Reducing polypyrimidine tract-binding protein 1 fails to promote neuronal transdifferentiation on HT22 and mouse astrocyte cells under physiological conditions

QIAN LI[1], WEI ZHANG[1,2], XIN-YU QIAO[1], CHONG LIU[1], JI-JI DAO[1], CHEN-MENG QIAO[3], CHUN CUI[3], YAN-QIN SHEN[3] and WEI-JIANG ZHAO[1]

[1]Department of Cell Biology, Wuxi School of Medicine, Jiangnan University, Wuxi, Jiangsu 214122; [2]Department of Pathogen Biology, Guizhou Nursing Vocational College, Guiyang, Guizhou 550081; [3]Department of Neurodegeneration and Neuroinjury, Wuxi School of Medicine, Jiangnan University, Wuxi, Jiangsu 214122, P.R. China

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Abstract. In contrast to prior findings that have illustrated the conversion of non-neuronal cells into functional neurons through the specific targeting of polypyrimidine tract-binding protein 1 (PTBP1), accumulated evidence suggests the impracticality of inducing neuronal transdifferentiation through suppressing PTBP1 expression in pathological circumstances. Therefore, the present study explored the effect of knocking down PTBP1 under physiological conditions on the transdifferentiation of mouse hippocampal neuron HT22 cells and mouse astrocyte (MA) cells. A total of 20 μM negative control small interfering (si)RNA and siRNA targeting PTBP1 were transfected into HT22 and MA cells using Lipo8000™ for 3 and 5 days, respectively. The expression of early neuronal marker βIII-Tubulin and mature neuronal markers NeuN and microtubule-associated protein 2 (MAP2) were detected using western blotting. In addition, βIII-tubulin, NeuN and MAP2 were labeled with immunofluorescence staining to evaluate neuronal cell differentiation in response to PTBP1 downregulation. Under physiological conditions, no significant changes in the expression of βIII-Tubulin, NeuN and MAP2 were found after 3 and 5 days of knockdown of PTBP1 protein in both HT22 and MA cells. In addition, the immunofluorescence staining results showed no apparent transdifferentiation in maker levels and morphology. The results suggested that the knockdown of PTBP1 failed to induce neuronal differentiation under physiological conditions.

Correspondence to: Professor Wei-Jiang Zhao, Department of Cell Biology, Wuxi School of Medicine, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, P.R. China
E-mail: weijiangzhao@jiangnan.edu.cn

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Introduction

Neuronal loss constitutes a pivotal factor propelling disease progression in cases of nervous system injury and neurodegenerative disorders (1). The loss of neurons disrupts intricate neural networks, leading to the dysregulation and interruption of neural signaling transduction (2). Additionally, the loss of neurons may result in the release of aberrant proteins, which can trigger neuroinflammation and neurotoxicity, further exacerbating damage and death of adjacent neurons. This creates a vicious cycle of neuronal degeneration (3). Neuronal loss precipitates tissue loss and atrophy in the brain, which not only impairs the functionality of the affected region but also has the potential to propagate to other brain areas, resulting in more extensive damage (4). As the disease advances, patients may encounter symptoms such as memory deterioration, cognitive impairment and motor dysfunction, which can significantly affect their quality of life (5). Hence, the restoration of depleted neurons has been a central area of focus and difficulty within the field of neurology (6).

The direct conversion of non-neuronal cells into functional neurons through the alteration of gene expression or transcription factors, known as cell reprogramming, presents a promising neural regenerative strategy for filling gaps in neural circuits (7). This neural regenerative strategy exhibits great potential for treating neurodegeneration, as it involves introducing specific transcription factors and molecular signals into glial cells during reprogramming. This process alters the expression profile and epigenetic status of the cells, gradually transforming them into neurons (8). The transformation of cells into neurons occurs in three stages (9): Induction, consolidation and maturation.

The induction phase involves the introduction of transcription factors (such as Achaete-scute homolog 1, POU class 3 homeobox 2 and myelin transcription factor 1-like) (10) or molecular signals (such as nerve growth factor and brain-derived neurotrophic factor) (11). Consequently, cellular transformation can be instigated, leading to a gradual alteration in the gene expression profile, ultimately initiating the

neuronal differentiation pathway (12). In the consolidation phase, the modulation of the neuronal differentiation pathway further facilitates the stable transformation of cells into neurons, accompanied by the expression of neuron-specific marker genes (13). Finally, during the maturation phase, these newly formed neurons establish synapses and participate in neuroelectrical signaling, exhibiting functional similarity to natural neurons (14).

Polypyrimidine tract-binding protein (PTBP)-1, also called hnRNPI or PTB, is a member of the hnRNP family of RNA-binding proteins. The PTBP gene family comprises PTBP1-3 as its primary constituents (15). PTBP1 has been demonstrated to function as a splicing inhibitor in regulating selective splicing, which is critical for neuronal growth and differentiation (16). PTBP1 is widely expressed in various types of cells, including but not limited to glial cells (17), neural progenitor cells (18), stem cells (19), cancer cells (20), fibroblasts (21) and lymphocytes (22), but is scarcely expressed in neurons (18). During neuronal development, the expression of PTBP1 decreases while the expression of PTBP2 increases (23). This alteration in expression levels results in the modification of the splicing of PTBP1-sensitive exons, ultimately leading to a reprogramming of neuronal splicing (24). As neuronal maturation progresses, PTBP2 levels experience a subsequent decrease, resulting in a secondary shift in the neuronal splicing pattern (25). Numerous studies have demonstrated that the reduction of PTBP1 expression levels, whether *in vivo* or *in vitro*, can facilitate the efficacious transdifferentiation of various cell types into fully functional neurons (26), including mouse cortex astrocytes, mouse striatal astrocytes (27), retinal Müller glia cells (28), mesenchymal stem cells (19), glioblastoma cells (29), HeLa, NT2, N2A, ARPE19 and MEF cells (21), HAFs (30) and rat OPCs (31).

