

# p53 siRNA promotes autophagy of U2OS cells through its target gene Rap2B

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**Abstract:** The present study aims to explore the effects of p53 and its target gene Rap2B on the autophagy of U2OS cells. U2OS cells were treated with siRNA against p53, Rap2B, and PLC $\epsilon$ . Relative expressions of p53, Rap2B, and PLC $\epsilon$  were determined using quantitative polymerase chain reaction (qPCR) and Western blotting, respectively. Levels of IP3 in the cells were determined using Enzyme-linked Immunosorbent Assay (ELISA). Levels of Ca<sup>2+</sup> were detected using Flow cytometry. Fluorescence microscopy was used to observe the autophagy of cells. Knockdown of p53 significantly decreased the expressions of Rap2B protein. Additionally, knockdown of p53 significantly decreased the mRNA levels of PLC $\epsilon$ . The knockdown of p53, Rap2B, and PLC $\epsilon$  significantly decreased the levels of intracellular IP3 and Ca<sup>2+</sup> and promoted autophagy of U2OS cells. Our results demonstrated that p53-Rap2B-PLC $\epsilon$ -IP3 signaling pathway regulated autophagy of U2OS cells.

## Introduction

Osteosarcoma is a primary bone malignancy, which is characterized by the direct formation of immature bone or osteoid tissue by the tumor cells. Limb osteosarcoma without metastasis is usually treated with amputation, and the 5-year survival rate is around 20% (Wang and Tian, 2010).

Tumor suppressor p53 can be stimulated and activated by many events including DNA damage, hypoxia, and proto-oncogene activation (Brooks and Gu, 2010). p53 activation results in the activation of a series of transcription factors, which further regulate cell cycle progression to repair damaged DNA, promote aging, or induce apoptosis (Vousden and Prives, 2009). As a target gene of p53, Rap2B belongs to the Ras family. It plays a molecular switch role in signal transduction and regulates a variety of cellular events, such as cell proliferation and differentiation. Rap2B is called the promoter gene of tumorigenesis (Zhang *et al.*, 2013; Paganini *et al.*, 2006; Bourne *et al.*, 1990; Bourne *et al.*, 1991). It promotes the activation of phospholipase C- $\epsilon$  (PLC $\epsilon$ ), leading to the breakdown of phosphatidylinositol 4,5-bisphosphate into the second messenger diacylglycerol and inositol 1,4,5-trisphosphate (IP3). The activation of protein kinase C accelerates the release of Ca<sup>2+</sup> from intracellular stores, affects

cell growth, differentiation, and gene expression (Kelley *et al.*, 2001; Kelley *et al.*, 2004; Song *et al.*, 2001; Song *et al.*, 2002; Jin *et al.*, 2001; Berridge *et al.*, 2000; Nishizuka, 1995). Autophagy is associated with ontogenesis, oxidative damage protection, the malignant proliferation of tumor cells, and neurodegenerative diseases (Todde *et al.*, 2009; Eskelinen and Saftig, 2009). Autophagy marker proteins consist of Apg12/Apg5 and microtubule-associated protein light chain 3 beta 1 (LC3), which is carried with a visualized green fluorescence protein (GFP) antibody. Our previous study applied a GFP-LC3 fusion protein to monitor autophagy formation (Di *et al.*, 2017). When the autophagy occurred, the GFP-LC3 fusion protein is translocated to the cell membrane, and then several bright green fluorescent spots are formed. One spot is equivalent to autophagy, so we can evaluate the activity level of autophagy by counting the number of spots.

It has been reported that cytoplasmic p53 inhibits autophagy (Chen *et al.*, 2012). The autophagy of cells is mainly regulated and maintained by mTORC1. The upstream key regulators of mTORC1 include PI3K-AKT, AMPK, and calcium-modulated signal transduction pathways (Wang and Li, 2010). cAMP activates downstream Rap2B to promote autophagy by transforming GDP into active GTP. These findings supported that Rap2B is correlated to autophagy (Mestre and Colombo, 2012). However, the relationship between p53-Rap2B and autophagy in osteosarcoma is still unknown. This study aims to investigate the roles of p53-Rap2B in the autophagy of osteosarcoma.

