Serine/Threonine-Protein Kinase PFTK1 Modulates Oligodendrocyte Differentiation via PI3K/AKT Pathway

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Abstract Oligodendrocytes (OLs) are derived oligodendrocyte progenitor cells (OPCs), and their differentiation is a tightly regulated process. It is known that cyclin-dependent kinases (CDKs) play an essential role as regulators of OPC differentiation. Here, we newly identified a CDK-like protein, PFTK1, to be involved in OPC differentiation. With serum-deprivation, OLN-93 undergoes OL differentiation, and PFTK1 expression is markedly decreased during differentiation. When PFTK1 is silenced, OL differentiation is potentiated, as suggested by the increase of various differentiation markers CNPase, MOG, CGT, and MBP, by qPCR and Western blotting analysis. Vice versa, PTTK1 overexpression has opposite effects on OL differentiation of OLN-93 in vitro. Next, the modulation mechanism underlying OL differentiation of OLN-93 was investigated. Significantly, PFTK1 silencing leads to the activation of PI3K/AKT pathway, but no activation of MAPK/ERK pathway. The inhibition of AKT by its specific inhibitor abrogates PFTK1 silencing-promoted OL differentiation, indicating that PFTK1 negatively regulates OL differentiation through PI3K/AKT pathway. Together, these findings indicate a novel role played by PFTK1 in OL development, thus presenting opportunities to establish therapeutic approaches in improving neurological recovery related to demyelinating disorders.

Keywords Oligodendrocyte · Oligodendrocyte progenitor cell · PFTK1 · Differentiation · Signaling pathway

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Introduction

Oligodendrocyte (OL) differentiation is tightly regulated, and each stage of the process follows a strict schedule (Tokumoto et al. 2002; He and Lu 2013). Differences in morphology and expression markers characterize the various stages of OL differentiation. Three stages are frequently observed when differentiation is induced in cell cultures. While oligodendrocyte progenitor cells (OPCs) are bipolar in appearance, pre-OLs are multiple process-bearing and non-myelinating mature OLs present with extensive branching networks (Friede 1973; Bradl and Lassmann 2010). These observations suggest the presence of highly specific signaling mechanism regulating the timing of OL differentiation. Elucidating regulatory molecules involved in the process will provide a better understanding of OL development in the CNS.

Cyclin-dependent kinases (CDKs) and their endogenous inhibitors (CKIs) play an essential role as regulators of differentiation onset within OPCs (Raff et al. 1998; Larocque et al. 2005). For example, CDK5 regulates differentiation of OPCs through the direct phosphorylation of paxillin (Miyamoto et al. 2007). Following the induction of differentiation, CDK5 is activated (Tang et al. 1998; Miyamoto et al. 2007), and its inhibitor inhibits OL differentiation. Differently, CDK2 activity was found to be decreased during OL differentiation (Tang et al. 1998). As for CDK inhibitors, p21^{Cip1} has been shown to be required for the differentiation of OLs, independently of cell cycle withdrawal (Zezula et al. 2001). Similarly, p27^{Kip1} also plays a central role in the initiation of OL differentiation, but its role is likely dependent on cell cycle withdrawal (Tikoo et al. 1998; Levine et al. 2000; Larocque et al. 2005).

PFTK1, also known as PFTAIRE-1, is a Cdc2-related serine/threonine protein kinase, showing 50 % amino acid identity with CDK5 (Besset et al. 1998). The gene is highly expressed in the brain, pancreas, kidney, heart, testis, and ovary. Cellular

localization of the mRNAs shows that PFTK1 is expressed in late pachytene spermatocytes in the testis and in postmitotic neuronal cells both in the brain and the embryo, indicating a potential role of PFTK1 in the process of meiosis as well as neuron differentiation. In addition, PFTK1 can promote the cell cycle as classical CDKs, and also regulate several pathways and cellular mechanisms as an oncogene (Pang et al. 2007; Jiang et al. 2009). Notably, CDK5 shares high similarity with PFTK1 and plays important roles in OL differentiation. However, very little is known about the role of PFTK1 in OL differentiation.

In this work, we aimed to investigate the potential role of PFTK1 in OL differentiation, using both ectopic gene expression and gene knockdown techniques. We also explored the regulatory mechanism of OL differentiation by PFTK1. Unraveling novel functions of PFTK1 would not only aid in better understanding of developmental biology pertaining to the CNS, but also be of therapeutic significance to numerous neurological diseases, such as spinal cord injuries and demyelinating diseases.

