RESEARCH COMMUNICATION

Silencing of the COPS3 Gene by siRNA Reduces Proliferation of Lung Cancer Cells Most Likely via Induction of Cell Cycle Arrest and Apoptosis

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Abstract

The COPS3 gene has stimulating effect on cell proliferation and progression of osteosarcomas and related cells. However, the features of COPS3 and its potential application as a therapeutic target in other cancers has not yet been studied. In this study, therefore, the effect of COPS3 silencing via COPS3 siRNA on lung cancer cell proliferation was examined. Expression levels of COPS3 gene in COPS3 siRNA infected cells and control siRNA infected cells were compared with real time PCR and Western blot analysis. Cell proliferation levels were comprehensively analyzed by MTT, BrdU incorporationy, and colony formation assays. For mechanistic assessment the effects of COPS3 silencing on cell cycle and apoptosis were analyzed using flow cytometry. Results showed that successful silencing of the COPS3 gene at both translational and transcriptional levels significantly reduced the proliferation and colony formation by lung cancer cells ($p<0.01$). Flow cytometry showed cell cycle arrest in the G0/G1 phase after COPS3 silencing, and more importantly, apoptosis was induced as a result of COPS3 knockdown, which negatively affected cell survival. Therefore, these results provide another piece of important evidence that the COPS3 gene expressed in lung cancer cells may play a critical role in stimulating proliferation. Down-regulation of COPS3 could significantly inhibit lung cancer cell growth, which was most likely mediated via induction of cell cycle arrest in G0/G1 phase and apoptosis.

Keywords: COPS3 - cell proliferation - cell cycle arrest - apoptosis - lung cancer

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Introduction

Lung cancer is one of the major causes of human death both in Asian and Western populations and thus has become a heavy burden disease in the world wide (Sharma et al., 2007). The number of lung cancer patients are increasing annually. Therapeutic approaches, such as chemotherapy, radiotherapy, and surgery have been widely used to treat lung cancer; however, these treatments offered little survival rate due to severe toxicity and drug resistance (Broker et al., 2002; Singh et al., 2008). Therefore, understanding of the carcinogenic mechanisms and genes involved in lung cancer cell's fast growth is of much importance in developing more precise treatments compared to conventional treatment methods. Recent discoveries in terms of the molecular basis of cancer brought fundamental changes in cancer treatment (Chen et al., 2011; Skrzypski et al., 2011). One of them is usage of small interfering RNA (siRNA) to manipulate the targeting genes for the prevention of certain cancer progression. Recently an exciting report by Singh et al. showed that siRNA-mediated silencing of nuclear factor erythroid-2–related factor 2 gene expression in non-small cell lung cancer inhibited tumor growth and increased efficacy of chemotherapy (Singh et al., 2008). Therefore, identification and silencing of genes related to the growth and survival of lung cancer cells would be an interesting area of research. To this end, lentivirus based short hairpin RNA (shRNA) is widely used to target specific gene in cancer cells (Luo et al., 2009).

COPS3 gene encodes a subunit of COP9 signalosome which is a highly conserved complex to regulat the ubiquitination and degradation of the p53 tumor suppressor (Wei et al., 2003). It is reported that COPS3 gene is required for the cell proliferation (Yan et al., 2003). Overexpression of COPS3 led to p53 protein degradation that is equivalent to inactivation of P53 by mutation (Bech-Otschir et al., 2001). Therefore, increased expression COPS3 expression was found in osteosarcomas (Henriksen et al., 2003; van Dartel et al., 2004; Yan

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et al., 2007), and cells derived from highly metastatic osteosarcomas also express high levels of COPS3 (Yan et al., 2011). Inhibition of COPS3 expression and function by siRNA was found to result in reduced proliferation and migration of highly metastatic osteosarcoma cells (Yan et al., 2011). These results suggest that COPS3 gene may be a target of genes that can be manipulated in order to prevent cancer cell growth or metastasis. However, these features were appreciated predominantly based on this one kind of cancer (osteosarcoma) and there was no information whether the above features of COPS3 were also true for other cancers or cancer cell lines.

