Cell Mol Neurobiol (2015) 35:669–677
DOI 10.1007/s10571-015-0162-1

ORIGINAL RESEARCH

Serine/Threonine Protein Phosphatase-5 Accelerates Cell Growth and Migration in Human Glioma

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Received: 11 December 2014 / Accepted: 30 January 2015 / Published online: 22 March 2015

Abstract Glioma is the most common type of primary central nervous system tumor. Ser/Thr protein phosphatase 5 (PP5) has been shown to regulate multiple signaling cascades that suppress growth and facilitate apoptosis in several human cancer cells. However, the role of PP5 in human gliomas remains unclear. Herein, the relationship between PP5 expression and glioma cell growth was investigated, and the therapeutic value of PP5 in glioma was further evaluated. We employed a short hairpin RNA targeting *PPP5C* gene to knock down PP5 expression in human glioma cell lines U251 and U373. Depletion of *PPP5C* via RNAi remarkably inhibited glioma cell proliferation and colony formation, and arrested cell cycle in the G0/G1 phase. Moreover, knockdown of PP5 markedly suppressed glioma cell migration, as determined by Transwell assay. Our findings suggest that *PPP5C* could be essential for glioma cell growth and serve as a promising therapeutic target in human gliomas.

Keywords Glioma · *PPP5C* · Proliferation · Migration

Xinglong Zhi and Hongqi Zhang have equally contributed to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10571-015-0162-1) contains supplementary material, which is available to authorized users.

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Introduction

The reversible protein phosphorylation (phosphorylation and dephosphorylation) plays an important role in most aspects of cell life, including cell growth regulation, differentiation, senescence, and apoptosis (Cohen 2001). Protein phosphatase catalyzes dephosphorylation in vivo, and there are three major types of protein phosphatases in humans, tyrosine phosphatase (PTPase) (Zhang 2002), serine/threonine phosphatase (PPase) (Mumby and Walter 1993) and dual specificity phosphatase (DSPase) (Camps et al. 2000). Serine/threonine protein phosphatase-5 (PP5, *PPP5C*) is one member of the protein serine/threonine phosphatase gene family which is broadly expressed in mammalian tissues. It is confirmed that PP5 comprises a regulatory tetratricopeptide repeat (TPR) domain and functions in signaling pathways in many cellular responses. Currently, the biological function of PP5 is not clear, and its elevated expression level is associated with the cancer development in breast, liver, and possibly other forms (Golden et al. 2004, 2008a, b; Ghobrial et al. 2005; Shirato et al. 2000; Fukuda et al. 2007). High levels of PP5 have been observed in human cancers, and constitutive PP5 overexpression aids tumor progression in mouse models of tumor development (Golden et al. 2004, 2008a, b). PP5 is found to be encountered in several proteins that regulate intra-cellular signaling cascades by hormones (i.e., glucocorticoids) or cellular stress. In glucocorticoids signaling, PP5 is associated with heat shock protein-90 (Hsp-90) which is glucocorticoids receptor (GR) (Chen et al. 1996; Silverstein et al. 1997; Davies et al. 2005), and can induce phosphorylation of p53 tumor suppressor protein (Zuo et al. 1998, 1999; Urban et al. 2003). In response to genomic cellular stress, especially when PP5 is down-regulated by siRNA or antisense oligonucleotides, several

stress-responsive proteins such as ASK1, PKcs, p53, Cdc37, Raf1, and GRs show rich phosphorylation (Vaughan et al. 2008; Mkaddem et al. 2009; Huang et al. 2004; Wechsler et al. 2004; Morita et al. 2001; Kutuzov et al. 2005). Phosphorylation of these responsive proteins can trigger apoptosis but apparently PP5 negatively regulates the processes (Morita et al. 2001; Kutuzov et al. 2005; Mkaddem et al. 2009; Huang et al. 2004). Taken together, previous studies have indicated that PP5 is a potentially important regulator of cellular signaling networks.

Glioma is the most common primary malignancy in the human central nervous system (CNS). Approximately, 13,000 deaths and 18,000 new cases of primary malignant brain and CNS tumors occur annually in the US (Ostrom et al. 2013). Four grades of gliomas are classified by histological progression: low-grade astrocytomas (Grade I–II), anaplastic astrocytomas (Grade III), and glioblastoma (GBM, Grade IV) (Kleihues et al. 1993, 2002). The five-year survival in low-grade gliomas is around 30–70 % depending on histology (Louis et al. 2007), but the patients with the most aggressive type of glioma, GBM, have the worst prognosis with a median survival of 9–12 months even after surgical resection, radiation therapy, and chemotherapy (Stupp et al. 2005). As a result of that, early diagnostics with treatment can provide comprehensive and preventive health care on glioma patients. Scientists are in aggressive needs to figure out new therapeutic target against human gliomas.