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## Material and Methods

### Cell culture

U2OS cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, 100 µg/mL penicillin, and 100 µg/mL streptomycin in the presence of 5% CO<sub>2</sub> at constant humidity.

### siRNA transfection

Scrambled siRNA and siRNA against p53, Rap2B, and PLCε were designed and synthesized by Invitrogen (Shanghai, China). The sequences of siRNAs are as follows: p53 siRNA forward: 5'-CUA CUU CCU GAA AAC AAC GTT -3'; and reverse: 5'-TCA GAC CTA TGG AAA CTA CTT-3'; Rap2B siRNA forward: 5'-UGA GCC UGG UGG GCA ACA ATT -3'; and reverse: 5'-UUG UUG CCC ACC AGG AUC ATT -3'; PLCε siRNA forward: 5'-GGC UGU CGA AGU GUA GAA UTT -3'; and reverse: 5'-AUU CUA CAC UUC GAC AGC CTT-3'; Scrambled siRNA forward: 5'-UUC UCC GAA CGU GUC ACG UTT-3'; and reverse: 5'-ACG UGA CAC GUU CGG AGA ATT-3'. The cells at a density of  $1 \times 10^5$  were seeded into a 6-well plate. When the cells reached 60% confluence, scrambled siRNA, and siRNA against p53, Rap2B and PLCε were transfected using silentFect Lipid Reagent (Bio-Rad, Hercules, CA), according to the manufacturer's protocol.

### Real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA was extracted from the U2OS cells using Trizol reagent (Invitrogen, CA, USA) and RNase-free DNase I was used to remove DNA contamination. cDNA was synthesized from total RNA using an Omniscript RT kit 50 (Qiagen, Valencia, CA) according to the document of the manufacturer. Primers for p53, Rap2B, PLCε, and endogenous control GAPDH were used to amplify these genes. The sequences are as follows. p53 forward: 5'-ATA GTG TGG TGG TGC CCT ATG A-3'; and reverse: 5'-TGT GAT GAT GGT GAG GAT GG-3'; Rap2B forward: 5'- ATG AGA GAG TAC AAA GTG GTG-3'; and reverse: 5'-TCA GAG GAT CAC GCA GG-3'; PLCε forward: 5'-GGG GCC ACG GTC ATC CAC-3'; and reverse: 5'-GGG CCT TCA TAC CGT CCA TCC TC-3'; GAPDH forward: 5'-CAA AGT TGT CAT GGA TGA CC-3'; and reverse: 5'-CCA TGG AGA AGG CTG GGG-3'. To analyze the accuracy of the PCR reaction, the Ct value was used. To evaluate the expressions of these genes,  $2^{-\Delta\Delta Ct}$  values were calculated. The p53, Rap2B, PLCε gene amplified within the linear region of the reaction amplification curves is shown as the Ct value. The expression values of p53, Rap2B, PLCε were normalized to the expression value of GAPDH before analysis.

### Luciferase reporter assay

U2OS cells were co-transfected siRNA against p53, Rap2B or PLCε siRNA with GFP-LC3 plasmids, using lipofectamine 2000. After co-transfection for 48 h, the medium was replaced by EBSS to induce autophagy. Autophagy was observed and photographed in each group at 0 and 8 h under fluorescence microscopy. GFP-LC3 fusion protein was used to trace autophagy formation. GFP-LC3 fusion protein was

dispersed in the cytoplasm in the absence of autophagy. Once autophagy occurred, GFP-LC3 fusion protein was translocated to the membrane of autophagy, and multiple bright green fluorescent spots were formed. One spot represented as autophagy. The levels of autophagy activity were evaluated by counting the number of spots.

### Measurement of intracellular IP3

The intracellular IP3 was measured using a human IP3 ELISA Kit according to the manufacturer's protocol (Beyotime, Shanghai, China). The absorbance of each well was determined at 450 nm using a microplate reader, and the concentrations of the intracellular IP3 were calculated.