The potential of cell transdifferentiation in regenerative medicine is significant; however, it is imperative to avoid being misled by the deceptive appearance of newly formed neurons resulting from inaccurate and unsuitable analysis. Wang *et al* have presented evidence that the transformation of dopaminergic neurons (DAns) into putative astrocytes induced by adeno-associated virus (AAV)-short hairpin PTBP1 does not stem from resident astrocytes, but rather from endogenous neurons that have been infected by AAV due to viral leakage (32). Additionally, Hoang *et al* (33) presented evidence indicating the absence of neurons derived from astrocytes in mice with Müller glia-specific Ptbp1 deletion. Similarly, Chen *et al* (34) employed a rigorous lineage-tracing approach to establish that the inhibition of PTBP1 does not successfully induce the transdifferentiation of astrocytes in the substantia nigra or striatum into DAns in a mouse model of Parkinson's disease induced by 6-hydroxydopamine. Additionally, the study observed leakage of AAV to neighboring neurons (34).

Cell transplantation of the central nervous system is a therapeutic approach that seeks to utilize exogenous cells for the purpose of restoring, substituting or enhancing impaired neurons or tissues within the brain or spinal cord of a patient (35). Glial cells, neurons and stem cells are among the commonly transplanted cell types in this treatment modality. Its application spans across a wide range of disease areas, including Parkinson's disease (36), Alzheimer's disease (37) and spinal cord injuries (38). Immortalized cells exhibit considerable promise as potential candidates for cell transplantation therapy due to their plasticity and unrestricted proliferation. It has been proposed that immortalized cell lines possess the ability to persist following transplantation into both intact and impaired brains, subsequently integrating into the neural circuitry and assuming functional roles (39).

The HT22 cells demonstrate favorable neuronal-like attributes, display exceptional gene-editing capabilities and are commonly employed as a cell line for modeling mouse hippocampal neurons, particularly in studies pertaining to neurological disorders and neuroprotection (40). The mouse astrocyte (MA) cells represent a murine astrocyte cell line with a fibrous-like morphology, effectively emulating the characteristics and functionalities of astrocytes (41).

In this experiment, siRNA was transfected into HT22 and MA cells for 3 and 5 days, respectively. Western blotting and immunofluorescence staining were used to detect the expression of early neuron markers βIII-Tubulin and mature neuron markers NeuN and MAP2. This experiment aimed to elucidate the involvement of PTBP1 in the differentiation and maturation mechanisms of HT22 and MA cells under physiological conditions, in order to provide the possibility for cell transplantation therapy to replace damaged neurons by transdifferentiation.

Materials and methods

Cell culture. Mouse hippocampal neuron HT22 cells were purchased from Procell Life Science & Technology Co., Ltd. (cat. no. CL-0595). MA cells were purchased from Jennio Biotech Co., Ltd. (cat. no. JNO-M0088), which were immortalized from mouse primary astrocytes (cat. no. 1800-57) provided by ScienCell Research Laboratories, Inc. HT22 cells and MA cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; cat. no. D211113; Shanghai BasalMedia Technologies Co., Ltd.) supplemented with 1% penicillin/streptomycin mixture (cat. no. C0222; Beyotime Institute of Biotechnology) and 10% fetal bovine serum (FBS; cat. no. 11011-8611; Zhejiang Tianhang Biotechnology Co., Ltd.). The cells were routinely incubated in an incubator containing 5% CO_2 at a temperature of 37˚C. When cells reached an 80-90% confluency, they were subcultured at a 1:2 ratio using trypsin (cat. no. J121002; Shanghai BasalMedia Technologies Co., Ltd.).

Cell transfection. The non-targeting negative control (NC) small interfering (si)RNA (sense, UUCUCCGAACGUGUC ACGUTT; antisense, ACGUGACACGUUCGGAGAATT) and PTBP1-siRNA (sense, GCAGCCAAUGGAAACGAU ATT; antisense, UAUCGUUUCCAUUGGCUGCTT) were synthesized by Shanghai GenePharma Co., Ltd. For western blot analysis, HT22 cells and MA cells were seeded on 24-well plates ($5x10^4$/well) and cultured to 70-80% confluence. For immunofluorescence staining, cover slides (cat. no. YA0350; Beijing Solarbio Science & Technology Co., Ltd.) were put into a 24-well plate and treated with 300 μl polylysine (cat. no. P2100; Beijing Solarbio Science & Technology Co., Ltd.) per well for 30 min at room temperature. The cover slides were washed with sterile water 2-3 times and dried in the air. Cells were seeded at a density of $5x10^3$ cells/well on the cover slides

and transfection was performed the next day. Each well was incubated with 500 μl of fresh DMEM containing 10% FBS and no penicillin/streptomycin. Subsequently, 20 μM siRNA and 0.8 μl Lipo8000™ (cat. no. C0533; Beyotime Institute of Biotechnology) transfection reagent was added to 25 μl DMEM without FBS and penicillin/streptomycin, and incubated for 20 min at room temperature. A mixture of siRNA and Lipo8000™ transfection reagent was added to each well, and the cells were continued to be cultured for 3 or 5 days before detection.

Immunofluorescence analysis. Immunofluorescence experiments were performed after 3 or 5 days of cell transfection. The cover slides were washed three times for 3 min with PBS at room temperature (cat. no. P1033; Beijing Solarbio Science & Technology Co., Ltd.), and the cover slides were fixed with 4% paraformaldehyde (cat. no. BL539A; Biosharp Life Sciences) for 15 min at room temperature. After washing with PBS, the cover slides were permeated with a 0.3% Triton X-100 (cat. no. PH0352; Phygene Biotech) solution for 20 min and washed again three times with PBS at room temperature. The cover slides were blocked with antibody dilution agent (cat. no. A1800; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 30 min and then incubated overnight at 4˚C with primary antibodies: Rabbit anti-PTBP1 antibody (1:500; cat. no. 101043-T46; Sino Biological, Inc.), mouse anti-PTBP1 antibody (1:50; cat. no. sc-515282; Santa Cruz Biotechnology, Inc.), mouse anti-βIII-Tubulin antibody (1:200; cat. no. Sc-80016; Santa Cruz Biotechnology, Inc.) and rabbit anti-NeuN antibody (1:200; cat. no. AF1072; Beyotime Institute of Biotechnology). After washing with PBS, the cover slides were incubated at room temperature with donkey anti-rabbit secondary antibody conjugated to Dylight® 594 (1:200; cat. no. ab96921; Abcam) and donkey anti-mouse secondary antibody conjugated to Dylight® 488 (1:200; cat. no. ab96875; Abcam) for 90 min. Subsequently, they were washed three times with PBS and added to anti-fluorescence quenching sealing solution (cat. no. P0131; Beyotime Institute of Biotechnology) for sealing. Stained sections were imaged using a ZEISS upright fluorescence microscope (Axio Imager Z2; Zeiss AG). Staining intensity was quantified by mean fluorescence intensity using Image J software (V1.6; National Institutes of Health).