RNA interference (RNAi) technique is a powerful tool to carry out loss-of-function assays. It provides a new approach to investigate cancer gene therapy (Kim et al. 2005; Guo et al. 2011). In this study, we employed a lentiviral vector-mediated RNAi system to achieve highly stable silencing of PP5. In this study, we are aiming to evaluate the biological function of PP5 protein (gene: *PPP5C*) and try to reveal its contribution to glioma progression. In order to verify that *PPP5C* could be an effective therapeutic target for glioma, we suppressed PP5 expression via RNAi in human glioma cell lines U251 and U373. To our knowledge, this is the first time that we provided evidence to demonstrate knockdown of endogenous PP5 expression could suppress the oncogenic properties of glioma cells and their invasion capacity in vitro. It is suggesting that *PPP5C* could be a promising therapeutic target for human gliomas.

Materials and Methods

Cell Culture

Human embryonic kidney cell line 293T (HEK293T) and human glioblastoma cell lines U251, U87MG, U373, A172, and U-118MG were obtained from Shanghai Institute of Cell Biology, the Chinese Academy of Sciences. U87MG cells were cultured in EMEM (Hyclone, Logan, UT, USA) supplemented with 10 % fetal bovine serum (FBS), 1 mM sodium pyruvate and 1 % non-essential amino acid at 37 °C in a humidified atmosphere of 5 % CO_2. U251, U373, A172, U-118MG, and HEK293T cells were maintained in DMEM (Hyclone, Logan, UT, USA) supplemented with 10 % FBS at 37 °C in a humidified atmosphere of 5 % CO_2.

Construction of Recombinant Lentivirus

The following oligonucleotides were synthesized. Two of short hairpin RNA (shRNA) sequences (5′-GAGAC AGAGA AGATT ACAGT ACTCG AGTAC TGTAA TCTTC TCTGT CTCTT TTT-3′, sequence 1) and (5′-CCACG AGACA GACAA CATGA ACTCG AGTTC ATGTT GTCTG TCTCG TGGTT TTT-3′, sequence 2) for human *PPP5C* gene (NM_001204284.1) were screened and validated to be candidate shRNAs. And the negative control shRNA was 5′-GCGGA GGGTT TGAAA GAATA TCTCG AGATA TTCTT TCAAA CCCTC CGCTT TTTT-3′. The stem-loop-stem oligos (shRNAs) were synthesized, annealed, and ligated into the AgeI/EcoRI-linearized pFH-L vector (Shanghai Hollybio, China). The lentiviral-based shRNA-expressing vectors were confirmed by DNA sequencing. The generated plasmids were named as pFH-L-shPPP5C-S1, pFH-L-shPPP5C-S2, and pFH-L-shCon.

HEK293T cells (1.0×10^6) were seeded into 10-cm dishes and cultured for 24 h to reach 70–80 % confluence. Two hours before transfection, the medium was replaced with serum-free DMEM. The plasmids including 10 μg of pFH-L-shPPP5C-S1/S2 or pFH-L-shCon, 7.5 μg of packaging vector pHelper-1.0, and 5 μg of expression plasmid pHelper-2.0 were added to 0.95 ml Opti-MEM and 50 μl of Lipofectamine 2000. The mixture was added to the cells and incubated for 8 h before replacing the medium with 10 ml of complete DMEM medium (supplemented with 10 % PBS). Lentiviral particles were harvested at 48 h after transfection. As the lentivirus carries green fluorescence protein (GFP), the viral titer was determined by end-point dilution assay through counting GFP-expressing cells under fluorescence microscope 96 h after infection.

RNA Extraction and Real-Time PCR

Human glioblastoma cells (U251 and U373) were pre-cultured and infected with recombinant lentivirus for 5 days. Total RNA was prepared using Trizol reagent (Gibco RL, Grand Island, NY, USA) according to the manufacturer's instruction. 5 mg of total RNA was used to

synthesize the first strand of cDNA using SuperScript II RT 200 U/ml (Invitrogen, Carlsbad, CA, USA). *PPP5C* mRNA expression was evaluated by real-time PCR on BioRad Connet Real-Time PCR platform (BioRad, Hercules, CA, USA) with SYBR Green PCR core reagents. The qPCR reaction system contains 2× SYBR premix ex taq 10 μl, plus forward and reverse primers (2.5 μM) 0.8 μl, cDNA template 5 μl, and ddH_2O 4.2 μl. *β-Actin* was applied as the internal reference. The following primers were synthesized and applied: *PPP5C*: 5′-CCCAA CTACTGCGACCAGAT-3′ as forward and 5′-CCCGTCA CCTCACATCATTC-3′ as reverse; *β-actin*: 5′-GTGGACA TCCGCAAAGAC-3′ as forward and 5′-AAAGGGTGTAA CGCAACTA-3′ as reverse. The reaction procedure was initiated with denaturation at 95 °C for 1 min and followed by 40 repeated cycles (denaturation at 95 °C for 5 s and annealing extension at 60 °C for 20 s). Results were presented as CT values, defined as the threshold PCR cycle number at which an amplified product was first detected. The average CT was calculated for both *PPP5C* and *β-actin*, and ΔCT was determined as the mean of the triplicate CT values for *PPP5C* minus the mean of the triplicate CT values for *β-actin*.

