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# Characterization of overexpression of the alternatively spliced isoform of the protein phosphatase 2A catalytic subunit in cells

Shen Tang [a], Yuyang Liu [d], Xinhang Wang [b, c], Ziwei Liang [b, c], Haiqing Cai [b, c], Laiming Mo [a], Deqiang Xiao [b], Songchao Guo [b], Yiqiang Ouyang [b], Bin Sun [b, c], Cailing Lu [b, c, **], Xiyi Li [b, c, *]

[a] School of Preclinical medicine, Guangxi Medical University, Nanning, Guangxi, 530021, China
[b] Guangxi Colleges and Universities Key Laboratory of Prevention and Control of Highly Prevalent Diseases, China
[c] School of Public Health, Guangxi Medical University, Nanning, Guangxi, 530021, China
[d] Hunan Provincial Center for Disease Control and Prevention, Changsha, Hunan, 410005, China

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ABSTRACT

PP2Acα2 is a recently discovered PP2Acα alternative splicing isoform that can be induced following serum withdrawal. It shows enhanced binding to immunoglobulin binding protein 1 and is overexpressed in chronic lymphocytic leukemia patients. Current knowledge concerning PP2Acα2 is limited. In this study, we induced and cloned PP2Acα2 from HL-60 cells and human lymphocytes, transfected them into human embryonic kidney 293 cells and constructed a stable overexpression cell line. We found that PP2Acα2 mRNA inhibits expression of its longer isoform PP2Acα mRNA but had no effect on the final protein expression and modification of this longer isoform. Moreover, PP2Acα2-overexpressed cells demonstrated increased expression of IGBP1, activated mTORC1 signaling to reduce basal autophagy and increased anchorage-independent growth. Our study provides new insights into the complex mechanisms of PP2A regulation.



## 1. Introduction

PP2A, a ubiquitously expressed serine/threonine phosphatase, has been implicated in multiple cellular functions, including cell cycle regulation, cell growth control, development, regulation of multiple signal transduction pathways, and cell mobility. PP2A consists of three subunits: the structural A subunit, regulatory B subunit, and catalytic C subunit. The A and C subunits form the PP2A core enzyme. The PP2A core enzyme associates with various regulatory B subunits (including B, B′, B″, B‴ four superfamilies) to form a heterotrimeric holoenzyme, which has full activity toward specific substrates [1]. The PP2A catalytic C subunit (PP2Ac) is a highly conserved protein. The C-terminus of PP2Ac has two types of modification, phosphorylation and methylation. Phosphorylation at Y307 and methylation at L309 have been demonstrated to greatly affect the activity of PP2A and ability of B to interact with AC, as well as modulate the functional specificity of PP2A [2]. PP2Ac has two isoforms, PP2Acα and PP2Acβ, which are encoded by different genes [3]. PP2Acα is approximately 10 times more abundant than PP2Acβ because expression from the PP2ACα gene promoter is 7- to 10-fold stronger than from the PP2Acβ gene promoter [4].

Recently, a novel PP2Acα alternative splicing isoform, PP2Acα2, was reported. This new isoform was initially identified in a yeast two-hybrid screen using TIPRL (TIP41, TOR signaling pathway regulator-like) as bait to screen a human leukocyte cDNA library [5]. It lacks an internal segment of exon 5 and has a molecular mass of approximately 26 kD. The alternatively spliced isoform has no phosphatase activity and displays enhanced binding to the immunoglobulin binding protein 1 (IGBP-1) regulatory subunit. The PP2Ac2 isoform is inducibly expressed following serum withdrawal in human peripheral blood mononuclear cells but is inhibited under normal culture conditions [5]. Since then, only a few studies have further explored its characteristics. Wang et al. reported that PP2Acα2 can be regulated by miR-3661, similar to its longer isoform [6]. A recent study found that in chronic lymphocytic leukemia

* Corresponding author. School of Public Health, Guangxi Medical University, Nanning, Guangxi, 530021, China.
** Corresponding author. School of Public Health, Guangxi Medical University, Nanning, Guangxi, 530021, China.
*E-mail addresses:* lcling78@163.com (C. Lu), xiyi.li2017@hotmail.com (X. Li).

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patients, PP2Acα2 displayed moderate to high overexpression compared to undetectable or very low levels in healthy donors [7], implying a possible important role of its regulation in physiological and pathological processes.

PP2A regulates many important signaling pathways, such as the mammalian target of rapamycin complex 1 (mTORC1) pathway [8], which regulates autophagy. IGBP-1, which was found to enhance binding to PP2Ac in PP2Acα2-overexpressed cells [5], is a component of the mammalian target-of-rapamycin (mTOR) pathway [9]. Moreover, PP2Acα2 can be induced under serum starvation conditions, implying its possible functional relationship to autophagy. Autophagy is a cellular pathway by which cytoplasmic macromolecules and organelles are delivered to lysosomes for degradation. Microtubule-associated protein light chain 3 (LC3) is an autophagy monitor marker that is widely used for autophagosome formation [10]. Measuring the conversion of LC3-I to LC3-II by immunoblotting in the presence and absence of inhibitors can detect the occurrence of autophagy [11]. Whether PP2Aα2 plays a role in mTOR signaling and as an autophagy monitor marker remains unknown.