### Measurement of intracellular calcium concentration (Ca<sup>2+</sup>)

The intracellular calcium concentration in U2OS cells was qualified by measuring the Ca<sup>2+</sup>-sensitive fluorescent dye Fluo 3-AM (Beyotime, Shanghai, China). After the cells were incubated in HBSS containing Fluo 3-AM for 1 h in the dark, a flow cytometer (BD Biosciences, San Jose, CA, USA) was used to analyze.

### Western blotting

Cells were lysed using RIPA buffer. After that, the cell lysate was centrifuged to remove the insoluble debris. The supernatant was collected, and protein concentrations were determined using a bicinchoninic acid (BCA) kit (Thermo Scientific, MA) according to the instructions of the manufacturer. Protein samples were separated by 10% SDS-PAGE gel, and then the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked using 5% non-fat milk. The membranes were incubated with primary antibody against p53, Rap2B, PLCε, and β-actin overnight at 4°C. After that, the membranes were incubated with peroxidase-conjugated secondary antibodies at room temperature. The expressions of protein were normalized to the internal control actin, and the results were analyzed using Bio-Rad Image J (Bio-Rad, CA, USA).

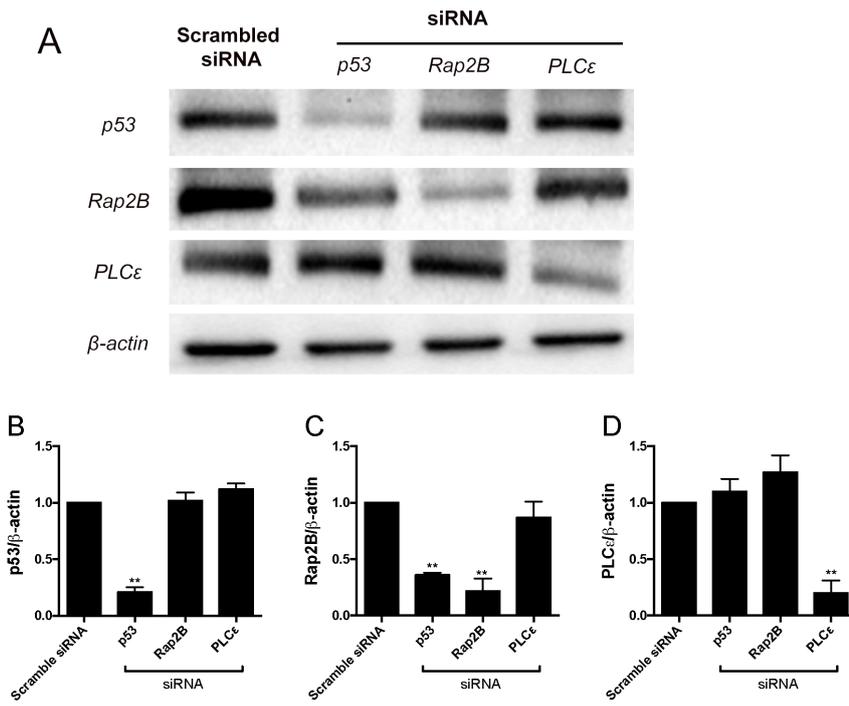
### Statistical analysis

Data are expressed as the means ± SD obtained from triplicates of experiments. One-way analysis of variance with multiple comparisons and Student-Newman-Keuls (SNK) test were performed. Differences were considered significant when *p*-values less than 0.05.

