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Original Research

Knockdown of protein phosphatase 5 (PPP5C) suppresses the growth of leukemic cell line U937

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Abstract: Serine/threonine protein phosphatase 5 (PPP5C) participates in multiple signaling pathways including cell cycle control and cell growth. PPP5C is involved in the progression of human breast cancer and hepatocellular carcinoma. However, its function in acute myelogenous leukemia (AML) remains unknown. In this study, we constructed a lentivirus system to knock down the expression level of *PPP5C* in leukemic cell line U937. Cell proliferation and cell cycles were assessed by MTT assay and flow cytometry respectively. Western blot was used to determine the level of caspase-3, PARP (poly ADP-ribose polymerase), CDK4 and CyclinD1. Knockdown of *PPP5C* suppressed the proliferation ability of U937 cells, and led to G0/G1 phase arrest, inducing cell apoptosis in U937 cells. The apoptosis of the U937 cells was associated with upregulating cleaved caspase-3 and PARP, and downregulating CDK4 and CyclinD1. In conclusions, PPP5C knockdown inhibits U937 cell proliferation and might be used as a potential therapeutic target for the treatment of leukemia.

Key words: Serine/threonine protein phosphatase 5, caspase-3, leukemia, apoptosis, cell cycle.

Introduction

Acute myelogenous leukemia (AML) is a hematological malignancy resulting from a variety of gene alterations which are essential for cell growth, differentiation, and cell death (1-3). Currently, chemotherapy and stem cell transplants are the primary therapeutic means of leukemia. Although these treatments can prolong the survival rate of patients with AML, treatment outcomes for most of the patients with AML have not improved in the past 20 years (4). Therefore, it's necessary to further explore the molecular pathogenesis mechanisms of this complex disease to improve the diagnosis and treatment approaches for AML.

Phosphorylation and dephosphorylation are implicated in cancer progression. Serine/threonine protein phosphatases (PPP), which is involved in dephosphoryalation regulation (5), plays a key role in regulation signaling networks that control cell growth, differentiation and cell apoptosis (6, 7). PPP5, coded by *PPP5C* gene, is a member of the PPP family of serine/threonine protein phosphatase (8). PPP5C is mostly expressed in human normal tissues and considered as a potentially important regulator that make cells respond appropriately to growth factors (9). In addition, PPP5 has been reported to be markedly elevated in proliferating cells in yeast (10) and rats (11). To date, several studies have shown that *PPP5C* is directly related to cancer progression. The expression of *PPP5C* has a positive correlation with human breast cancer and liver carcinoma (12, 13). Knockdown of *PPP5C* could suppress the growth of MCF-7 breast cancer cells (14). Moreover, knockdown of *PPP5C* by lentivirus markedly suppressed the proliferation and colony formation ability in hepatocellular carcinoma cells (15) and human glioma (16). Little is known about the role of *PPP5C* in the proliferation of AML.

To study the role of *PPP5C* in human AML, we employed a lentivirus system to specifically knockdown the expression of *PPP5C* in human leukemic cell line U937 cells. The effects of *PPP5C* on AML cell growth, as well as the underlying mechanism were investigated.

Materials and Methods

Cell Culture

Human leukemic cell line U937 and human embryonic kidney cell line 293T (HEK293T) were purchased from Chinese Academy of Sciences. U937 cells were cultured in RPMI-1640 (HyClone, USA) plus 10% fetal bovine serum (FBS, Biological Industries, Israel). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) plus 10% FBS. Both cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Vectors Construction and Lentivirus Packaging

Two short hairpin RNA (shRNA) sequences (S1, GAGACAGAGAAGATTACAGTACTCGAGTACTGTAATCTTCTCTGTCTCTTTT and S2, 5'-CCACGAGACAGACAACATGAACTCGAGTTCATGTTGTCTGTCTCGTGGTTTTT-3') targeting PPP5C gene and a control siRNA sequence (GCGGAGGGTTTGAAAGAATATCTCGAGATATTCTTTCAAACCCTCCGCTTTTTT) were designed according to the *PPP5C* sequence downloaded from NCBI (NM_001204284.1).

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Three shRNA fragments were cloned into a pFH-L vector (Shanghai Hollybio, China) by restriction enzyme sites *NheI* and *PacI*. HEK293T cells were plated in a six-well plate at a density of 50,000 cells/well, then transfected with the pFH-sh*PPP5C* or pFH-shCon vector, together with the envelope plasmid pVSVG-I and packaging plasmid pCMV△R8.92 (Shanghai Hollybio, China) by using Lipofectamine 2000 (Life Technologies, USA) according to the manufacturer's instructions. Two days after transfection, cell culture media was collected and concentrated. The generated recombinant lentivirus were termed as shCon, sh*PPP5C* (S1) and sh*PPP5C* (S2), and then stored at -80 °C.