Basal autophagy, defined as macroautophagic activity during cellular growth in normal medium containing nutrients in the absence of a measurable stimulus, is a catabolic process for the degradation of long-lived proteins and damaged organelles, as well as the recycling of cellular components. It plays important roles in organismal homeostasis. Autophagy suppresses tissue damage, chronic inflammation, the DNA damage response and genome instability, which are known to create an environment for cancer initiation [12,13]. Anchorage-independent growth is the ability of transformed cells to grow independently of a solid surface and is a hallmark of carcinogenesis. In this study, we induced and cloned PP2Acα2 from HL-60 cells and human lymphocytes, transfected them into human embryonic kidney 293 cells and constructed a stable overexpression cell line to study the PP2Aα2 effects.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

Promyelocytic leukemia (HL-60) cells, human embryonic kidney 293 (HEK293) cells and human neuroblastoma SK-N-SH cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology, China. HL-60 cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin. HEK293 cells and SK-N-SH cells were grown in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. All cells were grown in a stable environment with 5% $CO_2$ at 37 °C.

### 2.2. Human blood lymphocyte and monocyte separation

Isolation of lymphocytes and monocytes was conducted and modified as described previously. Briefly, human peripheral blood was collected from non-smoking, healthy donors. All donors signed an informed consent form, and the study was approved by the Guangxi Medical University Ethics Committee. Human peripheral blood mononuclear cells (PBMCs) were obtained using Ficoll gradient centrifugation. Ficoll was added to the blood diluted with an equal volume of 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum, and then the samples were centrifuged for 20 min. Next, the white interface fraction containing PBMCs was collected in 1640 medium containing 10% fetal bovine serum. After incubating for 2 h, monocytes were selected by adherence, and the non-adherent lymphocytes were washed out to another flask for subsequent experiments.

### 2.3. Vectors and transfection

The pcDNA3.0 vector and pCMV-Tag4A vector were digested with Bam HI and HindIII restriction enzymes and were used for coupled reactions of T4 DNA ligase after purification using the Universal DNA Purification Kit (Tiangen Bio. Beijing, China). Recombinant plasmids PP2Acα-pcDNA3.0 and PP2Acα2-pcDNA3.0 were generated by transformation with DH5a and were extracted using EndoFree Mini Plasmid Kit II (Tiangen Bio. Beijing, China). The PP2Acα-pcDNA3.0 and PP2Acα2-pcDNA3.0 sequences were verified by Invitrogen. HEK-293 and SK-N-SH cells were seeded into 6-well plates at a density of $5 \times 10^5$ per well. After 24 h of culture, the cells were transfected with 20 μg of plasmids pcDNA3.0, PP2Acα-pcDNA3.0 and PP2Acα2-pcDNA3.0 using the Lipofectamine®3000 Kit (Life Technologies, Carlsbad, CA, USA), followed by selection with 800 μg/ml G-418 sulfate (Sigma-Aldrich, Carlsbad, CA, USA) for three weeks. The expression of PP2Acα and PP2Acα2 was analyzed by RT-PCR and Western blotting.

### 2.4. Total RNA extraction and RT-PCR analysis

Total RNA was extracted from cells using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA quality and concentration were determined by spectrophotometry. cDNA was prepared using MMLV reverse transcriptase. Conventional PCR was performed using Mastercycler (Eppendorf)and Taq™ Hot Start Version (TaKaRa Bio, Dalian, China). The following primers were used for amplification: HPRT1, 5′-GTCGTGATTAGTGATGATG-3′ (sense), 5′-GTTCAGTCCTGTCCATAA-3′ (antisense); PP2Acα, 5′-GGATCCATGGACGAGAAGGTGTTCAC-3′ (sense), 5′-AAGCTTCAGGAAGTAGTCTGGGGTACGACG-3′ (antisense). Amplification was performed in 32 cycles at 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 1 min, followed by analyzed using 1.5% agarose gel electrophoresis.

### 2.5. Western blotting

Cells were lysed in Cell Lysis Buffer. After centrifugation, the total protein concentrations were determined using the Pierce®BCA Protein Assay Kit (Thermo, Waltham, MA, USA). Total proteins were analyzed by 10% SDS-PAGE, followed by transfer to PVDF membranes, which were then blocked with 5% dry milk in PBST for 1 h. The indicated antibodies diluted in PBST containing 2.5% dry milk were incubated with the membranes. The protein bands were detected by ECL and were quantified by ImageJ. Alpha View SA software was used to parse the grayscale values. Anti-PP2Ac antibody, anti-IGBP1 antibody and anti-alpha tubulin antibody were purchased from Abcam. Secondary antibodies were obtained from ZSGB-BIO (Beijing, China).

### 2.6. Soft agar assays

Base agar consisting of 0.5% agar was added to 1 × RPMI and 10% FCS. Next, top agar, comprising 0.5% agar, 2 × RPMI and 20% FCS, was mixed with 5000 cells/plate of transfected cells seeded in a 6-well plate. The plate was incubated at 37 °C in humidified incubator for 14 days. The colonies were counted using a dissecting microscope.

### 2.7. Cell cycle analysis

The cells were fixed in 70% ethanol, and, after washing with PBS, they were incubated with Vindelov solution [propidium iodide

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