Materials and Methods

Materials

Our chemicals and reagents were obtained commercially and used as received. Quick start Bradford reagent for measuring the protein concentration was obtained from Bio-Rad (USA). Antibodies against GCT and MBP were purchased from Millipore. Antibody against β-actin was from Sigma-Aldrich. Antibody against PFTK1 was from Santa Cruz Biotechnologies. The AKT inhibitor, LY294002, was purchased from Cell Signaling Technologies.

Plasmid Constructs

For silencing of the *pftk1* gene, the pSilencer™ 4.1-CMV expression vector system was employed. These siRNA-encoding DNA fragments were inserted in between *Bam*H I and *Hind* III restriction sites. A negative control siRNA template (scramble sequence) was served as the control for the knockdown experiments. Sequences for the siRNA oligos were as follows: 5′-GAT CCG ACG ACA CCA CCT TTG ATG TTC AAG AGA CAT CAA AGG TGG TGT CGT CAG A-3′ and 5′-AGC TTC TGA CGA CAC CAC CTT TGA TG T CTC TTG AAC ATC AAA GGT GGT GTC GTC G-3′ for PFTK1; 5′-GAT CCG AGC ACA TAT CCT CCG ATG TTC AAG AGA CAT CGG AGG ATA TGT GCT CAG A-3′ and 5′-AGC TTC TGA GCA CAT ATC CTC CGA TGT CTC TTG AAC ATC GGA GGA TAT GTG CTC G-3′ as a scramble siRNA-encoding oligo. The paired oligos were annealed and cloned into the sites of *Bam*H I/*Hind* III of psilencer 4.1-CMV neo (Ambion Inc.).

For overexpression of *pftk1* gene, the gene was amplified using rat OLN-93 cell complementary DNAs (cDNAs). Primer sequences used were as follows: Forward: 5′-CGG GAT CCA TGC AAC AGT ATA AAA GGG A-3′ and reverse 5′-ACC GCT CGA GCT ATC GAA CAG CAA GCA CTG A. The PCR products of *pftk1* cDNA were then cloned into *Bam*H I/*Xho* I sites of pcDNA4-Myc/His (Addgene) and confirmed by sequencing. Vector transfectant would hence express the c-myc epitope tag.

Cell Culture and Transfection

Rat OLN-93 cell line was obtained from Dr. Fengyi Liang (Department of Anatomy, National University of Singapore). Cells were cultured and maintained in Dulbecco's modified Eagle's medium (Cellgro, Herndon, VA) supplemented with 100 mg/L sodium pyruvate, 20 mM sodium bicarbonate, 5 mM Hepes, 1 % penicilin-streptomycin, and 10 % fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5 % CO_2. Cells were seeded in 75-cm^2 flasks and were passaged when 70–80 % confluency was attained.

For transfection of siRNA-expressing plasmids, 8 μg of DNA construct was introduced into OLN-93 cells cultured in 60-mm dish (70–80 % confluent) by electroporation using Neon™ Transfection System (Invitrogen) using 1400 mV in 30 ms. Cells were plated onto culture dishes with complete medium. Forty-eight hours following transfection, the culture medium was then replaced with complete media containing G418 (800 μg/ml) (Sigma, St. Louis, MO) or zeocin (800 μg/ml) as the selecting agent. After 1 week of selection, resistant cells were propagated, and then used for further experiments.

OL Differentiation

Cells were first seeded on growth medium (10 % FCS) for 2 days. They were then switched to differentiation medium consisting of serum-free medium and cultured for 4 days.

Real-time PCR

Total RNA was extracted using Trizol reagent following the manufacturer's instructions (Invitrogen), and reversely transcribed to cDNAs using SuperScript II reverse transcriptase (Invitrogen). Quantification of mRNA levels was measured by using real-time PCR system (ABI Prism7500, Applied Biosystems) and SYBR Green qPCR Master Mix (KAPA Biosystems). Gene-specific primers used for real-time PCR were shown in Table 1. The target mRNA level of control cells normalized to the level of rat GAPDH mRNA was set to 1.