Western blotting. HT22 and MA cells were lysed in RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) with phenyl methanesulfonyl fluoride (cat. no. P0100; Beijing Solarbio Science & Technology Co., Ltd.) and phosphatase inhibitors (cat. no. D7121; Beyotime Institute of Biotechnology) to prepare protein samples. The total proteins in the supernatant were quantified by BCA Protein Assay Kit (cat. no. AL006-01; ACE Biotech) Equal amounts of total protein from each sample (25 μg per sample) were loaded onto 10% SDS polyacrylamide gels. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (0.45 μm; Merck KGaA). After blocking for 1 h with 3% bovine serum albumin (cat. no. GC305010; Wuhan Servicebio Technology Co., Ltd.) at room temperature, the membrane was incubated overnight at 4˚C with appropriate primary antibodies: Rabbit anti-MAP2 antibody (1:1,000; cat. no. bs-1369R; BIOSS), rabbit anti-PTBP1 antibody (1:2,000; cat. no. 101043-T46; Sino Biological, Inc.), mouse anti-βIII-Tubulin antibody (1:1,000; cat. no. Sc-80016; Santa Cruz Biotechnology, Inc.), rabbit anti-NeuN antibody (1:1,000; cat. no. AF1072, Beyotime Institute of Biotechnology) and mouse anti-GAPDH antibody (cat. no. GB15002, Wuhan Servicebio Technology Co., Ltd.). After washing with PBS for 15 min at room temperature, secondary antibodies are added: Goat anti-rabbit antibody (cat. no. bs-40295G; BIOSS) and goat anti-mouse antibody (cat. no. bs-40296G; BIOSS) incubated for 2 h at room temperature, After washing with PBS for 15 min at room temperature, the blots were visualized using chemiluminescence (cat. no. P0018S; Beyotime Institute of Biotechnology). The protein bands were obtained using a gel imaging analysis system (Tanon-2500B; Tanon Science and Technology Co., Ltd.) After washing with PBS for 15 min at room temperature, the signal strength was quantified by densitometry using Image J software (V1.6, National Institutes of Health).

Statistical analysis. All data were presented as the mean ± standard error of the mean. Differences among groups were assessed using one-way ANOVA with Tukey's post hoc test. All statistical analyses were carried out using GraphPad Prism software (Dotmatics) version 8.0. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PTBP1 knockdown does not promote the maturation and differentiation of HT22 cells after 3 days. Existing reports have demonstrated a gradual decline in PTBP1 expression levels during neuronal development and maturation (24,25). To assess the potential of PTBP1 reduction in promoting neuronal maturation, mouse hippocampal HT22 neuronal cells were cultured in 24-well plates ($5x10^4$/well) and transfected with siRNA targeting PTBP1 *in vitro*. The expression of early neuronal markers βIII-Tubulin and mature neuronal markers NeuN and MAP2 was analyzed via western blotting after 3 days of incubation. The results showed that the expression level of PTBP1 in HT22 cells was significantly reduced after 3 days of transfection of PTBP1-siRNA compared with the mock control group and NC-siRNA group ($P<0.0001$; Fig. 1A and C). Due to the effect of cytotoxicity induced by the entry of siRNA into cells, the expression of the mature neuronal marker MAP2 was significantly reduced in the PTBP1-siRNA and NC-siRNA groups compared with the mock control group ($P<0.01$), but there was no significant alteration in the PTBP1-siRNA group compared with the NC-siRNA group ($P>0.05$; Fig. 1A and B). Furthermore, there were no significant changes noted in the early neuronal marker βIII-Tubulin and the mature neuronal marker NeuN among each group ($P>0.05$; Fig. 1D and E).

The cellular morphology of neurons undergoes a variety of changes as they proliferate and mature, including the growth of cell protrusions and the enlargement of nuclear bodies, which help neurons build complex networks of connections and support the functional development of the nervous system (42). To assess the changes in individual cell morphology and to show differences in the expression levels of neuronal markers *in situ*