The present study, therefore, was designed for two purposes. The first one was to define whether the features of COPS3 expression and function observed in osteosarcoma are also observable in lung cancer cells. To this end, we used two lung cancer cell lines to examine whether inhibition of COPS3 expression by its siRNA in lung carcinoma cells also provides an inhibitory effect on the cell proliferation. The second purpose was to preliminarily explore how silencing COPS3 affects the lung cancer cell proliferation by examining cell cycle and apoptosis.

Materials and Methods

Cell culture

Two lung cancer cell lines (A549 lung adenocarcinoma cell line and 95D metastatic lung cancer cell, both derived from epithelia cells) and human embryonic kidney cells (293T that was used for generating Lentiviruses) were used in this study. Except for 95D cell line, all were obtained from American Type Culture Collection (ATCC). The cells were maintained in penicillin/streptomycin treated DMEM supplemented with 10% FBS at 37 °C in humidified atmosphere of 5% CO_2.

Construction of COPS3 shRNA lentivirus

The sense and antisense strands of the siRNA for COPS3 were 5'- GCACATTCGATATGCAACA-3' and 5'-TGTTGCATATCGAATGTGC-3', respectively. The sense and antisense strands of non-silencing control siRNA were 5'-TTCTCCGAACGTGTCACGT-3' and 5'-ACGTGACACGTTCGGAGAA-3', respectively. The nucleotide sequences were inserted into the pFH1UGW-GFP vector (Hollybio, Shanghai, China) and the generated lentivirus based shRNA expressing vectors were confirmed by DNA sequencing. Lentiviruses were generated by transfection of 293T cells at 80% confluence with generated plasmids. At 2 h before the transfection the medium containing FBS was removed and then supplied with 200 μl of Opti-MEM containing 20 μg pFH1UGW-GFP-shCOPS3 or -shCTRL, 15 μg pCMV vector and 10 μg pVSVG plasmid and also with 15 μl of Lipofectamine 2000 to incubate for 8 h prior to replace with 10 ml of 10% FBS containing DMEM medium. The supernatant was collected after 48 h of transfection and lentiviral particles were harvested by ultra-centrifugation (4000 g) at 4 °C for 10 min. The collected virus particles were filtered through a 45 μm filter and the filtrate was centrifuged (4000 g at 4 °C) for 15 min to collect the viral concentrate.

COPS3 shRNA lentivirus and control lentivirus infection into the cancer cells

A549 and 95D cells were infected with COPS3 shRNA and control shRNA expressing lentivirus to create the silenced COPS3 gene group (si-COPS3) and non-silenced COPS3 gene group (si-CTRL) of cells. To this end, cells were seeded at 50,000 cells per well in 96 well plates and after 24 h of incubation the culture medium was replaced with Opti-MEM medium containing the appropriate amount of the virus. At 24 h after incubation the medium was replaced with fresh medium and the cells were further incubated for another 72 h. Successful transfection was observed through a fluorescence microscope for the green fluorescence protein expression.

RNA extraction and real-time PCR analysis

RNA was extracted from 5-day infected lung cancer cells. Cells were lysed with Trizol reagent (Invitrogen) and total RNA was extracted from the lysate using standard procedures. cDNA was synthesized from the extracted RNA using Promega M-MLV cDNA synthesis kit according to the manufactures instructions. The real time PCR analysis was carried out using the synthesized cDNA template to evaluate the COPS3 mRNA expression levels in si-COPS3 and si-CTRL infected cells. SYBR Green Master Mix Kit on DNA Engine Opticon™ System (MJ Research, Waltham, MA) was employed for the real-time PCR analysis. Actin was used as the reference. The forward and reverse primers of COPS3 are 5'-CGCTATTCTCACAGGTTCAG-3' and 5'-GCATCATAGGCTCCATTCTC-3' respectively. The forward and reverse primers of β-actin are 5'-GGCGGCACCACCATGTACCCT-3' and 5'-AGGGGCCGGACTCGTCATACT-3'. Relative gene expression levels were calculated using $2^{-\Delta\Delta CT}$ analysis.