Table 1 Primers used for real-time RT-PCR

Gene	Forward Primer	Reverse Primer
CGT	5′-CGTACTCCTAGAACACAGACT-3′	5′-AGTTACTGAAGGGGAGCTGTA-3′
MBP	5′-CTATAAATCGGCTCACAAGG-3′	5′-AGGCGGTTATATTAAGAAGC-3′
CNPase	5′-AGCTGCAGTTCCCTTTCCTTCA-3′	5′-TCATCGAGCACAAGAACCCTGA-3′
MOG	5′-TGCCTCTAGAAAACACCCCA-3′	5′-AGCACCTAGCTTGTTTGTGT-3′
GAPDH	5′-TGCCTCTAGAAAACACCCCA-3′	5′-CCCCACTTGATTTTGGAGGGA-3′

Image Acquisition

For morphological observations and immunofluorescence analysis, samples were observed under light or fluorescence microscope (Olympus 1X71).

Western Blotting

Cells were washed twice with PBS and then extracted with lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100, 1 mM PMSF, and Roche's complete protease inhibitors) and centrifuged at $14,000g$ for 20 min at 4°C. The protein concentration of cell lysates was determined using a Protein Assay Kit II (Bio-Rad). For Western blot, samples were separated by electrophoresis on 8–18 % SDS-PAGE, and transferred onto PVDF membranes (Millipore Corp.). After blocking with PBST (PBS with 0.1 % Tween-20) containing 5 % skim milk, the membranes were incubated with primary antibodies. They were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and developed using Millipore's chemiluminescence substrate. To determine the equivalence of protein amounts loaded among different samples, the developed membranes were stripped with a buffer consisting of 62.5 mM Tris–HCl (pH 6.7), 2 % SDS, and 100 mM 2-mercaptoethanol for 1 h, followed by incubation with reference antibody, such as anti-β-actin for further blotting. In some cases, immunoblots were quantified by measuring the immunoreactive protein band density with software ImageJ 1.48 (NIH, USA).

Statistical Analysis

All values were expressed in mean±standard deviation (SD). Student t test was applied to analyze the differences between groups, where $P<0.05$ was considered significant.

Results

PFTK1 is Downregulated During OL Differentiation and its Silencing Potentiates OL Differentiation of OLN-93 Cells

To elucidate the role of PFTK1 in OL differentiation, we first examined the expression of PFTK1 in OLN-93 cells by Western blotting assay. As shown in Fig. 1a, serum deprivation-induced OL differentiation led to a significant decrease of PFTK1 protein levels in a time-dependent way, suggesting a potential role of PFTK1 in OL differentiation.

Next, we used siRNA technique to silencing PFTK1 expression in OLN-93 cells. As shown in Fig. 1b, the introduction of PFTK1 siRNA (siPFTK1) strongly blocked the expression of PFTK1 compared to control siRNA (siCtl)-transfected cells. Under phase contrast microscope, PFTK1-knockdown cells appeared flattened and exhibited branched web-like extensions (Fig. 1c). This structure highly resembles that of mature OL. On the other hand, the control cells were rounder and bipolar, with thin cellular extension 1–3 times longer than their cell bodies (Fig. 1c). This is a characteristic of undifferentiated OPCs. At day 2 of differentiation, more extensive branching can be seen in PFTK1-silenced cells (Fig. 1c), whereas majority of the control cells remained undifferentiated. Even though these cells subsequently adopted differentiated morphology comparable with the PFTK1-silenced cells at day 4 of differentiation, we showed that the downregulation of PFTK1 significantly speeds up the OL differentiation process.

PFTK1 Silencing Increases the Expression of OL Differentiation Markers

Morphological analysis was insufficient to elucidate the role played by PFTK1 in OL differentiation. Therefore, Western blotting was performed to examine the effect of PFTK1 silencing on OL marker expression in OLN-93 cells (Fig. 2a). The markers examined included CNPase (2′,3′-cyclic nucleotide 3′-phosphodiesterase) and myelin oligodendrocyte glycoprotein (MOG). Compared with control siRNA-transfected cells, PFTK1 siRNA-transfected cells exhibited higher levels of OL markers, both CNPase and MOG giving 2.18 ($P<0.01$) and 2.02 times ($P<0.01$), respectively, than in control cells (Fig. 2a–c).