Western Blot Analysis

U251 and U373 cells were cultured and infected with recombinant lentivirus for 5 days. Cells were washed twice with ice-cold PBS and lysed in 2× SDS sample buffer (100 mM Tris–HCl (pH 6.8), 10 mM EDTA, 4 % SDS, 10 % Glycine). Equal amounts of proteins (30 μg) were loaded and separated on 10 % SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 300 mA for 1.5 h. Membranes were blocked and then probed with primary antibodies, mouse anti-PPP5C (1:3,000 dilution; Abcam, Cambridge, UK) or rabbit anti-GAPDH (1:60,000 dilution; Proteintech Group Inc., Chicago, IL, USA) overnight at 4 °C. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Dallas, Texas, USA) for 2 h at room temperature and then visualized by super ECL detection reagent (Applygen, Beijing, China).

MTT Viability Assay

Both U251 and U373 cells were cultured in 6-well plates and inoculated with recombinant lentiviruses. After 72 h of infection, cells were washed and re-cultured in 96-well plates with 2.0×10^3 cells per well (U251) or 2.5×10^3 cells per well (U373). MTT solution was added to each well and incubated at 37 °C for 4 h at different time points after lentivirus infection (1, 2, 3, 4, 5 days). Then the converted dye solubilized in acidic isopropanol (10 % SDS, 5 % isopropanol and 0.01 M HCl) was used and incubated at 37 °C for 10 min. The optical density was measured using microplate reader at the wavelength of 595 nm. The experiment was repeated at least three times.

Colony Formation Assay

Human glioblastoma cells U251 and U373 were cultured in 6-well plates and treated with recombinant lentiviruses. After 96 h of incubation, infected cells were washed, re-cultured in the prepared 6-well plates at a density of 800 cells per well (U251) or 500 cells per well (U373) and allowed to form natural colonies. After 13 days of incubation (U251) or 9 days of incubation (U373), treated glioblastoma cells were subjected to crystals violet staining. Subsequently, cells were washed and fixed by paraformaldehyde. Fixed cells were washed twice with PBS solution, treated with crystals violet for 10 min, washed 3 times by ddH_2O, and then photographed with a digital camera. The number of colonies (>50 cells/colony) was counted.

Flow Cytometry Analysis

U251 cells were cultured in 6-well plates and inoculated with recombinant lentiviruses at a MOI of 10. After 72 h of infection, cells were inoculated into 6-cm dishes at a density of 2.0×10^5 cells per dish. After 40 h incubation period, cells in each well were harvested and cell cycle was determined by propidium iodide (PI) staining method before cell density reached 80 % confluency. Tests were performed in triplicate for each sample, and analyses were performed by FAC Scan flow cytometer (Becton–Dickinson, San Jose, CA, USA) in accordance with the manufacturer's guidelines.

Cell Migration Assay

U251 and U373 cells infected with Lv-shPPP5C or Lv-shCon for 96 h and uninfected cells were harvested, and their ability to migrate in vitro was determined using a Transwell chamber (Corning, NY, USA). Cells were seeded into the upper chamber (3.0×10^4 cells/well of U251, 5.0×10^4 cells/well of U373) in 100 μl serum-free medium. Medium (1 ml) containing 20 % FBS was added to the lower chamber as a chemo-attractant. After incubation for 24 h at 37 °C in 5 % CO_2, the surface of the upper chamber was swabbed with cotton-tipped applicators to remove the cells that did not migrate, including dead cells. The viable cells that moved to the lower surface of the filter were fixed in 4 % paraformaldehyde and then stained with crystal violet. The migrated cells were counted in five random fields (100×) per filter under a light microscope,

and the amount of dissolved crystal violet was detected by the spectrometric absorbance at 570 nm.

Statistical Analysis

All statistical analyses were performed using SPSS13.0 software. The differences between groups were compared using Student's t test, and data were expressed as mean ± SD of three independent experiments. Statistical significant difference was accepted at $p < 0.05$.