Western blot analysis

The change in COPS3 protein expression level was detected by Western blotting. Cells were infected with the lentivirus containing si-COPS3 and si-CTRL for 5 days. The cells were then washed with cold PBS and lysed with radio-immune precipitation assay (RIPA) buffer [50 mM Tris (pH7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1%SDS] containing phenylmethyl sulfonylfluoride (PMSF) (1 mM) and protease inhibitors (2 μg/ml; Protease Inhibitor Cocktail Set III, Calbiochem) on ice for 30 min. The supernatant was collected after centrifuging the cell lysate (12,000 g for 15 min) and the protein content was measured by Lowry method. The protein concentration of each sample was adjusted to 2 μg/μl and 20 μl volume was electrophoresed on an 12% SDS-PAGE gel at 60 V for 4 h. the gel was transferred to polyvinylidene difluoride membrane and the protein levels were detected after antibody treatment using ECL kit (Amersham) by exposing to X-ray film. The bands obtained were quantified with an ImageQuant densitometric scanner (Molecular Dynamics).

Analysis of the effect of COPS3 silencing on cell proliferation

MTT analysis: Cells were seeded into a 96 well

plate at a concentration of 2000 cells/well on day 5 after infection. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis was performed in different time intervals (1, 2, 3, 4 and 5 days after incubation). Twenty μl of MTT solution (5 mg/ml) was added to each well and incubated at 37 °C for 4 h. Then the medium and MTT from the wells were removed and 200 µl of DMSO was added to each well. The optical density was measured using a micro-plate reader at 490 nm. Experiments were performed in triplicate.

Determination of cell proliferation using BrdU cell proliferation assay: After 5 days of infection with COPS3 siRNA and control siRNA, lung cancer cells were plated onto a 96-well plate at 5×10^3 cell density in 100 µl of medium and incubated for different time intervals (24 h and 72 h). After incubation bromodeoxyuridine (BrdU) was added into wells at a final concentration of 25 µmol/l and cells were incubated for another 8 h. During this time BrdU is incorporated into the cellular DNA. Then the cells were fixed and DNA was partially digested by nuclease treatment for 30 min at room temperature. Anti-BrdU monoclonal antibody (1:200 dilution) and 100 µl peroxidase conjugated goat anti-mouse secondary antibody were added and incubated for 1 h and 30 min at room temperature, respectively. Then 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate solution was added and incubated for 30 min in a dark environment at room temperature. The incorporated BrdU was detected and quantified by measuring the absorbance at 490 nm wavelength in a micro-plate reader. The absorbance at 490 nm is directly correlated to the level of BrdU incorporated into cellular DNA.

Colony forming assay: Lung cancer cells were infected with respective siRNA for 5 days and these infected cells were collected and seeded in 6 well plates at a density of 500 cells/well. The medium was changed every three days until 10 days of culture. Then cells were washed with PBS and fixed with 4% paraformaldehyde. Fixed cells were stained with freshly prepared diluted Giemsa stain for 20 min and then washed with double distilled water. Colonies with more than 50 cells were counted using a fluorescence microscope.

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry assay following popidium iodide (PI) staining. Infected cells were seeded on a 6 well plate at 1×10^6 cell density and incubated for 24 h. Cells were collected by adding trypsin, washed with ice cold PBS, and then fixed by suspending in 0.7 ml of 70% ethanol for 30 min at 4 °C. The ethanol was discarded by centrifugation and the PI (100 µg/ml) solution containing 10 μg/ml of DNase-free RNase A was added to stain the cell for 30 min. The cell suspension was filtered through a 50-μm nylon mesh, and the stained cell was analyzed by flow cytometer (FAS Calibur, BD Biosciences).

Detection of apoptosis

For detecting apoptosis, COPS3 siRNA and control siRNA infected cells were seeded onto a 6 well plate at a density of 5×10^5. After 5 days of incubation cells were collected by trypsin digesion and washed with ice cold PBS. After PBS was removed by centrifugation, cells were treated with 100 μl of binding buffer, followed with 5 μl of Annexin V solution and 5 μl of PI for incubation 5 min at RT in dark place. Total of 1×10^6 cells were analyzed using flow cytometer (FAS Calibur, BD Biosciences).

Statistical analysis

All data were expressed as mean ± SD of three independent experiments at least. Data were statistically analyzed using SPSS 16.0 software employing paired T test analysis method. $P < 0.05$ was considered as statistically significant.

