLa cells were seeded in 24 wells and transfected the following day with 200 ng of the reporter plasmid and 10 nM of the indicated Pre-miR™ miRNA Precursor Molecule (Ambion) by using Lipofectamine 2000 (Invitrogen). The Pre-miR™ Negative Control #1 (Ambion) was used as negative control. The Renilla and Firefly luciferase activities were measured 48 hours after transfection using the Dual Luciferase Reporter Assay System (Promega) and a Berthold luminometer (Berthold Technologies).

miRNA microarrays

Total RNA from progenitor cells and differentiated CG-4 cells was purified using the mirVana miRNA Isolation Kit (Ambion) and used for hybridization with a miRNA microarray (LC Sciences). The slides were scanned using an Axon GenePix 4000B microarray scanner (Axon Instruments). The microarray images were background subtracted using a local regression method and normalized to the statistical mean of all detectable miRNAs. The hierarchical clustering of miRNAs was done using GenePattern 2.0 (Reich et al., 2006). The miRNAs and microarrays were normalized and an average linkage clustering

was performed using the Euclidean distance algorithm. The probe content of the microarray chip included 233 rat miRNAs. The microarray data has been deposited in ArrayExpress, a public database of gene expression at EBI, under the accession number E-MEXP-1764.

miRNA real-time PCR

Total RNA was extracted and quantified at 260 nm using a Beckman Coulter DU series 600 spectrophotometer (Beckman Coulter, Fullerton, CA). For the first strand synthesis, 500 ng of total RNA was treated with Turbo DNase (Ambion) for 10 min at 37°C and cDNA synthesis was conducted using the mirVana qRT-PCR miRNA Detection Kit (Ambion). Semi-quantitative real-time PCR for miR-17, miR-19, miR-181, miR-191 and miR-125 was performed using mirVana specific primer sets (Ambion). The PCR were performed on a LightCycler System (Roche) using the Platinium SYBR Green qPCR SuperMix UDG (Invitrogen). A four step run protocol was used: (i) pre-incubation (2 min at 50°C) and denaturation (2 min at 95°C) (ii) amplification (15s at 95°C, 15s at 58°C, 15s at 72°C and 3s at 80°C for the acquisition) (iii) melting curve (55°C-95°C with a heating rate of 0.1°C/s) (iv) cooling step at 40°C. The PCR specificity was verified by the presence of a single peak in melting curve analysis and the presence of a single amplicon by agarose gel electrophoresis. Data was normalized to 5S ribosomal RNA level. The fold changes were expressed relative to the miRNA levels found in progenitor cells.

Immunoblotting

CG-4 cells were plated onto 6-well plates and protein lysates were obtained at regular intervals to quantify relative TPPP/p25 expression upon induction of cell differentiation or after siRNA transfection. Cell lysates were obtained using 150 μL of RIPA buffer supplemented with protease inhibitor mix (Sigma). After lysis, the samples were centrifuged (10 min, 15,000*g*) and the supernatants were stored at −70°C. Protein concentrations were determined by UV absorbance at 280 nm. The SDS/PAGE was followed by transfer onto a PVDF membrane using wet transfer equipment (Sigma). TPPP/p25 was detected using a rat polyclonal anti-TPPP/p25 antibody (Kovacs et al., 2007) and the optical density of the immunoreactive band obtained from the differentiated cell sample was compared with the one obtained from the dividing sample.

Immunocytochemistry

CG-4 cells or rat primary oligodendrocytes were fixed for 5 min using 0.25% glutaraldehyde (EM-grade, Sigma) and 4.0% paraformaldehyde (Sigma) in PBS. Cells were rinsed in PBS (3 × 5 min) and residual glutaraldehyde was quenched using 0.1 M NH_4Cl. The samples were blocked in 5% FCS for 30 min and sequentially incubated for 2 hours in the presence of the primary and secondary antibodies. The samples were washed three times with PBS between the first and the secondary antibody incubations. The rat polyclonal anti-TPPP/p25 antibody was used as previously described (Kovacs et al., 2007). The monoclonal antibody SMI 99 against MBP was obtained from Covance. The monoclonal antibodies ONS.1A6 against TUBB4 and DM1A against TUBA were both purchased from Sigma. Affinity purified secondary antibodies conjugated with Alexa 350, Alexa 488, and Texas Red against mouse, mouse and rat IgGs respectively, were obtained from Molecular Probes. Affinity purified secondary antibodies conjugated with Cy2 and Texas Red against rat and mouse IgGs, respectively, were purchased from Jackson Laboratories. Coverslips were mounted with GelMount (or CrystalMount) and sealed with Clarion (all reagents from Biomeda).

Imaging of the oligodendrocytes

Phase-contrast images were taken on Olympus CKX41 inverse microscope equipped with a LCAch 40x/0,55 objective using a mounted Olympus C5050Z camera. Fluorescent images

were taken on a Leica DMLS microscope equipped with C PLAN 100x/1.25 and HCX Fluotar 40x/0.75 objectives, using UV, I3 and N2.1 filter sets from Leica for DAPI or Alexa 350; GFP, Cy2 or Alexa 488; and Alexa 596, Texas Red; respectively. Images were acquired by a cooled CCD camera controlled by Windows version of Spot Advanced (version 4.0.9), (Diagnostic Instruments, Inc.).