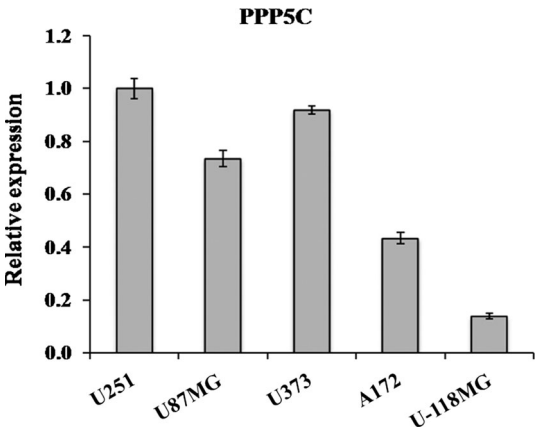

Fig. 1 Relative expression levels of *PPP5C* in various glioma cancer cell lines. qPCR analysis of *PPP5C* mRNA in human glioma cell lines (U251, U87MG, U373, A172, and U-118MG)

Results

Efficacy of Lentivirus-Mediated RNAi Targeting of *PPP5C*

PP5 protein (gene: *PPP5C*) is broadly expressed in various human glioma cell lines. Before we could move forward with RNAi study, we tried to screen and select the most appropriate cell line with highest intra-cellular PP5 expression. qPCR assay was carried out to better study the relative expression levels of *PPP5C* in glioma cell lines. As we could see from Fig. 1, we examined five glioma cell lines including U251, U87MG, U373, A172, and U-118MNG, whereas the highest expression level of *PPP5C* was observed in U251 cells, followed by U373 cells. Therefore, we employed U251 cells along with U373 cells for future investigation of the role of *PPP5C* in glioma progression.

To determine the silencing effect of lentivirus-mediated *PPP5C* RNAi on PP5 expression in U251 and U373 cells, we constructed both control lentivirus (Lv-shCon) and specific *PPP5C*-targeting lentivirus (Lv-shPPP5C-S1). We further constructed one more lentivirus (Lv-shPPP5C-S2) targeting *PPP5C* to avoid off-target effect. Both U251 and U373 cells were cultured and infected with Lv-shCon and Lv-shPPP5C, respectively. Non-infected parent cells were deemed as negative control (Con). To demonstrate the infection efficiency, we involved GFP tag which was embedded in lentivirus to provide visualized confirmation. As demonstrated in Fig. 2a, b, over 90 % of U251 and

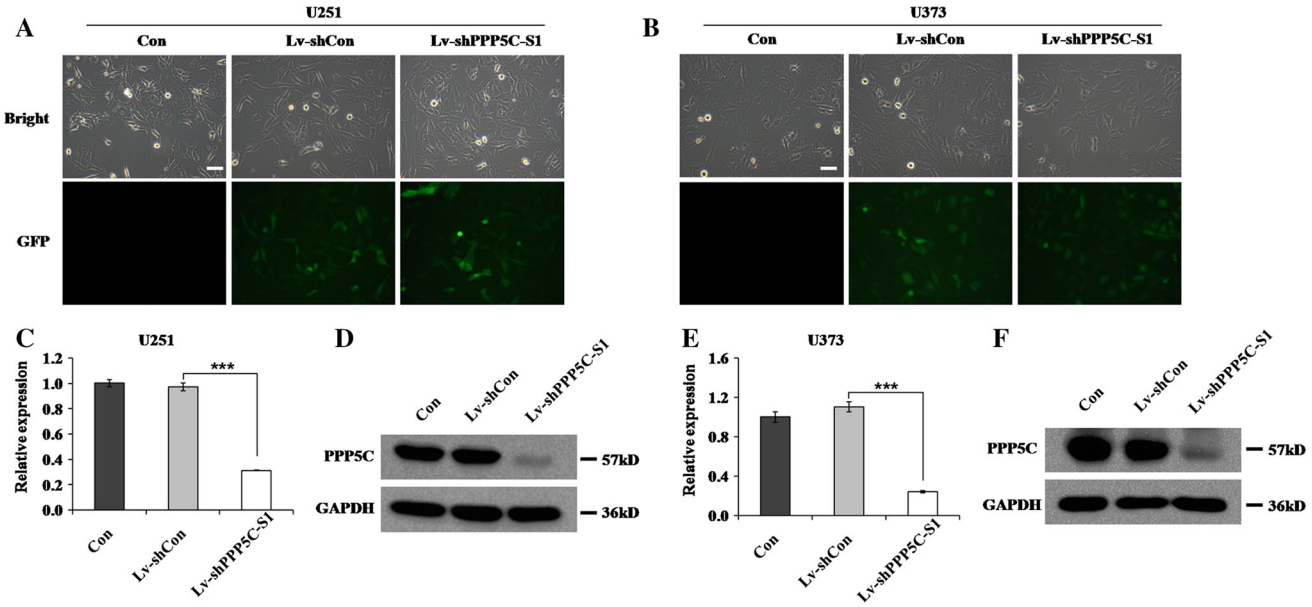

Fig. 2 Knockdown efficacy of *PPP5C* by lentivirus infection in glioma cells. **a**, **b** Microscopic images of U251 and U373 cells infected with lentivirus at MOI of 10, respectively (*scale bar* 100 μm). Visible GFP proteins proved that over 90 % of cells were successfully infected. qPCR analysis of *PPP5C* knockdown efficiency in U251 (**c**) and U373 cells (**e**). The transcription of *PPP5C* mRNA was significantly suppressed when infected with Lv-shPPP5C-S1. Western blot analysis of *PPP5C* knockdown efficiency in U251 (**d**) and U373 cells (**f**). ***$p < 0.001$