Lentivirus Infection

U937 cells were plated in 12-well plates at a density of 30,000 cells/well. Recombinant lentivirus (shCon, sh*PPP5C* (S1) and sh*PPP5C* (S2) were added at a multiplicity of infection (MOI) of 80. After infection for 96 h, cells were collected and observed under a fluorescence microscope to count the number of green fluorescent protein (GFP)-positive cells.

RNA Extraction and Quantitative RT-PCR Analysis

Primers (forward: 5'-CCCAACTACTGCGACCAGAT-3' and reverse: 5'-CCCGTCACCTCACATCATTC-3') for qRT-PCR were designed according to PPP5C sequence. Actin was used as control and its primers were 5'- GCGGAGGGTTTGAAAGAATATCTCGAGATATTCTTTCAAACCCTCCGCTTTTTT-3'. After 5 days' infection, total RNA was isolated from U937 cells using TRIzol reagent (Life Technologies, USA). And then cDNA was synthesized from 1 μg RNA. The mRNA expression of *PPP5C* was analyzed on a BioRad Connect Real-Time PCR platform using the 20 μl qPCR reaction mixtures (10 μl 2× SYBR Premix Ex Taq, 0.8 μl forward and reverse primers (2.5 μM), 5 μl cDNA, and 4.2 μl ddH_2O) according to the following the following qPCR reaction procedure: initial denaturation at 95°C for 1 min and 40 cycles of denaturation at 95°C for 5 s, annealing extension at 60°C for 20 s. Absorbance values were read at the extension stage. The statistical method $2^{-\Delta\Delta Ct}$ was used to compare the relative expression between *PPP5C* and actin values. Each experiment was repeated at least three times.

Western Blot Analysis

U937 cells were collected four days post lentivirus infection, then lysed in 2× sodium dodecyl sulphate (SDS) sample buffer (10 mM EDTA, 4% SDS, 10% glycine, 100 mM Tris-HCl buffer (pH 6.8) for 1 h at 4°C. Total proteins were extracted from the cell lysate by using a protein extraction kit. For each sample, 30 μg proteins were loaded on 10% SDS-PAGE gels and then transferred onto PVDF membranes (Millipore, USA) at 300 mA for 2 h. Proteins were detected by incubation with the indicated primary antibodies, including rabbit-anti PPP5C (1:1,000, 11715-1-AP, Proteintech Group, Inc. USA), rabbit-anti CDK4 (1:1000, 11026-2-AP, Proteintech Group, Inc. USA), rabbit-anti CyclinD1 (1:1000, 60186-1-1g, Proteintech Group, Inc. USA), rabbit-anti GAPDH (1:10000, 10494-1-AP, Proteintech Group, Inc. USA), rabbit-anti caspase-3 (1:500, #9661, Cell Signaling Technology) and rabbit-anti PARP (1:1000, #9542, Cell Signaling Technology), overnight at 4°C, followed by a goat anti-rabbit IgG (1:5000, SC-2054, Santa Cruz Biotechnology, USA) conjugated with horseradish peroxidase (HRP) at room temperature for 2 h. The fluorescence signal was detected using the catalysis of electrochemiluminescence (ECL) reagent with HRP. GAPDH was used as the internal standard. Each experiment was repeated at least three times.

MTT assay for cell viability

Four days after lentivirus infection, U937 cells (3, 000 cells/well) were re-seeded in a ninety-six well plate. Viable cell were analyzed using 3 - (4, 5 – dimethylthiazol - 2- yl)-2, 5 - diphenyltetrazolium bromide (MTT) method at day 1, 2, 3, 4, and 5. Briefly, for each well, cells were added with MTT solution and incubated for 4 h at 37°C. Then acidic isopropanol solution (10% SDS, 5% isopropanol and 0.01 M HCl) was added to each well to stop the reaction. After incubation for 10 min, the optical density was measured using a microplate reader (Epoch; BioTek, Winooski, VT, USA) at the wavelength of 595 nm. Each experiment was repeated at least three times.