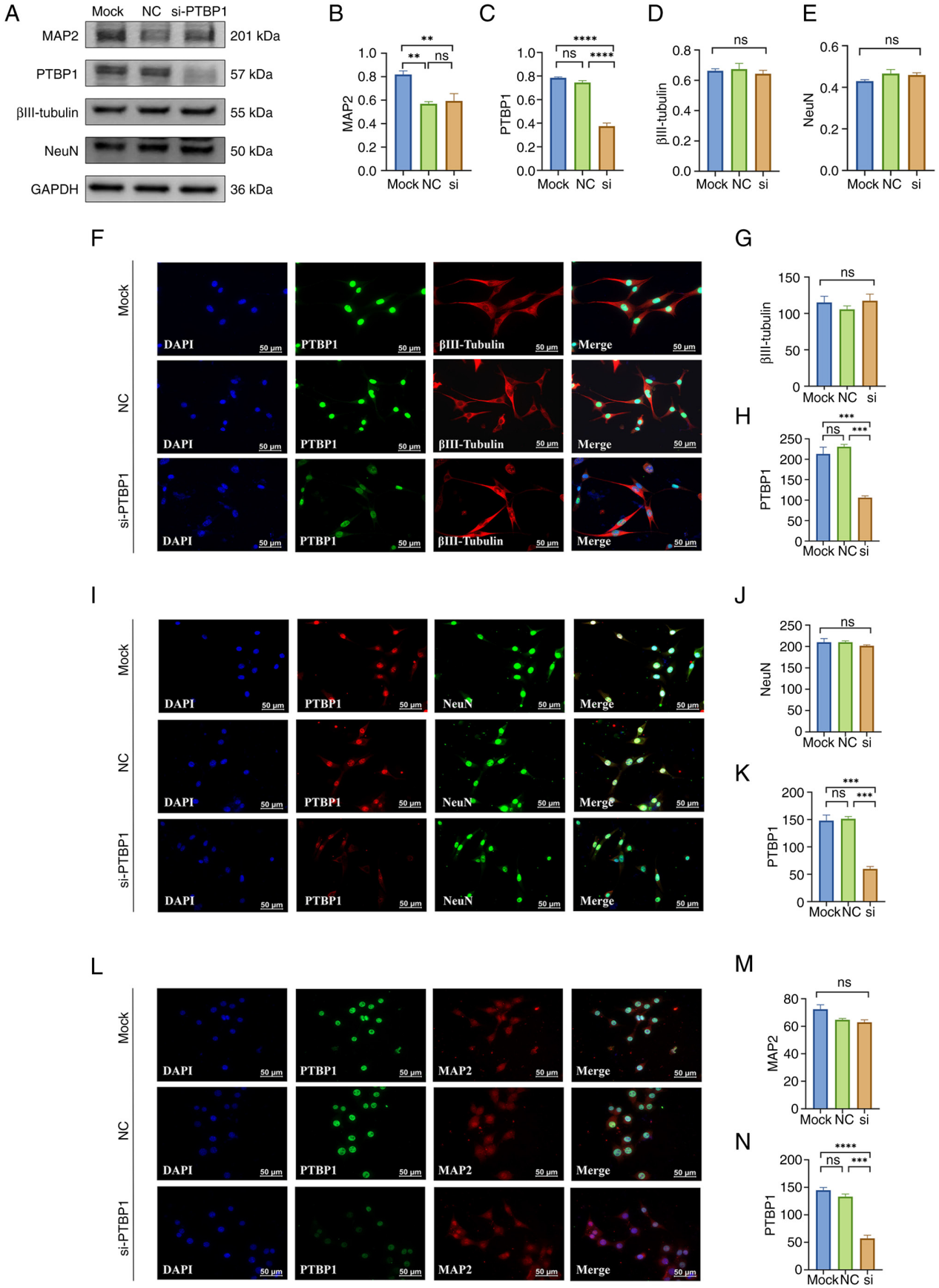

Figure 1. Effect of a 3-day decrease in PTBP1 protein level in HT22 cells on cell differentiation and maturation. (A) Representative western blotting of MAP2, PTBP1, βIII-Tubulin, NeuN and GAPDH (n=4 per group). Quantitative statistical analysis results of (B) MAP2, (C) PTBP1, (D) βIII-Tubulin, (E) NeuN. (F) Representative immunofluorescence staining images and quantitative statistical analysis results of (G) βIII-Tubulin and (H) PTBP1. (I) Representative immunofluorescence staining images and quantitative statistical analysis results of (J) NeuN and (K) PTBP1. (L) Representative immunofluorescence staining images and quantitative statistical analysis results of (M) MAP2 and (N) PTBP1 (n=3 per group). Scar bar, 50 μm. **P<0.01, ***P<0.001 and ****P<0.0001. PTBP1, polypyrimidine tract-binding protein 1; MAP2, microtubule-associated protein 2; NC, negative control; si, small interfering; ns, not significant.

after PTBP1 reduction, the present study used immunofluorescence experiments targeting βIII-tubulin, NeuN and MAP2 for detection and observed that PTBP1 protein was mainly localized in the nucleus and that PTBP1 protein expression levels in the nucleus of HT22 cells were significantly reduced after 3 days of cell transfection with PTBP1-siRNA (P<0.001, Fig. 1H; P<0.001, Fig. 1K; P<0.001, Fig. 1N), however, cellular immunofluorescence staining of βIII-tubulin, NeuN and MAP2 also revealed no significant differences in HT22 cell in both protein level and morphology (including protrusion length and nucleus size) after 3 days of cell transfection of siRNA targeting PTBP1 (P>0.05; Fig. 1F-N).

PTBP1 knockdown does not promote the maturation and differentiation of HT22 neurons after 5 days. In most research investigating the induction of cellular transdifferentiation based on PTBP1 protein deletion, a large number of cell lines with neuronal-like properties had been completely induced at 5-7 days (43,44), and to exclude the effect of the length of induction time, the present study extended the cell induction period to 5 days. The results of western blotting showed that PTBP1-siRNA transfection of HT22 cells for 5 days, the protein expression level of PTBP1 in HT22 cells was significantly reduced compared with the mock control group and NC-siRNA group (P<0.0001; Fig. 2A, C). However, the protein expression levels of βIII-Tubulin, NeuN and MAP2 did not change significantly (P>0.05; Fig. 2A-E). Immunofluorescence results showed that after 5 days of transfection of siRNA targeting PTBP1 in HT22 cells, the expression level of PTBP1 was significantly reduced compared with that in the mock control group and the NC-siRNA group (P<0.001, Fig. 2H; P<0.01, Fig. 2K; P<0.01, Fig. 2N), but immunocytofluorescence staining of βIII-Tubulin, NeuN and MAP2 also showed that there was no significant difference both in protein levels and morphology of HT22 cells (P>0.05; Fig. 2F-N).

PTBP1 knockdown does not promote MA cells differentiation after 3 days. The astrocyte, a type of glial cell widely distributed in the brain, is an important target for research into cell reprogramming because of its continuous self-renewal and high plasticity (41). A number of studies have shown that reducing the expression of PTBP1 in astrocytes can promote its transdifferentiation into functional neurons (45,46). To assess whether reducing PTBP1 expression levels in MA astrocytes can promote their reprogramming into neurons, MA cells were seeded into 24-well plates ($5x10^4$/well) for cell transfection *in vitro*. The expression of early neuronal markers βIII-Tubulin and mature neuronal markers NeuN and MAP2 were detected using western blotting. The results showed that the protein expression level of PTBP1 was significantly reduced in MA cells after 3-day of PTBP1-siRNA transfection compared with mock control and NC-siRNA groups (P<0.0001; Fig. 3A,C), but the early neuronal markers βIII-Tubulin, the mature neuronal marker NeuN and MAP2 were not significantly changed (P>0.05; Fig. 3A-E).