U373 cells were GFP positive after lentiviral infection, indicating that the transfection rate was satisfying. To further investigate the knockdown efficiency of *PPP5C*, we carried out real-time PCR and western blotting to study the expression levels of *PPP5C* in glioma cancer cells after infection. The non-silencing lentivirus encoded with irrelevant sequence had negligible effect on *PPP5C* expression, but *PPP5C*-silencing lentivirus (Lv-shPPP5C-S1) could remarkably down-regulate both mRNA and protein levels of *PPP5C*, by 67.9 and 78.0 % reduction in U251 and U373 cells, respectively (Fig. 2c–f). The other specific lentivirus, Lv-shPPP5C-S2, also had good performance in down-regulating the expression level of *PPP5C* by 81.8 % (Fig. S1a, b). Taken together, the lentiviruses we constructed ensured us the most efficient tool to knock down *PPP5C* expression in glioma cells.

Deprivation of PP5 Remarkably Inhibited the Proliferation Rate of Glioma Cells

To better understand the role of *PPP5C* in glioma cancer tumorigenesis, we examined the variation tendency of cell proliferation after lentivirus infection. We involved MTT assay due to its wonder testing sensitivity and dynamic range

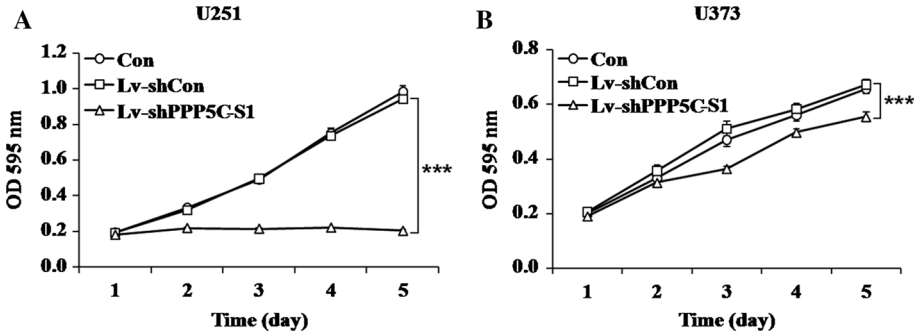

Fig. 3 Depletion of PP5 suppresses the proliferation rate in glioma cells. The monolayer growth rates of U251 (**a**) and U373 cells (**b**) from triplicate groups (Con, Lv-Con, and Lv-shPPP5C-S1) were determined by MTT assay. ***$p < 0.001$