Furthermore, qPCR was carried out to further assess the effect of PFTK1 on OL marker induction at mRNA levels. In addition to CNPase and MOG, two more OL markers, ceramide galactosyltransferase (CGT) and myelin oligodendrocyte glycoprotein (MBP), were also concluded in qPCR. Consistent with the results of Western blotting, all tested OL markers were significantly increased in PFTK1-silenced cells compared to control cells (Fig. 2d). Transcripts of CTG, MOG, CNPase, and MBP were increased to 4.6, 14.8, 2.7, and 5.3 times ($P<0.01$, $P<0.01$, $P<0.05$, and $P<0.01$,

Fig. 1 PFTK1 silencing potentiates OL differentiation of OLN-93 cells. **a** PFTK1 was downregulated during OL differentiation. **b** PFTK1 was silenced using siRNA technique, assessed by Western blotting. *siCtl* scramble siRNA-transfected cells, *siPFTK1* PFTK1-specific siRNA-transfected cells. **c** OLN-93 cells underwent OL differentiation with morphological changes when serum was deprived
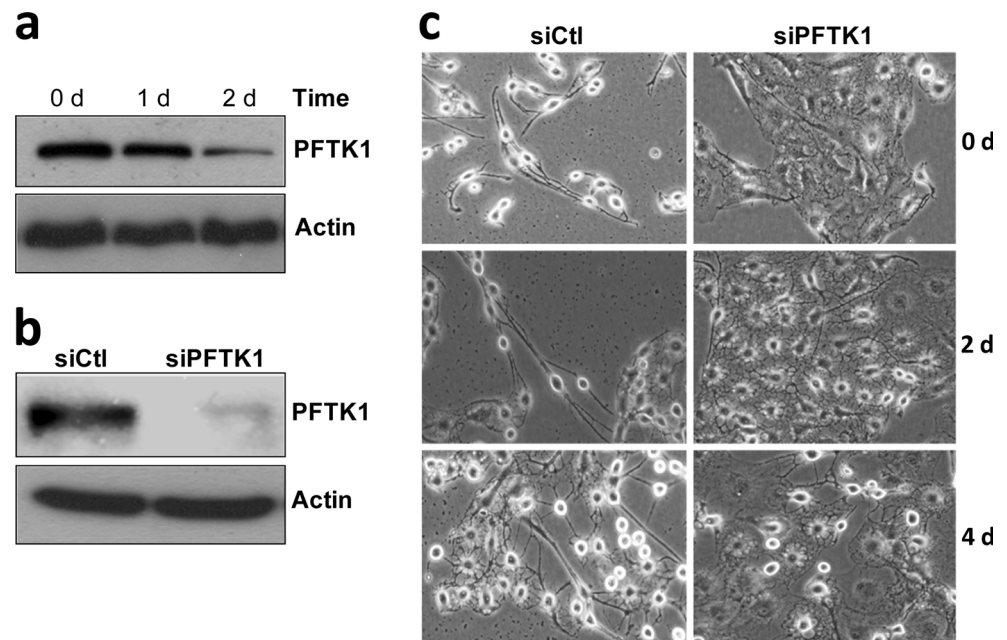
respectively) than those in control siRNA-transfected cells. Taken together, these data clearly suggest that PFRK1 acts negatively on OL differentiation of OLN-93 cells in vitro.

PFTK1 Overexpression Suppresses OL Differentiation of OLN-93 Cells

To further confirm the role of PFTK1 in OL differentiation, we examined the effect of PFTK1 overexpression on differentiation of OLN-93 cells. As shown in Fig. 3a, ectopic PFTK1 was overexpressed to a level much higher than endogenous PFTK1. At day 2 of differentiation, majority of the PFTK1-overexpressing cells displayed the undifferentiated bipolar morphology, while the control cells presented branched web-like structures (Fig. 3b). This signified lower extent of differentiation in the PFTK1-overexpressing cells. Hence, it was evident that OL differentiation is suppressed by the ectopic expression of PFTK1.