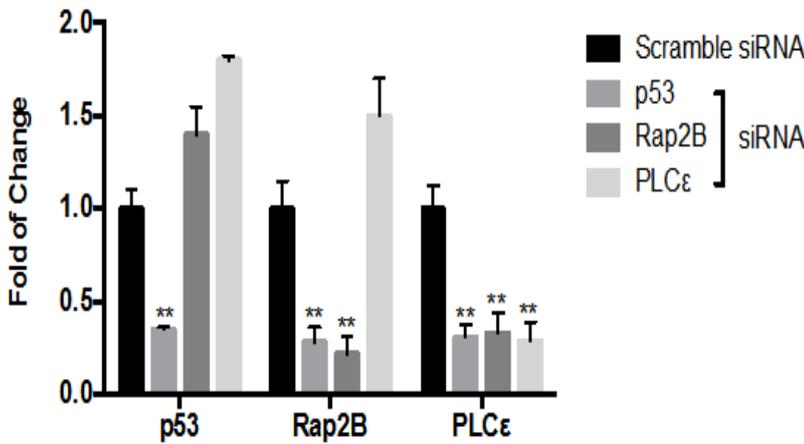
## Results

### siRNA silencing knocked down the expressions of p53, Rap2B, and PLCε in U2OS cells

First, U2OS cells were transfected with siRNA against p53, Rap2B, and PLCε. To ensure the knockdown efficiency, western blotting was used to examine the expressions of target genes, including p53, Rap2B, and PLCε. As shown in Figs. 1(A)–1(D), we observed that the proteins expressions of each target gene including p53, Rap2B, and PLCε were significantly decreased after the cells were transfected with siRNA. These results demonstrated that the expressions of p53, Rap2B, and PLCε were knocked down in U2OS cells.



**FIGURE 1.** U2OS cells were transfected with siRNA against Rap2B, p53 and PLCε, cells were harvested and analyzed by western blotting. All experiments were performed in triplicate. Data were presented as mean ± SD. \*\**P* < 0.01 as compared with scramble siRNA group (n = 3).



**FIGURE 2.** U2OS cells were transfected with siRNA against Rap2B, p53 and PLCε, the mRNA levels of each group were measured using qPCR. All experiments were performed in triplicate. Data were presented as mean ± SD. \*\**P* < 0.01 as compared with scramble siRNA group (n = 3).

The knockdown of p53 decreased the expressions of Rap2B protein level and had no effect on the expressions of PLCε

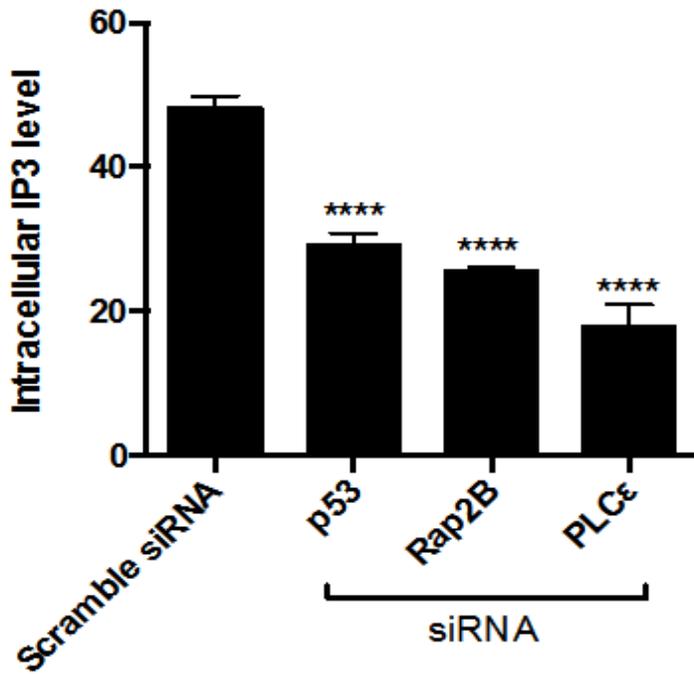
We then examined the protein expressions of p53, Rap2B, and PLCε after the cells transfected with siRNA. As shown in Figs. 1(C)–1(D), the expressions of Rap2B were significantly decreased in the cells transfected with p53 siRNA when compared with that in the cells transfected with scrambled siRNA (*p* < 0.01). However, we did not observe a decrease in expressions of PLCε. In addition, the protein expressions of PLCε were significantly decreased in cells transfected with PLCε siRNA, but not in cells transfected with siRNA against p53 and Rap2B (*p* < 0.01). Therefore, we inferred that Rap2B is a downstream target protein of p53.