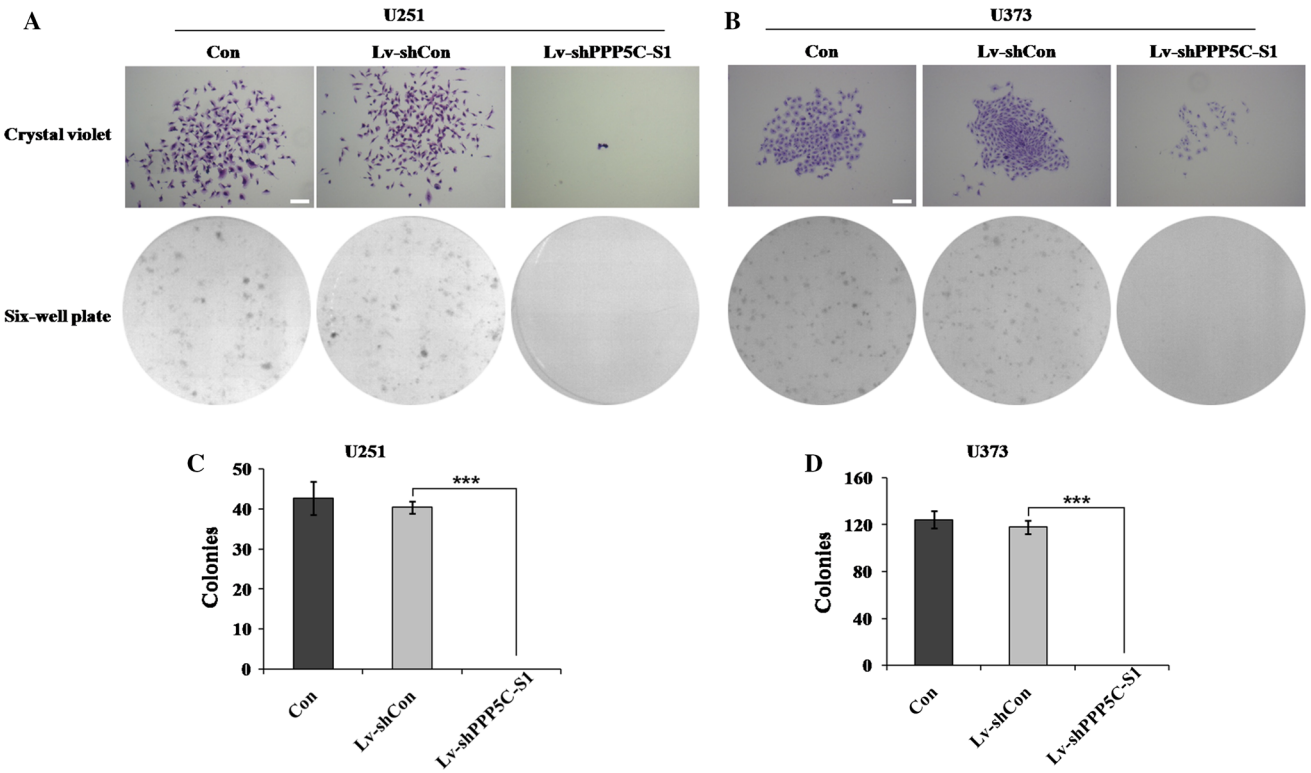

Fig. 4 Down-regulated expression of PP5 suppresses the colony formation capacity in glioma cells. Microscopic images of colonies formed in U251 (**a**) and U373 cells (**b**). Single colony was stained by crystal violet (*scale bar* 250 μm). The full vision of six-well plate under microscope showed significant colony formation inhibition in Lv-shPPP5C-S1-treated culture plate. Statistical analysis of colonies numbers utilizing crystal violet staining in U251 (**c**) and U373 cells (**d**). ***$p < 0.001$

to examine the cell proliferation rate after 3-day incubation. Line chart in Fig. 3 indicated that non-silencing cells had no obvious difference against control cells (Lv-shCon vs. Con), but meanwhile significant proliferation alteration was observed in the *PPP5C*-silenced cells (Lv-shPPP5C-S1 vs. Lv-shCon, $p < 0.001$). The similar result was observed in U251 cells after Lv-shPPP5C-S2 infection (Fig. S1c).

We subsequently cross evaluated the colony formation capacity in both U251 and U373 cells. As shown in Fig. 4, down-regulation of PP5 could lead to a significant reduction in colony formation capacity. The colony was remarkably smaller, and colony numbers were statistically fewer than control cells ($p < 0.001$), whereas there was no noticeable difference between non-silencing cells and control cells. The conformity of cell proliferation rate and colony formation capacity was surprising good when we treated U251 cells with Lv-shPPP5C-S1 or Lv-shPPP5C-S2. When U251 cells were infected with Lv-shPPP5C-S2, the cell proliferation rate was stalled and also the colony formation ability was mitigated (Fig. S1d, e). Collectively, knockdown of *PPP5C* by RNAi could markedly suppress the proliferation and colony formation ability of glioma cells.

Cell Cycle Arrest was Induced by Down-Regulation of PP5

To explore the potential mechanism of cell growth inhibition, we determined the cell cycle regulation alteration when U251 glioma cells were infected with Lv-shPPP5C. As we could see from Fig. 5a, cell distribution in cell cycles (G0/G1 phase, S phase, and G2/M phase) was significantly different in three groups (Con, Lv-shCon, and Lv-shPPP5C-S1). By contrast to control group, cells infected with Lv-shPPP5C-S1 were mostly distributed in the G0/G1 phase (61.4 %) and less distributed in the S phase (30.9 %) and G2/M phase (7.7 %) (Fig. 5b). Our results demonstrated that treatment with Lv-shPPP5C could remarkably induce G0/G1 cell cycle arrest ($p < 0.001$), but the cells infected with non-silencing lentivirus did not show any significant difference against non-