Cell transdifferentiation typically involves changes in cellular morphology to accommodate its new neuronal function. The star-shaped protrusions exhibited by astrocytes undergo a metamorphosis into elongated axons and branching dendrites, while the cell nucleus repositions itself towards one side of the axon (43). Consequently, newly generated neurons acquire electrophysiological characteristics and establish synapses, thereby actively engaging in neural network activity (43). Immunofluorescence results showed that after 3 days of transfection of siRNA targeting PTBP1 in MA cells, the expression level of PTBP1 was significantly reduced compared with that in the mock control group and the NC-siRNA group (P<0.0001, Fig. 3H; P<0.001, Fig. 3K; P<0.0001, Fig. 3N), but immunocytofluorescence staining of βIII-Tubulin, NeuN and MAP2 also showed no significant difference both in protein levels and morphology of MA cells (P>0.05; Fig. 3F-N). Furthermore, the nucleus size and position of MA cells, as well as the morphology of the star-shaped protrusions, showed no marked changes compared with the Mock and NC groups. This provides evidence that downregulation of PTBP1 did not induce differentiation.

PTBP1 knockdown does not promote MA cells differentiation after 5 days. The present study also extended the period of induced differentiation of MA astrocytes to 5 days in order to be able to observe evidence of glial cell conversion to neurons. However, the results of western blotting showed that significant reduction in the protein expression level of PTBP1 in MA cells (P<0.0001; Fig. 4A and C) after 5 days of transfection with PTBP1-siRNA did not result in significant changes in the protein expression levels of the neuronal markers βIII-Tubulin, NeuN and MAP2 (P>0.05; Fig. 4A-E). Immunofluorescence results showed that after 5 days of transfection of siRNA targeting PTBP1 in MA cells, the expression level of PTBP1 was significantly reduced compared with that in the mock control group and NC-siRNA group (P<0.001, Fig. 4H; P<0.001, Fig. 4K; P<0.0001, Fig. 4N), but immunocytofluorescence experiments targeting βIII-Tubulin, NeuN and MAP2 also showed no significant difference both in protein levels and morphology of MA cells (P>0.05; Fig. 4F-N), which was quite different from the results of previous studies (27,47).

Discussion

Numerous studies have been focusing on the potential of cell reprogramming as a means of converting resident glial cells into functional neurons in order to address deficiencies in neural circuits (48,49). The incorporation of novel neurons not only provides significant assets for the investigation of cerebral disorders, but also affords a prospect for the reparation or substitution of impaired neurons via reprogramming (46). Through the introduction of fresh neurons, individuals can reconstruct compromised neural connections, alleviate symptoms and potentially decelerate the advancement of the ailment (50). This treatment holds significant promise in alleviating or curing symptoms associated with neurodegeneration. Numerous studies have shown that after targeted knockdown of PTBP1 in mice, astrocytes derived from distinct brain regions undergo differentiation into distinct neuronal subtypes, which subsequently integrate into endogenous circuits (27,51). The experimental data demonstrate that these neonatal neurons exhibit identical electrophysiological characteristics and ameliorate disease states in animal models (52). Nevertheless, other studies have raised doubts regarding the impact of diminished PTBP1 on cell transdifferentiation. Research has