In addition, we also examined the mRNA levels of each target gene after the cells transfected with siRNA. As shown in Fig. 2, the knockdown p53 decreased the mRNA levels of

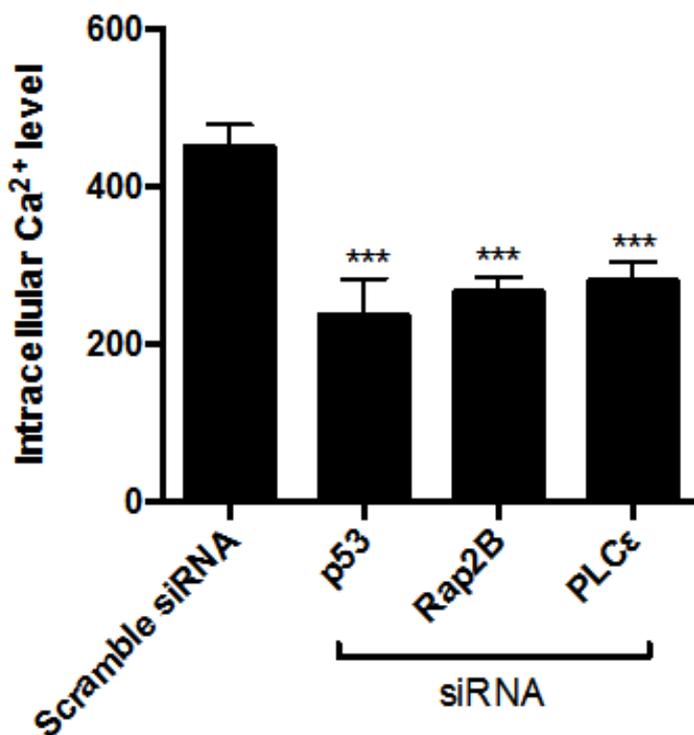
PLCε and Rap2B, indicating p53 regulated the expressions of PLCε and Rap2B. We also observed that the mRNA levels of PLCε were decreased in the cells transfected with Rap2B siRNA, suggesting that Rap2B regulated the expressions of PLCε. Interestingly, the mRNA levels of Rap2B and p53 did not change in the cells transfected with PLCε siRNA. The results demonstrated that Rap2B and PLCε are downstream target protein of p53.

The knockdown of p53 decreased the levels of intracellular IP3 through Rap2B

The levels of intracellular IP3 in knockdown cell lines were then determined using ELISA. As shown in Fig. 3, the levels of intracellular IP3 were significantly decreased in the cells transfected with siRNA against p53, Rap2B, or PLCε. Our results revealed that knockdown of either p53 or Rap2B



**FIGURE 3.** U2OS cells were transfected with siRNA against Rap2B, p53 and PLCε, then the levels of intracellular IP3 in each group were measured using an ELISA Kit. All experiments were performed in triplicate. Data were presented as mean  $\pm$  SD. \*\*\*\* $P$  < 0.0001 as compared with scramble siRNA group (n = 3).