Results

Infection of COPS3 siRNA can efficiently down-regulate COPS3 mRNA and protein expression

The successful infection to lung cancer cell lines A549 and 95D with COPS3 siRNA and control siRNA was confirmed through fluorescence microscope after 96 h of infection that the green fluorescence indicated the successful infection. As shown in Figure 1A more than 90% cells were infected and around 50% cells were infected in 95D cells. The expression level of COPS3 gene was analyzed by real-time PCR for COPS3 siRNA infected (si-COPS3) and control siRNA infected (si-CTRL) A549 and 95D cells, respectively. As indicated in Figure 1B the expression level of COPS3 mRNA was remarkably reduced by the COPS3 siRNA compared to control group in both lung cancer cells ($P<0.01$). Furthermore the protein expression levels were also analyzed by Western blotting, which demonstrated that the COPS3 expression is easily detected by Western blotting in both A549 and 95D lung cancer cells in control SiRNA group, and significant suppression of COPS3 protein expression was evident in cells treated with specific siRNA compared to control siRNA (Figure 1C). Therefore these results indicated that the successful infection with COPS3 siRNA to down-regulate both mRNA and protein expression of COPS3.

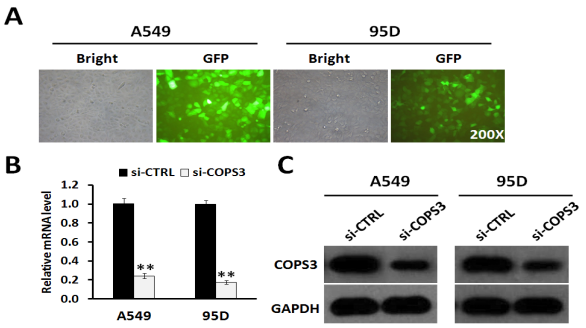

Figure 1. Knockdown of COPS3 Suppressed the COPS3 mRNA and Protein Expression Levels in Lung Cancer Cells. GFP fluorescence was indication of infected cells in A549 and 95D cells 96 h after infection with lentivirus containing COPS3 siRNA at the magnification of × 200 (A). Quantitative real time PCR data of COPS3 mRNA levels following the silencing COPS3 gene with SiRNA (B). Western blotting assay was used to detect the protein expression of COPS3 in two lung cancer cells (C). Data were presented as mean ± SD from three independent experiments with duplicate samples at least. **P<0.01 in comparison with control

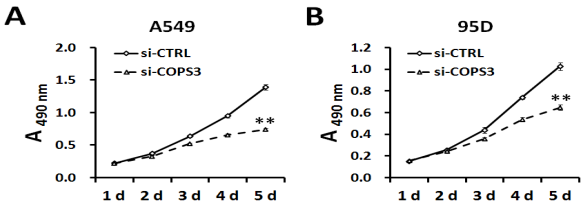

Figure 2. Effect of COPS3 Silencing on the Proliferation of Lung Cancer Cells. Cell proliferation in A549 (A) and 95D (B) cells were measured by MTT analysis. Data were presented as mean ± SD from three independent experiments with duplicate samples at least. **P<0.01 in comparison with control

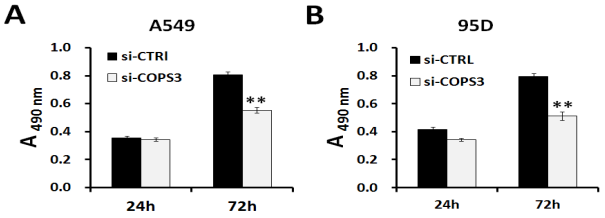

Figure 3. Effect of COPS3 Silencing on the DNA Synthesis of Lung Cancer Cells. Cell proliferation in A549 (A) and 95D (B) were measured by DNA synthesis with BrdU proliferation assay. Data were presented as mean ± SD from three independent experiments with duplicate samples at least. **P<0.01 in comparison with control

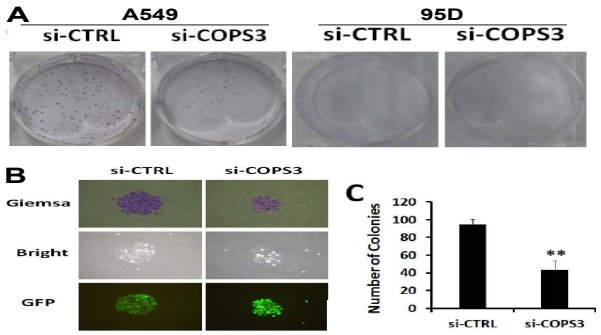

Figure 4. Effect of COPS3 Silencing on the Colony Forming Ability of Lung Cancer Cells. Cell colony formation capacity was examined for the colony with Giemsa staining (A, B), and also with bright filed and fluorescence microscopy (B). Panel A showed that there was no colony formation for D95 cells in the same condition as those for A542 cells that showed may colonies examined with Giemsa staining. Quantitative analysis of the numbers of colonies, accounted with Giemsa staining method, are presented as panel C. Data were presented as mean ± SD from three independent experiments with duplicate samples at least. **P<0.01 in comparison with control