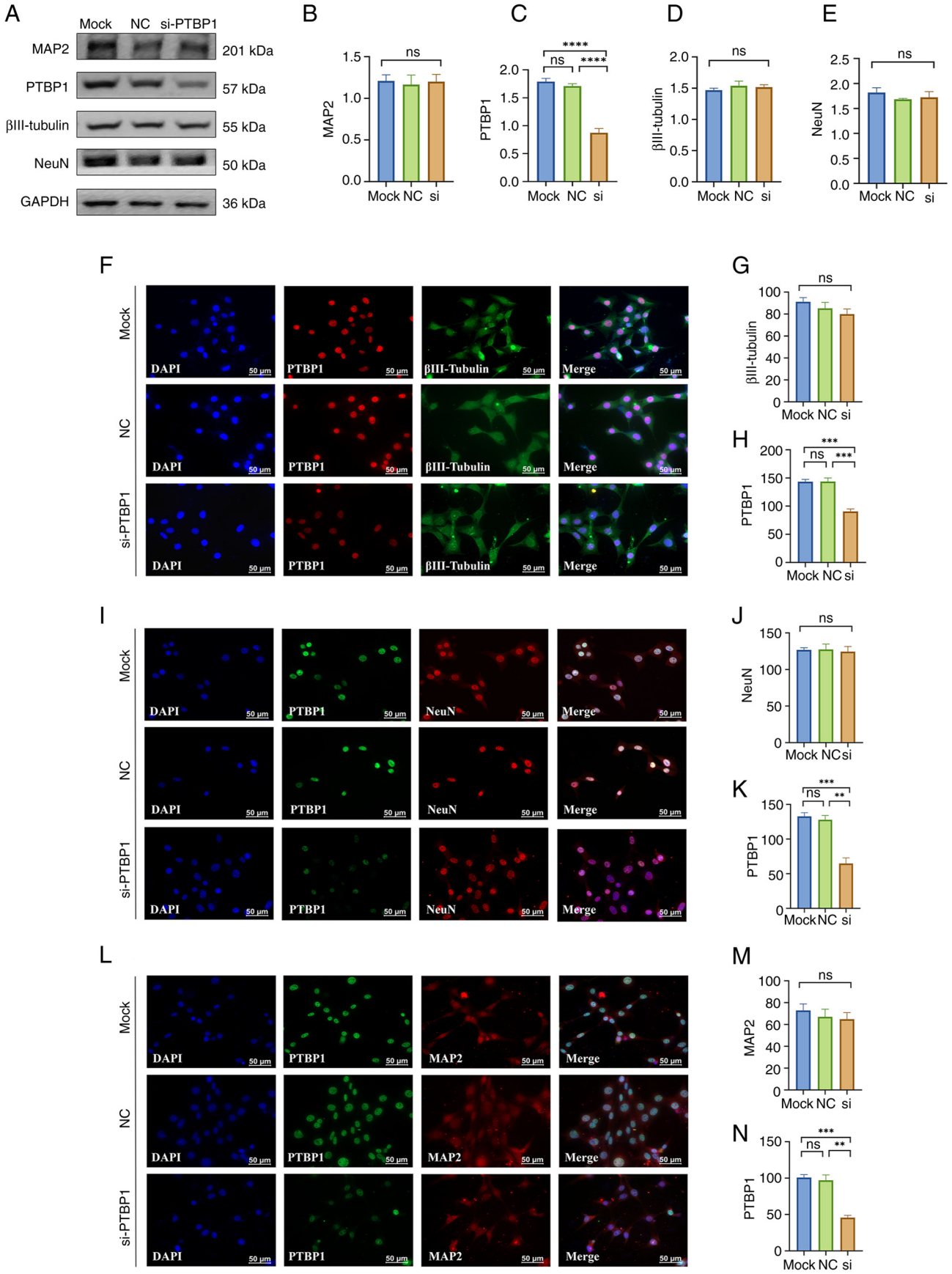

Figure 2. Effect of a 5 day decrease in PTBP1 protein level in HT22 cells on cell differentiation and maturation. (A) Representative western blotting of MAP2, PTBP1, βIII-Tubulin, NeuN and GAPDH (n=4 per group). Quantitative statistical analysis results of (B) MAP2, (C) PTBP1, (D) βIII-Tubulin and (E) NeuN. (F) Representative immunofluorescence staining images and quantitative statistical analysis results of (G) βIII-Tubulin and (H) PTBP1. (I) Representative immunofluorescence staining images and quantitative statistical analysis results of (J) NeuN and (K) PTBP1. (L) Representative immunofluorescence staining images and quantitative statistical analysis results of (M) MAP2 and (N) PTBP1 (n=3 per group). Scar bar, 50 μm. **P<0.01, ***P<0.001 and ****P<0.0001. PTBP1, polypyrimidine tract-binding protein 1; MAP2, microtubule-associated protein 2; NC, negative control; si, small interfering; ns, not significant.