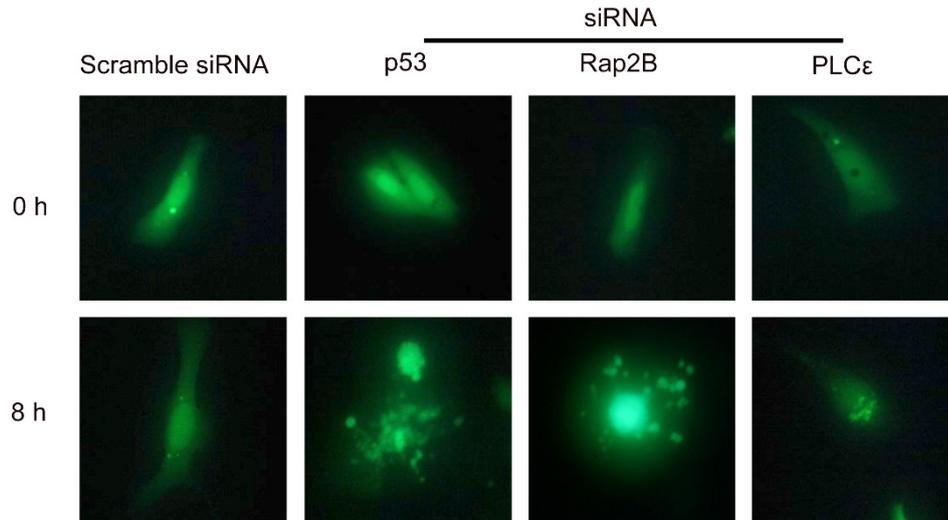


**FIGURE 4.** After U2OS cells were transfected with siRNA against Rap2B, p53 and PLCε, the levels of intracellular Ca<sup>2+</sup> were measured using flow cytometry. All experiments were performed in triplicate. Data were presented as mean  $\pm$  SD. \*\*\* $P$  < 0.001 as compared with scramble siRNA group (n = 3).

significantly decreased the levels of intracellular IP3 when compared with the cells transfected with scrambled siRNA. Additionally, we observed that the levels of intracellular IP3 in the cells transfected *Rap2B* siRNA are lower when compared with that in the cells transfected p53 siRNA. These results indicate that the knockdown of p53 decreased the levels of intracellular IP3 through a *Rap2B*-dependent pathway.

*The knockdown of p53 decreased the levels of intracellular Ca<sup>2+</sup>*  
It is known that IP3 interacts with a specific receptor located on the surface of the endoplasmic reticulum, resulting

in a rapid increase of intracellular Ca<sup>2+</sup> concentrations (Nowycky and Thomas, 2002; Carafoli, 2002). In order to determine the effects of p53-*Rap2B*-*PLCε* pathway on the levels of intracellular Ca<sup>2+</sup>, flow cytometry was performed in U2OS cells transfected siRNA against p53, *Rap2B*, and *PLCε*. As shown in Fig. 4, the knockdown of p53, *Rap2B*, or *PLCε* resulted in a significant decrease of intracellular Ca<sup>2+</sup> concentrations when compared with that of the cells transfected with scrambled siRNA. These results supported that the p53-*Rap2B* pathway played an important role in the regulation of the levels of intracellular Ca<sup>2+</sup>.



**FIGURE 5.** Fluorescence microscope was used to observe the autophagy after the U2OS cells were co-transfected scramble siRNA or siRNA against Rap2B, p53 and PLC $\epsilon$  with GFP-LC3 plasmids (400 $\times$ ). GFP-LC3 fusion protein was used to trace autophagy formation. GFP-LC3 fusion protein was dispersed in the cytoplasm in the absence of autophagy. Once autophagy occurred, GFP-LC3 fusion protein was translocated to the membrane of autophagy, and multiple bright green fluorescent spots were formed. One spot represented as autophagy.

#### *The knockdown of p53, Rap2B or PLC $\epsilon$ promoted autophagy in U2OS cells*

The U2OS cells were transfected with scrambled siRNA or siRNA against p53, Rap2B, or PLC $\epsilon$  and GFP-LC3 for 48 h, and then the medium was replaced by EBSS to induce cell autophagy. Autophagy in each group was observed under a fluorescence microscope after starvation induction for 0 h and 8 h, respectively. As shown in Fig. 5, we observed that the p53, Rap2B, or PLC $\epsilon$  knocked down group showed several bright green fluorescent spots. Each spot represents an autophagosome. Additionally, when compared with the control group, the numbers of intracellular autophagosomes in the cells which were transfected with siRNA against p53, Rap2B, or PLC $\epsilon$  were significantly increased. These results suggested that the knockdown of p53-Rap2B-PLC $\epsilon$  pathway promoted autophagy of tumor cells.