Effect of COPS3 knockdown on lung cancer cell proliferation

The effect of COPS3 suppression on lung cancer cell proliferation was analyzed by a few methods. First the cell proliferation was dynamically detected for 5 days with MTT assays. As shown in Figure 2A and B both cell lines showed a reduction in cell proliferation upon COPS3 suppression and on day 5 after infections, the cell proliferation of both cell lines was significantly lower in COPS3 silencing group than that in control (p<0.01).

BrdU cell proliferation assay that measures DNA synthesis was often used as a specific marker of cell proliferation. Results showed that both cell lines showed a significant inhibition of cell proliferation 72 h after infection (p<0.01) although there was no significant difference at 24 h after infection (Figure 3A and B).

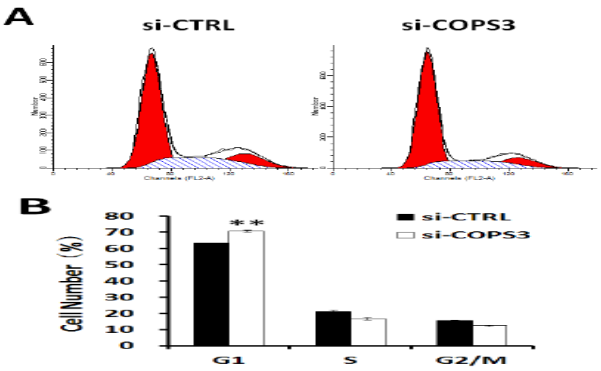

Figure 5. Effect of COPS3 Silencing on the Cell Cycle Distribution of A549 Lung Cancer Cells. Flow cytometry histograms for cell cycle as the raw data (A) and quantitative analysis (B) were obtained for A549 cells. Data were presented as mean ± SD from three independent experiments with duplicate samples at least. **P<0.01 in comparison with control

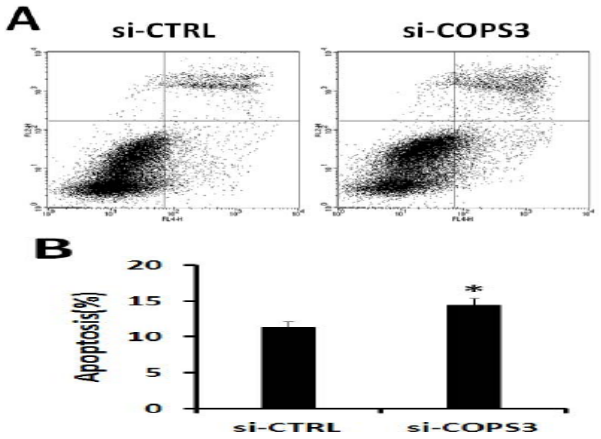

Figure 6. COPS3 Deletion Induced Apoptosis in A549 Lung Cancer Cells. The dot plots for apoptosis analysis as the raw data from flow cytometric analysis (A) and quantitative analysis of the apoptosis were provided for A549 cells. Data were presented as mean ± SD from three independent experiments with duplicate samples at least. **P<0.01 in comparison with control (B)

Furthermore, colony forming assay was performed to evaluate the effect of COPS3 knockdown on colony formation in lung cancer cells. We found that 95D cells do not have the ability to form colonies under the same condition for A549 cells that have formed remarkable colonies (Figure 4A). Therefore, for the colony assay we only used A549 cells. As depicted in Figure 4B the size and numbers of the colonies formed are significantly smaller in COPS3 knockdown group than that in control. Quantitative analysis confirmed that the number of colonies was over a 50% reduction in COPS3 knockdown group compared to control group (p<0.01, Figure 4C). Collectively, these results suggest that COPS3 gene is related to promoting tumor cell growth.