RESULTS

Transfection of miR-206 in oligodendrocytes down-regulates TPPP/p25

To better characterize miRNAs during oligodendrocyte development, we determined miRNA expression profiles of CG-4 cells by using miRNA microarrays. A pure population of bipolar oligodendrocyte progenitor cells that express the A2B5 ganglioside marker was generated under the proliferative conditions and compared with differentiated oligodendrocytes oligodendrocytes grown for 7 additional days. Total RNA from these two populations were hybridized to a miRNA microarray chip and 94 of 233 miRNAs were unambiguously detected (Fig. 1A). To validate the miRNA microarray data, real-time PCR analysis was done for three miRNAs known to be highly expressed in the brain (miR-125b, miR-181 and miR-191) and for two oncomirs (miR-17 and miR-19). The microarray and real-time PCR data for these 5 miRNAs were essentially similar (Fig. 1B). Of note, miR-206 was absent from progenitor cells and differentiated oligodendrocytes (Fig. 1A) and we previously determined that miR-206 was not found in postnatal oligodendrocytes purified from rat brains (Lau et al., 2008). However, TPPP/p25 was identified as target of mir-206 by in silico investigations (John et al., 2004). To validate that TPPP/p25 interacts with miR-206, CG-4 cells were transfected with a plasmid that contains the genomic sequence of miR-206 downstream the coding sequence of EGFP, which allowed us to track the simultaneous expression of EGFP and miR-206. The amount of TPPP/p25 in the miR-206-transfected CG-4 cells was determined at the progenitor stage after immunostaining for TPPP/p25 (Fig. 2A-C). The red fluorescence intensity of individual cell bodies showed that transfection of miR-206 lowered the basal level of TPPP/p25 by about 40% when compared with untransfected cells ($p<0.05$, *t*-test) (Fig. 2F). As a negative control, we verified that the level of TPPP/p25 in the presence of the empty plasmid was not different from what obtained with untransfected CG-4 cells (data not shown). Additionally, we determined that miR-206 affects the differentiation of oligodendrocytes. The CG-4 cells were induced to differentiate for 4 days after transfection with miR-206. The differentiation was not blocked after transfection with the empty plasmid, as shown by the appearance of arborized projections in the control oligodendrocytes and the appearance of red TPPP/p25 signal (Fig. 2D) while transfecting miR-206 impaired the differentiation into mature oligodendrocytes (Fig. 2E). For comparison, similar set of experiments was performed with primary oligodendrocytes (Fig. 2G, H). Non-arborized cell was characterized by lack of TPPP/p25 and MBP expression (Fig. 2H), suggesting that TPPP/p25 is important for oligodendrocyte differentiation. To confirm the functional interaction between miR-206 and TPPP/p25, we determined whether miR-206 inhibits TPPP/p25 expression through its 3'UTR.

miR-206 interacts with TPPP/p25 mRNA in vitro

The 3'UTR of mouse TPPP/p25 is 4336 bases long (Genbank accession number NM_182839) and the 3'UTR of human TPPP/p25 is 5239 bases long (Genbank accession number NM_007030). Only 283 nucleotides after the stop codon has been reported for the 3'UTR of rat TPPP/p25 (Genbank accession number NM_001108461), suggesting that the rat 3'UTR has not been cloned entirely yet. When human, mouse and rat sequences were aligned, the presence of 18 conserved nucleotides at the position +58 relative to the stop codon of the rat TPPP/p25 was noticed (Fig. 3A). *In silico* analysis predicted that this sequence was a binding site for miR-206 (Fig. 3B). Only four mismatches were observed in

the base pairing between the mouse/rat sequences and miR-206 while the human sequence led to three base mispairings (Fig. 3B). We therefore verified that miR-206 might act as a post-transcriptional regulator of TPPP/p25 expression. To validate that miR-206 interacts with TPPP/p25 *in vitro*, miR-206 was co-transfected in Hela cells with a luciferase reporter plasmid containing the 3'UTR of mouse TPPP/p25. As shown in Fig. 3C, a down-regulation of the normalized luciferase activity was observed with miR-206 while no effect was obtained with the miR-control. We also employed another reporter plasmid in which 88 nucleotides containing the predicted binding site of miR-206 found in the 3'UTR of rat TPPP/p25 was inserted downstream of the luciferase reporter. This short sequence was sufficient to mediate the down-regulation by miR-206 (Fig. 3C), thus suggesting that the predicted binding site of miR-206 was functional. To confirm the important role of TPPP/p25 in oligodendrocytes, we characterized TPPP/p25 expression profile during differentiation.

TPPP/p25 expression in differentiated CG-4 cells and rat primary oligodendrocytes

Fig. 4 illustrates representative pictures of both CG-4 cells and rat primary oligodendrocytes before and after differentiation. The undifferentiated CG-4 cells were bipolar (Fig. 4A) while pre-myelinating cells after differentiation showed complex arborization of the projections (Fig. 4B). The morphology of these cells corresponded to that of primary cells (Fig. 4C, D). Fluorescence of individual cell bodies after immunostaining for TPPP/p25 was determined and showed that dividing CG-4 cells exhibited significantly lower TPPP/p25 immunoreactivity when compared to differentiated CG-4 cells (Fig. 5A). To measure TPPP/p25 protein level during differentiation, total protein cell lysates were prepared from CG-4 cells before induction of cell differentiation and then daily for 4 days after induction. Immunoblot analysis showed that the expression of TPPP/p25 was up-regulated during CG-4 maturation (Fig. 5B).