Fig. 2 PFTK1 silencing promotes OL marker induction of OLN-93 cells. **a** When PFTK1 was silenced, OL marker induction was increased. **b**, **c** The levels of CNPase and MOG were expressed as a percentage of the level measured in siCtl cells. *CNPase* 2′,3′-cyclic nucleotide 3′-phosphodiesterase, *MOG* myelin oligodendrocyte glycoprotein. **d** qPCR analysis was performed for various markers during in vitro differentiation of cells. *CGT* ceramide galactosyltransferase, *MBP* myelin oligodendrocyte glycoprotein. *$P<0.05$ and ***$P<0.001$ (siPFTK1 vs siCtl)
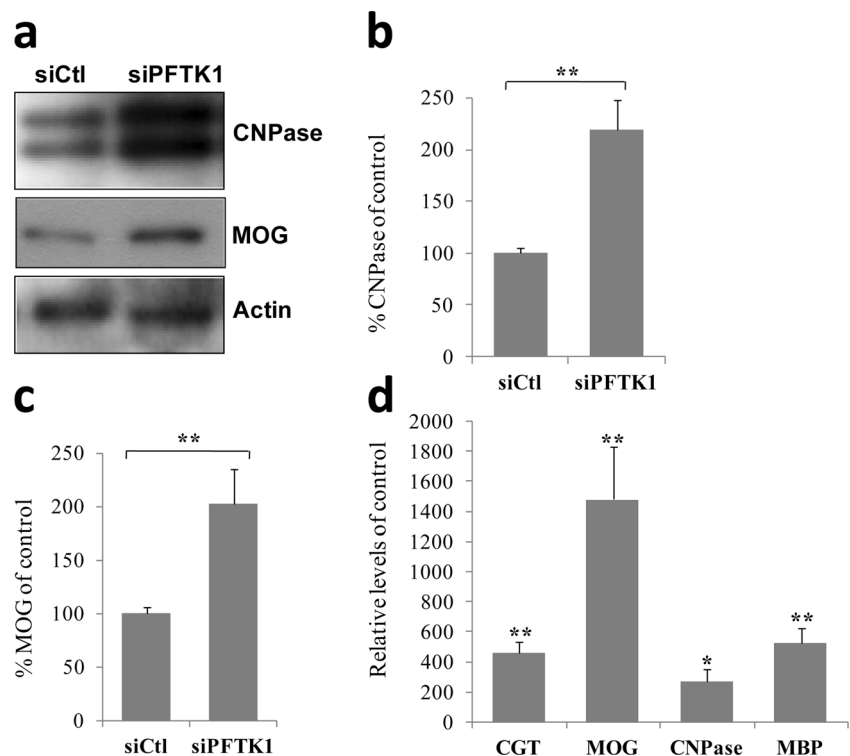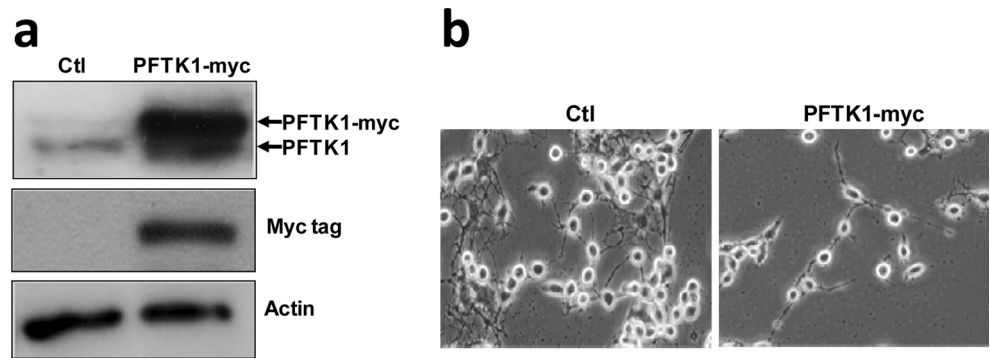
Fig. 3 PFTK1 overexpression inhibits OL differentiation of OLN-93 cells. **a** PFTK1 was overexpressed, assessed by Western blotting. **b** PFTK1-overexpressing OLN-93 cells exhibited a decreased OL differentiation when serum was deprived, compared to control cells


PFTK1 Overexpression Decreases the Expression of OL Differentiation Markers

Next, Western blotting were performed to further confirm the effect of PFTK1 overexpression on OL marker expression (Fig. 4a). The markers examined included CNPase and MBP. Compared with control vector-transfected cells, PFTK1-overexpressing cells exhibited lower levels of OL markers, both CNPase and MOG. The protein levels of CNPase and MOG were decreased to 52.9 % ($P<0.01$) and 45.9 % ($P<0.01$) of those in control vector-transfected cells, respectively (Fig. 4a–c).

Furthermore, qPCR was carried out to validate the effect of PFTK1 overexpression on OL marker expression. All the markers examined, namely CGT, CNPase, MOG, and MBP, were significantly lower in overexpressing cells, when compared to control cells. Transcripts of CTG, MOG, CNPase, and MBP were decreased to 23.4, 33.2, 61.3, and 19.9 % of those ($P<0.001$, $P<0.001$, $P<0.01$, and $P<0.001$, respectively) in control vector-transfected cells (Fig. 4d). Taken together, these results strongly suggest that PFTK1 plays a negative regulatory role in OL differentiation.