Effect of silencing COPS3 gene on the cell cycle of lung cancer cells

The above studies have indicated that COPS3 gene is required for lung cancer cell proliferation. To dissect how COPS3 affects cell proliferation, the cell cycle of the A549

cells were analyzed by flow cytometry after infection with COPS3 siRNA and control siRNA. Fig. 5A demonstrates the pattern of flow cytometry raw data for the cell cycle distribution between the control group and COPS3 gene silencing group. Quantitative data suggest that cells with COPS3 knockdown were predominantly at the G0/G1 phase while cells from control were predominantly at S phase and G2/M phase ($P<0.01$, $P<0.01$). This suggests that A549 lung cancer cells with the knockdown of COPS3 gene arrest at the cell cycle of G0/G1 phase.

Knockdown of COPS3 gene in A549 lung cancer cells induced apoptosis

The effect of knock-down COPS3 gene on apoptotic cell death was analyzed by flow cytometry following Annexin V-PI double staining in COPS3 siRNA and control siRNA infected A549 cells. The percentage of apoptotic cell number in si-COPS3 group were significantly higher than that in the control group (Figure 6A and B, $P<0.01$), suggesting that deletion of COPS3 gene can induce cell apoptotic death.

Discussion

Molecular based therapeutic targets are gaining much attention in cancer research over conventional cancer treatments such as chemotherapy and radiotherapy. Development of targeted cancer therapeutics at tumor specific proteins or genes has emerged as a novel strategy for cancer treatment. Finding of siRNA oligonucleotides as potent, sequence-selective gene inhibition tool has facilitated the expectation of developing targeted therapeutics (Fuchs et al., 2002; Paddison et al., 2003). Lentivirus-based vectors have been used successfully to deliver siRNA efficiently with longer gene silencing effects (Devi et al., 2006). In the present study efforts have been taken to identify the efficiency of COPS3 gene silencing through siRNA technology as a therapeutic target in treatment of lung cancer cells.

COPS3 gene, which is a part of highly conserved complex COP9 signalosome is studied in relevance to osteosarcoma. The overexpression of COPS3 has been reported as an alternative mechanism of degrading tumor suppressor gene p53 in osteosarcomas and thereby facilitating the development and progression of osteogenic sarcoma by regulating cell cycle, cell proliferation, apoptosis and metastasis (Henriksen et al., 2003). Therefore, COPS3 has been assumed as an oncogene which can regulate the growth and metastasis of osteosarcoma. However, there was no information whether COPS3 also play such roles in other cancers. The present study provides the first evidence that two lung cancer cells highly express COPS3 proteins (Figure 1C).

It is known that COPS3 targets the p53 protein via 26S proteasome degradation and the excessive degradation results in similar consequences to p53 mutation (Bech-Otschir et al., 2001), and mutations in p53 are a common factor in tumor progression (Gokgoz et al., 2001). Due to suppression of p53 the tumor cell survival rates may be increased in the cells with overexpression of COPS3 condition. In support of this notion, knockdown of COPS3 gene was reported to inhibit cell proliferation in osteosarcoma cells (Yan et al., 2011). Consistent with this early study, we also provide the first evidence here that knockdown COPS3 gene significantly inhibited the cell proliferation rate (Figsure 2 and 3) and colony forming potency (Figure 4) in lung cancer cells. Mechanistically we further demonstrated that deletion of COPS3 expression caused cell cycle arrest at G0/G1 phase (Figure 5) and also apoptotic cell death (Figure 6).

In summary, our study provided the evidence that COPS3 is highly expressed in lung cancer cells, which is important finding since it means that COPS3 overexpression is not only in osteosarcomas or osteosarcoma cell lines, but also in lung cancer cells. This implies that COPS3 may be also overexpressed in other tumor cells. Application of lentivirus-mediated siRNA can successfully knockdown COPS3 gene. This approach can efficiently induce lung cancer cell arrest at cell cycle G0/G1 phase along with apoptotic cell death, which results in a significant inhibition of lung cancer cell proliferation. Taken together with previous studies on the effect of COPS3 expression on osteosarcomas, we anticipate that targeting COPS3 gene will have a great potential to be considered as an efficient approach to prevent and/or treat certain tumors in clinics, at least for osteosarcomas and lung cancers, in the future.

Acknowledgements

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