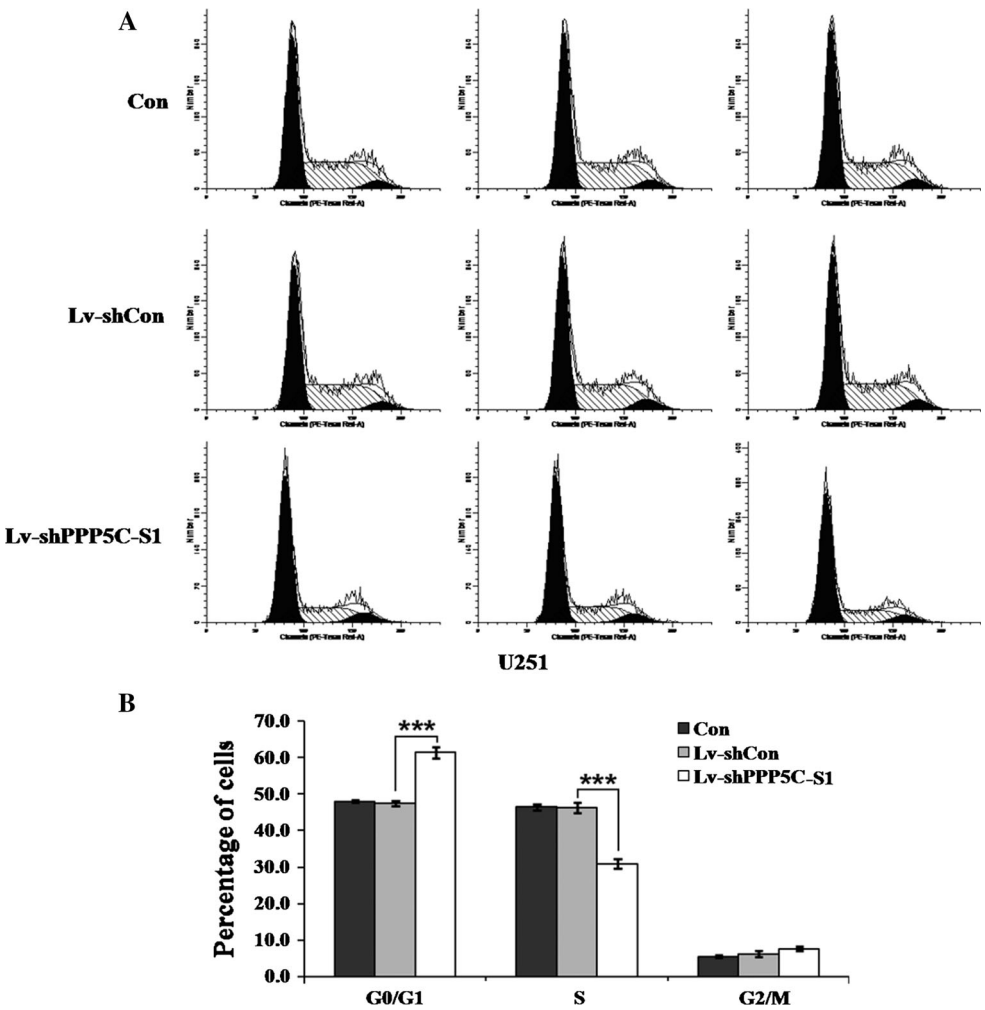

Fig. 5 Down-regulation of PP5 induces G0/G1 phase cell cycle arrest. **a** Cell cycle distribution of U251 cells was analyzed by flow cytometry. **b** The population of cells in G0/G1 phase was remarkably increased, accompanied by noticeable reduction in S phase. ***$p < 0.001$