Fig. 4 PFTK1 overexpression inhibits OL marker induction of OLN-93 cells. **a** When PFTK1 was over expressed (PFTK1-myc), OL marker induction was decreased. **b, c** The levels of CNPase and MOG were expressed as a percentage of the level measured in siCtl cells. **d** qPCR analysis was performed for various markers during in vitro differentiation of cells. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ (PFTK1-myc vs Ctl)
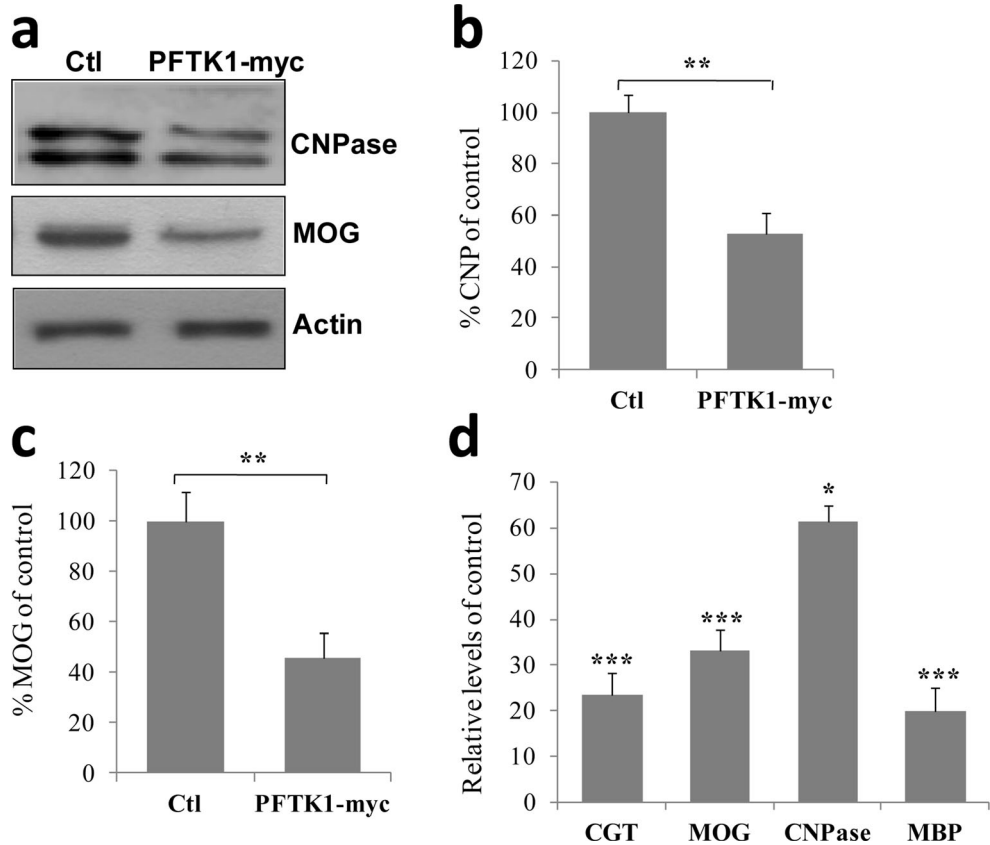
PFTK1 Silencing Potentiates OL Differentiation via Activation of PI3K/ATK Pathway

It is well known that MAPK/ERK and PI3K/ATK pathways play crucial roles in OL differentiation of OPCs (Ahrendsen and Macklin 2013; Wood et al. 2013). To elucidate how PFTK1 functions on OL differentiation, we examined the effect of PFTK1 silencing on these two important signaling pathways. As shown in Fig. 5, in PFTK1-silenced cells, the phosphorylation of AKT was increased up to 2.6 times ($P < 0.01$) than that in control siRNA-transfected cells. However, the phosphorylation of ERK1/2 remained unchanged (data not shown), suggesting that PFTK1 may affect the activation of PI3K/ATK pathway rather than MAPK/ERK pathway.

To determine whether the increased ATK activation in PFTK1-silenced cells is required for PFTK1-potentiated OL differentiation, we employed LY294002 (LY), an AKT-specific inhibitor to in vitro differentiation assay. As expected, the presence of LY (10 μM) inhibited AKT activation to a lower level than that of control cells (Fig. 5). As a result,

induction of OL differentiation markers CNPase and MOG potentiated by PFTK1 silencing was also decreased. In conclusion, these data indicate that PFTK1 negatively regulates OL differentiation via PI3K/ATK pathway in vitro.