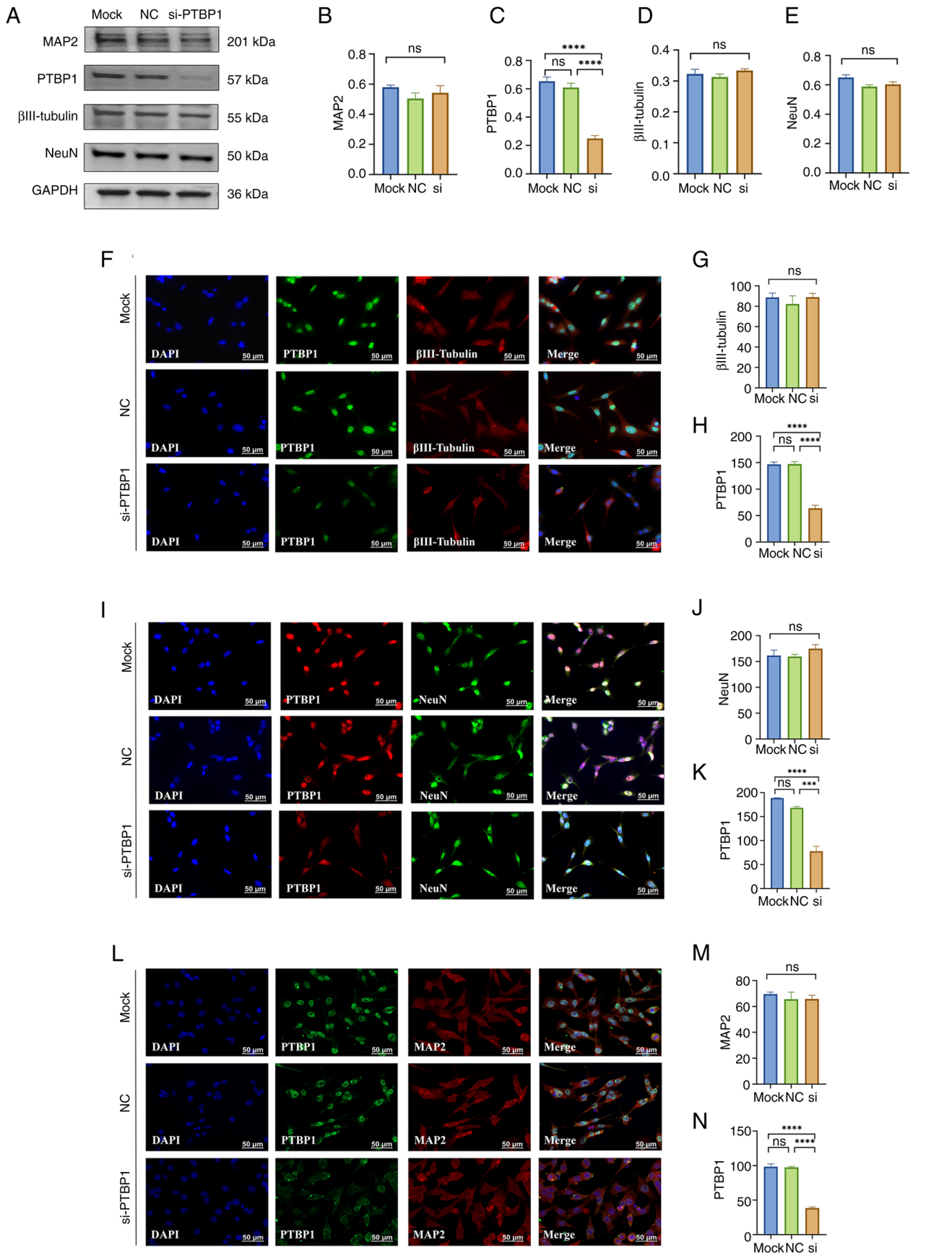

Figure 3. Effect of a 3 day decrease in PTBP1 protein level in MA cells on cell differentiation. (A) Representative western blotting of MAP2, PTBP1, βIII-Tubulin, NeuN and GAPDH (n=4 per group). Quantitative statistical analysis results of (B) MAP2, (C) PTBP1, (D) βIII-Tubulin and (E) NeuN. (F) Representative immunofluorescence staining images and quantitative statistical analysis results of (G) βIII-Tubulin and (H) PTBP1. (I) Representative immunofluorescence staining images and quantitative statistical analysis results of (J) NeuN and (K) PTBP1. (L) Representative immunofluorescence staining images and quantitative statistical analysis results of (M) MAP2 and (N) PTBP1 (n=3 per group). Scar bar, 50 μm. ***P<0.001 and ****P<0.0001. PTBP1, polypyrimidine tract-binding protein 1; MAP2, microtubule-associated protein 2; NC, negative control; si, small interfering; ns, not significant; MA, mouse astrocyte.

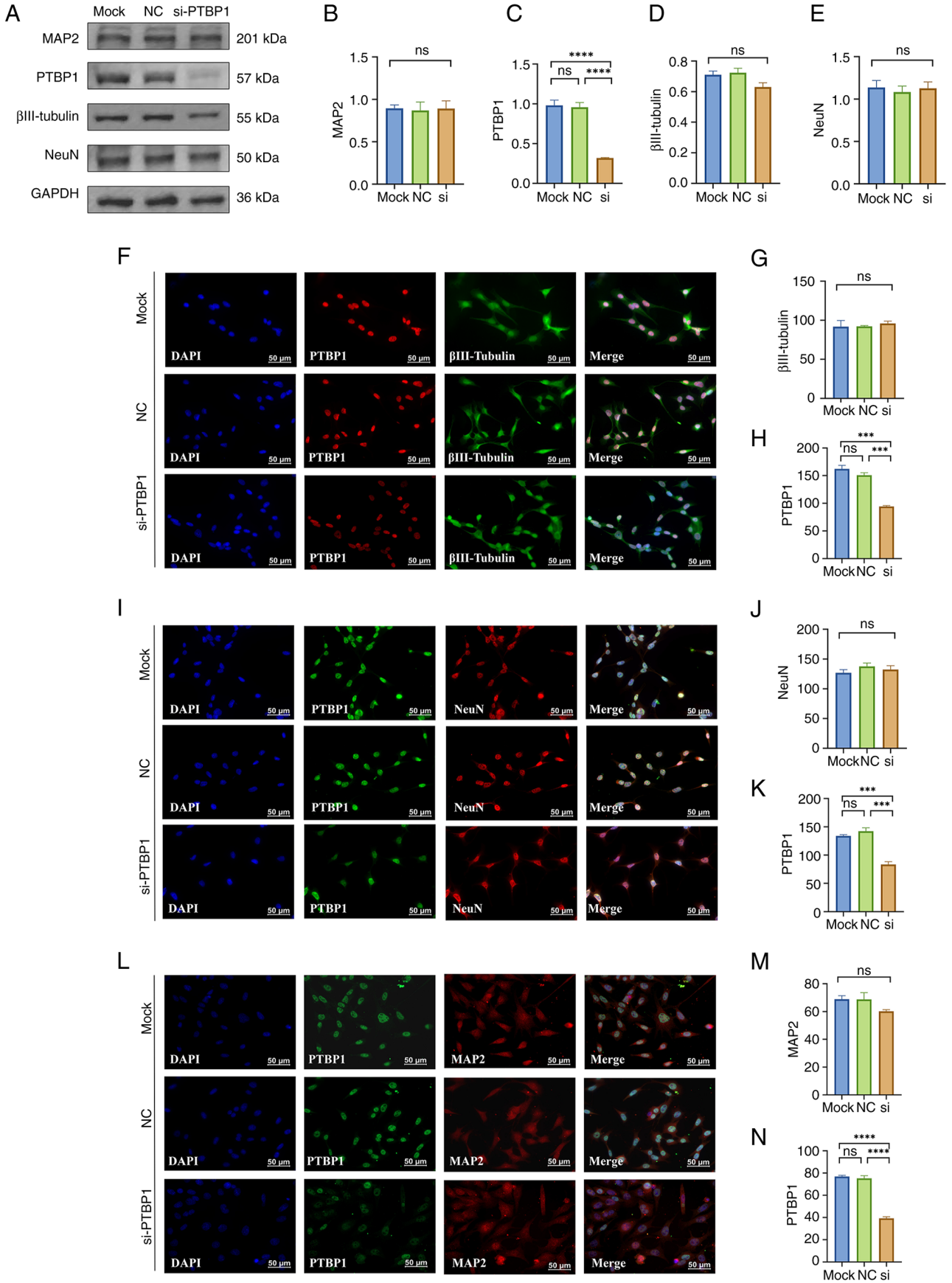

Figure 4. Effect of a 5 day decrease in PTBP1 protein level in MA cells on cell differentiation. (A) Representative western blotting of MAP2, PTBP1, βIII-Tubulin, NeuN and GAPDH (n=4 per group). Quantitative statistical analysis results of (B) MAP2, (C) PTBP1, (D) βIII-Tubulin and (E) NeuN. (F) Representative immunofluorescence staining images and quantitative statistical analysis results of (G) βIII-Tubulin and (H) PTBP1. (I) Representative immunofluorescence staining images and quantitative statistical analysis results of (J) NeuN and (K) PTBP1. (L) Representative immunofluorescence staining images and quantitative statistical analysis results of (M) MAP2 and (N) PTBP1 (n=3 per group). Scar bar, 50 μm. ***P<0.001 and ****P<0.0001. PTBP1, polypyrimidine tract-binding protein 1; MAP2, microtubule-associated protein 2; NC, negative control; si, small interfering; ns, not significant; MA, mouse astrocyte.

indicated that the reduction of PTBP1 does not inevitably result in neuronal differentiation, but rather may induce apoptosis or other alterations in cellular destiny (53,54). This suggests that the effect of reduced PTBP1 *in vivo* on cellular transdifferentiation is complex and influenced by other factors.