#### **Discussion**

Rap2B has drawn much attention in recent. It has been reported that the expressions of Rap2B protein are significantly increased in the cancer tissues and cell lines, including U2OS H1299 cells. Rap2B belongs to the Ras family. As a novel discovered target gene of p53, Rap2B has been identified to play an important role in p53-mediated signaling pathway (Zhang *et al.*, 2013; Paganini *et al.*, 2006; Machida *et al.*, 2004). Mestre and Colombo (2012) have demonstrated that the direct activation of Rap2B by cAMP is sufficient to inhibit autophagy through the mTOR pathway. However, the relationship between p53-Rap2B and autophagy is still unknown. Therefore, the present study aims to explore the effects of p53-Rap2B on autophagy in U2OS cells.

Bai and colleagues have revealed that PLC plays an important role in the formation of skin cancer induced by chemical carcinogens (Bai *et al.*, 2004). Interestingly, Rubinsztein and colleagues (Keiper *et al.*, 2004) have demonstrated the importance of EPAC and Rap2B in the

regulation of autophagy. Our previous study has elucidated that the mTOR-independent pathway regulated autophagy in which the direct activation of EPAC/Rap2B by cAMP activates PLC $\epsilon$  to upregulate the levels of IP3 and induce the release of Ca $^{2+}$  from the endoplasmic reticulum (Di *et al.*, 2017). In recent, the roles of p53 in the regulation of autophagy have been reported (Sui *et al.*, 2011; Saini *et al.*, 2019). Rap2B is the target gene of p53, and Rap2B can activate PLC $\epsilon$  and promote the release of downstream IP3 and Ca $^{2+}$ . Additionally, many studies have revealed that Rap2B plays an important role in tumorigenesis through the regulation of cell proliferation, adhesion, and migration (Miao *et al.*, 2019; Yi *et al.*, 2019). Herein, we speculate that the PLC $\epsilon$ -IP3-Ca $^{2+}$  pathway might be associated with p53-Rap2B dependent autophagy inhibition. In addition, the mechanisms of Rap2B in osteosarcoma cells are still unclear. Therefore, this study aims to investigate the roles of p53-Rap2B in the autophagy of osteosarcoma cells.

First, we verified the knockdown efficiency of siRNA against p53, Rap2B, and PLC $\epsilon$  in the U2OS cells. Interestingly, the results showed that the knockdown of p53 decreased the expressions of Rap2B, supporting that Rap2B is a target gene of p53. Furthermore, the knockdown of p53 also decreased the mRNA levels of PLC $\epsilon$ . Besides, the knockdown of p53 decreased the intracellular IP3 and Ca $^{2+}$  levels through Rap2B. Next, we investigated the effects of the knockdown of p53, Rap2B and PLC $\epsilon$  on the autophagy in U2OS cells. It has been reported that p53 inhibits autophagy, and Rap2B also indirectly affects autophagy through cAMP, and the regulation of autophagy is correlated with the calcium ion pathway to a certain extent, but their relationship with each other has not been reported.

In the present study, we confirmed Rap2B as a target gene of p53 and demonstrated that the knockdown of p53-Rap2B decreased the levels of intracellular IP3 and Ca $^{2+}$ . Our results also suggested that the knockdown of the p53-Rap2B-PLC $\epsilon$  pathway regulated autophagy induced by starvation.

Additionally, the results also revealed that the knockdown of p53 and Rap2B had no effect on the expressions of PLC $\epsilon$  on the protein level. We speculated that p53 and Rap2B regulated the mRNA levels of PLC $\epsilon$  other than its protein expressions. In summary, our results showed that the knockdown of p53 downregulated the PLC $\epsilon$ -IP3-Ca<sup>2+</sup> signal pathway through Rap2B to promote autophagy in U2OS cells. These results supported that the PLC $\epsilon$ -IP3-Ca<sup>2+</sup> signal pathway plays an important role in the autophagy of U2OS cells. Additionally, targeting PLC $\epsilon$ -IP3-Ca<sup>2+</sup> signal pathway might be an alternative method for the treatment of osteosarcoma.

### Conflict of Interest

The authors have declared that no competing interests exist.

### Acknowledgments

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