Differentiation of CG-4 cells was further confirmed by the immunopositivity for MBP, a well-established marker of mature oligodendrocytes (Fig. 6A, E). Notably, we found that both MBP and TPPP/p25 were extensively expressed in differentiated CG-4 cells as well as in primary oligodendrocytes (Fig. 6A, B, E and F). Therefore, CG-4 cells mimic the cellular properties of primary oligodendrocytes in respect to the involvement of TPPP/p25 in the process elongation. These observations were also in agreement with what observed for myelinating oligodendrocytes in developing rat brain (Skjoerringe et al., 2006). We next examined the cells using the antibody against class IV β-tubulin (TUBB4), a protein preferentially found in oligodendrocytes of developing brain (Terada et al., 2005). TUBB4 immunoreactivity was present in processes of differentiated CG-4 cells (Fig. 6C), including at their flattened distal endings. We also used an antibody against β-tubulin (TUBA) (Fig. 6D), an ubiquitous component of eukaryotic microtubule networks, and which was also found to be a major intracellular target of TPPP/p25 (Lehotzky et al, 2004). As shown in Fig. 7C and F, TPPP/p25 was aligned with a delicate network of microtubules in both differentiated CG-4 cells and primary oligodendrocytes. Co-localization was notably visualized at certain part of the cells. The increase of tubulin was also detected during the differentiation of oligodendrocytes, supporting the view that increase of cellular volume and surface of oligodendrocytes required increase amount of structural proteins during maturation, and concomitantly the relative fluorescence intensity of TPPP/p25 detected by immunostaining in differentiated CG-4 cells was estimated to be 5-10-fold higher than that of progenitor cells (Fig. 5A). These data support our previous notion that TPPP/p25, in conjunction with other microtubule associated proteins (MAPs) and microtubules could act as a MAP-like protein (Ovadi, J., 2008) which may manifest itself during the maturation of oligodendrocytes. The ability of TPPP/p25 to primarily target tubulin/microtubules also suggests that the association of the up-regulated TPPP/p25 with microtubules could be

important for the development of a highly organized cytoskeleton during the maturation of oligodendrocytes.

TPPP/p25 siRNAs inhibit the differentiation of oligodendrocytes

To further support the notion that TPPP/p25 plays a critical role during oligodendrocyte differentiation, we down-regulated TPPP/p25 expression in CG-4 cells using siRNAs. Three specific siRNAs which were originally designed to target the mouse TPPP/p25 transcript are depicted in Fig. 8A. The siRNA1 targets a conserved sequence between mouse and rat species which is localized in exon 3 of the TPPP/p25 gene. The siRNA5 and siRNA3 were selected from exon 1 and contain 1 and 2 mismatches respectively, resulting in A:C mispairings when paired with the rat TPPP/p25 mRNA (Fig. 8A). Since A:C mismatched base pairs are well tolerated for siRNA mediated gene silencing (Du et al., 2005), we tested the ability of all three siRNAs to down-regulate TPPP/p25 expression. First, we showed that each of these three siRNAs against TPPP/p25 were active. The progenitor cells were transfected with the different siRNAs and 24 hours after transfection, immunoblotting analyses showed that TPPP/p25 was down-regulated (Fig. 8B). To maximize the effects of the siRNAs, a second transfection step was done 2 days after the first transfection. Subjecting cells to two rounds of transfection yielded a further increase in number of undifferentiated cells up to about four-fold (Fig. 8B). As a negative control, we verified that the level of α-tubulin (TUBA) was not affected after transfection of the siRNAs (Fig. 8B). Next we investigated the functional effect of down-regulating TPPP/p25 in differentiated CG-4 cells. After transfecting the progenitor cells with siRNAs, differentiation was induced by incubating the cells in the Oligo media. After 4 days, immunofluorescence analysis demonstrated that the reduction of TPPP/p25 by siRNAs led to significant increase in the number of progenitor-like cells with faint TPPP/p25 labelling, which was associated with the decrease of number of CG-4 cells with highly arborized structure (Fig. 8D-E). To make unambiguous the effect of TPPP/p25 expression on the proliferation of CG-4 cells, samples were treated with 5’-ethynyl-2’-deoxyuridine (EdU), which can be incorporated into the newly synthesized DNA of replicating cells and detected by a click-reaction with azide-substituted stains (Salic and Mitchison, 2008). CG-4 cells transfected with siRNA3 (Fig. 8F) showed increased number of dividing cells when compared with the control (Fig. 8G). Both the total number of nuclei stained with DAPI (blue) and the total number of proliferating cells (red) were increased after transfection of siRNA3. Proliferation indexes of samples were calculated as 0.34 for siRNA1, 0.53 for siRNA3, 0.32 for siRNA5, and 0.20 for the negative control. Primary oligodendrocytes were also transduced with a virus expressing either siRNA-T1 or either siRNA-T3, two additional TPPP/p25 siRNAs designed against the rat TPPP/p25. Immunofluorescence analysis showed qualitatively similar result for both siRNAs, namely, that down-regulation of TPPP/p25 impeded the differentiation of oligodendrocytes, as suggested by the decrease of MBP and TPPP/p25 labelling as illustrated for siRNA-T3 in Fig. 8H, I.

DISCUSSION

In this study, we show that TPPP/p25 is dynamically regulated in oligodendrocytes, and that its up-regulation in differentiated oligodendrocytes is crucial for process extension. This observation is in agreement with a recent study showing TPPP/p25 up-regulation during differentiation of cultured rat brain oligodendrocytes (Goldbaum et al., 2008). Control of TPPP/p25 protein level during oligodendrocyte maturation is important since increased TPP/p25 levels led to cytoplasmic structures resembling inclusion bodies, in agreement with *in vitro* experiments showing that substoichiometric concentrations of TPPP/p25 induce aberrant tubulin formation (Tirian et al., 2003; Lehotzky et al., 2004). Notably, myelin disruption and accumulation of TPPP/p25 within cytoplasmic inclusions is detected in