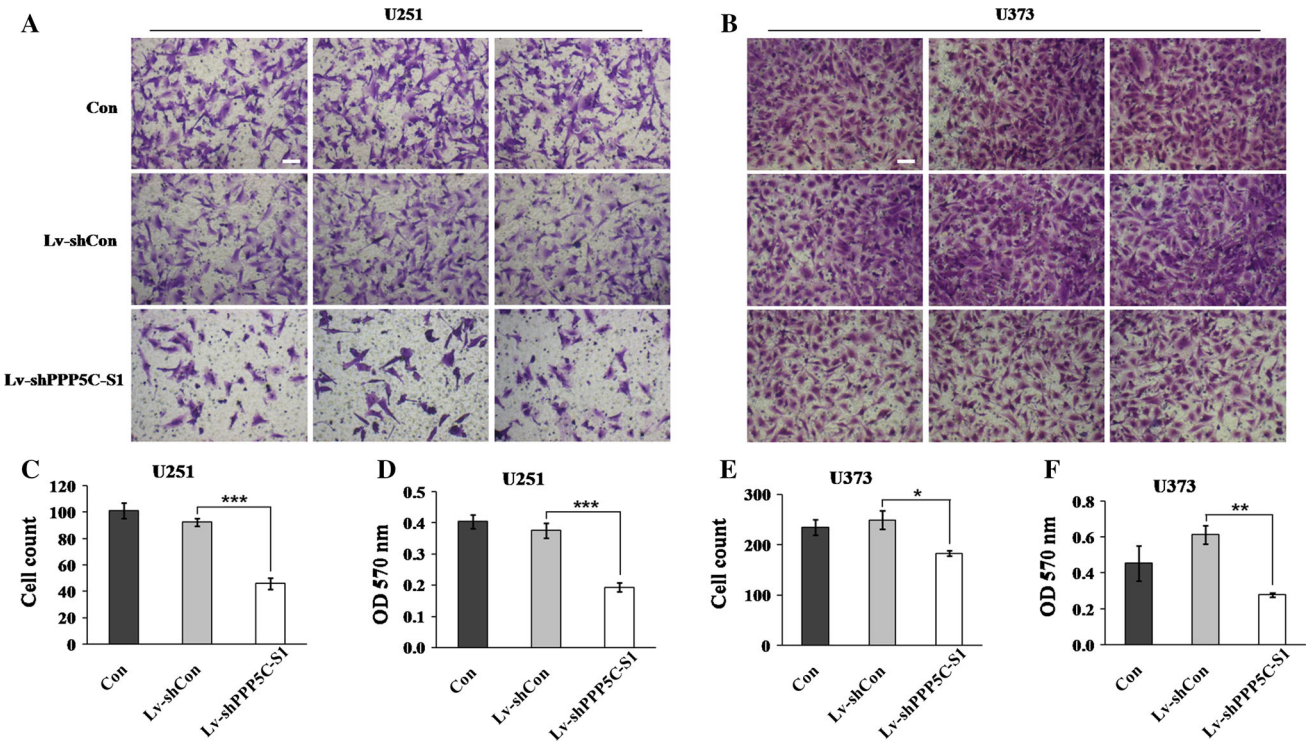

Fig. 6 Knockdown of PP5 mitigated glioma cell migration by lentivirus infection. Microscopic images of migrated U251 (**a**) and U373 cells (**b**) (*scale bar* 100 μm). Cell counting of migrated U251 (**c**) and U373 cells (**e**). Quantitative analysis of migrated U251 (**d**) and U373 cells (**f**) by destaining and reading optical density at 570 nm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

infected cells. These findings are in agreement with cell growth inhibition, which suggest that *PPP5C* could modulate glioma cell growth via cell cycle control.

Impact of Down-Regulation of PP5 Expression on Cell Migration

To determine the role of *PPP5C* in glioma cell migration, we employed transwell chamber assay after 72 h lentiviral infection. As we could see from Fig. 6, the migrative ability of Lv-shCon-infected cells did not significantly differ from non-infected cells. But meanwhile, the migration ability of glioma cells infected with Lv-shPPP5C-S1 was remarkably lower than those of non-infected and Lv-shCon-infected cells ($p < 0.001$ in U251 cells and $p < 0.01$ in U373 cells, respectively, Lv-shCon vs. Lv-shPPP5C-S1). Therefore, we proved that down-regulation of *PPP5C* could mitigate the migration of glioma cells.

Discussion

Glioma is the most common malignant tumor over the world. It has a worse prognosis especially when it turns to Grade IV due to its aggression and metastasis. Therefore, developing novel therapeutic method for gliomas has attracted great attention. In the current study, we firstly found that *PPP5C* is widely expressed in multiple human glioma cell lines. Thus two shRNA vectors for *PPP5C* were constructed to suppress PP5 expression in glioma cell lines U251 and U373 so as to determine its role in glioma malignancy progress.

Our results indicated that transfection with *PPP5C*-targeting shRNA significantly suppressed cell proliferation and colony formation capacity. Moreover, down-regulation of *PPP5C* in U251 cells significantly increased the cell percentage in G0/G1 phase, but concomitantly decreased the cell percentage in S phase. Therefore, knockdown of *PPP5C* inhibited glioma cell proliferation possibly via G0/G1 phase cell cycle arrest. Alternatively, when PP5 is suppressed, the cells could die in S phase and/or G2 M phase. However, the molecular mechanism of whether PP5 regulates cell cycle progression and apoptosis in glioma is poorly understood. Previous studies with siRNA and antisense oligonucleotides demonstrate that a decrease of PP5 expression can also result in G1-growth arrest, GR/p53-dependent increase in p21 expression (Zuo et al. 1998, 1999), altered DNA-PKcs signaling (Wechsler et al. 2004), DNA-damage checkpoint failure (Zhang et al. 2005) and prolonged activation of ASK1 signaling (Zhou et al. 2004; Morita et al. 2001). In particular, PP5 has been shown to act as a suppressor of ASK1 (Matsuzawa et al. 2002;

Ferguson et al. 2003; Morita et al. 2001; Zhou et al. 2004; Huang et al. 2003, 2004), p53 (Zuo et al. 1998; Urban et al. 2003), and DNA-PKcs (Wechsler et al. 2004). Further investigation should be carried out to elucidate the regulation mechanisms of PP5 in cell cycle control and apoptosis.

Furthermore, the effect of PP5 on glioma cell invasion was investigated in this study. We proved that down-regulation of *PPP5C* remarkably mitigated cell migration in both U251 and U373 cell lines, which suggested that PP5 could also play an essential role in glioma metastasis.

To the best of our knowledge, this is the first study to demonstrate the correlation between *PPP5C* and glioma progression. Our data provided evidence that reduced PP5 expression was correlated with reduced proliferation and impeded migration of glioma cells, underlying a connection between down-regulation of PP5 and stalled glioma progression. This study extends our knowledge of the basic biological mechanism of *PPP5C* in glioma in vitro. As a result, we speculate that *PPP5C* could be an potential therapeutic target against human gliomas.

Conflict of interest The authors declare that they have no conflict of interest.

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