Flow Cytometry Analysis for Cell Cycle

Four days after lentiviral infection, U937 cells were reseeded in a twelve-well plate at a density of 100, 000 cells/well, and cultured for another four days, then harvested for cell cycle analysis. Briefly, the cells were fixed with 75% ethanol overnight and then washed twice with ice-cold 2% phosphate buffer saline (PBS, HyClone, USA). Cell pellets were then spun down, resuspended in ice-cold RNase-containing (1:100) PBS buffer, and stained with propidium iodide (PI, Sigma, USA) for 30 minutes. The distribution of cells in different cell cycle phases (G0/G1, S and G2/M) was analyzed using a flow cytometer (FACSCalibur, Becton, Dickinson and Company, USA) following the manufacturer's guidelines. Each experiment was repeated at least three times.

Apoptosis assay

The U937 cells (100000 cells per orifice) were seeded into 12 orifice plate and incubated overnight. After infection for 6 days, the cells were collected, washed with PBS and subjected to Annexin V-APC/7-AAD double staining following the instruction of Apoptosis Assays Kit (KeyGEN Biotech, Nanjing, China), and then analyzed by flow cytometry (FCM). Each experiment was repeated at least three times.

Statistical Analysis

All data were analyzed using SPSS version 13.0 software and expressed as means ± standard deviation (SD) from at least three independent experiments. Statistical analysis was evaluated by Student's t-test, $P < 0.05$ was considered statistically significant.

Results

Knockdown of PPP5C in U937 cells by RNA interference

In order to study the functions of *PPP5C*, we specifically knocked down the expression of *PPP5C* in U937 cells based on lentivirus-mediated system. As shown in

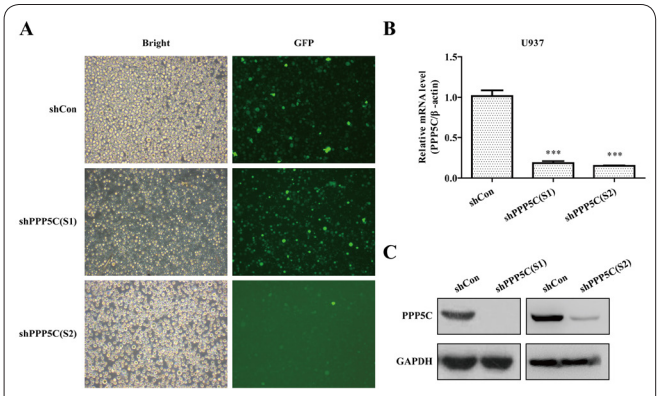

Figure 1. Images recorded under a fluorescence microscope of U937 (A); Knockdown efficiency of PPP5C levels analyzed by quantitative real-time RT-PCR (B) and Western Blot (C). ***p <0.001; Scale bars, 10.

Figure 1A, most of the U937 cells had GFP positive signals after infection by recombinant lentivirus (shCon, sh*PPP5C* (S1) and sh*PPP5C* (S2) for 96 h. To further confirm the interference effect, qRT-PCR and Western blot assays were performed to analyze the mRNA and protein levels of *PPP5C* in sh*PPP5C* and shCon infected U937 cells. Quantitative PCR assay showed a decrease of *PPP5C* mRNA in shPPP5C infected U937 cells (Figure 1B). The relative mRNA expression level of *PPP5C* in the shPPP5C group was only one-fifth of that in the shCon group ($p < 0.001$). Similar trends were observed in *PPP5C* protein expression levels by Western blot assay (Figure 1C).

Suppressing PPP5C inhibited the viability of U937 cells

As showed in Figure 2, the absorbance value at 595 nm of shCon showed quicker growth rate compared with sh*PPP5C* infected cells. Specifically, the cell proliferation rates in sh*PPP5C*(S1) and sh*PPP5C*(S2) groups were significantly reduced by 62.6% and 71.2% in comparison with shCon group in U937 cells at day 5, respectively ($p < 0.001$, Figure 2A and B).

PPP5C knockdown blocked U937 cell cycle

As shown in Figure 3A and B, there was an increase in the percentage of cells at G0/G1 phase ($p < 0.001$), and an decrease in the percentage of cells at S ($p < 0.01$) and G2/M phases ($p < 0.05$) in sh*PPP5C* (S1) infected U937 cells compared with those in shCon infected U937 cells. More cells at sub-G1 phase were detected in sh*PPP5C* (S1) infected U937 cells, much higher than

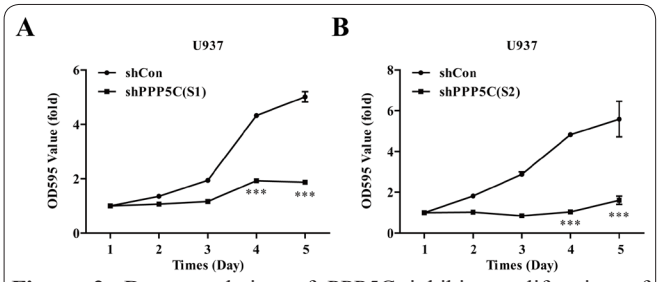

Figure 2. Downregulation of PPP5C inhibits proliferation of U937. The proliferation of U937 cells with different treatments shCon, shPPP5C(S1) or shPPP5C(S2) was determined by MTT assay, respectively (A, B). ***$p < 0.001$.