Multiple System Atrophy (MSA). In MSA brains, TPPP/p25 strongly co-localizes with α-Synuclein (SNCA) and up to 50% of oligodendrocytes within affected fibre tracts show abnormal distribution of TPPP/p25 (Song et al., 2007). The present study reinforces the necessity for proper expression levels of TPPP/p25 during oligodendrocyte development, since down-regulation of TPPP/p25 blocks oligodendrocyte differentiation. This importance of correct expression level was demonstrated by two different types of "knock-down" experiments in which TPPP/p25 expression was negatively regulated by either specific siRNAs complementary to segments of the coding region of the mRNA or by miR-206, a miRNA targeting a sequence found in the 3'UTR of TPPP/p25 mRNA. Moreover, we found that extensive down-regulation of TPPP/p25 by siRNAs resulted in significant increase in the number of progenitor cells. This phenomenon can be explained by the absence of process formation, resulting in a block at an undifferentiated stage. Alternatively, we recently demonstrated that only differentiated oligodendrocytes, but not rapidly dividing oligodendrogliomas, express TPPP/p25 in brain tissue (Preusser et al., 2007). This suggests an inhibitory effect of TPPP/p25 on cell division and we hypothesize that abnormal diminution of TPPP/p25 by siRNAs may block the exit of the cell cycle. Our study using EdU label to detect the newly synthesized DNA of replicating cells strengthened the inhibitory role of TPPP/p25 on the proliferation of progenitor oligodendrocytes. Further experiments are needed to clarify whether TPPP/p25-dependent differentiation coupled with an anti-proliferative effect is a more general phenomenon or if it is restricted to oligodendrocytes.

A number of miRNAs have been isolated from vertebrate nervous systems (Kosik, 2006) and a crucial role in neuronal maturation, synapse development and plasticity have been documented (Mehler and Mattick, 2006). *In silico* prediction of binding sites for several miRNAs on TPPP/p25 mRNA suggests the existence of a complex regulatory framework which includes miR-206. In the present study, we did not detect miR-206 in CG-4 cells, consistent with previous reports showing enrichment of miR-206 in skeletal myocytes (McCarthy, 2008). However, the observations that Tac1, a neurotransmitter gene, is a target of miR-206 and that a SNP found in the human mir-206 gene shows allelic association with schizophrenia (Hansen et al., 2007) suggest a potential role of miR-206 in post-transcriptional regulation of neural cells. Interestingly, TPPP/p25 mRNA was increased in developing neuronal cells, although at less extent than in oligodendrocytes (Cahoy et al., 2008). However, recent studies identified TPPP/p25 protein in neuronal cells or astrocytes only in neurological diseases (Kovacs et al., 2007; Song et al., 2007), thus reinforcing the idea of a post-transcriptional regulation of TPPP/p25 in neuronal cells.

The miRNA microarray analysis did not reveal miR-1 in CG-4 cells, another miRNA that belongs to the same family as miR-206 and also predicted to regulate TPPP/p25. This observation is in agreement with the specific expression of miR-1 in both cardiac and skeletal muscles (Chen et al., 2006). Recent expression analysis of predicted miR-1/ miR-206 targets revealed that these transcripts tend to be expressed in non-muscle tissues, in patterns that are mutually exclusive with miR-1/miR-206 expression. Together, these observations support the hypothesis that miR-1/ miR-206 blocks detrimental mRNAs that are promiscuously expressed during muscle development (Stark et al., 2005). Of note, TPPP/p25 mRNA has been detected in the atrial chamber of the heart (ArrayExpress database, accession number E-GEOD-1479). Because TPPP/p25 is a brain-specific protein (Takahashi et al., 1993), it is thus tempting to hypothesis that TPPP/p25 transcripts are subjected to "quality control" and are negatively regulated in cardiac muscle cells. A recent study shows that miR-206 occupies a cytoplasmic location in cultured myoblasts and its level increases during differentiation into myotubes (Politz et al., 2006), suggesting that myotubes do not require TPPP/p25 and that other actin-related proteins may play dominant role in the ultrastructural organization of their contractile networks.

Ensheathment of axons with myelin is essential for the normal function of the CNS. During myelination, oligodendrocyte lineage cells alter the transcriptional rate of numerous genes in a highly coordinated manner (Nielsen et al., 2006). The large and membranous sheet of oligodendrocytes contains a complex cytoskeletal network containing microtubules and actin-binding proteins associated with actin filaments. In a recent study, the Kelch-related protein MRP2/HL1 was found to promote process elongation of oligodendrocytes (Jiang et al., 2007). The significantly increase of MRP2/HL1 in differentiated oligodendrocytes when compared with progenitor cells parallels the up-regulation of TPPP/p25. Our study reinforces the importance of molecular mechanisms controlling the reorganization of the cytoskeleton by showing that differentiation of oligodendrocytes is TPPP/p25-dependent and that its down-regulation reduces the process extension. The observation that TPPP/p25 is not only co-localized with the microtubule network but is also found at the plasma membrane, where it is thought to mediate interactions between MBP and tubulin (Wenning et al., 2008), suggests that TPPP/p25 may possess additional properties and specific roles in these MBP immunopositive sheaths. Overall, the proper up-regulation of TPPP/p25 protein level during differentiation may be essential for the dynamics of cytoskeletal rearrangement through its binding to microtubules, which are integral to the structural stability and plasticity of the oligodendrocyte cytoskeleton.

Acknowledgments

This work was supported by FP6–2003-LIFESCIHEALTH-I: BioSim and by the Hungarian National Scientific Research Fund Grants OTKA T-067963 to J. Ovádi. This study was supported by intramural funds from NINDS to L.D. Hudson. We thank Jo Ann Berndt (NINDS) for expertise in oligodendrocyte cell culture. We greatly acknowledge Prof. László Buday and Dr. András Illés of Semmelweis University Medical School for their valuable advice/assistance in siRNA experiments.