PTBP1 plays a crucial role in the regulation of exon splicing, and selective splicing can modulate gene expression (55). A decrease in PTBP1 levels may result in alterations in exon splicing, thereby impacting the expression of numerous genes (25). These genes may participate in diverse cellular processes, including cell differentiation and neuronal development, as well as other cellular functions (56). Consequently, the regulation of gene expression following PTBP1 reduction may be intricate, potentially leading to ambiguous or inconsistent outcomes in neuronal differentiation. Secondly, the impact of diminished PTBP1 expression may be contingent upon the specific cell type and prevailing environmental circumstances, and the differentiation of cells is also influenced by the activated and resting states of the cells (57). Different cell types may exhibit different responses following a decrease in PTBP1 levels, with certain cells potentially promoting the differentiation of neurons, while others may display opposing or alternative effects (29,58). Wang *et al* (29) have provided evidence that the downregulation of PTBP1 can induce neural differentiation in glioblastoma multiforme cells through the activation of UNC5B receptors, consequently impeding the proliferation of cancer cells both *in vitro* and *in vivo*. However, Xie *et al* (58) have provided evidence through lineage tracing that the conversion of Müller glial cells into retinal ganglion cells does not occur subsequent to PTBP1 downregulation, achieved either by CRISPR-CasRx or small hairpin RNA. Furthermore, discrepancies in the extent and manner of PTBP1 suppression across investigations may contribute to the conflicting findings. Diverse methodologies, including ASO, CRISPR-CasRx, AAV, shRNA and small molecules, have been utilized to diminish PTBP1 expression in various studies (21,32,58-60). Disparities in the efficacy and precision of these techniques may result in incongruous research outcomes. Therefore, the imperative for the advancement of cell reprogramming demands a meticulous and methodical evaluation approach to ascertain and track the origin of newly generated neurons (32).

In contrast to previous studies examining PTBP1 *in vivo* during neuronal transdifferentiation (27,61), the present study aimed to induce the transdifferentiation of immortalized cells into neurons under physiological conditions, with the ultimate goal of facilitating transplantation. Due to its resemblance to the natural cell developmental process, cell transdifferentiation under physiological conditions yields neurons that exhibit characteristics more akin to normal neurons (62). Consequently, this approach mitigates the detrimental impact of foreign implantation or exogenous factors on the body, thereby augmenting the biological compatibility of cell therapy and transplantation (35). The current study employed highly specific and highly efficient siRNA to conduct *in vitro* protein knockdown in cell lines, thereby reducing interference from the complex metabolic and physiological processes of the organism and avoiding erroneous labeling caused by viral vector leakage previously reported (34). siRNA was used to downregulate the expression of PTBP1 in mouse hippocampal neuronal HT22 cells for a duration of 3 and 5 days. Subsequently, western blotting and immunofluorescence staining were conducted to evaluate the neuronal differentiation and maturation in HT22 cells. Furthermore, the process of astrocyte-to-neuron transdifferentiation was investigated through the downregulation of PTBP1 expression in mouse astrocytic MA cells for a duration of 3 and 5 days under physiological conditions. However, the mechanisms of differentiation resulting from the decreased PTBP1 expression in HT22 and MA cells were not observed. The outcomes of the present study corroborate previous studies indicating that, in both physiological and pathological contexts, the downregulation of PTBP1 did not result in the transformation of astrocytes into neurons in the mouse brain (63).

One primary constraint of the present study pertained to the exclusive utilization of immortalized cell lines as *in vitro* models to investigate the transdifferentiation mechanism targeting PTBP1, without employing more physiologically relevant human cell types for in-depth validation, such as primary human neurons and primary astrocytes. The cultivation of primary human cells can better mimic their natural physiological environment *in vivo*, typically preserving their inherent biological characteristics to a greater extent and displaying varying functions and properties depending on the brain region from which they are extracted (64). The cultivation of immortalized cell lines cannot fully replicate the intricate microenvironments present within the human brain. However, they offer unlimited growth potential and provide an ample cell source (65). The immortalized cell lines also demonstrate similarities to primary cells, including comparable cell morphology, gene expression, expression of specific markers, electrophysiological characteristics and participation in the regulation of the extracellular environment (66). Immortalized cells also allow for standardized production and amplification, ensuring consistent quality of cell therapy for each patient (35). These advantages make it more feasible to use immortalized cells to transdifferentiate neurons for transplantation therapy. Although the present study did not present evidence of cell transdifferentiation into neurons in this experiment, this research also adds to the search for medical regeneration. These results indicate that the transdifferentiation of non-neuronal cells into neurons necessitates the resolution of specific technical obstacles. Moreover, in some specific research investigations (27,51), the reduction of PTBP1 expression improves the brain pathology and behavioral performance in mice, suggesting that PTBP1 downregulation may exert beneficial effects on brain conditions via mechanisms unrelated to transdifferentiation.

In brief, the promotion of cell transdifferentiation into neurons following PTBP1 reduction presents certain inconsistencies, which may arise from the intricate regulation of PTBP1, variations in experimental conditions and cell types and variances in the extent and means of PTBP1 reduction. Further investigation is necessary to elucidate the precise mechanism underlying the impact of PTBP1 reduction on cell transdifferentiation, thus presenting a more dependable theoretical foundation for the implementation of cell therapy and regenerative medicine.

In conclusion, the process of neuronal transdifferentiation is highly intricate, and is influenced by a multitude of factors. Additional investigation is necessary to elucidate the molecular

mechanisms that govern cell fate and differentiation. Despite the inability of PTBP1 knockdown to promote neuronal maturation and differentiation under normal physiological circumstances, the combined administration of PTBP1 down-regulation alongside crucial adjuncts, such as small molecules, may hold promise for facilitating the transdifferentiation of immortalized cells into neurons in forthcoming research (67).

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

WJZ, QL, CMQ, CC and YQS designed the general concept of the study. QL performed the experiments. WZ, XYQ, CL and JJD curated and analyzed the data. QL wrote the original draft manuscript, WJZ, CMQ, CC and YQS made revisions and improvements to the draft. WJZ and QL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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