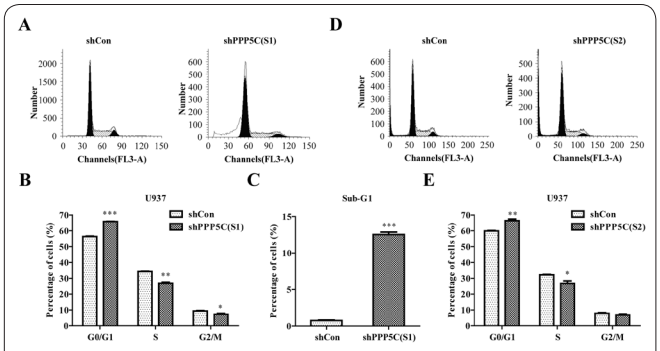

Figure 3. Cell cycle distribution determined by flow cytometer (A, D); Statistical analysis of cell cycle in the PPP5C knockdown group of U937 (B, E); The respective percentage of U937 cells in the sub-G1 phase (C). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

those in shCon infected cells (Figure 3C, $p < 0.001$). Similar to the results in sh*PPP5C*(S1) group, the percentage of U937 cells in G0/G1 phase was also increased ($p < 0.01$) while decreased in S phase ($p < 0.05$) compared to the shCon group in sh*PPP5C*(S2) group (Figure 3D and E).

PPP5C knockdown induced U937 cells apoptosis

As shown in Figure 4A and B, there were more cells at early apoptosis (Annexin V+/7-AAD-) and late apoptosis (Annexin V+/7-AAD+) stages in sh*PPP5C*(S1) infected U937 cells, as compared to those in shCon infected U937 cells ($p < 0.001$). In addition, similar results were examined in sh*PPP5C*(S2) group. The apoptotic cell percentage especially late apoptosis was also remarkably increased in sh*PPP5C*(S2) group in comparison with that in shCon group ($p < 0.001$, Figure 4C and D).

PPP5C knockdown altered the expression of cell cycle regulatory and apoptotic factors in U937 cells

Figure 5 shows that sh*PPP5C* (S1) treatment enhanced the expression levels of cleaved caspase-3 and cleaved PARP, and suppressed the expression levels of CDK4 and CyclinD1 in U937 cells.

Discussion

PPP5C, as PPP family of serine/threonine protein phosphatase, has been reported to promote cell proliferation and is closely related to the development of

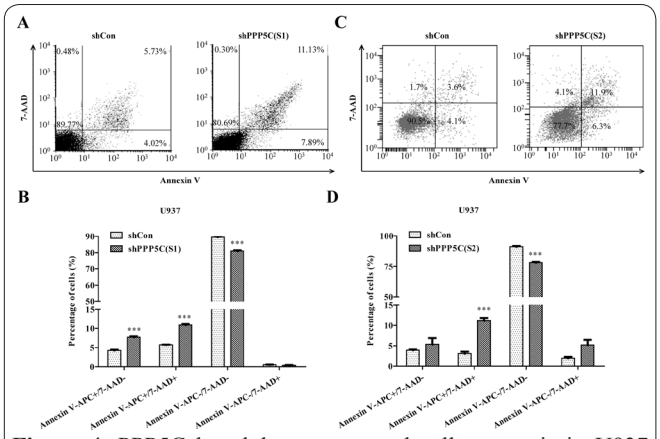

Figure 4. PPP5C knockdown promoted cell apoptosis in U937 cells as described by flow cytometry (A, C). Statistical analysis of apoptotic cells including early-stage (Annexin V+/7-AAD-) and late-stage (Annexin V+/7-AAD+) apoptosis (B, D). ***$p < 0.001$.