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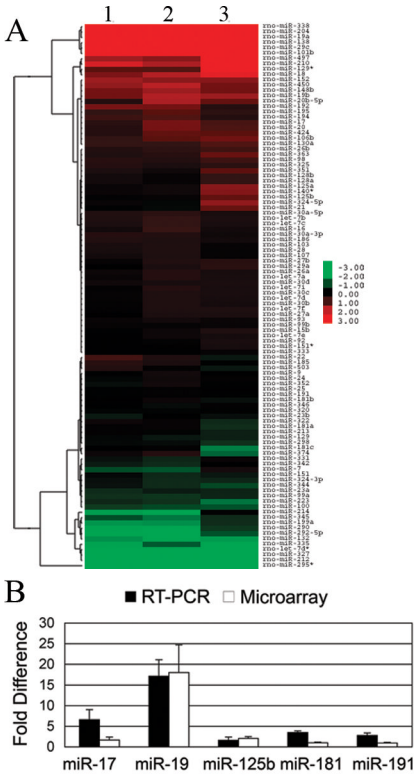

Fig. 1. miRNA expression profiles during differentiation of CG-4 cells

(A) Detection of miRNAs by miRNA microarrays in three independent experiments. The heatmap displays the $\log^2$ transformation of the relative fold change in expression levels of miRNAs during differentiation. Up-regulated miRNAs with increased levels are highlighted in red while down-regulated miRNAs are shown in green. The middle of the heatmap contains miRNAs showing little change in expression levels during maturation. (B) Real-time PCR analysis of miR-17, miR-19, miR-125b, miR-181 and miR-191 were compared with the miRNA microarray data. The fold differences were calculated with respect to oligodendrocyte progenitor cells before differentiation. Normalization was done with 5S ribosomal RNA. Error bars represent the s.e.m. n= 3.

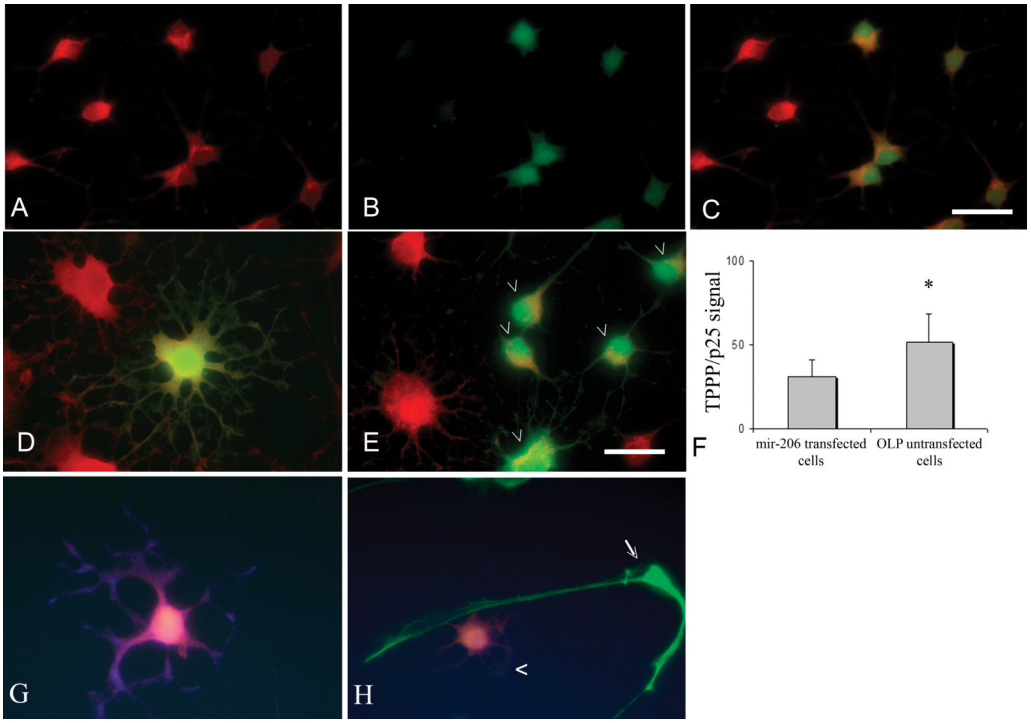

Fig. 2. Effect of miR-206 on the expression of TPPP/p25 in CG-4 cells and rat primary oligodendrocytes

(A-C) CG-4 progenitor cells were transfected with the miR-206 expression plasmid and processed for immunocytochemistry after 24 hours. (A) Expression of TPPP/p25 (red) in transfected and untransfected progenitor cells. (B) miR-206 expressing cells were identified by the co-expression of EmGFP (green channel). (C) Merged picture of A and B. (F) The red signal intensity of individual cellular bodies of transfected and untransfected cells was measured using ImageJ ($p<0.05$, *t*-test). (D-E) CG-4 cells were transfected with the miR-206 expression plasmid or the empty plasmid, and cultured in Oligo medium for 96 hours to allow differentiation. (D) Differentiated CG-4 cell transfected with the empty plasmid (green). (E) Cells transfected with miR-206 (green) showed absence of projections with complex branching (arrowheads). (G) A rat primary oligodendrocyte transduced with the virus control was immunostained for MBP (blue) and TPPP/p25 (red) after 4 days of differentiation. CopGFP was localized mostly in the nucleus. (H) Representative picture of a cell over-expressing miR-206 which did not show arborized morphology. The cell was negative for TPPP/p25 and MBP (arrow). Note the presence of MBP-weak signal at the periphery of a faint $TPPP^+$ and $copGFP^+$ cell (arrowhead). Scale bar = 25 μm in panels A-C, and 10 μm in panels D, E, G, and H.