Discussion

PFTK1 has recently been characterized as a novel CDK member that regulates cell procession and cell proliferation. Ectopic expression of PFTK1 promotes cell proliferation, whereas knocking down PFTK1 expression causes cell cycle arrest at G1. Mechanically, PFTK1 specifically interact with cyclin D3 and formed a ternary complex with p21^{Cip1} in mammalian cells (Shu et al. 2007). It also physically interacts with cyclin Y to promote noncanonical Wnt signaling (Sun et al. 2014). Thus, PFTK1 is even recognized as an oncogene, evidenced by its role to promote invasiveness and cell motility in hepatocellular carcinoma or predict esophageal squamous

Fig. 5 PFTK1 silencing induces AKT activation and then OL differentiation. a Western blotting were performed to assess the effect of PFTK1 silencing on AKT activation and OL marker induction in the absence or presence of AKT inhibitor, LY294002 (LY, 10 μM). b–d The levels of phosphorylated AKT, CNPase, and MOG were expressed as a percentage of the level measured in siCtl cells. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$
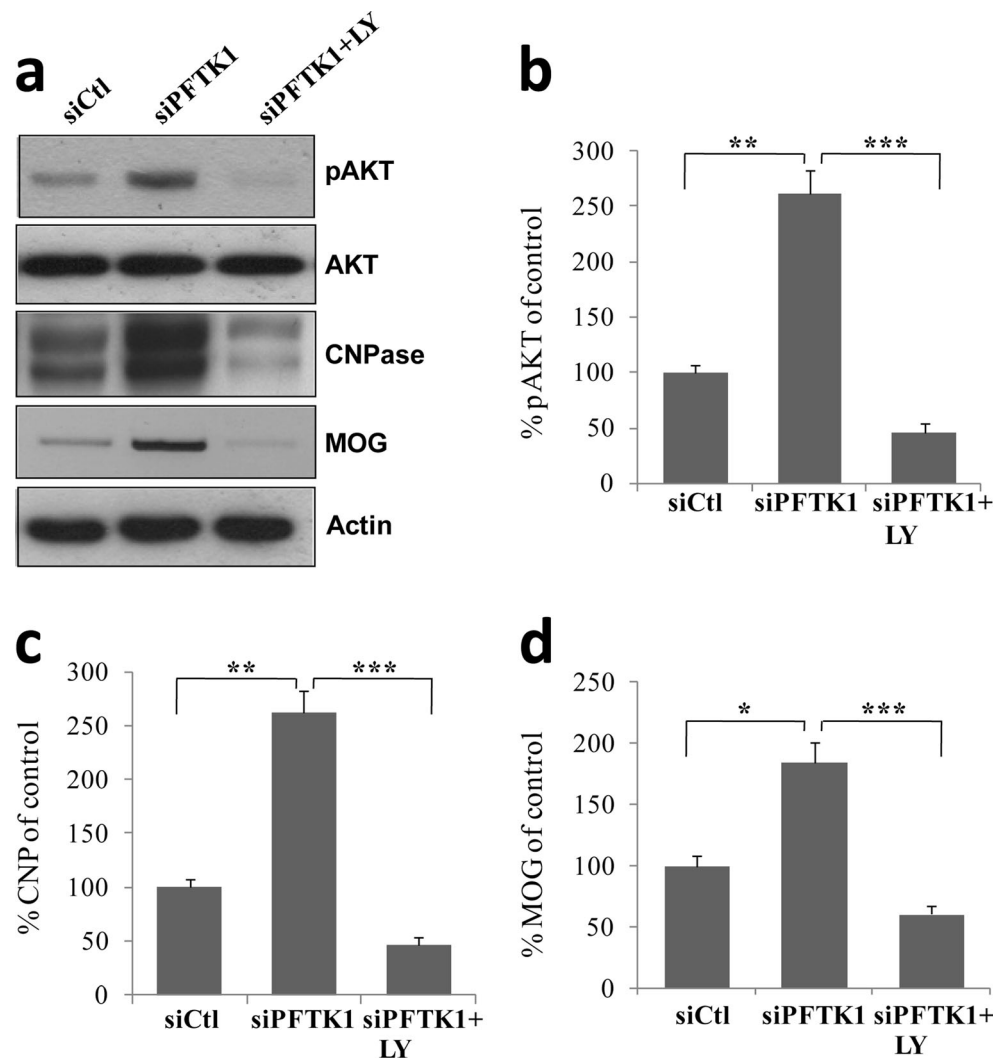
cell carcinoma (Pang et al. 2007; Leung et al. 2011; Miyagaki et al. 2012). However, little is known about the role of PFTK1 in OL differentiation of OPCs, despite other CDK members, such as CDK2 and CDK5, have been implicated in OL differentiation. Here, we presented clear evidence for its suppression effect on OL differentiation for the first time. Furthermore, we demonstrated that PFTK1 inhibits OL differentiation through PI3K/ATK pathway.