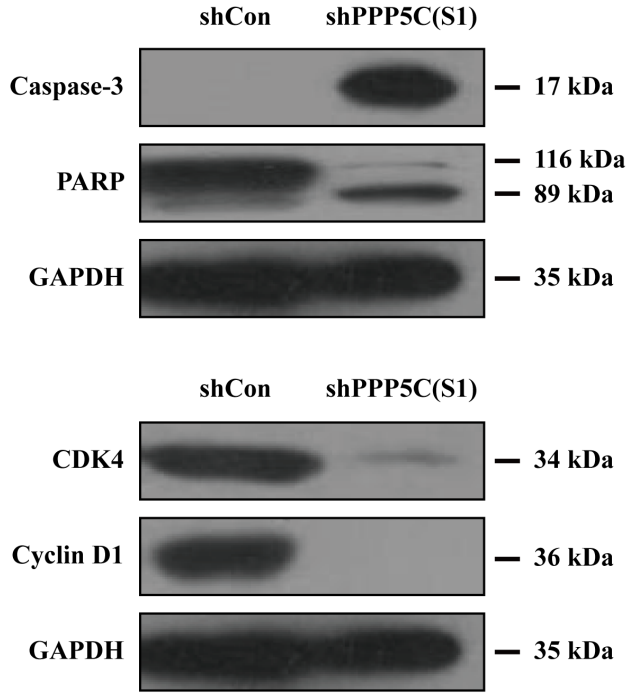

Figure 5. Effect of PPP5C knockdown on the expression of cell cycle and apoptotic regulators in U937 cells, including CDK4, cyclin D1, cleaved caspase-3 and PARP, as determined by Western blot analysis. GAPDH was used as internal control.

cancer, such as breast cancer (14), hepatocellular carcinoma (15) and glioma (16). But its functional role in AML remains unclear. In the present study, lentivirus-mediated shRNA was used to knock down the expression of *PPP5C* at transcription and translation levels. Knockdown of *PPP5C* suppressed cell viability in U937 cells. In addition, PPP5C silencing blocked cell cycle at G0/G1 phase and promoted cell apoptosis in U937 cells. Furthermore, Western blot analysis showed that knockdown of *PPP5C* reduced the expression levels of Cyclin D1 and CDK4, and enhanced the expression levels of cleaved caspase-3 and cleaved PARP.

Previous studies indicate *PPP5C* is involved in cell cycle regulation, which has a strong link with carcinogenesis (17). Cell cycle consists of four distinct sequential phases (G0/G1, S, G2, and M), which primarily regulates cellular proliferation by altering expression levels of cyclin-dependent kinases (CDKs) (18). Cell cycle arrest occurs partly due to the loss of cyclin expression and CDK activity (19). As one of the main checkpoints of cell cycle, G0/G1 phase is responsible for initiation of DNA replication, which is strongly regulated by the combined activity of cyclinD/CDK4 (20, 21). Therefore, downregulation of cyclin D1 and CDK4 was closely associated with G0/G1 phase arrest in cell cycle regulation. Moreover, *PPP5C* has been shown to affect phosphorylation dependent circuit of cell cycle regulatory protein Chk1 (22). Based on these evidences, we could speculate that the proliferation inhibition of PPP5C knockdown on U937 cells might be due to the inhibitory activity of cyclin D1/CDK4 complexes.

Our study also showed that knockdown of *PPP5C* promoted cell apoptosis via upregulating expression levels of cleaved caspase-3 and PARP. Caspase-3 is the key enzyme required in the caspase cascade activation (23), as the central part of cell apoptosis. PARP, as the specificity substrate of cleaved caspases, plays an important role in inducing cell apoptosis (24). Therefore, activating caspase cascade may be the molecular mechanism of *PPP5C* knockdown induced cell apoptosis in U937 cells.

In conclusion, knockdown of PPP5C inhibits proliferation of U937 cells by arresting G0/G1-phase of cell cycle, and promoting cell apoptosis via activating the caspase-cascades and downregulating the expression of Cyclin D1 and CDK4. These results suggest that PPP5C may be used as a therapeutic target for AML.

Author's Contribution

GYL conceived of the study, and participated in its design and coordination, data collection and analysis, helped to draft the manuscript. JZL conceived of the study, and participated in its design and coordination, data collection and analysis, helped to draft the manuscript. ZL conceived of the study, and participated in its design and coordination, data collection and analysis, helped to draft the manuscript. SJL participated data collection and analysis, and drafting of the manuscript. GZL participated data collection and analysis, and drafting of the manuscript. TWX participated data collection and analysis, and drafting of the manuscript. JXW participated data collection and analysis, and drafting of the manuscript. LXW participated data analysis, and drafting of the manuscript. MH participated data collection and analysis, and drafting of the manuscript. All authors read and approved the final manuscript."

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