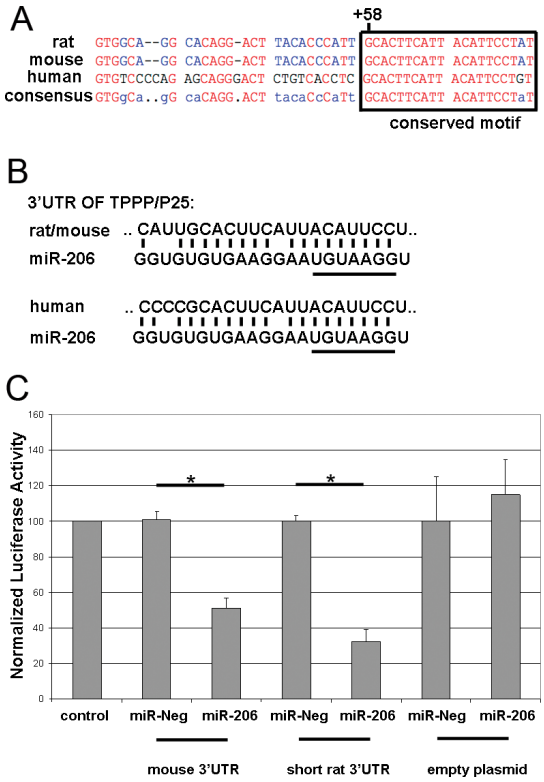

Fig. 3. TPPP/p25 and miR-206 interact in vitro

(A) Determination of a conserved nucleic acid motif in the 3'UTR of TPPP/p25. The alignment of rat, mouse and human TPPP/p25 sequences reveals the presence of 18 nucleotides 5'-GCACTTCATTACATTCCT-3' that are conserved between the three species at position +58 after the end of the stop codon. The conserved motif is highlighted in the box. High consensus color = red. Low consensus color = blue. Neutral color = black. (default options of the Multalin software). (B) Alignments of 3'UTR of TPPP/p25 with miR-206. The conserved motif found in the 3'UTR of TPPP/p25 was interrogated for the presence of miRNA binding sites using the TargetScan software. A hit for miR-206 was obtained and the predicted base pairing is shown. The rat and the mouse TPPP/p25 led to four base mispairings (indicated by *) whereas the human TPPP/p25 sequence resulted in three mismatched bases. The seed sequence of miR-206 is underlined and corresponds to the positions 2–8 at the 5′ end of the miRNA that determine the specificity of the interaction. (C) The luciferase reporter plasmid containing the 3'UTR of mouse TPPP/p25 was co-transfected in Hela cells with miR-206 and compared with the co-transfection with the miRNA negative control (miR-Neg.). The luciferase activity was down-regulated by 50% in the presence of miR-206 (*$p<0.05$, *t*-test, when compared with miRNA control). No difference was found between the cells that were transfected with the luciferase reporter alone (control) and Hela co-transfected with the miRNA negative control. The down-regulation was recapitulated with the luciferase construct containing the short sequence derived from the 3'UTR of rat TPPP/p25 (*$p<0.05$, *t*-test, when compared with miRNA control) while no effect was observed when the empty reporter plasmid was employed. Error bars represent the s.e.m. n= 3.

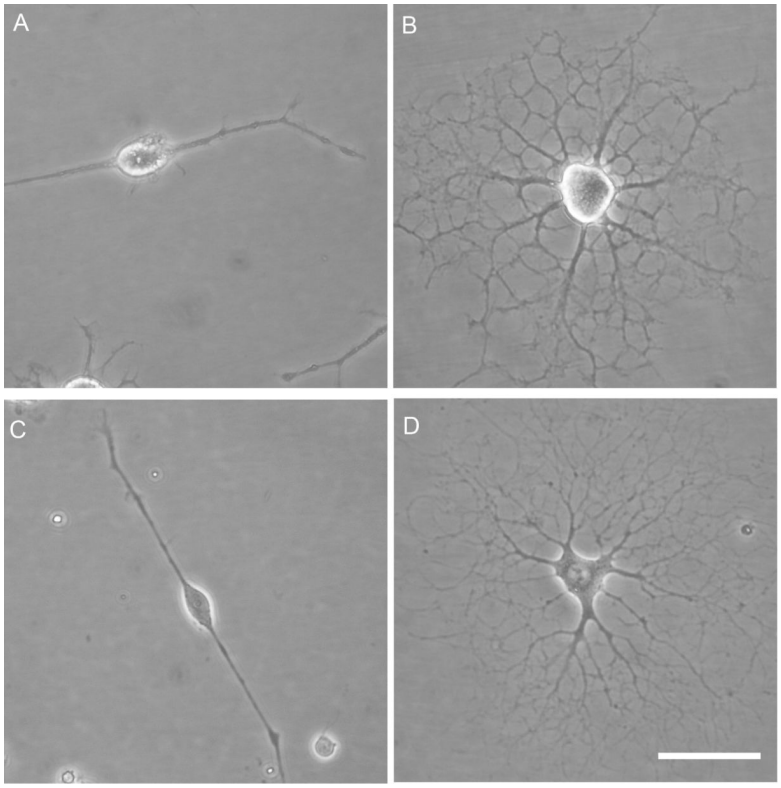

Fig. 4. Phase-contrast view of CG-4 cells and rat primary oligodendroglial precursor cells and oligodendrocytes

Cells were plated on PLO-coated vessels and pictures were taken before and after differentiation *in vitro*. Oligodendroglial precursor CG-4 cells (A) and oligodendrocyte progenitor cells (C) show bipolar morphology. Differentiated rat CG-4 cells (B) and oligodendrocyte cells (D) show similar morphology with highly arborized processes. Scale bar = 20 μm.