The OL differentiation involves several steps and is tightly coupled to cell cycle withdrawal. CDKs and their endogenous inhibitors (CKIs) controlling cell cycle withdrawal, thus they play roles in OL differentiations. The most studied CDKs are CDK2 and CDK5 (Tang et al. 1998; Ghiani and Gallo 2001; Frederick and Wood 2004; Miyamoto et al. 2007). CDK5 is activated following OL differentiation induction and its inhibitor suppresses OL differentiation, suggesting a positive role of CDK5 in OL differentiation regulation (Miyamoto et al. 2007). On the contrary, CDK2 may play a negative role in OL differentiation, because there is a fall in CDK2 activity when OPC begin to differentiate both in vitro and in vivo. In our present study, we demonstrated that PFTK1 silencing promotes cultured OLN-93 OPCs to differentiate, whereas ectopic PFTK1 expression inhibits cells to differentiate. These data suggest PFTK1 may play a different role from CDK5, but similar to CDK2. Interestingly, there are still some different behaviors between PFTK1 and CDK2 in OL differentiation. In our study, we found a significant decrease of PFTK1 protein level when OPCs undergoes OL differentiation. However, CDK2 protein level remains unchanged during the whole OL differentiation, although its activity is downregulated (Tang et al. 1998). The different behaviors between PFTK1 and CDK2 suggest that PFTK1 gene regulation may be required for OL differentiation, whereas CKD2 activity inhibition is sufficient for OL differentiation. Future research should be done to further explore the underlying different mechanisms.

Previous works showed that AKT and ERK are important signaling pathways that regulate OL differentiation and myelination in vitro and in vivo (Ahrendsen and Macklin 2013; Wood et al. 2013). EEK signaling regulates transition of early progenitors to the late progenitor stage and, as a consequence, to the immature OL stage, but not the transition of immature OL to the mature OL stage (Guardiola-Diaz et al. 2012). In contrast, AKT signaling is required for sequential transition of immature OLs to the mature OL stage. Thus, we tried to examine the effect of PFTK1 on these two signaling pathways. We find that AKT signaling is required for OLN-93 cell differentiation rather than ERK signaling. PFTK1 may inhibit its differentiation through downregulating AKT signaling, as shown by the facts that PFTK1 silencing leads to AKT activation and then promoting OL differentiation, whereas AKT inhibitor abrogates this effect. Our finding is considerably consistent with previous observations. Namely, AKT

signaling is required for transition of immature OLs to the mature OL stage. Because one recent study has pointed out that OLN-93 cell line represents a late immature OL rather than a real OPC (Buckinx et al. 2009).

OPCs not only could be differentiated into OLs, but also have the potential to differentiate into other type neural cells. Studies have shown that it is possible to generate neurons from OPCs (Gregori et al. 2002; Virard et al. 2006; Lyssiotis et al. 2007). Since PFTK1 is expressed in great amounts in postmitotic neurons (Besset et al. 1998), it is reasonable to believe that it might be involved in neuronal differentiation. However, from the results of experiments that we have conducted (data now shown), evidence showed that PFTK1 does not have the ability to induce neuronal differentiation of OPCs. Using a neuronal differentiation protocol including NGF and RA, we were not able to identify any effect of PFTK1 on the induction of neuronal marker expression.

In summary, our results show that PFTK1 is sufficient as an inhibitor of OL differentiation. PFTK1 is shown to be able to alter the gene expression of several OL markers, CGT, CNPase, MOG and MBP, as well as confer morphological changes in lineage-committed OPCs. In addition, this CDK-like protein could affect OL differentiation via regulating PI3K/ATK signaling pathway. Taken together, these findings provide a better understanding of the functional roles played by PFTK1. They not only strengthen our knowledge on OL development, but also open up new doors to therapeutic applications associated with neurological diseases, in particular demyelinating disorders.

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