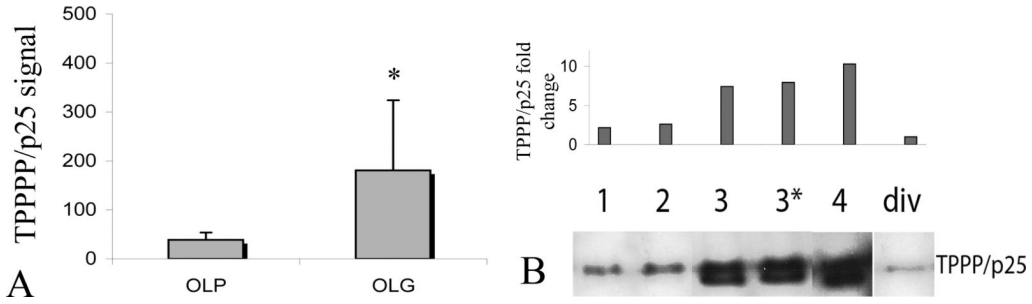

Fig. 5. Increase of TPPP/p25 during differentiation of CG-4 cell line

(A) Increase of TPPP/p25 expression in differentiated cells (OLG) *vs*. dividing progenitor cells (OLP) was detected after TPPP/p25 immunostaining (*$p<0.05$, *t*-test). Differentiated cells were analyzed 4 days after induction of differentiation. The quantification of the TPPP/p25 signal in individual cell bodies was done by using ImageJ. Error bars represent s.e.m. (B) The amount of TPPP/p25 was detected by immunoblotting after 1, 2, 3 or 4 days of differentiation. In sample 3*, extra addition of CNTF at day 2 did not further increase TPPP/p25 level. Analysis of blot was done by using ImageJ and optical densitometry of the immunoreactive band was used to calculate fold change of TPPP/p25 protein level.

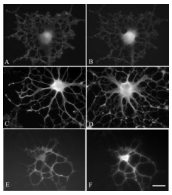

Fig. 6. TPPP/p25 expression in differentiated CG-4 cells and rat primary oligodendrocytes

(A) MBP is unevenly distributed in CG-4 cells, mostly at the plasma membrane and some intense patch-like patterns are detectable. (B) The same differentiated CG-4 cell shows intense labelling of TPPP/p25 in soma and in their processes, with faint staining on flattened membranes found at the end of the arborized processes.(C) Class IV β-tubulin (TUBB4) shows intense labelling on microtubular network in CG-4 cells, notably in the flattened membranous parts and at the end of the highly organized structures. (D) Microtubular network staining with anti α-tubulin (TUBA) antibody in differentiated CG-4 cells. (E) MBP staining of a rat primary oligodendrocyte cultured for 6 days. (F) TPPP/p25 staining of the same oligodendrocyte shown in (E). The pattern and the intracellular distributions of MBP and TPPP/p25 are similar in differentiated CG-4 cells and rat primary oligodendrocytes. Scale bar = 10 μm.

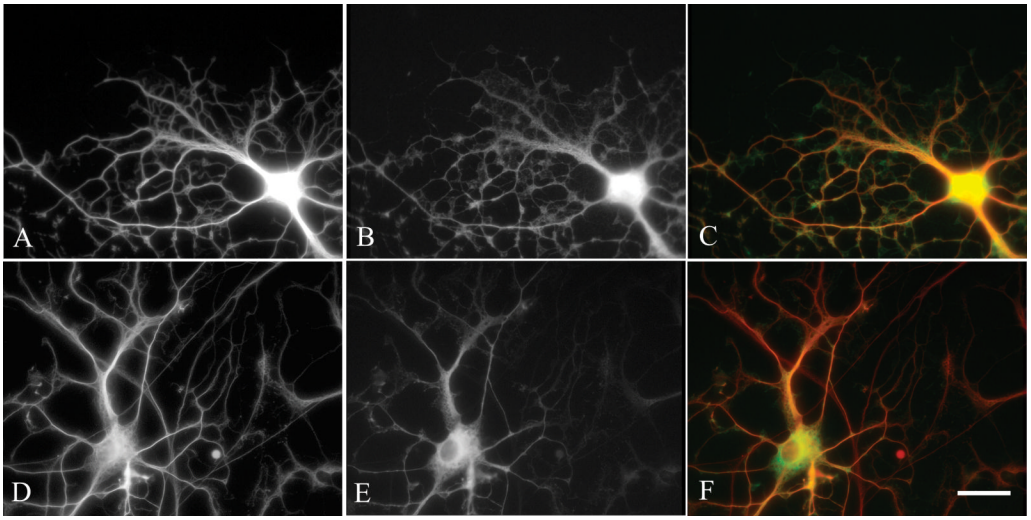

Fig. 7. TPPP/p25 co-localization with microtubules in differentiated CG-4 cells and rat primary oligodendrocytes

There is partial co-localization of TUBA and TPPP/p25 in CG-4 cells and rat primary oligodendrocytes. (A-C) Differentiated CG-4 cell was immunostained for tubulin (red) (A) and TPPP/p25 (green) (B). (C) Merged picture of A and B. (D-F) Rat primary oligodendrocyte stained for tubulin (red) (D) and TPPP/p25 (green) (E). (F) Merged picture of D and E. Scale bar = 10 μm.

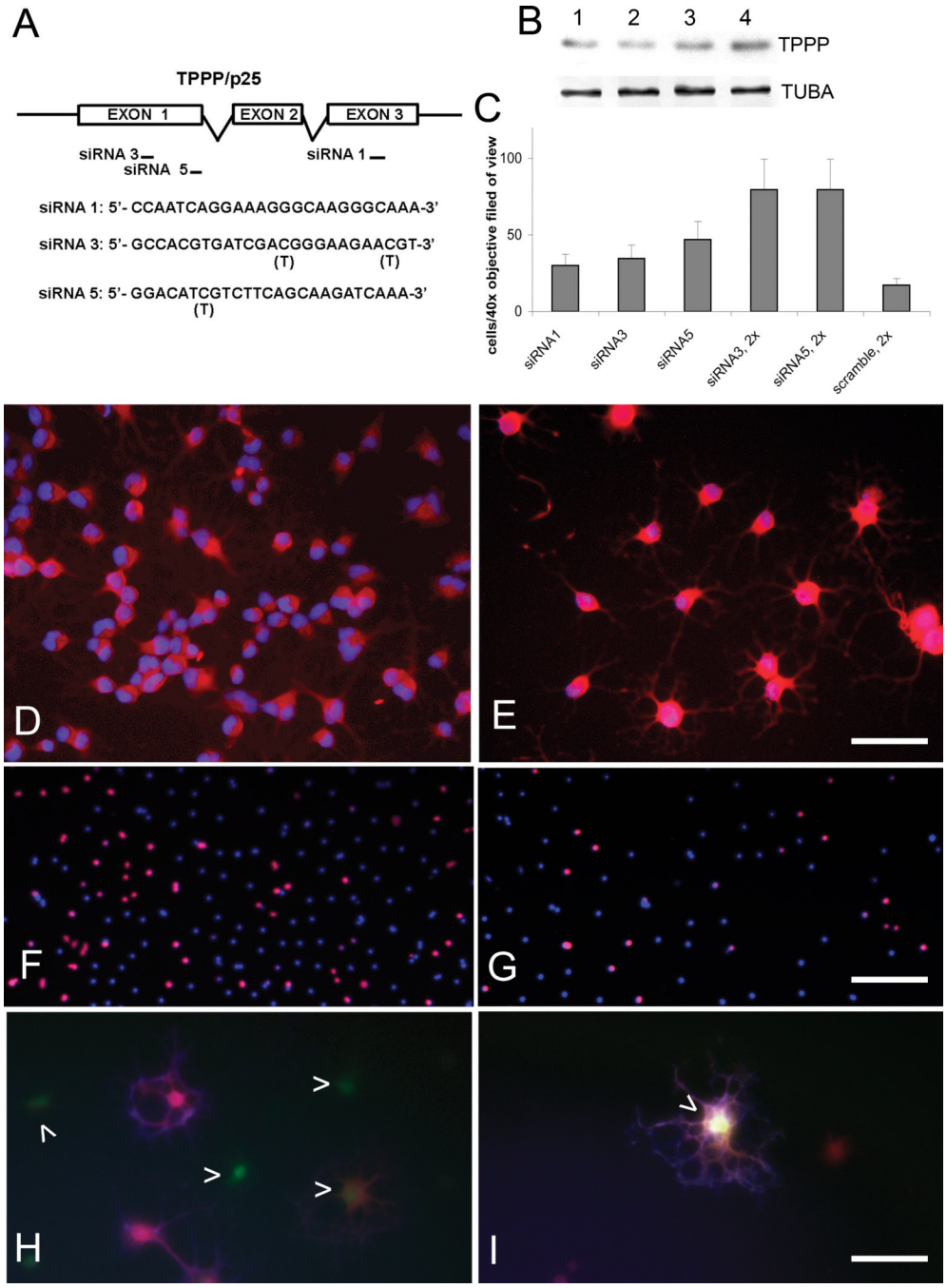

Fig. 8. Effect of TPPP/p25 siRNAs on the differentiation and proliferation of CG-4 cells and rat primary oligodendrocytes

(A) Localization of the target sites. The siRNA1 targets exon 3 of TPPP/p25 whereas two sequences of exon 1 were selected to design siRNA3 and siRNA5. The three rat target sequences are found below the diagram showing the exon-intron structure of TPPP/p25 gene. The nucleotides (T) in brackets below the rat sequences highlight the differences between rat and mouse. The siRNAs were originally designed based on the mouse sequence, resulting in A:C base mismatches between TPPP/p25 and the antisense strand of the siRNA when used with the rat cDNA. (B) Immunoblotting after transfection of the siRNAs. A representative experiment illustrates the decrease of TPPP/p25 caused by the presence of the

siRNA1, −3, −5 (lanes 1, 2 and 3 respectively) when compared with the scramble control (lane 4). Immunoblotting with the α-tubulin (TUBA) antibody shows equal protein loading in each lane. (C) The cells were transfected with each of the three siRNAs against TPPP/p25 and incubated for 96 hours in the Oligo media. The cells were immunostained for TPPP/p25 and oligodendrocytes were identified by morphological criteria. The overall number of cells per microscopic field was counted to evaluate the effect of the siRNAs on the proliferation of CG-4 cells. The samples marked as 2x in (C) were subjected to a second round of transfection after 48 hours. (D-E) The differentiation of the double transfected cells with siRNA3 (D) was inhibited, resulting in an increase of the cell number when compared with the scramble control (E). (F) CG-4 cells transfected with siRNA3 shows increased number of dividing cells when compared with the control (G). (D-G) Nuclei were stained with DAPI (blue) and the dividing cells were labelled with EdU incorporation (red). (H-I) Depletion of TPPP/p25 inhibits the differentiation of rat primary oligodendrocytes. Rat primary oligodendrocytes were transduced with the siRNA-T3 expressing virus (H) or the control virus (I) and immunostained for MBP (blue) and TPPP/p25 (red) (H) Two untransfected and differentiated oligodendrocytes (MBP^+ and $TPPP/p25^+$) are shown as internal control. Four transfected ($copGFP^+$) cells (indicated by arrowheads) weakly stained for MBP and TPPP/p25. (I) A MBP^+, $copGFP^+$ and $TPPP/p25^+$ oligodendrocyte in the control sample is shown by arrowhead. Scale bar = 25 μm in panels D, E, H, and I; = 100 μm in panels F and G.